The Kinetic Effects of the Binding of Mitochondrial Creatine Kinase to Chicken Heart Inner Mitochondrial Membranes

By

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ABSTRACT

The present study examines the relative contributions of the outer mitochondrial membrane, and the binding of mitochondrial creatine kinase (MiMi-CK) to the inner mitochondrial membrane (IMM), to the preferential coupling of MiMi-CK to oxidative phosphorylation in chicken heart mitochondria. Mitochondrial creatine kinase (EC 2.7.3.2) was purified to homogeneity from chicken ventricle using a procedure which makes use of an Agarose-Hexane-ADP column run under conditions where a MiMi-CK active site transition-state analog is formed. The homogeneous enzyme has a Mr = 86,000 $\pm$ 5,000 and a specific activity of 124 IU/mg.

An examination of the binding process shows that the MiMi-CK:IMM interaction is ionic in nature. The binding process is dependent on the pH of the incubation medium with increased binding at lower pH values; binding is dependent on the protonation of a group(s) with an apparent pKa value of 6. Extrapolating titrations of mitoplasts with MiMi-CK gives a maximum of 14.6 IU bound per nmole cytochrome $\underline{aa}_3$ on the IMM. This value corresponds to 1.12 moles MiMi-CK per mole cytochrome $\underline{aa}_3$ or 0.33 mole MiMi-CK per mole inner membrane nucleotide translocase.

The kinetic parameters of MiMi-CK were examined using either intact mitochondria or mitoplasts. When MiMi-CK is coupled to the nucleotide translocase, a K_a value for $MgATP^{-2}$ of 36 $\mu\underline{M}$ is obtained. This K_a value is three fold lower than that measured using mitoplasts under conditions where about 70% of the enzyme is bound to the IMM (100 $\mu\underline{M}$), or for that of soluble enzyme (125 $\mu\underline{M}$). The apparent nucleotide

translocase K_m value for ADP decreases from 20 μM to 10 μM in the presence of 50 mM creatine only when intact mitochondria are used. Using these different kinetic constants and coupling enzyme theory, one can correctly predict the steady state concentrations of ATP and ADP indicating that the measured K_m values reflect the solution kinetic parameters of the system. These results show that preferential coupling is dependent on the presence of the outer mitochondrial membrane in chicken heart mitochondria.

To my wife Janet
for her patience
and her love.

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ABBREVIATIONS

Ap_5A: P',P_5-Di(adenosine-5')pentaphosphate

BB-CK: brain type creatine kinase

BICINE: N,N-bis(2-hydroxyethyl)glycine

BSA: bovine serum albumin

CK: creatine kinase

DTT: dithiothreitol

EGTA: ethyleneglycol-bis-(2-aminoethyl ether) N,N,N',N'-tetraacetic acid

G6PdH: glucose 6-phosphate dehydrogenase

Hepes: N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid

IMM: inner mitochondrial membrane

IU: 1 µmole of substrate converted per minute

MES: 2-(N-morpholino)ethanesulfonic acid

MiMi-CK: mitochondrial creatine kinase

MM-CK: muscle type creatine kinase

MOPS: 3-(N-morpholino)propanesulfonic acid

NaDOC: sodium deoxycholate

OMM: outer mitochondrial membrane

PGM: phosphoglucomutase

PMSF: phenylmethylsulfonyl fluoride

TPCK: N-tosyl-phenyalanine chloromethyl ketone

INTRODUCTION

The localization of the mitochondrial isozyme of creatine kinase (MiMi-CK) in between the inner and outer mitochondrial membranes reprotedly provides the enzyme with preferential access to the ATP generated by oxidative phosphorylation. This preferential access is demonstrated by lower MiMi-CK kinetic constants for $MgATP^{-2}$ when the enzyme is coupled to oxidative phosphorylation as compared with the kinetic constants of the soluble enzyme by direct assay of the products. Preferential access is also demonstrated by trapping experiments where ATP generated by oxidative phosphorylation is utilized preferentially over the solution ATP.

Two prevailing hypotheses can account for the observed preferential access. A. Binding of MiMi-CK to the nucleotide translocase aligns the active sites of the two enzymes such that MiMi-CK sees a locally high ATP concentration. B. The outer mitochondrial membrane limits the diffusion of the nucleotides so that the localization of MiMi-CK in the inter membrane space exposes the enzyme to a higher ATP concentration than is found in the cytosol.

In order to distinguish between these two hypotheses, chicken heart mitochondria which contain a low amount of MiMi-CK were examined for the relative contribution of the binding of MiMi-CK to the inner mitochondrial membrane (IMM), and the outer mitochondrial membrane to functional coupling. Chicken heart mitochondria are used so that the rate limiting step in the coupled MiMi-CK:nucleotide translocase reaction is MiMi-CK. In order to measure the kinetic constants for bound MiMi-CK the conditions under which MiMi-CK is bound to the IMM had to be defined. Examining the binding process required the determination of the dissociation constant for the enzyme, a value which required the titration of IMM with homogeneous MiMi-CK.

This dissertation presents the results of an examination of the preferential coupling of MiMi-CK to oxidative phosphorylation in chicken heart mitochondria. The dissertation is organized into seven separate chapters. The first chapter is a review of the pertinent literature. Chapters II to VI are written as separate papers, each with their own Introduction, Materials and Methods, Results, Discussioo and References sections. The last chapter is a general discussion and summary.

Chapters II and III deal with coupled enzyme theory and are presented to give a background to the results presented in Chapter VI. Chapters IV and V contain the results of the purification and binding studies of MiMi-CK. Chapter VI presents the results of the experiments on the preferential coupling of MiMi-CK to oxidative phosphorylation.

Chapter I

Literature Review

Eppenberger et al. (1, 2) first discovered the existence of two cytosolic creatine kinase (EC 2.7.3.2) isozymic variants. These isozymes, M (muscle type) and B (brain type) are located in various tissues in different species. The M isozyme is found in mature mammalian and avian skeletal muscle and mammalian myocardium, the B isozyme in mammalian brain, neural tissue, and embryonic skeletal muscle and avian myocardium (1 - 3). A third isozymic form, Mi (mitochondrial type), is found in large amounts in human, beef, and rat heart, as well as rat brain, skeletal muscle, and intestinal muscle mitochondria (4, 5). The largest amount of mitochondrial creatine kinase per mitochondrion is found in chicken breast muscle, a pure white fiber muscle (6). Initially the Mi isozyme was not found in chicken heart tissue (7) but subsequent studies have confirmed that the isozyme is present at about 1/20 of the chicken breast muscle concentration (6).

The assignment of creatine kinase as B or M type is based on the initial localization of these enzymes in brain and skeletal muscle of rabbit (8). The differentiation of these isozymes is based on their migration in starch gel electrophoresis (7, 9), cellulose acetate electrophoresis at pH 8.8 (6, 10) or isoelectric focusing (11). At pH 8.8, the B type migrates quickly to the positive terminal, the M type is neutral or slightly positive, and the Mi type moves toward the negative terminal. This general pattern is seen for all species although the

extent of migration may differ.

Studies from Kaplan's laboratory (1, 2, 9) demonstrated that intact creatine kinase is a dimer. They showed this by renaturing mixtures of M and B type enzymes from various tissue sources and animal species. The resulting starch gel electrophoretic patterns showed three protein bands which are assigned as the original dimeric MM and BB enzymes and a mixed type, MB, with a pI value exactly in between the MM and BB isozymes. Analyzing several of these creatine kinase mixtures shows that the subunits mix in equal (1:1) stoichiometries. Determining the molecular weight of the intact enzyme by exclusion chromatography, and of the subunits by SDS-polyacrylamide electrophoresis (12 - 16), confirms the dimeric structure of creatine kinase. Because of the above results, muscle type creatine kinase is abbreviated as MM-CK, brain type BB-CK and mitochondrial type MiMi-CK. These abbreviations will be used throughout the rest of this dissertation. Interestingly, a unique isozyme of creatine kinase which is a monomer with a Mr of 147,000 is located in the tail of sea urchin sperm (17).

Although the intact enzyme is a dimer, isolated subunits apparently have a specific activity equal to that of the intact enzyme (18) suggesting that a dimeric structure is not necessary for catalytic activity (19 - 21). In the dimeric enzyme the subunits show negative cooperativity with respect to $MgADP^-$ binding (22) indicating that conformational changes in one subunit are transmitted to the other.

Individual cytosolic subunits readily join with one another both *in vivo* (7) and *in vitro* (1, 2) to form the MB isozyme but the mitochondrial subunit does not hybridize with either of the two cytosolic forms (13, 14, 23 - 25). Amino acid sequence data and studies of

antibody cross-reactivity show that the cytosolic subunits are similar in their N and C terminals (26 - 28), and that antibodies directed against one cytosolic subunit react with the other (14, 29). The mitochondrial isozyme, on the other hand, is clearly different: antibodies against the cytosolic isozymes do not react with the mitochondrial isozyme (10, 13, 14, 29) and N-terminal sequence analysis shows a large difference between cytosolic and mitochondrial sequences (27, 28). The molecular weight of the M subunits appears to be identical at Mr = 43,000 for the human, canine, rabbit mouse and bovine enzymes but the B subunit molecular weights are different: human, 44,500, canine, 46,000, rabbit, 44,000, and mouse, 49,000 (29). The molecular weight values for dimeric MiMi-CK are reported as follows: beef heart, 65,000 (15), human heart, 82,000 (14) and 84,000 (25), and dog heart, 84,000 (23).

Kinetic Studies of Creatine Kinase

Creatine kinase catalyzes the reversible transfer of the phosphoryl group from ATP to creatine as shown in equation I-1 (30, 31).

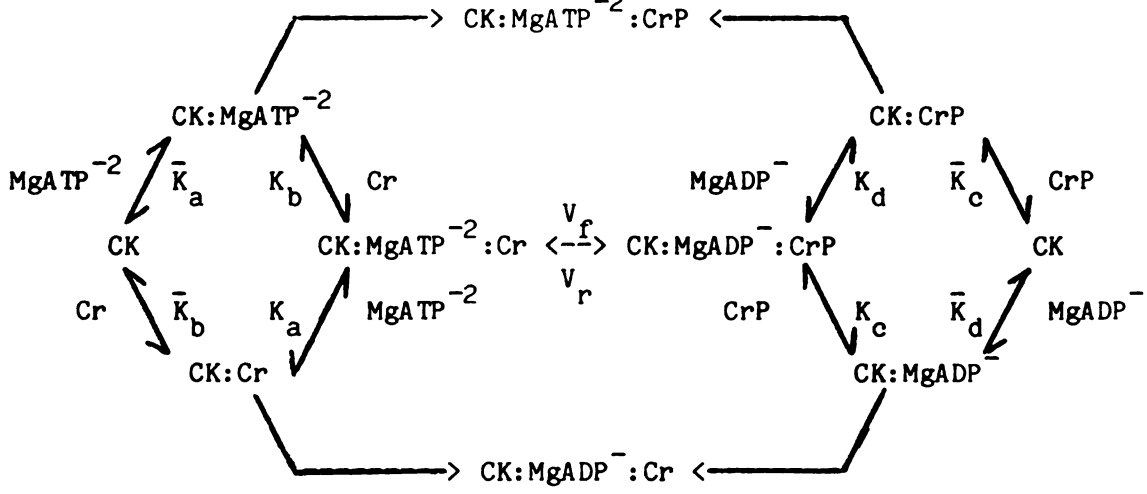
[I-1] $MgATP^{-2}$ + creatine $\longleftrightarrow$ $MgADP^{-}$ + creatine $phosphate^{-2}$ + H^{+}

The kinetic properties of the enzyme were established using purified skeletal muscle (M isozyme, 1, 32) and brain (B isozyme, 2) preparations. The pH optimum for the forward reaction (creatine phosphate synthesis) is approximately 8 - 9 and for the reverse direction (creatine synthesis) is about 7 (33, 34). The effect of divalent cations on the reaction kinetics showed that $MgATP^{-2}$ and $MgADP^{-}$ are the substrates for creatine kinase (33, 35, 36) but other divalent cations such as Mn^{+2} (the rate is

about 75% of the rate in the presence of Mg^{+2}) and Ca^{+2} (the rate is about 25% of the rate in the presence of Mg^{+2}) can effectively substitute for Mg^{+2} in both the forward (37) and reverse (38) directions. Other nucleoside diphosphates are capable of binding the enzyme but all (except deoxy ADP) have a five to ten fold lower affinity and V_{max} value (39).

The kinetic mechanism was determined by using classical kinetic studies. Product inhibition data indicate that for the forward reaction $MgADP^-$ is competitive with $MgATP^{-2}$ and non-competitive with creatine, and creatine phosphate is competitive with creatine and non-competitive with $MgATP^{-2}$ (33, 38). A similar pattern, seen for the reverse reaction, combined with the postulated existence of the two dead end complexes shown in equation I-2 (38), and substrate binding data (8, 36) indicate that the reaction obeys a rapid equilibrium random mechanism. Later studies have confirmed this for the mitochondrial isozyme as well (40).

[I-2]



In equation I-2 Cr and CrP represent creatine and creatine phosphate,

respectively. The constants $\bar{K}_a$, $\bar{K}_b$, $\bar{K}_c$, and $\bar{K}_d$ are the dissociation constants for $MgATP^{-2}$, Cr, CrP and $MgADP^-$ binding to the enzyme and the constants K_a, K_b, K_c and K_d represent the dissociation constants in the presence of the other substrate.

Although several of the initial kinetic studies were carried out in chloride containing buffers, later observations of the effects of anions such as Cl^-, HPO_4^{-2}, and SO_4^{-2} demonstrated that these ions are competitive inhibitors of creatine phosphate and non-competitive inhibitors of $MgADP^-$ (35, 36). A further examination of this phenomenon showed that the chloride ion sits in between the substrates in the CK:$MgADP^-$:Cr dead end complex to form a very stable quaternary complex (37). Planar anions such as NO_3^- and NO_2^- are the most effective in stabilizing the complex: the K_D value for $MgADP^-$ binding to the dead end complex is seven fold lower in the presence of Cl^- and thirty fold lower in the presence of NO_3^-. The above results led Milner-White and Watts (37) to propose that planar anions mimic a planar phosphoryl group and thus $MgADP^-$:anion:Cr forms a transition state analog. This explanation accounts for the large decrease in the K_D values for $MgADP^-$ in the presence of Cl^- and NO_3^-.

Studies of the active site show the presence of a cysteine sulfur group which must be reduced for maximal enzyme activity (38, 41) but which apparently does not participate directly in the reaction sequence (42). A histidyl residue (43, 44) and a lysyl residue (45) which are apparently involved in the reaction sequence have been reported. The reaction occurs by a direct in line transfer of the phosphoryl group from one substrate to the other in the active site (46, 47); a phosphorylated enzyme intermediate could not be detected (33, 34).

Cellular localization of creatine kinase

Many "soluble" enzymes bind reversibly to cellular structures (48). These enzymes, termed ambiquitous to describe their dual bound/free status (49), frequently exhibit altered kinetics when in the bound versus free state (48). Both M type and Mi type creatine kinase isozymes are ambiquitous. Muscle type creatine kinase apparently binds to the M-line of skeletal muscle tissue as demonstrated by the localization of antibodies directed against MM-CK by immunofluorescence (50) and by the release of creatine kinase activity from muscle fibers after treatment with antibodies directed against the M line proteins (51). In vitro studies of MM-CK binding to myosin and to myosin subfragments generated by protease action, confirm that MM-CK binds to the tail portion of myosin (52, 53) but the number of MM-CK binding sites was not measured. Washing myofibrils with low ionic strength buffer shows that 5% to 10% of the total cytoplasmic creatine kinase activity is bound to the M-line of myofibrils in chicken pectoralis (54, 55). Brain type creatine kinase apparently does not bind to chicken heart myofibrils (54, 55).

Examining the effect of increasing salt concentrations on heart mitochondria and outer membrane stripped mitochondira (mitoplasts) led Scholte et al. (56) and Jacobus and Lehninger (5) to localize MiMi-CK on the outside of the inner mitochondrial membrane (IMM). These results were later confirmed by Iyengar and Iyengar (57) using sonicated mitochondria and detergents to localize the enzyme. Despite these findings, studies of the binding and release of MiMi-CK are still performed with intact mitochondria (6, 58 - 61). They show that MiMi-CK

is released from the mitochondria by increasing ionic strength. Presumably, the release of the enzyme from mitochondria is due to a ruptured outer mitochondrial membrane which permits the passage of MiMi-CK through large holes and consequently the data on the release of MiMi-CK from the IMM are not easily interpretable. Furthermore, the above studies (58 - 61) fail to provide information regarding optimal binding conditions, the dissociation constant for MiMi-CK, or the number of MiMi-CK binding sites on the IMM. A study by Hall and DeLuca (62) examined the binding of semi-purified MiMi-CK to phosphate extracted mitochondria. Although they observed saturable binding, they failed to quantitate the data, nor did they demonstrate that the enzyme associated exclusively with the IMM. Interestingly, sulfhydryl reagents such as *para*-chloromercuribenzoate release the enzyme from mitochondria (59, 60) but whether sulfhydryl residues participate directly in the binding process has not been demonstrated (61).

Recent attempts to define the binding site for MiMi-CK on the IMM led Muller *et al.* (64) to conclude that MiMi-CK binds to cardiolipin in the IMM. Their studies showed that MiMi-CK bound to cardiolipin vesicles but not to phosphatidylcholine vesicles. They also demonstrated that MiMi-CK is released from mitoplasts by adriamycin (63, 64), a cardiolipin-binding drug. Their data was later supported by Schlame and Augustin (65) who treated mitochondria with phospholipase A_2 and phospholipase C and compared the amount of MiMi-CK which was solubilized. Since only phospholipase A_2 released MiMi-CK, they concluded that cardiolipin was the MiMi-CK binding site. The above results are strengthened by the observation that MiMi-CK binds liver mitoplasts (which contain no MiMi-CK). This latter observation suggests that the

binding factor for MiMi-CK is present in mitochondria which do not have endogeneous MiMi-CK (62, 64).

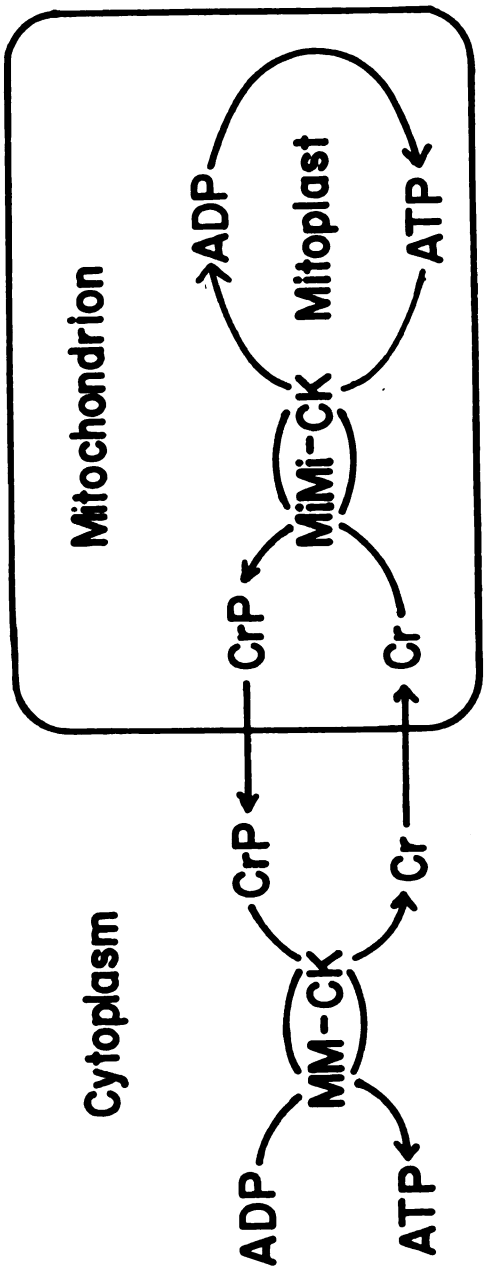
However, not all the data indicates that the MiMi-CK receptor in the IMM is cardiolipin. Experiments by Vial et al. (58) show that only 6% of the total MiMi-CK binds to sonicated mitoplasts (inverted vesicles) under conditions where 51% of the total MiMi-CK binds to mitoplasts. If MiMi-CK binds only to cardiolipin on the IMM, then the inside of the IMM should bind at least the same amount of MiMi-CK as the cytosolic side of the IMM; the matrix side has three times more cardiolipin than the cytosolic side (67). Furthermore, Kuznetsov and Saks (66), who report a MiMi-CK:translocase ratio of around 1:1, suggest that MiMi-CK binds directly to the translocase, and not to cardiolipin (67).

The Creatine Phosphate Shuttle

The importance of creatine, and the creatine kinase reaction (equation I-1) to muscle bioenergetics is demonstrated by the high concentration of creatine phosphate found in muscle tissue (68, 69) and the direct correlation between creatine phosphate breakdown and muscle contraction (30, 70). Although initial investigators believed that creatine phosphate was the direct energy source for muscle contraction, the discovery of ATP (71, 72), and the observation that ATP stimulated actomyosin contraction (73 - 75) showed that adenine nucleotides were involved in the contractile process. These results, coupled with the measurement of ATP hydrolysis in contracting muscle tissue poisoned with 2,4-dinitrofluorobenzene (which inhibits creatine kinase activity) demonstrated that ATP directly supplies the energy for contraction (76).

In the present model of muscle contraction, ATP hydrolysis

Figure I-1: Schematic representation of the creatine phosphate shuttle.


Mitochondrion
ADP
Mitoplast
ATP
MiMi-CK
CrP
Cr
CrP
Cr
Cytoplasm
MM-CK
ADP
ATP

indirectly provides the source of energy for contraction; the largest free-energy change results from inorganic phosphate release from the S-1 protein (actin dependent myosin ATPase) after ATP hydrolysis (77, 78). Regeneration of ATP at the myosin ATPase can be accomplished by adding external ATP (73 - 75) or creatine phosphate (79) as shown by the initial experiments on muscle contraction, 69 - 80).

The first evidence for the creatine phosphate shuttle came from experiments which show that ATP and creatine phosphate are compartmentalized and not in equilibrium as suggested by the above experiments. Studies by Gudbjarnson et al. (82) and Seraydarian et al. (83, 84) demonstrate that the ability of ischemic heart muscle or cultured heart muscle cells to contract correlates with the concentration of creatine phosphate and not ATP. Gudbjarnson et al. (82) assayed creatine phosphate and ATP concentrations at different time points after the onset of ischemia. At the one minute time point, the heart stopped beating even though over 80% of the original ATP concentration remained. Interestingly, at one minute, over 70% of the creatine phosphate had been hydrolyzed. This indicated to the authors that a "functional compartmentation of ATP and creatine phosphate" existed in dog heart muscle since it appears that not all of the ATP has access to the myosin ATPase. These conclusions are supported by Seraydarian (85) using frog sartorious muscle. She showed that fatigued muscle (which does not respond to electrical stimulus) still has more than 80% of its original ATP but less than 30% of its creatine phosphate. The proposal for compartmentation of creatine phosphate was strengthened by pulse-chase experiments with [1-^{14}C] creatine which suggested that two separate pools of creatine phosphate exist in heart tissue (see 86).

The above data led Bessman to propose the existence of a creatine phosphate shuttle in muscle tissue (86, 87). The central idea of this shuttle mechanism is that creatine phosphate serves as a high energy phosphate carrier which shuttles between the site of ATP synthesis (mitochondria) and ATP breakdown (myosin ATPase). Thus the mitochondrial creatine kinase and muscle creatine kinase, in mammalian muscle, serve different functions within the cell (Figure I-1). When muscle contracts, MM-CK rephosphorylates the resulting ADP using creatine phosphate as a substrate. The creatine produced by MM-CK diffuses to the mitochondria where MiMi-CK uses ATP to rephosphorylate creatine. The MiMi-CK isozyme also provides ADP to the respiratory system stimulating oxidative phosphorylation. Support for the creatine phosphate shuttle comes from the localization of M type and Mi type creatine kinase near the energy consuming and energy producing centers in the cell, and from kinetic studies of the interaction between the creatine kinase reaction and muscle contraction or oxidative phosphorylation.

MiMi-CK and Oxidative Phosphorylation

The effect of the localization of MiMi-CK in the inter membrane space of mitochondria was studied initially by Jacobus and Lehninger (5). They demonstrated that MiMi-CK could accept the ATP released from oxidative phosphorylation and provide the nucleotide translocase with ADP so that creatine increases the post ADP-stimulated respiration rate (state 4). Although they observed that creatine also increases the ADP-stimulated respiration rate (state 3) at lower ADP concentrations, thus showing that MiMi-CK generates a locally high ADP concentration near the nucleotide translocase, they concluded simply that the role of MiMi-CK in

the heart tissue is to maintain a high steady state ADP concentration near the translocase.

A direct demonstration of the kinetic effect of MiMi-CK localization in the inter membrane space is provided by Saks et al. (88) who showed that creatine phosphate synthesis is greater in the presence of oxidative phosphorylation that in its absence (when oligomycin A is added) even though the total ATP concentration is equal in both cases. This result suggests that a locally high ATP concentration exists near the MiMi-CK active site, as was shown for the nucleotide translocase (5). Measuring the MiMi-CK K_m for $MgATP^{-2}$ (in the presence of a finite creatine concentration) confirms the above conclusion: the K_m value is 37 μM in the presence of oxidative phosphorylation and 200 μM in its absence even when the external ATP is regenerated by adding phospho(enol)pyruvate and pyruvate kinase to the suspension (89 - 91). This latter K_m value is similar to the K_m value of 145 μM determined for semi-purified MiMi-CK (89). These results suggested to Jacobus and Saks that MiMi-CK prefers the ATP generated by oxidative phosphorylation over that added externally to the suspension (90).

In order to test the kinetic results obtained in Saks' and Jacobus' laboratories, Erickson-Viitanen et al. (92, 93) measured the contribution of mitochondrial versus cytosolic ATP to the formation of creatine phosphate. Mitochondrial ATP was labelled by starting the reactions with $H_3{}^{32}PO_4$ so that the creatine phosphate formed from oxidative phosphorylation would be radioactive. The specific activity of creatine phosphate generated by MiMi-CK in the presence of increasing concentrations of unlabelled ATP, compared with the specific activity of the solution ATP, shows that at external ATP concentrations of greater

that 0.1 mM, less than 10% of the creatine phosphate produced comes from ATP generated by oxidative phosphorylation. However, below this value, the percentage of creatine phosphate derived from radioactive ATP (mitochondrial in origin) rises sharply to about 50% (92).

The above results were challenged by Altschuld and Brierley (94) and Borrebaek (95) who failed to observe differences in the K_m values measured with oxidative phosphorylation or with a pyruvate kinase-phospho(enol)pyruvate regenerating system. However the data of Erickson-Viitanen et al. (92) can explain this apparent discrepancy. Both Altschuld and Brierley (94) and Borrebaek (95) used high external concentrations of ATP to start their reactions and had low rates of ATP synthesis. Under these conditions Erickson-Viitanen et al. (92) observed a very low specific activity of creatine phosphate indicating that preferential access of MiMi-CK to the ATP generated by oxidative phosphorylation cannot be observed using the system of Altschuld and Brierley (66) or Borrebaek (95).

The apparent increased activity of MiMi-CK in the space between the inner and outer mitochondrial membranes, shown by lower K_m values for ATP (89 - 91), and increased rates of creatine phosphate production (89, 90), also results in an increased ADP concentration in the inner membrane space. This is shown by Moreadith and Jacobus (96) who measured the effect of increasing atractyloside concentrations on the rate of respiration in the presence and absence of 20 mM creatine. Unfortunately their results are difficult to interpret because the concentration which inhibited respiration by 50% is the same when either 20 mM creatine or hexokinase (coupled to agarose beads) and glucose are added (based on the percentage of oxygen consumption in the absence of added atractyloside).

However, a direct measurement of the nucleotide translocase K_m for ADP shows that the K_m value for ADP decreases from 13.2 μM to 2.9 μM in the presence of 50 mM creatine (97). This result is confirmed by Barbour et al. (98) who also demonstrated that the exchange of matrix ADP with external ATP is slower when creatine is present, suggesting that active MiMi-CK creates a localized pool of ADP which does not equilibrate with the bulk solution as quickly as MiMi-CK turns over.

The question of whether the outer membrane (87, 92, 93) or direct binding of MiMi-CK to the nucleotide translocase (88 - 90, 96, 99, 100, 101) is responsible for the decrease in the K_m value for $MgATP^{-2}$ is still unresolved. Bessman's group (87, 92, 93) argue that the presence of the outer membrane is necessary to create an unstirred layer which is responsible for maintaining a locally high ATP concentration in the inter membrane space. The contribution of the outer membrane to the coupling between the translocase and MiMi-CK is demonstrated by a higher apparent K_m value for $MgATP^{-2}$ when the outer mitochondrial membrane is present (in the presence of oligomycin A) and a failure to observe preferential access of MiMi-CK for the ATP generated by oxidative phosphorylation in the absence of the outer mitochondrial membrane (93). These results suggested to Erickson-Viitanen et al. (93) that the outer membrane is responsible for the preferred access of MiMi-CK for ATP derived from oxidative phosphorylation.

The preferential access of MiMi-CK for ATP generated by oxidative phosphorylation may be the result of a diffusion barrier for $MgATP^{-2}$ which prevents its mixing with the solution $MgATP^{-2}$ (102 - 104). This barrier may result from either the creation of an unstirred layer around the IMM as is seen for whole cells (105), or the unequal ion distribution

which surrounds poly ionic surfaces (106). The outer mitochondrial membrane may act to stabilize these layers or may be a diffusional barrier to the movement of certain ions (107 - 110).

Contrary to Erickson-Viitanen et al. (92, 93), Saks and Jacobus and cowerkers (40, 87 - 91, 96, 99, 100) maintain that preferential access results from the direct binding of MiMi-CK to the nucleotide translocase. Although the data do not support the direct transfer of ATP from the nucleotide translocase to MiMi-CK (111), they believe that the active sites of the two enzyme are close enough such that MiMi-CK can react with the ATP released by the nucleotide translocase before it diffuses into the surrounding solution. In this way, MiMi-CK will have an apparent lower K_m value for $MgATP^{-2}$ when preferentially coupled to oxidative phosphorylation. This substrate channelling has apparently been observed in large enzyme complexes which perform more than one reaction (48).

In an experiment designed to measure if the outer mitochondrial membrane could act as a diffusion barrier to ATP, Moreadith and Jacobus (96) measured the concentration of atractyloside required to inhibit liver mitochondrial respiration by 50% when respiration is initiated by UDP plus ATP or ADP. Since nucleoside diphosphokinase is present in the inner membrane space of liver mitochondria, if the outer mitochondrial membrane acts as a diffusion barrier then it should require more atractyloside to inhibit respiration when nucleoside diphosphokinase substrates (ATP and UDP) are used to initiate respiration as opposed to the situation when ADP alone is used. Their results apparently show that the amount of atractyloside required to inhibit 50% of respiration is identical for both cases, the respiration rates in the absence of

atractyloside are much lower when UDP plus ATP are used (96). This result, they conclude, shows that the direct binding of MiMi-CK to translocase, and not the outer mitochondrial membrane, is responsible for the observed lowering of the translocase K_m value for ADP. However, Bessman agrues that the result suggests that the outer membrane is responsible for coupling because the concentration of atractyloside which inhibits respiration by 50% is clearly different when the curves are plotted as a percentage of respiration in the absence of atractyloside (87).

The results of Hall and DeLuca (101) and Bennett et al. (6), who examined creatine phosphate production as a function of increasing phosphate concentrations using mitochondria, show that the production of creatine phosphate decreases with increasing phosphate, suggesting that free enzyme in the inner membrane space is not as effective as bound enzyme in accepting ATP from the translocase.

A more direct measure of the contribution of the outer mitochondrial membrane to preferential coupling is provided by experiments using mitoplasts. The K_m values for $MgATP^{-2}$ (at infinite creatine concentrations), measured in the presence and absence of oxidative phosphorylation, still show an apparent decrease when MiMi-CK is coupled to oxidative phosphorylation (100). However, Wenger et al. (61) point out that neither Saks et al. (100) nor Erickson-Viitanen et al. (93) indicate the percent of MiMi-CK bound to the inner mitochondrial membrane under the conditions of the experiment. Based on the concentration of inorganic phosphate required to release 50% of the MiMi-CK from mitoplasts (6) and the ionic strength of the assay media for both experiments, almost all of the enzyme should be free (58, 59, 61). The

significance of the coupling observed by Saks et al. (100) is thus in question (61).

MM-CK and Myosin ATPase

Measuring tension development in cardiac fiber bundles (112) and skeletal muscle fiber bundles (113) which have been treated to increase the membrane permeability to small molecules such as creatine phosphate shows that tension develops at a faster rate and at 100 fold lower ATP concentrations in the presence of creatine phosphate (114). This result suggested to Vesker and Kaplel'ko (112) that a locally high ATP concentration was generated around the myosin ATPase as a result of MM-CK action. In agreement with this hypothesis, Savabi et al. (113) report that the measured K_m value of MM-CK for ATP is lower when contraction is initiated by creatine phosphate versus ADP (113) showing that ATP concentrations are higher near the myosin ATPase than in bulk solution. The actin activated magnesium ATPase activity is also significantly higher when creatine phosphate is added to the medium (54). These results suggest that, similarly to the translocase:MiMi-CK case, binding of MM-CK to the M-line of muscle tissue results in a higher ATP concentration near the myosin ATPase. The converse, that MM-CK has a better access to ADP generated by the myosin ATPase, also appears to be true (115, 116).

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Chapter II

Theory and Practical Application of Coupled Enzyme Reactions: One and Two Auxiliary Enzymes

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Abstract

An extended and practical set of equations which describe coupled enzyme reactions is presented. The mathematical treatment relies on two assumptions: (a) the rate of the primary enzyme reaction is constant and (b) the reverse reactions are negligible. The treatment leads to the development of new equations which relate the time required for the concentration of a reaction intermediate to reach a defined fraction of its steady-state concentration to the kinetic parameters of the enzymes when mutarotation of one of the intermediates does not occur. The new equations reduce to those previously derived when the steady state concentration of the intermediate is small compared to its K_m value. A method for minimizing the cost of the two auxiliary enzyme system is also provided.

Introduction

Enzymologists commonly use two types of assay systems to monitor the course of an enzyme reaction: the end point assay and the continuous assay. For these assays to be correct, it is of paramount importance

that the steady state is achieved before the initial substrate concentration changes significantly. This is a trivial consideration when the product or substrate of the reaction can be monitored directly, but it becomes a major concern when auxiliary enzymes are used to detect the appearance of product.

The equations relating the velocity of an enzyme to the rate of appearance of a detectable product resulting from subsequent (auxiliary) enzyme reactions were first developed by McClure (1) for systems involving two auxiliary enzymes. These were later extended by others (2-7) to include multienzyme systems, and methods to minimize the cost of a two auxiliary enzyme system (3,4).

The expressions, derived by several investigators (1-4), relate the velocity of the initial enzyme and the kinetic parameters of the auxiliary enzymes to the time required (t_F, lag time) for the observed velocity to reach a defined fraction of the initial enzyme velocity. The derivations rely on the fulfillment of three assumptions: (a) the rate of product formation of the first enzyme is constant with respect to time ($dv_1/dt = 0$), (b) the steady state concentration(s) of the intermediate(s) is (are) much less that the apparent Michaelis constant for the subsequent auxiliary enzyme(s), and (c) only initial velocity conditions are considered (the reverse reactions are negligible). Whereas none of the above conditions are necessary for the development of a generalized theory for coupled enzyme reactions, the first and third conditions are easily achieved by selecting appropriate substrate concentrations. These expressions allow calculation of the amount of coupling enzyme(s) needed to shorten the lag time so that the steady-state condition may be achieved while initial velocity conditions still

apply ($d[ES]/dt = 0$).

Other investigators (6,7) recently developed expressions to describe the time course of coupled enzyme reactions when the intermediate concentration is greater than 0.01 K_m of the coupling enzyme. Easterby's (6) elegant analysis in terms of the transition time, τ, (see Figure II-1), although theoretically sound, is not practical as it does not define the time taken for the rate of the reaction to achieve a defined fraction of the steady state rate. Furthermore, the practical value, t_F (the lag time), is not related to τ by a simple mathematical expression: in order to obtain t_F, it is necessary to solve a system of differential equations. For the case involving two coupling enzymes, the differential equations can only be solved by numerical methods.

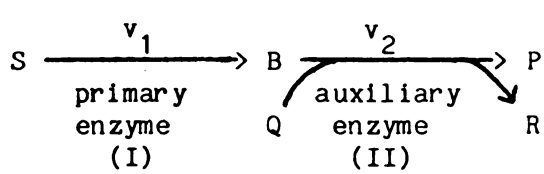
The purpose of this paper is two fold: (a) to provide a simple method for calculating the amount of coupling enzymes required for a valid assay when the concentration of the intermediate(s) is greater than 0.01 K_m and (b) to provide a method for minimizing the amount and cost of the coupling enzymes required for the two coupling enzyme system.

Theory

One Auxiliary Enzyme

The general form of a coupled enzyme reaction is as follows:

Scheme 1



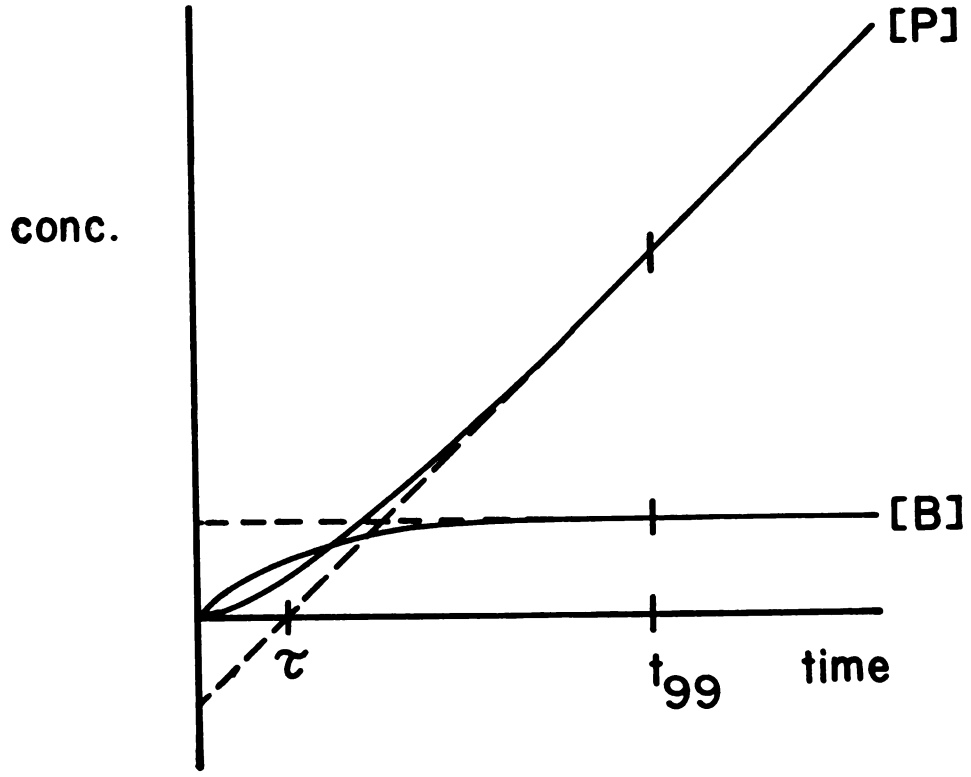
where v_1 is the rate of the enzyme under study and v_2 is the rate of the auxiliary enzyme. Since the auxiliary enzyme usually catalyses a two

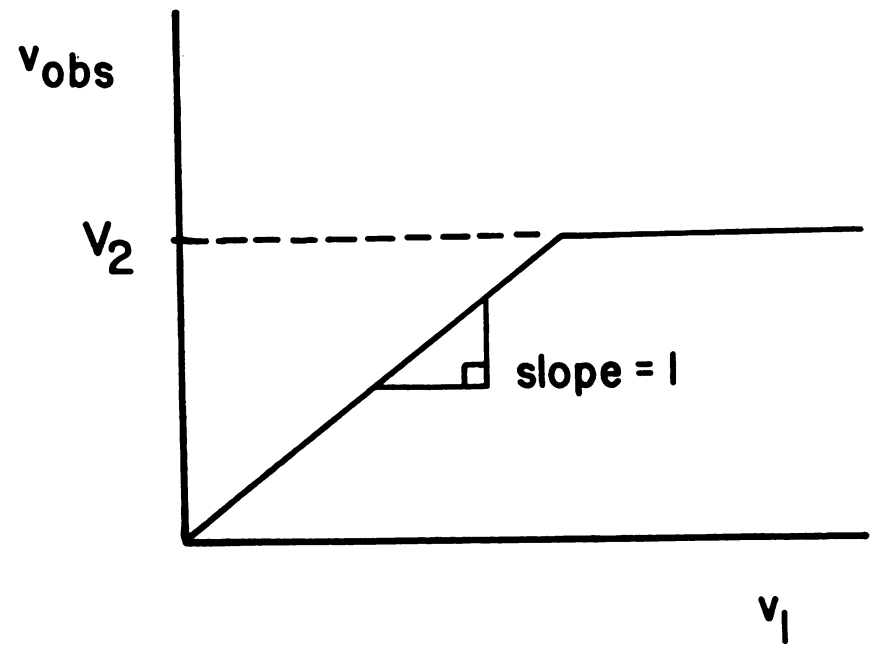
Figure II-1: Relationship between product concentration and time for a theoretical enzyme assay.

The solid line designated with P represents the time course for the appearance of product: the dashed line on the P curve is an extrapolation from the steady state rate for the appearance of product to the time axis. The intersection of the extrapolated dashed line with the time axis defines Easterby's τ value (6). The solid line designated B represents the time course for the appearance of the intermediate B: the dashed line from the B curve represents the concentration of B in the steady state. The time at which the concentration of the intermediate is 99% of its steady state value is t_{99}.

Figure II-2: Theoretical prediction from Equation II-7.

Note $v_1 = v_{obs}$ for all values of $v_1 < V_2$. The figure presupposes measurements at infinite time.


conc.
[P]
[B]
τ
t_{99}
time


v_{obs}
V_2
slope = 1
v_1

substrate reaction, it is represented above as B + Q ---> P + R where R and Q are generally the oxidized or reduced forms of the pyridine nucleotides, NADP(H) or NAD(H).

We will begin by assuming that the first reaction obeys the initial velocity condition in the derivation of the Briggs-Haldane steady-state equation:

$$[II\text{-}1] \qquad d[E_1S]/dt = 0 \quad \text{and} \quad v_1 = V_1[S]/(K_S + [S])$$

where $[E_1S]$ is the enzyme-substrate complex of enzyme I, v_1 is the velocity of enzyme I, V_1 is the V_{max} for enzyme I and K_S is the K_m for substrate S.

The rate of disappearance of B is given by:

$$[II\text{-}2] \qquad -d[B]/dt = V_2[B]/(K_B + [B])$$

where V_2 is the apparent maximal velocity for enzyme II at finite concentrations of the second substrate (Q) and K_B is the Michaelis constant for B. The relationship between V_2 and the velocity of enzyme II at infinite concentrations of B and Q ($V_{2,max}$) is given by Equation II-3:

$$[II\text{-}3] \qquad V_2 = V_{2,max}\,[Q]/(K_Q + [Q])$$

where K_Q is the apparent Michaelis constant for Q. V_2 is then expressed in concentration terms, units per assay or units per milliliter; its value may be changed either by adding more auxiliary enzyme to the assay

or by increasing the concentration of the substrate Q as shown by Equation II-3.

The observed steady state velocity of a coupled enzyme reaction is simply the velocity of enzyme II:

$$[II\text{-}4] \qquad v_{obs} = V_2 [B]_{ss}/(K_B + [B]_{ss})$$

When the steady state concentration of B is less than 0.01 K_B, Equation II-4 simplifies to the relationship derived by others (1-4).

$$[II\text{-}5] \qquad v_{obs} = V_2 [B]_{ss}/K_B$$

In order to calculate the elapsed time before the onset of steady state, we must first obtain an expression for formation of B in terms of time (t). The expression shown in Equation II-6 is the difference between the rate of formation of B (v_1) and the rate of its destruction ($V_2[B]/(K_B + [B])$)

$$[II\text{-}6] \qquad d[B]/dt = v_1 - V_2 [B]/(K_B + [B])$$

At steady state, when $d[B]/dt = 0$, Equations II-6 and II-4 predict:

$$[II\text{-}7] \qquad v_1 = v_{obs} = d[P]/dt = d[R]/dt$$

Equation II-7 implies that when the reaction enters steady state, the observed rate will be that of enzyme I. This is depicted graphically in Figure II-2. A plot of v_{obs} versus v_1, gives a straight line with a

slope of 1 until the rate of enzyme I is equal to the rate of enzyme II; at this point and at all values of v_1 in excess of V_2, the observed rate equals V_2. By holding v_1 and V_2 constant with respect to time, Equation II-6 gives Equation II-8.

$$[II\text{-}8] \quad \int \frac{(K_B + [B])\, d[B]}{v_1 K_B + [B](v_1 - V_2)} = \int dt$$

Integration of Equation II-8 assuming [B] = 0 when t = 0 gives the complete equation for [B] at any time t

$$[II\text{-}9] \quad (K_B v_1 - \Phi[B])\exp(\Phi[B]/K_B V_2) = K_B v_1 \exp(-t\Phi^2/K_B V_2)$$

Where $\Phi = V_2 - v_1$. When $v_1 << V_2$ and $[B] << K_B$, Equation II-9 gives Equation II-10, the equation obtained by others (1-4).

$$[II\text{-}10] \quad [B] = \frac{K_B v_1}{V_2}\,[1 - \exp(-tV_2/K_B)]$$

Following McClure (1) we define:

$$[II\text{-}11] \quad F_B = [B]/[B]_{ss}$$

where [B] is the concentration of the intermediate, B, at any time t and $[B]_{ss}$ is the concentration of B in the steady state given by:

$$[II\text{-}12] \quad [B]_{ss} = v_1 K_B/(V_2 - v_1) = v_1 K_B/\Phi$$

Combining Equations II-9, II-11 and II-12 we obtain

$$[II\text{-}13] \qquad t_{FB} = \frac{-K_B}{\Phi^2}[F_B v_1 + V_2 \ln(1-F_B)]$$

Where t_{FB} is the lag time before B reaches a defined fraction of its steady state concentration as given by Equation II-11. If $v_1 << V_2$, we obtain the previously derived equation (see reference 1)

$$[II\text{-}14] \qquad t_{FB} = \frac{-K_B}{V_2} \ln(1-F_B)$$

Figure II-3 shows the relationship between the lag time (t_{FB}, with $F_B = 0.99$) calculated from Equation II-13 and the ratio v_1/V_2. Note that as the ratio v_1/V_2 increases above 0.1, the time required for B to achieve 99% of its steady state concentration increases dramatically. If F_B is selected as 0.95, the actual lag times are shorter but follow the same trends as seen for $F_B = 0.99$. Thus v_1/V_2 should be less than 0.1 if the steady state rate is to be reached under conditions when the steady state assumptions are still valid. This is particularly important when kinetic data for enzyme I are examined at concentrations of substrate below the K_m value.

The t_{FB} value in Equation II-13 reflects the time required for the intermediate concentration to achieve a defined fraction (F_B) of its steady state concentration ($[B]_{ss}$). This is in contrast to the earlier equations developed by Storer and Cornish-Bowden (5) and Easterby (6) where the lag time (t_{FB}) reflects the time taken for the observed velocity to achieve a defined fraction of the steady state velocity. A comparison of Equation II-13 and the relationship given by Storer and Cornish-Bowden (5) shows that the calculated lag times differ by 3% at

most. However, in contrast to the expression developed by Storer and Cornish-Bowden, Equation II-13 allows the direct calculation of V_2 or v_1 when either v_1 or V_2, t_{FB} and K_B are known. Rearranging Equation II-13 in terms of V_2 we obtain:

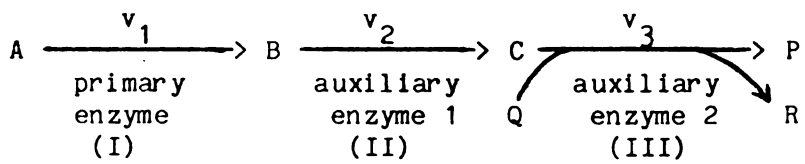
$$V_2^2 + V_2[\ln(1-F_B)K_B/t_{FB} - 2v_1] + v_1^2 + F_BK_Bv_1/t_{FB} = 0$$

Thus the amount of enzyme II (V_2 in units) required for a defined lag time (t_{FB}) is easily calculated.

Two Auxiliary Enzymes

The general form of a two auxiliary enzyme reaction can be written as follows:

Scheme 2



Following the treatment developed for the one auxiliary enzyme system, we obtain

[II-15] $$d[C]/dt = V_2[B]/(K_B + [B]) = V_3[C]/(K_C + [C])$$

where K_C is the K_m for C and V_3 is the apparent maximal velocity of auxiliary enzyme III at finite substrate concentrations, or

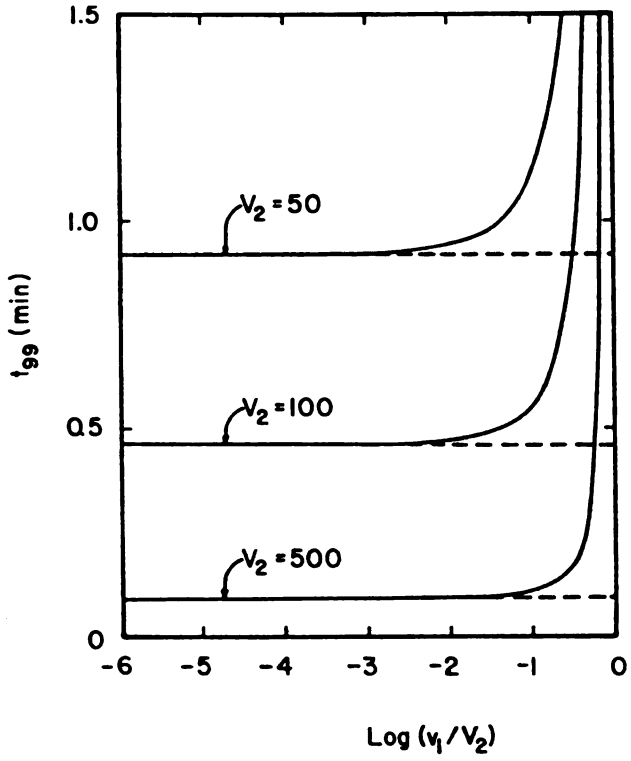
[II-16] $$V_3 = V_{3,max}\,[Q]/(K_Q + [Q])$$

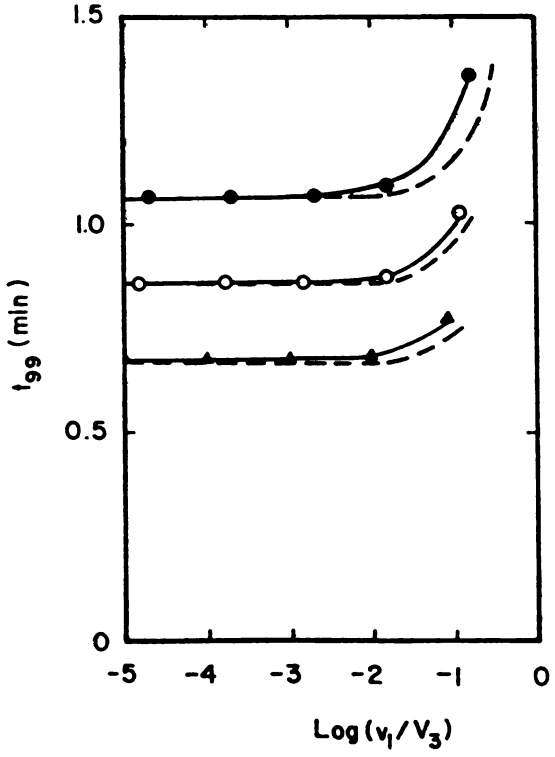
Figure II-3: Theoretical relationship between $\log(v_1/V_2)$ and the time required for B to reach 99% of its steady state value.

