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THE ROLE OF HEXOKINASE IN THE REGULATION OF GLUCOSE METABOLISM IN RAT BRAIN

By

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ABSTRACT

THE ROLE OF HEXOKINASE IN THE REGULATION OF GLUCOSE METABOLISM IN RAT BRAIN

by

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Rat brain hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1.) binds to the outer mitochondrial membrane through interactions with porin, the protein that forms the structural pores through which metabolites enter and exit the mitochondrion. This location gives hexokinase preferential access to the ATP made in the mitochondria and allows an efficient recycling of the ADP formed by the hexokinase reaction back to oxidative phosphorylation.

This project was undertaken to study the physiological consequences of the interactions between hexokinase and the mitochondria and their importance in the role of hexokinase as a regulator of Glc metabolism in rat brain. Mitochondria from rat brain, containing naturally bound hexokinase, were used to study the function of the enzyme under various experimental conditions. Spectrophotometric techniques were used to monitor ATP production by the adenylate kinase reaction, the creatine kinase reaction and oxidative phosphorylation, as well as the rate of ATP utilization by mitochondrial hexokinase with these ATP sources.

Functional studies showed that, with oxidative phosphorylation as the source of ATP, mitochondrially bound

hexokinase does not respond to gradual changes in extramitochondrial ATP concentration, and it obtains its ATP from one or both of two intramitochondrial compartments located at the contact sites between the inner and outer mitochondrial membranes. Hexokinase activity is regulated by the ATP concentration in these compartments which in turn depends directly on the rate of ATP production by oxidative phosphorylation. The adenylate kinase reaction does not significantly contribute to the ATP used by hexokinase or the ATP present in the intramitochondrial compartments. ATP from the creatine kinase reaction has access to these compartments only during active oxidative phosphorylation.

Hexokinase forms a link between the initial and final steps of aerobic metabolism of Glc. This provides a way to regulate the introduction of Glc into glycolysis, depending on the varying rate of ATP production by oxidative phosphorylation, thus preventing the accumulation of toxic lactate.

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To my wife Marisa

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LIST OF ABREVIATIONS

A_2P_5	P^1P^5,-Di(adenosine-5') pentaphosphate
BKA	bongkrekic acid
BSA	bovine serum albumin
CAT	carboxyatractyloside
Cr	creatine
DEAE	diethylaminoethyl
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol bis(ß-aminoethyl ether) N,N'-tetraacetic acid
Glc-6-P	glucose-6-phosphate
Glc-6-PDH	glucose-6-phosphate dehydrogenase
HEPES	N-2-hydroxyethylpiperazineethanesulfonic acid
HRP	horseradish peroxidase
MOPS	3-[N-morpholino] propanesulfonic acid
NMR	nuclear magnetic resonance
NEM	N-ethylmaleimide
PCr	phosphocreatine
SAND	sulfosuccinimidyl 2-(m-azido-o-nitrobenzamido)-ethyl-1,3'-dithiopropionate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
RCR	respiratory control ratio

Chapter I

Literature Review

Introduction

Rat brain hexokinase (ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1.), catalyzes the initial step in glucose metabolism and plays an important role in the regulation of glycolysis (1,2). In brain, the majority of hexokinase is bound to the mitochondria (3-5) and this association with the energy producing organelles allows the enzyme to form a link between the initial and the final steps of oxidative metabolism.

The major goals of the present work have been to characterize the interactions between the mitochondria and hexokinase and to determine the role these interactions play in the function of hexokinase as a regulator of glucose utilization in brain. In general, this was done by monitoring spectrophotometrically, ATP utilization by mitochondrially bound hexokinase under various experimental conditions and with various sources of ATP. Digitonin fractionation of mitochondria was used to determine the location of hexokinase within specific regions of the outer mitochondrial membrane.

The present chapter will provide information about the structure and function of hexokinase, especially hexokinase I, relevant features of mitochondria, and the interaction between mitochondria and hexokinase.

MAMMALIAN HEXOKINASES

Nomenclature

Four distinct isozymes of hexokinase exist in mammalian tissues, most commonly designated as I, II, III and IV in order of increasing electrophoretic mobility toward the anode (6). Gonzalez *et al.* (7) have designated these isozymes as A, B, C, and D, respectively, based in their order of elution from DEAE cellulose columns. Throughout this thesis, I will use the roman numeral designation.

Hexokinase isozymes

Hexokinase I is the main focus of the present thesis and will be discussed in greater detail later in this chapter. Structural characteristics, tissue distribution, kinetic parameters and other physiologically relevant features of each of the other three isozymes will be discussed at this time. Relevant differences between the isozymes will be emphasized.

Hexokinase II

Hexokinase II has a molecular weight of approximately 100 kDa. It is the predominant form in insulin sensitive tissues like adipose tissue, muscle, and mammary gland (8). Its levels depend on insulin availability, with a significant decrease in activity seen in muscle extracts from diabetic rats (9). No effect on type I is observed in diabetic rat tissues (9).

The type II isozyme appears to be involved in providing Glc-6-P for glycolysis, as well as for the synthesis of storage forms like glycogen and lipids (8). It is inhibited by Glc-6-P, the product of the reaction; but in contrast to type I, which is inhibited almost instantaneously, in type II there is a pronounced time lag before a complete response to Glc-6-P is observed (10,11). Also, in contrast to the type I isozyme, in which inorganic phosphate antagonizes Glc-6-P inhibition, Glc-6-P inhibition of type II is not antagonized by inorganic phosphate (10).

Hexokinase II, as well as hexokinase I, are present in particulate fractions of various tissue homogenates (8,9). It has been determined that, although most of the hexokinase I is associated with the mitochondria, hexokinase II is associated with both mitochondria and microsomes (9). Kurokawa *et al.* (12) have shown that both isozymes compete for a common site on the mitochondrial membrane, and that chymotryptic treatment of both isozymes rendered them non-bindable without affecting their catalytic activity (12). This indicated that both isozymes might have a common chymotrypsin sensitive structural feature that is involved in binding. Polakis and Wilson (13) later found that the N-terminus of hexokinase I was very hydrophobic and contained cleavage sites for chymotrypsin. With the amino acid sequence deduced from cloned cDNA by Thelen and Wilson (14) a similar hydrophobic N-terminus was identified in hexokinase II.

The deduced amino acid sequence of hexokinase II shows striking similarities between the N- and C-terminal halves (14). This had been previously reported to be the case for type I (15) and

type III (16), and is consistent with the view that mammalian hexokinase isozymes I-III evolved by duplication and fusion of a gene that coded for an ancestral hexokinase. More about this when hexokinase I is discussed below.

Hexokinase III

Hexokinase III is the least studied of the hexokinase isozymes. It has not been found to be the predominant form in any one tissue and reports indicate that it is found mainly in the soluble form in tissue homogenates (8,9). However, Preller and Wilson (17) reported weak association with the nuclei of several rat tissues. In tissue slides, using a laser scanning confocal microscope, hexokinase III was seen in the periphery of the nucleus. However, when the nuclei, were isolated, the hexokinase dissociated and was found in the supernatant of homogenates. This was taken to indicate a weak interaction between the nuclei and the enzyme.

The sequence has been deduced from cloned cDNA (16) and, like types I and II, shows extensive similarity between sequences in the N- and C-terminal halves (16); but in contrast to types I and II, type III does not have the N-terminal sequence required by these isozymes to bind to the mitochondria.

Hexokinase IV

Hexokinase IV, also known as glucokinase, is present in liver and the insulin producing ß cells of the pancreas (8). In additon, mRNA for glucokinase has been identified in pituitary cells (18,19). The transcript found in islet cells and in pituitary cells has an

extended 5'-end relative to the liver transcript thought to arise from a tissue specific splicing (18). In liver and in islet cells (but not in pituitary cells), a high K_m Glc transporter protein (GLUT-2) is also expressed and thought to be part, with glucokinase, of the system that senses blood Glc levels.

Glucokinase has a molecular weight of about 50 kDa and is not inhibited by Glc-6-P at physiological concentrations (20). Some of the kinetic parameters of the isozymes are depicted in Table I (20). It is important to point out that although the K_m for ATP is similar for all four isozymes, the K_m for Glc is considerably higher for glucokinase than for the other three isozymes. The physiological significance is that the K_m of glucokinase for Glc is similar to the normal Glc concentration in blood; this allows the enzyme to respond to blood Glc levels that are higher than normal to regulate the magnitude of insulin secretion (21,22).

Hexokinase I

Hexokinase I from rat brain is the main focus of this thesis and therefore the rest of this chapter will address the most important characteristics of this isozyme and its interactions with the mitochondria.

Structure

Rat brain hexokinase (Type I) is a single polypeptide with a molecular weight of approximately 100 kDa (8). The cDNA has been cloned and the amino acid sequence deduced (15). It was shown that

Table I

Comparison of the various kinetic parameters for the mammalian hexokinase isozymes[a].

	Hexokinase			
Parameter	I	II	III	IV
K_m glucose	0.04[b]	0.13	0.02	4.50
K_m ATP	0.42	0.07	1.29	0.49
K_i Glc-6-P vs. ATP	0.026	0.021	0.074	15.0

a- Table adapted from Ureta (20), and references therein.

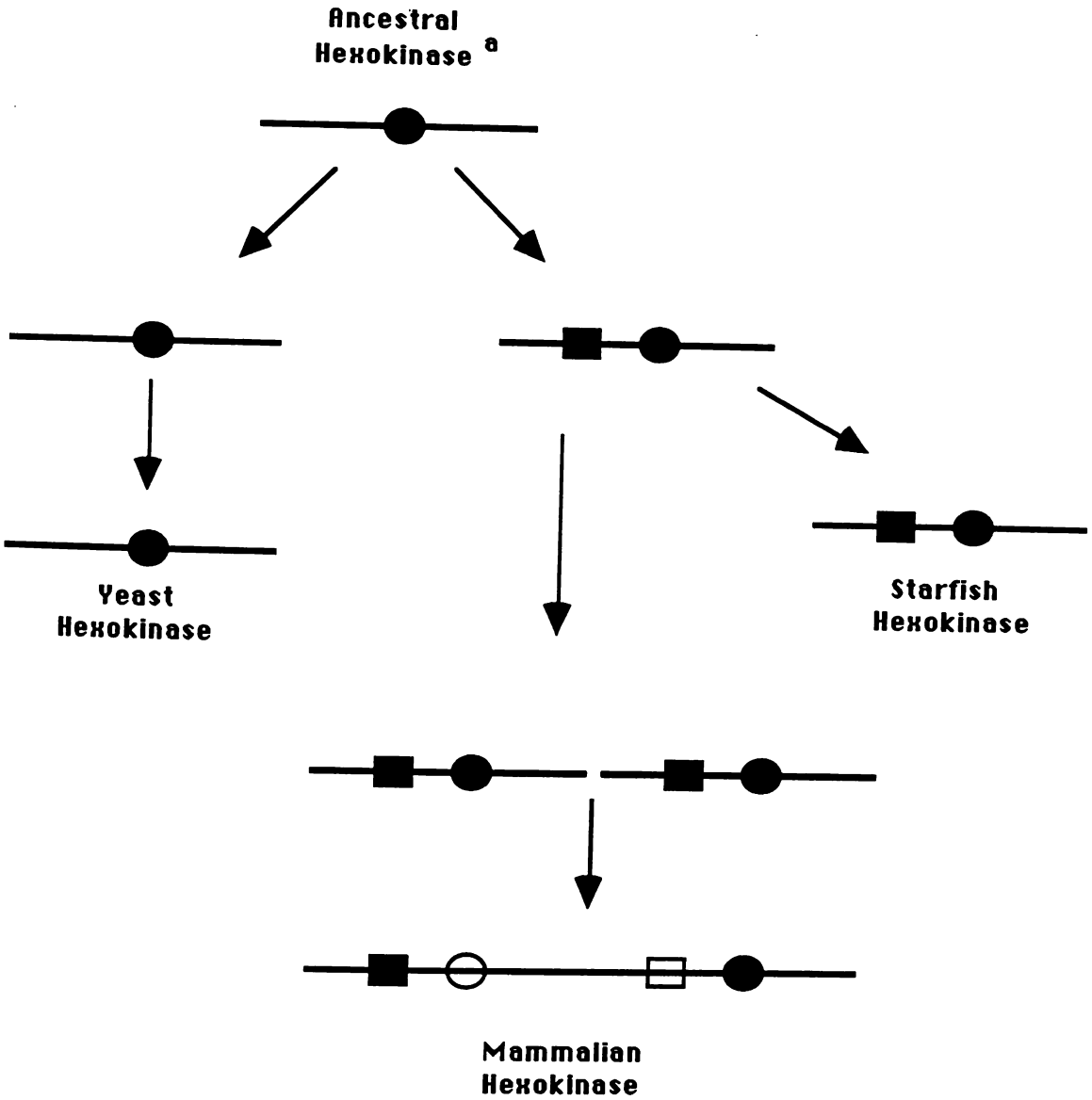
b- All apparent kinetic constant values are expressed in mM.