The t_{99} values were calculated from Equation II-13. Each curve represents a different value of V_2 as shown in the figure.

Figure II-4: Relationship between t_{99} and $\log(v_1/V_3)$.

The values of t_{99} were obtained with KINFIT 4 (8) using Equation II-6 and II-15. K_B = 10 μM, V_2 = 100 units, K_C = 10 μM. ●, V_3 = 50 units; o, V_3 = 66.6667 units; ▲, V_3 = 99 units. A reaction volume of 1 mL is assumed. The solid lines are calculated using Equation II-22. The dashed lines are from Equation II-21a.


1.5
1.0
0.5
0
t_{99} (min)
V_2 = 50
V_2 = 100
V_2 = 500
-6
-5
-4
-3
-2
-1
0
Log (v_1 / V_2)


1.5
1.0
0.5
0
t_{99} (min)
-5
-4
-3
-2
-1
0
Log (v_1 / V_3)

where $V_{3,max}$ is the velocity at infinite substrate concentration and K_Q is the Michaelis constant for Q. When $d[C]/dt = 0$ (i.e. $[B] = [B]_{ss}$ and $[C] = [C]_{ss}$) Equations II-6 and II-15 predict that

$$[II\text{-}17] \qquad v_{obs} = V_3[C]_{ss}/(K_C + [C]_{ss}) = v_1$$

and thus, in agreement with Easterby (8), we obtain the general relationship

$$[II\text{-}18] \qquad [I]_{jss} = v_1K_j/(V_j - v_1)$$

where $[I]_{jss}$ is the steady state concentration of the jth intermediate, and K_j and V_j are the K_m and V_{max} for the enzyme following the appearance of I_j.

Because Equation II-15 cannot be integrated by conventional methods, an exact solution for the intermediate [C], and consequently, an exact value of t_{FC} cannot be obtained as was done so conveniently for the case of one auxiliary enzyme. We, therefore, developed equations to approximate the concentration of C at any time t. To do this, equations to approximate the concentration of the intermediate for the one auxiliary enzyme case were first developed. These equations were obtained by rewriting Equation II-6 as two separate equations, each defining an upper and lower limit for the concentration of B over the time course of the experiment. Using the solutions to these equations (see Appendix), it was possible to obtain an approximate solution for the two auxiliary enzyme system described by Equation II-15. As with the one auxiliary enzyme system, we obtain two equations which define an upper

and lower limit for the concentration of the intermediate ([C]). The lower limit is defined by Equation II-19a and the upper limit by Equation II-19b:

[II-19a]

$$[C_-] = [C]_{ss}\left[\frac{\Psi}{V_3} + \frac{V_2K_B\Psi}{\Phi(K_CV_2 - K_BV_3)}\exp(-V_2t/K_B) - \frac{K_C\Phi}{\Phi K_C - \Psi K_B}\exp(-V_3t/K_C)\right]$$

[II-19b]

$$[C_+] = [C]_{ss}\left[1 + \frac{\Psi K_B}{\Phi K_C - \Psi K_B}\exp(-\Phi t/K_B) - \frac{\Phi K_C}{\Phi K_C - \Psi K_B}\exp(-\Psi t/K_C)\right]$$

where $\Psi = V_3 - v_1$. When $V_3 >> v_1$ and $V_2 >> v_1$, we can obtain, from Equations II-19a and II-19b, the equation for the pseudo-first order case originally derived by McClure (1):

[II-20]

$$[C]_{ss} = \frac{v_1}{k_3} - \frac{v_1}{(k_3 - k_2)}\left[\exp(-k_2t_{FC}) + \frac{k_2}{k_3}\exp(-k_3t_{FC})\right]$$

where $k_2 = V_2/K_B$ and $k_3 = V_3/K_C$. Table II-1 shows a comparison of the t_{FC+} and t_{FC-} values obtained from Equations II-19a and II-19b when $\Phi/K_B = 0.48\ \Psi/K_C$. The calculations reveal that the t_{FC-} value is the better estimate and we therefore provide the following equations which apply when the indicated conditions are valid. When $\Phi/K_B \geq 2\Psi/K_C$:

[II-21a]

$$t_{FC-} = -K_C\ln[(1-F_C)(1 - \Psi K_B/\Phi K_C)]/\Psi$$

When $2\Phi/K_B \leq \Psi/K_C$:

[II-21b] $$t_{FC-} = -K_B \ln[(1-F_C)(1 - \Phi K_C/\Psi K_B)]/\Phi$$

A more precise estimate of t_{FC} can be obtained by a numerical solution of Equation II-22, an empirical equation developed from McClure's equation 10 (1) (our Equation II-20). We reasoned that equations for the two enzyme case could be developed which have the same relationship to each other as Equation II-13 and II-14 have for the one enzyme case. Starting with Equation II-20, which was developed for the two enzyme system when V_2 and $V_3 >> v_1$, and substituting Φ/V_2 for V_2 and Ψ/V_3 for V_3 and multiplying the term $(1-F_C)$ by $\exp(F_C v_1/V_3)$, we obtain the following equation:

[II-22]

$$(1- \frac{\Phi^2 K_C V_3}{\Psi^2 K_B V_2})(1-F_C)\exp(F_C v_1/V_3) = \exp(-t_{FC}\Phi^2/V_2 K_B) - \frac{\Phi^2 K_C V_3}{\Psi^2 K_B V_2}\exp(-t_{FC}\Psi^2/V_3 K_C)$$

Arguments supporting the usefulness of Equation II-22 are: (a) the variable (in this case t_{FC}) shows a symmetrical dependence on V_2, K_B, V_3 and K_C (this is expected as lowering either V_2 or V_3 will affect the lag time) (b) the expression contains the term F_C only because we are looking for the time taken for the final intermediate in the reaction sequence to reach a defined fraction of its steady state concentration. At this time, F_B is very close to 1.0. Table II-1 shows a comparison of the calculated concentrations of C using Equations II-21 and II-22. Equation II-22 gives a better estimate but a computer solution is necessary (see Appendix). An analysis of Equation II-22 over a wide range of V_2, V_3, K_B and K_C values shows that the error in calculating [C] is usually less than 5% for any value of V_2, V_3, K_B and K_C for which

Table II-1 Comparison of t_{FC} Values Calculated by Various Methods for the Two Auxiliary Enzyme System.

F_C	t_{FC-}	%Error	t_{FC+}	%Error	t_{FC} (emp)	%Error	t_{FC} (exact)
	(min)		(min)		(min)		(min)
0.901172	1.64235	-3.4	1.88333	10.8	1.75903	3.5	1.7
0.915877	1.73185	-3.8	2.0625	14.6	1.86035	3.4	1.8
0.92841	1.82148	-4.1	2.29137	20.6	1.96045	3.2	1.9
0.939087	1.9112	-4.4	2.63252	31.6	2.06005	3.0	2.0
0.948179	2.00101	-4.7	3.5278	68.6	2.16064	2.9	2.1
0.955918	2.09087	-5.0	*	-	2.26000	2.8	2.2
0.962505	2.18077	-5.2	*	-	2.36000	2.6	2.3
0.968109	2.27071	-5.4	*	-	2.46100	2.5	2.4
0.972877	2.36068	-5.6	*	-	2.56100	2.4	2.5
0.976933	2.45066	-5.7	*	-	2.66110	2.4	2.6
0.980382	2.54066	-5.9	*	-	2.75900	2.2	2.7
0.983317	2.63066	-6.0	*	-	2.86130	2.2	2.8
0.985812	2.72068	-6.2	*	-	2.99590	2.0	2.9
0.987934	2.81069	-6.3	*	-	3.05660	1.9	3.0
0.989739	2.90071	-6.4	*	-	3.15430	1.8	3.1
0.991274	2.99072	-6.5	*	-	3.26270	1.9	3.2

Note: t_{FC-}, t_{FC+} and t_{FC} (emp) were calculated using Equations II-21, II-19a and II-22, respectively. t_{FC}(exact) was obtained from a Runge-Kutta numerical integration of Scheme 2. The F_C values were obtained from the t_{FC}(exact) solution using $F_C = [C]/[C]^{C}_{ss}$ and $[C]^{C}_{ss} = 0.263$ μM. Other values are as follows: $v_1 = 1$ μM/min, $V_2 = 10$ μM/min, $V_3 = 20$ μM/min, $K_B = 5$ μM, $K_C = 5$ μM. The asterisk (*) denotes the case when $F_C \geq \Psi/V_3$.

$\Phi^2/V_2K_B \neq \Psi^2/V_3K_C$. It should be noted, however, that the estimate of t_{FC} is always greater than the true value of t_{FC}, and improves as F_C increases.

Figure II-4 shows the t_{99} values generated by Equations II-6 and II-15 (symbols), Equation II-21a (dashed lines) and Equation II-22 (solid lines). The t_{99} values given by the symbols in Figure II-4 are exact values obtained by numerical integration of Equations II-6 and II-15. The estimate obtained from Equation II-22 is always better than the t_{FC}-analysis. When $\Psi/K_C = \Phi/K_B$, Equation II-19a or II-22 must be solved numerically for t_{FC}. When Equation II-19a is used, the following value of $[C_+]$ must be used:

[II-23] $$[C_+] = F_{C+}\,[C]_{ss}$$

The reader is cautioned against the use of an equation of the form

[II-24] $$t_{FC} = K_C \ln(1-F_C)/\Psi$$

which can be derived from Equations II-19a and II-19b (see Appendix) or 22 when $\Phi/K_B = \Psi/K_C$: the results obtained from Equation II-24 are then in error by at least 30% as compared to the true value obtained by numerical integration (8). Therefore, the curves corresponding to $V_2 = V_3 = 100$ and $K_C = K_B = 10$ in Figure II-4 are calculated by assuming $V_3 = 99$ and solving Equation II-19a or II-22 for t_{FC} by a method similar to that shown in the Appendix. It should also be noted that interchanging V_2 for V_3 and K_B for K_C results in the same t_{99} value. This is reflected by Equations II-19a and II-19b and Equation II-22. Thus Figure II-4 would

be identical, if we plotted t_{99} versus $\log(v_1/V_3)$ instead of versus $\log(v_1/V_2)$.

Minimum Values of V_2 and V_3:

For a reaction involving two auxiliary enzymes, the value of t_{FC} can be obtained from the above analysis only when both V_2 and V_3 are known. Furthermore, Equations II-19a and II-19b and II-22 show that a wide range of V_2 and V_3 values can be obtained for a single t_{FC} value. Therefore, in order to select a single value of V_2 and V_3, it is useful to derive a function which will minimize the concentration of each coupling enzyme required to give a selected t_{FC} value at the minimum cost. For a reaction involving two auxiliary enzymes, the total cost per assay is simply the sum of the concentration of the enzyme in units (V_2 or V_3) multiplied by the price per unit (P_2 or P_3).

[II-25] $$\text{Cost} = P_2V_2 + P_3V_3$$

Because the functions which describe the lag time are not explicit for V_2 or V_3, we use the transition time (τ) function (see Figure II-1). From Easterby (6) we know that for a system of coupling enzymes.

[II-26] $$\tau = \Sigma K_j/(V_j - v_1)$$

Thus for two enzymes:

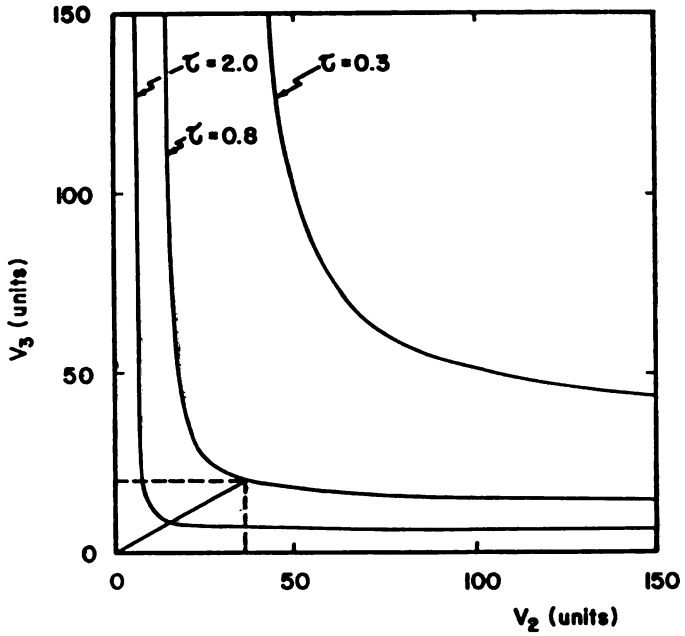
[II-27] $$\tau = K_B/\Phi + K_C/\Psi$$

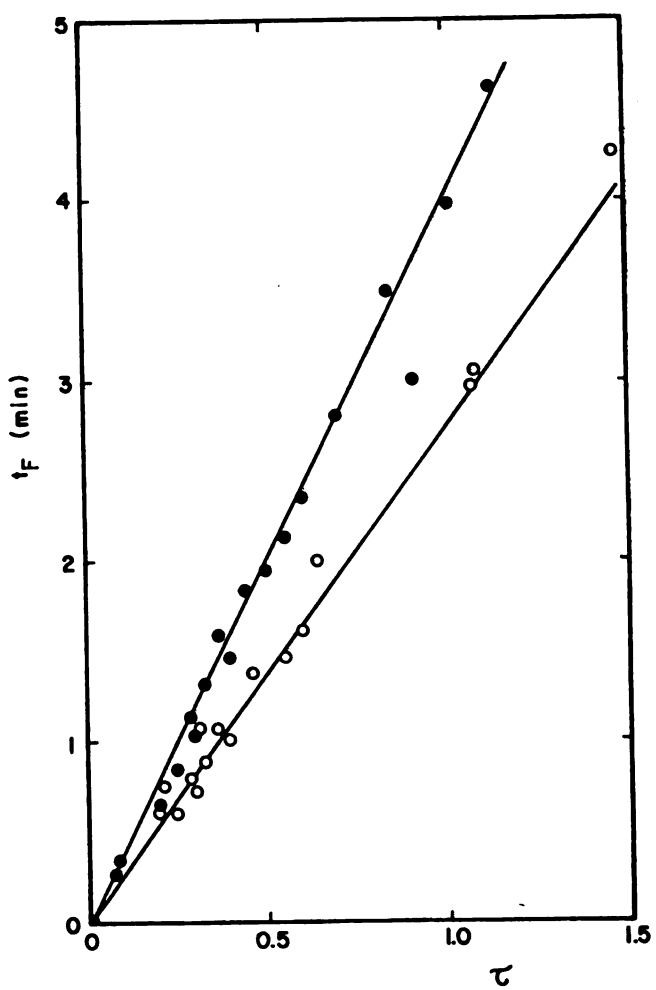
Figure II-5: Graphical representation of the cost minimization technique.

The three curves, generated by Equation II-28, represent the relationship between V_2 and V_3 for various values of τ. The slope of the straight solid line is defined by δ from Equation II-29 with $P_2/P_3 = 3$ and $\tau = 0.8$ min. The dotted lines are extrapolations of the point of intersection of the functions from Equation II-28b and Equation II-29 to the axis. A reaction volume of 1 mL is assumed.

Figure II-6: Relationship between t_{FC} and τ.

The value of t_{FC} was calculated using Equation II-22 and τ (min) was obtained from Equation II-27. The solid lines represent the line of best fit through the points. ●, relationship between t_{99} and τ; o, relationship between t_{90} and τ.


150
100
50
0
0
50
100
150
V_3 (units)
V_2 (units)
τ = 2.0
τ = 0.8
τ = 0.3


5
4
3
2
1
0
0
0.5
1.0
1.5
t_F (min)
τ

which gives after rearrangement

[II-28a] $$V_2 = v_1 + K_B\Psi/(\tau\Psi - K_C)$$

[II-28a] $$V_3 = v_1 + K_C\Phi/(\tau\Phi - K_B)$$

Substituting Equations II-28a and II-28b into Equation II-25, taking the derivative of cost with respect to V_2 and V_3 (holding τ constant), and setting $dCost/dV_2 = 0$ and $dCost/dV_3 = 0$, we obtain the following relationship:

[II-29] $$\delta = \frac{V_2}{V_3} = \frac{P_3P_2(\tau v_1 + K_B) + P_3(P_2P_3K_CK_B)^{1/2}}{P_2P_3(\tau v_1 + K_C) + P_2(P_2P_3K_CK_B)^{1/2}}$$

where δ is the slope of the line of minimum cost as depicted in Figure II-5. The minimum values of V_2 and V_3 required to give a specified τ value occur at the point of intersection between Equation II-28a or II-28b and II-29. This point is given by Equation II-30:

[II-30] $$V_2 = \frac{R_1 + (R_2^2 + \delta K_B K_C)^{1/2}}{2\tau}$$

where $$R_1 = \tau v_1 + K_B + \delta(K_C + \tau v_1)$$

$$R_2 = \tau v_1 + K_B - \delta(K_C + \tau v_1)$$

and $V_3 = V_2/\delta$. Figure II-5 shows a plot of V_2 versus V_3 derived from Equation II-28b. Note that the value of δ differs for each τ value as shown in Equation II-29.

Although the functions described above minimize the τ value (and consequently are useful for minimizing V_2 and V_3), the τ value has no

practical use. However, a plot given in Figure II-6 of t_{99} and t_{95} values (obtained with Equation II-22) versus τ (calculated from Equation II-27) demonstrates that an approximate linear relationship between the two parameters can be obtained. Thus τ can be calculated from the t_{FC} values by $t_{99} \approx 4.28\tau$ and $t_{90} \approx 2.79\tau$.

The relationships between t_{99}, t_{90} and τ are obtained from a least square analysis of the points shown in Figure II-6. It must be stated that the actual values of t_F/τ can vary considerably from the above relationships and that these numbers serve only as an approximate guide. Once appropriate values of V_2 and V_3 are obtained, one must return to Equations II-19a and II-19b or II-22 to obtain an exact lag time.

DISCUSSION

To set up a successful coupled enzyme reaction, one needs to know the amount of coupling enzyme to add to give as short a lag time as possible consistent with a reasonable cost. Most investigators simply add excess coupling enzyme to an assay and confirm that the observed rate is directly proportional to the amount of primary enzyme added. While this is satisfactory for a limited number of assays, it is not cost effective.

A short lag time is desirable for at least two reasons: (a) the shorter the lag time, the lower the steady state concentration of product intermediates and the smaller the affect, if product inhibits, and (b) the shorter the lag time, the less substrate is consumed before the steady state rate is observed. However, the shorter the lag time, the more coupling enzyme required. Therefore, it is important to be able to

calculate the amount of coupling enzyme to add for an assumed but defined reasonable lag time.

Previous theoretical treatments of coupled enzyme reactions relied on three assumptions: (a) the rate of the primary enzyme is constant, (b) the reverse reactions are negligible and (c) sufficient coupling enzyme is added so that the steady state concentration(s) of intermediate(s) is (are) small or $(K_B + [B]) = K_B$. The first assumption forms the basis of all initial velocity measurements; adding a coupling enzyme would not be expected to invalidate it. The second assumption is considered valid because coupling enzymes usually displace the equilibrium of the primary enzyme by removing substrate. Because the third assumption restricts the usefulness of available equations, the equations in this paper were developed with the assumption that $(K_B + [B]) \neq K_B$.

Previous investigators also assumed that the coupling enzymes obey Michaelis-Menten kinetics even though the concentration of intermediates in the coupling system increases from zero to some steady state value with time; Michaelis-Menten kinetics assumes $d[S]/dt = 0$. To test this assumption, Equation II-6 and the differential equations that describe the reactions in Scheme 3 were integrated numerically and found to give identical values for v_{obs} at $F_B > 0.9$ (unpublished observations). Thus Michaelis-Menten kinetics adequately describe coupled enzyme systems.

Scheme 3

$$S \xrightarrow{v_1} B \underset{k_2}{\overset{k_1 E}{\rightleftarrows}} EB \xrightarrow{k_3} E + \text{product}$$

The equations for calculating the units of coupling enzyme(s) necessary to produce a defined lag time, t_F, when v_1 is known and when $(K_B + [B]_{ss}) \neq K_B$, are presented for both the one coupling enzyme system (Equation II-13) and for the two coupling enzyme system (Equation II-19a and II-19b and II-22). The difficulties encountered when incorrect lag times are calculated, when it is assumed that $(K_B + [B]_{ss}) = K_B$, are discussed in more detail in the accompanying paper (9). A method for minimizing the total concentration of V_2 and V_3, and thus the cost of the assay in the two coupling enzyme system is also provided. To proceed, the investigator must first select a desired lag time, t_{FC}, and them estimate a transition time, τ, from the relationship shown in Figure II-6. The minimum concentrations of V_2 and V_3 are calculated using Equations II-29 and II-30 and the selected τ value. The correct lag time must then be recalculated using Equation II-19a or II-19b or equation II-22 and the new values of V_2 and V_3.

Acknowledgment

S.B. wishes to express his thanks to Dr. P. Nicholls for helpful discussions during the initial stages of this paper.

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Appendix

One Auxiliary Enzyme

The equations which approximate the concentration of B over the time course of the experiment were obtained by rewriting Equation II-6 as two separate equations

[II-A1a] $d[B_+]/dt = v_1 - k_+[B_+]$

[II-A1b] $d[B_-]/dt = v_1 - k_-[B_-]$

where $[B_+]$ and $[B_-]$ are the upper and lower estimates of the true concentration of [B]. $[B_+]$ and $[B_-]$ are defined by the appropriate choice of the constants k_+ and k_-. Because the constant k_+ must satisfy the condition $[B_+] \geq [B]$, Equation II-A1a dictates that k_+ must be equal to the smallest value for the expression $V_2/(K_B + [B])$ (see Equation II-6) for any time t or

[II-A2a] $$k_+ = V_2/(K_B + [B]_{ss}) = \Phi/K_B$$

Conversly k_- must be equal to the largest value of $V_2/(K_B + [B])$ or:

[II-A2b] $$k_- = V_2/K_B \quad \text{(McClure's (1) assumption)}$$

It is readily seen that with these assignments, the condition

$$k_+ \leq V_2/(K_B + [B]) \leq k_-$$

is satisfied and consequently

[II-A3] $$d[B_-]/dt \leq d[B]/dt \leq d[B_+]/dt$$

is always true. Integration of Equations II-A1a and II-A1b gives two equations of the general type:

[II-A4] $$[B] = v_1/k + Z_1 \exp(-kt)$$

where the value of Z_1 is obtained by selecting the initial (t=0) condition. This initial condition must satisfy the imposed criterion that $t_{FB+/-}$ is the best estimate of the true t_{FB} value. Selecting $[B_+]_{t=0} = 0$ we obtain $Z_{1+} = v_1 K_B/\Phi$, and Equation II-A1a becomes:

[II-A5a] $$[B_+] = \frac{v_1 K_B}{\Phi} (1 - \exp(-\Phi t/K_B))$$

If we choose $[B_-]_{t=0} = 0$, Equation II-A1b becomes McClures's

equation 2 (our Equation II-10). However a better estimate of t_{FB} can be obtained if we let $Z_{1-} = Z_{1+}$ (i.e. $[B_-]_{t=0} < 0$). This gives Equation II-A5.

$$[\text{II-A5b}] \qquad [B_-] = \frac{v_1 K_B}{V_2} - \frac{v_1 K_B}{\Phi} \exp(-V_2 t/K_B)$$

and it follows from Equation II-A5a and b that for any time t

$$\frac{v_1 K_B}{\Phi}(1-\exp(-\Phi t/K_B)) \geq [B] \geq \frac{v_1 K_B}{V_2} - \frac{v_1 K_B}{\Phi}\exp(-V_2 t/K_B)$$

Figure II-A1a shows the behavior of $[B]/K_B$ calculated by Equations II-9, II-A5a and II-A5b versus the reaction time t. In order to solve for t_{FB} given in Figure II-A1b as a function of F_B, we define:

$$F_B = [B]/[B]_{ss} = [B]\Phi/v_1 K_B$$

and solve Equation II-A5a and II-A5b for t_{FB}. This leads to Equation II-A6 where t_{FB-} is derived from Equation II-A5a and t_{FB+} from Equation II-A5b:

$$[\text{II-A6}] \qquad t_{FB-} = \frac{-K_B}{\Phi}\ln(1 - F_B) \leq t_{FB} \leq \frac{-K_B}{V_2}\ln(\frac{\Phi}{V_2} - F_B) = t_{FB+}$$

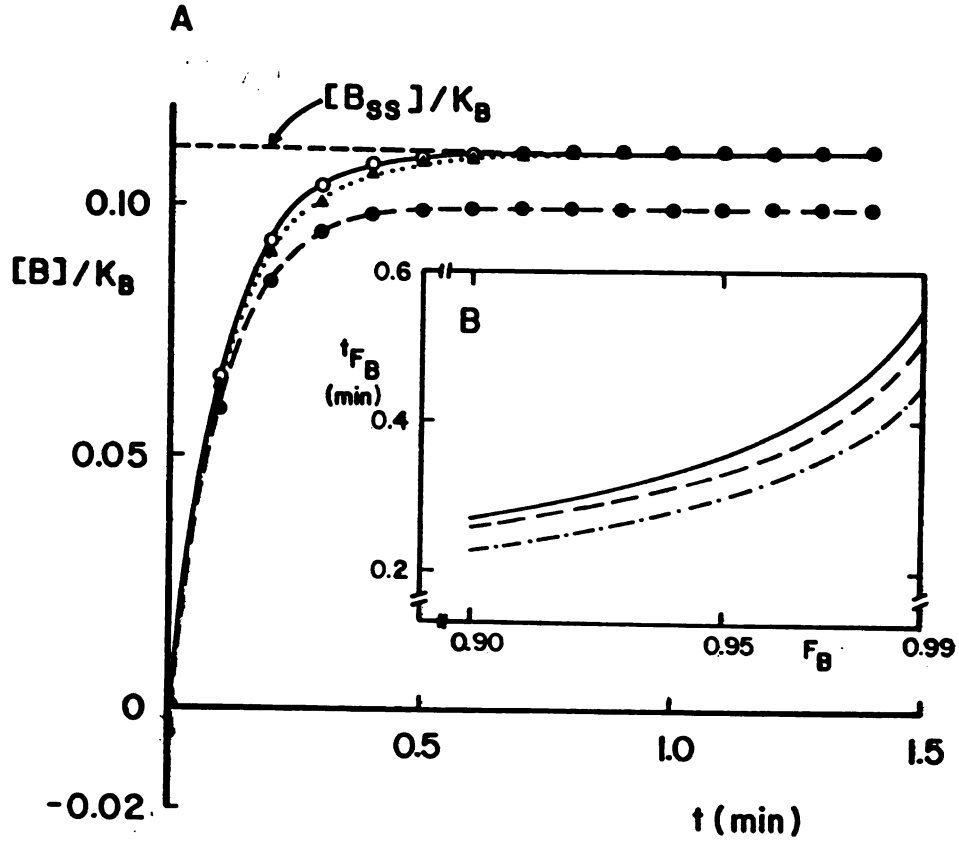
Figure II-A1b shows a comparison of t_{FB} values calculated by Equations II-13, II-14 and II-A6. The exact values of t_{FB} are calculated with Equation II-13. The values obtained from Equation II-14, which apply when $V_2 >> v_1$, are also shown for comparison. The best estimates of [B] and t_{FB} are derived from the B_+ analysis. The values of t_{FB-}

Figure II-A1: a.Graph of $[B]/K_B$ versus reaction time calculated by three methods.

●, $[B_-]/K_B$ (see Equation II-A5b); o, $[B_+]/K_B$ (see Equation II-A5a) and ▲, $[B]/K_B$ from Equation II-11. v_1 = 1 μM/min, V_2 = 10 μM/min.

b.Plot of t_{FB} versus F_B calculated from various equations.

Solid line (t_{FB} exact) from Equation II-13. The dashed line (t_{FB_-}) is from Equation II-A6. The dotted-dashed line is from Equation II-14 (McClure's estimation (1)). v_1 = 1 μM/min, V_2 = 10 μM/min.


A
[B_SS]/K_B
0.10
[B]/K_B
0.05
0
-0.02
0.5
1.0
1.5
t (min)
B
t_FB (min)
0.6
0.4
0.2
0.90
0.95
0.99
F_B

(dashed line, Figure II-A1b) are always slightly low due to the fact that the value of [B] calculated by the B_+ analysis is always greater that the true value of [B] and consequently the calculated value of [B] achieves the true steady state concentration faster. The values of t_{FB+} are always infinite since the concentration of B defined by Equation II-A5, and the parameters indicated in the figure legend, never achieve $[B]_{ss}$; $(\Phi/V_2 - F_B)$ is always less than or equal to zero for $F_B \geq 0.9$.

Two Auxiliary Enzymes

Having defined the values k_+ and k_- for the one enzyme case we can proceed to estimate the value of t_{FB} for the two enzyme case by rewriting Equation II-15 as:

[II-A7a] $$d[C_+]/dt = k_+[B_+] - r_+[C_+]$$

[II-A7b] $$d[C_-]/dt = k_-[B_-] - r_-[C_-]$$

with k_- and k_+ defined as above. Following the above rationale, we choose $r_+ = \Psi/K_C$ and $r_- = V_3/K_C$ where $\Psi = V_3 - v_1$. Integration of Equation II-A7 gives an equation of the general form:

[II-A8] $$[C] = \frac{v_1}{r} + \frac{kZ_1}{(k-r)}\exp(-tk) + Z_2\exp(-tr)$$

By selecting an appropriate initial or final condition such that the t_{FC} estimate is close to the true t_{FC} value, Equations II-19a and II-b are obtained (refer to the text).

Program minimum

A program written in the BASIC language useful for calculating the units of enzyme II and III required to give a valid assay at a minimum cost for a two enzyme coupled assay system given by scheme 2, and the lag time before the steady state is achieved (see Equation II-22).

```
100 PRINT "THIS IS PROGRAM MINIMUM. IT WILL CALCULATE THE MINIMUM AMOUNT"
200 PRINT "OF ENZYMES II AND III TO ADD IN ORDER TO MINIMIZE THE TOTAL
    COST"
300 PRINT "OF A COUPLED ENZYME ASSAY. IT CAN ALSO CALCULATE THE LAG TIME
    FOR"
400 PRINT "GIVEN VALUES OF V2 AND V3."
500 PRINT
600 REM*****************************************************************
700 REM
800 REM THE PROGRAM FIRST ASKS YOU TO SELECT OPTION A OR B
900 REM OPTION A CALCULATES THE MINIMUM AMOUNT OF ENZYMES II AND III TO
1000 REM ADD WHEN GIVEN AN INITIAL ESTIMATE OF THE LAG TIME. IT THEN
1100 REM RECALCUCLATES AN APPROXIMATE LAG TIME BASED ON EQUATION II-22
1200 REM
1300 REM*****************************************************************
1400 PRINT "WHAT DO YOU WANT TO DO ?"
1500 PRINT "----------------------"
1600 PRINT "(A) CALCULATE THE MINIMUM COST AND LAG TIME."
1700 PRINT
1800 PRINT "(B) CALCULATE THE LAG TIME ONLY.  ";
1900 INPUT A$
2000 IF A$ <> "A" AND A$ <> "B" THEN 1400
2100 REM*****************************************************************
2200 REM
2300 REM THE PROGRAM NEXT ASKS YOU TO ENTER THE FOLLOWING VARIABLES:
2400 REM KB AND KC (AS K2 AND K3), FC (AS F), V1 (AS A)
2500 REM
2600 REM*****************************************************************
2700 PRINT
2800 PRINT "ENTER KB, KC (MICROMOLAR) ";
2900 INPUT K2,K3
3000 PRINT
3100 PRINT "ENTER FC ";
3200 INPUT F
3300 IF F <= 0 OR F >= 1 THEN 3100
3400 PRINT
3500 PRINT "ENTER THE VELOCITY OF ENZYME I (MICROMOLAR/MIN) ";
3600 INPUT A
3700 REM*****************************************************************
3800 REM
```

```
3900 REM IF OPTION A IS SELECTED , THE PROGRAM JUMPS TO SUBROUTINE
4000 REM MINIMUM TO CALCULATE THE VALUES OF V2 AND V3.
4100 REM IF OPTION B IS SELECTED, THE PROGRAM JUMPS TO SUBROUTINE
4200 REM VELOCITY AND ASKS FOR V2 AND V3
4300 REM
4400 REM*****************************************************************
4500 IF A$="A" THEN GOSUB 8800
4600 IF A$="B" THEN GOSUB 13000
4700 REM*****************************************************************
4800 REM
4900 REM NEXT THE PROGRAM CALCULATES THE VALUE OF TIME (T) BY GUESSING
5000 REM ITS VALUE AND CHECKING IF THE VALUE T1 IS ZEROED.
5100 REM IF IT IS NOT, THE PROGRAM RETURNS TO LINE 6300.
5200 REM THE VALUE T1 IS CALCULATED BY SUBTRACTING THE LEFT AND RIGHT
5300 REM SIDES OF THE EQUATION IN THE SUBROUTINE. WHEN LS=RS THE VALUE
5400 REM OF T HAS BEEN FOUND AND T1=0.
5500 REM Q IS A POINTER SET ONLY IF THE INEQUALITY IN LINE 7600 NEEDS
5600 REM TO BE REVERSED. THIS MAY OCCUR IF THE EQUATION IS SOLVED IN A
5700 REM DIFFERENT MANNER DEPENDING ON THE PARAMETERS.
5800 REM
5900 REM*****************************************************************
6000 Q=0
6100 T=5
6200 E=LOG(T/2)/LOG(2)+1
6300 GOSUB 11500
6400 T1=INT(T1*10000)
6500 IF T<9.99 AND T>0.01 THEN 6800
6600 Q=Q+1.1
6700 GOTO 6100
6800 IF Q<2 THEN 7200
6900 PRINT
7000 PRINT "THE PROBLEM IS UNSOLVABLE !!"
7100 GOTO 13900
7200 IF T1<>0 THEN 7600
7300 PRINT
7400 PRINT "THE TIME REQUIRED TO REACH ";F;" STEADY STATE IS: ";T;"
     MINUTES."
7500 GOTO  13900
7600 IF Q<1 AND T1<0 THEN 7900
7700 IF Q>1  AND T1>0 THEN 7900
7800 T=0-T
7900 E=E-1
8000 T = ABS(2^E+T)
8100 GOTO 6300
8200 REM*****************************************************************
8300 REM
8400 REM THIS IS SUBROUTINE MINIMUM. THIS SUBROUTINE USES EQUATION
8500 REM II-29 TO CALCULATE V2 AND V3 MINIMUM.
8600 REM
8700 REM*****************************************************************
8800 PRINT
8900 PRINT "ENTER THE APPROX. VALUE OF T";F*100;" DESIRED (MIN.) ";
```

```
9000 INPUT B
9100 J=16.5556*F-12.11
9200 B1=B/J
9300 PRINT
9400 PRINT "ENTER THE COST OF ENZYME II, III (COST/UNIT) ";
9500 INPUT P2,P3
9600 A6=P3*(P2*(B1*A+K2)+SQR(P2*P3*K2*K3))
9700 A7=P2*(P3*(B1*A+K3)+SQR(P2*P3*K2*K3))
9800 A8=A6/A7
9900 R1=A*B1+K2+A8*(K3+B1*A)
10000 R2=V1*B1+K2-A8*(K3+B1*V1)
10100 V2=(R1+SQR(R2^2+K2*A8*K3))/(2*B1)
10200 V3=V2/A8
10300 PRINT
10400 PRINT "THE MINIMUM VALUES OF V2 AND V3 ARE:
10500 PRINT
10600 PRINT "V2 = ";V2, "V3 = ";V3
10700 RETURN
10800 REM*************************************************************
10900 REM
11000 REM THIS IS SUBROUTINE EQUATION. THE EQUATION YOU WISH TO
11100 REM SOLVE GOES HERE. THE VALUE T1 MUST BE RETURNED
11200 REM SO THAT THE PROGRAM WILL FIND THE CORRECT LAG TIME (T).
11300 REM
11400 REM*************************************************************
11500 C1=(V2-A)^2
11600 C2=(V3-A)^2
11700 IF (C1/V2*K2) <> (C2/V3*K3) THEN 12000
11800 V3=V3-V3/100
11900 GOTO 11500
12000 L=EXP(-T*C1/(V2*K2))-C1*K3*V3/(C2*K2*V2)*EXP(-T*C2/(V3*K3))
12100 R=(1-C1*K3*V3/(C2*K2*V2))*(1-F)*EXP(F*A/V3)
12200 T1=L-R
12300 RETURN
12400 REM*************************************************************
12500 REM
12600 REM THIS IS SUBROUTINE VELOCITY.
12700 REM THIS IS THE INPUT FOR V2 AND V3 IF OPTION B WAS CHOSEN
12800 REM
12900 REM*************************************************************
13000 PRINT
13100 PRINT "ENTER V2, V3 (MICROMOLAR/MIN) ";
13200 INPUT V2,V3
13300 RETURN
13400 REM*************************************************************
13500 REM
13600 REM THIS IS THE END OF THE PROGRAM
13700 REM
13800 REM*************************************************************
13900 END
```

Chapter III

Theory and Practical Application of Coupled Enzyme Systems:
One and Two Coupling Enzymes with Mutarotation of an Intermediate

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Abstract

This paper provides equations to calculate the elasped time before the concentration of the final intermediate, in a sequence of coupled enzymatic reactions, achieves a defined fraction of its steady state concentration when one of the intermediates undergoes mutarotation. The equations can be used to predict lag times for systems involving one coupling enzyme, as is the case when hexokinase or phosphoglucomutase activity is monitored using glucose 6-phosphate dehydrogenase as the auxiliary enzyme, or for systems of two coupling enzymes, as is the case when the activity of enzymes producting ATP (such as creatine kinase) are monitored by coupling the production of ATP to hexokinase and glucose 6-phosphate dehydrogenase. The theoretical aspects of the assay have been verified using hexokinase (as the primary enzyme) and glucose 6-phosphate dehydrogenase (as the coupling enzyme). A method of cost minimization, based on the above relationships, is also provided.

Introduction

It is common practice for enzymologists to use auxiliary enzymes as a means to assay the activity of an enzyme when its product is not detectable by conventional techniques. When coupling enzymes are employed, the observed velocity is not constant over the time course of an experiment: it increases until it approximates the rate of the primary enzyme. The final steady state rate is achieved after a defined lag time (see Figure III-1). It is important that the experimenter be capable of calculating this period, because the observed velocity does not accurately reflect the rate of the primary enzyme before a lag period has elapsed.

One of the most common coupling enzyme systems is the hexokinase-glucose 6-phosphate dehydrogenase system which is often used to monitor ATP production. The product of the hexokinase reaction, glucose 6-phosphate, undergoes mutarotation at the carbon one position giving a mixture of α and β enantiomers (1). Because glucose 6-phosphate dehydrogenase reacts only with the β-enantiomer, the observed lag time (t_{99}, Figure III-1) will depend not only on the kinetic constants of the coupling enzymes, but on the rate of interconversion of the α and β enantiomers as well (2).

Cleland (2) has analysed the hexokinase:glucose 6-phosphate dehydrogenase system and presented equations to calculate the transition time, τ (see Figure III-1), which take into account the mutarotation of glucose 6-phosphate. Although theoretically sound, the equations are not practical as they do not define the time which must elapse before the primary enzyme rate is approximated by the observed rate. The practical time, t_F (t_{99}, see Figure III-1), is not directly obtainable as the

equations which accurately define the coupled enzyme systems can not be differentiated. We have thus applied a method for approximating the solutions to these equations which was developed in the previous paper (3). These equations have been verified using hexokinase and phosphoglucomutase as the primary enzymes, and glucose 6-phosphate dehydrogenase as the coupling enzyme. A method of cost minimization is also provided for the system involving 2 coupling enzymes.

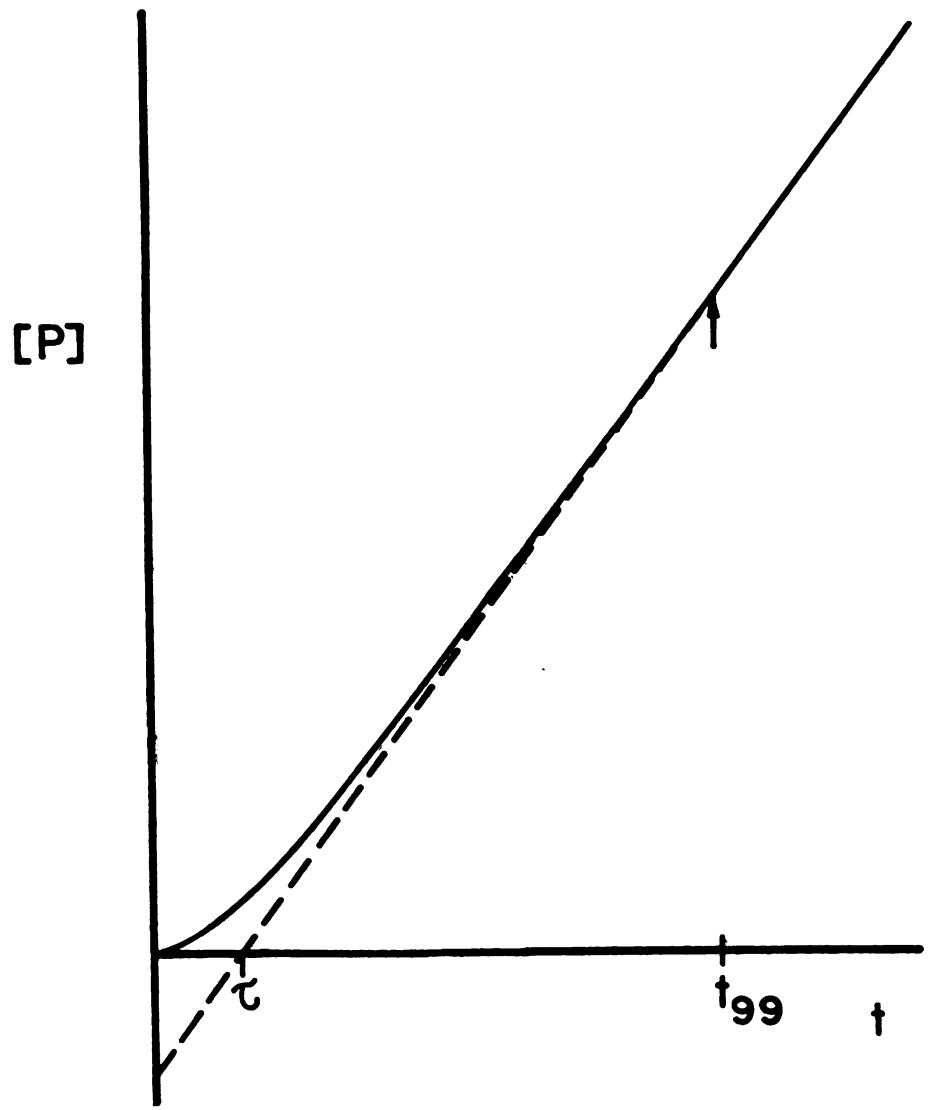
Materials and Methods

Rabbit muscle phosphoglucomutase in 2.5 M $(NH_4)_2SO_4$, yeast hexokinase in 3.2 M $(NH_4)_2SO_4$ and yeast glucose 6-phosphate dehydrogenase (type IX) were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. The phosphoglucomutase solution was diluted with 5 mM citrate (pH 7.2) prior to use. Addition of the diluted phosphoglucomutase or hexokinase solution did not significantly alter the ionic strength of the assay. All other chemicals were purchased from Sigma and were of the highest quality available. Spectrophotometric measurements were carried out on a Beckman model DU spectrophotometer with a Gilford model 222 photomultiplier at 340 nm using a $\Delta\varepsilon$ for NADPH[4] of 6.23×10^3 $M^{-1}cm^{-1}$. All substrate stock solution concentrations were measured using 10 µg of glucose 6-phosphate dehydrogenase (S.A. = 330 I.U./mg) and 0.66 µg of phosphoglucomutase (S.A. = 210 I.U./mg) or 2 µg hexokinase (S.A. = 321 I.U./mg) at 340 nm prior to each set of experiments.

4. Abbreviations used: NADP(H); nicotinamide adenine dinucleotide phosphate (reduced form), PGM; phosphoglucomutase, G6PdH; glucose 6-phosphate dehydrogenase

Figure III-1: Theoretical time course for a one enzyme coupled assay involving mutarotation of the intermediate as shown in Scheme 1.

The solid line represents accumulation of product and the dashed line is the asymptote to the curve at $t = \infty$. The time taken for $[\beta]$ to reach 99% of $[\beta]_{ss}$ is indicated. The transition time, τ, is also shown. See text for details.

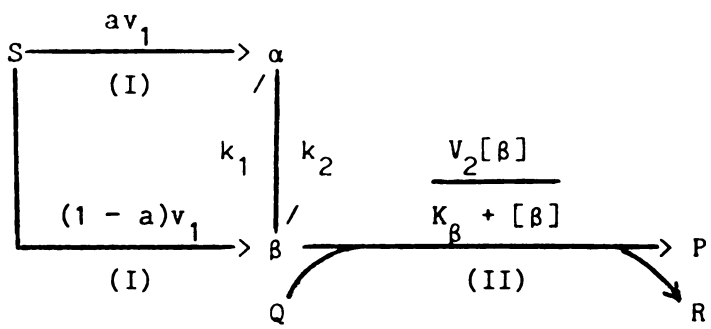

[P]
τ
t99
t

Theory

One Auxiliary Enzyme

A general scheme for a one enzyme coupled reaction in which the intermediate undergoes mutarotation is shown below.

Scheme 1



Where α and β denote the concentrations of the two forms of the intermediate. The constant, a, is the fraction of the total velocity of enzyme I which results in the formation of the intermediate α. When enzyme I is hexokinase and S is glucose, a = 0.4 (1), because both the α and β forms of glucose react with equal facility. The value of a then reflects the equilibrium between α and β glucose. If enzyme I is phosphoglucomutase (PGM), a = 1.0 as PGM reacts only with the α form of glucose 1-phosphate. The rate constants for isomerization of the α and β forms are represented by the values k_1 and k_2. For glucose 6-phosphate, Cleland (2) gives values of: $k_1 = 3.8\ min^{-1}$ and $k_2 = 2.2\ min^{-1}$. v_1 represents the rate of the initial enzyme (the enzyme under study) and is assumed to be constant over the time course of the experiment. If we assume that the reverse reactions of enzyme I and II are negligible and that v_1 is constant, we can define the change in the concentrations of the intermediate over time by equations 1a and 1b.

[III-1a] $d[\alpha]/dt = av_1 + k_2[\beta] - k_1[\alpha]$

[III-1b] $d[\beta]/dt = (1-a)v_1 + k_1[\alpha] - k_2[\beta] - V_2[\beta]/(K_\beta + [\beta])$

with V_2 defined as

[III-2] $V_2 = V_{2,max}\,[Q]/(K_Q + [Q])$

where $V_{2,max}$ is the maximal velocity for enzyme II. If K_B is the measured K_m of enzyme II for a mixture of α and β enantiomers, then (see reference 2):

[III-3] $K_\beta = K_B/(1 + k_2/k_1)$

Note that V_2 represents the concentration of enzyme II in units $min^{-1}mL^{-1}$ or μM/min. To obtain an exact solution of Equation III-1, we assume that $V_2 >> v_1$, and then define

[III-4] $m = V_2/K_\beta$

Equations 1a and 1b then become:

[III-5a] $d[\alpha]/dt = av_1 + k_2[\beta] - k_1[\alpha]$

[III-5b] $d[\beta]/dt = (1 - a)v_1 + k_1[\alpha] - (k_2 + m)[\beta]$

Integrating equations 5a and b gives

[III-6a] $[\alpha] = [\alpha]_{ss} + C_1\exp(r_1t) - ([\alpha]_{ss} + C_1)\exp(r_2t)$

[III-6b] $$[\beta] = [\beta]_{ss} + C_2\exp(r_1 t) - ([\beta]_{ss} + C_2)\exp(r_2 t)$$

where: $$r_1 = \{-(k_1 + k_2 + m) - [(k_1 + k_2 + m)^2 - 4k_1 m]^{1/2}\}/2$$

$$r_2 = \{-(k_1 + k_2 + m) + [(k_1 + k_2 + m)^2 - 4k_1 m]^{1/2}\}/2$$

$$[\alpha]_{ss} = v_1(a/k_1 + k_2/k_1 m)$$

$$[\beta]_{ss} = v_1/m$$

$$C_1 = (av_1 + [\alpha]_{ss} r_2)/(r_1 - r_2)$$

$$C_2 = [(1-a)v_1 + [\beta]_{ss} r_2]/(r_1 - r_2)$$

In order to calculate the time required for [β] to achieve a defined fraction of $[\beta]_{ss}$, we define: $F_\beta = [\beta]/[\beta]_{ss}$. Substituting $F_\beta[\beta]_{ss}$ for [β] in Equation III-6b gives an expression which relates the time required for [β] to achieve F_β of $[\beta]_{ss}$ in terms of the enzyme kinetic parameters.

[III-7] $$[\beta]_{ss}(F_\beta - 1) = C_2\exp(r_1 t_{F\beta}) - ([\beta]_{ss} + C_2)\exp(r_2 t_{F\beta})$$

Equation III-7 is not explicit in terms of $t_{F\beta}$, but can be solved by either of two methods: a computer solution of Equation III-7 can be obtained by numerical methods or by the following approximation. Numerical analysis of Equation III-7 reveals that for $k_1 > 0$ and $k_2 > 0$ an accurate value ($\pm$ 0.01%) of $t_{F\beta}$ can be obtained from Equation III-8.

[III-8] $$t_{F\beta} = \frac{1}{r_2} \ln[(1-F_\beta)[\beta]_{ss}/([\beta]_{ss} + C_2)]$$

which is derived from Equation III-7 by assuming $r_1/r_2 \geq 2$.

Equation III-7 was obtained by integration of Equation III-1

assuming that $V_2 >> v_1$. If v_1 is greater than 0.01 V_2, then the error in assuming that $(K_\beta + \beta) = K_\beta$ becomes significant so that the above assumption is no longer valid. However, an approximate solution can be obtained by rewriting Equation III-1b so that the term $(K_\beta + [\beta])$ is constant. We can then obtain two equations which define an upper (β_+) and lower (β_-) for the term $(K_\beta + [\beta])$. Integration and subsequent numerical analysis of these equations shows that the upper limit yields the best approximation (see Appendix). Solution of this equation for the lag time $(t_{F\beta})$ gives Equation III-9. The lag time is denoted by the term $t_{F\beta-}$ as the β_+ analysis gives the best estimate.

$$[III\text{-}9] \qquad t_{F\beta-} = \frac{1}{r_2} \ln[(1-F_\beta)[\beta]_{ss}/([\beta]_{ss} + Z_{1+})]$$

where $Z_{1+} = [(1-a)v_1 + [\beta]_{ss}r_2]/(r_1 - r_2)$ and r_1 and r_2 are defined in Equation III-5 with $m = \Phi/K_\beta$. $[\beta]_{ss}$ is given by Equation III-10:

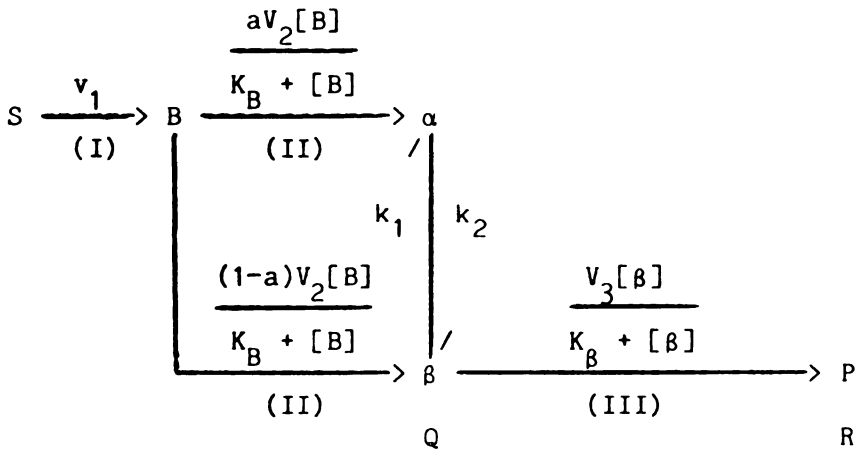
$$[III\text{-}10] \qquad [\beta]_{ss} = v_1 K_\beta/\Phi$$

where $\Phi = V_2 - v_1$. The analysis shown in the Appendix indicates that the $t_{F\beta-}$ approximation from Equation III-9 can be used for values of v_1/V_2 up to 0.2. After this point the error is in excess of 10%.