tryptic digestion cleaved the enzyme giving three fragments: a 10 kDa N-terminal fragment that contains a sequence critical in the binding of hexokinase to mitochondria (13), a 40 kDa C-terminal fragment that contains the binding sites for Glc and ATP (23, 24) and is responsible for the catalytic activity of the enzyme, and a 50 kDa central fragment. More recent work by White and Wilson (25,26), using tryptic digestion under denaturing conditions, showed that the enzyme is composed of two domains of approximately the same size. The C-terminal half is catalytic and the N-terminal half regulatory. Both domains contain binding sites for ATP, hexoses and hexose-6-phosphates. However, in the intact enzyme, the hexose site in the N-terminal half and the Glc-6-P binding site in the C-terminal half appear to be "masked" and, therefore, are nonfunctional (23,26). Figure 1 shows the current model for the evolution of hexokinase. This enzyme appears to have evolved by gene duplication and fusion from a 50 kDa protein similar to that found in present day organisms like yeast that, during the evolutionary process, had acquired the ability to respond to Glc-6-P.

Binding to mitochondria and regulation of hexokinase

Most of the hexokinase in rat brain is bound to the mitochondria (8). It has been suggested (27) that levels of the mitochondrially bound enzyme correlate with the relative

Figure 1. Proposed evolutionary relationship between the hexokinases. The catalytic site is represented by a filled circle, and the regulatory site by a filled square. According to this model, a 50 kDa ancestral hexokinase without a regulatory site evolved in two directions. One remained insensitive to regulation by Glc-6-P, leading to the present day yeast hexokinase. The second one developed a Glc-6-P binding site without changes in molecular weight. A protein with these characteristics is found in present day starfish. The 100 kDa mammalian hexokinase evolved from this Glc-6-P sensitive protein by gene duplication. This molecule contains catalytic and regulatory binding sites in both halves, but in each half one is functional (filled) and the other is nonfunctional (open) (25).



a Modified from White and Wilson (25).

dependence of the tissue on blood-borne Glc as a substrate for energy metabolism. This observation was based on studies with ten different tissues in which levels of phosphoglucomutase, taken as an indication of the importance of glycogen metabolism, and the hexokinase : fumarase ratios (an indication of the proportion of the hexokinase bound to mitochondria) were compared. In general, an inverse relationship between the content of phosphoglucomutase of a tissue and the hexokinase fumarase ratio of the mitochondria of that tissue was found. The high levels of bound hexokinase in brain and the relatively low content of phosphoglucomutase are certainly consistent with this proposal since, under normal circumstances, brain is entirely dependent on blood-borne Glc.

Hexokinase binds to the outer mitochondrial membrane in a Glc-6-P dependent manner; binding of this metabolite to hexokinase promotes solubilization of the enzyme (5). The solubilization by Glc-6-P is antagonized by inorganic phosphate (10,11) and binding is enhanced by Mg^{++} (5).

Binding makes the enzyme more active by decreasing the K_m for ATP and increasing the K_i for Glc-6-P (8). The decrease in K_m for ATP is in the order of two-to-five fold and, coupled with the decrease in sensitivity to inhibition by Glc-6-P, gives the bound enzyme a significant kinetic advantage in competing for available ATP. Another advantage, possibly the most important one, appears to be the fact that hexokinase binds to the pores through which ATP and ADP enter and exit the mitochondria. This gives the enzyme preferential access to the ATP exiting the mitochondria (28-36) and allows it to recycle the ADP back to the oxidative phosphorylation

apparatus more efficiently than enzymes located in the cytosol (34,35).

Considering the changes in the kinetic parameters outlined previously, hexokinase has been proposed to exist in an equilibrium between the bound (more active) and the cytosolic (less active) forms (37). According to this model, the equilibrium, and therefore the catalytic activity of the enzyme, is regulated by changes in the levels of metabolites, especially Glc-6-P and inorganic phosphate. This proposal was supported by evidence that the bound and putatively more active form increases during states like ischemia, that increase utilization of Glc (8), and decreases during states like anesthesia, characterized by lower Glc utilization (38,39). Modifications to this model were later made (8) to include an intermediate form between the bound and the completely dissociated forms of the enzyme. This form is thought to interact with the mitochondria through Mg^{++}, an interaction that cannot be disrupted by Glc-6-P even though the enzyme is inactivated. Binding of hexokinase in this inactive form allows the enzyme to go back to the active state more rapidly when Glc-6-P levels decrease or inorganic phosphate levels increase.

Molecular basis for hexokinase binding to mitochondria

The molecular basis for binding has been studied by several investigators (12,13,29,40-45) and it is thought to involve both electrostatic (44) and hydrophobic interactions (13,33). The electrostatic interactions appear to be mediated by divalent cations (probably Mg^{++}) forming a bridge between negative charges on the

membrane and on the enzyme. It has been shown that, if the pH is increased beyond the pI of the enzyme (pI=6.3), the solubilization of the enzyme is increased probably due to the increase in negative charges (44). Ionic strength also affects binding (44). Neutral salts enhance hexokinase binding at ionic strengths of less than 0.02 M, probably by screening negative charges on the membrane and the enzyme, therefore decreasing the repulsive forces. At higher ionic strengths, the attractive electrostatic forces as disrupted resulting in dissociation of the enzyme (44).

Hydrophobic interactions between the N-terminus of the protein and the outer mitochondrial membrane play a very important role in hexokinase binding. Polakis and Wilson (13) showed that binding of hexokinase to mitochondria is prevented by cleaving the N-terminus of the enzyme with chymotrypsin. This indicated that this hydrophobic N-terminal sequence is critical for the binding of hexokinase to the mitochondria, and later it was shown that a segment of the N-terminus is actually inserted into the membrane core (40).

A protein purified by Felgner *et al.* (41), later shown to be identical to porin (42,43), was also found to be essential for specific and reversible binding of hexokinase. It has a subunit molecular weight of 31,000, determined by SDS-gel electrophoresis, and it was found to confer on lipid vesicles the ability to bind hexokinase in a Glc-6-P sensitive manner (41). The specifics of its interaction with the enzyme are not known, but it is thought to interact with the enzyme's N-terminal sequence inserted into the membrane core.

The disposition of hexokinase at the membrane surface has been determined by Smith and Wilson (45). A panel of monoclonal antibodies against rat brain hexokinase was used to determine the orientation of the hexokinase molecule on the mitochondrial membrane. The epitopes for these monoclonal antibodies were localized to various regions of the N-terminal half of the enzyme and the ability of these antibodies to block binding of soluble hexokinase to liver mitochondria, as well as their ability to recognize mitochondrially bound hexokinase, were examined. The antibodies that were able to block binding and did not recognize bound hexokinase were assumed to recognize epitopes on the surface of hexokinase that are in contact with the membrane. The disposition of the enzyme on the mitochondrial membrane was deduced by relating the results of these experiments to the proposed hexokinase I structure (15), which is based on the structure of yeast hexokinase that has been determined by x-ray crystallographic methods (46). There are extensive sequence similarities between the two hexokinase I domains and yeast hexokinase, so the proposed structure for hexokinase I was developed by fusing two molecules of yeast hexokinase (15). Each one of the two domains of hexokinase I (as well as yeast hexokinase) contains a "large" and a "small" lobe. The large lobe of the N-terminal domain, the domain that had already been implicated in the binding of the enzyme to mitochondria (13,40), is thought to be in contact with the mitochondrial membrane. As previously mentioned, the N-terminus is inserted into the membrane core (40).

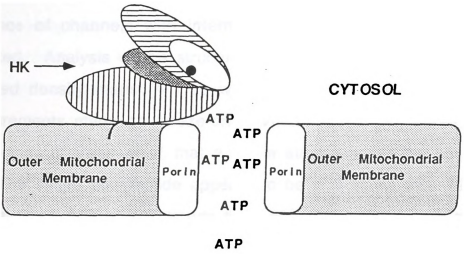
The arrangement of hexokinase with respect to the pores is not known. Figure 2 is a model of the disposition of hexokinase on the membrane based on results from Smith and Wilson (45), the proposed structure of hexokinase I (15) and on calculations of the size of hexokinase and the pore. This figure depicts how the catalytic domain of the enzyme could be situated at the entrance of the pore giving it a kinetic advantage as discussed below under "Physiological implications of the binding of hexokinase to the mitochondria."

Xie and Wilson (47), using a crosslinking agent, found that brain hexokinase bound to liver mitochondria is present either as a monomer or as a tetramer. No evidence of dimers or trimers was found. The physiological significance of these findings is, at the present time, not clear. More studies need to be done to try to quantitate the relative amount of each one of these forms and to determine their location to specific regions of the outer mitochondrial membrane. Some initial work toward this goal is included in this thesis.

Outer mitochondrial membrane channels

The outer mitochondrial membrane separates the metabolic processes of the cytosol from those of the mitochondrion. Exchange of metabolites between the two cellular compartments occurs through channels formed by a protein known as porin, also referred to as voltage-dependent, anion selective channel (VDAC). This protein has a subunit molecular weight of approximately 30 kDa (41-43).

Figure 2. Model of the disposition of bound hexokinase on the outer mitochondrial membrane. Hexokinase has an N-terminal domain and a C-terminal domain, each one with a molecular weight of about 50 kDa. According to the proposed structure of hexokinase (15), each domain contains a "large" and a "small" lobe. The large lobe of the N terminal domain (vertical cross-hatching), interacts with the outer mitochondrial membrane surface (45), and a hydrophobic N-terminal sequence is inserted into the core of the membrane (47). The C-terminal domain of the enzyme, containing the ATP binding site, lies over the opening of the pore, giving the enzyme preferential access to the ATP exiting the mitochondrion. The proposed location of the ATP binding site is depicted by the black dot on the "small" lobe of the C-terminal domain (white). The "small" lobe of the N-terminal domain (gray) and the "large" lobe of the catalytic domain (horizontal crosshatching) are also depicted in the figure.