Two Auxiliary Enzymes

The most common system utilizing two coupling enzymes involves the hexokinase:glucose 6-phosphate dehydrogenase system represented below:

Scheme 2



with a defined as before. We begin by deriving the equations which describe Scheme 2 for the case when $K_B >> [B]$ and $K_\beta >> [\beta]$ (pseudo first order analysis). The following equations use $m_1 = V_2/K_B$ and $m_2 = V_3/K_\beta$.

[III-11a] $$d[B]/dt = v_1 - m_1[B]$$

[III-11b] $$d[\alpha]/dt = am_1[B] - k_1[\alpha] + k_2[\beta]$$

[III-11c] $$d[\beta]/dt = (1-a)m_1[B] + k_1[\alpha] - k_2[\beta] - m_2[\beta]$$

Equations 11a, 11b and 11c can be solved for the concentrations of all intermediates: B, α and β (see Appendix). Since we are looking for the time required for the final intermediate to achieve a defined fraction of its steady state value, we present the solution for the intermediate β only.

[III-12] $$[\beta] = [\beta]_{ss} + D_1\exp(-m_1t) + D_2\exp(s_1t) + D_3\exp(s_2t)$$

Where: $[\beta]_{ss} = v_1/m_2$

$R_T = k_1 + k_2 + m_2$

$$s_1 = [-R_T - (R_T^2 - 4k_1m_2)^{1/2}]/2$$

$$s_2 = [-R_T + (R_T^2 - 4k_1m_2)^{1/2}]/2$$

$$X_0 = K_C v_1/\Psi + av_1/k_1$$

$$X_1 = K_B(m_2D_1 + v_1)/\Phi$$

$$D_1 = [k_1[\beta]_{ss} - (1-a)v_1]/R_T$$

$$D_2 = ([\beta]_{ss} + D_1 + S_2(X_0 + X_1)/m_2)/(s_2/s_1-1)$$

$$D_3 = -([\beta]_{ss} + D_1 + D_2)$$

$$\Psi = V_3 - v_1$$

Note that, as is the case for the one auxiliary enzyme system, if K_C is the measured K_m of enzyme III for a mixture of α and β enantiomers then $K_\beta = K_C/(1+k_2/k_1)$. For the case when K_β and K_B are not much greater than $[\beta]$ and $[B]$, respectively, Equation III-12 cannot be integrated. We, therefore, present equations to approximate $t_{F\beta}$ for the two auxiliary enzyme case. Following the rationale for the one auxiliary enzyme case, we can obtain two equations which define an upper and lower limit for the concentration of β. Numerical analysis reveals that the β_+ analysis yields the most accurate results and consequently Equation III-13 is the more accurate:

$$[\beta_+] = [\beta]_{ss} + D_1\exp(-\Phi t/K_B) + D_2\exp(s_1t) + D_3\exp(s_2t) \qquad [III-13]$$

with D_1, D_2, D_3, s_1 and s_2 obtained from Equation III-12 with $m_2 = \Psi/K_\beta$. If $k_1 = 3.8\ min^{-1}$ and $k_2 = 2.23\ min^{-1}$, it can be shown that for all

values of $\Psi/K_\beta > 0$, $s_1/s_2 \geq 2$. Equation III-13 then reduces to:

[III-14] $$[\beta_+] = [\beta]_{ss} + D_1\exp(-\Phi t/K_B) + D_3\exp(s_2 t)$$

The value $t_{F\beta-}$ is approximated by two relationships under defined conditions. When $s_2 \leq -\Phi/2K_B$:

[III-15a] $$t_{F\beta-} = \ln\{(1-F_\beta)[\beta]_{ss}/([\beta]_{ss} + D_1 + D_2)\}/s_2$$

and when $s_2 \geq -\Phi/2K_B$:

[III-15b] $$t_{F\beta-} = -K_B \ln[(F_\beta-1)[\beta]_{ss}/D_1]/\Phi$$

The conditions defining the application of Equation III-15 show that the $t_{F\beta}$ value depends upon Ψ/K_β or Φ/K_B depending upon which coupling enzyme is limiting. As more of enzyme II is added, Equation III-15a predicts the correct $t_{F\beta-}$ value and when enzyme III is in excess, Equation III-15b predicts the correct $t_{F\beta-}$ value. If neither of the above conditions applies, then Equation III-14 should be solved numerically to determine the value of $t_{F\beta-}$ using: $[\beta_-] = F_\beta[\beta]_{ss}$ and solving for t in a manner similar to that shown by Brooks et al. (3). A practical limit on the use of equations 14 and 15 must also be imposed. When either v_1/V_2 or v_1/V_3 is greater than 0.2, the calculated value of $t_{F\beta}$ is in error by about 15%. This therefore reflects the limit of the technique.

Minimum Concentration of Each Coupling Enzyme

For systems involving two auxiliary enzymes as defined above, the transition time, τ, (see Figure III-1 and also reference 4), is given by

[III-16] $$\tau = a/k_1 + K_B/\Phi + K_C/\Psi$$

By solving for V_2 or V_3, Equation III-16 gives

[III-17a] $$V_2 = v_1 + \Psi K_B/(\tau\Psi - a\Psi/k_1 - K_C)$$

[III-17b] $$V_3 = v_1 + \Phi K_C/(\tau\Phi - a\Phi/k_1 - K_B)$$

In order to minimize the total enzyme concentration, we use the convenient cost function, which is the sum of the price per unit of enzyme multiplied by the concentration of enzyme (see references 2 and 3), or;

[III-18] $$\text{Cost} = P_2V_2 + P_3V_3$$

We can substitute Equation III-17 into Equation III-18 and obtain the total cost as a function of either V_2 or V_3. Taking the derivative of these functions with respect to V_2 or V_3 and setting this value to zero gives two equations which are continuous for all values of V_2 and V_3. The ratio of V_2 to V_3 gives the slope of the line of minimum cost.

$$[III\text{-}19] \qquad \delta = \frac{V_2}{V_3} = \frac{P_2P_3(K_B + \tau v_1 - v_1 a/k_1) + P_3(P_2P_3K_BK_C)^{1/2}}{P_2P_3(K_C + \tau v_1 - v_1 a/k_1) + P_2(P_2P_3K_BK_C)^{1/2}}$$

The value of V_2 which minimizes the above function is given by the intersection of Equation III-19 and III-17b:

$$[III\text{-}20] \qquad V_2 = \frac{R_1 + [R_1^2 - 4R_2(\tau k_1 - a)]^{1/2}}{2(\tau k_1 - a)}$$

Where: $R_1 = v_1(1 + \delta)(k_1\tau - a) + k_1(K_B + \delta K_C)$

$R_2 = v_1^2\delta(k_1\tau - a) + \delta v_1 k_1(K_B + K_C)$

Since the τ value has no practical use, we use two empirical relationships which represent the average value of the ratio $t_{F\beta}/\tau$: $t_{99} = 4.58\tau$ and $t_{90} = 2.38\ \tau$ (see also reference 3). These relationships can be used to obtain an approximate τ value for a desired value of $t_{F\beta}$. Using the τ value, one can obtain minimum values of V_2 and V_3 which correspond to the τ value. One must then return to Equation III-15 and calculate the correct $t_{F\beta}$ value using the calculated V_2 and V_3 values. This will ensure that an accurate lag time has been defined.

Results

To test the theoretical expressions developed in the previous section, a coupled enzyme system involving either PGM or hexokinase as the primary enzyme and glucose 6-phosphate dehydrogenase (G6PdH) as the secondary enzyme was examined. These systems were chosen for their convenience and lack of product inhibition. To apply the theories outlined above, it is necessary to know K_B, the Michaelis constant of glucose 6-phosphate for G6PdH. Prior to the present studies, a kinetic

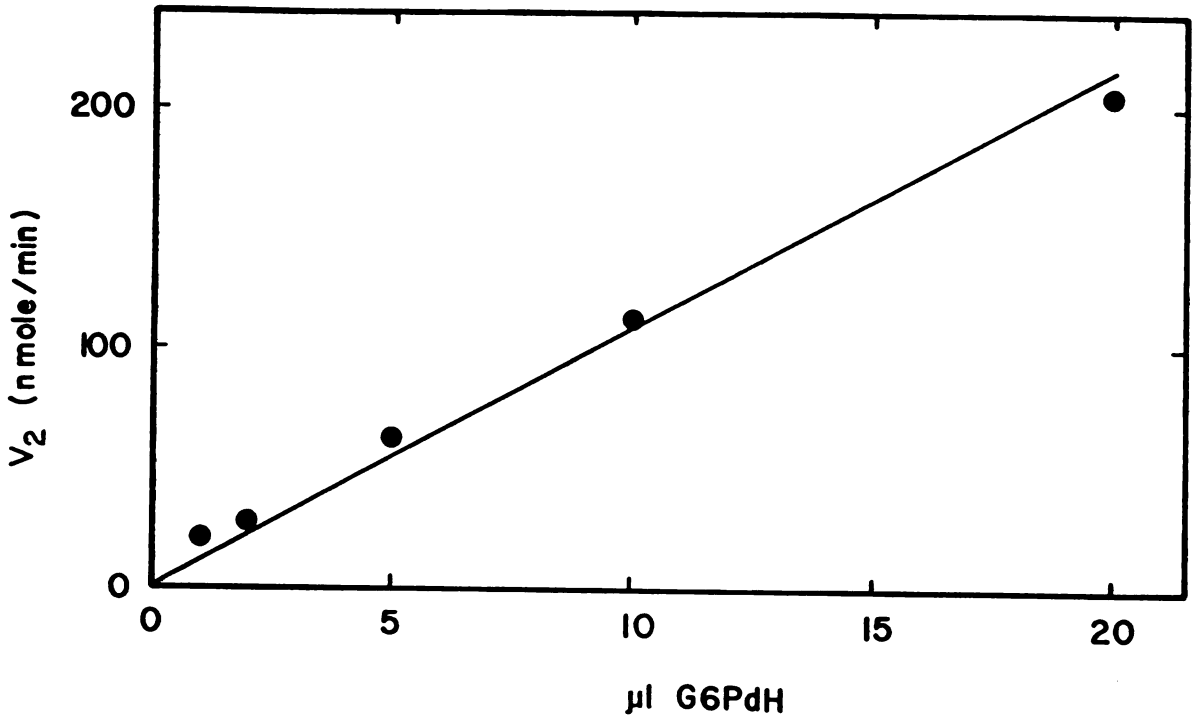
Figure III-2: Graph of V_2 calculated from Equation III-23 versus the volume of glucose 6-phosphate dehydrogenase added to the reaction mixture.

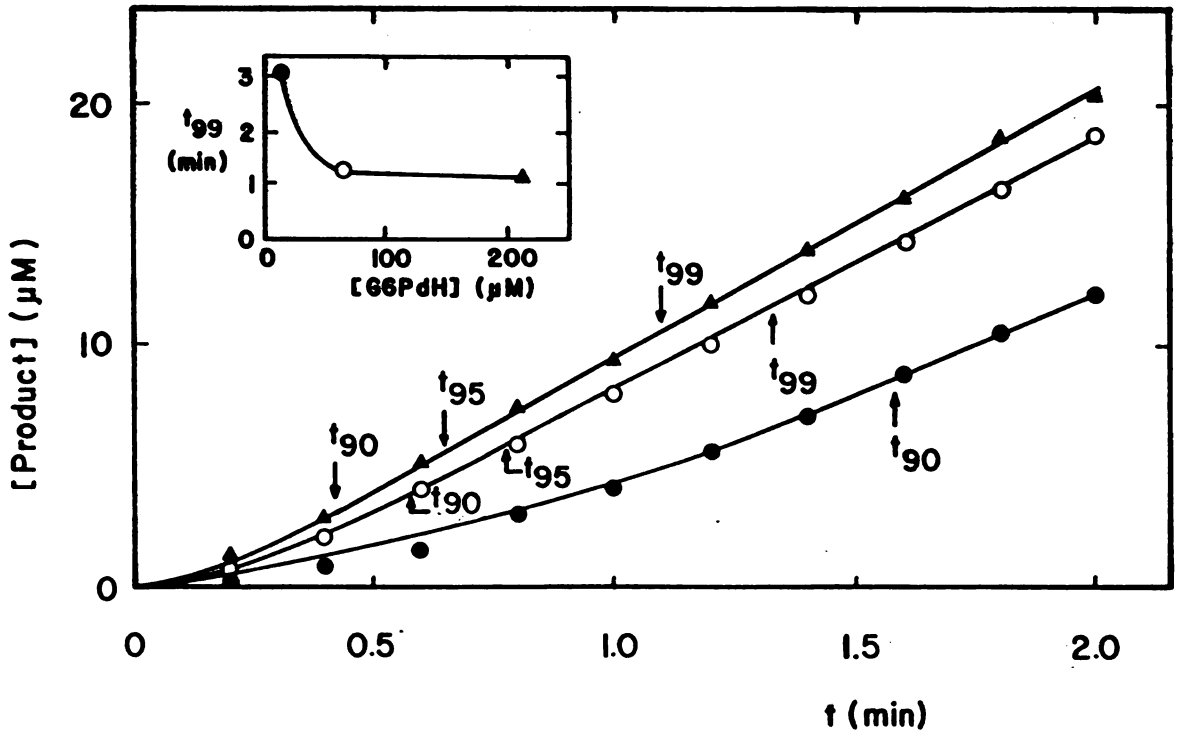
The reaction mixture contained: 10 mM KMOPS (pH 7.2), 10 mM $MgCl_2$, 1 mM EDTA, 170 μM $NADP^+$, 0.5 mM glucose and 350 μM ATP. The temperature was 30°C and the assay was monitored at 340 nm. The total volume is 1 mL. For each assay, 0.17 μg of hexokinase was added. The final reaction velocity was 10 nmoles/min.

Figure III-3: Comparison of theoretical and actual results for the hexokinase-glucose 6-phosphate dehydrogenase (G6PdH) system.

Various amounts of G6PdH were added to 0.17 μg hexokinase and the assay monitored at 340 nm. Substrate concentrations were: 170 μM $NADP^+$, 500 μM glucose and 350 μM ATP. Other conditions are given in the legend to Figure III-2. The solid lines are theoretical lines obtained as described in the text.

The inset shows the t_{99} values for the three cases. V_{obs} = 10 ± 0.5 nmoles/min. ▲; 20 μL G6PdH (V_2 = 250 nmole/min, t_{99} = 1.1 min); ○, 5 μL G6PdH (V_2 = 63 nmole/min, t_{99} = 1.4 min); ●, 2 μL G6PdH (V_2 = 24 nmole/min, t_{99} = 3.5 min)


200
100
0
V_2 (n mole/min)
0
5
10
15
20
µl G6PdH


20
10
0
[Product] (µM)
0
0.5
1.0
1.5
2.0
t (min)
t_{90}
t_{95}
t_{99}
t_{90}
t_{95}
t_{90}
t_{99}
t_{90}
3
2
1
0
t_{99} (min)
0
100
200
[G6PdH] (µM)

assay was conducted to determine the value of K_B. Under the conditions outlined in the legend of Figure III-3, we obtained: K_B = 6.1 μM. The value of V_2 can also be obtained from this experiment. However, if K_B is known and the experimenter wishes only to determine the value of V_2 in a single assay, he may proceed as follows. The assay for hexokinase obeys a reaction of the type shown in Scheme 1. At steady state $d[\alpha]/dt = 0$ and $d[\beta]/dt = 0$ and equations 1a and 1b predict that (see also references 2 and 4):

[III-21a] $$[\beta]_{ss} = K_\beta v_1/\Phi$$

[III-21b] $$[\alpha]_{ss} = av_1/k_1 + k_2K_\beta v_1/k_1\Phi$$

The transition time, τ, is simply the sum of the steady state concentrations of the intermediates divided by the initial velocity (2,4); and thus equations 21a and 21b give:

[III-22] $$\tau = a/k_1 + K_B/\Phi$$

which rearranges to give Equation III-23

[III-23] $$V_2 = v_1 + K_B/(\tau - a/k_1)$$

so that a plot of V_2 calculated with Equation III-23 using measured values of τ (see Figure III-1) versus the amount of enzyme II added (at constant v_1) will give the specific activity for the coupling enzyme as shown in Figure III-3.

Using the values obtained from Figure III-3, it is possible to construct time courses for each enzyme assay using the value for the observed rate at $t = t_{99}$ (v_1) and the following analysis. The rate of product appearance is given by Equation III-24:

[III-24] $$d[P]/dt = k_+[\beta_+]$$

Substituting the value of $[\beta_+]$ from Equation III-28 into Equation III-24 and subsequent integration gives the value of [P] for any time t:

[III-25] $$[P_+] = k_+\{[\beta]_{ss}t + \frac{Z_1}{r_1}(\exp(r_1t)-1) - \frac{(Z_1+[\beta]_{ss})}{r_2}(\exp(r_2t)-1)\}$$

The continuous curves in Figure III-4 are constructed using Equation III-25. It is apparent that the theoretically derived time courses (continuous curves) and the observed time courses (symbols, Figure III-4) are in good agreement. Using Equation III-9, we estimate t_{99} values of: t_{99} (V_2 = 250 $\mu\underline{M}$/min) = 1.1 min, t_{99} (V_2 = 63 $\mu\underline{M}$/min) = 1.3 min and t_{99} (V_2 = 24 $\mu\underline{M}$/min) = 3.4 min. The values are in good agreement with the actual t_{99} values obtained from computer simulated progress curves using equations 1a and 1b (5). Note that accurate rates are obtained from the experimental progress curves after waiting the required period of time. Figure III-4 also shows the t_{90} and t_{95} values: the curves appear to be linear after t_{95} minutes so that it is hard to distinguish between the rate at t_{95} and t_{99} minutes.

Figure III-5A shows the results of another experiment using PGM as the primary enzyme and G6PdH as the coupling enzyme. For this experiment various amounts of PGM were added to a reaction mix containing

Figure III-4a: Titration of G6PdH with PGM.

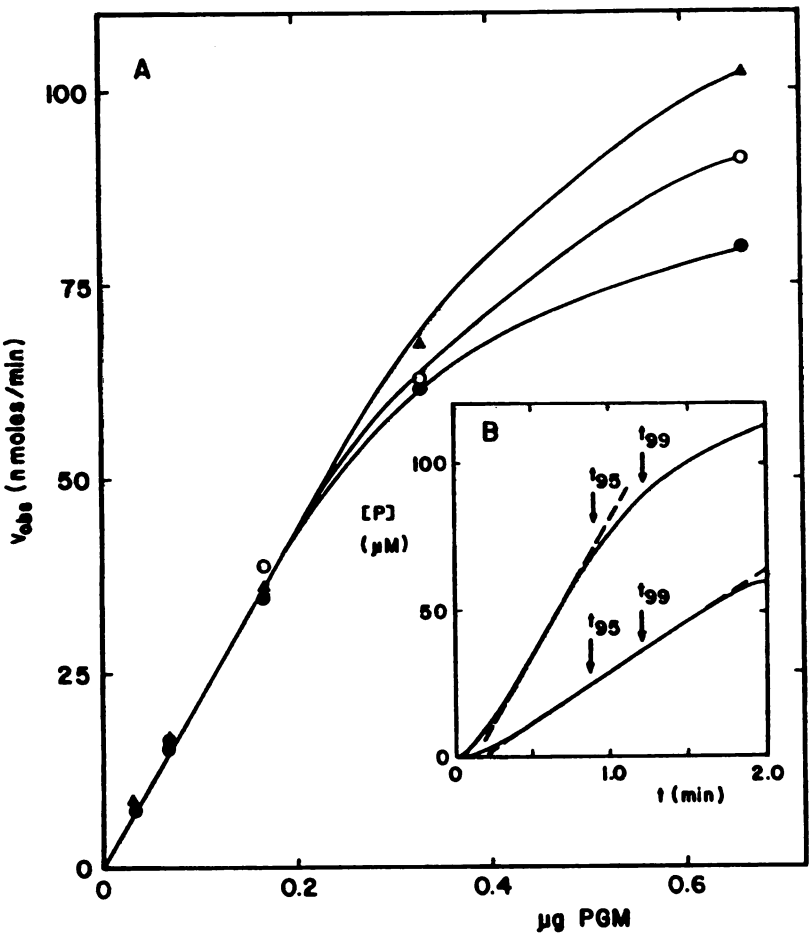
Various amounts of PGM were added to either 1 unit (●), 2 units (o) or 10 units (▲) of G6PdH and the rates measured when the observed velocity was linear. Substrate concentrations were: 30 μM glucose 1-phosphate, 170 μM $NADP^+$ and 10 μM glucose 1,6 diphosphate. Other conditions are given in the legend of Figure III-3.

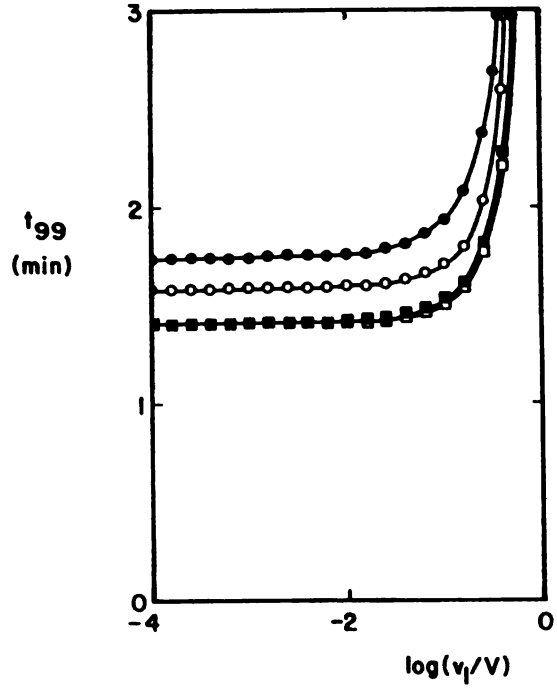
Figure III-4b: Experimental progress curves for measurement of PGM enzyme activity.

Top curve, v_{obs} = 79 nmoles/min. Bottom curve, v_{obs} = 34 nmoles/min. The v_{obs} values were obtained from the slope of the dashed lines. t_{95} and t_{99} values (calculated with Equation III-9) are indicated. V_2 = 1 unit. Other conditions are given in the legend of Figure III-3.

Figure III-5: Relationship between t_{99} and v_1/V_2 or v_1/V_3.

The values of t_{99} were obtained using KINFIT 4 (5) and Equation III-11. Closed symbols represent $\log(v_1/V_3)$ versus t_{99} with V_2 = 100 μM/min, $K_B = K_C$ = 10 μM. ●, V_3 = 50 μM/min; ■, V_3 = 95 μM/min. Open symbols represent $\log(v_1/V_2)$ versus t_{99} with V_3 = 100 μM/min, $K_B = K_C$ = 10 μM. o, V_2 = 50 μM/min; □, V_2 = 95 μM/min.


A
100
75
50
25
0
0
0.2
0.4
0.6
µg PGM
v_{obs} (n moles/min)
B
100
50
0
0
1.0
2.0
t (min)
[P]
(µM)
t_{99}
t_{95}
t_{99}
t_{95}


3
2
1
0
-4
-2
0
t_{99}
(min)
$\log(v_i/V)$

fixed amounts of G6PdH. Note that, contrary to the expected linear relationship, the observed velocity (v_{obs}) is not linear with respect to the amount of PGM added. The initial velocities plotted in Figure III-5A are the slopes of experimental progress curves (Figure III-5B) when the observed rate appeared to be constant. It appears as if the rate of the primary enzyme decreases with time resulting in decreased v_{obs} at the longer times. This behaviour was not observed for concentrations of PGM below 0.2 µg/mL. The calculated t_{95} value for this system, using Equation III-9, is 0.8 minutes. Figure III-5B shows that the reaction velocity depicted by the upper curve is no longer constant at 0.8 minutes and, therefore, an accurate v_1 value cannot be obtained. When v_1 was less than 40 nmoles/min, however, the progress curve depicted by the bottom curve was still linear at t_{95} minutes. Thus, it is important to calculate t_{95} values for coupled enzyme reactions to insure that slopes are measured at the proper point in a progress curve.

Discussion

The equations presented here represent, for the first time, a method for calculating the lag time when the kinetic parameters of the coupling enzyme(s) are known and when one of the intermediates undergoes mutarotation. If one wishes to specify a lag time, the equations can be inverted to obtain the concentration of the coupling enzymes necessary to produce the desired lag period. The development of the equations relies on two assumptions: (a) the rate of the primary enzyme is constant and (b) the reverse reactions are negligible. A third assumption is also implied, i.e., that either v_1/V_2 or v_1/V_3 does not exceed 0.2. This

latter assumption is necessary as the $t_{F\beta}$ estimates are based on approximations of the value $V_2/(K_\beta + [\beta])$ or $V_3/(K_\beta + [\beta])$. Thus the third assumption gives practical limits to the equations.

The results obtained with the hexokinase-G6PdH system demonstrate the usefulness of the equations. Using the $t_{F\beta-}$ values calculated from Equation III-9, accurate primary enzyme rates were obtained from experiments where the coupling enzyme G6PdH was varied (all observed rates were identical) (see Figure III-4). Furthermore, experimentally accurate lag periods ($\pm$ 10%) were calculated even for the case where $v_1/V_2 = 0.5$. A cost minimization technique is described which relies on the minimization of the transition time, τ, as defined by Cleland (2). Although the τ value is not practical, an empirical relationship between τ and $t_{F\beta-}$ is presented. This allows an estimation of a τ value from a desired $t_{F\beta}$ value. Minimum values of V_2 and V_3 can then be calculated and used to obtain a more accurate $t_{F\beta}$ value. It is important to recalculate the $t_{F\beta}$ value after V_2 and V_3 have been obtained as the empirical relationships defined are only approximations. Thus the initial $t_{F\beta}$ may not be the same as the recalculated value.

An interesting result was obtained when the exact lag time, calculated with Equation III-11, was plotted against $\log(v_1/V_3)$ or $\log(v_1/V_2)$. Figure III-2 shows that the t_{99} values differ depending on whether V_2 is greater than V_3 or the reverse is true. When $V_3 > V_2$ (and $K_\beta = K_B$), the lag time is shorter. This is because the system in scheme 2 is not symmetrical about the intermediate β. It can also be shown that α does not achieve 0.9 or 0.99 of its steady state concentration when $[\beta]/[\beta]_{ss} = 0.9$ or 0.99 and $a=0.4$, even though the observed velocity is equal or greater than 0.9 or 0.99 times the initial velocity. When a =

1.0, the intermeditate α does achieve the indicated F_β value because it is an obligatory intermediate in reaction Scheme 2.

Finally it should be noted that, when the intermediates undergo mutarotation, the observed lag time ($t_{F\beta}$) increases condiderably compared to the case when intermediates do not mutarotate. The t_F values in Figure III-5B were obtained by assuming that, with the PGM-G6PdH system, mutarotation of one of its intermediates did occur. If the calculations were made assuming that mutarotation did not occur as outlined in the preceeding paper (3), we obtained a t_{95} value of 0.06-0.08 minutes (depending on v_1). Thus one might assume that the linear portion of the progress curves accurately reflected the primary enzyme velocity. However, the proper calculation shows that t_{95} = 0.8 minutes. At this time, the observed rate is obviously not linear and consequently other factors (such as decreasing enzyme activity) may be contributing to the observed velocity. This underscores the importance of calculating t_F values derived from a model which accurately reflects the reaction scheme.

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Appendix

One Auxiliary Enzyme

In order to obtain an approximate equation for the lag time, we rewrite Equation III-1b so that the term $V_2/(K_\beta + [\beta])$ is constant. Equations 1a and 1b then yield an explicit value for $t_{F\beta}$. Therefore, by choosing constant values of $V_2/(K_\beta + [\beta])$ which approximate the true value of β at any time t, we can rewrite Equation III-1b as:

[III-A1a] $$d[\beta_+]/dt = (1-a)v_1 + k_1[\alpha] - [\beta](k_2 + k_+)$$

[III-A1b] $$d[\beta_-]/dt = (1-a)v_1 + k_1[\alpha] - [\beta](k_2 + k_-)$$

where β_+ and β_- reflect the largest and smallest possible values of β. The term k_+ is now defined as the smallest value of $V_2/(K_\beta + [\beta])$ such that $d[\beta_+]/dt$ is always greater than $d[\beta]/dt$ or:

$$k_+ = V_2/(K_\beta + [\beta]_{ss}) = \Phi/K_\beta$$

where $\Phi = V_2 - v_1$. Conversely k_- is the largest value of $V_2/(K_\beta + [\beta])$ or $k_- = V_2/K_\beta$. Using these approximations we now integrate equations 26a, 26b and 1a. This integration gives two equations of the general form:

[III-A2] $$[\beta] = v_1/k + Z_1\exp(r_1t) + Z_2\exp(r_2t)$$

with r_1 and r_2 as defined in Equation III-5 and with $m = k_+$ or k_-. Z_1 and Z_2 are obtained by selecting an appropriate initial (t=0) condition.

Selecting $[\beta_+]_{t=0} = 0$ gives:

[III-A3] $$[\beta_+] = [\beta]_{ss} + Z_{1+}\exp(r_1 t) - Z_{2+}\exp(r_2 t)$$

where $Z_{1+} = [(1-a)v_1 + [\beta]_{ss} r_2]/(r_1 - r_2)$, $Z_{2+} = -([\beta]_{ss} + Z_{1+})$ and r_1 and r_2 are defined by Equation III-5 with $m = \Phi/K_\beta$. $[\beta]_{ss}$ is given by Equation III-10.

If we choose $[\beta_-]_{t=0} = 0$, Equation III-A2 gives Equation III-5 once again. However, a better estimate of $t_{F\beta}$ can be obtained if we let $k = k_-$ and $Z_2 = Z_{2+}$ in Equation III-A2 (i.e. $[\beta_-]_{t=0} < 0$). Equation III-A2 then gives

[III-A4] $$[\beta_-] = v_1 K_\beta / V_2 + Z_{1-}\exp(r_1 t) - ([\beta]_{ss} + Z_{1+})\exp(r_2 t)$$

where $Z_{1-} = [(1-a)v_1 + v_1 K_\beta r_2 / V_2]/(r_1 - r_2)$ and r_1 and r_2 are obtained from Equation III-5 with $m = V_2/K_\beta$.

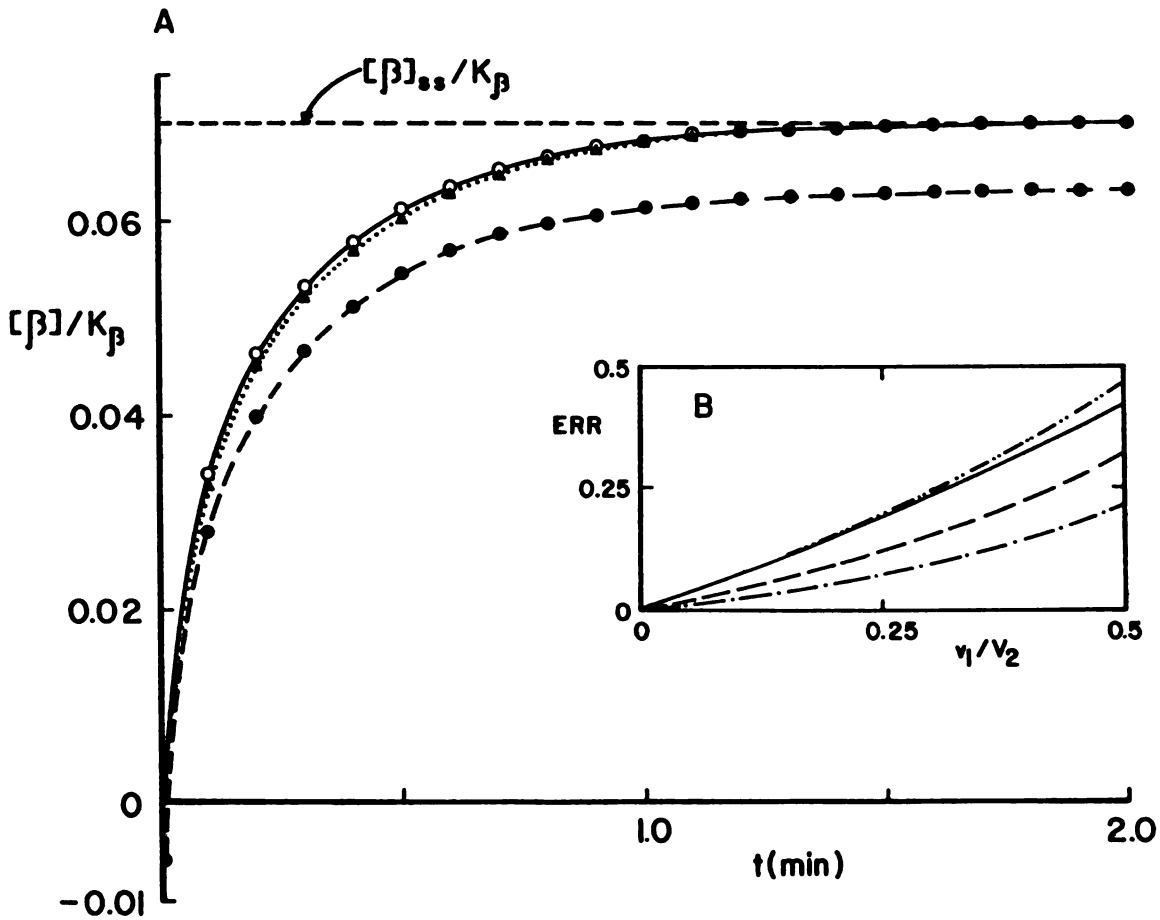
Figure III-A1a shows a comparison of the time courses for $[\beta]/K_\beta$ exact (Equation III-1), $[\beta_-]/K_\beta$ (Equation III-A4) and $[\beta_+]/K_\beta$ (Equation III-A3). The curves are drawn for $V_2 = 100$ μM/min and $v_1 = 10$ μM/min. Note that both $[\beta]/K_\beta$ exact (closed triangles) and $[\beta_+]/K_\beta$ (open circles) tend toward $[\beta]_{ss}/K_\beta$ when $t \to \infty$, the value of $[\beta_-]/K_\beta$ (closed circles) tends toward $v_1 K_\beta / V_2$. If v_1/V_2 decreases, all curves tend toward $[\beta]_{ss}/K_\beta$ because $v_1 K_\beta / \Phi$ tends toward $v_1 K_\beta / V_2$ as v_1/V_2 decreases. This is clearly shown in Figure III-A1b where the error in $t_{F\beta}$ is plotted as a

Figure III-A1a: Theoretical time courses for the accumulation of [β] calculated from three sources.

●, Equation III-A4 (β_-); o, Equation III-A3 (β_+) and ▲, $[\beta]/K_\beta$ exact from KINFIT 4 estimation (5).

Figure III-A1b: Relationship between the error obtained from the $t_{F\beta}$ analysis and the true $t_{F\beta}$ value as a function of V_1/V_2.

Solid line: $K_\beta/V_2 = 1$; dashed line, $K_\beta/V_2 = 0.1$, dotted-dashed line, $K_\beta/V_2 = 0.01$; double-dotted dashed line; first order approximation (see Equation III-6). The value of ERR is given by: ERR = $[t_{F\beta}(\text{true}) - t_{F\beta}(\text{estimate})]/t_{F\beta}(\text{true})$.


A
$[\beta]_{ss}/K_\beta$
0.06
$[\beta]/K_\beta$
0.04
0.02
0
-0.01
1.0
2.0
t(min)
B
ERR
0.5
0.25
0
0
0.25
0.5
v_1/V_2

function of v_1/V_2 for various values of K_β/V_2 as indicated in the figure legend. As K_β/V_2 decreases, the error in the determination of $t_{F\beta}$ decreases (compare solid line, dashed line and dotted-dashed line). The estimate from Equation III-6, which assumes that $V_2 >> v_1$ ($m = V_2/K_\beta$, double-dotted-dashed line, Figure III-A1b), has an error considerably greater than that for the $t_{F\beta-}$ estimate (dashed line).

The values of $t_{F\beta-}$ and $t_F\beta+$ are also obtained from equations A3 and A4 by defining F_β as before with $[\beta]_{ss}$ given by Equation III-10 when $r_1/r_2 \geq 2$

[III-A5a] $$t_{F\beta-} = \ln[(1-F_\beta)[\beta]_{ss}/([\beta]_{ss} + Z_{1+})]/r_2$$

[III-A5b] $$t_F\beta+ = \ln[(\Phi/V_2 - F_\beta)[\beta]_{ss}/([\beta]_{ss} + Z_{1+})]/r_2$$

Two Auxiliary Enzymes

Solving Equation III-11 for all concentrations of intermediates gives:

[III-A6a] $$[B] = [B]_{ss} - [B]_{ss}\exp(-m_1 t)$$

[III-A6b] $$[\alpha] = [\alpha]_{ss} + C_1\exp(-m_1 t) + C_2\exp(s_1 t) + C_3\exp(s_2 t)$$

and Equation III-12. By defining boundary conditions of $[B]_{t=0} = [\alpha]_{t=0} = [\beta]_{t=0} = 0$, we obtain:

$$[B]_{ss} = v_1/m_1$$

$$[\alpha]_{ss} = av_1/k_1 + k_2v_1/k_1m_2$$

$$C_1 = (av_1 + [\beta]_{ss}m_2 + [B]_{ss}k_2)/R_T$$

$$C_2 = (v_1 + s_1[\beta]_{ss} + D_1(m_2+s_1) + (X_0+X_1)(k_1+s_2))/(s_1-s_2)$$

$$C_3 = -([\alpha]_{ss} + C_1 + C_2)$$

where $m1 = V_2/K_B$, $m_2 = V_3/K_\beta$ and $[\beta]_{ss}$, D_1, X_0, X_1, s_1 and s_2 as defined by Equation III-12.

Following the rationale for the one auxiliary enzyme case, we choose approximations for the term $V_3/(K_\beta + [\beta])$ which will allow integration of the resulting differential equations. Thus, as before, we choose r_+ and r_- values which represent the highest and lowest value for the expression $V_3/(K_\beta + [\beta])$:

$$r_- = V_3/K_\beta \quad \text{and} \quad r_+ = \Psi/K_\beta$$

where $\Psi = V_3 - v_1$. Using the k_+ and k_- values from the one coupling enzyme case, we can obtain two equations which represent an upper and lower limit for the concentration of β over the time course of the experiment. Computer simulation (5) indicated that Equation III-13 is the more accurate and it is, therefore, presented in the text.

Chapter IV

Characterization of Chicken Atrium Mitochondrial Creatine Kinase Purified Using Transition State Analog Affinity Chromatography

Abstract

A method for preparing homogeneous mitochondrial creatine kinase from chicken ventricle is presented. The two column procedure, which can be completed in two days, uses Procion Red-agarose and Agarose-Hexane-ADP column chromatography. The latter column is run under conditions which promote the formation of a transition-state analog. The enzyme is a dimer composed of two 43,000 molecular weight subunits. The sequence of the first N-terminal 20 amino acids shows that the enzyme is different from the cytosolic isozymes but similar to human mitochondrial creatine kinase. The enzyme has an extinction coefficient of $\varepsilon_{280} = 2.1 \pm 0.4$ $mL \cdot mg^{-1} \cdot cm^{-1}$ and a specific activity of 124 IU/mL. The kinetic constants for the chicken heart mitochondrial isozyme are comparable to values for the canine and beef heart enzymes.

Introduction

Creatine kinase (EC 2.7.3.2) exists in nature in several isozymic forms (1). Two cytosolic subunits, M (muscle)[3] and B (brain), dimerize to form three different cytoplasmic isozymes: MM, BB and the hybrid MB (2). The MM isozyme of creatine kinase is found in mature skeletal muscle and mammalian myocardium, the BB isozyme in mammalian brain,

neural tissue and embryonic skeletal muscle and avian myocardium (1); and the hybrid MB creatine kinase appears in mammalian heart and skeletal muscle (1).

An additional CK isozyme, positively charged at pH 8.8, was initially reported in rat heart and brain mitochondria (3). Later studies revealed that human, beef, and rat heart, as well as rat brain, skeletal muscle and intestinal muscle mitochondria contain significant amounts of the mitochondrial isozyme of creatine kinase (MiMi-CK). Nominal amounts of MiMi-CK appear in rat and rabbit liver, kidney and testes (4). Although initial reports indicated that chicken heart mitochondria did not contain MiMi-CK (5), subsequent studies have confirmed the presence of the isozyme in small amounts (6). MiMi-CK is associated with the outer surface of the inner mitochondrial membrane in all tissues containing the isozyme (4, 7-10). Although all CK isozymes are dimeric and have similar kinetic constants (11, 12), the MiMi-CK subunits are different from the cytoplasmic CK subunits as shown by amino acid composition analysis (12-14), N-terminal sequence analysis (15, 16), lack of antisera cross reactivity (13, 14, 17, 18) and the absence of hybridization with the cytoplasmic subunits to form hetero-dimeric enzyme (13, 14, 17, 18).

Previously published purification procedures for MiMi-CK take advantage of an ionic-strength-dependent release of this enzyme from the inner mitochondrial membrane (12-14, 17-20). Incubating intact

3. Abbreviations used: B; brain type creatine kinase, BICINE; N,N-bis(2-hydroxyethyl)glycine, BSA; bovine serum albumin, CK; creatine kinase, Hepes; N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid, IU; 1 μmole of substrate converted per minute, M; muscle type creatine kinase, MiMi-CK; mitochondrial creatine kinase, MOPS; 3-(N-morpholino)propanesulfonic acid, NaDOC; sodium deoxycholate, PMSF; phenylmethylsulfonyl fluoride, TPCK; N-tosyl-L-phenylalanine chloromethyl ketone.

mitochondria in 100 mM sodium phosphate releases the enzyme (20) which is subsequently purified by anion exchange, gel filtration, and ATP-affinity chromatography (12-14, 17-20). Several of these procedures are rather lengthy and incorporate unnecessary steps. Varying specific activities (12, 14, 17) indicate that proteolytic products or enzymes with altered activity may be present in the final enzyme preparation, even though a single band is obtained after SDS-polyacrlyamide gel electrophoresis.

This paper presents a procedure for the purification of homogeneous chicken ventricle MiMi-CK using dye-ligand affinity and transition state analog chromatography. This two column procedure can be completed in approximately two days and gives a high yield as compared to previous procedures. The amino acid content, 20 N-terminal amino acid sequence, extinction coefficient, kinetic parameters, molecular weight and subunit composition are reported.

Materials and Methods

Materials: All chemicals, enzymes and creatine kinase assay kits were obtained from Sigma Chemical Co. (St. Louis MO) unless otherwise specified. Agarose-Hexane-adenosine 5'diphosphate, type 3 (Agarose-Hexane-ADP) was obtained from P.L. Biochemicals, Inc. (Milwaukee, WI). Sephraphore III cellulose acetate electrophoresis strips were purchased from Gelman Sciences, Inc. (Ann Arbor, MI). Common laboratory chemicals were reagent grade or better. NaDOC was prepared from deoxycholic acid recrystallized from hot 80% acetone. Chickens were obtained from the Department of Animal Science, Michigan State University.

Enzyme Assays and Protein Determinations: CK activity was determined spectrophotometrically at 340 nm at 30°C using the CK assay mix from Sigma (21, 22). The concentration of cytochrome aa_3 was determined from the differences in absorbance of the reduced minus oxidized spectra at 602 minus 630 nm ($\Delta\varepsilon$ = 24 $mM^{-1}cm^{-1}$, 23). Protein concentrations were determined by fluorescamine assays (24) using bovine serum albumin as a standard.

Cellulose Acetate Electrophoresis: CK isozymes were separated on 2.5 X 17 cm Gelman Sephraphore III cellulose polyacetate electrophoresis strips in 0.06 M Tris-barbital, pH 8.8, 25 mM 2-mercaptoethanol as previously described (20). The electrophoresis buffer contained 1% Triton X-100 to prevent CK from sticking to the strips. Electrophoresis proceeded for two hours at 300 V at 6°C. Following electrophoresis, the strips were stained for CK activity as previously described (25).

Polyacrylamide Gel Electrophoresis: Homogeneity and subunit molecular weights of crude and purified MiMi-CK were determined using sodium dodecyl sulfate-polyacrylamide gel (10% polyacrylamide, 0.26% bisacrylamide) electrophoresis (SDS-PAGE) in the presence of 2-mercaptoethanol according to the method of Laemmli (26). Molecular weight standards were a mixture of bovine serum albumin (68,000), chicken egg albumin (43,000), glyceraldehyde 3-phosphate dehydrogenase (36,000) and β-lactoglobulin (18,000). After electrophoresis, the gels were fixed for 1 hour in 10% acetic acid, stained for 2 hours in 0.25% Coomassie brillant blue R dissolved in 50% methanol plus 7.5% acetic acid and destained in 25% methanol plus 10% acetic acid.

Sequencing and Amino Acid Analysis: The sequence of the first 6 N-terminal amino acids and the total amino acid composition of MiMi-CK were provided by the Michigan State University Macromolecular Structure Facility. The first 20 N-terminal amino acid sequence was graciously provided by Dr. A. W. Strauss (Washington University School of Medicine, St. Louis, Mo).

The amino acid composition was determined as follows. A sample of protein was hydrolyzed under nitrogen in the presence of 6 N constant boiling HCl and phenol at 110°C for 24 hours. The sample was then neutralized by adding 10 µL of a 2:2:1 mixture (by volume) of ethanol:water:triethylamine, dried under vacuum and the neutralization procedure repeated once. The amino acid composition was measured, after derivitization with phenyisothiocyanate, on a PICO·TAG (Waters Instruments) column and the concentration obtained by peak integration. The results are reported as the mean of two runs. In order to prevent cysteine oxidation, the protein was carboxymethylated with iodoacetic acid according to Gracey (27) prior to amino acid composition analysis. No attempt was made to analyze for tryptophan.

Equilibrium Centrifugation: The molecular weight of native MiMi-CK was determined with a Beckman airfuge as previously described (28, 29). Including tritiated water in the protein solution made it possible to accurately determine the volume of each succeeding sample in the equilibrium gradient as suggested by Nickerson et al. (30).

Carboxypeptidase Y digestions: Time dependent digestions of MiMi-CK with carboxypeptidase Y were performed by incubating 75 µg of carboxypeptidase Y with 170 µL of 0.53 µg/mL MiMi-CK in 10 mM MES, 2% glycerol, 25 mM 2-mercaptoethanol (pH 6.0) and and 20 µM pepstatin A (31) at room temperature. The reaction was stopped at the indicated time points by adding PMSF to a final concentration of 2 mM.

Determining Kinetic Constants: Kinetic parameters for MiMi-CK were determined using coupled enzyme reactions at 30°C. The forward reaction (creatine phosphate production) was measured using excess pyruvate kinase and lactate dehydrogenase in a buffer containing 10 mM MOPS, 2.5 mM magnesium acetate, 0.1 mM EDTA, 50 mM potassium acetate, 2.5 mM dithiothreitol, 2.5% glycerol, 0.48 mM NADH, and 1.8 mM phospho(enol)pyruvate at pH 7.0. The reverse reaction (creatine production) was measured using hexokinase and glucose 6-phosphate dehydrogenase in a buffer containing 25 mM MOPS, 75 mM sucrose, 225 mM mannitol, 3.5 mM magnesium acetate, 1 mM EDTA, and 2 mM dithiothreitol at pH 7.4. The amount of coupling enzymes needed to perform these assays was determined using Equations II-22 and II-30 (Chapter II) for pyruvate kinase and lactate dehydrogenase, and Equations III-14 and III-20 (Chapter III) for hexokinase and glucose 6-phosphate dehydrogenase. The $MgATP^{-2}$ and $MgADP^{-}$ concentrations were calculated using the equilibrium constants for the formation of these species (32).

Purification Procedure

All purification steps were performed at room temperature unless otherwise noted. Glycerol or Triton X-100 was used throughout this study

to prevent loss of enzyme by adsorption to the walls of vessels or chromatographic matrices (35). The pH of all buffers was measured at room temperature.

Isolating Mitochondria and Preparing Mitoplasts: Mitochondria were isolated from eight 6-8 week old white leghorn chickens in ice cold 75 mM sucrose, 222 mM mannitol, 5 mM EDTA, 25 mM 2-mercaptoethanol, 2 mM PMSF, 0.1 mM TPCK (Buffer A). Extracting the mitochondria twice increased the yield (23). Mitoplasts were prepared from mitochondria using digitonin (Chapter IV).

Releasing MiMi-CK from Mitoplasts: After washing mitoplasts with Buffer A, the pellet was resuspended in 15 mL of 50 mM sodium phosphate, 10 mM Tris (pH 8.0) in Buffer A and allowed to incubate at 30°C for 7 minutes (see Chapter V). The mitoplasts were centrifuged at 8,000 x g for 10 minutes and the supernatant was saved. This procedure was repeated and the pooled supernatants were added to 70 mL of 25 mM 2-mercaptoethanol, 1.43% NaDOC, 0.36% (w/v) Triton X-100 and the pH adjusted to 8.2. This solution has a specific activity of 1.5 IU/mg (Table IV-1).

Procion Red-agarose Chromatography: The 5 mL Procion Red-agarose column was washed with 50 ml of 1% (w/v) sodium dodecyl sulfate and subsequently with 50 mL each of solutions made up of 30:5:5:80, 85:5:5:5 and 20:0:0:80 of acetone:triethylamine:acetic acid:water (33). The column was then washed with 50 mL water and equilibrated with 20 mM Tris-HCl (pH 8.2), 1 mM EDTA, 25 mM 2-mercaptoethanol, 0.25% (w/v) Triton X-

100, 2 mM PMSF and 0.1 mM TPCK (Buffer B) plus 1% (w/v) NaDOC. The sample from step b was loaded directly onto the Procion Red-agarose column and washed with 50 mL 1% NaDOC in Buffer B to remove contaminating BB-CK and other non-binding proteins. MiMi-CK is then eluted from the column with 2.5 M NaCl in Buffer B. Occasionally the first fraction contains a red pigment which persists during subsequent purification steps if not removed. For this reason, it is useful to collect 3 or 4 small fractions (1-2 mL each) at the beginning of the elution and discard those with a red pigment. The fractions containing activity (normally the first 40 mL) are pooled and dialyzed overnight against two changes (750 mL each) of 20 mM Hepes (pH 7.4), 1 mM EDTA, 25 mM 2-mercaptoethanol, and 0.25% (w/v) Triton X-100 (Buffer C) at 6^{o}C to remove the NaCl. The pool of CK activity from the Procion Red-agarose column contains only the MiMi-CK isozyme (data not shown) and has a specific activity of 11 IU/mg (Table IV-1).

Affinity Chromatography: The 2 mL Agarose-Hexane-ADP column was washed in the same manner as the Procion Red-agarose column and equilibrated with 50 ml of Buffer C. Following dialysis of the sample from the Procion Red-agarose column, the pool of MiMi-CK activity was applied to the Agarose-Hexane-ADP column and the column was washed with 50 mL of Buffer C to remove non-bound proteins. Washing with 50 mL Buffer C containing 5% glycerol instead of Triton X-100 removes Triton X-100 from the final sample. The disappearance of Triton X-100 can be followed by monitoring the drop size until it appears constant (change of surface tension). The column is then washed with 50 mL of 20 mM BICINE (pH 8.0), 25 mM 2-mercaptoethanol, 2% (v/v) glycerol, 20 mM creatine, 30 mM KNO_3, 2 mM

Table IV-1: Purification summary for the preparation of MiMi-CK.

Purification Step	Volume (mL)	[Protein] (mg/mL)	Total activity (IU)	Specific[a] activity (IU/mg)	Yield (%)
Crude solution	200	11.9	11,200	---	100[b]
Mitochondria	5	29.7	146	1	33
Released enzyme	100	0.36	53	2	12
Post Procion Red-agarose	40	0.11	46	11	10.3
Final enzyme	3.1	0.10	34	350[c]	7.5

Note: a. The specific activity is based on the fluorescamine procedure using BSA as a standard . b. 100 percent yield is based on the observation that 4% of the total ventricular CK activity is due to the mitochondrial enzyme (data not shown). c. Determining the protein concentration by several spectrophotometric methods gives a specific activity of 124 IU/mg (see Table IV-2).

Figure IV-1: ADP (Transition state analog) column profile.

The dialyzed peak fractions from the Procion Red-agarose step were loaded on a 2 mL Hexane Agarose-ADP column in Buffer C. The column was washed with Buffer C and Buffer C containing 5% (v/v) glycerol instead of TX-100. Washing with Buffer D (arrow) shows a large release of protein (o) and a small release of enzyme activity (●). After the activity has decreased to zero, the enzyme is eluted with a 20 mL gradient of 0 - 0.6 M NaCl. The NaCl concentration (□) is estimated from the conductivity of the fractions. Fractions at a volume of 156.8 to 167 mL were pooled to give the final purified enzyme.

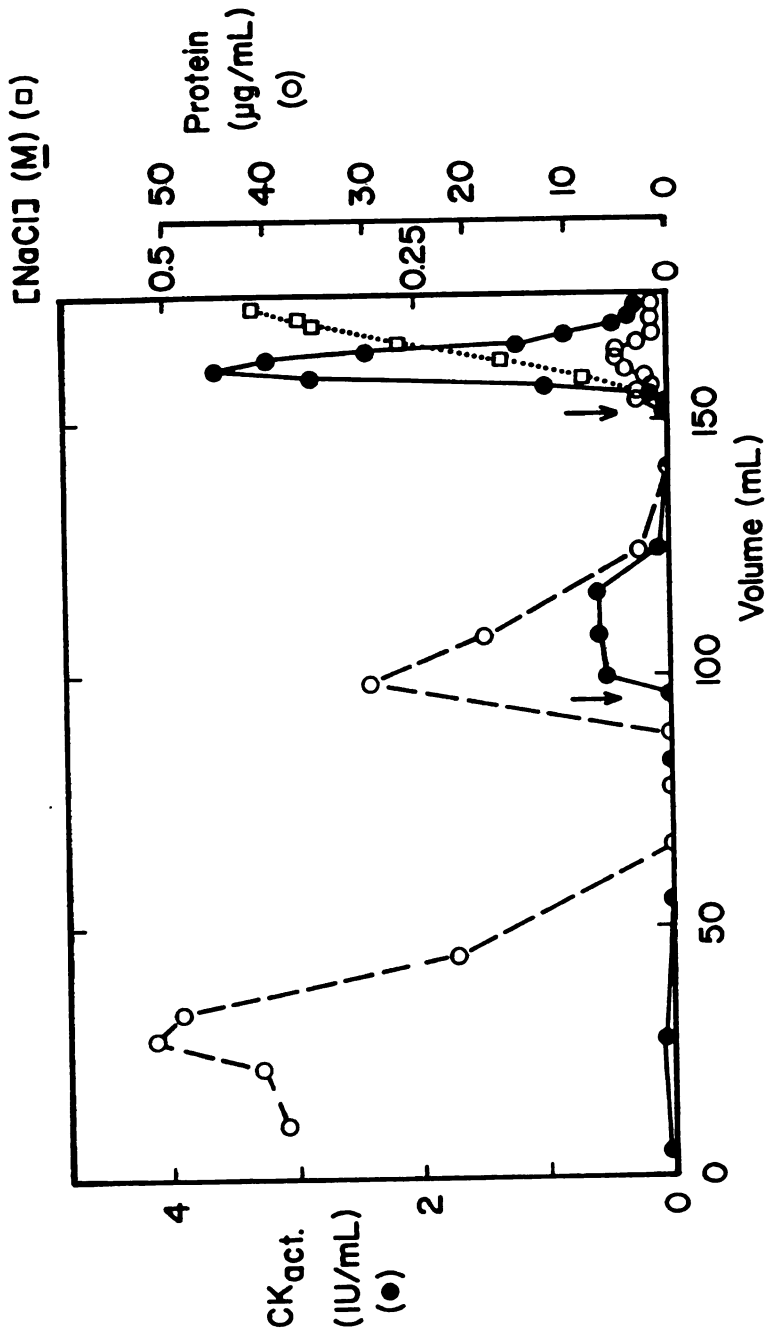

[NaCl] (M) (□)
Protein (μg/mL) (○)
0.5
0.25
0
50
40
30
20
10
0
150
100
50
0
Volume (mL)
4
2
0
CK act. (IU/mL) (●)

Figure IV-2: SDS-Polyacrylamide Gel Electrophoresis of Samples at various stages of purification.

Samples were loaded on a 10% polyacrylamide gel according to the procedure outlined in the Methods section. Lanes 1,2 7 and 8 are standards with molecular weights indicated on the Figure. Other lanes are as follows: Lane 3, enzyme released from the mitoplasts; Lane 4, dialyzed post Procion Red-agarose peak fractions; Lane 5, proteins released from Hexane Agarose-ADP column after washing with Buffer D; Lane 6, purified MiMi-CK.


1 2 3 4 5 6 7 8

$MgCl_2$ (Buffer D, see reference 36). This wash releases approximately 25% of the bound enzyme (Table IV-1). The CK activity in the eluant should be zero before the NaCl gradient is applied (Figure IV-1). Purified MiMi-CK is eluted in a single peak from the Agarose-Hexane-ADP column with a 20 mL gradient of 0-0.6 M NaCl in Buffer D. Approximately 1 mL fractions are collected. Contaminating protein, when present, is located in the first few fractions of the gradient and consequently these fractions are monitored closely for specific activity.

Concentrating and Storing the Enzyme: The pooled fractions from the ADP column are placed in a dialysis bag which is surrounded by solid sucrose suspended on a piece of cheese cloth in a covered chamber. The solution is allowed to concentrate for 2-3 hours and is then dialyzed against 1 liter of 10 mM MOPS (pH 7.2), 2% (v/v) glycerol, 25 mM 2-mercaptoethanol, and 0.1 mM EDTA overnight at $6^{o}C$. The enzyme can be stored at $4^{o}C$ for longer than 4 months without noticeable loss of kinetic or binding activity provided the 2-mercaptoethanol concentration is kept high (sealed tubes with minimal opening and closing). This enzyme has a specific activity of 350 IU/mg when the protein is measured by fluorescamine (Table IV-1) using BSA as a standard.

Results

Procion Red-Agarose Chromatography: Chromatographing MiMi-CK on a Procion Red-agarose column in the presence of Triton X-100 requires the addition of 1% (w/v) NaDOC because MiMi-CK fails to bind to Procion Red-agarose in 0.25% (w/v) Triton X-100. The NaDOC presumably forms mixed charged micelles with Triton X-100 which excludes the dye from the

micelle (37). The Procion Red dye is now free to react with MiMi-CK: BB-CK does not bind to the column. Procion Red-agarose chromatography, like cibacron Blue-agarose chromatography (35) affords a convenient method for separating BB-CK from MiMi-CK. Note that the elution buffer contains NaCl but not NaDOC which is not required for the elution as it functions to enable MiMi-CK binding to the dye.

Transition State Analog Affinity Chromatography: Using an Agarose-Hexane ATP affinity column, as originally used by Hall et al. (12, 20) to purify bovine heart MiMi-CK, failed to yield homogeneous enzyme. However, using an Agarose-Hexane-ADP column and conditions which promote the formation of a transition state analog yielded homogeneous enzyme. The initial wash of the column with Buffer D results in a 25% loss of the enzyme activity (Figure IV-1, Table IV-1). This loss is not due to overloading of the column because the dialyzed enzyme fails to bind to a clean Agarose-Hexane-ADP column equilibrated with Buffer D. This non-binding enzyme was not studied further. Combining fractions with a specific activity greater than 200 IU/mg gives a preparation which shows a single band on an SDS-polyacrylamide gel (Figure IV-2).

Purity and Extinction Coefficient: Three independent criteria show that the purified enzyme is homogeneous. A single band is present after SDS-polyacrylamide electrophoresis of 15 μg of enzyme (lane 6, Figure IV-2). The specific activity of the peak fractions from the ADP column were constant (Figure IV-1). The first cycle of the amino acid sequencing yielded a single N-terminal amino acid >98% pure.

Determining the specific activity of MiMi-CK depends on the choice

Table IV-2: Measuring MiMi-CK protein by spectrophotometric methods.

Procedure (reference)	Extinciton coefficient ($mL \cdot mg^{-1} \cdot cm^{-1}$)	Protein concentration BSA ($\mu g/mL$)	MiMi-CK ($\mu g/mL$)
Fluorometric	-----	80.0[a]	10.4 ± 0.2
Spectrophotometric:			
A_{205} (38)	32.3	87.2	31.5
$A_{215}-A_{225}$ (39)	6.94	81.6	29.5
$A_{224}-A_{233}$ (40)	4.74	70.5	28.3
A_{280} (41)	2.43[b]	80.1	28.8
A_{280} (41)	2.52[c]	79.8	27.7
Mean spectrophotometric:		79.8 ± 6.0	29.2 ± 1.5

Note: MiMi-CK was dialyzed against 50 mM sodium phosphate (pH 7.4) containing 5% (v/v) glycerol for 4 hours to remove 2-mercaptoethanol. The BSA solution was prepared in 50 mM sodium phosphate (pH 7.4) containing 5% (v/v) glycerol. a. BSA at 80 µg/mL was used as a basis for both the fluorescamine and spectrophotometric methods. b and c. The extinction coefficients for these cases were calculated using b: $\epsilon_{280} = 34.148*(A_{280}/A_{205})-0.02$, and c: $\epsilon_{280} = 30.0*(A_{280}/A_{207})-0.05$.

Table IV-3: Amino acid composition of MiMi-CK.

Amino Acid	% of total[a]	Amino Acid	% of total[a]
Asp + Asn	13.1	Tyr	0.4
Glu + Gln	9.7	Val	4.7
Ser	6.0	Met	1.6
Gly	19.2	Cys	1.8
His	2.5	Ile	1.6
Arg	7.2	Leu	6.0
Thr	4.8	Phe	1.1
Ala	9.4	Lys	2.8
Pro	8.1		

Note: a. The amino acid composition is presented as a percentage of the total ananlyzed (tryptophan is not included).

Figure IV-3: N-Terminal sequences for creatine kinase from chicken tissues.

Chicken muscle (M) and brain (B) amino sequences are taken from reference 43. Cytoplasmic CK amino acids which are identical to the mitochondrial isozyme appear in bold type. See Methods section for experimental details. The 11 N-terminal amino acids from human heart MiMi-CK sequence are taken from reference 17.

Isozyme:

```
          1                   5                  10                  15
M:  Pro-Phe-Ser-Ser-Thr-His-Asn-Lys-His-Lys-Leu-Lys-Phe-Ser-Ala-

B:  Pro-Phe-Ser-Asn-Ser-His-Asn-Leu-Leu-Lys-Met-Lys-Tyr-Ser-Val-

Mi: (chicken)       Thr-Val-His-Glu-Lys-Arg-Lys-Leu-Phe-Pro-Pro-

Mi: (human)         Glu-Val-Cys-Glu-Cys-Thr-Ser-Leu-Phe-Pro-Pro
                     1                  5                  10

          16                  20                  25                  30
M:  Glu-Glu-Glu-Phe-Pro-Asp-Leu-Ser-Lys-His-Asn-Asn-(His)-Met-Ala

B:  Asp-Asp-Glu-Tyr-Pro-Asp-Leu-Ser- ? -His-Asn-Asn-(His)-Met-Ala

Mi: Ser-Ala-Asp-Tyr-Pro-Asp-Leu-Arg-Lys
                    15                  20
```

of both the method used to measure the protein concentration and the type of protein used for the standard curve (39 - 41). In order to minimize the problem of varied responses of proteins, we chose to measure the protein concentration using spectrophotometric procedures which are largely independent of the type of protein standard. The results of these measurements are presented in Table IV-2. Protein determinations using the fluorescamine method are included for comparison. Note that the response of MiMi-CK to fluorescamine is very different from BSA; fluorescamine underestimates the protein concentration by approximately 2.8 fold. The spectrophotometric methods give a specific activity for the pure enzyme of 124 IU/mg using the Sigma creatine kinase assay kit in the direction of creatine synthesis at pH 6.9. The extinction coefficients for the purified protein are $\varepsilon_{280} = 2.1 \pm 0.4$ mL$\cdot$mg$^{-1}\cdot$cm^{-1}, and $\varepsilon_{205} = 31.7 \pm 2.5$ mL$\cdot$mg$^{-1}\cdot$cm^{-1}.

Amino Acid Composition and Sequences: The amino acid composition of purified MiMi-CK, in percentages of the total amino acids present in the analysis, is presented in Table IV-3. Note that both glutamine and asparagine have been hydrolyzed to the carboxylic acids and are included with glutamic acid and aspartic acid, respectively.

The N-terminal sequence of the homogeneous enzyme is presented in Figure IV-3. For comparison the N-terminal sequences of the M and B isozymes from chicken and from human mitochondria are also presented. Although extensive homology exists between the two cytosolic subunits in the first 30 amino acids (19 amino acids are identical), the 20 amino acids from the N terminal of the chicken heart mitochondrial enzyme are clearly different from the cytosolic enzymes. The seven cytosolic CK

amino acids which are identical to the mitochondrial isozyme are indicated in bold type. Note that the MiMi-CK sequence has been shifted by 5 amino acids so that the greatest homology exists between the three sequences. When the 10 N-terminal amino acids for the two mitochondrial CK enzymes are compared to one another, 6 out of ten are identical (shown by underlining).

C-Terminal Studies: Figure IV-4 shows the results of experiments to define a binding domain for MiMi-CK. Treating intact protein with carboxypeptidase Y in the presence of an endopeptidase inhibitor pepstatein A (31) results in a rapid decrease in the catalytic activity but little, if any, alteration in the ability of MiMi-CK to bind to mitoplasts. These results agree with previous studies which localized active site essential amino acid(s) for the cytoplasmic M form of the enzyme near the C-terminus (42, 43).

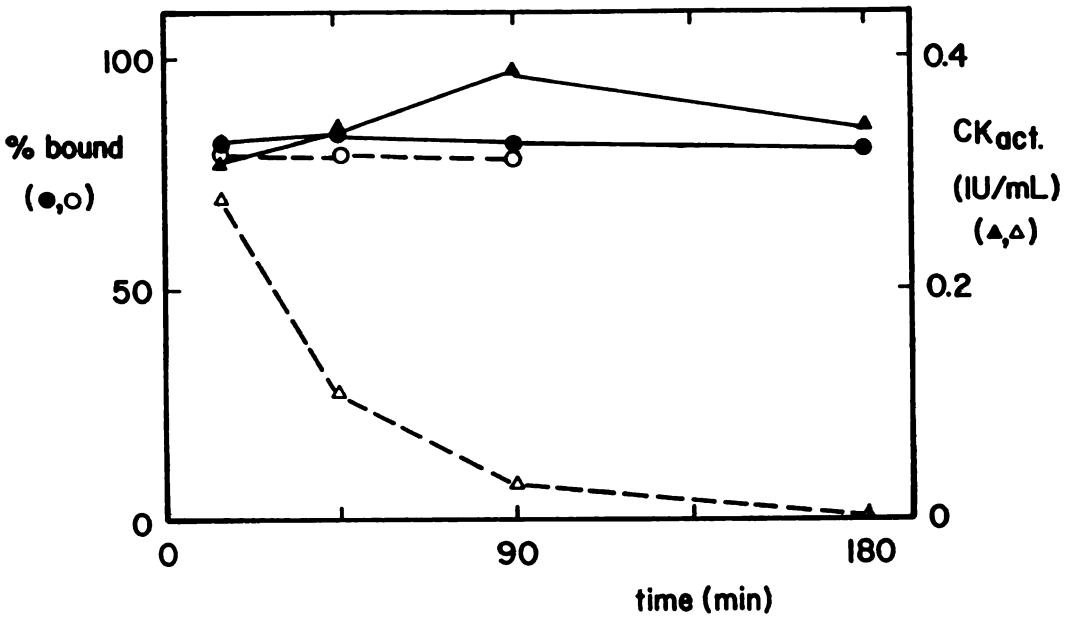
Kinetic Studies: Kinetic analysis of the purified enzyme gives results which are consistent with a rapid equilibrium random ordered mechanism identical to other creatine kinase isozymes. The kinetic constants for the various processes are presented in Table IV-4. The $\bar{K}_a$ value for creatine phosphate measured in our system is about 10 times higher than that reported by Hall et al. (13) but is close to the values reported for other CK enzymes (11). The K_m values for $MgATP^{-2}$ are approximately five fold lower than the cytosolic enzyme values in agreement with previous values for rat heart MiMi-CK (4).

Figure IV-4: Kinetic activity and binding ability of carboxypeptidase Y treated MiMi-CK.

After treatment of MiMi-CK with carboxypeptidase Y, approximately 0.7 IU of enzyme was added to mitoplasts (0.43 nmole cytochrome aa_3, prepared according to 36) in 0.1 mL of 75 mM sucrose, 225 mM mannitol, 0.2% BSA, 2 mM dithiothreitol and 10 mM MES (pH 6.4). The tubes were incubated at 30°C for 10 minutes, centrifuged at 8,000 x g for 10 minutes and the supernatants removed and assayed for enzyme activity. The percent bound represents the supernatant activity divided by the total activity present. The control tubes are represented by the solid symbols and the protease treated samples are represented by the open symbols.

Figure IV-5: Determining the molecular weight of MiMi-CK by equilibrium sedimentation.

MiMi-CK (0.16 IU) was placed in 150 µL of 12 mM HEPES, 25 mM 2-mercaptoethanol, 1 mMEDTA, 0.2 (w/v) dextran and 0.1 µCi of ^3H_2O (pH 7.4). The sample was centrifuged for 25.5 hours at 29.85 K rpm in a Beckman Airfuge at 6°C. Samples were carefully removed, the radioactivity counted to determine their volume and assayed for CK activity.


100
50
0
% bound
(●,○)
0.4
0.2
0
CK act.
(IU/mL)
(▲,△)
0
90
180
time (min)

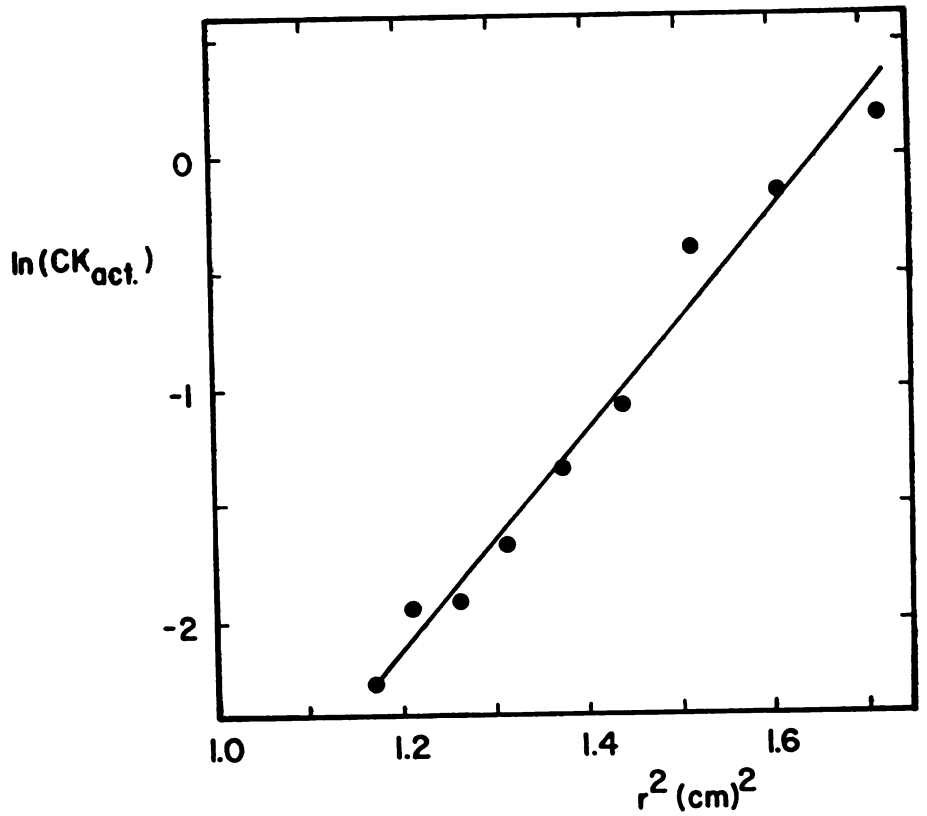

0
-1
-2
ln(CK act.)
1.0
1.2
1.4
1.6
r² (cm)²

Table IV-4: Kinetic constants for MiMi-CK.

Constant	Value (mM)
Forward reaction (pH 7.0):	
$\bar{K}_a$ ($MgATP^{-2}$)	0.25 ± 0.03
K_a ($MgATP^{-2}$)	0.13 ± 0.02
$\bar{K}_b$ (creatine)	20 ± 3
K_b (creatine)	10 ± 1
V_f	200 ± 20 IU/mg
Reverse reaction (pH 7.4):	
$\bar{K}_c$ (creatine phosphate)	6 ± 2
K_c (creatine phosphate)	2 ± 0.4
$\bar{K}_d$ ($MgADP^-$)	0.08 ± 0.02
K_d ($MgADP^-$)	0.03 ± 0.01
V_r	210 ± 34 IU/mg

Note. The constants are defined in Equation I-2. Reaction conditions are described in the Materials and Methods section. For the forward reaction, the creatine concentrations were 50 mM, 35 mM, 20 mM, 10 mM, 7 mM, 5 mM, and 3 mM and the MgATP concentrations were 790 μM, 561 μM, 370 μM, 197 μM, 98 μM, 56 μM, and 35 μM. For the reverse reaction creatine phosphate concentrations were 28 mM, 21 mM, 17.5 mM, 14 mM, 12.6 mM, 10.5 mM, 7.0 mM, 5.6 mM, 3.5 mM, 2.1 mM, and 0.7 mM; MgADP concentrations were 212 μM, 162 μM, 109 μM, 55 μM, 22 μM, and 11 μM. The magnesium nucleotide concentrations were calculated as described by Storer and Cornish-Bowden (32). The data were analyzed using a computer program (see Appendix A) and the results are presented as ± one standard deviation.

Molecular Weight Studies: A molecular weight of 84,000 ± 5,000 was determined by equilibrium centrifugation according to Pollet et al (29). This molecular weight agrees well with the subunit molecular weight estimated from its mobility relative to a standard protein mixture on a 10% acrylamide gel (Figure IV-2). Like other CK isozymes, the enzyme is a dimer consisting of subunits of 43,000 molecular weight.

Discussion

The protease inhibitors PMSF and TPCK are included in the initial stages of the preparation to prevent partial degradation of the protein which may give erroneous amino acid composition and N-terminal sequence results. These two inhibitors are used because of previous studies which indicated that proteases sensitive to these inhibitors are present in mitochondria (45, 46).

Although Procion Red-Agarose column chromatography increases the specific activity by only a small amount, this step has been retained for two reasons: a) it separates the cytosolic isozyme from the mitochondrial isozyme (33) and b) it removes proteins which contaminate the final preparation if this step is omitted. If MiMi-CK is prepared using only Agarose-Hexane-ADP, the resulting enzyme has a reddish color and low specific activity (data not shown). Deoxycholate is included in the initial steps of this procedure to allow the binding of the enzyme to the dye ligand column in Triton X-100 (37).

Several initial attempts to purify chicken ventricle MiMi-CK resulted in a preparation which was only approximately 85% pure and had a low specific activity. In an attempt to increase the specific activity, several different chromatographic matricies, which were used in other

MiMi-CK preparations, were assayed. These included: carboxymethyl Sephadex (13), phenyl Sepharose CL-4B (44), Cibacron Blue-agarose (35), amino-heptane Sepharose, Sephadex G-75 and Biogel P-200. These procedures either failed to increase the specific activity of the final enzyme or resulted in the loss of more than 80% of the enzyme.

The success of the Agarose-Hexane-ADP column depends on the use of a transition state analog complex obtained by mixing ADP, KNO_3 and creatine in the presence of $MgCl_2$. Here the nitrate ion mimics the planar transition state phosphate configuration and a strong complex is formed (36). The ADP column permits immobilization of the enzyme to the column under conditions which prevent the binding of two major contaminants (Figure IV-2). Note that a large amount (25%) of the activity is lost during this step. Although the reason for this loss is unexplained, it is probable that the released enzyme has an altered active site because it fails to bind under conditions which promote the formation of a transition state analog.

A comparison of the characteristics of MiMi-CK from four different animal mitochondria is presented in table IV-5. The molecular weight and number of subunits are the same for all four species but the specific activity varies considerably between species. This variation may be, in part, due to the different methods used to measure activity and protein concentration. However the specific activities measured using the commerical kits are more constant. The pI values for the chicken heart is similar to that of the dog heart (as estimated by the direction of enzyme migration at pH 8.8 on cellulose acetate electrophoresis) but much higher than the human heart enzyme. The kinetic constants for the chicken heart enzyme are closer to those

Table IV-5: Characteristics of MiMi-CK purified from various sources.

	Chicken Heart[a]	Human Heart[b]	Beef Heart[c]	Dog Heart[d]
Mr	86,000	84,000[e]	64,000	84,000
subunits	2	2	2	2
pI	> 9	6.8	N.D.	> 9
Specific Activity				
CrP_i synthesis	202	15 (T?)	N.D.	N.D.
Cr synthesis	124 (30^oC)[f]	45 (T?)[g]	111 (30^oC)[h]	310 (30^oC)
	210 (30^oC)[i]	410 (30^oC)[j]		
$A^{1\%}_{280}$	21 ± 4	11.7	N.D.	N.D.
Apparent K_m values[k]				
$MgATP^{-2}$	0.125 mM	1.7 mM	0.56 mM	N.D.
MgADP	0.031 mM	0.15 mM	0.015 mM	N.D.
Cr	10 mM	8 mM	4.5 mM	N.D.
CrP_i	2 mM	3 mM	0.31 mM	N.D.

Note. a. data from this study, b. from Grace et al. (14) and Blum et al. (17) c. from Hall et al. (12), d. from Roberts and Grace (13) and Roberts (18), e. Grace et al. (14) report a Mr = 66,000 by filtration on Sephacryl S-200 but measure a subunit size of 41,000 on SDS polyacrylamide gels, f. determinded using CPK single vial assay (Sigma) pH 6.8, g. determinded using CK/UV-assay (Merck), pH 6.8, h. determined using CPK-MaxPak (Calbiochem) pH 6.8, i. determined from V_{max} at pH 7.4, j. determined by Rosalki method (48) at pH 6.8, k. measured at saturating concentrations of the other substrate, N.D. not determined.

measured for the beef heart enzyme and other non purified MiMi-CK enzymes (4) than the human heart enzyme which appears to have values similar to the MM isozyme (11).

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Chapter V

Association of Avian Mitochondrial Creatine Kinase with the Inner Mitochondrial Membrane

Abstract

The stoichiometry and dissociation constant for the binding of homogeneous chicken heart mitochondrial creatine kinase (MiMi-CK) to mitoplasts was examined under a variety of conditions. The effect of salts and substrates on the release of MiMi-CK from mitoplasts suggests that this interaction is ionic in nature. Adriamycin competitively inhibits the binding of MiMi-CK to mitoplasts suggesting that MiMi-CK and adriamycin compete for the same binding site. However fluorescence measurements show that adriamycin binds to MiMi-CK suggesting that this latter process, and not the binding of adriamycin to the IMM, may be responsible for the observed competition between MiMi-CK and adriamycin. Titrating mitoplasts with homogeneous MiMi-CK at different pH values shows that the binding of MiMi-CK to mitoplasts can be described by a pH dependent equilibria involving a group(s) on either the membrane or the enzyme with a pKa of about 6. Extrapolating these titrations to infinite MiMi-CK concentration gives 14.6 IU bound per nmole cytochrome $\underline{aa}_3$ corresponding to 1.12 moles MiMi-CK/mole cytochrome $\underline{aa}_3$ assuming that pure enzyme has a specific activity of 124 IU/mg. Chicken heart mitochondria contain, after isolation, 2.86 $\pm$ 0.42 IU/nmole cytochrome $\underline{aa}_3$ showing that only 22% of the MiMi-CK

binding sites are occupied in intact mitochondria. Titrating respiring mitoplasts with carboxyatractyloside gives a value of 3.3 moles ADP/ATP nucleotide translocase per mole cytochrome $\underline{aa}_3$. Therefore, chicken heart mitoplasts can bind about 1 mole of MiMi-CK per 3 moles nucleotide translocase maximally; in normal chicken heart mitochondria about 1 mole of MiMi-CK is bound per 13 moles nucleotide translocase.

Introduction

The mitochondrial isozyme of creatine kinase (MiMi-CK, adenosine-5'-triphosphate creatine phosphotransferase; EC 2.7.3.2)[3] is located on the outer surface of the inner mitochondrial membrane (IMM) (1,2) and is demonstrably different from either of the cytosolic forms as shown by lack of antibody cross reactivity and hybridization studies (3). Evidence for the participation of this isozyme in Bessman's creatine phosphate shuttle (4,5) comes from studies showing that both MiMi-CK and the adenine nucleotide translocase have a lower apparent K_m value for the nucleotide substrate when both enzymes function in a coupled reaction (6-12). These studies also demonstrate increased rates of creatine phosphate synthesis are at identical solution ATP concentrations when the MiMi-CK reaction is coupled to oxidative phosphorylation as compared to the soluble enyzme. The data suggest that MiMi-CK, presumably bound near the adenine nucleotide translocase on the IMM, may have preferential access to the ATP produced by

3. Abbreviations used: Ap_5A; P',P^5-Di(adenosine-5')pentaphosphate, BSA; bovine serum albumin, DTT; dithiothreitol, EGTA; ethyleneglycol-bis-(2-aminoethyl ether) N,N,N',N'-tetraacetic acid, IMM; inner mitochondrial membrane, MiMi-CK; mitochondrial creatine kinase, MES; 2-(N-morpholino)ethanesulfonic acid, MOPS; 3-(N-morpholino)propanesulfonic acid.

oxidative phosphorylation. The ADP produced by the creatine kinase reaction is then taken up into the mitochondrial matrix via the adenine nucleotide translocase and the creatine phosphate diffuses out of the mitochondria. This sequential utilization of substrates may have an important role in muscle bioenergetics.

Dystrophic chicken breast muscle mitochondria contain significantly lower amounts of MiMi-CK when compared to age-matched control birds (14). Interestingly, these mitochondria show both a decreased rate of creatine phosphate production and a lower ATP trapping efficiency. To ascertain the quantitative significance of the reduced level of MiMi-CK on the functional coupling between MiMi-CK and the nucleotide translocase in muscle mitochondria, we examined the function of MiMi-CK in chicken heart mitochondria because they contain only 5% as much MiMi-CK as normal breast muscle mitochondria (11).

Prior to undertaking this study, however, it is necessary to determine the conditions which influence the binding of MiMi-CK to the IMM. Previous studies of this interaction are either difficult to interpret (13, 15-18) or not sufficiently complete to allow a detailed description of the optimal binding conditions (14, 19). Although results of the studies of the release of MiMi-CK from whole mitochondria (15-17) are difficult to interpret in light of the fact that MiMi-CK interacts with the outer surface of the IMM, MiMi-CK can be solubilized from either whole mitochondria or from mitoplast preparations by increasing ionic strength (12, 14-17). Other evidence provided by Muller et al (18) also shows that adriamycin, a cationic lipid (20-22), releases MiMi-CK presumably from the negatively charged cardiolipin head groups on the inner membrane (23). Schlame and

Augustin (19) confirmed the binding site by looking at the release of MiMi-CK induced by the action of phospholipases.

This paper reports the effects of salts, enzyme substrates, pH, and adriamycin on the binding of MiMi-CK to IMM. Studying the binding of homogeneous MiMi-CK provides a direct determination of the effect of pH and adriamycin on the stoichiometry and dissociation constant for its interaction with IMM. The effect of adriamycin on the binding of MiMi-CK is more complex than previously reported (18,19).

Materials and Methods

Preparation of Avian Heart Mitoplasts: Mitochondria were prepared daily from avian hearts as previously decribed (24). The final mitochondrial pellet in 1 mL total volume of S/M buffer (75 mM sucrose, 225 mM mannitol, 0.1 mM EGTA, 0.2% fatty acid free BSA) gives a suspension 5-8 μM in cytochrome aa_3 as determined from the difference between the absorbance of the reduced (602 nm) and the oxidized (630 nm) forms ($\Delta\varepsilon$ = 24 $mM^{-1}cm^{-1}$, 24). Mitoplasts were prepared fresh each day essentially as described by Allman et al (25). Digitonin, 0.6 mg per nmole cytochrome aa_3, was added dropwise to the resuspended pellet with rapid mixing. The resulting suspension was incubated for 10 minutes on ice, diluted 10 fold with S/M buffer, and centrifuged at 8,000 X g for 10 minutes. The mitoplast pellet was subsequently washed two times with S/M buffer and resuspended in a final volume of 0.85 mL of S/M buffer. Using 0.6 mg digitonin/nmole cytochrome aa_3 results in a mitoplast preparation with less than 5% of the original monoamine oxidase activity and adenylate kinase activity. The malate dehydrogenase activity associated with the mitoplast pellet was $\geq$ 95%

of the original mitochondrial value and the respiratory control ratios were $\geq$ 7 demonstrating intact inner membranes. Mitoplasts contain 2.66 $\pm$ 0.34 IU MiMi-CK per nmole cytochrome aa_3 compared to 2.86 $\pm$ 0.42 IU in intact chicken heart mitochondria.

Purification of MiMi-CK: MiMi-CK was prepared from eight 6-8 week old white leghorn chicken hearts according to the procedure of Chapter IV. The final enzyme solution was dialyzed against 10 mM MOPS, 25 mM 2-mercaptoethanol, 2% glycerol and stored at 4°C for greater than 4 months without noticeable loss of binding ability or catalytic activity provided the 2-mercaptoethanol concentration remains high.

Titrating Mitoplasts with MiMi-CK: Unless otherwise indicated, the binding of MiMi-CK to mitoplasts was performed in 75 mM sucrose, 225 mM mannitol, 10 mM MOPS (pH 7.4), 25 mM 2-mercaptoethanol, 0.2% BSA and 50 µM Ap_5A. Mitoplasts, buffer, and various effectors (final volume 0.1 mL), were mixed in 0.4 mL Eppendorf centrifuge tubes and incubated at 30°C for 10 minutes. The tubes were spun at 8,000 X g for 10 minutes at 25°C and the supernatants were immediately removed and assayed for creatine kinase activity. Pelleted activities were not commonly measured but initial experiments demonstrated that the activities of the pellets and supernatants always added up to approximately 100% of the initial activity.

Titrating Adenine Nucleotide Translocase with Carboxyatractyloside: This procedure was performed essentially according to Forman and Wilson (26). From 0 to 8 moles of carboxyatractyloside per mole cytochrome

$\underline{aa}_3$ were added to mitoplasts (approximately 0.1 nmole cytochrome $\underline{aa}_3$) and incubated 2-3 minutes at 30°C in a 1.75 mL respiration chamber containing 5.8 m$\underline{M}$ pyruvate, 2.9 m$\underline{M}$ L-malate, in 225 m$\underline{M}$ mannitol, 75 m$\underline{M}$ sucrose, 20 mM inorganic phosphate pH 7.4. Oxygen consumption was initiated by the addition of 400 nmoles ADP. The rates of oxygen consumption (Clarke electrode, Yellow Springs Instruments) were recorded as the rate after the addition of ADP (state 3) minus the rate prior to ADP addition (pre-state 3).

Titrating Mitoplasts with Adriamycin: Solutions containing from 8 to 250 $\mu\underline{M}$ adriamycin were incubated with mitoplasts (0.35 $\mu\underline{M}$ cytochrome $\underline{aa}_3$) in 75 m$\underline{M}$ sucrose, 225 m$\underline{M}$ mannitol, 10 m$\underline{M}$ MOPS (pH 7.4), 25 m$\underline{M}$ 2-mercaptoethanol, and 0.2% BSA. Stock solutions of adriamycin were prepared fresh each day and proctected from light. Adriamycin concentrations were low enough so that insignificant amounts of the aggregated forms were present (22). The decrease in the concentration of adriamycin in the supernatant and its increase in the pellets was monitored using an ε = 13,200 $\underline{M}^{-1}cm^{-1}$ at 480 nm (28). The pellets were solubilized in Triton X-100 and sonicated for 10 minutes prior to measurement. Both procedures gave a concentration of bound adriamycin which did not deviate by more than 5%.

Other Procedures: Protein concentrations were determined by the fluorescamine procedure using BSA as a standard (28). Creatine kinase activities were measured by adding an aliquot of the enzyme solution to 0.3 mL of Sigma CK assay mix and recording the absorbance at 340 nm at 30°C. Malate dehydrogenase activity was measured in a 1 mL reaction

mix containing 0.25 mM NAD^+, 0.1 mM malate in 0.1 M Tris buffer pH 8.8 and recording the absorbance changes at 340 nm. Previously published procedures were used to monitor monoamine oxidase (29) and N-acetly glucoseaminidase (30) activity.

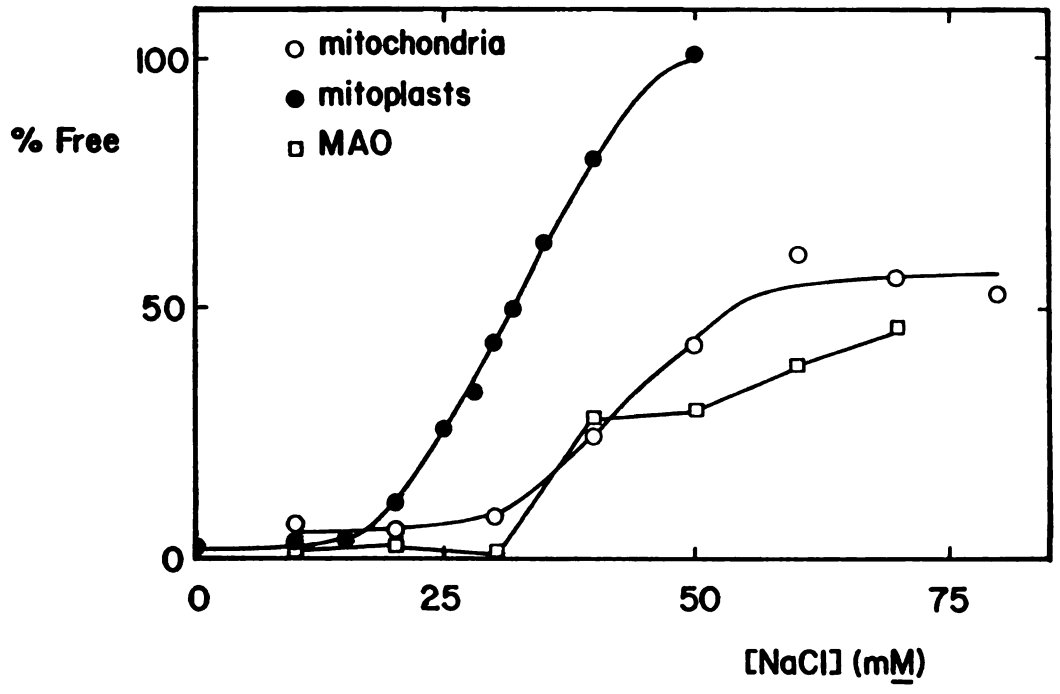
Results

Effects of Neutral Salts on the Interaction of MiMi-CK with Mitochondria and Mitoplast Preparations: The data in Figure V-1 shows that 50 mM NaCl releases greater than 95% of the endogeneous MiMi-CK from mitoplasts. However, when intact mitochondria are titrated with NaCl, less than 50% of the enzyme is released at 50 mM NaCl; 80 mM NaCl only releases 60%. The release of monoamine oxidase activity approximately parallels the release of MiMi-CK from intact mitochondria suggesting that the outer membrane interferes with the complete release of MiMi-CK. Cellulose acetate electrophoresis confirms that MiMi-CK is released (data not shown).

Figure V-2 and Table V-1 show the effect of other salts on releasing MiMi-CK from mitoplasts. Contrary to previously published results (16) showing that more than 20 min is required to establish an equilibrium between free and mitochondrial bound MiMi-CK at a given salt concentration, the equilibrium position is established in less than 2 minutes with mitoplast preparations. The solubilization process is readily reversible; dialyzing suspensions of mitoplasts with solubilized MiMi-CK in 50 mM phosphate against a buffer without phosphate results in rebinding of the enzyme to the IMM (data not shown). The concentration of several salts causing release of 50% of the total MiMi-CK activity are tabulated in Table V-1: the C_{50} values

Figure V-1: Release of MiMi-CK from mitochondria and mitoplasts.

Percent of total enzyme activity found in the supernatant liquid after incubating either mitochondria (0.14 nmole cytochrome $\underline{aa_3}$) or mitoplasts (0.093 nmole cytochrome $\underline{aa_3}$) are plotted as a function of the NaCl concentation. MiMi-CK (●, o) and monoamine oxidase activity (□) were assayed as described in the Methods section. The assay conditions were pH = 7.4, T = 30°C.


mitochondria
mitoplasts
MAO
% Free
100
50
0
0
25
50
75
[NaCl] (mM)

are expressed in molarities, the C_{IS} values are expressed as ionic strength. Although the C_{50} values vary from 50 mM for LiCl to 3.9 mM for sodium pyrophosphate, the C_{IS} values fall within a much narrower range (20-54 mM) suggesting that ionic strength is primarily responsible for the release of the enzyme and that the interaction of MiMi-CK with IMM is primarily electrostatic. However, since monovalent salts with lower viscosity B coefficients (KSCN, KCl) are slightly more effective at removing MiMi-CK from the IMM than salts with higher viscosity B coefficients (LiCl, $(CH_3)_4NCl$) (31) there may be a small non-polar effect in the binding.

The data in Table V-2 show that none of the various salts specifically release MiMi-CK from the membrane. Most of the specific activity values of MiMi-CK solubilized by several salts at concentrations equivalent to 2 times the C_{50} value are near 3 IU/mg consistent with the lack of a specific ion effect.

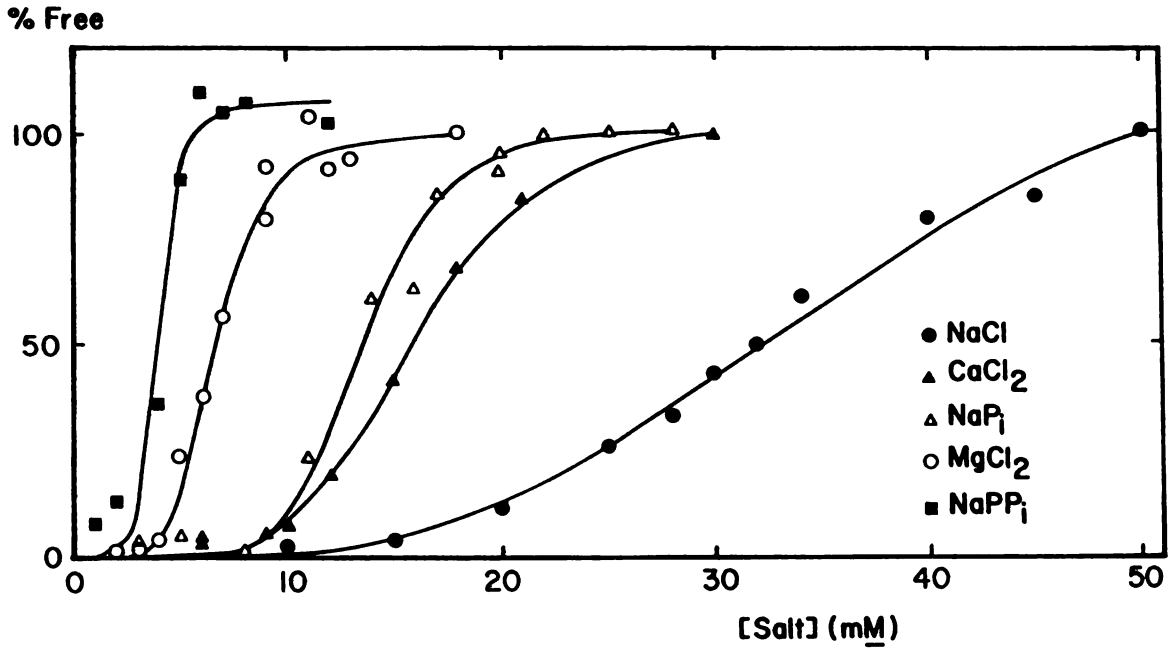
Effect of Substrates on the Interaction of MiMi-CK with Mitoplasts:
The effect of increasing substrate concentrations on the distribution between free and IMM bound MiMi-CK is presented in Figure V-3 and Table V-3. The C_{50} value for creatine phosphate is similar to the C_{50} value for sodium phosphate suggesting that the effect of creatine phosphate on releasing MiMi-CK is primarily due to an increase in ionic strength and not to a specific interaction with the enzyme. The lack of a significant effect of creatine, a neutral zwitterionic species which does not contribute to the ionic strength of the medium, on releasing MiMi-CK from mitoplasts is consistent with previous conclusions that electrostatic interactions are primarily responsible for the binding of

Figure V-2: Effect of increasing salt concentrations on the percentage of MiMi-CK released from mitoplasts.

Various concentrations of salts were added to mitoplasts (1.0 to 1.6 nM cytochrome aa_3) at pH 7.4. Supernatant liquids were assayed for activity after centrifugation as described in the Methods section.

Figure V-3: Release of MiMi-CK from mitoplasts with increasing substrate concentrations.

Conditions are identical to those described in Figure V-2.


% Free
100
50
0
0
10
20
30
40
50
[Salt] (mM)
NaCl
CaCl2
NaPi
MgCl2
NaPPi

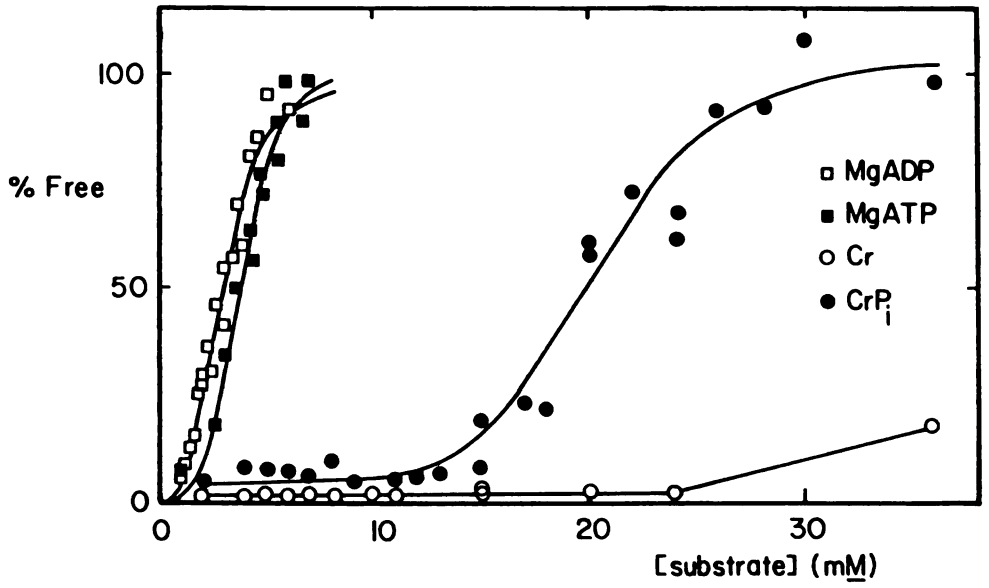

100
% Free
50
0
0
10
20
30
[substrate] (mM)
MgADP
MgATP
Cr
CrPi

Table V-1: C_{50} and C_{IS} values for the release of MiMi-CK from mitoplasts by various salts at pH 7.4.

Salts	C_{50} (mM)	C_{IS} (mM)
LiCl	50 ± 3	50
$(CH_3)_4NCl$	37 ± 1	37
NaCl	35 ± 1	35
KCl	31 ± 2	31
RbCl	30 ± 1	30
CsCl	30 ± 5	30
KSCN	25 ± 2	25
$MgCl_2$	7 ± 2	20
$CaCl_2$	16 ± 2	48
$BaCl_2$	8 ± 2	24
Na_2SO_4	18 ± 1	54
sodium phosphate	14 ± 1	35
sodium pyrophosphate	4 ± 1	25

Note: C_{50} values are the salt concentrations at which 50% of the endogeneous MiMi-CK is present in the supernatant liquid after centrifugation (see Methods section). The C_{IS} values are the ionic strength of the salt solution at the C_{50} value.

MiMi-CK with IMM. Adenine nucleotides also appear to promote release of MiMi-CK by increasing ionic strength. The nucleotides alone solubilize MiMi-CK at lower concentrations than the 1:1 nucleotide:$MgCl_2$ mixture even though the free nucleotide binds the enzyme with a much lower affinity (32). Furthermore, solutions containing 1:1 mixtures of nucleotide and $MgCl_2$ have lower ionic strengths than free nucleotide (33).

Stoichiometry and Dissociation Constant for the Interaction of MiMi-CK with Mitoplasts: Before determining the binding parameters for the interaction of MiMi-CK with mitoplasts, it was necessary to show that MiMi-CK interacts with mitoplasts and not with possible contaminating organelles such as lysosomes. To do this, mixtures of mitochondria or mitoplasts and exogenous MiMi-CK were centrifuged on a continuous sucrose gradient in order to separate the various components of the organelle preparation. As shown in Figure V-4a, MiMi-CK activity follows the peak of malate dehydrogenase activity (mitochondrial marker) and not the lysosomal marker N-acetyl glucosaminidase; the ratio of CK activity to malate dehydrogenase activity (MDH) is constant across the peak fractions. Figure V-4b shows the sedimentation profile for mitoplasts. Note that protein, malate dehydrogenase and creatine kinase migrate in the sucrose gradient as a single band. Also note that N-acetyl glucosaminidase activity is very low in the mitoplast preparations showing that digitonin treatment lyses the lysosomes as was previously reported (34).

A study of the interaction of purified MiMi-CK with mitoplasts as a function of pH gave the results shown in Figure V-5. Here the

Table V-2: Specific activity of MiMi-CK released by various salts at 2 X C_{50} concentrations.

Salt	Concentration[a]	Specific activity
	(mM)	(IU/mg)
NaCl	60	2.57
KCl	60	3.12
$MgCl_2$	14	3.10
$BaCl_2$	32	1.14
sodium phosphate	27	3.21
sodium pyrophosphate	8	3.21
Na_2SO_4	36	2.61
creatine phosphate	40	2.79
MgADP[b]	6	1.97
MgATP[b]	7	2.08
sodium deoxycholate[c]	0.5%	0.83

Note: a. The salt concentrations in these experiments were equal to 2 times the C_{50} value. b. $MgCl_2$ and nucleotide were mixed in a 1:1 molar ratio. c. Sodium deoxycholate at 0.5% solubilizes the mitoplasts so that the specific activity is based on the total mitoplast protein.