HK
CYTOSOL
ATP
ATP
ATP
ATP
ATP
ATP
Outer Mitochondrial Membrane
Porin
Porin
Outer Mitochondrial Membrane

Peng *et al.* (48) have suggested that the pore consists of a single polypeptide. This conclusion was based on studies with wild-type *Saccharomyces cereviciae* that expressed VDAC having ion selectivities different from those found in the wild-type. These strains revealed the presence of wild-type and mutant VDAC channels with approximately the same frequency. No channels with intermediate selectivity were observed, indicating that a single protein forms a pore; if VDAC were an oligomeric channel, the presence of channels with intermediate selectivity would be expected. Analysis of the structure of the pore based on frozen-hydrated density maps (44) as well as recent electron scattering measurements made on freeze-dried VDAC arrays (50) agree with the findings of Peng *et al.* that a single subunit forms the pore.

The single polypeptide appears to be a ß-barrel with 19 strands tilted 35° or 13 strands tilted 60° (51). The lumen of the pore has been calculated to be about 2.5 to 3 nm.

Functionally, these channels are voltage gated (52) and are open at low membrane potentials, but they switch to partially closed states in response to elevated voltages (negative or positive) and certain macromolecular modulators (53,54). These changes in size affect the flux of metabolites in and out of the mitochondrion (52). The open channels are anion selective; but a change to the closed state makes them less permeable or even impermeable to metabolites such as ATP and they become cation selective (55). The pore has a diameter in the open state of about 3 nm and in the closed state of about 1.8 nm (52).

Physiological implications of the binding of hexokinase to the mitochondria.

As mentioned previously, binding makes hexokinase more active, not only because of a favorable change in the kinetic parameters but because binding gives the enzyme preferential access to intramitochondrial ATP. Brdiczka *et al.* (56) suggested that hexokinase obtains its ATP from two intramitochondrial compartments, one located at the contact sites between the inner and the outer mitochondrial membranes and the other in the intermembrane space. It has also been suggested (57,58) that hexokinase preferentially binds at these areas of close contact between the two membranes. Dorbani *et al.* (58), using digitonin fractionation, suggested the existence of three distinct domains differing in their cholesterol content within the outer membrane of rat brain mitochondria: (a) cholesterol-rich domains, as evidenced by the release of adenylate kinase at low digitonin concentrations, with practically no release of monoamine oxidase; (b) domains of moderate cholesterol content contain the major portion of the monoamine oxidase and include pore structures that have less affinity for hexokinase (58); and (c) cholesterol-free domains at the contact sites contain porin molecules to which most of the hexokinase is bound (58).

ONTACT SITES

Contact sites between the inner and the outer mitochondrial membranes were first observed by Hackenbrock in the late 1960's). Since then, they have been the focus of many studies. Their

hree main functions are: (a) the import of proteins into the
itochondria (60); (b) the transfer of lipids between the inner and
ne outer membranes (61,62); and (c) the site of
icrocompartmentation including oxidative phosphorylation, the
DP/ATP translocase and kinases like creatine kinase and
exokinase (63). This compartmentation appears to give the kinases
referential access to ATP made by oxidative phosphorylation and
ay participate in the regulation of energy metabolism and energy
ansfer from the matrix to the cytosol.

Contact sites appear to be stable structures that have been
olated from mitochondria from liver (64) and brain (56,57). On
ensity gradient centrifugation they show intermediate density
etween the inner and the outer membranes and they contain marker
nzymes normally found in both mitochondrial membranes (64).

Ardail *et al.* (64) have analyzed the lipid content and fluidity
the outer and the inner membranes as well as the contact sites.
ese investigators reported the existence of two populations of
ntact sites in liver mitochondria. One is enriched in inner and the
er in outer membrane components, but they both contain
noamine oxidase and cytochrome c oxidase activities. Their
olesterol to phospholipid ratio is lower than that of the outer
embrane. Of the two populations of contact sites, the "inner
embrane contact sites" have a lower cholesterol to phospholipid
io and a lower degree of saturation leading to increased fluidity.
e significance of these findings is that there might be different
ntact sites that have distinct functions.

Moran *et al.* (65) performed electrophysiological studies of contact sites from rat brain mitochondria. These investigators found that there are channels at the contact sites that are not voltage-dependent and can remain open at physiological potentials during mitochondrial respiration. This could be important in performing some of the functions hypothesized for these regions, such as the import of protein and the interactions between cytosolic and mitochondrial energy producing pathways.

In short, contact sites are distinct domains that appear to be the regions of interactions between the mitochondria and the cytoplasm.

DIGITONIN FRACTIONATION OF MITOCHONDRIA

The development of techniques to separate the various mitochondrial fractions and to identify each of them by the presence of marker enzymes has facilitated the study of mitochondria tremendously. Digitonin fractionation has been used extensively as a method for selective disruption of the outer mitochondrial membrane and has been applied in studies using mitochondria from several different tissues (57,66,67-71) including rat brain (57,58,71).

Digitonin forms complexes with membrane cholesterol which disrupt the integrity of the membrane (72), and it has been shown in liposomes that digitonin binding is linearly dependent on the concentration of cholesterol in the membrane (72). As mentioned before, contact sites have a lower cholesterol to phospholipid ratio than the outer membrane; and, therefore, it is to be expected that

outer membrane would be more susceptible than contact sites to
uption by digitonin. Digitonin fractionation of mitochondria
n tumor (67), kidney (68), liver (66,69,70) and brain (57,58,71)
shown that although there is some variability in the digitonin
centration needed to solubilize the various marker enzymes
ng the different studies, the pattern of solubilization appears to
very similar. In general, 3-5 times more digitonin is needed to
upt the outer membrane of mitochondria from tumor and brain
n of mitochondria from kidney and liver (58,67,68). Hexokinase
tionates with monoamine oxidase in tumor mitochondria and in
chondria from liver containing exogenously bound tumor
okinase. In brain and kidney, however, 60-80 % of the enzyme
ains bound after monoamine oxidase has been fully released
68). This has been interpreted as an indication that most of the
okinase is bound at the contact sites in a cholesterol poor domain
. Adenylate kinase, located in the intermembrane space (66,73),
ne first enzyme to be released, indicating partial disruption of
outer membrane. Damage to the inner mitochondrial membrane,
ndicated by the release of matrix or inner mitochondrial
brane markers, generally starts at concentrations equal to or
er than those needed to fully solubilize outer membrane
kers.

Parry *et al.* (71,74) have proposed that hexokinase in brain
rentially binds a second receptor present in microsomes or
contaminating cell membranes of mitochondrial preparations.
gitonin fractionation studies, these investigators obtained
s that, in general, agree with fractionation studies from other

atories; however, their interpretation of these results is
ent. The release of hexokinase after treatment with
asing concentrations of digitonin does not coincide with the
se of monoamine oxidase in brain, but it does in tumor
nondria. When the monoamine oxidase has been almost
letely released, about 70-80 % of the hexokinase is still
nt in the mitochondrial pellet of brain. These investigators
these results to indicate that brain hexokinase is not bound to
nitochondria but tumor hexokinase is. It is difficult to believe
30 % of the hexokinase is located in "contaminants" present in a
hondrial preparation that has been characterized biochemically
with electron microscopy to be a preparation practically free of
otosomes and other membranal contaminants (75). Also, during
fractionation experiments, Parry *et al.* noted that brain
kinase fractionated with NADPH cytochrome c reductase, an
ne that they took to be a microsomal marker. As will be shown
in this thesis, I was not able to reproduce these results.
ver, there are reports that this enzyme is present in the outer
nondrial membrane (76,77).

TINE KINASE

Another kinase that is important in the regulation of energy
olism and that appears to form a compartment with oxidative
norylation is creatine kinase.

Creatine kinase catalyzes the reversible reaction:

$$Mg\text{-}ADP + PCr^{2-} + H^{+} \text{<------>} Mg\text{-}ATP + Cr$$

Phosphocreatine is a high energy compound that is present in concentrations in tissues with high energy demand. In skeletal le, the concentration is 20-35 mM and in brain 5-10 mM. This ound is able to replenish cellular ATP much faster than tive phosphorylation, and it keeps ADP concentration low g sudden bursts of energy demand (78).

Creatine kinase is present in the cell in two separate artments (78): (a) in the cytosol, close to the energy utilizing ons, where it catalyzes the formation of ATP and creatine; and the mitochondria, close to oxidative phosphorylation, from it obtains ATP for the formation of PCr and ADP. There are cytoplasmic and two mitochondrial isozymes. Both lasmic and mitochondrial isozymes form dimers with an ximate molecular weight of 80-86 kDa (78). Cytoplasmic ne kinase is composed of two different subunits M-CK and B- M standing for muscle and B for brain) that can combine to form MB and BB dimers (79). The two mitochondrial forms (Mi_a and differ in their pI: Mi_a-CK has a pI of 8.4-9.0, and that of Mi_b-CK -9.5 (78). Furthermore, their tissue distribution is different. K is present in brain, and Mi_b-CK is found in cardiac muscle

Wyss *et al.* (81), using denaturation and subsequent ration, found that Mi_a-CK can form dimers with Mi_b-CK and M- d heterooctamers with Mi_b-CK; Mi_b-CK cannot form dimers ther M-CK or B-CK. These multimeric forms between the isozymes are probably not seen in vivo since these isozymes

resent at different cellular locations and in different tissues. ver, it has been reported that, in vivo, the mitochondrial zymes form octamers as well as dimers and are located in the nembranal space and at the contact sites between the inner and uter mitochondrial membranes (78). This location allows the tion of functional coupling between the mitochondrial CK and tive phosphorylation, giving CK preferential access to nitochondrial ATP (82-85). Creatine kinase, especially in the neric form, interacts with the inner (82,86) as well as with the membranes (83,87), and it has been suggested that these ners are able to induce and stabilize contact sites (88).

From the foregoing discussion, it can be seen that both kinase and creatine kinase appear to have a special relationship oxidative phosphorylation and to form part of a compartment at the mitochondrial level. The interactions, if between hexokinase and creatine kinase at this level are not Some aspects of these interactions will be addressed later in hesis.