Table V-3: C_{50} and C_{IS} values for the release of MiMi-CK from mitoplasts by enzyme substrates.

Substrate	Charge	C_{50} (mM)	C_{IS} (mM)
ADP	-3	3.1 ± 1	15.5
ATP	-4	1.3 ± 1	21.4
creatine	0	????	---
creatine phosphate	-1.8	21 ± 3	50.7
MgADP[a]	several species	2.1 ± 1	14.4
MgATP[a]	several species	3.5 ± 1	14.5

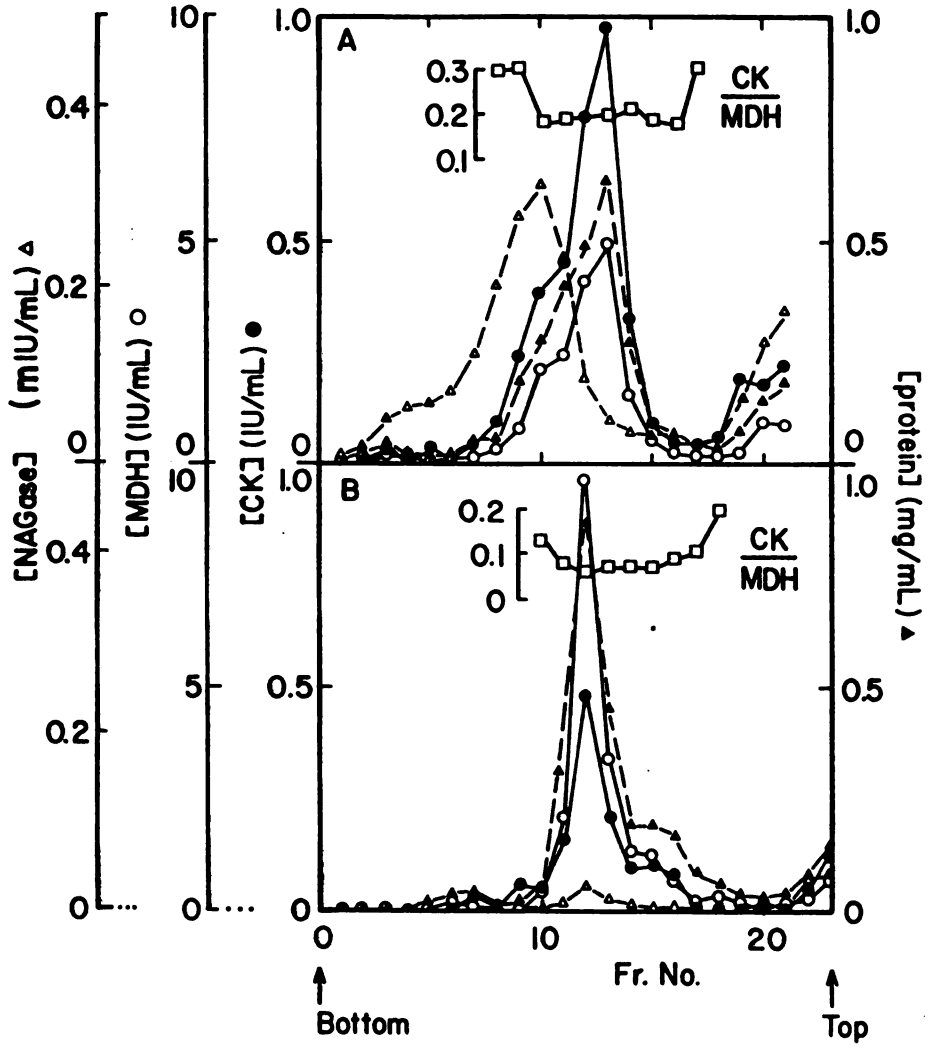
Note: C_{50} values are the concentrations at which 50% of the endogeneous MiMi-CK is present in the supernatant liquid after centrifugation (see Methods section). C_{IS} values are the ionic strength of the salt solution at the C_{50} value. a. $MgCl_2$ and nucleotides were mixed in a 1:1 ratio. The concentration of MgADP and MgATP at the C_{50} values are calculated from the equilibrium constants for these species (33). The C_{IS} values represent the total ionic strength of the solution which contains several salt species.

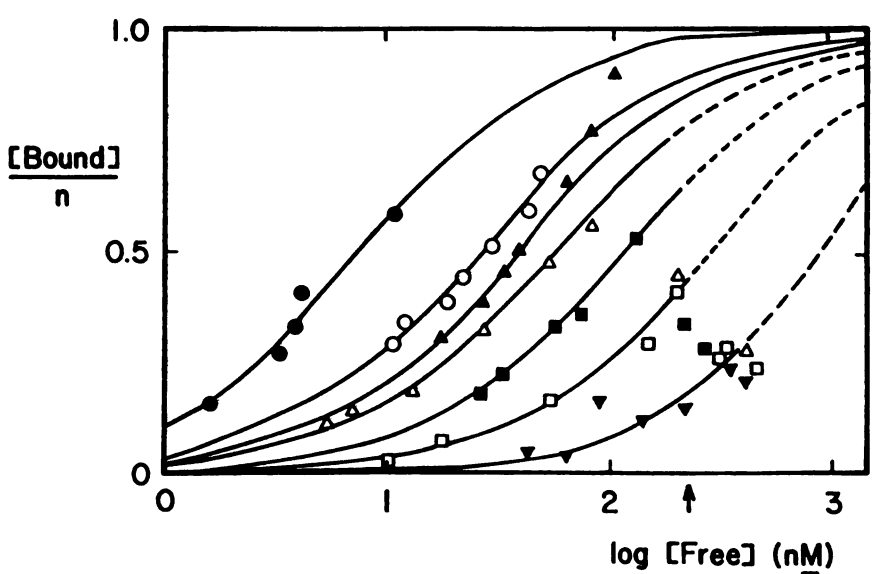
Figure V-4: Association of MiMi-CK with mitochondrial membranes.

MiMi-CK (97 nmoles) was added to (A) mitochondria or (B) mitoplasts (2.6 nmole cytochrome aa_3) in 75 mM sucrose, 225 mM mannitol, 0.2% BSA, 10 mM MOPS, and 2mM DTT (pH 7.4) in a total volume of 1.0 mL. This solution was layered over a 26 mL 30-65% sucrose (w/v) gradient (over a 3 mL 70% (w/v) sucrose cushion). Samples were then centrifuged at 25,000 rpm in a Spinco SW 25.1 rotor for 3 hours at 8°C. Fractions 1.5 mL were collected and assayed for MiMi-CK (●), malate dehydrogenase (o), lysosomal N-acetyl-β-d-glucosaminidase (▲) and protein (Δ). The ratio of MiMi-CK to malate dehydrogenase activity is shown over the peak fractions.

Figure V-5: Binding of purified MiMi-CK to mitoplasts: Klotz plot.

Varying amounts of MiMi-CK (final concentration 19 to 600 nM) were added to a fixed concentration of mitoplasts (0.29 μM to 0.43 μM cytochrome aa_3 depending on the experiment) at a final pH value of 5.9; ●, pH 6.2; o, pH 6.4; ▲,pH 6.8; Δ, pH 7.1; ■, pH 7.4; □, or pH 7.9; ▽. The buffer consisted of 7 mM MOPS, 7 mM MES, 7 mM Tris, 75 mM sucrose, 225 mM mannitol, 0.2% BSA, 2% glycerol, 25 mM 2-mercaptoethanol, and 50 μM Ap_5A. Other conditions are described in the Methods section. The lines are theoretical curves drawn using parameters obtained from regression analysis (38).


A
B
CK
MDH
[NAGase] (mIU/mL) △
[MDH] (IU/mL) ○
[CK] (IU/mL) ●
[protein] (mg/mL) ▲
Fr. No.
Bottom
Top


[Bound]
n
log [Free] (nM)

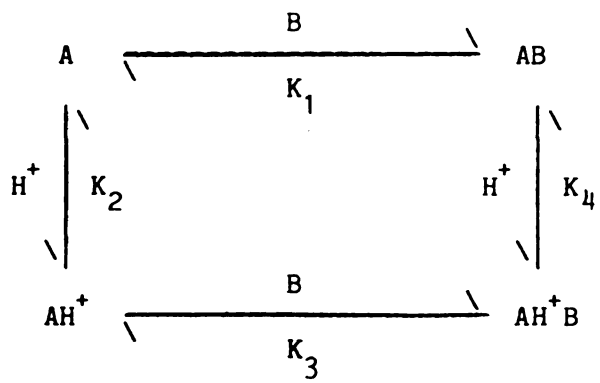
number of moles of MiMi-CK bound to the IMM per mole cytochrome oxidase ($\bar{v}$), divided by the maximum number of moles of MiMi-CK bound per mole cytochrome oxidase (n) is plotted as a function of the log of the free enzyme concentration according to Equation V-1.

[V-1] $$\bar{v}/n = [\text{MiMi-CK}]_{free}/([\text{MiMi-CK}]_{free} + K_D^{app})$$

The value of n was determined for each pH value from a weighted linear regression analysis of the data using a computer program (Appendix A). Several points are evident from Figure V-5. First, the binding is described by a rectangular hyperbola. Second, the lower the pH value, the greater the affinity of MiMi-CK for the IMM. Third, for any given pH value, when free MiMi-CK is above 330 n$\underline{M}$, the amount of enzyme bound is no longer described by Equation V-1: this altered binding behavior is presumably due to the highly cooperative concentration dependent polymerization of the enzyme (35).

The increased affinity of MiMi-CK for the IMM at lower pH values can be mathematically described by the simplified equilibria of Equation V-2 (see 36). In this system, protonation of A results in an altered affinity for B.

[V-2]



In Equation V-2 K_1 and K_3 are the dissociation constants for B binding to A in the unprotonated (A) or protonated (AH^+) form and K_2 and K_4 represent the dissociation constants for protons (H^+) binding to A in the free (A) or bound (AB) form. Solving Equation V-2 gives a general equation which relates the bound and free concentrations of A by a function similar to that of Equation V-1 where the K_D^{app} value is given by Equation V-3.

$$[V\text{-}3] \qquad K_D^{app} = \frac{[A + AH^+][B]}{[AB + AH^+B]} = \frac{K_3 (K_2 + [H^+])}{(K_4 + [H^+])}$$

Because of the complexity of the MiMi-CK:IMM system, it is difficult to determine whether MiMi-CK, or the IMM binding site, or both interactants are protonated. Irrespective of the mechanism of the protonation reaction, the binding equilibria can be described by Equation V-2. In order to determine the four equilibria governing the binding reaction, the pH dependent apparent K_D values (K_D^{app}), are plotted in Figure V-6a as a function of the proton concentration. The error bars in Figure V-6 represent $\pm$ 1 standard deviation obtained from the least squares fit of the data in Figure V-5 (see legend to Figure V-5). Estimating a value for K_1 from the limiting pK_D values at high pH values is technically difficult because of the weak binding at high pH values. However, values for K_2 and K_3 can be estimated using Equation V-4 which is derived from Equation V-3 by assuming $[H^+] >> K_4$.

$$[V\text{-}4] \qquad K_D^{app} = K_3 + K_2 K_3/[H^+]$$

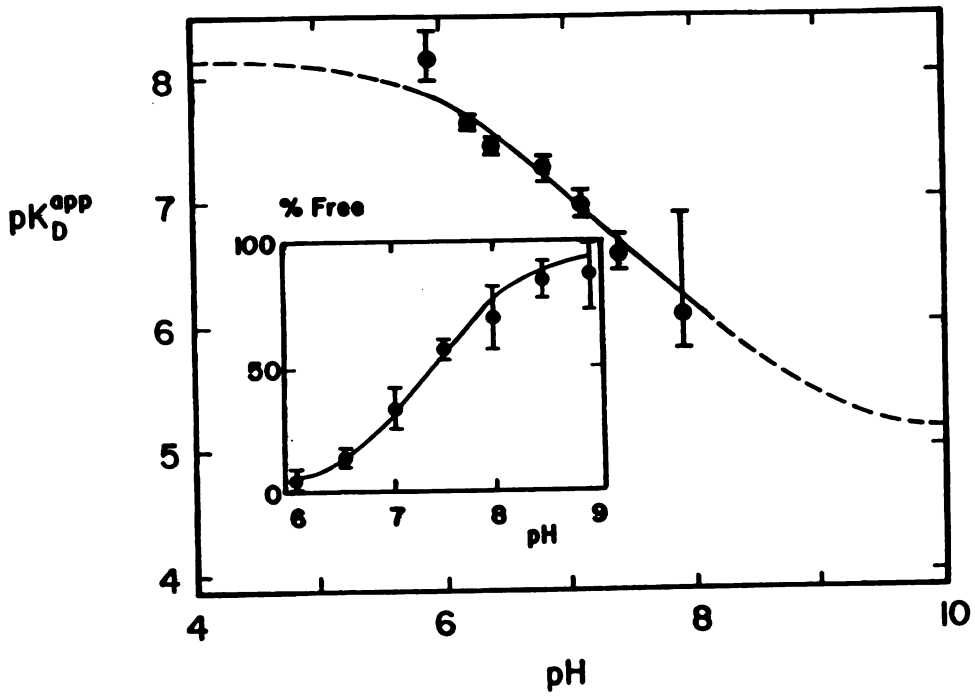
a plot of Equation V-4, given in Figure V-6b, demonstrates the validity

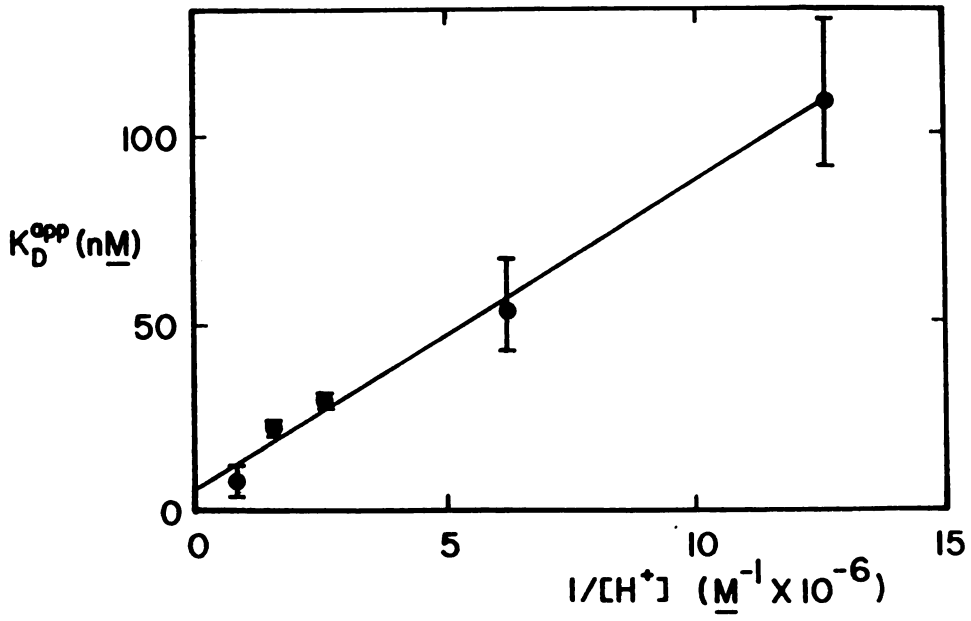
Figure V-6: Effect of pH on the apparent K_D value for MiMi-CK binding to mitoplasts.

A: Conditions are as described in Figure V-4. Error bars represent ± 1 standard deviation of the K_D value obtained from a computer analysis of the reciprocal plot (36). The curve is theoretically derived as described in the text.

Inset: Release of endogeneous MiMi-CK from mitoplasts as a function of pH. Mitoplasts (0.14 nmole of cytochrome aa_3) were incubated in a medium consisting of 75 mM sucrose, 225 mM mannitol, 0.2% BSA, 2% glycerol, 25 mM 2-mercaptoethanol, 50 µM Ap_5A, 7 mM MOPS, 7 mM MES, 7 mM Tris, 35 mM NaCl and maintained at a constant ionic strength by adding NaCl. This mixture was incubated at 30°C for 10 minutes, centrifuged, and the supernatant activity assayed.

B: Plot of the log of the K_D^{app} value against the reciprocal of the hydrogen ion concentration used to determine the values of K_2 and K_3. See the text for details.


pK_D^{app}
8
7
6
5
4
% Free
100
50
0
6
7
8
9
pH
4
6
8
10
pH


K_D^{app} (nM)
100
50
0
0
5
10
15
$1/[H^+]$ ($M^{-1} \times 10^{-6}$)

of this assumption up to a pH value of 7.1. Linear regression analysis gives $pK_2 = 6 \pm 0.65$ and $K_3 = 8 \pm 2$ n$\underline{M}$. Estimates of the pK_4 and K_1 values are obtained by a nonlinear iterative computer fit of the results using the values for pK_2 and K_3. The theoretical line of Figure V-6a is drawn using a value of $pK_4 = 9$, $pK_2 = 6$ and $K_3 = 8$ n$\underline{M}$. A value of $K_1 = 8$ $\mu\underline{M}$ can then be obtained from the linkage function: $K_1K_4 = K_2K_3$.

Confirmation of the mooel given by Equation V-2 comes from an experiment in which the distribution between free and bound MiMi-CK is measured as a function of the pH of the medium in the presence of 35 m$\underline{M}$ NaCl (see inset, Figure V-6a). Note that the percent of the total enzyme in the supernatant (% Free) decreases with decreasing pH values in agreement with Figure V-5. The value of K_D^{app} for the binding of MiMi-CK in the presence of 35 m$\underline{M}$ NaCl at pH 7.4 can be calculated using Equation V-2 and the concentration of free MiMi-CK when 50% of the total enzyme is bound (see Figure V-2). Assuming that the K_1 and K_3 but not pK_2 and pK_4 are affected by 35 m$\underline{M}$ NaCl gives a value of $K_3 = 45$ n$\underline{M}$ under these conditions in contrast to 8 n$\underline{M}$ at 0 m$\underline{M}$ NaCl. The theoretical curve shown in the inset of Figure V-6a is drawn using $K_3 = 45$ n$\underline{M}$, $pK_2 = 6$ and $pK_4 = 9$.

Regression analysis of the data of Figure V-5 to Equation V-2 shows that 14.6 ± 1 IU of MiMi-CK is bound per nmole cytochrome $\underline{aa}_3$ on the inner membrane. Assuming that pure MiMi-CK has a specific activity of about 124 IU/mg (Chapter IV) indicates that approximately 0.92 moles MiMi-CK/mole cytochrome $\underline{aa}_3$ can be bound maximally. Preparations of intact heart mitochondria contain approximately 2.86 IU MiMi-CK/nmole cytochrome $\underline{aa}_3$ indicating that only 20% of the MiMi-CK binding sites

are occupied. Carboxyatractyloside titration of respiring mitoplasts (see Materials section) gives a value of 3.3 moles nucleotide translocase per mole cytochrome $\underline{aa}_3$ a value which has been found by others (43). Therefore at infinite concentrations of MiMi-CK, about 1 mole of MiMi-CK is bound per 3.6 moles nucleotide translocase on the IMM. Intact chicken heart mitochondria contain about 1 mole MiMi-CK per 13 moles nucleotide translocase. If the specific activity in Chapter IV is low, then the ratio of MiMi-CK to nucleotide translocase would be lower.

Effect of adriamycin: Adding adriamycin to mitoplasts with endogeneous MiMi-CK releases the enzyme associated with the IMM in agreement with previously published reports (18, 19, 23). The data in Figure V-7 suggest that adriamycin and MiMi-CK compete directly for the same IMM binding site. Further investigation of this interaction, however, shows that the reaction mechanism is not as simple as previously suggested (18, 19, 23). Adriamycin inhibits the binding of MiMi-CK with a K_i of 60 $\mu\underline{M}$. This binding constant is obtained by correcting the total adriamycin concentration for the amount bound to mitoplasts. Adriamycin binding to mitoplasts has a K_D value of 116 $\mu\underline{M}$ and a maximum of 737 $\pm$ 154 moles/mole cytochrome $\underline{aa}_3$ (data not shown). This latter stoichiometry is approximately 8 times the number of cardiolipin sites available on the IMM (Appendix A) suggesting that adriamycin dissolves in the IMM (38) or binds to integral membrane proteins. In addition, adding adriamycin to MiMi-CK results in an increase in the fluorescence of adriamycin consistent with its binding to MiMi-CK with a $K_D = 12 \pm 5$ $\mu\underline{M}$ (Figure V-8). Binding adriamycin, presumably to a nonpolar site on

Figure V-7: Effect of adriamycin on the binding of purified MiMi-CK to mitoplasts.

Mitoplasts (0.25 μM in cytochrome aa_3) were incubated in the presence of 0-200 μM adriamycin and titrated with MiMi-CK (final concentration varied from 19 to 333 nM). The buffer was 10 mM MES (pH 6.8), 225 mM mannitol, 75 mM sucrose, 0.2% BSA, 2% glycerol, 25 mM 2-mercaptoethanol and 50 μM Ap_5A.

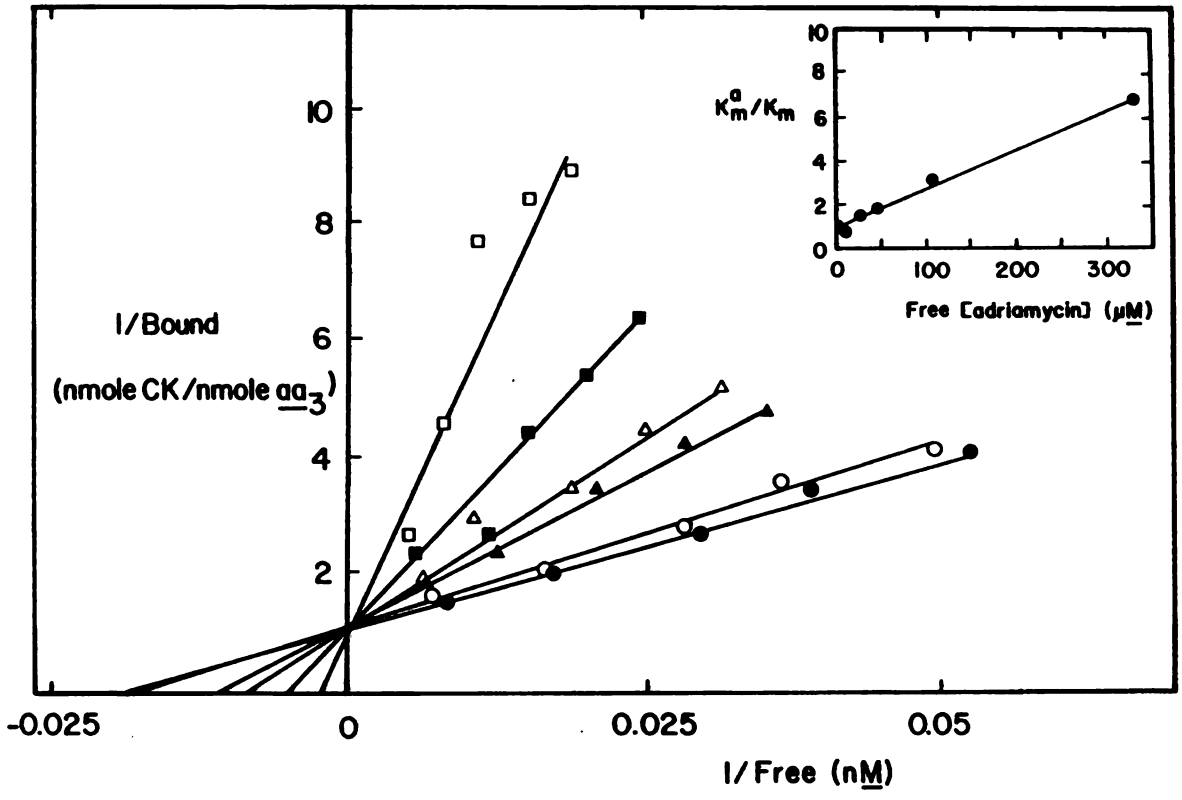
Inset shows a plot of ratio K_m^{app}/K_m versus adriamycin concentration. The slope of the line gives K_i = 60 μM.

The total concentration of adriamycin is as follows: ●, 0 μM, o, 20 μM, ▲, 50 μM, Δ, 100 μM, ■, 200 μM, and □, 500 μM.

Note that the curve for 500 μM adriamycin was fit by eye.

Figure V-8: Binding of Adriamycin to MiMi-CK.

Ordinate shows reciprocal change in fluorescence (arbitrary units) and the abscissa shows the log of the adriamycin concentration (μM). Approximately 13 nM MiMi-CK was incubated with 1 to 50 μM adriamycin and the fluorescence recorded at 580 nm after excitation at 480 nm (39). The fluorescence response represents the difference between the control tubes (without MiMi-CK) and the sample tubes (with MiMi-CK). The different symbols represent different experiments. See the text for additional details.


10
8
6
4
2
I/Bound
(nmole CK/nmole aa3)
-0.025
0
0.025
0.05
I/Free (nM)
K_m^a/K_m
10
8
6
4
2
0
0
100
200
300
Free [adriamycin] (μM)

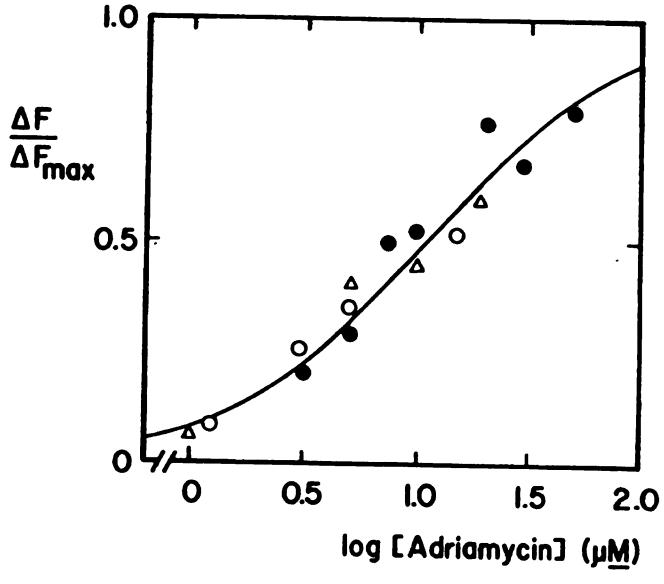
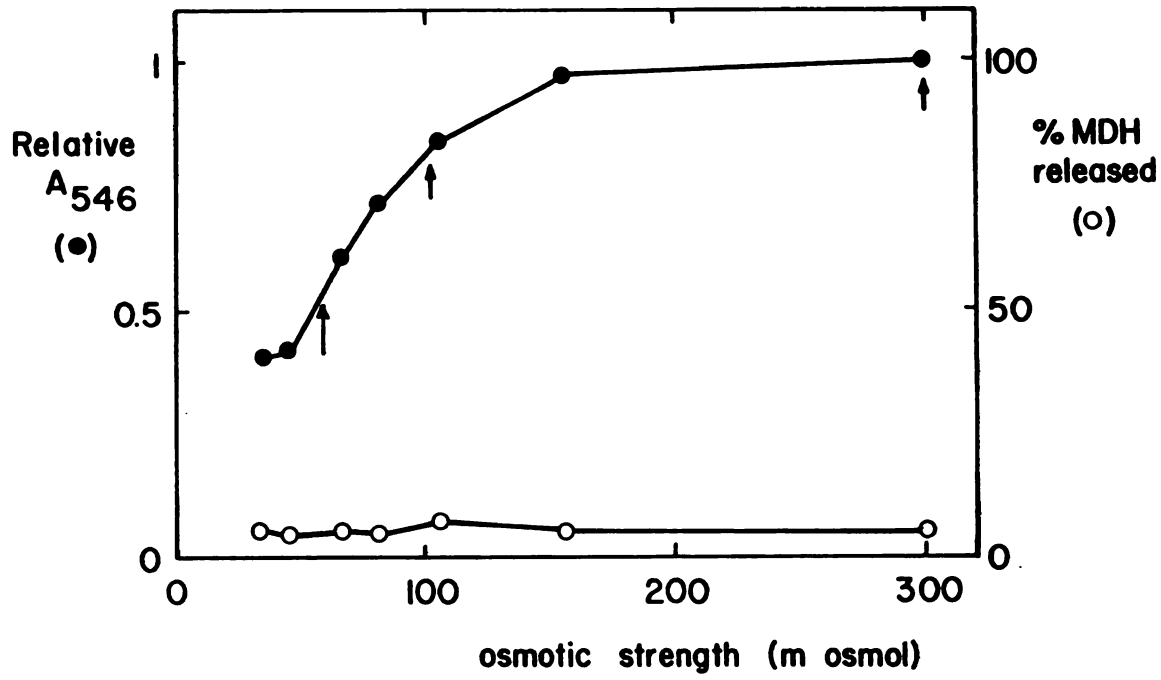

1.0
0.5
0
$\frac{\Delta F}{\Delta F_{max}}$
0
0.5
1.0
1.5
2.0
log [Adriamycin] (μM)

Figure V-9: Swelling of mitoplasts at reduced osmolalities.

Mitoplasts (0.408 μM in cytochrome aa_3) were incubated in various dilutions of 75 mM sucrose, 225 mM mannitol, 10 mM MOPS, 2 mM DTT (pH 7.4) in a total volume of 0.7 mL and the change in absorbance was monitored at 546 nm according to Packer (40). Malate dehydrogenase activity was assayed as described in the Methods section.


Relative
A_{546}
(●)
1
0.5
0
100
% MDH
released
(○)
50
0
0
100
200
300
osmotic strength (m osmol)

MiMi-CK, increases its fluorescence by analogy with the increase in daunomycin fluorescence in nonpolar methanol solutions (39). This solvent effect is confirmed for adriamycin which has the same fluorophore as daunomycin (data not shown).

Effect of osmotic strength: Previous studies of the release of MiMi-CK from mitochondria suggest that decreasing osmotic strength solubilizes the enzyme (16, 17). Similar effects are not observed with chicken heart mitoplasts. Decreasing the osmotic strength of the support medium results in an expansion of the mitoplasts as shown by lower A_{546} values (Figure V-9)(40). Note that an insignificant amount of malate dehydrogenase was released showing that the expansion was not sufficient to rupture the inner membrane. When mitoplasts were titrated with MiMi-CK at three different osmotic strengths, indicated by the arrows in Figure V-9, identical values for K_D^{app} and the maximal number of binding sites per mole cytochrome $\underline{aa}_3$ were obtained (data not shown). Therefore, decreasing the osmotic strength does not appear to affect the total number of available binding sites for these mitoplast preparations.

Discussion

Recent reports from other laboratories (7, 9) on the coupling of mitochondrial oxidative phosphorylation with MiMi-CK activity show that both MiMi-CK and nucleotide translocase preferentially accept substrates from each other even when the outer membrane is removed (12). The present study was initiated in order to obtain a more quantitative assessment of this functional coupling. However, before

this could be initiated, it was necessary to determine the effect of buffer salts, substrates and ionic strength on the stoichiometry and dissociation constant for the binding of MiMi-CK to IMM under a variety of conditions.

The interaction between MiMi-CK and the IMM is primarily due to electrostatic forces as illustrated by the following results. First, at pH 7.4, relatively low salt concentrations ($\leq$ 50 mM ionic strength) release the enzyme in a reversible manner. The release of enzyme is nearly independent of the ion as shown by the similarities in the ionic strengths required to release 50% of the total MiMi-CK activity (Table V-1). Second, the concentration of ionic substrates or nucleotides causing the release of 50% of the MiMi-CK from the IMM are in the same ionic strength range as the neutral salts. When expressed in molarities, the values range from 1.3 mM for ADP to 50 mM for LiCl. Third, the neutral zwitterionic substrate creatine is ineffective in causing release of the enzyme even though creatine phosphate has a C_{50} of 21 mM. MiMi-CK released from the IMM by ionic substrates or neutral salts at their C_{50} values has nearly the same specific activity (Table V-2) indicating the lack of a specific substrate or ion effect. Although the concentrations of salts in the range of the C_{50} values given in Tables 1 and 3 exert little effect on the bulk phase water structure (41, 42), the C_{50} values of the simple 1:1 salts are not identical but vary from 50 to 25 mM and follow the order LiCl>NaCl>KCL>RbCl=CsCl>KSCN. This order is the same as the order of their viscosity B coefficients (31) suggesting that the differences between the C_{50} values reflect a hydrophobic component to the interaction. The differences between the salts may be a reflection of

their ability to penetrate non-polar areas of the complex and disrupt ionic interactions (41, 42).

Studying the binding of homogeneous MiMi-CK to mitoplasts under other conditions revealed a strong pH dependence: MiMi-CK binds approximately 1000 times more strongly at pH 6 than at pH 9. Although the data can be modeled by a simple protonation scheme (Equation V-2), the process is almost certainly more complex. The electrostatic nature of the binding reaction suggests that the MiMi-CK, which is positively charged at pH 7.4 as evidenced by its migration to the cathode in an electric field at pH 8.8, is the protonated species. A decreased pH value would either neutralize a negative charge or result in an extra positive charge on the protein. Neutralizing a negative charge or producing a positive charge on the IMM would not be expected to increase its affinity for MiMi-CK.

Previous studies showed that adriamycin releases MiMi-CK from IMM (23) and that MiMi-CK binds to vesicles containing cardiolipin (18). However we were not able to demonstrate the binding of homogeneous MiMi-CK to either asolectin or cardiolipin:phosphatidyl choline (1:3, w/w) vesicles under conditions where mitoplasts bind MiMi-CK. This result suggested that adriamycin may not prevent binding of MiMi-CK by interacting with cardiolipin but that other mechanisms may account for the competitive inhibition with adriamycin. Further investigation showed that the fluorescence of adriamycin increases when added to MiMi-CK, similar to its response when placed in a hydrophobic medium (39). Assuming that the fluorescence enhancement is due to an adriamycin:MiMi-CK complex gave a K_D = 12 $\mu\underline{M}$ (Figure V-8). This value is smaller than the the K_i for adriamycin inhibition of MiMi-CK binding

to the IMM (60 μM) but the latter value may reflect the concentration of MiMi-CK binding sites on the IMM in the assay mixture. The binding of adriamycin to MiMi-CK and the lack of binding of enzyme to cardiolipin vesicles suggests that the inhibition by adriamycin is not due to its binding to cardiolipin in the IMM. However, whether MiMi-CK binds to a protein of other charged groups in the membrane cannot be discerned by these data. Two lines of evidence also suggest that, contrary to previous suggstions (4, 5), MiMi-CK does not interact with ADP/ATP nucleotide translocase. First, only 0.34 moles of MiMi-CK bind maximally per mole of nucleotide translocase and second, adding up to 5 moles of atractyloside or carboxyatractyloside per mole of nucleotide translocase does not release MiMi-CK from IMM (data not shown).

The stoichiometry for the binding of MiMi-CK to IMM is not consistent with that recently reported by Kuznetov and Saks (43). Using an oxidized ADP analog affinity label they report that an equivalent of 1 mole MiMi-CK per mole of nucleotide translocase is present in intact chicken heart mitochondria. Using their value of 0.8 units MiMi-CK per mg mitochondrial protein, which is in agreement with our results (Table V-2), gives a specific activity for homogeneous MiMi-CK of 8 IU/mg. This value is much lower than those reported by others (33) and the value of 124 IU/mg which we find (Chapter IV). Perhaps other proteins in the IMM bind the ADP affinity label.

Acknowledgments

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Chapter VI

Preferential Coupling of Mitochondrial Creatine Kinase to Chicken Heart Nucleotide Translocase in Mitochondria and Mitoplasts

Abstract

Preferential coupling of mitochondrial creatine kinase (MiMi-CK) to nucleotide translocase in chicken heart mitochondrial preparations is demonstrated. Using intact mitochondria to measure the MiMi-CK K_m value for $MgATP^{-2}$ (at saturating creatine) gives a value of 36 μM when MiMi-CK is coupled to oxidative phosphorylation. This K_m value is three fold lower than that of MiMi-CK measured with mitoplasts under conditions where about 70% of the MiMi-CK is bound to the inner mitochondrial membrane (IMM). The K_m value for $MgATP^{-2}$ is also three fold higher when the soluble enzyme is assayed using pyruvate kinase and lactate dehydrogenase as the coupling enzymes. The nucleotide translocase K_m value for ADP decreases from 20 μM to 10 μM in the presence of 50 mM creatine only when the outer mitochondrial membrane is present. The observed K_m differences can be used to calculate the concentration of ATP and ADP under steady state conditions showing that the observed differences in the kinetic constants accurately reflect the enzyme activities of MiMi-CK under the different conditions. These data indicate that, in the chicken heart system, and similar to the rabbit heart system, intact mitochondria are necessary to observe preferential coupling.

Introduction

Mitochondrial creatine kinase (MiMi-CK)[3] is localized in the inter membrane space of mitochondria presumably adsorbed to the outer surface of the inner membrane (1,2). Because of this location, several approaches have been used to demonstrate that MiMi-CK has preferential access to ATP generated by oxidative phosphorylation. Saks et al. (5,6) and Jacobus and Saks (7) report a 7 - 10 fold lower K_m value for $MgATP^{-2}$ (at finite creatine) when MiMi-CK is coupled to oxidative phosphorylation. Moreadith and Jacobus (8) and Barbour et al. (9) also see a two fold lower nucleotide translocase K_m value for ADP in the presence of creatine. ^{32}P partitioning experiments by Erickson-Viitanen et al. (10, 11) show that up to 50% of the creatine phosphate is derived from ATP generated by oxidative phosphorylation when the bulk ATP concentration is below 0.1 mM. These experiments indicate that at low ATP concentrations, MiMi-CK has a preferential access to ATP produced by oxidative phosphorylation over ATP in the bulk solution.

Two different hypotheses have been advanced to account for the preferential coupling of the nucleotide translocase and MiMi-CK. Saks et al. (5, 6, 12), Jacobus and Saks (7), and Jacobus (13) argue that MiMi-CK is bound to the nucleotide translocase such that the rate of ATP utilization by MiMi-CK is faster than its diffusion into the surrounding solution; the nucleotide translocase does not transfer ATP directly to MiMi-CK as atractyloside does not inhibit MiMi-CK activity (23). This hypothesis is supported by Saks et al. (6) who show

3. Abbreviations used: Ap_5A; diadenosine 5' pentaphosphate, IU; 1 μmole of substrate converted per minute, IMM; inner mitochondrial membrane, MiMi-CK; mitochondrial creatine kinase, MOPS; 3-(N-morpholino)propanesulfonic acid, OMM; outer mitochondrial membrane.

preferential coupling in rat heart mitochondria even when the outer mitochondrial membrane is removed by digitonin.

Contrary to the data from Jacobus and Saks' laboratories, Erickson-Viitanen et al. (10, 11) show preferential coupling only with intact mitochondria. They argue that the outer membrane acts as a partial diffusion barrier limiting the efflux of newly synthesized ATP and influx of medium ATP (11).

Using chicken heart mitochondria and mitoplasts, we have reexamined the importance of the outer membrane and the binding of MiMi-CK to the inner membrane to coupling of MiMi-Ck and oxidative phosphorylation. The data show that MiMi-CK is preferentially coupled to oxidative phosphorylation in intact mitochondria but not in mitoplasts, even though 70% of the total MiMi-CK is bound. We are also able to show that normal enzyme coupling kinetic theory (Chapter II and III) can be used to predict the steady state concentration of the intermediates ADP and ATP.

Materials and Methods

General: All chemicals, enzymes and creatine kinase assay kits were obtained from Sigma Chemical Co. (St. Louis MO) unless otherwise specified. Common laboratory chemicals were reagent grade or better. Chickens were obtained from the Department of Animal Science, Michigan State University as newly hatched chicks and maintained on Chick G0125 (Kent Feed Inc., Muscatine IA). MiMi-CK was purified from chicken heart mitochondria as described in Chapter IV. Its concentration is determined from activity measurements using a specific activity of 124 IU/mg.

Preparation of Mitochondria and Mitoplasts: Mitochondria and mitoplasts were prepared as described in Chapter IV. Washed mitochondria were prepared by suspending mitochondria one time in 50 mM NaCl to remove MiMi-CK which may be solubilized under the experimental conditions due to broken outer mitochondrial membranes. Thirty percent of the creatine kinase activity was lost during this wash. Washed mitoplasts were prepared by incubating mitoplasts in 50 mM phosphate, 10 mM Tris (pH 8.0) at $0^{o}C$ for 7 minutes. This procedure removes approximately 90% of the MiMi-CK and allows the addition of a high mitoplast concentration so that approximately 70% of the MiMi-CK present is bound to the IMM. The concentration of cytochrome aa_3 was determined from the differences in the absorbance of the reduced minus oxidized spectrum at 602 minus 630 nm ($\Delta\varepsilon$ = 24 $mM^{-1}cm^{-1}$, 18).

Enzyme Assays: Monoamine oxidase and malate dehydrogenase activities were measured as described in Chapter IV. The kinetic constants of homogeneous soluble MiMi-CK were analyzed as previously described in Chapter III. Because the conditions used to determine the kinetic constants of MiMi-CK release the enzyme from the IMM (Chapter V), the kinetic constants of MiMi-CK bound to the IMM in the presence of oligomycin A and of MiMi-CK in intact mitochondria were determined by following the production of ADP by end point assays. The assays were performed by incubating washed mitochondria or washed mitoplasts in 1 mL of 75 mM sucrose, 222 mM mannitol, 0.2% bovine serum albumin, 2.5 mM phosphate, 10 mM MOPS, 2 mM magnesium acetate (pH 7.0, Buffer A). The reaction, which contained varying amounts of creatine, was initiated by

adding a 1:1 mixture of magnesium acetate and ATP. At 0.5, 1, and 1.5 minutes, 0.3 mL aliquots were removed and added to 30 µL 3 N trichloroacetic acid. The solutions were centrifuged for 3 minutes at 8,000 X g, and 250 µL of the supernatant was quickly removed and neutralized by adding KOH. To this solution, 678 µL of 50 mM MOPS, 4 mM $MgCl_2$, 0.2 mM EDTA, 100 mM KCl (pH7.0), 10 µL of 0.1 M phospho(enol)pyruvate, and 10 µL of 67 mM NADH were added. The difference in the absorbance before and after the addition of pyruvate kinase and lactate dehydrogenase gives the ADP concentration. The rates of ADP production are linear over the time course of the experiment.

Kinetic parameters for MiMi-CK coupled to oxidative phosphorylation were measured using an oxygen electrode apparatus (Yellow Springs Instruments) in Buffer A plus 5 mM pyruvate and 2.5 mM malate. The reaction was initiated by the addition of a 1:1 molar mixture of magnesium acetate and ATP. The state 3 rates are corrected from velocities measured in the absence of creatine. The lag time for the coupled reaction was always less that 4 minutes.

The kinetic parameters of the nucleotide translocase, in the presence and absence of creatine, were determined using an oxygen electrode apparatus with the normal sensitivity increased six fold. The reactions were initiated by adding various amounts of ADP to Buffer A containing 5 mM pyruvate, 2.5 mM malate, and mitochondria or mitoplasts spiked with 0.38 IU MiMi-CK per assay so that the final ratio of enzyme was 3.3 IU/nmole cytochrome aa_3.

Measuring Creatine Phosphate and ATP Concentrations: An aliquot (10 μL) of 27 mM ATP plus 27 mM magnesium acetate was added to a suspension of mitochondria or mitoplasts in 1.5 mL Buffer A plus 5 mM pyruvate and 2.5 mM malate. At time 0.5, 1.0, 1.5, and 2.0 minutes, a 0.3 mL aliquot was removed and added to 0.3 mL boiling water (19). The samples were allowed to boil for 1 to 2 minutes, cooled on ice for 10 minutes and centrifuged for 3 minutes at 8,000 X g. The supernatants (0.25 mL) were removed and made 14 mM in glucose, 3 mM in magnesium acetate, 25 mM in 2-mercaptoethanol, 20 mM in Hepes, 4% (v/v) in glycerol and 0.1 mM in EDTA. The concentration of ATP was calculated from changes in absorbance at 340 nm after adding 1 mM $NADP^+$, hexokinase and glucose 6-phosphate dehydrogenase. Creatine phosphate was measured after adding creatine kinase.

Theory

The coupling of MiMi-CK to the oxidative phosphorylation is a special case of the two coupled enzyme system presented in Scheme 1 of Chapter II. The equations presented in Chapters II and III show that, when the rate of the primary enzyme is constant, the steady state concentration of each intermediate is dependent on the rate and K_m value of the coupling enzyme; the higher the coupling enzyme concentration or the lower the coupling enzyme K_m value, the lower the intermediate concentration. When MiMi-CK is coupled to oxidative phosphorylation, the substrate and product for translocase (e_2 in Figure VI-1) are the product and substrate for MiMi-CK (e_1 in Figure VI-1). Consequently the rate of the primary enzyme (v_1) cannot be fixed as the reaction tends toward steady state as was done so

conveniently for the general coupled enzyme systems of Chapters II and III.

The concentration of ADP at any time t can be expressed as

[VI-1] $$d[ADP]/dt = v_1 - v_2$$

which says that the change in ADP over time is a function of the difference between the rate of ADP formation (v_1) and the rate of ADP utilization (v_2). Substituting the Michaelis-Menten relationships for these rates, we obtain:

[VI-2] $$d[ADP]/dt = V_1[ATP]/(K_1 + [ATP]) - V_2[ADP]/(K_2 + [ADP])$$

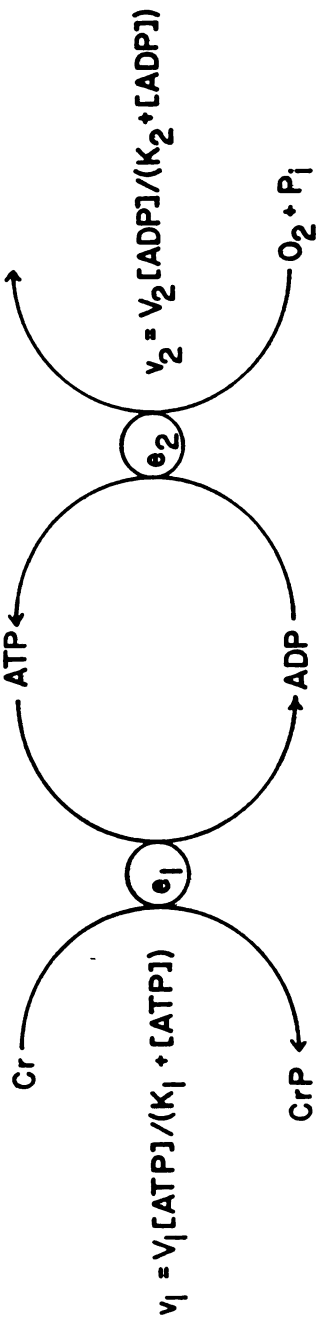
where V_1 and V_2 are the maximal velocities of MiMi-CK and oxidative phosphorylation respectively. K_1 is the MiMi-CK K_m for $MgATP^{-2}$ in the presence of a finite amount of creatine and K_2 is the measured K_m of oxidative phosphorylation for ADP. Since the nucleotide translocase uses only free ADP and MiMi-CK uses only $MgATP^{-2}$, the constants α and β, which reflect the binding constants of ADP and ATP for magnesium, are defined as:

$$\alpha = 1 - [MgADP^-]/[ADP_T] \qquad \beta = [MgATP^{-2}]/[ATP_T]$$

The value α thus represents the fraction of ADP which reacts with the translocase and the value β represents the fraction of ATP which reacts with MiMi-CK. The values of $[ADP_T]$ and $[ATP_T]$ represent the total concentrations of ADP and ATP. If we define the total nucleotide

Figure VI-1: Coupling the synthesis of creatine phosphate to oxidative phosphorylation through MiMi-CK.

e_1 represents MiMi-CK and e_2 represents oxidative phosphorylation.


$v_2 = V_2[ADP]/(K_2+[ADP])$
$O_2 + P_i$
e_2
ATP
ADP
e_1
Cr
CrP
$v_1 = V_1[ATP]/(K_1+[ATP])$

concentration as $A_T = [ADP_T] + [ATP_T]$, Equation VI-2 can be written as Equation VI-3.

$$[VI\text{-}3] \qquad \frac{d[ADP]}{dt} = \frac{V_1 * \beta(A_T - [ADP])}{K_1 + \beta A_T - \beta[ADP]} - \frac{V_2 * \alpha[ADP]}{K_2 + \alpha[ADP]}$$

Solving Equation VI-3, and a similar equation for the rate of change of ATP with respect to time for the steady state condition ($d[ADP]/dt = 0$) gives Equations VI-4 and VI-5.

$$[VI\text{-}4] \qquad 0 = \Phi[ADP]_{ss}^2 + [ADP]_{ss}(V_1K_2/\alpha + V_2K_1/\beta - A_T\Phi) - A_TV_1K_2/\alpha$$

$$[VI\text{-}5] \qquad 0 = \Phi[ATP]_{ss}^2 - [ATP]_{ss}(V_1K_2/\alpha + V_2K_1/\beta + A_T\Phi) + A_TV_2K_1/\beta$$

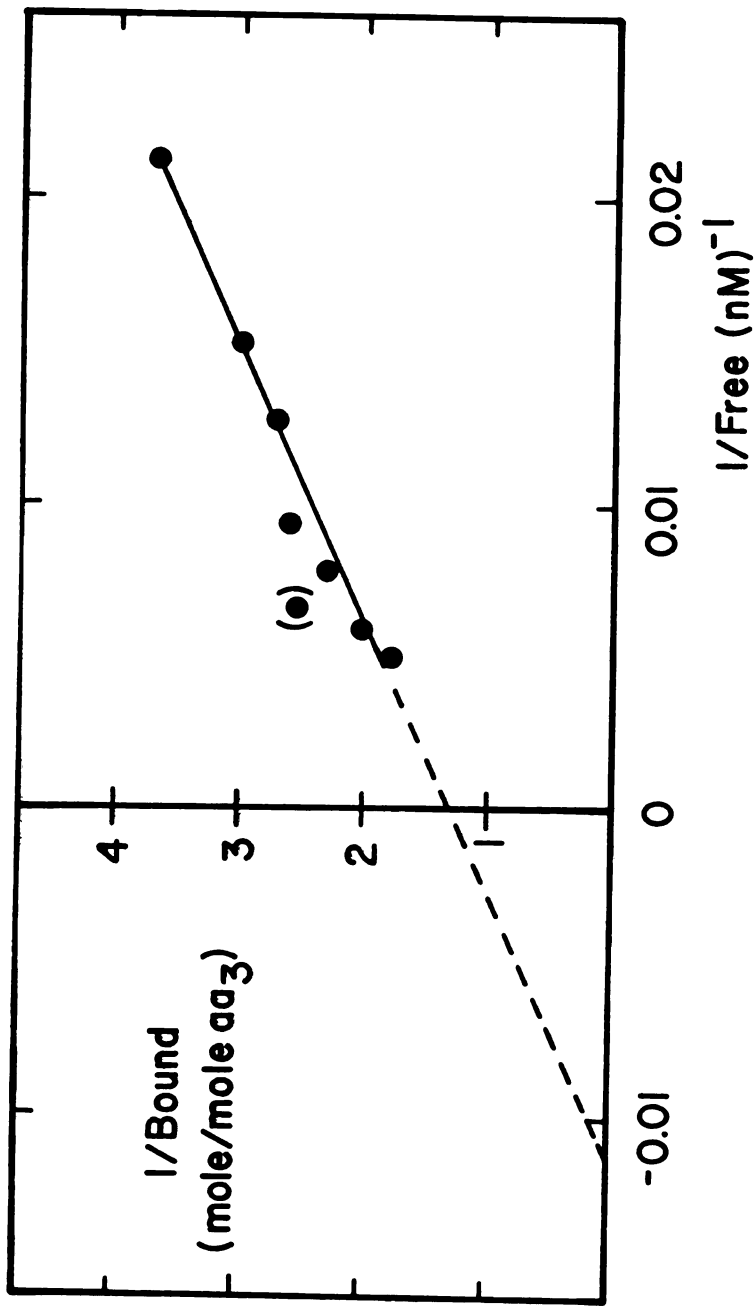
where $\Phi = V_1 - V_2$. Equations VI-4 and VI-5 enable one to calculate the concentration of ADP or ATP for any concentration of e_1 and e_2 provided K_1 and K_2 are known. Equation VI-4 and VI-5 predict that as e_2 increases, $[ADP]_{ss}$ increases and $[ATP]_{ss}$ decreases in a non-linear fashion. If $V_2 = 0$, Equations VI-4 and VI-5 predict that $[ADP_{ss}] = 0$ and $[ATP]_{ss} = A_T$. Also as $V_2 \to \infty$, Equations VI-4 and VI-5 predict that $[ADP]_{ss} \to A_T$ and $[ATP]_{ss} \to 0$.

Results

Binding of MiMi-CK to Mitoplasts: In order to assess the preferential coupling of bound MiMi-CK to oxidative phosphorylation, it is necessary to define the conditions for the binding of MiMi-CK to the IMM. To measure the distribution of free and bound MiMi-CK under the conditions of our experiments, homogeneous MiMi-CK was added to mitoplasts and the

Figure VI-2: Binding of MiMi-CK to the inner mitochondrial membrane.

MiMi-CK (free concentration from 47 to 204 nM) was incubated with mitoplasts (0.01 nmole cytochrome aa_3) for 10 minutes at 30°C in 100 µL of 5 mM pyruvate, 2.5 mM malate, 0.5 mM magnesium acetate, 0.5 mM ADP, 50 mM creatine, 10 mM MOPS, 25 mM 2-mercaptoethanol, 75 mM sucrose, 225 mM mannitol, 2% bovine serum albumin and 2.5 mM phosphate (pH 7.0). The concentration of free MiMi-CK was measured in the supernatants following centrifugation at 8,000 x g for 10 minutes. The amount of MiMi-CK bound was obtained by substracting the total activity from the free activity. The line was fit with a linear regression program (Appendix A) using the weighting scheme suggested by Wilkinson (22). The value in parentheses was omitted from the analysis as it lies 2 standard deviations outside the calculated line.


1/Bound
(mole/mole aa_3)
4
3
2
1
0
-0.01
0.01
0.02
1/Free $(nM)^{-1}$

free and bound concentrations of enzyme plotted according to Equation VI-6 (Figure VI-2)

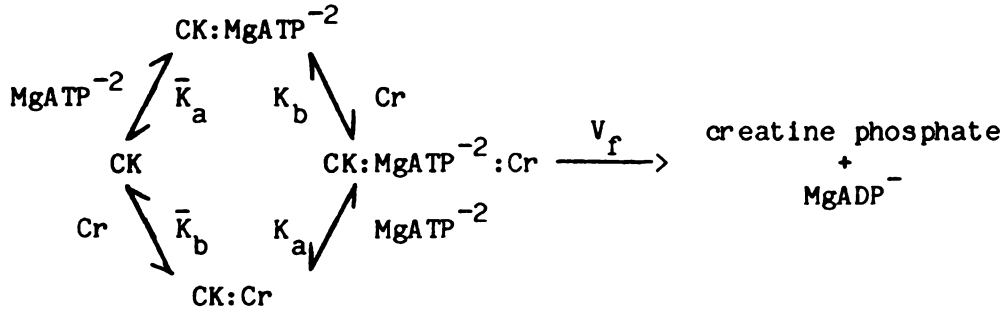
[VI-6] $$\bar{v}/n = [\text{MiMi-CK}]_F/(K_D + [\text{MiMi-CK}]_F)$$

where $[\text{MiMi-CK}]_F$ is the free concentration of MiMi-CK and $\bar{v}/n$ is the fractional saturation of the available binding sites on the IMM. A K_D value of 87 $\pm$ 12 n$\underline{M}$ and the maximal number of binding sites, 0.87 $\pm$ .06 mole MiMi-CK/mole cytochrome $\underline{aa}_3$ agree with the values given in Chapter IV. Using these parameters in conjunction with Equation VI-6 allows one to calculate the amount of MiMi-CK bound and the number of binding sites on the IMM which are occupied under the conditions of the steady state experiments. Measuring the kinetic parameters of MiMi-CK requires the presence of 2 m$\underline{M}$ magnesium acetate; this concentration has a negligible effect on the binding of MiMi-CK to the IMM. However, adding 1 m$\underline{M}$ magnesium acetate plus ATP (highest substrate concentrations used in the assay) caused a further 10% release of MiMi-CK from the IMM (see Chapter V). Thus, when the MgATP concentration is close to 1 m$\underline{M}$, approximately 60% of the total MiMi-CK is bound to the inner mitochondrial membranes under the experimental conditions of Figure VI-3.

Kinetic Constants of MiMi-CK: Figures VI-3a and VI-3b present the intercepts and slopes of the primary plots of 1/velocity and 1/[creatine] for MiMi-CK under a variety of conditions as a function of the log (base 10) of the $MgATP^{-2}$ concentrations. The kinetic constants obtained from these data are presented in Table VI-1 and defined in

Equation VI-7.

[VI-7]



Measuring the K_a and $\bar{K}_a$ values using intact mitochondria with the translocase as the coupling enzyme shows that the K_a and $\bar{K}_a$ values are approximately three fold lower than those measured with mitoplasts, even though 78% of the MiMi-CK is bound to the IMM when mitoplasts are used (compare open and closed squares, Figures VI-3a and VI-3b). The K_a and $\bar{K}_a$ values for the soluble enzyme are similar to the bound enzyme suggesting that the outer membrane is responsible for the preferential coupling.

The effect of the OMM on the kinetic parameters of MiMi-CK is further indicated by measuring the kinetic parameters in the presence of oligomycin A. In this case, respiration is inhibited and the reaction is measured by following ADP concentration over time. The K_a and $\bar{K}_a$ values for MgATP^{-2} are six fold higher using intact mitochondria but are identical when mitoplasts are used even though 69% of the MiMi-CK is associated with the IMM. Note that the K_b and $\bar{K}_b$ values for creatine are identical for all five conditions (Table VI-1).

Kinetic Constants for Oxidative Phosphorylation: Determining the nucleotide translocase K_m value for ADP by measuring the respiration rate in the presence of increasing concentrations of creatine as a

Table VI-1: Kinetic constants in mM for MiMi-CK.

Constant	Soluble Enzyme	Mitochondria + oxi. phos.	Mitoplasts + oxi. phos.	Mitochondria + Oligo. A	Mitoplasts + Oligo. A
$\bar{K}_a$ ($MgATP^{-2}$)	0.25 ± 0.03	0.08 ± 0.03	0.19 ± 0.02	1.3 ± 0.2	0.29 ± 0.06
K_a ($MgATP^{-2}$)	0.13 ± 0.02	0.04 ± 0.01	0.10 ± 0.02	0.80 ± 0.12	0.13 ± 0.02
$\bar{K}_b$ (creatine)	20 ± 6	24 ± 3	19 ± 1	13 ± 2	16 ± 4
K_b (creatine)	10 ± 1	9 ± 3	10 ± 2	9 ± 1	7 ± 3
V_{max} (IU/mg)	200 ± 8	150 ± 20	170 ± 20	150 ± 15	190 ± 20

Note. Conditions as in the legend to Figure VI-3.

Figure V-3: Measuring the kinetic parameters for the synthesis of creatine phosphate by MiMi-CK.

The figures are the secondary plots of the slopes ($mM*mg*IU^{-1}$) and intercepts (mg/IU) of the primary plots of 1/velocity versus 1/[creatine]. The curves are obtained from a linear regression analysis (Appendix A) using weighting suggested by Wilkinson (22).

In experiments b to e, mitochondria or mitoplasts were incubated for 2-4 minutes at 30°C in 75 mM sucrose, 225 mM mannitol, 0.2% bovine serum albumin, 2 mM magnesium acetate, 0.1 mM EDTA, 2.5 mM phosphate, and 10 mM MOPS, pH 7.0 (Buffer B). The creatine concentrations were 50 mM, 35 mM, 20 mM, 10 mM, 7 mM, 5 mM, and 2 mM for experiments a, d and e and 50 mM, 20 mM, 10 mM, 5 mM, and 2 mM for experiments b and c. The reactions were initiated by adding a 1:1 molar mixture of magnesium acetate and ATP, the $MgATP^{-2}$ concentrations are calculated following Storer and Cornish-Bowden (21). The state three rates were subtracted from rates measured in the absence of creatine. The bottom axis is used with the data for experiment b.

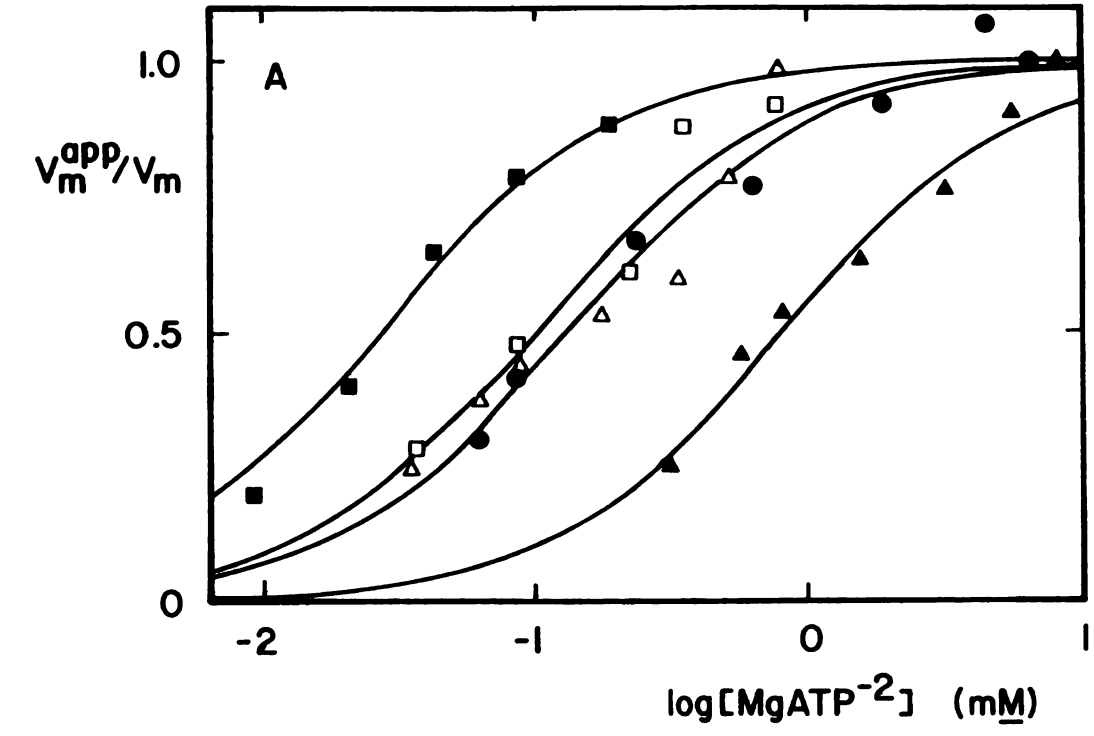
a. (●) MiMi-CK (0.19 IU) was added to 1.0 mL of reaction mix as described in the Methods section. $MgATP^{-2}$ varied from 63 µM to 6.4 mM.

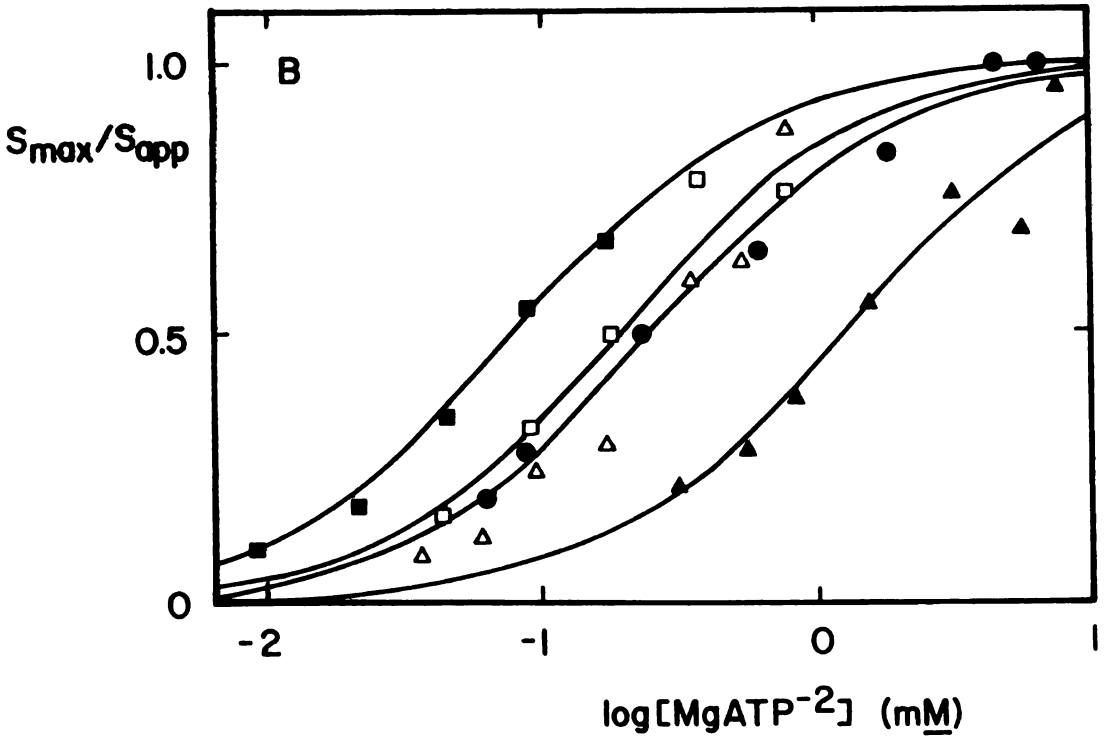
b. (■) Washed mitochondria (0.21 nmole cytochrome aa_3, 0.4 IU MiMi-CK) were added to 1.75 mL of Buffer B plus 5 mM pyruvate and 2.5 mM malate. $MgATP^{-2}$ varied from 9 µM to 173 µM.

c. (□) Washed mitoplasts (2.7 nmole cytochrome aa_3, 0.36 IU MiMi-CK) were added to 1.75 mL of Buffer B plus 5 mM pyruvate and 2.5 mM malate. $MgATP^{-2}$ varied from 36 µM to 790 µM.

d. (▲) Washed mitochondria (0.1 nmole cytochrome aa_3, 0.2 IU MiMi-CK) were added to 1.0 mL of Buffer B plus 4 µg/mL oligomycin A. $MgATP^{-2}$ varied from 0.32 mM to 7.9 mM.

e. (Δ) Washed mitoplasts (0.24 nmole cytochrome aa_3, 0.07 IU MiMi-CK) were added to 1.0 mL of Buffer B plus 4 µg/mL oligomycin A. $MgATP^{-2}$ varied from 36 µM to 790 µM.


A
1.0
0.5
0
V_m^{app}/V_m
-2
-1
0
1
log[MgATP^{-2}] (mM)


B
1.0
0.5
0
S_{max}/S_{app}
-2
-1
0
1
log[MgATP^{-2}] (mM)

function of ADP shows that it decreases to 10 μM, about half of the K_m value measured in the absence of creatine (Figure VI-4b) in agreement with the results of Barbour et al. (9) and Jacobus et al. (20). Removing of the outer membrane abolishes this effect. It is important to note, however, that only 20% of the available IMM binding sites for MiMi-CK are occupied and that MiMi-CK activities are equal to the oxidative phosphorylation rate when mitoplasts are used to determine the translocase K_m value for ADP.

Rates of Creatine Phosphate Synthesis: Figure VI-5 is a plot of creatine phosphate and ATP concentrations as a function of time. Although the ATP_{ss} concentrations are nearly identical for all three cases, the rates of creatine phosphate synthesis (v_{obs}) are clearly different (see Table VI-2). When MiMi-CK is coupled to oxidative phosphorylation in intact mitochondria, the steady state rate of creatine phosphate synthesis is nearly twice that seen for the soluble enzyme (squares, Figure VI-5) or enzyme bound to mitoplasts (triangles, Figure VI-5). Since the concentration of MiMi-CK is identical in all three cases, the different rates of creatine phosphate synthesis are the result of different apparent K_m values for $MgATP^{-2}$ (measured at 100 mM creatine) under the conditions of the experiment. These apparent K_m values can be calculated from the ATP concentrations and the maximal activity of MiMi-CK by inverting the Michaelis-Menten equation as shown below.

$$[VI\text{-}8] \qquad K_m^{app} = [ATP]_{ss}(V_1 - v_{obs})/v_{obs}$$

Table VI-2: Determination of the MiMi-CK K_m value for ATP and the ATP steady state concentration.

Condition	v_{obs}	$[ATP]_{ss}$	apparent K_m	calculated $[ATP]_{ss}$
a. Mitochondria	80 μM/min	150 μM	16 μM	155 μM
Mitoplasts				
b. free	46 μM/min	127 μM	117 μM	142 μM
c. bound	52 μM/min	140 μM	99 μM	149 μM

Note. v_{obs} is the rate of creatine phosphate synthesis at steady state. The apparent K_m values are calculated according to Equation VI-8. The value of V_1, which represents the maximal rate of creatine phsophate synthesis, is 88.6 μM/min under the conditions of the experiment. The $[ATP]_{ss}$ values are calculated using K_1 = apparent K_m, and the following values: a. V_2 = 174 μM/min, K_2 = 10 μM and $[ATP]_{ss}$ in the absence of creatine = 169 ± 3 μM, b. V_2 = 91 μM/min, K_2 = 20 μM and $[ATP]_{ss}$ in the absence of creatine = 167 ± 3 μM, c. V_2 = 204 μM/min, K_2 = 20 μM and $[ATP]_{ss}$ in the absence of creatine = 155 ± 5 μM. K_2 and V_2 are the K_m value and maximal rate of ATP synthesis measured in the absence of creatine. Note that the decreased value of $[ATP]_{ss}$ (as compared to the initial value of 170 μM ATP) is due to the action of an endogeneous ATPase. This lower ATP concentration is taken as the total nucleotide concentration when the calculated $[ATP]_{ss}$ values are obtained using equation VI-5. The values are measured under the conditions of Figure VI-4. α = 0.766 and β = 0.854 under these conditions (21).

Table VI-3: Steady state ADP concentrations for different mitochondrial preparations.

Condition	K_1	$[ADP]_{ss}$ obs.	$[ADP]_{ss}$ calc.
	(μM)	(μM)	(μM)
Mitochondria	36	10.6 ± 1	11.9
Mitoplasts			
free	125	18.2 ± 0.8	15.4
bound	100	6.5 ± 1.1	6.8

Note. Washed mitochondria (0.11 nmole cytochrome aa_3, 0.2 IU MiMi-CK), mitoplasts (0.07 nmole cytochrome aa_3, 0.2 IU MiMi-CK), or washed mitoplasts (0.72 nmole cytochrome aa_3, 0.2 IU MiMi-CK) were incubated in 1.5 mL 75 mM sucrose, 225 mM mannitol, 0.2% bovine serum albumin, 0.5 mM magnesium acetate, 10 mM MOPS, 2.5 mM phosphate, 5 mM β-hydroxybutyrate, 100 mM creatine (pH 7.0) for 2 minutes prior to addition of 80 μM ADP. The respiration of washed mitoplasts was inhibited to 30% of normal by adding 3.1 mole carboxyatractlyoside per mole cytochrome aa_3. The values represent the mean of two determinations. The $[ADP]_{ss}$ values were calculated from Equation VI-4 using the following values: V_1 = 44 μM/min, and a. V_2 = 57 μM/min, K_2 = 10 μM, and $[ADP]_{ss}$ in the absence of creatine = 1 ± .3 μM, b. V_2 = 36 μM/min, K_2 = 20 μM, and $[ADP]_{ss}$ in the absence of creatine = 2 ± .7 μM, a. V_2 = 80 μM/min, K_2 = 20 μM, and $[ADP]_{ss}$ in the absence of creatine = 3 ± .4 μM. α = 0.766 and β = 0.854 under these conditions (21). The definitions of V_1, V_2, and K_2 are presented in the legend to Table VI-2 and in the Theory section.

where V_1 and K_m^{app} are the V_{max} and apparent K_m for MiMi-CK measured in the presence of 100 mM creatine. The value of V_1, reported in the legend to Table VI-2, is the maximal rate of creatine phosphate synthesis measured under the conditions of the experiment. The apparent K_m values are presented in Table VI-2.

Using the apparent K_m values determined above, the V_1 values for the system, and the rate and K_m value for oxidative phosphorylation measured for this experiment (V_2 and K_2 values, see Theory), permits us to calculate the steady state ATP concentrations using Equation VI-5. The results of this calculation, and the kinetic constants for the two enzymes are presented in Table VI-2 as well. These values are obtained by assuming that the value of ATP_{ss} in the absence of creatine is the total nucleotide concentration. This latter assumption corrects for the presence of an ATPase. Although the calculated ATP_{ss} values are slightly higher than the measured values, they agree well with one another.

Measuring ADP Concentrations: In order to measure the effect of different kinetic constants on the steady state concentration of ADP, the three different mitochondrial preparations were allowed to go into steady state and the concentration of ADP measured. The establishment of steady state is confirmed by measurement of an identical ADP concentration at 5 and 6 minutes after the reaction is initiated. The $[ADP]_{ss}$ concentrations presented in Table VI-3 have been subtracted from those measured in the absence of creatine, and thus represent the steady state concentrations due to the activity of MiMi-CK and not to an ATPase activity (see legend to Table VI-3). Equation VI-4 is used

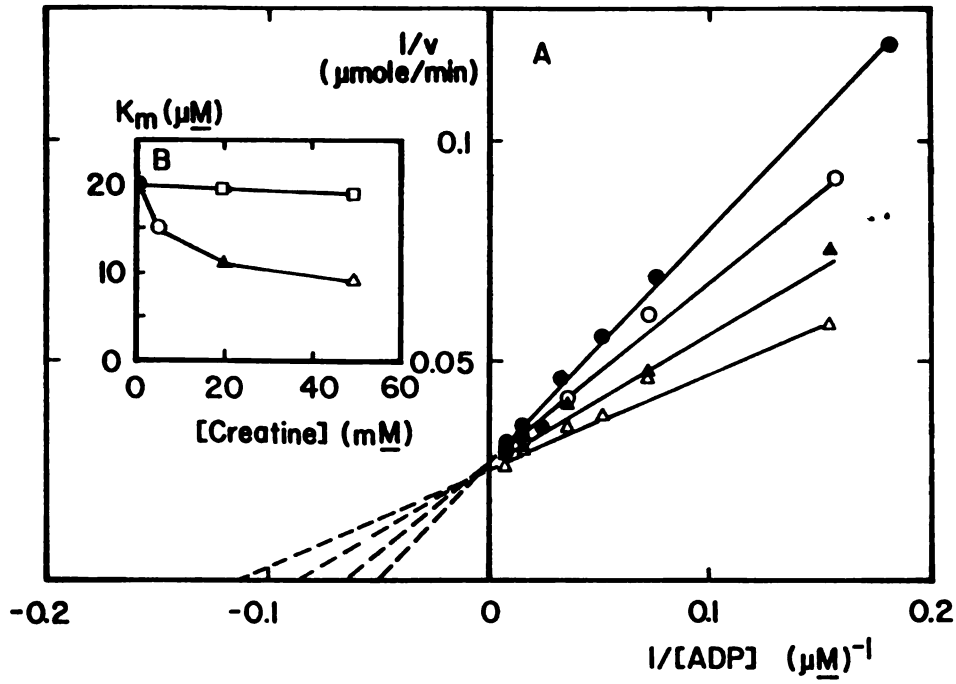
Figure VI-4: Effect of increasing creatine concentrations on the V_{max} and K_m for the nucleotide translocase.

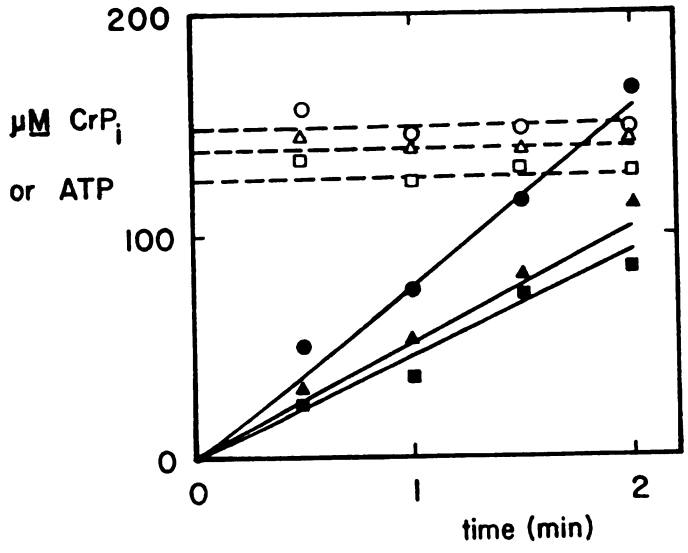
a. Mitochondria (0.021 nmole cytochrome $\underline{aa}_3$, 0.062 IU MiMi-CK) or mitoplasts (0.018 nmole cytochrome $\underline{aa}_3$, 0.058 IU MiMi-CK) plus 0.38 IU MiMi-CK were incubated in 1.75 mL Buffer B at 30°C for 2-4 minutes. The reaction was initiated by adding from 6 $\mu\underline{M}$ to 195 $\mu\underline{M}$ ADP. The creatine concentrations were 0 $m\underline{M}$ (●), 5 $m\underline{M}$ (o), 20 $m\underline{M}$ (▲), and 50 $m\underline{M}$ (Δ). The observed maximum velocity corresponds to 320 nmole O_2/min/nmole cytochrome $\underline{aa}_3$.

b. Plot of the K_m value versus the concentration of creatine for mitochondria (various symbols) or mitoplasts (□).

Figure VI-5: Rates of creatine phosphate synthesis with mitochondria and mitoplasts.

Washed mitochondria (●, 0.18 nmole cytochrome $\underline{aa}_3$, 0.4 IU MiMi-CK), mitoplasts (▲, 0.13 nmole cytochrome $\underline{aa}_3$, 0.4 IU MiMi-CK), or washed mitoplasts (■, 1.4 nmole cytochrome $\underline{aa}_3$, 0.4 IU MiMi-CK) were incubated in 1.5 mL Buffer B at 30°C for 1-2 minutes in the presence of 5 $m\underline{M}$ β-hydroxybutyrate. The reaction was started by adding a 1:1 mixture of magnesium acetate and ATP which corresponds to 170 $\mu\underline{M}$ total ATP. The open symbols are the ATP concentrations, the closed symbols are the creatine phosphate concentrations. The respiration of washed mitoplasts was inhibited to 30% of normal by adding 3.1 mole carboxyatractlyoside per mole cytochrome $\underline{aa}_3$. The values are the mean of two determinations. The rates of creatine phosphate synthesis are theoretical lines representing the calculated creatine phosphate concentrations using a fourth order Runge-Kutta numerical integration of Equation VI-4.


1/v
(μmole/min)
A
Km (μM)
B
20
10
0
0
20
40
60
[Creatine] (mM)
0.1
0.05
-0.2
-0.1
0
0.1
0.2
1/[ADP] (μM)⁻¹


200
μM CrPi
or ATP
100
0
0
1
2
time (min)

to calculate the $[ADP]_{ss}$ concentration using the measured values for this system (see legend to Table VI-3) and the apparent K_m values for ATP which are listed in Table VI-3. Equation VI-4 predicts values of $[ADP]_{ss}$ which are close to those measured directly. These data support the observations made earlier that the OMM is the responsible for the preferential coupling of MiMi-CK in chicken heart mitochondria.

Discussion

The chicken heart mitochondria:MiMi-CK system appears similar to the rabbit heart (10, 11) and rat heart (5 - 7, 12 - 14) systems in that MiMi-CK and the translocase are preferentially coupled. We define preferential coupling as a decreased K_m of an enzyme for a substrate, when measured in the presence of another coupling enzyme, versus the K_m value determined by a direct measurement of the enzyme activity. When K_a and $\bar{K}_a$ values for MiMi-CK are compared, the values are 3 fold lower when determined by coupling the reaction to oxidative phosphorylation as compared to the values measured for free enzyme and enzyme bound to mitoplasts (Table VI-1). While these differences are small, they do accurately predict the steady state concentrations of ATP and ADP measured directly (Table VI-2 and VI-3). The kinetic differences also translate into different rates of creatine phosphate synthesis when the bulk steady state ATP concentration is maintained near 140 $\mu\underline{M}$ (Figure VI-4); the rate of creatine phosphate production for intact mitochondria is twice that of the free enzyme and approximately 1.7 times that of the enzyme bound to the IMM. Although the $[ADP]_{ss}$ values in Table VI-3 are higher for the bound enzyme (experiment b), this is a result of different V_2 values for the two experiments (different rates

of oxidative phosphorylation). Extrapolating these results to the case where both V_1 and V_2 values are identical shows that the effect of lower K_m values for translocase and MiMi-CK is to increase the steady state solution concentration of ADP; the steady state concentration of ADP increases two fold when the system is preferentially coupled. These results show that the kinetic constants measured under the various conditions can be used as practical kinetic constants for MiMi-CK to predict concentrations of the relevant substrates in the bulk solution.

The lack of an effect of the OMM on the K_b and $\bar{K}_b$ values for creatine suggests that the outer membrane probably stabilizes an unstirred layer which exists in between the IMM and the OMM apparently by creating a partial diffusion barrier to charged species only. This result has been previously observed for ATP (11), ADP (24) and for individual ion species such as magnesium and inorganic phosphate (25, 26). The diffusion barrier results in an increased concentration of ATP and ADP in the inter membrane space versus the bulk concentration when preferential coupling occurs.

The magnitude of the concentration increase can be estimated by assuming that the K_m values are true dissociation constants. If the true K_m value is that measured in the absence of a diffusion barrier and the observed K_m value is that measured in the presence of a diffusion barrier, then the ratio of the concentrations of substrate outside the barrier to inside the barrier (S_o/S_i) is defined by Equation VI-9.

[VI-9] $$S_o/S_i = \text{real } K_m/\text{observed } K_m$$

For the MiMi-CK:translocase system, we can calculate that the $MgATP^{-2}$ concentration is 3.3 fold higher in the presence of respiration (see Figure VI-3) and the ADP concentration is two fold higher in the presence of creatine (see Figure VI-4).

The preferential coupling seen in the above experiments is absent when the outer mitochondrial membrane is removed by digitonin treatment, even though about 70% of the MiMi-CK is associated with the IMM. These results are different from those of Saks et al. (6) who observed preferential coupling in the absence of the outer membrane. However, if we assume that the rat heart system is similar to the chicken heart system in binding characteristics, only 10% of the MiMi-CK is bound to the IMM under the conditions of Saks et al. (see Chapter V). Furthermore, as rat heart mitochondria contain approximately 9 times more MiMi-CK than chicken heart mitochondria (M. DeLuca, personal communication), the rate limiting step in the experiments of Saks et al. (6) when MiMi-CK is coupled to the translocase, should not be MiMi-CK but oxidative phosphorylation. Comparing the MiMi-CK activity in the experiments of Saks et al. (6) to the amount of enzyme previously reported for this system (27), shows that they have approximately 10% of the activity which should be present. Lastly, if we interpret the results of their experiments in terms of the the direct binding of MiMi-CK to nucleotide translocase, it is the activities of the individual proteins, and not the overall (solution) activities which are important in determining if preferential coupling occurs. They must directly demonstrate that the turnover number of the nucleotide translocase is greater than that of MiMi-CK so that the rate limiting

step is not the nucleotide translocase but MiMi-CK in their experiments.

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Chapter VII

GENERAL CONCLUSION

Chicken heart MiMi-CK binds to the outside of the inner mitochondrial membrane (IMM). Increasing ionic strength causes the release of enzyme. Only 50% is released from intact mitochondria while 95% is released from mitoplasts. Titrating mitoplasts with homogeneous MiMi-CK results in the binding of 1 mole of MiMi-CK per 3 moles of nucleotide translocase with a K_D = 200 n$\underline{M}$ at pH 7.4. The binding is also pH dependent; increasing pH decreases the affinity. Measuring the K_D values as a function of pH indicates that a group(s) with a pKa of about 6 must be protonated for binding to occur.

The binding of MiMi-CK on the outside of the inner membrane results in a preferential access of MiMi-CK for ATP released by the translocase and of translocase for ADP generated by MiMi-CK. The preferential coupling of these two enzymes is shown by a three fold lower K_a and $\bar{K}_a$ value for $MgATP^{-2}$ when the reaction is coupled to oxidative phosphorylation. These lower values translate into higher ADP and lower ATP steady state concentrations in the presence of creatine showing that the measured K_a values reflect the solution kinetics of the system: lower apparent K_a values result in higher activity and higher steady state ADP levels. Thus the translocase and MiMi-CK enzymes behave as two coupled enzymes with different solution K_m values under different conditions. When coupled to oxidative phosphorylation, creatine phosphate synthesis is greater at identical

solution ATP concentrations and the translocase activity is higher at identical solution ADP concentrations.

The outer mitochondrial membrane is responsible for the observed lowering of the K_a and $\bar{K}_a$ values: digitonin prepared mitoplasts show a much smaller preferential coupling even though more than 70% of the enzyme is bound to the IMM under the conditions of the experiment. This latter suggests that the chicken heart outer mitochondrial membrane stabilizes a diffusional barrier to ATP which becomes the rate limiting step when solution ATP is used to measure the kinetic constants of MiMi-CK. This conclusion is strengthened by the measurement of a K_a value for $MgATP^{-2}$ of 800 $\mu\underline{M}$ using intact mitochondria in the presence of oligomycin A as compared to 125 $\mu\underline{M}$ for soluble homogeneous MiMi-CK.