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Chapter II

Hexokinase of Rat Brain Mitochondria: Relative Importance of denylate Kinase and Oxidative phosphorylation as Sources of substrate ATP, and Interaction with Intramitochondrial Compartments of ATP and ADP

Abstract

nteractions between intramitochondrial ATP-generating, requiring processes and ATP-requiring, ADP-generating orylation of Glc by mitochondrially bound hexokinase (ATP:D- 6-phosphotransferase, EC 2.7.1.1.) have been investigated well-coupled mitochondria isolated from rat brain. ADP ted by mitochondrially bound hexokinase was more effective ulating respiration than was ADP generated by hexokinase ated from the mitochondria, and pyruvate kinase was less e as a scavenger of ADP generated by the mitochondrially hexokinase than was the case with ADP generated by the ated enzyme. These results indicate that ADP generated by ochondrially bound enzyme is at least partially sequestered ected toward the mitochondrial oxidative phosphorylation us. Under the conditions of these experiments, the maximum ATP production by oxidative phosphorylation was mately 10-fold greater than the maximum rate of ATP ion by the adenylate kinase reaction. Moreover, during of active oxidative phosphorylation, adenylate kinase made ctable contribution to ATP production. Thus, adenylate does not represent a major source of ATP for hexokinase o actively phosphorylating mitochondria. With adenylate as the sole source of ATP, a steady state was attained in

hich ATP formation was balanced by utilization in the hexokinase eaction. In contrast, when oxidative phosphorylation was the ource of ATP, a steady state of Glc phosphorylation was attained, ut it was equivalent to only about 40-50% of the rate of ATP roduction and thus there was a continued net increase in ATP oncentration in the system. Rates of Glc phosphorylation with ATP enerated by oxidative phosphorylation exceeded those seen with quivalent levels of exogenously added ATP. Moreover, at total ATP oncentrations greater than approximately 0.2 mM, hexokinase bound actively phosphorylating mitochondria was unresponsive to ntinued slow increases in ATP levels; acute increase in ATP (by ddition of exogenous nucleotide) did, however, result in increased exokinase activity. The relative insensitivity of mitochondrially ound hexokinase to extramitochondrial ATP suggested dependence an intramitochondrial pool (or pools) of ATP during oxidative osphorylation. Two intramitochondrial compartments of ATP were entified based on their selective release by inhibitors of electron nsport or oxidative phosphorylation. These compartments were stinguished by their sensitivity to inhibitors and the kinetics with ich they were filled with ATP generated by oxidative osphorylation. Exogenous glycerol kinase competed effectively h mitochondrially bound hexokinase for extramitochondrial ATP, h relatively low levels of glycerol kinase completely inhibiting osphorylation of Glc In contrast, with ATP supplied by oxidative osphorylation, the inhibition was markedly biphasic, with much her levels of glycerol kinase required to completely suppress xokinase activity. These results suggest that glycerol kinase was

competing for two distinct compartments of ATP available to mitochondrially bound hexokinase.

Introduction

Under normal conditions, Glc represents virtually the sole ubstrate supporting the energy metabolism required for function of e brain, with the rate of Glc consumption being closely coupled to europhysiological activity (1). Hexokinase (ATP:D-hexose 6-hosphotransferase, EC 2.7.1.1.) catalyzes the initial step in the etabolism of Glc, and regulation of hexokinase plays a major role governing the rate of cerebral Glc utilization reviewed in Ref. (2)]. c is metabolized almost exclusively via aerobic glycolysis (1), a ocess that requires the cooperative action of the glycolytic thway and intramitochondrial oxidative reactions.

Although glycolysis and glycolytic enzymes (3) are generally sociated with the cytoplasm, much of the hexokinase in brain is und to the mitochondria (3-6). Mitochondrially bound hexokinase s also been reported in other normal tissues reviewed in Ref. (2)]. has been suggested (7) that levels of the mitochondrially bound zyme are correlated with relative dependence of the tissue on od-borne Glc as a substrate for glycolytic metabolism; thus, the remely high levels found in brain reflect its unique dependence on substrate. High levels of mitochondrially bound hexokinase are o characteristic of highly glycolytic, rapidly growing tumor cell s (8).

Hexokinase is thought to bind to the pore structure in the outer ochondrial membrane, through which metabolites, including

adenine nucleotides, enter and exit the mitochondrion (9-11). This physical association provides a topological basis for interaction between the ATP-requiring, ADP-generating hexokinase reaction and intramitochondrial ADP-requiring, ATP-generating processes (12-20). For example, it has been reported that hexokinase bound to the mitochondria from liver (12), heart (13), diaphragm (14), skeletal muscle (15), pancreatic islets (16), ascitis tumor (17), or hepatoma cells (18) preferentially uses (has "privileged access" to) ntramitochondrially generate ATP as a substrate.

Considering the probable metabolic importance of mitochondrially bound hexokinase in cerebral energy metabolism (2), and despite the fact that the existence of mitochondrially bound hexokinase was first noted in brain (4) and that, of all the (normal) issues thus-far studied, brain mitochondria have by far the highest evel of bound hexokinase (7), there have been remarkably few studies on the interactions between hexokinase and ATP generation n brain mitochondria. Perhaps the most relevant is the study by nui and Ishibashi (21) demonstrating that efficient use of ntramitochondrially generated ATP did indeed depend on physical ssociation of the enzyme with the mitochondria. We thus initiated study seeking further insight into the adenine nucleotide mediated nteractions between hexokinase and ATP-generating systems of rain mitochondria. One question of particular interest was the xtent to which hexokinase, bound to brain mitochondria, utilizes TP generated by oxidative phosphorylation or by the adenylate nase reaction. Studies with tumor mitochondria (18, 22, 23) have

suggested that this may depend greatly on the particular tumor as well as the concentration of ADP offered as substrate.

In terms of potential physiological significance, the most striking conclusion from the present study was that during active oxidative phosphorylation, mitochondrial hexokinase of brain exhibited a primary reliance on intramitochondrial compartments as a source of ATP, and is unresponsive to gradual changes in extramitochondrial levels of this substrate. Two intramitochondrial compartments of ATP were demonstrated, although the physical basis for this compartmentation has not yet been established. These results can be related to recent studies by other investigators and provide further information relevant to the ultimate understanding of the metabolic significance of mitochondrially bound hexokinase.

Materials and Methods

Materials. ATP, ADP, A_2P_5 CAT, Ficoll 400, yeast hexokinase, pyruvate kinase, chymotrypsin, and glycerol kinase were obtained from Sigma Chemical Co. (St. Louis, MO). Glc-6-P dehydrogenase was a product of Boheringer Mannheim (Indianapolis, IN). Both the BCA Protein Assay Reagent and the bovine serum albumin standard were purchased from Pierce Chemical Co. (Rockford IL.). Other chemicals were of reagent grade and obtained from various commercial sources.

Assays for hexokinase, chymotrypsin, and protein. Hexokinase activity was measured spectrophotometrically, coupling Glc-6-P formation to production of NADPH, monitored at 340 nm, in the presence of excess Glc-6-P dehydrogenase (24). Chymotrypsin was assayed as described by Hummel (25), and protein determined using the BCA method (Pierce Chemical Co., Rockford IL.) with bovine serum as standard.

Brain mitochondria. Brains were obtained from Sprague-Dawley rats (150-250 g) of either sex, and "nonsynaptic" mitochondrial prepared by the method of Lai and Clark (26); the original homogenization was performed with a Teflon-glass homogenizer (size C, Thomas Scientific, Swedsboro NJ). The final mitochondrial suspension contains 7.9 ± 0.4 mg protein and

3.0 ± 0.3 units hexokinase activity per milliliter (mean ± SD for 25 preparations).

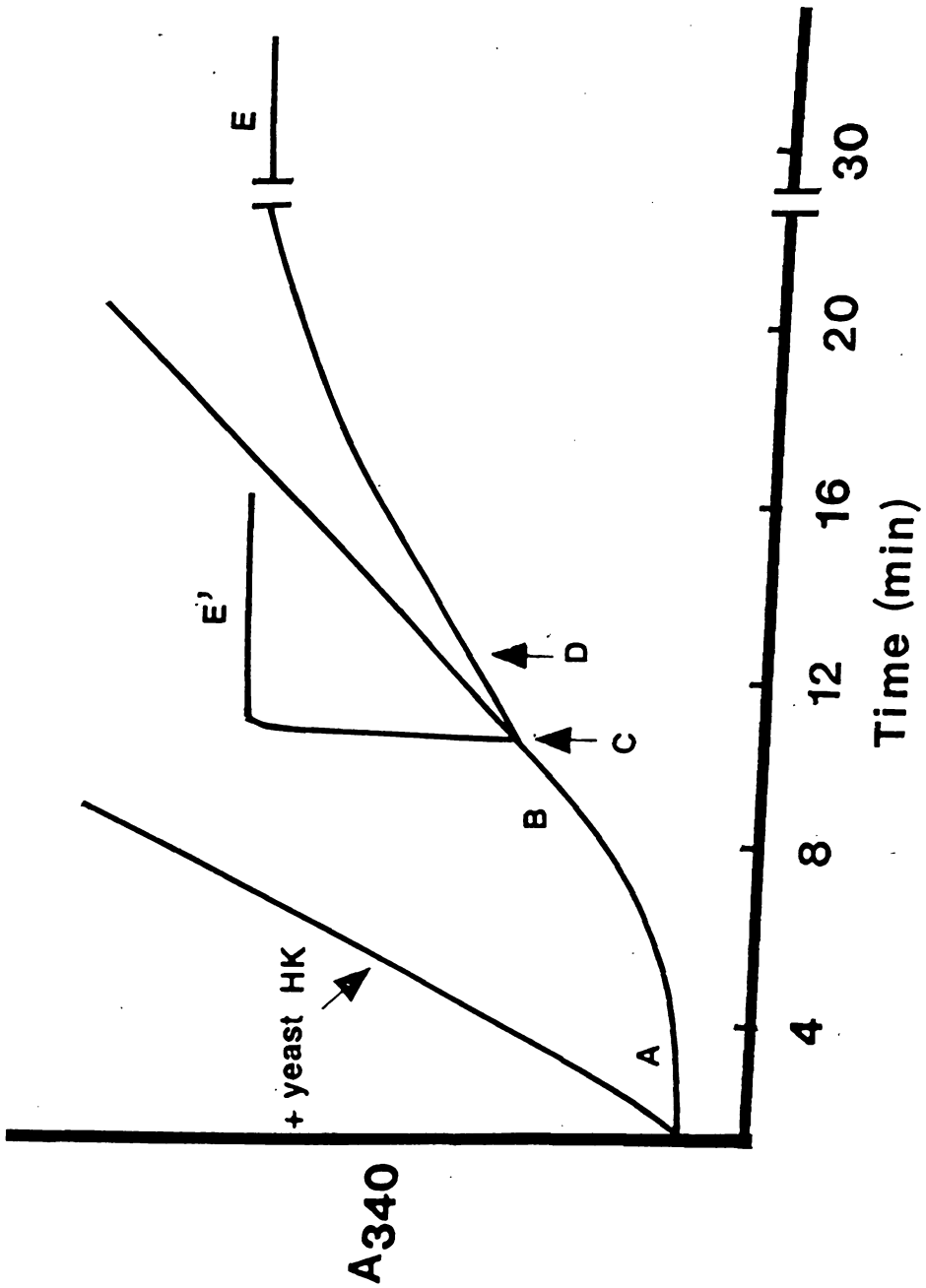
Respiratory rates were determined using a Gilson Oxy-5 oxygraph. Reactions were carried out in "incubation medium" (26) prepared by combining (per 100 ml final volume of incubation medium) 30 ml 0.5 M KCl, 10 ml 1 mM EGTA, 10 ml 0.1 M Tris-Cl, pH 7.4, and 5 ml 0.1 M Tris-phosphate, pH 7.2: the final pH was adjusted to 7.4 with KOH. Substrates were 5 mM pyruvate plus 2.5 mM malate. The RCR for mitochondria used in this study was 4.2 ± 0.7 (mean ± SD for 23 preparations).

Measurement of ATP production by mitochondria and ATP utilization by bound hexokinase. Reactions were carried out at 25° C in incubation medium (see above) supplemented with 5 mM Glc, 5 mM $MgCl_2$, 5 mM pyruvate, 2.5 mM malate, 0.63 mM NADP, and one unit of Glc-6-P dehydrogenase in a final volume of 1 ml. Unless indicated otherwise, the reactions received 10 µl of mitochondrial suspension (approx. 0.08 mg mitochondrial protein), with 0.24 mM ADP added to initiate ATP formation. Selective formation of ATP via either the adenylate kinase reaction oxidative phosphorylation was obtained by inclusion of either 5 mM KCN or 0.1 mM A_2P_5, respectively, in the reaction. KCN is well known as an inhibitor of electron transport (27), while the multisubstrate analog, A_2P_5, is a potent inhibitor (competitive vs both ATP and AMP) of adenylate kinase (28). These concentrations of inhibitors were shown to completely block ATP formation by the susceptible path. The rate of ATP utilization by endogenous mitochondrial hexokinase was

measured by following the absorbance at 340 nm (NADPH formation via the coupled Glc-6-P dehydrogenase reaction). Total ATP production was monitored in the same way but with inclusion of excess (3 u) yeast hexokinase in the reaction.