APPENDICIES

Appendix A

This is the program listing for WILMAN4, written in basic, which allows the calculation of kinetic parameters from initial velocity data.

```
10 REM WILMAN4: Written by S.P.J. Brooks
20 LG = 2.302585093#
30 PP$="press any key to continue"
40 PPP$="press 'c' to continue or 'p' to print"
50 PPPP$="Is the printer turned on ?"
60 PPPPP$="                                        "
70 EE$="************************************************************"
80 CLS:LOCATE 1,1,0:WIDTH 40:COLOR 15,4:LOCATE 10,13:PRINT"          "
90 LOCATE 11,13:PRINT" WILMAN 4 ":LOCATE 12,13,1:PRINT"          "
100 LOCATE 19,1:PRINT"copyright 1985, M.S.U."
110 LOCATE 21,1,0:PRINT PP$
120 ANYKEY$=INKEY$:IF ANYKEY$="" THEN 120
130 DIM S(40),VEL(40),RESID(40),RVM(1600),KBV(1600),W(40),LPY$(75,45),
    A$(45),PT(40)
140 COLOR 7,0:CLS:LOCATE 1,1,0:PRINT:PRINT:PRINT"This program
    calculates the Michaelis"
150 PRINT:PRINT"constant (Km) and Vmax from substrate"
160 PRINT:PRINT"concentrations and initial velocities"
170 PRINT:PRINT"according to one of four different"
180 PRINT:PRINT"estimation methods."
190 PRINT:PRINT:PRINT"This program also tests for the"
200 PRINT:PRINT"presence of outliers based on criteria"
210 PRINT:PRINT"suggested by B. Mannervik, Meth."
220 PRINT:PRINT"Enzymol., 87, 370-390 (1982)."
230 PRINT:PRINT:PRINT PP$-
240 ENT$=INKEY$: IF ENT$="" THEN 240:Z4=0:N1=0
250 REM*******************************************************
260 REM
270 REM MENU
280 REM
290 REM*******************************************************
300 CLS:LOCATE 2,1,0:COLOR 15,4:PRINT"You may do any of the following:"
310 COLOR 7,0:PRINT:PRINT"     1. Input new data pairs"
320 PRINT:PRINT"     2. Add data to existing data"
330 PRINT:PRINT"     3. Edit and/or review the data"
340 PRINT:PRINT"     4. Calculate Km and Vmax;"
350 PRINT"        plot data and residuals."
360 PRINT"        (with and without outliers)"
370 PRINT:PRINT"     H. Help menu"
380 PRINT:PRINT"     E. Exit the program"
390 ANS$=INKEY$: IF ANS$="" THEN 390
400 IF ANS$="e" OR ANS$="E" THEN 6230
410 IF ANS$="h" OR ANS$="H" THEN 5560
420 A1= VAL(ANS$): IF A1<1 OR A1> 4 OR A1<>INT(A1) THEN 390
430 IF N1<1 AND A1=1 OR N1>.1 THEN 470
440 LOCATE 20,1,0:COLOR 15,4:PRINT"You must enter data PRIOR
    ":PRINT"to selection of this option":COLOR 7,0:PRINT:PRINT PP$
```

```
450 AKEY$=INKEY$:IF AKEY$ = "" THEN 450
460 LOCATE 20,1,0:PRINT"                                    ":PRINT"
                    ":PRINT:PRINT"                                    ":GOTO 390
470 ON A1 GOTO 480,480,790,2120
480 REM*************************************************************
490 REM
500 REM option #1: Enter data pairs
510 REM option #2: Add data to existing data
520 REM
530 REM*************************************************************
540 IF A1=2 THEN 620
550 IF N1<1 THEN 660
560 CLS:LOCATE 11,5:COLOR 15,4:PRINT"NOTE: This will erase all"
570 LOCATE 12,5:PRINT"previously entered data !!"
580 PRINT:PRINT:PRINT"Press 'r' to return to the menu"
590 PRINT"Press 'c' to continue"
600 ANS$=INKEY$:IF ANS$<>"c" AND ANS$<>"C" AND ANS$<>"r" AND ANS$<>"R"
    THEN 600
610 COLOR 7,0:IF ANS$="r" OR ANS$="R" THEN 250 ELSE 660
620 IF N1>=40 THEN CLS:COLOR 15,4:LOCATE 11,4:PRINT"40 data pairs is
    the limit !!":COLOR 7,0:PRINT:PRINT:GOTO 760
630 ES=N1+1:IF N1<12 THEN EN=13:GOTO 670
640 IF N1<27 THEN EN=27:GOTO 670
650 IF N1<=40 THEN EN=40:GOTO 670
660 EN=13:ES=1:LI=0
670 IF A1=2 THEN LI=N1
680 CLS:COLOR 15,1:PRINT"press '*' after entering last data pair"
690 LOCATE 3,1:PRINT" pair # ":LOCATE 3,14:PRINT" [S] ":LOCATE 3,26:
    PRINT" v "
700 FOR I=ES TO EN:COLOR 15,11LOCATE I+5-LI,1:PRINT I:COLOR 7,0:LOCATE
    I+5-LI,14:INPUT S$
710 IF S$="*" THEN 750 ELSE S(I)=VAL(S$)
720 LOCATE I+5-LI,26:INPUT VEL(I):NEXT I
730 IF I=14 THEN EN=27:ES=14:LI=13:GOTO 680
740 IF I=28 THEN EN=40:ES=28:LI=27:GOTO 680
750 N1=I-1:COLOR 15,1:PRINT:PRINT N1"data pairs have been entered":
    GOSUB 5430
760 COLOR 7,0:PRINT:PRINT PP$:Z4=0
770 ANS$=INKEY$:IF ANS$="" THEN 770
780 GOTO 250
790 REM*************************************************************
800 REM
810 REM OPTION #3: Review the data
820 REM OPTION #5: List data in plot format
830 REM OPTION #7: List residuals
840 REM
850 REM*************************************************************
860 IF A1<>5 THEN 930
870 CLS:LOCATE 4,1,0:COLOR 15,1:PRINT"In what format do you want the
    data ?":COLOR 7,0
880 LOCATE 7,1:PRINT"(a) v versus [S]"
```

```
890 PRINT:PRINT"(b) 1/v versus 1/[S]"
900 PRINT:PRINT"(c) v/[S] versus v"
910 PRINT:PRINT"(d) v/Vmax versus log([S]/Km)"
920 O4$=INKEY$:IF (O4$<"a" OR O4$>"d") AND (O4$<"A" OR O4$>"D") THEN
    920
930 IF N1<=13 THEN EN=N1:ES=1:LI=0:GOTO 950
940 EN=13:ES=1:LI=0
950 CLS:LOCATE 1,1,0:COLOR 15,1:IF A1=3 THEN PRINT:PRINT" PAIR #":
    LOCATE 2,13:PRINT" [S] ":LOCATE 2,26:PRINT" v "
960 IF A1<>5 THEN 1020
970 PRINT:PRINT" pair # ":LOCATE 2,12
980 IF O4$="a" OR O4$="A" THEN PRINT" [S] ":LOCATE 2,25:PRINT" v "
990 IF O4$="b" OR O4$="B" THEN PRINT" 1/[S]":LOCATE 2,25:PRINT" 1/v "
1000 IF O4$="c" OR O4$="C" THEN PRINT" v ":LOCATE 2,25:PRINT" v/[S] "
1010 IF O4$="d" OR O4$="D" THEN PRINT" log([S]/Km) ":LOCATE 2,25:
     PRINT" v/Vmax "
1020 IF A1=7 AND PN>0 THEN COLOR 15,4:PRINT"Pair #";PN;"removed from
     S.D. calculation":COLOR 15,1
1030 IF A1=7 THEN LOCATE 2,1:PRINT" # ":LOCATE 2,8:PRINT" resid.
     ":LOCATE 2,22:PRINT" % 2S.D.":LOCATE 2,32:PRINT" >2S.D.? "
1040 PRINT:FOR I=ES TO EN
1050 COLOR 15,1:LOCATE 3+I-LI,2:IF A1=3 OR A1=5 THEN PRINT I;
1060 COLOR 7,0:IF A1=3 THEN PRINT TAB(12) S(I) TAB(25) VEL(I):GOTO 1170
1070 IF A1<>5 THEN 1130
1080 IF O4$="a" OR O4$="A" THEN STEMP = S(I):VTEMP = VEL(I)
1090 IF O4$="b" OR O4$="B" THEN STEMP = 1/S(I):VTEMP = 1/VEL(I)
1100 IF O4$="c" OR O4$="C" THEN STEMP = VEL(I):VTEMP = VEL(I)/S(I)
1110 IF O4$="d" OR O4$="D" THEN STEMP = LOG(S(I)/KM)/LG:VTEMP =
     VEL(I)/VM
1120 PRINT TAB(10) STEMP TAB(24) VTEMP:GOTO 1170
1130 PC=INT(RESID(I)/(SQR(SE)*2)*1000)/10
1140 COLOR 15,1:LOCATE 3+I-LI,1:PRINT I;
1150 COLOR 7,0:PRINT TAB(6) RESID(I) TAB(23) PC:IF ABS(PC)<100 THEN
     1170
1160 COLOR 15,4:LOCATE 3+I-LI,35:PRINT" * ":COLOR 7,0
1170 NEXT I: IF A1=3 THEN 1430
1180 REM*******************************************************
1190 REM
1200 REM continue listing the data
1210 REM
1220 REM*******************************************************
1230 IF N1=I-1 THEN 1340
1240 LOCATE 4+I-LI:PRINT PP$
1250 ANS$=INKEY$:IF ANS$="" THEN 1250
1260 IF N1<=27 AND I<28 THEN EN=N1:ES=13:LI=12:GOTO 950
1270 IF N1>27 AND I<28 THEN EN=27:ES=13:LI=12:GOTO 950
1280 EN=N1:ES=27:LI=26:GOTO 950
1290 REM*******************************************************
1300 REM
1310 REM stop listing the data
1320 REM
```

```
1330 REM************************************************************
1340 IF A1=7 AND N1+1=I THEN COLOR 15,1:PRINT:PRINT:PRINT"sequence =
       ";BB$:PRINT AA$:COLOR 7,0
1350 IF (A1=7 AND AAA1 =0) OR A1=5 THEN 1390
1360 PRINT:PRINT PP$
1370 ANS$=INKEY$:IF ANS$="" THEN 1370
1380 CLS:GOTO 2230
1390 PRINT:PRINT PPP$
1400 ANS$=INKEY$:IF ANS$<>"c" AND ANS$<>"C" AND ANS$<>"p" AND ANS$<>"P"
     THEN 1400
1410 IF ANS$="p" OR ANS$="P" THEN 1750
1420 COLOR 7,0:GOTO 3650
1430 REM************************************************************
1440 REM
1450 REM continue with option #3
1460 REM
1470 REM************************************************************
1480 LOCATE 5+I-LI,1:PRINT"Press 'e' to edit data"
1490 PRINT"Press 'd' to delete data"
1500 PRINT"Press 'c' to continue"
1510 ANS$=INKEY$: IF ANS$<>"c" AND ANS$<>"C" AND ANS$<>"e" AND
     ANS$<>"E" AND ANS$<>"d" AND ANS$<> "D" THEN 1510
1520 IF ANS$<>"c" AND ANS$<> "C" THEN 1570
1530 IF I=N1+1 THEN COLOR 7,0:WIDTH 40:GOTO 250
1540 IF I=14 AND N1<27 THEN EN=N1:ES=13:LI=12:GOTO 950
1550 IF I=14 AND N1>27 THEN EN=27:ES=13:LI=12:GOTO 950
1560 EN=N1:ES=27:LI=26:GOTO 950
1570 Z4=0:LOCATE I+5-LI:COLOR 15,4:PRINT"Which data pair do you want
     to"
1580 COLOR 7,0
1590 PRINT"     - -                    "
1600 PRINT"                            "
1610 LOCATE 6+I-LI,1:COLOR 15,4
1620 IF ANS$<>"e" AND ANS$<>"E" THEN 1690
1630 INPUT"edit  ";N2
1640 IF N2<ES OR N2>EN THEN 1610
1650 LOCATE 3+N2-LI,2:PRINT N2
1660 LOCATE 3+N2-LI,13:INPUT" ";S(N2)
1670 LOCATE 3+N2-LI,25:INPUT" ";VEL(N2)
1680 COLOR 7,0:GOSUB 5430: GOTO 790
1690 INPUT"delete ";N2:COLOR 7,0
1700 IF N2<1 OR N2>N1 THEN 1610
1710 N1=N1-1:FOR I=N2 TO N1:VEL(I)=VEL(I+1):S(I)=S(I+1):NEXT I: GOTO
     790
1720 REM************************************************************
1730 REM
1740 REM print results to line printer
1750 REM
1760 REM************************************************************
1770 IF A1=7 THEN LOCF=8+I-LI ELSE LOCF=4+I-LI
1780 LOCATE LOCF:PRINT PPPPP$:COLOR 15,4:LOCATE LOCF:PRINT PPPP$:COLOR
```

```
    7,0
1790 PRINT:PRINT PP$
1800 ANS$=INKEY$:IF ANS$=""THEN 1800
1810 LPRINT:LPRINT EE$
1820 IF A1=7 THEN 1950
1830 LPRINT:LPRINT"Data listing:":LPRINT"------------"
1840 LPRINT:LPRINT"pair #" TAB(10) "[substrate]" TAB(27) "velocity";
1850 IF O4$="a" OR O4$="A" THEN LPRINT TAB(44) "  "
1860 IF O4$="b" OR O4$="B" THEN LPRINT TAB(44) "1/[substrate]" TAB(61)
    "1/velocity"
1870 IF O4$="c" OR O4$="C" THEN LPRINT TAB(44) "velocity/[substrate]"
1880 IF O4$="d" OR O4$="D" THEN LPRINT TAB(44) "log([sub.]/Km)" TAB(61)
    "velocity/Vmax"
1890 LPRINT"------" TAB(10) "-----------" TAB(27) "--------";
1900 IF O4$="a" OR O4$="A" THEN LPRINT TAB(44) "  "
1910 IF O4$="b" OR O4$="B" THEN LPRINT TAB(44) "----------" TAB(61) "--
    ----------"
1920 IF O4$="c" OR O4$="C" THEN LPRINT TAB(44) "------------------"
1930 IF O4$="d" OR O4$="D" THEN LPRINT TAB(44) "--------------" TAB(61)
    "-------------"
1940 GOTO 1980
1950 LPRINT:LPRINT:LPRINT"Results of WILMAN4 calculations:  ";CC$
1960 LPRINT:LPRINT:LPRINT"pair #" TAB(17) "[substrate]" TAB(34)
    "velocity" TAB(51) "residual" TAB(68) "% of 2 S.D."
1970 LPRINT"------" TAB(17) "-----------" TAB(34) "--------" TAB(51) "-
    -------" TAB(68) "----------"
1980 LPRINT: FOR I=1 TO N1
1990 IF A1=7 THEN PC=INT(RESID(I)/(SQR(SE)*2)*1000)/10:GOTO 2060
2000 LPRINT I TAB(10) S(I) TAB(27) VEL(I);
2010 IF O4$="a" OR O4$="A" THEN LPRINT TAB(44) " "
2020 IF O4$="b" OR O4$="B" THEN LPRINT TAB(44) 1/S(I) TAB(61)1/VEL(I)
2030 IF O4$="c" OR O4$="C" THEN LPRINT TAB(44) VEL(I)/S(I)
2040 IF O4$="d" OR O4$="D" THEN LPRINT TAB(44) LOG(S(I)/KM)/LG TAB(61)
    VEL(I)/VM
2050 GOTO 2070
2060 LPRINT I TAB(17) S(I) TAB(34) VEL(I) TAB(51) RESID(I) TAB(68) PC
2070 NEXT I:IF A1=5 THEN 2100
2080 IF PN>0 THEN LPRINT:LPRINT"Pair #";PN;"removed from analysis"
2090 LPRINT:LPRINT"sequence of residuals (in order of increasing
    velocity) = ";BB$:LPRINT AA$
2100 LPRINT:LPRINT EE$
2110 GOTO 3650
2120 REM************************************************************
2130 REM
2140 REM Option #4: Km and Vmax estimation --> leads to sub-menu
2150 REM
2160 REM************************************************************
2170 CLS:LOCATE 1,1,0
2180 PN=0:IF Z4=0 THEN 2310
2190 CLS:LOCATE 6,1:COLOR 15,4:PRINT"Do you wish to eliminate outliers"
2200 PRINT"(y=yes, n=no) ?":COLOR 7,0
```

```
2210 ANS4$=INKEY$: IF ANS4$<>"y" AND ANS4$<>"Y"AND ANS4$<>"n" AND
     ANS4$<>"N" THEN 2210
2220 IF ANS4$="n" OR ANS4$="N" THEN Z2=2:PN=0:GOTO 2310
2230 AAA1=0:COLOR 15,1:LOCATE 10,1:PRINT"Enter outlier pair #":COLOR
     7,0
2240 COLOR 15,3:PRINT:PRINT:PRINT"(To display residuals and possible"
2250 PRINT"outliers enter -1)"
2260 PRINT:PRINT"(To calculate Km and Vmax without"
2270 PRINT"removing an outlier enter 0)":COLOR 7,0
2280 LOCATE 10,23:COLOR 15,1:INPUT PN:COLOR 7,0
2290 IF PN<-1 OR PN>N1 THEN LOCATE 10,23:PRINT"          ":GOTO 2280
2300 IF PN = -1 THEN A1=7:AAA1=1:GOTO 4390
2310 CLS:PRINT:COLOR 15,2:PRINT"Parameter estimation method desired ?"
2320 COLOR 7,0:PRINT:PRINT"(a) Linear regression (Wilkinson)"
2330 PRINT"    (Biochem J., 1961, 80, 324)":PRINT"    ";
2340 COLOR 15,1:PRINT"(w = Vmax^2*v^2/(Km+[S])^2))":COLOR 7,0
2350 PRINT:PRINT"(b) Linear regression (C.-Bowden)*":PRINT"    ";
2360 COLOR 15,1:PRINT"(w = Vmax*v^2/(Km+[S])[S])":COLOR 7,0
2370 PRINT:PRINT"(c) Linear regression (J. & Lumry)"
2380 PRINT"    (C. R. Trav. Lab. Carls., 1961,)"
2390 PRINT"     32, 185)   ";
2400 COLOR 15,1:PRINT"(w = v^2/[S]^2)":COLOR 7,0
2410 PRINT:PRINT"(d) Non-parameter estimate (C.-Bowden)*"
2420 PRINT"    ";
2430 COLOR 15,4:PRINT"Note !!";
2440 COLOR 7,0:PRINT" Different velocity measure-"
2450 PRINT"    ments at a single substrate conc."
2460 PRINT"    are not allowed with this option !!"
2470 PRINT:PRINT:PRINT"* (Principals of Enzyme Kinetics,(1976)"
2480 PRINT" Butterworths Pub. Co.)"
2490 F$=INKEY$:IF (F$<"A" OR F$>"D") AND (F$<"a" OR F$>"d") THEN 2490
2500 IF F$="a" OR F$="A" THEN CC$="Linear regression (Wilkinson)"
2510 IF F$="b" OR F$="B" THEN CC$="Linear regression (Intermed.)"
2520 IF F$="c" OR F$="C" THEN CC$="Linear regression (J. & Lumry)"
2530 IF F$="d" OR F$="D" THEN CC$="Non-parameter (C.-Bowden)":GOTO 2810
2540 REM*******************************************
2550 REM
2560 REM Linear regression estimation
2570 REM
2580 REM*******************************************
2590 CLS:KO=0:N3=0
2600 E1=0:E2=0:E3=0:E4=0:E5=0
2610 FOR I=1 TO N1
2620 IF I=PN THEN 2730
2630 IF N3=0 AND (F$="a" OR F$="A") THEN W(I)=VEL(I)^4/S(I)^2
2640 IF N3=0 AND (F$="b" OR F$="B") THEN W(I)=VEL(I)^3/S(I)^2
2650 IF N3>0 AND (F$="a" OR F$="A") THEN W(I)=VEL(I)^2*VM^2/(KM+S(I))^2
2660 IF N3>0 AND (F$="b" OR F$="B") THEN
     W(I)=VEL(I)^2*VM/((KM+S(I))*S(I))
2670 IF F$="c" OR F$="C" THEN W(I) = VEL(I)^2/S(I)^2
2680 E1=E1+W(I)
```

```
2690 E2=E2+W(I)*S(I)^2
2700 E3=E3+W(I)*S(I)
2710 E4=E4+W(I)*S(I)^2/VEL(I)
2720 E5=E5+W(I)*S(I)/VEL(I)
2730 NEXT I
2740 KM=(E5*E2-E3*E4)/(E1*E4-E3*E5)
2750 VM=(E1*E2-E3^2)/(E1*E4-E3*E5)
2760 IF F$="c" OR F$="C" THEN 3110
2770 KT=ABS(INT((KM-KO)/KM*10000)):IF KT<1 THEN DD$="":GOTO 3110
2780 KO=KM:N3=N3+1
2790 IF N3<10 THEN 2600
2800 DD$="Not converged by 10th iteration !!":GOTO 3110
2810 REM***********************************************
2820 REM
2830 REM Non-parameter estimation
2840 REM
2850 REM***********************************************
2860 CLS:DD$="":K=0:FOR I=1 TO N1: IF I=PN THEN 2920
2870 FOR J=1 TO N1: IF J=PN THEN 2910
2880 IF I=J THEN 2910
2890 K=K+1:RVM(K)=(S(J)/VEL(J)-S(I)/VEL(I))/(S(J)-S(I))
2900 KBV(K)=S(I)*(1/VEL(I)-RVM(K))
2910 NEXT J
2920 NEXT I:GAP =INT(K/2)
2930 FLG=1:FOR I=1 TO K-GAP: IF RVM(I)<=RVM(I+GAP) THEN 2950
2940 TEMP=RVM(I):RVM(I)=RVM(I+GAP):RVM(I+GAP)=TEMP:FLG=0
2950 NEXT I:IF FLG=0 THEN 2930
2960 GAP=INT(GAP/2):IF GAP>0 THEN 2930
2970 IF K/2=INT(K/2) THEN I1=K/2:VM=.5*(1/RVM(I1)+1/RVM(1+I1)):J1=2
2980 IF K/2<>INT(K/2) THEN I1=INT(K/2)+1:VM=1/(RVM(I1)):J1=1
2990 GAP=INT(K/2)
3000 FLG=1:FOR I=1 TO K-GAP:IF KBV(I)<=KBV(I+GAP) THEN 3020
3010 TEMP=KBV(I):KBV(I)=KBV(I+GAP):KBV(I+GAP)=TEMP:FLG=0
3020 NEXT I:IF FLG=0 THEN 3000
3030 GAP=INT(GAP/2):IF GAP>0 THEN 3000
3040 IF J1=1 THEN KM=VM*KBV(I1)
3050 IF J1=2 THEN KM=VM*(KBV(I1)+KBV(I1+1))/2
3060 REM***********************************************
3070 REM
3080 REM Print results to the screen
3090 REM
3100 REM***********************************************
3110 CLS:PRINT:PRINT"Results: "; CC$
3120 PRINT:COLOR 15,4:PRINT DD$:COLOR 7,0
3130 SS=0
3140 IF F$="d" OR F$="D" THEN E1=0:E2=0:E3=0
3150 FOR I=1 TO N1:IF I=PN THEN 3220
3160 IF F$<>"d" AND F$<>"D" THEN 3190
3170 W(I) = VEL(I)^2*VM/((KM + S(I))*S(I))
3180 E1=E1+W(I):E2=E2+W(I)*S(I)^2:E3=E3+W(I)*S(I)
3190 ERRORF = S(I)/VEL(I) - S(I)/VM - KM/VM
```

```
3200 RESID(I) = -ERRORF*SQR(W(I))
3210 SS = SS + W(I)*ERRORF^2
3220 NEXT I:IF PN=0 THEN 3270
3230 IF F$="a" OR F$="A" THEN W(PN)=VEL(PN)^2*VM^2/(KM+S(PN))^2
3240 IF F$="b" OR F$="B" OR F$="d" OR F$="D" THEN
     W(PN)=VEL(PN)^2*VM/((KM+S(PN))*S(PN))
3250 IF F$="c" OR F$="C" THEN W(PN)=VEL(PN)^2/S(PN)^2
3260 RESID(PN)= (S(PN)/VM+KM/VM-S(PN)/VEL(PN))*SQR(W(PN))
3270 BT = E1*E2 - E3^2
3280 PE=0:IF PN>0 THEN PE=1
3290 SE=SS/(N1-2-PE)
3300 VK=SQR(VM^2*SE*(E2+2*KM*E3+KM^2*E1)/BT)
3310 VV=SQR(VM^4*SE*E1/BT)
3320 VS=SQR(SE*E2/BT)
3330 IF PN>0 THEN COLOR 15,4:LOCATE 4,1:PRINT"pair #";PN;"removed from
     analysis":COLOR 7,0
3340 COLOR 15,1:LOCATE 6,1:PRINT"Km = ";KM
3350 LOCATE 8,1:PRINT"Vmax = ";VM
3360 LOCATE 10,1:PRINT"Slope (Km/Vmax) = ";KM/VM:COLOR 7,0
3370 COLOR 15,2:LOCATE 13,1:PRINT"std. dev. of Km = ";VK
3380 LOCATE 15,1:PRINT"Std. dev. of Vmax = ";VV
3390 LOCATE 17,1:PRINT"Std. dev. of slope = ";VS:COLOR 7,0
3400 IF F$="d" OR F$="D" THEN COLOR 15,3:LOCATE 19,1:PRINT"** Note: std
     devs. are approximations !!":COLOR 7,0
3410 IF PN=1 THEN KML1=2 ELSE KML1=1
3420 IF PN=N1 THEN KML2=N1-1 ELSE KML2=N1
3430 KML=S(KML1)/KM*100:KMH=S(KML2)/KM*100
3440 COLOR 15,1:LOCATE 21,1:PRINT USING "[S] varies from ###.#% to
     ###.#% of Km"; KML, KMH:COLOR 7,0
3450 LOCATE 23,1:PRINT PPP$
3460 Z4=1:ANS$=INKEY$:IF ANS$<>"c" AND ANS$<>"C" AND ANS$<>"p" AND
     ANS$<>"P" THEN 3460
3470 IF ANS$="c" OR ANS$="C" THEN 3650
3480 REM************************************************************
3490 REM
3500 REM print to line printer
3510 REM
3520 REM************************************************************
3530 LOCATE 21,1:PRINT PPPPP$+" ":LOCATE 23,1:PRINT PPPPP$
3540 COLOR 15,4:LOCATE 21,1:PRINT PPPP$:COLOR 7,0:LOCATE 22,1:PRINT PP$
3550 ANS$=INKEY$:IF ANS$="" THEN 3550
3560 LPRINT:LPRINT EE$
3570 LPRINT:LPRINT"Results of WILMAN4 calculations:  ";CC$
3580 LPRINT:LPRINT "Km = ";KM;" +/- ";VK
3590 LPRINT"Vmax = ";VM;" +/- ";VV
3600 LPRINT"Km/Vmax = ";KM/VM;" +/- "VS
3610 LPRINT:LPRINT:LPRINT USING "[S] varies from ###.#% to ###.#% of
     Km"; KML, KMH
3620 IF F$="d" OR F$="D" THEN LPRINT:LPRINT"** Note: std. devs. are
     approximations !!"
3630 IF PN>0 THEN LPRINT:LPRINT"Pair #";PN;"removed from analysis"
```

```
3640 LPRINT:LPRINT EE$
3650 REM************************************************************
3660 REM
3670 REM Sub-menu
3680 REM
3690 REM************************************************************
3700 CLS:LOCATE 2,1,0:COLOR 15,4:PRINT"You may do any of the
     following:"
3710 COLOR 7,0:PRINT:PRINT"     4. Recalculate Km and Vmax"
3720 PRINT:PRINT"     5. List data in plot format"
3730 PRINT:PRINT"     6. Plot the data"
3740 PRINT:PRINT"     7. List residuals"
3750 PRINT:PRINT"     8. Plot residuals"
3760 PRINT:PRINT"     R. Return to main menu"
3770 ANS$=INKEY$: IF ANS$="" THEN 3770
3780 IF ANS$="r" OR ANS$="R" THEN 250
3790 A1= VAL(ANS$): IF A1<4 OR A1> 8 OR A1<>INT(A1) THEN 3770
3800 A11=A1-3:ON A11 GOTO 2120,790,3810,4390,4390
3810 REM************************************************************
3820 REM
3830 REM OPTION #6: Plot the data
3840 REM
3850 REM************************************************************
3860 CLS:LOCATE 4,1,0:COLOR 15,1:PRINT"How do you want to plot the data
     ?":COLOR 7,0
3870 LOCATE 7,1:PRINT"(a) v versus [S]"
3880 PRINT:PRINT"(b) 1/v versus 1/[S]"
3890 PRINT:PRINT"(c) v/[S] versus v"
3900 PRINT:PRINT"(d) v versus log[S]"
3910 O6$=INKEY$:IF (O6$<"a" OR O6$>"d") AND (O6$<"A" OR O6$>"D") THEN
     3910
3920 TEMP=1:TEMP1=1:IF PN=1 THEN TEMP=2:TEMP1=2
3930 FOR I=2 TO N1:IF PN=I THEN 3970
3940 IF O6$="a" OR O6$="A" THEN IF VEL(I)>VEL(TEMP) THEN TEMP=I
3950 IF O6$="b" OR O6$="B" THEN IF 1/VEL(I)>1/VEL(TEMP) THEN TEMP=I
3960 IF O6$="d" OR O6$="D" THEN IF
     ABS(LOG(S(I)/KM)/LG)>ABS(LOG(S(TEMP)/KM)/LG)THEN TEMP=I
3970 NEXT I:TEMP2=N1:IF N1=PN THEN TEMP2=N1-1
3980 IF O6$ ="a" OR O6$="A" THEN B1=S(TEMP2):B2=VEL(TEMP)
3990 IF O6$ ="b" OR O6$="B" THEN B1=1/S(TEMP1):B2=1/VEL(TEMP)
4000 IF O6$ ="c" OR O6$="C" THEN B1=VM:B2=VM/KM
4010 IF O6$ ="d" OR O6$="D" THEN B1=ABS(LOG(S(TEMP)/KM)/LG):B2=1
4020 CLS:SCREEN 1,0:COLOR 0,1
4030 IF PN>0 THEN PRINT"Pair #";PN;"removed from the graph"
4040 IF O6$="a" OR O6$="A" THEN LOCATE 9,1:PRINT" v":LOCATE
     20,28:PRINT"[S]"
4050 IF O6$="b" OR O6$="B" THEN LOCATE 9,1:PRINT" 1":PRINT" -":PRINT"
     v":LOCATE 20,28:PRINT"1/[S]"
4060 IF O6$="c" OR O6$="C" THEN LOCATE 9,1:PRINT" v":PRINT"---
     ":PRINT"[S]":LOCATE 20,28:PRINT"v"
4070 IF O6$="d" OR O6$="D" THEN LOCATE 9,1:PRINT"  v":PRINT" ----
```

```
    ":PRINT" Vmax":LOCATE 20,28:PRINT"(log[S]/Km)"
4080 LOCATE 22,1:PRINT PP$ 4090 LINE (30,150)-(300,147),2,BF
4100 A=33:IF O6$="d" OR O6$="D" THEN A=166
4110 LINE (A,10)-((A-3),147),2,BF
4120 FOR I=1 TO N1:IF PN = I THEN 4180
4130 IF O6$="a" OR O6$="A" THEN XCORD=S(I)/B1*267+33:YCORD=147-
    VEL(I)/B2*137
4140 IF O6$="b" OR O6$="B" THEN XCORD=(1/S(I))/B1*267+33:YCORD=147-
    (1/VEL(I))/B2*137
4150 IF O6$="c" OR O6$="C" THEN XCORD=VEL(I)/B1*257+33:YCORD =147-
    (VEL(I)/S(I))/B2*127
4160 IF O6$="d" OR O6$="D" THEN
    XCORD=33+133.5*(B1+LOG(S(I)/KM)/LG)/B1:YCORD =147-VEL(I)/VM*137
4170 CIRCLE (XCORD,YCORD),2,1,,,1
4180 NEXT I
4190 IF O6$<>"a" AND O6$<>"A" THEN 4250
4200 FOR XCORD = 33 TO 300 STEP 4
4210 XXX1=(XCORD-33)*B1/267:XXX2=(XCORD+4-33)*B1/267
4220 YCORD1= 147-137*(VM*XXX1/(KM + XXX1))/B2
4230 YCORD2= 147-137*(VM*XXX2/(KM + XXX2))/B2
4240 LINE (XCORD,YCORD1) - (XCORD+4,YCORD2),3:NEXT XCORD:GOTO 4370
4250 IF O6$<>"d" AND O6$<>"D" THEN 4320
4260 FOR XCORD = 33 TO 300 STEP 4
4270 XXX1=KM*EXP(((XCORD-33)*B1/133.5-B1)*LG)
4280 XXX2=KM*EXP(((XCORD-29)*B1/133.5-B1)*LG)
4290 YCORD1= 147-137*(VM*XXX1/(KM + XXX1))/VM
4300 YCORD2= 147-137*(VM*XXX2/(KM + XXX2))/VM
4310 LINE (XCORD,YCORD1) - (XCORD+4,YCORD2),3:NEXT XCORD:GOTO 4370
4320 IF O6$="c" OR O6$="C" THEN 4360
4330 CORD1=147-(1/VM)/B2*137
4340 CORD2=147-(1/(VM/B1/(KM+1/B1)))/B2*137
4350 LINE(33,CORD1)-(300,CORD2),3:GOTO 4370
4360 LINE(33,20)-(290,147)
4370 ANS$=INKEY$:IF ANS$="" THEN 4370
4380 SCREEN 0,1:COLOR 7,0:GOTO 3650
4390 REM************************************************************
4400 REM
4410 REM Option #7: List residuals
4420 REM Option #8: Plot residuals
4430 REM
4440 REM************************************************************
4450 FOR I=1 TO N1:PT(I)=I:NEXT I
4460 FOR I=1 TO N1-1: I1=I:LEAST = VEL(PT(I))
4470 FOR J=1+I TO N1:IF VEL(PT(J))<LEAST THEN I1=J:LEAST=VEL(PT(J))
4480 NEXT J
4490 IF I1<>I THEN TEMP=PT(I):PT(I)=PT(I1):PT(I1)=TEMP
4500 NEXT I
4510 CLS:M9=0:N9=0:U9=0:A2=0:BB$=""
4520 FOR I=1 TO N1: IF PT(I)=PN THEN 4570
4530 IF RESID(PT(I))<0 THEN M9=M9+1:A3=1:BB$=BB$+"-"
4540 IF RESID(PT(I))>0 THEN N9=N9+1:A3=2:BB$=BB$+"+"
```

```
4550 IF RESID(PT(I))=0 THEN BB$=BB$+"0"
4560 IF A3<>A2 THEN U9=U9+1
4570 A2=A3:NEXT I
4580 IF M9+N9<8 THEN AA$="Too few points to analyse randomness":GOTO
     4750
4590 IF N9<M9 THEN TEMP=N9:N9=M9:M9=TEMP
4600 IF M9<2 THEN FT=0:GOTO 4740
4610 FT=0:FOR I=2 TO U9:K9=I/2
4620 IF K9<>INT(K9) THEN K9=(I+1)/2:GOTO 4660
4630 A3=M9-1:A4=K9-1:GOSUB 5300:C1=C9
4640 A3=N9-1:A4=K9-1:GOSUB 5300:C2=C9
4650 FU = 2*C1*C2:GOTO 4710
4660 A3=M9-1:A4=K9-1:GOSUB 5300:C1=C9
4670 A3=N9-1:A4=K9-2:GOSUB 5300:C2=C9
4680 A3=M9-1:A4=K9-2:GOSUB 5300:C3=C9
4690 A3=N9-1:A4=K9-1:GOSUB 5300:C4=C9
4700 FU=C1*C2+C3*C4
4710 FT=FT+FU:NEXT I
4720 A3=N9+M9:A4=M9:GOSUB 5300: FT=FT/C9:Z9=1
4730 IF FT>.05 THEN AA$="The sequence is random (p>0.95)"
4740 IF FT<=.05 THEN AA$="The sequence is non random (p>0.95)"
4750 IF A1=7 THEN 790
4760 REM************************************************************
4770 REM
4780 REM Option #8: Plot residuals (continued)
4790 REM
4800 REM************************************************************
4810 TEMP=1:TEMP1=1:IF PN=1 THEN TEMP=2:TEMP1=2
4820 B1=VEL(TEMP):B2=ABS(RESID(TEMP1))
4830 FOR I=TEMP TO N1:IF I=PN THEN 4860
4840 IF ABS(RESID(I))>B2 THEN B2=ABS(RESID(I))
4850 IF VEL(I)>B1 THEN B1=VEL(I)
4860 NEXT I:SCREEN 1,0
4870 COLOR 0,1:PRINT"Sequence = ";BB$:PRINT AA$
4880 EN=8:IF PN>0 THEN PRINT"Pair #";PN;"removed from graph":EN=7
4890 FOR I=1 TO EN:PRINT:NEXT I:PRINT"R":PRINT"e":PRINT"s":PRINT"i";
4900 PRINT TAB(28)"velocity":PRINT"d"
4910 LOCATE 23,1:PRINT"Press 'c' to continue or 'p' to plot"
4920 LINE (33,30)-(30,175),2,BF
4930 LINE (30,104)-(300,101),2,BF
4940 P4=N1:IF PN=N1 THEN P4=N1-1
4950 FOR I=1 TO N1:IF I=PN THEN 4980
4960 XCORD = 33 + VEL(PT(I))/B1*267
4970 YCORD =102 - (RESID(PT(I))/B2)*72:CIRCLE (XCORD,YCORD),2,1,,,1
4980 NEXT I
4990 ANS$=INKEY$:IF ANS$<>"c" AND ANS$<>"C" AND ANS$<>"p" AND ANS$<>"P"
     THEN 4990
5000 IF ANS$="c" OR ANS$="C" THEN SCREEN 0,1:COLOR 7,0:GOTO 3650
5010 LOCATE 23,1:PRINT"Is the printer on ? (press any key)  "
5020 ANS$=INKEY$:IF ANS$="" THEN 5020
5030 REM************************************************************
```

```
5040 REM
5050 REM plot residuals on line printer
5060 REM
5070 REM************************************************************
5080 CLS:FOR I=0 TO 40: A$(I)="    ":NEXT I
5090 A$(18)=" r  ":A$(17)=" e  ":A$(16)=" s  ":A$(15)=" i  ":A$(14)=" d
     ":A$(13)=" u  ":A$(12)=" a  ":A$(11)=" l  "
5100 FOR X=0 TO 70:FOR Y=0 TO 30:LPY$(X,Y)=" ":NEXT Y:NEXT X
5110 FOR X=1 TO 70:IF INT(X/10)=X/10 THEN A1$="|" ELSE A1$="-"
5120 LPY$(X,15)=A1$:NEXT X
5130 FOR Y=0 TO 30:IF INT(Y/3)=Y/3 THEN A1$="-" ELSE A1$="|"
5140 LPY$(0,Y)=A1$:NEXT Y
5150 FOR I=1 TO N1:IF I=PN THEN 5180
5160 Y=(RESID(PT(I))+B2)/B2*14.5:X=VEL(PT(I))/B1*70
5170 LPY$(INT(X+.5),INT(Y+.5))="O"
5180 NEXT I
5190 LPRINT:LPRINT:LPRINT EE$:LPRINT:LPRINT"Graph of residuals versus
     velocity:":LPRINT"----------------------------------
     ":LPRINT:LPRINT
5200 FOR Y=30 TO 0 STEP -1
5210 LP5$="":FOR X=0 TO 70:LP5$=LP5$+LPY$(X,Y):NEXT X
5220 LPRINT " "+A$(Y)+LP5$:NEXT Y
5230 LPRINT:LPRINT"
     velocity"
5240 LPRINT:LPRINT"x interval = ";B1/7:LPRINT"y interval = ";B2/15*3
5250 LPRINT:LPRINT"Residuals calculated using:  ";CC$
5260 LPRINT:LPRINT"sequence of residuals = ";BB$:LPRINT AA$
5270 IF PN>0 THEN LPRINT:LPRINT"Pair #";PN;"removed from analysis"
5280 LPRINT:LPRINT EE$
5290 SCREEN 0,1:COLOR 7,0: GOTO 3650
5300 REM************************************************************
5310 REM
5320 REM Factorial subroutine
5330 REM
5340 REM************************************************************
5350 J1=1:J2=1:J3=1:A5=(A3-A4)
5360 IF A3<=0 THEN A3=1
5370 IF A4<=0 THEN A4=1
5380 IF A5<=0 THEN A5=1
5390 FOR I3= A3 TO 1 STEP -1:J1=J1*I3:NEXT I3
5400 FOR I3 =A4 TO 1 STEP -1:J2=J2*I3:NEXT I3
5410 FOR I3=A5 TO 1 STEP -1:J3=J3*I3:NEXT I3
5420 C9=J1/(J2*J3):RETURN
5430 REM************************************************************
5440 REM
5450 REM Sorting subroutine
5460 REM
5470 REM************************************************************
5480 FOR I3=1 TO N1-1:I1=I3:LE=S(I3)
5490 FOR I4=I3+1 TO N1:IF S(I4)<LE THEN I1=I4:LE = S(I4)
5500 NEXT I4: IF I3=I1 THEN 5520
```

```
5510 I5=S(I1):I6=VEL(I1):S(I1)=S(I3):VEL(I1)=VEL(I3):S(I3)=I5:
     VEL(I3)=I6
5520 NEXT I3:RETURN
5530 REM*************************************************************
5540 REM
5550 REM Option #H: Help menu
5560 REM
5570 REM*************************************************************
5580 CLS:LOCATE 3,1,0:COLOR 15,3:PRINT"You may obtain help on any of
     the"
5590 PRINT"the following:":COLOR 7,0
5600 PRINT:PRINT:PRINT"(A) Removing outliers"
5610 PRINT:PRINT"(B) Residual equations"
5620 PRINT:PRINT"(C) Km and Vmax estimation"
5630 PRINT:PRINT"(D) Return to the menu"
5640 ANS$=INKEY$:IF (ANS$<"A" OR ANS$>"D") AND (ANS$<"a" OR ANS$>"d")
     THEN 5640
5650 IF ANS$="d" OR ANS$="D" THEN 250
5660 IF ANS$<>"a" AND ANS$<>"A" THEN 5840
5670 CLS:LOCATE 2,1,0:COLOR 15,3:PRINT"Removing outliers:":COLOR 7,0
5680 PRINT:PRINT"Outliers are defined as data which are"
5690 PRINT"two times greater than the experimental"
5700 PRINT"standard deviation. They are indicated"
5710 PRINT"by the presence of an '*' in the far"
5720 PRINT"right hand column of option #7.  Once"
5730 PRINT"outliers are identified, return to #4"
5740 PRINT"and indicate which value you wish to"
5750 PRINT"remove (you can remove only one"
5760 PRINT"outlier at a time).  The new values"
5770 PRINT"of Vmax and Km are calculated without"
5780 PRINT"the outlier.  Option #7 now shows the"
5790 PRINT"new residuals."
5800 PRINT:PRINT"See B. Mannervik, 1982, Meth.  Enzymol.,"
5810 PRINT"vol. 87, pp 370-390.":PRINT:PRINT PP$
5820 ANS$=INKEY$:IF ANS$="" THEN 5820
5830 GOTO 5560
5840 IF ANS$<>"b" AND ANS$<>"B" THEN 6010
5850 CLS:LOCATE 2,1,0:COLOR 15,3:PRINT"Residual equations:":COLOR 7,0
5860 PRINT:PRINT"The following equation defines the"
5870 PRINT"residual shown in option #7 and #8:"
5880 PRINT:COLOR 15,1:PRINT"resid = u * d"
5890 COLOR 7,0:PRINT:PRINT"where d = -Vmax*v*e/(Km + [S])"
5900 PRINT"      e = [S]/v - Km/Vmax - [S]/Vmax"
5910 PRINT"(see menu C for a definition of u)"
5920 PRINT:PRINT"One standard deviation (S.D.) is"
5930 PRINT"defined as follows:"
5940 PRINT:COLOR 15,1:PRINT"S.D. = SQR[SUM(u * d^2)/(n - p)]"
5950 COLOR 7,0:PRINT:PRINT"where n is the number of data points"
5960 PRINT"      p is the number of parameters"
5970 PRINT"        (for our case p=2)"
5980 PRINT:PRINT PP$
```

```
5990 ANS$=INKEY$:IF ANS$="" THEN 5990
6000 GOTO 5560
6010 CLS:LOCATE 2,1,0:COLOR 15,3:PRINT"Km and Vmax estimations:"
6020 COLOR 7,0:PRINT:PRINT"Km and Vmax are estimated using"
6030 PRINT"either one of the three linear"
6040 PRINT"regression procedures or by the"
6050 PRINT"non-parametrical method. The"
6060 PRINT"weighting factor, u, is defined as:"
6070 PRINT:COLOR 15,1:PRINT"Wilkinson: u = 1":COLOR 7,0
6080 PRINT:COLOR 15,1:PRINT"C.-Bowden: u = (Km + [S])/Vmax*[S]":COLOR
     7,0
6090 PRINT:COLOR 15,1:PRINT"J. & Lumry: u = (Km +
     [S])^2/VM^2*[S]^2":COLOR 7,0
6100 PRINT:PRINT"The non-parametrical method"
6110 PRINT"calculates Km and Vmax estimates"
6120 PRINT"and uses the median value:"
6130 PRINT:COLOR 15,1:PRINT"Vmax(i,j) = [sj - si]/[sj/vj - si/vi]"
6140 PRINT"Km(i,j) = si*[Vmax(i,j)/vi - 1]":COLOR 7,0
6150 PRINT:PRINT PP$
6160 ANS$=INKEY$:IF ANS$="" THEN 6160
6170 GOTO 5530
6180 REM************************************************************
6190 REM
6200 REM Option #E: End the program
6210 REM
6220 REM************************************************************
6230 WIDTH 80:COLOR 7,0:CLS:END
```

APPENDIX B

This is a listing of the program LAGTIME, written in basic, which calculates the amount of coupling enzyme(s) needed to produce a desired lag time and the lag time for defined conditions.

```
100 REM Written by S.P.J.Brooks
200 CLS:WIDTH 40: COLOR 15,4
300 LOCATE 11,7,0
400 PRINT"                          "
500 LOCATE ,7
600 PRINT" PRACTICALS OF COUPLING  "
700 LOCATE ,7
800 PRINT"                          "
900 LOCATE ,7
1000 PRINT"     ENZYME THEORY       "
1100 LOCATE ,7
1200 PRINT"                         "
1300 LOCATE 20,1:PRINT"Copyright 1985, M.S.U."
1400 LOCATE 22,1:PRINT"Press any key to continue"
1500 ANYKEY$=INKEY$:IF ANYKEY$="" THEN 1500
1600 COLOR 7,0,0:CLS:WIDTH 80:LOCATE 1,1,0
1700 PRINT:PRINT:PRINT:PRINT "This program calculates the parameters
     required to set up a"
1800 PRINT "successful coupled enzyme assay.  Before one begins, a
     knowledge"
1900 PRINT"of the primary enzyme rate and the Km of the coupling
     enzyme(s)"
2000 PRINT"are necessary.  You may then calculate the units of
     coupling"

2100 PRINT"enzyme(s) needed to obtain a predefined lag time or
     calculate "
2200 PRINT"the lag time when the units of coupling enzyme(s) are
     known."
2300 PRINT:PRINT"The equations are based on theory developed by S.P.J.
     Brooks,"
2400 PRINT"T. Espinola and C.H. Suelter, Canadian Journal of
     Biochemistry and"
2500 PRINT"Cell Biology, 62, 945-955 and 956-963 (1984)."
2600 PRINT"                --"
2700 PRINT"Four different assay systems can be analyzed: one and two
     coupling"
2800 PRINT"enzymes in which the first intermediate may or may not
     mutarotate."
2900 PRINT:PRINT:PRINT"Press any key to continue"
3000 ANYKEY$=INKEY$:IF ANYKEY$="" THEN 3000
3100 K1=3.8:K2=2.2:A=.4
3200 REM*****************************************************************
3300 REM
3400 REM MENU
3500 REM
```

```
3600 REM*****************************************************************
3700 WIDTH 40:CLS:LOCATE 1,1,0
3800 PRINT:PRINT:PRINT:PRINT:PRINT"What do you want to do ?"
3900 PRINT:PRINT:PRINT"(A) Calculate lag times for given"
4000 PRINT"    values of V2 and/or V3"
4100 PRINT:PRINT"(B) Calculate the amount of coupling"
4200 PRINT"    enzyme to add to obtain a desired"
4300 PRINT"    lag time"
4400 PRINT:PRINT"(C) Review the reaction schemes"
4500 PRINT"    (alter some intrinsic parameters)"
4600 PRINT:PRINT"(D) Exit the program"
4700 ANYKEY$=INKEY$:IF (ANYKEY$<"A" OR ANYKEY$>"D") AND (ANYKEY$<"a" OR
     ANYKEY$>"d") THEN 4700
4800 IF ANYKEY$="D" OR ANYKEY$="d" THEN 48400
4900 IF ANYKEY$="A" OR ANYKEY$="a" THEN CAL=1 ELSE CAL=2
5000 IF ANYKEY$="C" OR ANYKEY$="c" THEN CAL =3
5100 WIDTH 40:CLS:LOCATE 1,1,0
5200 PRINT:PRINT:PRINT:PRINT:PRINT"Which system are you using ?"
5300 PRINT:PRINT:PRINT:PRINT:PRINT"(1) One coupling enzyme"
5400 PRINT:PRINT"(2) Two coupling enzymes"
5500 PRINT:PRINT"(3) One coupling enzyme with"
5600 PRINT"    mutarotation"
5700 PRINT:PRINT"(4) Two coupling enzymes with"
5800 PRINT"    mutarotation"
5900 ANYKEY$=INKEY$:SYS=VAL(ANYKEY$):IF SYS<1 OR SYS>4 THEN 5900
6000 IF CAL=3 THEN 29300
6100 ON SYS GOTO 6200,10000,14500,17800
6200 REM*****************************************************************
6300 REM
6400 REM ONE ENZYME WITH NO MUTAROTATION
6500 REM
6600 REM*****************************************************************
6700 CLS:WIDTH 80:LOCATE 1,1,0:COLOR 15,5
6800 PRINT:PRINT"ONE ENZYME WITH NO MUTAROTATION:":COLOR 7,0
6900 PRINT:INPUT"Enter the primary enzyme rate (mM/min) ";V1
7000 PRINT:INPUT"Enter the KB value for the coupling enzyme (mM) ";KB
7100 PRINT:INPUT"Enter the value of FB ";FB
7200 IF FB<0 OR FB>=1 THEN 7100
7300 IF CAL = 2 THEN 7900
7400 PRINT:INPUT"Enter the value of V2 (mM/min) ";V2
7500 TFB = -KB/(V2-V1)^2*(FB*V1+V2*LOG(1-FB))
7600 IF TFB<=0 THEN PRINT:GOSUB 28200: GOTO 8700
7700 COLOR 15,1:PRINT
7800 PRINT "THE TIME REQUIRED TO REACH ";FB;" STEADY STATE IS: ";TFB;"
     MINUTES.":GOTO 8700
7900 PRINT:INPUT"Enter the desired lag time (min) ";TFB
8000 SRB=LOG(1-FB)*KB/TFB-2*V1:SRC=V1^2+FB*KB*V1/TFB
8100 IF SRB^2-4*SRC<0 THEN PRINT:GOSUB 28200:GOTO 8700
8200 SRU=SQR(SRB^2-4*SRC): ANS1=(-SRB-SRU)/2:ANS2=(-SRB+SRU)/2
8300 IF ANS1>V1 THEN V2=ANS1
8400 IF ANS2>V1 THEN V2=ANS2
```

```
8500 IF ANS1<V1 AND ANS2<V1 THEN PRINT:GOSUB 28200:GOTO 8700
8600 COLOR 15,1:PRINT:PRINT:PRINT V2;"mM/min of coupling enzyme is
     necessary to obtain the desired lag time"
8700 COLOR 7,0:PRINT:PRINT:PRINT"Enter A to return to menu"
8800 PRINT"Enter B to keep primary enzyme rate"
8900 PRINT"Enter C to keep primary enzyme rate, and KB of coupling
     enzyme"
9000 PRINT"Enter D to keep primary enzyme rate, KB of coupling enzyme,
     and FB"
9100 ANYKEY$=INKEY$:IF (ANYKEY$<"A" OR ANYKEY$>"D") AND (ANYKEY$<"a" OR
     ANYKEY$>"d") THEN 9100
9200 IF ANYKEY$="A" OR ANYKEY$="a" THEN 3200
9300 CLS:WIDTH 80:LOCATE 1,1,0:COLOR 15,5
9400 PRINT:PRINT"ONE ENZYME WITH NO MUTAROTATION:":COLOR 7,0
9500 PRINT:PRINT"Primary enzyme rate = "V1"mM/min"
9600 IF ANYKEY$="b" OR ANYKEY$="B" THEN 7000
9700 PRINT:PRINT"Coupling enzyme Km (value of KB) = ";KB;"mM"
9800 IF ANYKEY$="c" OR ANYKEY$="C" THEN 7100
9900 PRINT:PRINT"FB = ";FB:GOTO 7300
10000 REM*****************************************************************
10100 REM
10200 REM TWO ENZYMES WITH NO MUTAROTATION
10300 REM
10400 REM*****************************************************************
10500 CLS:WIDTH 80:LOCATE 1,1,0:COLOR 15,5
10600 PRINT:PRINT"TWO ENZYMES WITH NO MUTAROTATION:":COLOR 7,0
10700 PRINT:INPUT"Enter the rate of the primary enzyme (mM/min) ";V1
10800 PRINT:INPUT"Enter the values of KB, KC (mM) ";KB,KC
10900 PRINT:INPUT"Enter the value of FC ";FC
11000 IF FC<0 OR FC>=1 THEN 10900
11100 IF CAL=1 THEN PRINT:INPUT"Enter the values of V2, V3 (mM/min)
      ";V2,V3:PRINT:GOTO 12700
11200 PRINT:PRINT "Enter the desired value of t";FC*100;"(min.) ";
11300 INPUT TFCA
11400 TAU=TFCA/(149/9*FC-12.11)
11500 PRINT:INPUT"Enter the cost of enzyme II, III (cost/unit) ";P2,P3
11600 IF P2<0 OR P3<0 THEN 11500
11700 TOPF=P3*(P2*(TAU*V1+KB)+SQR(P2*P3*KB*KC))
11800 BOTF=P2*(P3*(TAU*V1+KC)+SQR(P2*P3*KB*KC))
11900 DELTA=TOPF/BOTF
12000 R1=V1*TAU+KB+DELTA*(KC+TAU*V1)
12100 R2=V1*TAU+KB-DELTA*(KC+TAU*V1)
12200 V2=(R1+SQR(R2^2+DELTA*KB*KC))/(2*TAU)
12300 V3=V2/DELTA
12400 IF V2<0 OR V3<0 THEN GOSUB 28200:GOTO 12900
12500 COLOR 15,1:PRINT:PRINT "V2 = ";V2;"mM/min","V3 = ";V3;"mM/min"
12600 PRINT:PRINT"Using these values of V2 and V3:"
12700 TSTART=40:TM=79.999:TL=.001:T=TSTART
12800 GOSUB 22200:IF ERR1>0 THEN 13200
12900 COLOR 15,1
13000 PRINT "THE TIME REQUIRED TO REACH ";FC;" STEADY STATE IS: ";T;"
```

```
      MINUTES."
13100 PRINT"ERROR = +/- 10 %"
13200 COLOR 7,0:PRINT:PRINT"Enter A to return to menu"
13300 PRINT"Enter B to keep the primary enzyme rate"
13400 PRINT"Enter C to keep primary enzyme rate, KB, and KC"
13500 PRINT"Enter D to keep primary enzyme rate, KB, KC, and FC"
13600 ANYKEY$=INKEY$:IF (ANYKEY$<"A" OR ANYKEY$>"D") AND (ANYKEY$<"a"
      OR ANYKEY$>"d") THEN 13600
13700 IF ANYKEY$="A" OR ANYKEY$="a" THEN 3200
13800 CLS:WIDTH 80:LOCATE 1,1,0:COLOR 15,5
13900 PRINT:PRINT"TWO ENZYMES WITH NO MUTAROTATION:":COLOR 7,0
14000 PRINT:PRINT"Primary enzyme rate = ";V1;"mM/min"
14100 IF ANYKEY$="b" OR ANYKEY$="B" THEN 10800
14200 PRINT:PRINT"KB = ";KB;"mM","KC = ";KC;"mM"
14300 IF ANYKEY$="C" OR ANYKEY$="c" THEN 10900
14400 PRINT:PRINT"FC = ";FC: GOTO 11100
14500 REM*****************************************************************
14600 REM
14700 REM ONE ENZYME WITH MUTAROTATION
14800 REM
14900 REM*****************************************************************
15000 CLS:WIDTH 80:LOCATE 1,1,0:COLOR 15,5
15100 PRINT:PRINT"ONE ENZYME WITH MUTAROTATION:":COLOR 7,0
15200 PRINT:INPUT"Enter the primary enzyme rate (mM/min) ";V1
15300 PRINT:INPUT"Enter the KB value for the coupling enzyme (mM) ";KB
15400 PRINT:INPUT"Enter the value of FB ";FB
15500 IF FB<0 OR FB>=1 THEN 15400
15600 IF CAL = 1 THEN 16100
15700 PRINT:PRINT"Enter the desired value of t";FB*100;"(min.) ";
15800 INPUT TFDESIRED
15900 TL=.01:TM=499.99:TSTART=250:T=TSTART:GOSUB 22200:IF ERR1>0 THEN
      16500
16000 COLOR 15,1:PRINT:PRINT T;"mM/min of coupling enzyme is necessary
      to obtain the desired lag time":GOTO 16500
16100 PRINT:INPUT"Enter the value of V2 (mM/min) ";V2
16200 T=V2:GOSUB 25700
16300 COLOR 15,1:PRINT
16400 PRINT "THE TIME REQUIRED TO REACH ";FB;" STEADY STATE IS:
      ";TFBETA;" MINUTES."
16500 COLOR 7,0:PRINT:PRINT:PRINT"Enter A to return to menu"
16600 PRINT"Enter B to keep primary enzyme rate"
16700 PRINT"Enter C to keep primary enzyme rate, and KB of coupling
      enzyme"
16800 PRINT"Enter D to keep primary enzyme rate, KB of coupling enzyme,
      and FB"
16900 ANYKEY$=INKEY$:IF (ANYKEY$<"A" OR ANYKEY$>"D") AND (ANYKEY$<"a"
      OR ANYKEY$>"d") THEN 16900
17000 IF ANYKEY$="A" OR ANYKEY$="a" THEN 3200
17100 CLS:WIDTH 80:LOCATE 1,1,0:COLOR 15,5
17200 PRINT:PRINT"ONE ENZYME WITH MUTAROTATION:":COLOR 7,0
17300 PRINT:PRINT"Primary enzyme rate = ";V1;"mM/min"
```

```
17400 IF ANYKEY$="b" OR ANYKEY$="B" THEN 15300
17500 PRINT:PRINT"Coupling enzyme Km (value of KB) = ";KB;"mM"
17600 IF ANYKEY$="c" OR ANYKEY$="C" THEN 15400
17700 PRINT:PRINT"FB = ";FB:GOTO 15600
17800 REM*****************************************************************
17900 REM
18000 REM TWO ENZYMES WITH MUTAROTATION
18100 REM
18200 REM*****************************************************************
18300 CLS:WIDTH 80:LOCATE 1,1,0:COLOR 15,5
18400 PRINT:PRINT"TWO ENZYMES WITH MUTAROTATION:":COLOR 7,0
18500 PRINT:INPUT"Enter the rate of the primary enzyme (mM/min) ";V1
18600 PRINT:INPUT"Enter the values of KB, KC (mM) ";KB,KC
18700 PRINT:INPUT"Enter the value of FC ";FC
18800 IF FC<0 OR FC>=1 THEN 18700
18900 IF CAL=1 THEN PRINT:INPUT"Enter the values of V2, V3 (mM/min)
      ";V2,V3:PRINT:GOTO 20400
19000 PRINT:PRINT "Enter the desired value of t";FC*100;"(min.) ";
19100 INPUT TFCA
19200 TAU=TFCA/(220/9*FC-19.62)
19300 PRINT:INPUT"Enter the cost of enzyme II, III (cost/unit) ";P2,P3
19400 IF P2<0 OR P3<0 THEN 19300
19500 TOPF= P2*P3*(KB+TAU*V1-V1*A/K1)+ P3*SQR(P2*P3*KB*KC)
19600 BOTF= P2*P3*(KC+TAU*V1-V1*A/K1)+ P2*SQR(P2*P3*KB*KC)
19700 DELTA = TOPF/BOTF
19800 R1 = V1*(1+DELTA)*(K1*TAU-A) + K1*(KB+DELTA*KC)
19900 R2 = V1^2*DELTA*(K1*TAU-A) + DELTA*V1*K1*(KB+KC)
20000 V2=(R1+SQR(R1^2-4*R2*(TAU*K1-A)))/(2*(TAU*K1-A)):V3=V2/DELTA
20100 IF V2<=0 OR V3<=0 THEN GOSUB 28200: GOTO 20900
20200 COLOR 15,1:PRINT:PRINT "V2 = ";V2;"mM/min","V3 = ";V3;"mM/min"
20300 PRINT:PRINT"Using these values of V2 and V3:"
20400 TSTART=40:TM=79.999:TL=.001:T=TSTART
20500 GOSUB 22200:IF ERR1>0 THEN 20900
20600 COLOR 15,1
20700 PRINT "THE TIME REQUIRED TO REACH ";FC;" STEADY STATE IS: ";T;"
      MINUTES."
20800 PRINT"ERROR = +/- 10 %"
20900 COLOR 7,0:PRINT:PRINT"Enter A to return to menu"
21000 PRINT"Enter B to keep the primary enzyme rate"
21100 PRINT"Enter C to keep primary enzyme rate, KB, and  KC"
21200 PRINT"Enter D to keep primary enzyme rate, KB, KC, AND FC"
21300 ANYKEY$=INKEY$:IF (ANYKEY$<"A" OR ANYKEY$>"D") AND (ANYKEY$<"a"
      OR ANYKEY$>"d") THEN 21300
21400 IF ANYKEY$="A" OR ANYKEY$="a" THEN 3200
21500 CLS:WIDTH 80:LOCATE 1,1,0:COLOR 15,5
21600 PRINT:PRINT"TWO ENZYMES WITH MUTAROTATION:":COLOR 7,0
21700 PRINT:PRINT"Primary enzyme rate = "V1"mM/min"
21800 IF ANYKEY$="b" OR ANYKEY$="B" THEN 18600
21900 PRINT:PRINT"KB = ";KB;"mM","KC = ";KC;"mM" 22000 IF ANYKEY$="C"
      OR ANYKEY$="c" THEN 18700
22100 PRINT:PRINT"FC = ";FC: GOTO 18900
```

```
22200 REM*****************************************************************
22300 REM
22400 REM SUBROUTINE MINIMUM: FINDS THE VALUE OF T
22500 REM
22600 REM*****************************************************************
22700 Q=0:ERR1=0:ERR2=0
22800 E=LOG(T/2)/LOG(2)+1
22900 ON SYS GOSUB 24400,24400,25200,26500:IF ERR2>0 THEN ERR1=1:RETURN
23000 T1=INT(T1*10000)
23100 IF T>TM OR T<TL THEN Q=Q+1.1:T=TSTART: GOTO 22800
23200 IF Q>2 THEN PRINT:PRINT:GOSUB 28200: ERR1=1:RETURN
23300 IF T1=0 THEN RETURN
23400 IF T1>1E+10 AND E<.01 THEN Q=Q+1.1:T=TSTART:GOTO 22800
23500 IF Q<1 AND T1<0 THEN 23800
23600 IF Q>1 AND T1>0 THEN 23800
23700 T=0-T 23800 E=E-1:T=ABS(2^E+T): GOTO 22900
23900 REM*****************************************************************
24000 REM
24100 REM EQUATION FOR TWO ENZYMES WITH NO MUTAROTATION
24200 REM
24300 REM*****************************************************************
24400 C1=(V2-V1)^2:C2=(V3-V1)^2
24500 IF(C1/V2*KB)<>(C2/V3*KC) THEN 24800
24600 V3=V3-V3/100
24700 GOTO 24400
24800 L=EXP(-T*C1/(V2*KB))-C1*KC*V3/(C2*KB*V2)*EXP(-T*C2/(V3*KC))
24900 R=(1-C1*KC*V3/(C2*KB*V2))*(1-FC)*EXP(FC*V1/V3)
25000 T1=R-L 25100 RETURN
25200 REM*****************************************************************
25300 REM
25400 REM EQUATION FOR ONE ENZYME WITH MUTAROTATION
25500 REM
25600 REM*****************************************************************
25700 KBETA=KB/(1+K2/K1):V2=T
25800 M=(V2-V1)/KBETA:BETASS=V1/M:SRB=K1+K2+M:SRC=4*K1*M
25900 SR=SRB^2-SRC:IF SR<0 THEN ERR2=1:RETURN
26000 R1=(-SRB-SQR(SR))/2:R2=(-SRB+SQR(SR))/2
26100 Z1PLUS=((1-A)*V1+BETASS*R2)/(R1-R2)
26200 TFBETA = (1/R2)*LOG((1-FB)*BETASS/(BETASS+Z1PLUS))
26300 T1=TFDESIRED-TFBETA
26400 RETURN
26500 REM*****************************************************************
26600 REM
26700 REM EQUATION FOR TWO ENZYMES WITH MUTAROTATION
26800 REM
26900 REM*****************************************************************
27000 PHI=V3-V1:THETA=V2-V1:KBETA=KC/(1+K2/K1)
27100 M2=PHI/KBETA:BETASS = V1/M2
27200 RTOTAL=K1+K2+M2
27300 S1=(-RTOTAL-SQR(RTOTAL^2-4*K1*M2))/2
27400 S2=(-RTOTAL+SQR(RTOTAL^2-4*K1*M2))/2
```

```
27500 D1=(K1*BETASS-(1-A)*V1)/RTOTAL
27600 X0=KC*V1/PHI+A*V1/K1
27700 X1=KB*(M2*D1+V1)/THETA
27800 D2=(BETASS+D1+S2*(X0+X1)/M2)/(S2/S1-1)
27900 D3=-(BETASS+D1+D2)
28000 T1= BETASS*(1-FC)+D1*EXP(-THETA*T/KB)+D3*EXP(S2*T)
28100 RETURN
28200 REM*****************************************************************
28300 REM
28400 REM SUBROUTINE FOR ERRORS IN CALCULATIONS
28500 REM
28600 REM*****************************************************************
28700 COLOR 15,4
28800 PRINT"                                          "
28900 PRINT"YOUR ANSWER CANNOT BE CALCULATED !!"
29000 PRINT"PLEASE CHECK YOUR INPUT VALUES !!  "
29100 PRINT"                                          "
29200 COLOR 7,0:RETURN
29300 REM*****************************************************************
29400 REM
29500 REM OPTION #C: REVIEW THE REACTION SCHEMES
29600 REM
29700 REM*****************************************************************
29800 ON SYS GOTO 29900,31700,34500,42400
29900 CLS:WIDTH 80:LOCATE 1,1,0:COLOR 15,5
30000 PRINT:PRINT"ONE ENZYME WITH NO MUTAROTATION:":COLOR 15,0
30100 PRINT:PRINT:PRINT"THE REACTION IS AS FOLLOWS:"
30200 PRINT"                                        V2 * [B]"
30300 PRINT"                                        --------"
30400 PRINT"                          v1            KB + [B]"
30500 PRINT"         ";
30600 COLOR,4:PRINT"SUBSTRATE ------------> B -------------> PRODUCT "
30700 PRINT:COLOR ,0:PRINT"                       ";
30800 COLOR ,1:PRINT"ENZYME I";
30900 COLOR ,0:PRINT"         ";
31000 COLOR ,1:PRINT"ENZYME II":COLOR 0,7
31100 PRINT:PRINT:PRINT"ASSUMPTIONS:":COLOR 15,0
31200 PRINT:PRINT"(1) v1 is constant"
31300 PRINT"(2) reverse reaction is negligible":COLOR 7,0
31400 PRINT:PRINT:PRINT"Press any key to return to the menu"
31500 ANYKEY$=INKEY$:IF ANYKEY$="" THEN 31500
31600 GOTO 3700
31700 CLS:WIDTH 80:LOCATE 1,1,0:COLOR 15,5
31800 PRINT:PRINT"TWO ENZYMES WITH NO MUTAROTATION:":COLOR 15,0
31900 PRINT:PRINT:PRINT"THE REACTION IS AS FOLLOWS:":PRINT:PRINT
32000 PRINT"                V2 * [B]          V3 * [C]"
32100 PRINT"                --------          --------"
32200 PRINT"    v1          KB + [B]          KC + [C]":COLOR 15,4
32300 PRINT"S--------> B -------------> C -------------->
      PRODUCT":COLOR,0
32400 PRINT"                                ";
```

```
32500 COLOR,4:PRINT"/";
32600 COLOR,0:PRINT"             ";
32700 COLOR,4:PRINT"\"
32800 COLOR,0:PRINT" ";
32900 COLOR ,1:PRINT"ENZYME I";
33000 COLOR ,0:PRINT"      ";
33100 COLOR ,1:PRINT"ENZYME II";
33200 COLOR ,0:PRINT"     ";
33300 COLOR,4:PRINT"Q";
33400 COLOR,0:PRINT"   ";
33500 COLOR ,1:PRINT"ENZYME III";
33600 COLOR,0:PRINT"  ";
33700 COLOR,4:PRINT"> R":COLOR 0,7
33800 PRINT:PRINT:PRINT"ASSUMPTIONS:":COLOR 15,0
33900 PRINT:PRINT"(1) v1 is constant"
34000 PRINT"(2) reverse reactions are negligible"
34100 PRINT"(3) Fc >= .90":COLOR 7,0
34200 PRINT:PRINT"Press any key to return to the menu"
34300 ANYKEY$=INKEY$:IF ANYKEY$="" THEN 34300
34400 GOTO 3700
34500 CLS:WIDTH 80:LOCATE 1,1,0:COLOR 15,5
34600 PRINT:PRINT"ONE ENZYME WITH MUTAROTATION:":COLOR 15,0
34700 PRINT:PRINT"THE REACTION IS AS FOLLOWS:":PRINT
34800 PRINT"         a * v1":PRINT"   ";
34900 COLOR 15,4:PRINT"S ------------> ALPHA"
35000 COLOR,0:PRINT"     ";
35100 COLOR,4:PRINT"|";
35200 COLOR,0:PRINT"   ";
35300 COLOR,1:PRINT"ENZYME I";
35400 COLOR,0:PRINT"    ";
35500 COLOR,4:PRINT"|";
35600 COLOR,0:PRINT" ";
35700 COLOR,4:PRINT"|\"
35800 COLOR,0:PRINT"     ";
35900 COLOR,4:PRINT"|";
36000 COLOR,0:PRINT"               ";
36100 COLOR,4:PRINT"|";
36200 COLOR,0:PRINT" ";
36300 COLOR,4:PRINT"|"
36400 COLOR,0:PRINT"     ";
36500 COLOR,4:PRINT"|";
36600 COLOR,0:PRINT"            k1";
36700 COLOR,0:PRINT" ";
36800 COLOR,4:PRINT"|";
36900 COLOR,0:PRINT" ";
37000 COLOR,4:PRINT"|";
37100 COLOR,0:PRINT" k2       V2 + [BETA]"
37200 COLOR,0:PRINT"     ";
37300 COLOR,4:PRINT"|";
37400 COLOR,0:PRINT"               ";
37500 COLOR,4:PRINT"|";
```

```
37600 COLOR,0:PRINT" ";
37700 COLOR,4:PRINT"|";
37800 COLOR,0:PRINT"          --------------"
37900 COLOR,0:PRINT"     ";
38000 COLOR,4:PRINT"|";
38100 COLOR,0:PRINT" (1-a) * v1    ";
38200 COLOR,4:PRINT"\|";
38300 COLOR,0:PRINT" ";
38400 COLOR,4:PRINT"|";
38500 COLOR,0:PRINT"          KBETA + [BETA]"
38600 COLOR,0:PRINT"     ";
38700 COLOR,4:PRINT"`----------->  BETA ------------------------->
      PRODUCT"
38800 COLOR,0:PRINT"                           ";
38900 COLOR,4:PRINT"/";
39000 COLOR,0:PRINT"                          ";
39100 COLOR,4:PRINT"\"
39200 COLOR,0:PRINT"          ";
39300 COLOR,1:PRINT"ENZYME I";
39400 COLOR,0:PRINT"       ";
39500 COLOR,4:PRINT"Q";
39600 COLOR,0:PRINT"           ";
39700 COLOR,1:PRINT"ENZYME II";
39800 COLOR,0:PRINT"       ";
39900 COLOR,4:PRINT"> R":COLOR 0,7
40000 LOCATE 18,1:PRINT"ASSUMPTIONS:":COLOR 15,0
40100 PRINT:PRINT"(1) v1 is constant           (2) reverse reaction is
      negligible"
40200 PRINT"(3) F BETA >= 0.9":COLOR 7,0
40300 PRINT:PRINT"Press any key to continue"
40400 ANYKEY$=INKEY$:IF ANYKEY$="" THEN 40400
40500 LOCATE 18,1:COLOR 0,7:PRINT"The following values are defined:";
40600 COLOR 15,0:PRINT"  "
40700 PRINT:PRINT"a = ";A;",    k1 = ";K1;"(per min.),    k2 =
      ";K2;"(per min.)          "
40800 PRINT"                                        "
40900 COLOR 7,0:PRINT"Press c to change a value"
41000 PRINT"Press m to return to the menu"
41100 ANYKEY$=INKEY$:IF ANYKEY$<>"c" AND ANYKEY$<>"C" AND ANYKEY$<>"M"
      AND ANYKEY$<>"m" THEN 41100
41200 IF ANYKEY$="M" OR ANYKEY$="m" THEN 3200
41300 LOCATE 18,1:COLOR 0,7:PRINT"Which value do you want to
      change:":COLOR 15,0
41400 PRINT:PRINT"(A) a,       (B) k1,         (C) k2
      "
41500 PRINT:PRINT"                                  "
41600 PRINT"                                "
41700 ANYKEY$=INKEY$:IF (ANYKEY$<"a" OR ANYKEY$>"c") AND (ANYKEY$<"A"
      OR ANYKEY$>"C") THEN 41700
41800 LOCATE 20,1:PRINT"                                                "
41900 PRINT"Enter the new value of ";
```

```
42000 IF ANYKEY$="a" OR ANYKEY$="A" THEN INPUT"a ";A
42100 IF ANYKEY$="B" OR ANYKEY$="b" THEN INPUT"k1 ";K1
42200 IF ANYKEY$="C" OR ANYKEY$="c" THEN INPUT"k2 ";K2
42300 GOTO 40500
42400 CLS:WIDTH 80:LOCATE 1,1,0:COLOR 15,5
42500 PRINT:PRINT"TWO ENZYMES WITH MUTAROTATION:":COLOR 15,0
42600 PRINT:PRINT"THE REACTION IS AS FOLLOWS:":PRINT
42700 PRINT"                         V2 * [B]"
42800 PRINT"                    a * --------"
42900 PRINT"       v1                KB + [B]":PRINT"   ";
43000 COLOR 15,4:PRINT"S ------> B ---------------------> ALPHA"
43100 COLOR,0:PRINT"    ";
43200 COLOR,1:PRINT"ENZYME I";
43300 COLOR,0:PRINT"   ";
43400 COLOR,4:PRINT"|";
43500 COLOR,0:PRINT"      ";
43600 COLOR,1:PRINT"ENZYME II";
43700 COLOR,0:PRINT"        ";
43800 COLOR,4:PRINT"|";
43900 COLOR,0:PRINT" ";
44000 COLOR,4:PRINT"|\"
44100 COLOR,0:PRINT"               ";
44200 COLOR,4:PRINT"|";
44300 COLOR,0:PRINT"                        ";
44400 COLOR,4:PRINT"|";
44500 COLOR,0:PRINT" ";
44600 COLOR,4:PRINT"|"
44700 COLOR,0:PRINT"               ";
44800 COLOR,4:PRINT"|";
44900 COLOR,0:PRINT"         V2 * [B]   k1";
45000 COLOR,0:PRINT" ";
45100 COLOR,4:PRINT"|";
45200 COLOR,0:PRINT" ";
45300 COLOR,4:PRINT"|";
45400 COLOR,0:PRINT" k2    V3 * [BETA]"
45500 COLOR,0:PRINT"               ";
45600 COLOR,4:PRINT"|";
45700 COLOR,0:PRINT" (1-a) * --------      ";
45800 COLOR,4:PRINT"|";
45900 COLOR,0:PRINT" ";
46000 COLOR,4:PRINT"|";
46100 COLOR,0:PRINT"      --------------"
46200 COLOR,0:PRINT"               ";
46300 COLOR,4:PRINT"|";
46400 COLOR,0:PRINT"         KB + [B]     ";
46500 COLOR,4:PRINT"\|";
46600 COLOR,0:PRINT" ";
46700 COLOR,4:PRINT"|";
46800 COLOR,0:PRINT"      KBETA + [BETA]"
46900 COLOR,0:PRINT"               ";
47000 COLOR,4:PRINT"`--------------------->  BETA --------------------->
```

```
      PRODUCT"
47100 COLOR,0:PRINT"                                                    ";
47200 COLOR,4:PRINT"/";
47300 COLOR,0:PRINT"                    ";
47400 COLOR,4:PRINT"\"
47500 COLOR,0:PRINT"                       ";
47600 COLOR,1:PRINT"ENZYME II";
47700 COLOR,0:PRINT"           ";
47800 COLOR,4:PRINT"Q";
47900 COLOR,0:PRINT"       ";
48000 COLOR,1:PRINT"ENZYME III";
48100 COLOR,0:PRINT"    ";
48200 COLOR,4:PRINT"> R"
48300 COLOR 0,7:GOTO 40000
48400 REM*****************************************************************
48500 REM
48600 REM THIS IS THE END OF THE PROGRAM
48700 REM
48800 REM*****************************************************************
48900 COLOR 7,0:WIDTH 80:END
```