Typical reaction progress curves are shown in Fig. 1. Various regions relevant to understanding the presentation below are indicated in this figure. After a brief incubation to permit thermal equilibration, the reaction was started by addition of ADP at time zero. *In the presence of* excess yeast hexokinase (as when total ATP production was being measured), there was only a brief lag before ATP production achieved a steady state rate that persisted throughout the experimental period. *In the absence of* excess yeast hexokinase (as when the ATP utilization by endogenous mitochondrially bound hexokinase was being measured), the results were quite different. After a rather prolonged (approx. 6 min.) transition period (A), a steady state (B) was attained which, in the absence of further additions, persisted for at least 25-30 min; rates during the transition period were determined by taking the tangents to the tracing at the specified times. In some experiments, inhibitors were added at specific times of reaction (e.g., Point C). The "postinhibitor rate" is defined as that immediately following addition of inhibitor (region labeled D in the figure); typically, this remained constant for 1-2 min then slowly decreased as ATP formed prior to addition of inhibitor was consumed. ATP present

Figure 1. Representation of typical tracings obtained during the measurements of Glc phosphorylation, coupled to NADPH formation (monitored at 340 nm) via the Glc-6-P dehydrogenase reaction supported by intramitochondrially generated ATP. This is a composite figure, illustrating several parameters of interest in the present study. Measurement of *total* ATP production was performed in the presence of excess yeast hexokinase; initiation of ATP production by addition of ADP was followed by a brief lag before attainment of a steady state rate of Glc phosphorylation. In the absence of added yeast hexokinase, there was an extended transient phase (A) prior to attainment of a steady state of Glc phosphorylation (B). Addition of inhibitors (C) of ATP formation resulted in a decreased rate (D) which slowly diminished as ATP formed prior to addition of inhibitor was consumed by endogenous hexokinase activity, reaching a final absorbance value (E); the difference between A_{340} at E and at the time of inhibitor addition (C) was used to calculate ATP present at the time of inhibitor addition. If excess yeast hexokinase was added with the inhibitor, the same final A_{340} value (E') was attained, but much more quickly.


A340
+ yeast HK
A
B
C
D
E'
E
4
8
12
16
20
30
Time (min)

at the time of inhibitor addition (which may include both extramitochondrial ATP and that in intramitochondrial compartments) was calculated from the difference between the final absorbance (E) and that at the time of inhibitor addition. If yeast hexokinase was already present in the reaction mixture (i.e., the total rate of ATP production was being monitored), or if it was added simultaneously with the inhibitor, the increase in absorbance due to consumption of previously formed ATP was completed much more quickly (though as described below the kinetics depend on the inhibitor added) but the final absorbance (E') was the same as that seen if endogenous mitochondrial hexokinase was used to consume the ATP.

Treatment of mitochondria with Glc-6-P to remove the bound hexokinase. The product of the hexokinase reaction, Glc-6-P, induces release of the mitochondrially bound enzyme (5, 24). The mitochondrial suspension was diluted 1:5 in "isolation medium" (26) containing 250 mM sucrose,).1 mM EGTA, and 5 mM Mops, pH adjusted to 7.4 with KOH. The suspension was made 1 mM in Glc-6-P, incubated for 5 min at room temperature, then centrifuged at 14,500 g for 10 min. The supernatant was carefully removed and the mitochondrial pellet resuspended in isolation medium to restore the original mitochondrial protein concentration. Hexokinase activity was assayed in both supernatant and pellet. Treatment with Glc-6-P reduced the mitochondrial hexokinase content to 11 ± 2 % of the original activity (mean ± SD for nine preparations) without

significant effect on the RCR (4.0 ± 0.5, mean ± SD for seven preparations).

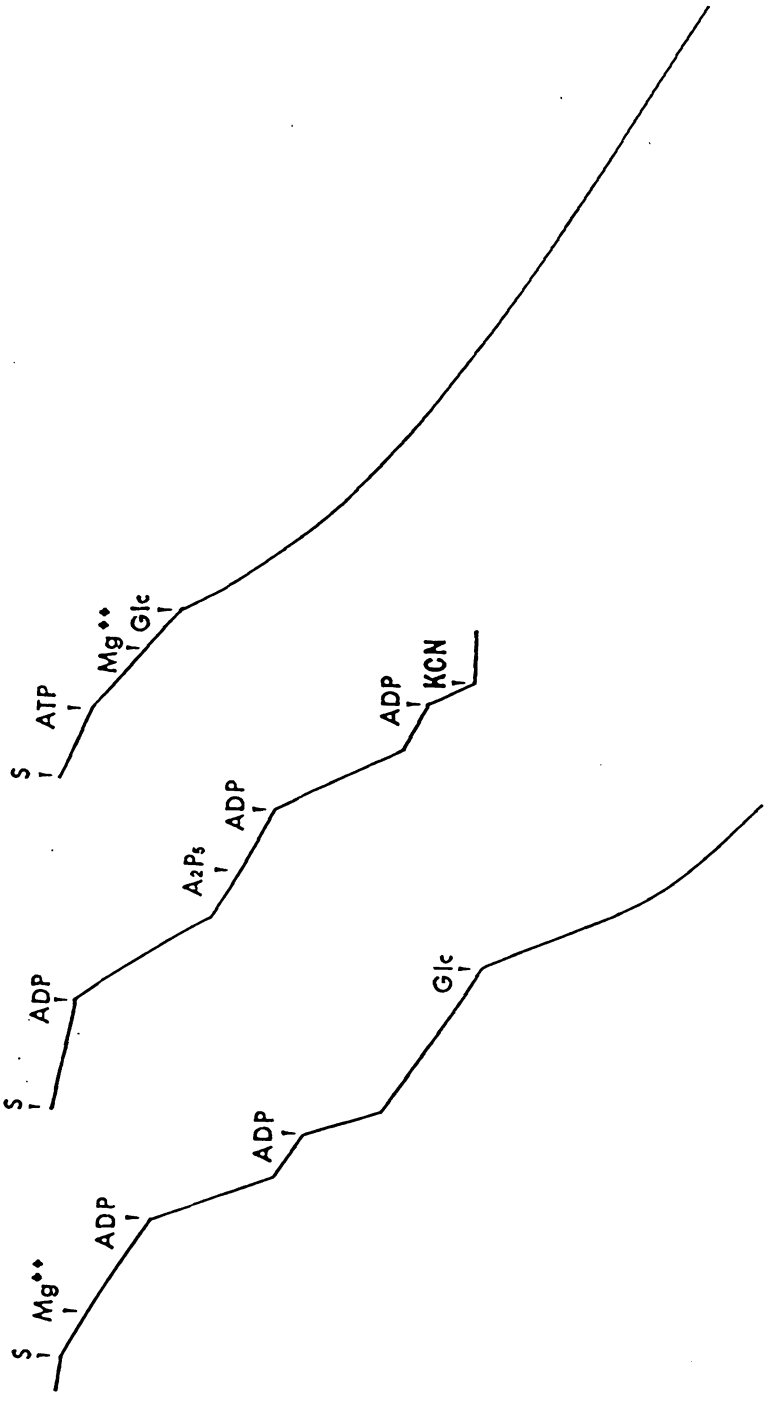
Treatment of hexokinase with chymotrypsin. Treatment of hexokinase with chymotrypsin leads to loss of the ability of the enzyme to bind to mitochondria, but has no effect on catalytic activity; this has been shown to result from the selective modification of a hydrophobic N-terminal sequence which is critical for binding (29). Purified rat brain hexokinase (24), 0.25 mg/ml in 50 mM Hepes, 0.5 mM EDTA, 10 mM thioglycerol, pH 7.5, was incubated with chymotrypsin (0.2 μg chymotrypsin for 20 μg hexokinase) at room temperature for 1 h. The enzyme was then chromatographed on a column of Sephadex G-75 equilibrated with this same buffer. Fractions (0.5 ml) were collected and assayed for hexokinase and chymotrypsin. Peak fractions, containing hexokinase activity but no detectable chymotrypsin activity, were combined.

Results

Mitochondrial respiration. Various pertinent aspects of the respiratory activity of the brain mitochondria used in this study are illustrated by the results shown in Fig. 2. In contrast to the observation of Moore and Jobsis (19), addition of low concentrations of Mg^{++} (required to form the chelated form of ATP, used as substrate by hexokinase) had no detectable effect on State 4 respiration (Fig. 2 Tracing A). Otherwise, these results were similar to those of Moore and Jobsis, including the observed RCR and the marked stimulation of respiration by addition of Glc. With respect to the latter, it is important to note that it occurred virtually immediately after addition of Glc, reflecting the rapid burst of ADP formation via the hexokinase reaction. Respiration then slowed to a steady state rate, indicating a balance between ADP formation by hexokinase and reutilization of ADP by mitochondrial oxidative phosphorylation; this steady state rate was well above State 4 rates, and persisted until the oxygen was virtually exhausted.

As shown in Tracing B (Fig. 2), addition of the adenylate kinase inhibitor, A_2P_5, at 0.1 mM concentration had no effect on either State 3 or State 4 respiration, while addition of 5 mM KCN led to virtually immediate cessation of oxygen uptake.

Figure 2. Respiratory measurements. Reactions were carried out as described under Materials and Methods. Initially, each reaction mixture contained mitochondria (approx. 0.8 mg protein) in incubation medium. Additions were made as indicated: S substrate (5 mM pyruvate plus 2.5 mM malate); $MgCl_2$, 3 mM; ADP, 0.08 mM in all cases except for the second addition in tracing A, which was 0.04 mM; Glc, 3 mM; KCN, 5 mM; A_2P_5, 0.1 mM; ATP 1 mM.

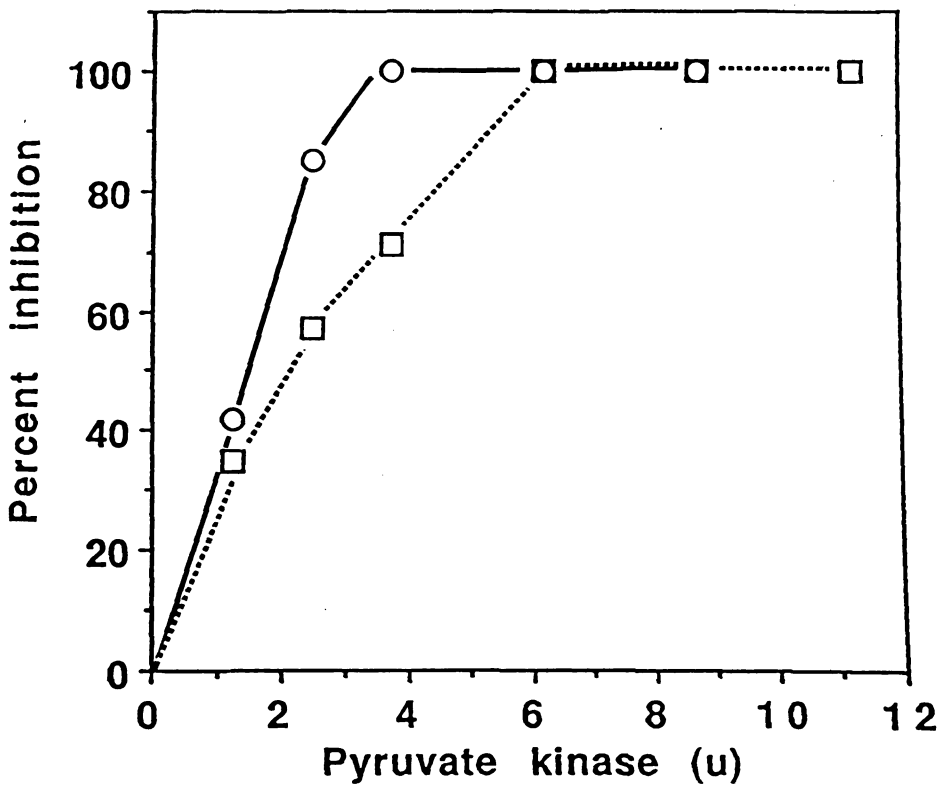

A
S
Mg++
ADP
ADP
B
S
ADP
A2P5
ADP
ADP
Glc
C
S
ATP
Mg++
Glc
ADP
KCN

Relative effectiveness of ADP produced by mitochondrially bound hexokinase vs nonbound enzyme in stimulation of respiration. Tracing C in Fig. 2 shows that addition of ATP resulted in only a modest increase in respiration rate. Subsequent addition of Glc resulted in the expected burst of respiration due to formation of ADP by the hexokinase reaction. This then slowed to a steady state rate which persisted until the oxygen was essentially exhausted. The ratio of the respiration rate immediately prior to Glc addition and that immediately following addition of this substrate was taken as a measure of the effectiveness with which ADP generated by the hexokinase reaction stimulated respiration. In four measurements with a single mitochondrial preparation, containing endogenously bound hexokinase, this ratio was 2.42 ± 0.04. Similar experiments were also done with this same mitochondrial preparation after treatment with Glc-6-P (and now containing only about 10% of the original endogenously bound hexokinase), supplemented with the nonbindable, chymotrypsin-treated enzyme to bring the total hexokinase concentration in the reaction vessel to the same level. In three determinations, the stimulation ratio was 1.71 ± 0.02. Very similar values were obtained in experiments with two other mitochondrial preparations. Thus, ADP generated by the mitochondrially bound hexokinase is considerably more effective in supporting respiration.

An alternative approach to determining the effectiveness with which ADP generated in the hexokinase reaction was redirected to oxidative phosphorylation was to examine the ability of an extramitochondrial ADP requiring enzyme to compete for the ADP.

The experiment was performed essentially as depicted in Tracing C of Fig. 2 except that 1.5 mM phosphoenolpyruvate was present in the reaction medium. After allowing the steady state to be established subsequent to Glc addition, sequential additions of pyruvate kinase were made, the new steady state rate being noted after each addition. This "titration" of the ADP was continued until addition of pyruvate kinase produced no further reduction in the rate; at this point, the observed rate closely approximated that seen prior to ATP addition. The inhibition of respiration after each addition of pyruvate kinase was expressed as a percentage of the difference between the rates with no added pyruvate kinase (0 % inhibition) and those with excess pyruvate kinase (100 % inhibition), with result shown in Fig. 3. With endogenously bound hexokinase, pyruvate kinase was substantially less effective at inhibiting respiration than was the case with Glc-6-P treated mitochondria supplemented with nonbindable, chymotrypsin-treated enzyme to maintain a constant hexokinase level in the reaction vessel. Thus, the ADP generated in the reaction catalyzed by the mitochondrially bound enzyme is preferentially available to the intramitochondrial oxidative phosphorylation apparatus. However, sequestration of the ADP is obviously not complete since it remains accessible to extramitochondrial pyruvate kinase.

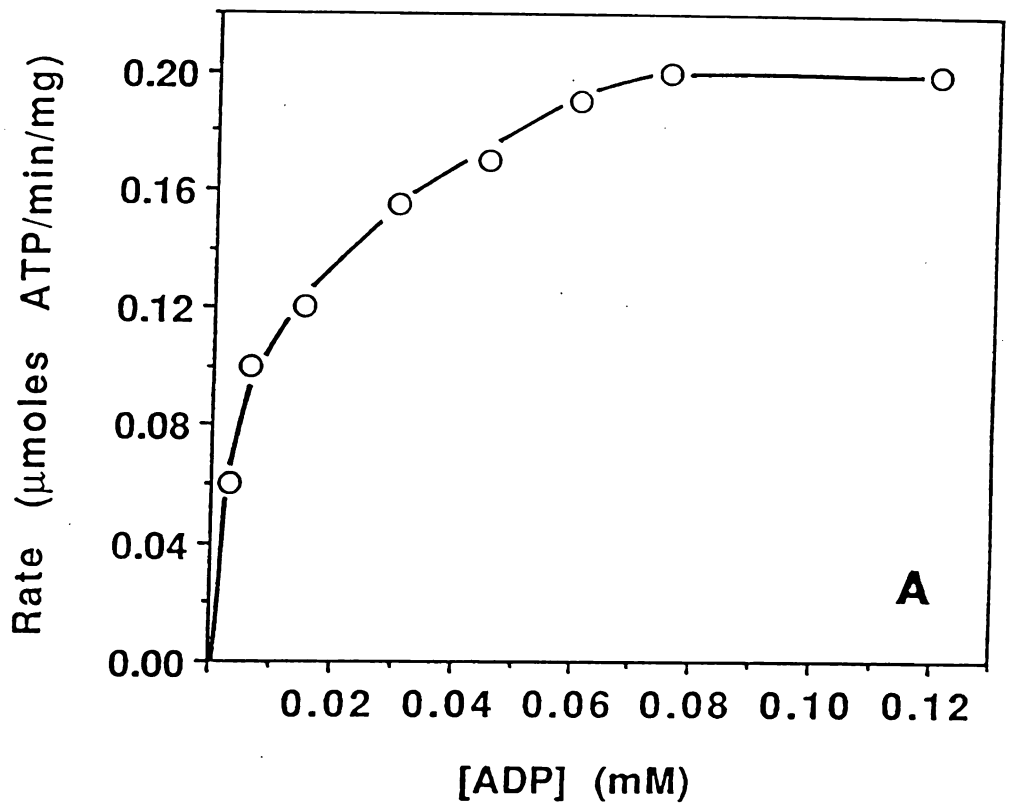
Figure 3. Inhibition of ADP-stimulated respiration by addition of pyruvate kinase. The experiment was conducted as described in the text. Briefly, the ability of increasing concentrations of pyruvate kinase to inhibit coupled respiration, with ADP generated by activity of the mitochondrially bound hexokinase (□) or by hexokinase that was predominantly located in the extramitochondrial medium (o) was determined.


Percent Inhibition
100
80
60
40
20
0
0
2
4
6
8
10
12
Pyruvate kinase (u)

Production of ATP by oxidative phosphorylation and adenylate kinase. Steady state rates of ATP production via either oxidative phosphorylation or the adenylate kinase reaction, measured in the presence of excess yeast hexokinase, were determined as a function of ADP concentration (Figs. 4A and 4B). These results demonstrate that the apparent K_m for ADP is much lower for oxidative phosphorylation than for adenylate kinase; similar results have been reported for tumor mitochondria (22,23). The results shown in Fig. 4 also demonstrate that, with saturating levels of ADP, the rate of ATP production by oxidative phosphorylation is approximately 10-fold greater than that attained by adenylate kinase.

To determine the contribution of adenylate kinase and oxidative phosphorylation to total ATP production under the conditions used for these experiments, KCN and A_2P_5 were added, both together and separately (Table I). Inclusion of A_2P_5 in the reaction had no effect on the rate of ATP production, indicating that adenylate kinase was making a negligible contribution in the presence of active oxidative phosphorylation. It is thus apparent that, when ATP is being generated by oxidative phosphorylation, adenylate kinase cannot represent a significant source of ATP for hexokinase. When KCN was included in the reaction, a modest rate of ATP production by adenylate kinase was observed. Addition of KCN subsequent to addition of A_2P_5, or vice versa, resulted in total inhibition of ATP production, as was also the case if both inhibitors were included from the beginning of the reaction.

Figure 4. ATP production by oxidative phosphorylation and adenylate kinase as a function of ADP concentration. Steady state rates of ATP production, coupled to production of Glc-6-P by addition of excess yeast hexokinase, are expressed as a function of ADP concentration used to initiate ATP formation. A, ATP produced by oxidative phosphorylation. B, ATP produced by the adenylate kinase reaction.


Rate (μmoles ATP/min/mg)
0.20
0.16
0.12
0.08
0.04
0.00
0.02 0.04 0.06 0.08 0.10 0.12
[ADP] (mM)
A

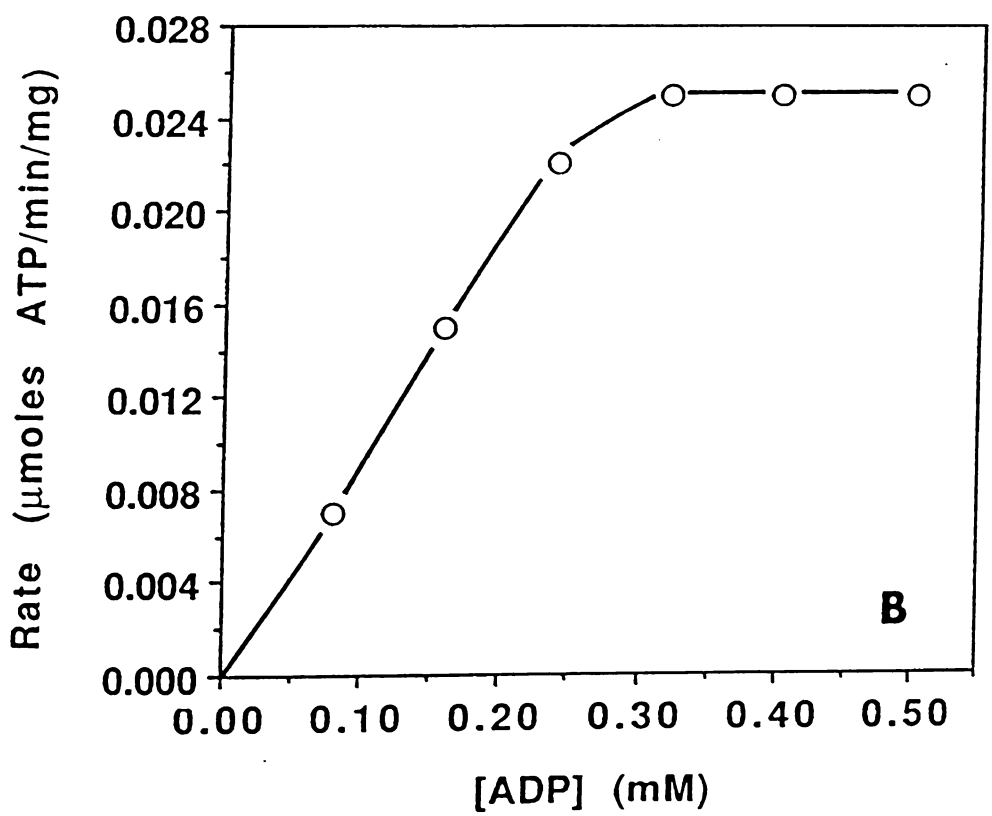

Rate (μmoles ATP/min/mg)
0.028
0.024
0.020
0.016
0.012
0.008
0.004
0.000
0.00 0.10 0.20 0.30 0.40 0.50
[ADP] (mM)
B

TABLE I

Contribution of Oxidative Phosphorylation and Adenylate Kinase to ATP Production by Rat Brain Mitochondria

Addition	Rate[a] (μmol G6P/min/mg protein)
None	0.22± 0.03
A_2P_5	0.23± 0.03
KCN	0.04± 0.01
KCN + A_2P_5	0.0

[a]Mean ± SD for 13 measurements with five different mitochondrial preparations.