APPENDIX C

This is the listing for NONLIN, a progran, written in basic, which allows one to calculate the parameters (up to 6) of a non-linear function.

```
10 REM NONLIN: Written by S.P.J. Brooks
20 LG = 2.302585093#
30 PP$="press any key to continue"
40 PPP$="press 'c' to continue or 'p' to print"
50 PPPP$="Is the printer turned on ?"
60 PPPPP$="                                                    "
70 EE$="**********************************************************************
   ************"
80 CLS:LOCATE 1,1,0:WIDTH 40:COLOR 15,4:LOCATE 10,16:PRINT"          "
90 LOCATE 11,16:PRINT" NONLIN ":LOCATE 12,16:PRINT"          "
100 LOCATE 19,1:PRINT"copyright 1985, M.S.U."
110 LOCATE 21,1,0:PRINT PP$
120 ANYKEY$=INKEY$:IF ANYKEY$="" THEN 120
130 DIM RESID(40),W(40),LPY$(75,45),A$(45),PT(40),X(41),Y(41),P(40,6),
    PPWP(6,6),PPW(6,40),YN(70),Q(6),B(6),VB(6),YHAT(41),BMAX(6),BMIN(6),
    BI(6)
140 COLOR 7,0:CLS:LOCATE 1,1,0:PRINT:PRINT:PRINT"This program calculates
    the values of"
150 PRINT:PRINT"parameters (max. of 6) for non-linear"
160 PRINT:PRINT"equations. This is accomplished using"
170 PRINT:PRINT"the Gauss-Newton algorithm adapted"
180 PRINT:PRINT"from J. Fox in Linear Statistical"
190 PRINT:PRINT"Models and Related Models (1984),"
200 PRINT:PRINT"John Wiley & Sons, N. Y."
210 PRINT:PRINT:PRINT PP$
220 ENT$=INKEY$: IF ENT$="" THEN 220:NN=0:KK=0:FLAG6=0
230 REM*******************************************************
240 REM
250 REM MENU
260 REM
270 REM*******************************************************
280 CLS:LOCATE 1,1,0:COLOR 15,4:PRINT"You may do any of the following:"
290 COLOR 7,0:PRINT:PRINT"     1. Input new data pairs"
300 PRINT:PRINT"     2. Add data to existing data"
310 PRINT:PRINT"     3. Edit and/or review the data"
320 PRINT:PRINT"     4. Enter parameter estimates"
330 PRINT:PRINT"     5. Calculate parameters,"
340 PRINT"        plot data and residuals."
350 PRINT:PRINT"     H. Help menu/enter equation"
360 PRINT:PRINT"     E. Exit the program"
370 PRINT:PRINT"     F. Use data already on file"
380 LOCATE 20,6,0:COLOR 15,2:PRINT"Did you remember to   ":LOCATE
    21,6,0:PRINT"enter your EQUATION ??":COLOR 7,0
390 ANS$=INKEY$: IF ANS$="" THEN 390
400 IF ANS$<>"f" AND ANS$<>"F" THEN 460
410 OPEN "NDATA" AS #1 LEN =8
```

```
420 FIELD #1,4 AS Q1$, 4 AS Q2$:GET #1, 1:NN = CVI(Q1$)
430 FOR I=1 TO NN:RECN=I+1:GET #1, RECN:X(I)=CVS(Q1$):Y(I)=CVS(Q2$)
440 NEXT I:CLOSE #1
450 LOCATE 18,9,0:COLOR 15,3:PRINT"File data has been entered":COLOR
    7,0
460 IF ANS$="e" OR ANS$="E" THEN 5560
470 IF ANS$="h" OR ANS$="H" THEN 4980
480 A1= VAL(ANS$): IF A1<1 OR A1> 5 OR A1<>INT(A1) THEN 390
490 IF A1=4 AND NN<.5 THEN 520
500 IF A1<>5 THEN 580
510 IF A1=5 AND NN>0 AND KK>0 THEN 580
520 LOCATE 20,1,0:COLOR 15,4
530 IF NN=0 THEN PRINT"You must enter data PRIOR  ":PRINT"to selection
    of this option":GOTO 550
540 PRINT"You must enter parameter estimates":PRINT"PRIOR to selection
    of this option "
550 COLOR 7,0:PRINT:PRINT PP$
560 AKEY$=INKEY$:IF AKEY$ = "" THEN 560
570 LOCATE 20,1,0:PRINT"                                          ":PRINT"
    ":PRINT:PRINT"                                ":GOTO 380
580 ON A1 GOTO 590,590,1170,900,2040
590 REM************************************************************
600 REM
610 REM option #1: Enter data pairs
620 REM option #2: Add data to existing data
630 REM
640 REM************************************************************
650 IF A1=2 THEN 730
660 IF NN<1 THEN 770
670 CLS:LOCATE 11,5:COLOR 15,4:PRINT"NOTE: This will erase all"
680 LOCATE 12,5:PRINT"previously entered data !!"
690 PRINT:PRINT:PRINT"Press 'r' to return to the menu"
700 PRINT"Press 'c' to continue"
710 ANS$=INKEY$:IF ANS$<>"c" AND ANS$<>"C" AND ANS$<>"r" AND ANS$<>"R"
    THEN 710
720 COLOR 7,0:IF ANS$="r" OR ANS$="R" THEN 230 ELSE 770
730 IF NN>=40 THEN CLS:COLOR 15,4:LOCATE 11,4:PRINT"40 data pairs is
    the limit !!":COLOR 7,0:PRINT:PRINT:GOTO 860
740 ES=NN+1:IF NN<12 THEN EN=13:GOTO 780
750 IF NN<27 THEN EN=27:GOTO 780
760 IF NN<=40 THEN EN=40:GOTO 780
770 EN=13:ES=1:LI=0
780 IF A1=2 THEN LI=NN
790 CLS:COLOR 15,1:PRINT"enter '*' after entering last data pair"
800 LOCATE 3,1:PRINT" pair # ":LOCATE 3,14:PRINT" X ":LOCATE
    3,26:PRINT" Y "
810 FOR I=ES TO EN:COLOR 15,1:LOCATE I+5-LI,1:PRINT I:COLOR 7,0:LOCATE
    I+5-LI,14:INPUT X$
820 IF X$="*" THEN 860 ELSE X(I)=VAL(X$)
830 LOCATE I+5-LI,26:INPUT Y(I):NEXT I
840 IF I=14 THEN EN=27:ES=14:LI=13:GOTO 790
```

```
850 IF I=28 THEN EN=40:ES=28:LI=27:GOTO 790
860 NN=I-1:COLOR 15,1:PRINT:PRINT NN"data pairs have been
    entered":GOSUB 5310
870 COLOR 7,0:PRINT:PRINT PP$
880 ANS$=INKEY$:IF ANS$="" THEN 880
890 GOTO 230
900 REM*************************************************************
910 REM
920 REM OPTION #4: Enter parameter estimates
930 REM
940 REM*************************************************************
950 IF KK<.5 THEN FOR I=1 TO 6:BMIN(I)=-1000:BMAX(I)=1000:NEXT I
960 IF NN>6 THEN KMAX=6 ELSE KMAX=NN-1
970 CLS:LOCATE 3,1,0:PRINT"Enter the number of
    constants":PRINT"(parameters): maximum =";KMAX;
980 INPUT KK
990 IF KK<1 OR KK>KMAX THEN 970
1000 COLOR 15,1:PRINT:PRINT"Enter the initial values for:":COLOR 7,0
1010 PRINT:PRINT:FOR I=1 TO KK:PRINT"B("I")   ";
1020 INPUT BI(I):NEXT I
1030 CLS:PRINT:PRINT"The maximum and minimum values for":PRINT"the
     constants have been set at:"
1040 COLOR 15,1:LOCATE 6,2:PRINT" # ":LOCATE 6,15:PRINT" max ":LOCATE
     6,28:PRINT" min ":COLOR 7,0
1050 FOR I=1 TO KK:LOCATE 7+I,2:PRINT I:LOCATE 7+I,15:PRINT
     BMAX(I):LOCATE 7+I,28:PRINT BMIN(I):NEXT I
1060 LOCATE 7+I+2,1:PRINT"Are all values correct (y or n)":PRINT"If all
     values are incorrect enter 'a'"
1070 ENT$=INKEY$:IF ENT$<>"y" AND ENT$<>"Y" AND ENT$<>"n" AND ENT$<>"N"
     AND ENT$<>"a" AND ENT$<>"A" THEN 1070
1080 IF ENT$="y" OR ENT$="Y" THEN 230
1090 IF ENT$<>"a" AND ENT$<>"A" THEN 1120
1100 FOR I=1 TO KK:LOCATE 7+I,1:PRINT"
     "
1110 LOCATE 7+I,2:COLOR 15,4:PRINT I:LOCATE 7+I,15:INPUT BMAX(I):LOCATE
     7+I,28:INPUT BMIN(I):COLOR 7,0:NEXT I:GOTO 1060
1120 LOCATE 7+I+2,1:PRINT"                                    "
1130 LOCATE 7+I+2,1:INPUT"Which pair number is incorrect   ";PN
1140 LOCATE 7+I+2,1:PRINT"                                          ":IF
     PN<1 OR PN>KK THEN GOTO 1130
1150 LOCATE 7+PN,1:PRINT"                                        "
1160 LOCATE 7+PN,2:COLOR 15,4:PRINT PN:LOCATE 7+PN,15:INPUT
     BMAX(PN):LOCATE 7+PN,28:INPUT BMIN(PN):COLOR 7,0:GOTO 1060
1170 REM*************************************************************
1180 REM
1190 REM OPTION #3: Review the data
1200 REM OPTION #7: List residuals
1210 REM
1220 REM*************************************************************
1230 IF NN<=13 THEN EN=NN:ES=1:LI=0:GOTO 1250
1240 EN=13:ES=1:LI=0
```

```
1250 CLS:LOCATE 1,1,0:COLOR 15,1:IF A1=3 THEN PRINT:PRINT" PAIR
     #":LOCATE 2,13:PRINT" X ":LOCATE 2,26:PRINT" Y "
1260 IF A1=7 THEN LOCATE 2,1:PRINT" # ":LOCATE 2,8:PRINT" resid.
     ":LOCATE 2,22:PRINT" % 2S.D.":LOCATE 2,32:PRINT" >2S.D.? "
1270 PRINT:FOR I=ES TO EN
1280 COLOR 15,1:LOCATE 3+I-LI,2:IF A1=3 THEN PRINT I;
1290 COLOR 7,0:IF A1=3 THEN PRINT TAB(12) X(I) TAB(25) Y(I):GOTO 1340
1300 PC=INT(RESID(I)/(SQR(SE)*2)*1000)/10
1310 COLOR 15,1:LOCATE 3+I-LI,1:PRINT I;
1320 COLOR 7,0:PRINT TAB(6) RESID(I) TAB(23) PC:IF ABS(PC)<100 THEN
     1340
1330 COLOR 15,4:LOCATE 3+I-LI,35:PRINT" * ":COLOR 7,0
1340 NEXT I: IF A1=3 THEN 1550
1350 REM************************************************************
1360 REM
1370 REM continue listing the data
1380 REM
1390 REM************************************************************
1400 IF NN=I-1 THEN 1460
1410 LOCATE 4+I-LI:PRINT PP$
1420 ANS$=INKEY$:IF ANS$="" THEN 1420
1430 IF NN<=27 AND I<28 THEN EN=NN:ES=13:LI=12:GOTO 1250
1440 IF NN>27 AND I<28 THEN EN=27:ES=13:LI=12:GOTO 1250
1450 EN=NN:ES=27:LI=26:GOTO 1250
1460 REM************************************************************
1470 REM
1480 REM stop listing the data
1490 REM
1500 REM************************************************************
1510 IF NN+1=I THEN COLOR 15,1:PRINT:PRINT:PRINT"sequence = ";BB$:PRINT
     AA$:COLOR 7,0
1520 PRINT:PRINT PPP$
1530 ANS$=INKEY$:IF ANS$<>"c" AND ANS$<>"C" AND ANS$<>"p" AND ANS$<>"P"
     THEN 1530
1540 IF ANS$="p" OR ANS$="P" THEN 1850 ELSE COLOR 7,0:GOTO 3500
1550 REM************************************************************
1560 REM
1570 REM continue with option #2
1580 REM
1590 REM************************************************************
1600 LOCATE 5+I-LI,1:PRINT"Press 'e' to edit data"
1610 PRINT"Press 'd' to delete data"
1620 PRINT"Press 'c' to continue"
1630 ANS$=INKEY$: IF ANS$<>"c" AND ANS$<>"C" AND ANS$<>"e" AND
     ANS$<>"E" AND ANS$<>"d" AND ANS$<> "D" THEN 1630
1640 IF ANS$<>"c" AND ANS$<> "C" THEN 1690
1650 IF I=NN+1 THEN 230
1660 IF I=14 AND NN<27 THEN EN =NN:ES=13:LI=12:GOTO 1250
1670 IF I=14 AND NN>27 THEN EN =27:ES=13:LI=12:GOTO 1250
1680 EN=NN:ES = 27:LI=26:GOTO 1250
1690 LOCATE I+5-LI:COLOR 15,4:PRINT"Which data pair do you want to"
```

```
1700 COLOR 7,0
1710 PRINT"                              "
1720 PRINT"                              "
1730 LOCATE 6+I-LI,1:COLOR 15,4
1740 IF ANS$<>"e" AND ANS$<>"E" THEN 1820
1750 INPUT"edit  ";N2
1760 IF N2<ES OR N2>EN THEN 1730
1770 COLOR 7,0:LOCATE 3+N2-LI,1:PRINT"                                        "
1780 COLOR 15,4:LOCATE 3+N2-LI,2:PRINT N2
1790 LOCATE 3+N2-LI,12:INPUT" ";X(N2)
1800 LOCATE 3+N2-LI,25:INPUT" ";Y(N2)
1810 COLOR 7,0:GOSUB 5310: GOTO 1170
1820 INPUT"delete ";N2:COLOR 7,0
1830 IF N2<1 OR N2>NN THEN 1730
1840 NN=NN-1:FOR I=N2 TO NN:Y(I)=Y(I+1):X(I)=X(I+1):NEXT I:GOSUB
     5310:GOTO 1170
1850 REM*************************************************************
1860 REM
1870 REM print results to line printer
1880 REM
1890 REM*************************************************************
1900 LOCF=8+I-LI
1910 LOCATE LOCF:PRINT PPPPP$:COLOR 15,4:LOCATE LOCF:PRINT PPPP$:COLOR
     7,0
1920 PRINT:PRINT PP$
1930 ANS$=INKEY$:IF ANS$=""THEN 1930
1940 LPRINT:LPRINT EE$
1950 LPRINT:LPRINT:LPRINT"Results of NONLIN calculations:  ";CC$
1960 LPRINT:LPRINT:LPRINT"pair #" TAB(17) "X value" TAB(34) "Y value"
     TAB(51) "residual" TAB(68) "% of 2 S.D."
1970 LPRINT"------" TAB(17) "-------" TAB(34) "-------" TAB(51) "------
     --" TAB(68) "----------"
1980 LPRINT: FOR I=1 TO NN
1990 PC=INT(RESID(I)/(SQR(SE)*2)*1000)/10
2000 LPRINT I TAB(17) X(I) TAB(34) Y(I) TAB(51) RESID(I) TAB(68) PC
2010 NEXT I
2020 LPRINT:LPRINT:LPRINT"sequence of residuals = ";BB$:LPRINT AA$
2030 LPRINT:LPRINT EE$:GOTO 3500
2040 REM*************************************************************
2050 REM
2060 REM Option #5: Parameter estimation --> leads to sub-menu
2070 REM
2080 REM*************************************************************
2090 CLS:LOCATE 3,1,0:PRINT"What type of weighting ?"
2100 COLOR 7,0:LOCATE 6,1:PRINT"(a) constant absolute error"
2110 PRINT"    (constant standard deviation)":PRINT"    ";
2120 COLOR 15,1:PRINT"w = 1":COLOR 7,0
2130 LOCATE 10,1:PRINT"(b) constant relative error"
2140 PRINT"    (proportional standard deviation)":PRINT"    ";
2150 COLOR 15,1:PRINT"w = (1/y)^2":COLOR 7,0
2160 LOCATE 15,1:PRINT"(c) Intermediate case":PRINT"    ";
```

```
2170 COLOR 15,1:PRINT"w = 1/y":COLOR 7,0
2180 O6$=INKEY$:IF (O6$<"a" OR O6$>"c") AND (O6$<"A" OR O6$>"C") THEN
     2180
2190 FOR I=1 TO NN
2200 IF O6$="a" OR O6$="A" THEN W(I)=1:CC$="constant absolute error"
2210 IF O6$="b" OR O6$="B" THEN W(I)=(1/Y(I))^2:CC$="constant relative
     error"
2220 IF O6$="c" OR O6$="C" THEN W(I)=1/Y(I):CC$="inbetween case"
2230 NEXT I:FOR I=1 TO KK:B(I)=BI(I):NEXT I:GOSUB 5460:SS=0:FOR I=1 TO
     NN
2240 SS=SS+W(I)*(Y(I)-YHAT(I))^2:NEXT I:C=100:SSO=SS:ITER=0:ER$="":CLS
2250 ITER=ITER+1:IF ITER>=20 THEN ER$="Maximum of 20 iterations
     !!":GOTO 3120
2260 LOCATE 10,4:PRINT"iteration #";ITER:LOCATE 13,4:PRINT"SS
      =";SS:LOCATE 14,4:PRINT"# to zero =";C:LOCATE 16,4:PRINT"Press
      'q' to quit"
2270 REM*******************************************
2280 REM
2290 REM Calculate a P(NN*KK) partial derivative matrix
2300 REM
2310 REM*******************************************
2320 FOR J=1 TO KK: B(J)=1.02*B(J):GOSUB 5460
2330 FOR I=1 TO NN:YN(I)=YHAT(I):NEXT I
2340 B(J)=B(J)*.98/1.02:GOSUB 5460
2350 B(J)=B(J)/.98
2360 FOR I=1 TO NN:P(I,J)=(YN(I)-YHAT(I))/(.04*B(J)):NEXT I
2370 NEXT J
2380 REM*******************************************
2390 REM
2400 REM Calculate the values of Q (matrix corrections)
2410 REM
2420 REM (1) Calculate the P'WW'P matrix labelled as PPWP(KK*KK)
2430 REM
2440 REM*******************************************
2450 FOR I=1 TO KK:FOR J=1 TO NN
2460 PPW(I,J)=P(J,I)*W(J):NEXT J:NEXT I
2470 FOR I=1 TO KK:FOR J=1 TO KK:PPWP(I,J)=0:FOR K=1 TO NN
2480 PPWP(I,J)=PPWP(I,J)+PPW(I,K)*P(K,J):NEXT K:NEXT J:NEXT I
2490 REM*******************************************
2500 REM
2510 REM (2) Invert the PPWP matrix
2520 REM
2530 REM*******************************************
2540 IF KK=1 THEN PPWP(1,1)=1/PPWP(1,1):GOTO 2890
2550 FOR L=1 TO KK:DD=0
2560 FOR K=1 TO KK:DD=DD+PPWP(L,K)*PPWP(L,K):NEXT K
2570 DD=SQR(DD):NEXT L
2580 FOR L=1 TO KK:YN(L+20)=L:NEXT L
2590 L=0
2600 L=L+1:IF L>KK THEN 2780
2610 CC=0:M=L
```

```
2620 FOR K=L TO KK: IF ABS(CC)>=ABS(PPWP(L,K)) THEN 2640
2630 M=K:CC=PPWP(L,K)
2640 NEXT K
2650 IF L=M THEN 2700
2660 K=YN(M+20):YN(M+20)=YN(L+20):YN(L+20)=K
2670 FOR K=1 TO KK:S=PPWP(K,L)
2680 PPWP(K,L)=PPWP(K,M):PPWP(K,M)=S
2690 NEXT K
2700 PPWP(L,L)=1
2710 FOR M=1 TO KK:PPWP(L,M)=PPWP(L,M)/CC:NEXT M
2720 FOR M=1 TO KK:IF L=M THEN 2760
2730 CC=PPWP(M,L):IF CC=0 THEN 2760
2740 PPWP(M,L)=0
2750 FOR K=1 TO KK:PPWP(M,K)=PPWP(M,K)-CC*PPWP(L,K):NEXT K
2760 NEXT M
2770 GOTO 2600
2780 L1=0
2790 L1=L1+1:IF L1>KK THEN 2890
2800 IF YN(L1+20)=L1 THEN 2790
2810 M=L1
2820 M=M+1
2830 IF YN(M+20)=L1 THEN 2850
2840 IF KK>M THEN 2820
2850 YN(M+20)=YN(L1+20)
2860 FOR K=1 TO KK: CC=PPWP(L1,K)
2870 PPWP(L1,K)=PPWP(M,K):PPWP(M,K)=CC:NEXT K
2880 YN(L1+20)=L1:GOTO 2790
2890 REM*******************************************
2900 REM
2910 REM (3) Calculate the P'WW'E matrix labelled as YN(KK)
2920 REM
2930 REM*******************************************
2940 GOSUB 5460
2950 FOR I=1 TO KK:YN(I)=0:FOR K=1 TO NN
2960 YN(I)=YN(I)+PPW(I,K)*(Y(K)-YHAT(K)):NEXT K:NEXT I
2970 REM*******************************************
2980 REM
2990 REM calculate the parameters and check the results
3000 REM
3010 REM*******************************************
3020 ML=1:FOR I = 1 TO KK:Q(I)=0:YN(I+KK)=B(I):FOR K=1 TO KK
3030 Q(I)=Q(I)+PPWP(I,K)*YN(K):NEXT K:NEXT I:ITERA=0
3040 C=0:FOR I=1 TO KK:C=ABS(Q(I)/B(I))+C:B(I)=YN(I+KK)+ML*Q(I):IF
     B(I)<BMIN(I) OR B(I)>BMAX(I) THEN 3090
3050 NEXT I:GOSUB 5460:SS=0:FOR I=1 TO NN:SS=SS+W(I)*(Y(I)-
     YHAT(I))^2:NEXT I
3060 QU$=INKEY$:IF QU$<>"q" AND QU$<>"Q" THEN 3080
3070 FOR I=1 TO KK:B(I)=YN(I+KK):NEXT I:GOTO 3120
3080 IF SS<SSO THEN 3110
3090 ITERA=ITERA+1:IF ITERA>=20 THEN ER$="Local minimum found !!":GOTO
     3070
```

```
3100 ML=ML/2:GOTO 3040
3110 SSO=SS:IF C> .00001 THEN 2250
3120 REM*****************************************
3130 REM
3140 REM Run has converged !!!
3150 REM
3160 REM*****************************************
3170 SE=SSO/(NN-KK):FOR I=1 TO KK:VB(I)=SQR(ABS(SE*PPWP(I,I))):NEXT I
3180 CLS:PRINT:PRINT:COLOR 15,4:PRINT"Results:";
3190 COLOR 7,0:PRINT "  "+CC$:PRINT:COLOR 15,4:PRINT ER$:COLOR
     7,0:PRINT
3200 PRINT:FOR I=1 TO KK:PRINT "B(";I;") =";B(I);"  +/-";VB(I):NEXT I
3210 PRINT:PRINT:PRINT PPP$
3220 ANS$=INKEY$:IF ANS$<>"c" AND ANS$<>"C" AND ANS$<>"p" AND ANS$<>"P"
     THEN 3220
3230 IF ANS$="c" OR ANS$="C" THEN 3500
3240 REM*************************************************************
3250 REM
3260 REM print to line printer
3270 REM
3280 REM*************************************************************
3290 LOCATE 9+I,1:PRINT PPPPP$:COLOR 15,4:LOCATE 9+I,1:PRINT
     PPPP$:COLOR 7,0
3300 PRINT PP$
3310 ANS$=INKEY$:IF ANS$="" THEN 3310
3320 LPRINT:LPRINT EE$
3330 LPRINT:LPRINT"Results of NONLIN calculations:  ";CC$
3340 LPRINT:LPRINT:LPRINT TAB(1) "pair #" TAB(20) "X value" TAB(40) "Y
     value" TAB(60) "Yhat"
3350 LPRINT TAB(1) "------" TAB(20) "-------" TAB(40) "-------" TAB(60)
     "----"
3360 LPRINT:FOR I=1 TO NN:LPRINT TAB(3) I TAB(20) X(I) TAB(40) Y(I)
     TAB(60) YHAT(I):NEXT I
3370 LPRINT:LPRINT:LPRINT "# iterations = ";ITER,"SS = ";SS:LPRINT
3380 LPRINT:FOR I=1 TO KK:LPRINT "B(";I;") =";B(I);"  +/-";VB(I):NEXT I
3390 LPRINT:LPRINT"The covariance matrix is as follows:"
3400 LPRINT:LPRINT"              var(1,1),    var(1,2),    var(1,3),
3410 LPRINT:FOR I=1 TO KK:LPRINT"var("I",1)";
3420 IF KK=1 THEN LPRINT"  ";PPWP(I,1)
3430 IF KK=2 THEN LPRINT"  ";PPWP(I,1);"  ";PPWP(I,2)
3440 IF KK=3 THEN LPRINT"  ";PPWP(I,1);"  ";PPWP(I,2);"  ";PPWP(I,3)
3450 IF KK=4 THEN LPRINT"  ";PPWP(I,1);"  ";PPWP(I,2);"  ";PPWP(I,3);"
     ";PPWP(I,4)
3460 IF KK=5 THEN LPRINT"  ";PPWP(I,1);"  ";PPWP(I,2);"  ";PPWP(I,3);"
     ";PPWP(I,4);"  ";PPWP(I,5)
3470 IF KK=6 THEN LPRINT"  ";PPWP(I,1);"  ";PPWP(I,2);"  ";PPWP(I,3);"
     ";PPWP(I,4);"  ";PPWP(I,5);"  ";PPWP(I,6)
3480 NEXT I
3490 LPRINT:LPRINT EE$
3500 REM*************************************************************
3510 REM
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```
3520 REM Sub-menu
3530 REM
3540 REM************************************************************
3550 CLS:LOCATE 2,1,0:COLOR 15,4:PRINT"You may do any of the
     following:"
3560 COLOR 7,0:PRINT:PRINT"     5. Recalculate parameters"
3570 PRINT:PRINT"     6. Plot the data"
3580 PRINT:PRINT"     7. List residuals"
3590 PRINT:PRINT"     8. Plot residuals"
3600 PRINT:PRINT"     R. Return to main menu"
3610 ANS$=INKEY$: IF ANS$="" THEN 3610
3620 IF ANS$="r" OR ANS$="R" THEN 230
3630 A1= VAL(ANS$): IF A1<5 OR A1> 8 OR A1<>INT(A1) THEN 3610
3640 A11=A1-4:ON A11 GOTO 2040,3650,4130,4130
3650 REM************************************************************
3660 REM
3670 REM OPTION #6: Plot the data
3680 REM
3690 REM************************************************************
3700 CLS:LOCATE 4,1,0:COLOR 15,1:PRINT"How do you want to plot the data
     ?":COLOR 7,0
3710 LOCATE 7,1:PRINT"(a) Y versus X"
3720 PRINT:PRINT"(b) 1/Y versus 1/X"
3730 PRINT:PRINT"(c) Y versus log(X)"
3740 PRINT:PRINT"(d) log(Y) versus log(X)"
3750 O6$=INKEY$:IF (O6$<"a" OR O6$>"d") AND (O6$<"A" OR O6$>"D") THEN
     3750
3760 TEMP = 1:TEMP1=1:FOR I=2 TO NN
3770 IF O6$="b" OR O6$="B" THEN IF 1/Y(I)>1/Y(TEMP) THEN TEMP=I
3780 IF O6$<>"b" AND O6$<>"B" THEN IF Y(I)>Y(TEMP) THEN TEMP =I
3790 IF O6$="d" OR O6$="D" THEN IF Y(I)<Y(TEMP1) THEN TEMP1=I
3800 NEXT I 3810 IF O6$ ="a" OR O6$="A" THEN B1=X(NN):B2=Y(TEMP)
3820 IF O6$ ="b" OR O6$="B" THEN B1=1/X(1):B2=1/Y(TEMP)
3830 IF O6$ ="c" OR O6$="C" THEN B1=LOG(X(1))/LG:B2=Y(TEMP):
     B3=LOG(X(NN))/LG
3840 IF O6$ ="d" OR O6$="D" THEN B1=LOG(X(1))/LG:B2=LOG(Y(TEMP))/LG:
     B3=LOG(X(NN))/LG:B4=LOG(Y(TEMP1))/LG
3850 CLS:SCREEN 1,0:COLOR 0,1
3860 IF O6$="a" OR O6$="A" THEN LOCATE 9,1:PRINT" Y":LOCATE
     20,28:PRINT"X"
3870 IF O6$="b" OR O6$="B" THEN LOCATE 9,1:PRINT" 1":PRINT" -":PRINT"
     Y":LOCATE 20,28:PRINT"1/X"
3880 IF O6$="c" OR O6$="C" THEN LOCATE 9,1:PRINT" Y":LOCATE
     20,28:PRINT"log(X)"
3890 IF O6$="d" OR O6$="D" THEN LOCATE 9,1:PRINT" 1":PRINT" o":PRINT"
     g":PRINT:PRINT" Y":LOCATE 20,28:PRINT"log(X)"
3900 LOCATE 22,1:PRINT PP$
3910 LINE (30,150)-(300,147),2,BF
3920 LINE (33,10)-(30,147),2,BF
3930 FOR I=1 TO NN
3940 IF O6$="a" OR O6$="A" THEN XCORD=X(I)/B1*267+33:YCORD=147-
```

```
     Y(I)/B2*137
3950 IF O6$="b" OR O6$="B" THEN XCORD=1/X(I)/B1*267+33:YCORD=147-
     1/Y(I)/B2*137
3960 IF O6$="c" OR O6$="C" THEN XCORD=33+267*(LOG(X(I))/LG-B1)/(B3-
     B1):YCORD =147-Y(I)/B2*137
3970 IF O6$="d" OR O6$="D" THEN XCORD=33+267*(LOG(X(I))/LG-B1)/(B3-
     B1):YCORD =137-(LOG(Y(I))/LG-B4)/(B2-B4)*127
3980 CIRCLE (XCORD,YCORD),2,1,,,1
3990 NEXT I
4000 FOR XCORD = 34 TO 300 STEP 4
4010 IF O6$="a" OR O6$="A" THEN XXX1=(XCORD-33)*B1/267:XXX2=(XCORD-
     29)*B1/267
4020 IF O6$="b" OR O6$="B" THEN XXX1=267/(XCORD-33)/B1:XXX2=267/(XCORD-
     29)/B1
4030 IF O6$="c" OR O6$="C" THEN XXX1=EXP(LG*(B1+(XCORD-33)*(B3-
     B1)/267)):XXX2=EXP(LG*(B1+(XCORD-29)*(B3-B1)/267))
4040 IF O6$="d" OR O6$="D" THEN XXX1=EXP(LG*(B1+(XCORD-33)*(B3-
     B1)/267)):XXX2=EXP(LG*(B1+(XCORD-29)*(B3-B1)/267))
4050 FLAG6=1:JJ=41:X(JJ)=XXX1:GOSUB 5520:YYY1=YHAT(JJ)
4060 X(JJ)=XXX2:GOSUB 5520:YYY2=YHAT(JJ):FLAG6=0
4070 YCORD1= 147-137*YYY1/B2:YCORD2=147-137*YYY2/B2
4080 IF O6$="b" OR O6$="B" THEN YCORD1=147-137/YYY1/B2:YCORD2=147-
     137/YYY2/B2
4090 IF O6$="d" OR O6$="D" THEN YCORD1 =137-(LOG(YYY1)/LG-B4)/(B2-
     B4)*127:YCORD2=137-(LOG(YYY2)/LG-B4)/(B2-B4)*127
4100 LINE (XCORD,YCORD1) - (XCORD+4,YCORD2),3:NEXT XCORD
4110 ANS$=INKEY$:IF ANS$="" THEN 4110
4120 SCREEN 0,1:COLOR 7,0:GOTO 3540
4130 REM************************************************************
4140 REM
4150 REM Option #7: List residuals
4160 REM Option #8: Plot residuals
4170 REM
4180 REM************************************************************
4190 CLS:FOR I=1 TO NN:RESID(I)=SQR(W(I))*(Y(I)-YHAT(I)):PT(I)=I:NEXT I
4200 FOR I=1 TO NN-1: I1=I:LEAST = Y(PT(I))
4210 FOR J=1+I TO NN:IF Y(PT(J))<LEAST THEN I1=J:LEAST=Y(PT(J))
4220 NEXT J
4230 IF I1<>I THEN TEMP=PT(I):PT(I)=PT(I1):PT(I1)=TEMP
4240 NEXT I
4250 M9=0:N9=0:U9=0:A2=0:BB$=""
4260 FOR I=1 TO NN: IF RESID(PT(I))<0 THEN M9=M9+1:A3=1:BB$=BB$+"-"
4270 IF RESID(PT(I))>0 THEN N9=N9+1:A3=2:BB$=BB$+"+"
4280 IF A3<>A2 THEN U9=U9+1
4290 A2=A3:NEXT I
4300 IF M9+N9<8 THEN AA$="Too few points to analyse randomness":GOTO
     4470
4310 IF N9<M9 THEN TEMP=N9:N9=M9:M9=TEMP
4320 IF M9<2 THEN FT=0:GOTO 4460
4330 FT=0:FOR I=2 TO U9:K9=I/2
4340 IF K9<>INT(K9) THEN K9=(I+1)/2:GOTO 4380
```

```
4350 A3=M9-1:A4=K9-1:GOSUB 5180:C1=C9
4360 A3=N9-1:A4=K9-1:GOSUB 5180:C2=C9
4370 FU = 2*C1*C2:GOTO 4430
4380 A3=M9-1:A4=K9-1:GOSUB 5180:C1=C9
4390 A3=N9-1:A4=K9-2:GOSUB 5180:C2=C9
4400 A3=M9-1:A4=K9-2:GOSUB 5180:C3=C9
4410 A3=N9-1:A4=K9-1:GOSUB 5180:C4=C9
4420 FU=C1*C2+C3*C4
4430 FT=FT+FU:NEXT I
4440 A3=N9+M9:A4=M9:GOSUB 5180: FT=FT/C9:Z9=1
4450 IF FT>.05 THEN AA$="The sequence is random (p>0.95)"
4460 IF FT<=.05 THEN AA$="The sequence is non random (p>0.95)"
4470 IF A1=7 THEN 1170
4480 REM*************************************************************
4490 REM
4500 REM Option #8: Plot residuals (continued)
4510 REM
4520 REM*************************************************************
4530 B1=Y(1):B2=ABS(RESID(1))
4540 FOR I=2 TO NN
4550 IF ABS(RESID(I))>B2 THEN B2=ABS(RESID(I))
4560 IF Y(I)>B1 THEN B1=Y(I)
4570 NEXT I:SCREEN 1,0
4580 COLOR 0,1:PRINT"Sequence = ";BB$:PRINT AA$
4590 FOR I=1 TO 8:PRINT:NEXT I:PRINT"R":PRINT"e":PRINT"s":PRINT"i";
4600 PRINT TAB(28)" Y ":PRINT"d"
4610 LOCATE 23,1:PRINT PPP$
4620 LINE (33,30)-(30,175),2,BF
4630 LINE (30,104)-(300,101),2,BF
4640 FOR I=1 TO NN
4650 XCORD = 33 + Y(PT(I))/B1*267
4660 YCORD =102 - (RESID(PT(I))/B2)*72:CIRCLE (XCORD,YCORD),2,1,,,1
4670 NEXT I
4680 ANS$=INKEY$:IF ANS$<>"c" AND ANS$<>"C" AND ANS$<>"p" AND ANS$<>"P"
     THEN 4680
4690 IF ANS$="c" OR ANS$="C" THEN SCREEN 0,1:COLOR 7,0:GOTO 3500
4700 LOCATE 23,1:PRINT PPPPP$:LOCATE 22,1:PRINT PPPP$:LOCATE 23,1:PRINT
     PP$
4710 ANS$=INKEY$:IF ANS$="" THEN 4710
4720 REM*************************************************************
4730 REM
4740 REM plot residuals on line printer
4750 REM
4760 REM*************************************************************
4770 SCREEN 0,1:COLOR 7,0:CLS:FOR I=0 TO 40: A$(I)="    ":NEXT I
4780 A$(18)=" r  ":A$(17)=" e  ":A$(16)=" s  ":A$(15)=" i  ":A$(14)=" d
     ":A$(13)=" u  ":A$(12)=" a  ":A$(11)=" l  "
4790 FOR X=0 TO 70:FOR Y=0 TO 30:LPY$(X,Y)=" ":NEXT Y:NEXT X
4800 FOR X=1 TO 70:IF INT(X/10)=X/10 THEN A1$="|" ELSE A1$="-"
4810 LPY$(X,15)=A1$:NEXT X
4820 FOR Y=0 TO 30:IF INT(Y/3)=Y/3 THEN A1$="-" ELSE A1$="|"
```

```
4830 LPY$(0,Y)=A1$:NEXT Y
4840 FOR I=1 TO NN
4850 Y=(RESID(PT(I))+B2)/B2*14.5:X=Y(PT(I))/B1*70
4860 LPY$(INT(X+.5),INT(Y+.5))="O"
4870 NEXT I
4880 LPRINT:LPRINT:LPRINT EE$:LPRINT:LPRINT"Graph of residuals versus
     velocity:":LPRINT"----------------------------------
     ":LPRINT:LPRINT
4890 FOR Y=30 TO 0 STEP -1
4900 LP5$="":FOR X=0 TO 70:LP5$=LP5$+LPY$(X,Y):NEXT X
4910 LPRINT " "+A$(Y)+LP5$:NEXT Y
4920 LPRINT:LPRINT"                                                    Y value"
4930 LPRINT:LPRINT"x interval = ";B1/7:LPRINT"y interval = ";B2/15*3
4940 LPRINT:LPRINT"Residuals calculated using:  ";CC$
4950 LPRINT:LPRINT"sequence of residuals = ";BB$:LPRINT AA$
4960 LPRINT:LPRINT EE$:GOTO 3500 4970 SCREEN 0,1:COLOR 7,0: GOTO 3500
4980 REM*************************************************************
4990 REM
5000 REM Option #H: Help menu
5010 REM
5020 REM*************************************************************
5030 CLS:LOCATE 2,1,0:PRINT"Equations are entered starting at line"
5040 PRINT"     in the following format:"
5050 PRINT:COLOR 15,1:PRINT"yhat(  ) = f{B(1), B(2), ..., x(  )}
5060 COLOR 7,0:PRINT:PRINT"An example is as follows:"
5070 PRINT:COLOR 15,1:PRINT"     yhat(  ) = B(1)+exp(B(2)*x(  ))":COLOR
     7,0
5080 PRINT:PRINT"Note that the variable is always =   "
5090 COLOR 15,4:LOCATE 5,6:PRINT "jj":LOCATE 5,33:PRINT "jj":LOCATE
     9,11:PRINT "jj":LOCATE 9,33:PRINT "jj":LOCATE
     11,36:PRINT"jj":COLOR 7,0
5100 PRINT"and that the equation has a line number."
5110 PRINT"To enter more than one equation, number"
5120 PRINT"the lines in increments of 1. For"
5130 PRINT"example:     ,     ,     , ..."
5140 PRINT:PRINT PP$
5150 COLOR 15,2:LOCATE 3,1:PRINT "5520":LOCATE 9,1:PRINT "5520":LOCATE
     16,10:PRINT "5520":LOCATE 16,16:PRINT"5521":LOCATE
     16,22:PRINT"5522":COLOR 7,0
5160 ANS$=INKEY$:IF ANS$="" THEN 5160
5170 GOTO 230
5180 REM*************************************************************
5190 REM
5200 REM Factorial subroutine
5210 REM
5220 REM*************************************************************
5230 J1=1:J2=1:J3=1:A5=(A3-A4)
5240 IF A3<=0 THEN A3=1
5250 IF A4<=0 THEN A4=1
5260 IF A5<=0 THEN A5=1
5270 FOR I3= A3 TO 1 STEP -1:J1=J1*I3:NEXT I3
```

```
5280 FOR I3 =A4 TO 1 STEP -1:J2=J2*I3:NEXT I3
5290 FOR I3=A5 TO 1 STEP -1:J3=J3*I3:NEXT I3
5300 C9=J1/(J2*J3):RETURN
5310 REM*************************************************************
5320 REM
5330 REM Sorting subroutine
5340 REM
5350 REM*************************************************************
5360 FOR I3=1 TO NN-1:I1=I3:LE=X(I3)
5370 FOR I4=I3+1 TO NN:IF X(I4)<LE THEN I1=I4:LE = X(I4)
5380 NEXT I4: IF I3=I1 THEN 5400
5390 I5=X(I1):I6=Y(I1):X(I1)=X(I3):Y(I1)=Y(I3):X(I3)=I5:Y(I3)=I6
5400 NEXT I3
5410 OPEN "NDATA" AS #1 LEN = 8
5420 FIELD #1, 4 AS Q1$, 4 AS Q2$:LSET Q1$=MKI$(NN):PUT #1, 1
5430 FOR I=1 TO NN:LSET Q1$=MKS$(X(I)):LSET Q2$=MKS$(Y(I)):RECN=I+1
5440 PUT #1, RECN:NEXT I:CLOSE #1
5450 RETURN
5460 REM*************************************************
5470 REM
5480 REM Equations go here
5490 REM
5500 REM*************************************************
5510 FOR JJ=1 TO NN:FLAG6=0
5520 REM
5530 REM
5540 IF FLAG6=1 THEN RETURN
5550 NEXT JJ:RETURN
5560 REM*************************************************************
5570 REM
5580 REM Option #E: End the program
5590 REM
5600 REM*************************************************************
5610 WIDTH 80:COLOR 7,0:CLS:END
```

APPENDIX D

Published and Submitted Papers

Brooks, S.P.J., Espinola, T., and Suelter, C.H. (1984) Theory and Practical Application of Coupled Enzyme Reactions: One and Two Auxiliary Enzymes, Can. J. Biochem. Cell Biol. 62, 945-955.

Brooks, S.P.J., Espinola, T., and Suelter. C.H. (1984) Theory and Practical Application of Coupled Enzyme Systems: One and Two Coupling Enzymes with Mutarotation of an Intermediate, Can. J. Biochem. Cell Biol. 62, 956-963.

Brooks, S.P.J., and Suelter, C.H. (1986), Estimating Enzyme Kinetic Parameters: A Computer Program for Linear Regression and Non-Parametric Analysis, Int. J. Biomed. Comp. in press.

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Brooks, S.P.J., and Suelter, C.H. (1986), Association of Avian Mitochondrial Creatine Kinase with the Inner Mitochondrial Membrane, submitted to Arch. Biochem. Biophys.