Relative rates of ATP production and ATP utilization by mitochondrially bound hexokinase. Steady state rates of total ATP production (measured in the presence of excess yeast hexokinase) and ATP utilization by endogenous mitochondrially bound hexokinase, with either adenylate kinase or oxidative phosphorylation as ATP-generating source, are compared in Table II. In both cases, the steady state rate of ATP utilization was approximately half of the rate of ATP production.

Although the results shown in Table II are typical, it should be mentioned that occasional preparations of mitochondria displayed considerably less effectiveness in utilizing the ATP produced via the adenylate kinase reaction, with Glc phosphorylation rates by endogenous hexokinase being approximately 20 % (cf. 48 % in Table II) of the total rate of ATP production. In these preparations, rates of ATP production by either oxidative phosphorylation or adenylate kinase and fractional utilization of ATP produced by oxidative phosphorylation were similar to the values given in Table II; other characteristics (RCR, hexokinase content) were also indistinguishable from mitochondrial preparations normally obtained. We do not understand the basis for the less effective coupling of hexokinase to ATP production by adenylate kinase in these atypical mitochondrial preparations; its sporadic appearance has precluded linking it to any specific variation in methodology or reagents.

The ATP utilized by bound hexokinase may be captured as it exits the mitochondria, or it may be ATP that has already

Table II

Comparison of Rate of ATP Utilization by Mitochondrially Bound Hexokinase with rate of ATP Production from Intramitochondrial Sources.

ATP source	Utilization[a]	Production[a]	Utilization/ production (%)
Adenylate kinase	0.015 ± 0.006	0.029 ± 0.004	48 ± 9
Oxidative phosphorylation	0.08 ± 0.02	0.19 ± 0.06	43 ± 11

Note. [a] Rate of Glc phosphorylation by mitochondrial hexokinase and total rate of ATP production are given in μmol/min/mg mitochondrial protein. Mean ± SD for nine determinations, each with different mitochondrial preparation.

esca
the
that
mito
intra
that
Glc
mito
the
con
inte
kina

the
mito
tota
ade
adc
ATI
intr
Acc
and
oxi
pre
spe
poi

escaped from the mitochondria and is now supplied to the enzyme by the surrounding medium. Other results, presented below, indicate that when ATP is generated by oxidative phosphorylation, mitochondrially bound hexokinase exhibits a primary reliance on intramitochondrial compartments of ATP. It therefore seems likely that, when ATP is supplied by oxidative phosphorylation, most of the Glc phosphorylation reflects capture of ATP as it leaves the mitochondria. It follows that the "efficiency" of this capture is in the range of 40-50 %. Additional results consistent with the conclusion are presented below. However, as will become evident, interpretation of the results with production of ATP by adenylate kinase is more complicated.

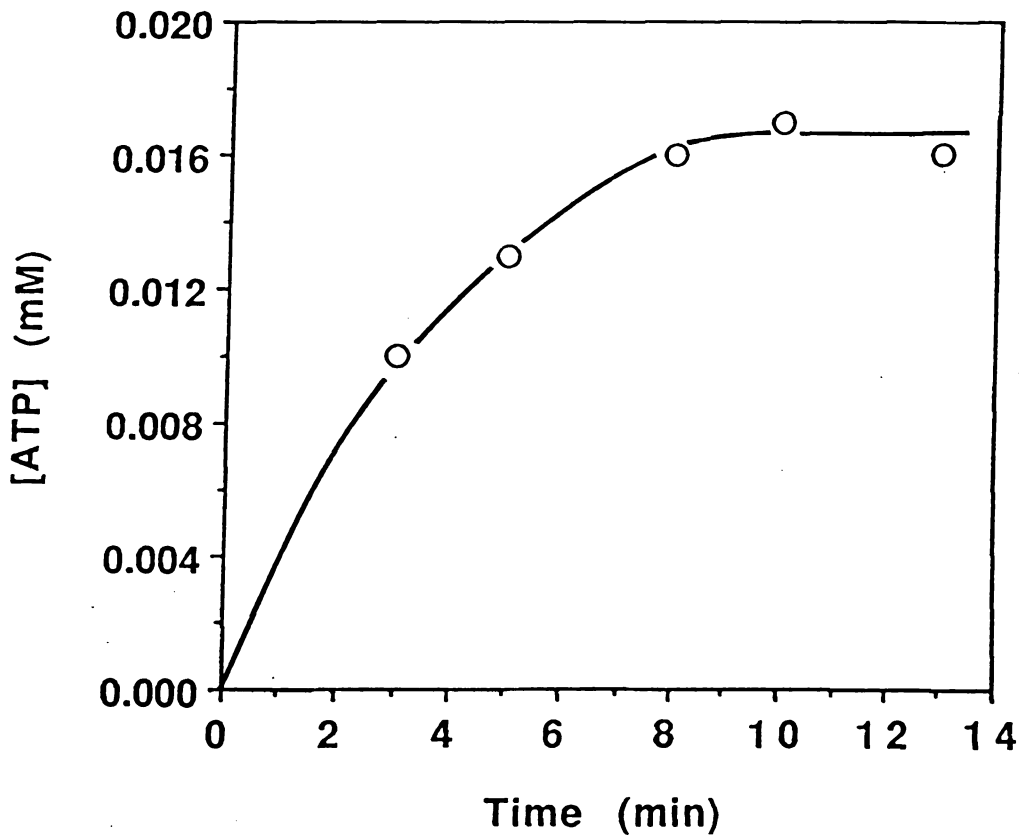
Disposition of ATP not captured by hexokinase during exit from the mitochondria. As noted above, rates of Glc-6-P production by mitochondrially bound hexokinase corresponded to about 50 % of total ATP production by either oxidative phosphorylation or adenylate kinase (Table II). This implies that (in the absence of added yeast hexokinase) there should be a *continued* accumulation of ATP (corresponding to the 50 % not utilized by hexokinase) in intramitochondrial pools and/or the surrounding medium. Accumulated ATP was determined by addition of yeast hexokinase and the complementary inhibitor (e.g., KCN if ATP production by oxidative phosphorylation were being studied, A_2P_5 already being present in such experiments-- see Materials and Methods) at specified times after initiation of the reaction (i.e., at various points along the reaction curve depicted in Fig.1); that the combined

inhibitors did totally inhibitors did totally inhibit further ATP was confirmed by the observation of a total absence of further Glc phosphorylation after the initial burst attributable to consumption of the previously formed ATP (i.e., in the region labeled E' in Fig. 1).

The results of a typical experiment in which ATP was being produced by adenylate kinase are shown in Fig. 5. They are not in accord with the expectation stated above, and accordingly, the results in Table II cannot be interpreted as reflecting the efficiency with which ATP formed by adenylate kinase is captured by bound hexokinase (i. e., as done above for ATP produced by oxidative phosphorylation). In contrast to the predicted continued rise in ATP, the ATP concentration rose during the initial phase of the reaction but then leveled off at a value that represents a steady state in which ATP production by adenylate kinase equaled ATP utilization by endogenous mitochondrially bound hexokinase. It is thus evident, that in the absence of yeast hexokinase, the rate of ATP production by adenylate kinase must be approximately half that seen in the presence of excess yeast hexokinase (Table II).

Why would the presence of excess yeast hexokinase increase the rate of ATP production by adenylate kinase? It can be expected that added yeast hexokinase would serve as an effective scavenger of ATP escaping from the mitochondria, with the result that the ATP concentration in the extramitochondrial solvent space would be maintained at negligible levels. Indeed, it was shown that, with the amount of yeast hexokinase used,

Figure 5. ATP concentration as a function of time after initiation of ATP production by the adenylate kinase reaction. Total ATP present at various times was determined as described under Materials and Methods this includes ATP present in the extramitochondrial medium as well as that present in the intramitochondrial compartments.

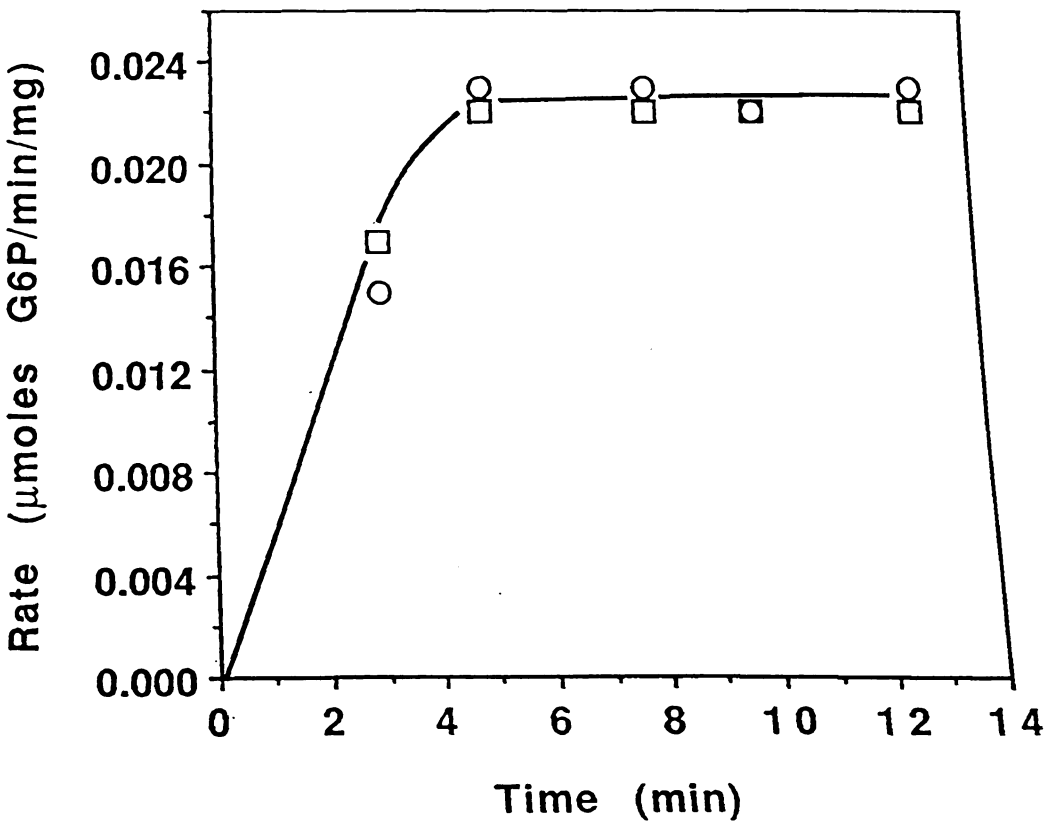

0.020
0.016
0.012
0.008
0.004
0.000
[ATP] (mM)
0
2
4
6
8
10
12
14
Time (min)

only submicromolar levels of exogenously added ATP were required to produce rates of Glc phosphorylation equivalent to those seen these experiments (e. g., as in the measurement of total ATP production, Table II). Thus, by effectively regenerating substrate ADP for the adenylate kinase reaction, yeast hexokinase may increase the net rate of ATP production by adenylate kinase. This also implies rapid interchange of nucleotides between the extramitochondrial space and the intramitochondrial compartment containing adenylate kinase.

The rate of Glc phosphorylation at various times after initiation of ATP production by adenylate kinase is shown in Fig. 6. As noted above (Fig. 1), the rate increased steadily during a transition period, than achieved a steady state value at a time that approximated that required for stabilization of ATP levels (Fig. 5). The postinhibitor rates (see Fig. 1 and related comments in text) following addition of A_2P_5 at various times along the reaction progress curve were indistinguishable from those seen at the time of inhibitor addition (Fig. 6) These results indicate that, under these conditions, Glc phosphorylation is dependent either on ATP from the surrounding medium or on ATP from an intramitochondrial pool that rapidly equilibrated with the latter. In either case, the concentration of ATP available to the bound hexokinase would not be markedly affected in the period immediately after addition of inhibitor.

The results obtained in similar experiments, but with ATP being produced by oxidative phosphorylation, were quite different. In contrast to the leveling off of ATP levels seen with

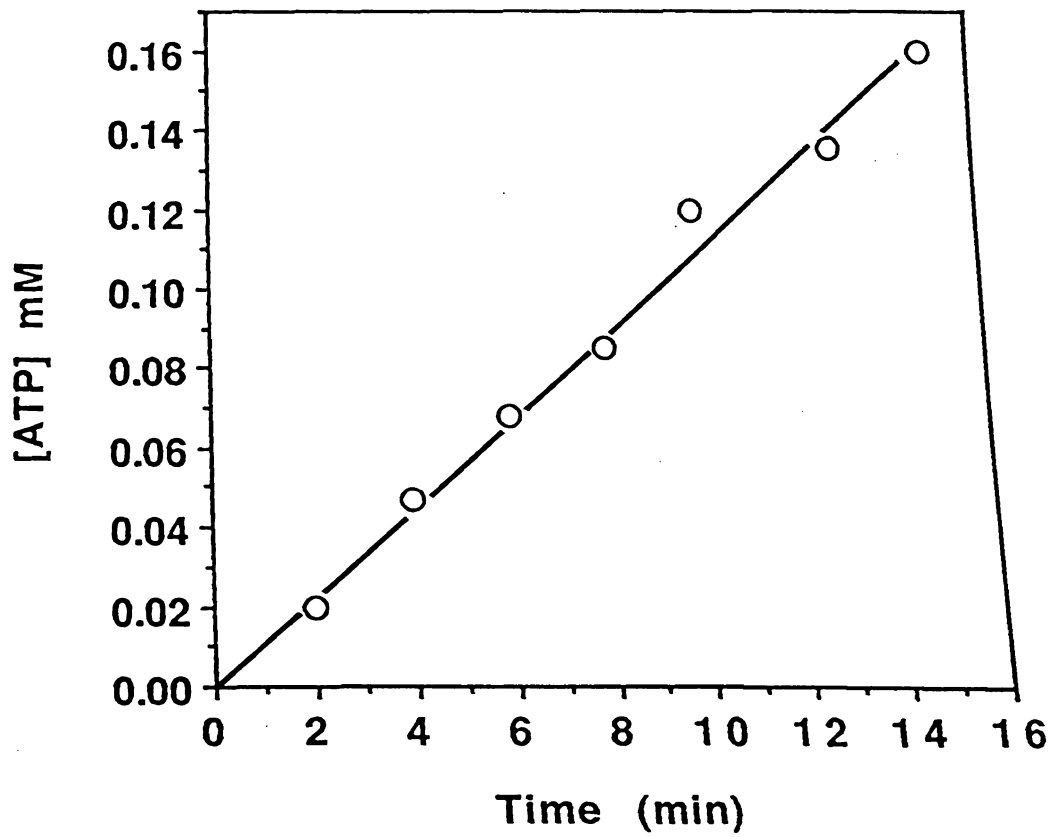
Figure 6. Rate of Glc phosphorylation with ATP generated by the adenylate kinase reaction. ATP generation was initiated by addition of ADP at zero time. At various times thereafter (when ATP concentrations were as shown in Fig. 5), the rate of Glc phosphorylation immediately before (□) and after (o) addition of A_2P_5 was determined as described in the text.


Rate (μmoles G6P/min/mg)
0.024
0.020
0.016
0.012
0.008
0.004
0.000
0
2
4
6
8
10
12
14
Time (min)

adenylate kinase as the source (Fig. 5), ATP levels continued to rise linearly throughout the experimental period (Fig. 7). The rate of ATP accumulation (with 0.08 mg mitochondrial protein in the reaction) calculated from the results in Fig. 7 is 0.13 μmol/min/mg protein, slightly greater than the rate (0.08 μmol/min/mg protein) at which ATP is being utilized by mitochondrially bound hexokinase (Table II), and the sum of these rates is in close agreement with the rate at which ATP is produced (Table II). This is consistent with the above interpretation of the results in Table II, i. e., at steady state, approximately 40 % of the ATP being produced by oxidative phosphorylation is captured by hexokinase as the ATP exits the mitochondrion, with the remainder accumulating. A second notable feature of the results in Fig. 7 is that production and accumulation of ATP proceeds virtually immediately from the time of initiation of oxidative phosphorylation by addition of ADP. In contrast, there is a marked transition period before Glc phosphorylation enters the steady state (Fig. 1). We believe that this lag in initiation of Glc phosphorylation can be attributed to the time required to fill intramitochondrial compartments of ATP, upon which mitochondrially bound hexokinase depends; this concept will be further developed below.

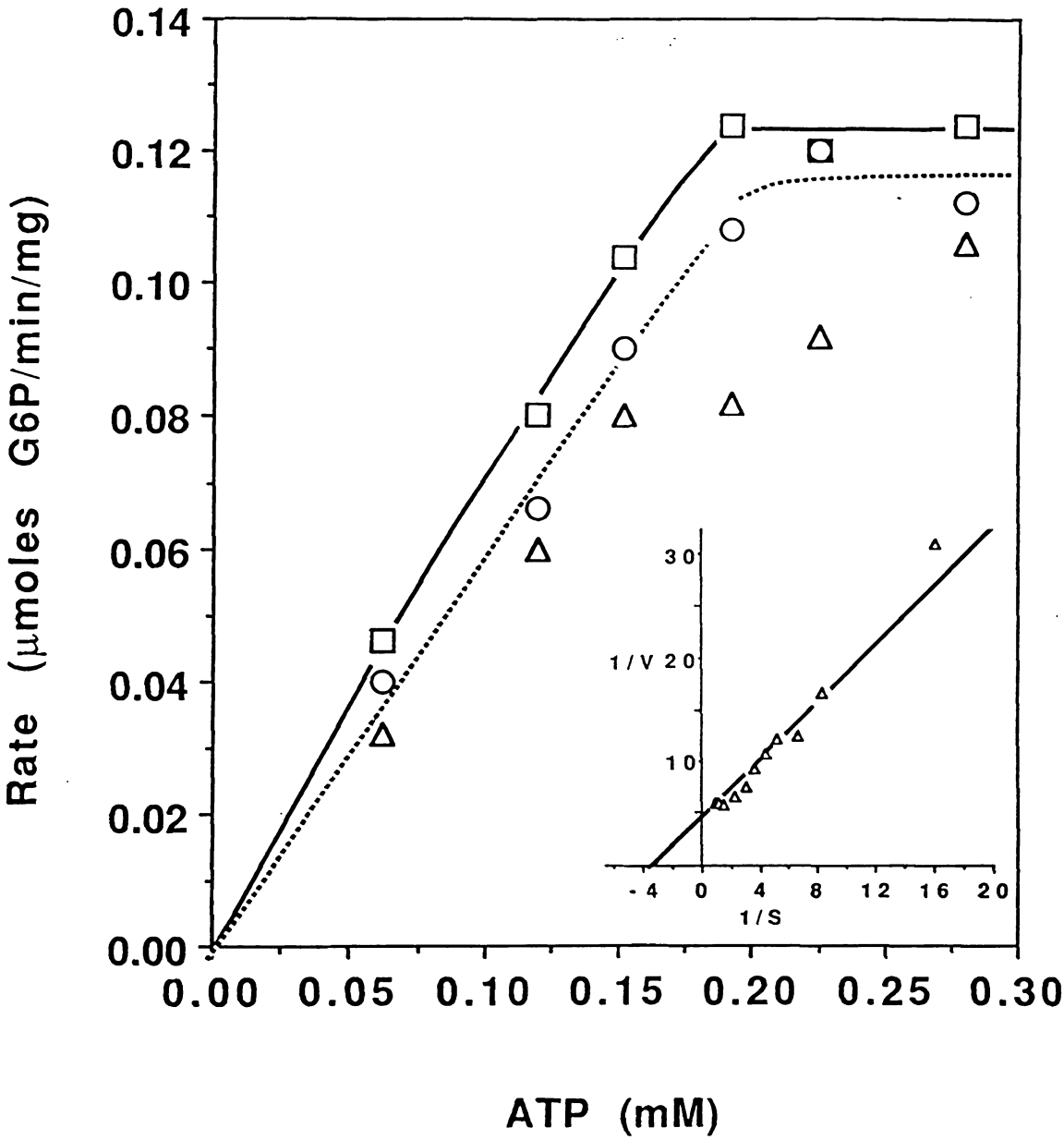
If extramitochondrial ATP represented a major source of substrate for hexokinase, then it would be expected that directly supplying mitochondrial hexokinase with exogenous ATP (by addition of ATP to the medium in the absence of production by intramitochondrial processes) would lead to rates of Glc

ure 7. ATP concentration as a function of time after initiation of
P production by oxidative phosphorylation. Total ATP present at
ous times was determined as described under Materials and
thods; this includes ATP present in the extramitochondrial
dium as well as that present in the intramitochondrial
npartments.


0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
[ATP] mM
0
2
4
6
8
10
12
14
16
Time (min)

phosphorylation indistinguishable from those with equivalent amounts of ATP generated by oxidative phosphorylation. Shown in Fig. 8 are the results of such an experiment. When ATP was added directly, with ADP required for oxidative phosphorylation deleted from the reaction medium, the observed rate of Glc phosphorylation increased in accord with Michaelis-Menten kinetic behavior, and the enzyme was found to have a K_m (for ATP) approximately 0.25 mM under the conditions of these experiments (inset, Fig. 8) In contrast, with total ATP concentrations of 0-0.2 mM *generated by oxidative phosphorylation*, the rates of Glc phosphorylation were substantially greater than those with equivalent amounts of exogenous ATP as substrate. This is consistent with the view that hexokinase is preferentially using a sequestered pool of ATP generated by this intramitochondrial process, in which the effective ATP concentration is considerably greater than would be the case were the ATP to be uniformly distributed in available solvent spaces. Further increases in levels of ATP produced by oxidative phosphorylation -to levels greater than approximately 0.2 mM- were not accompanied by corresponding increases in hexokinase activity, even though the ATP present was far from what would be saturating levels of exogenously added ATP. Moreover, the maximum rate of Glc phosphorylation supported by endogenously generated ATP was only about half of the V_{max} attained with exogenously added ATP. Halting further production of ATP by addition of KCN resulted in a modest decrease in hexokinase activity but the rates still exceeded those seen with equivalent amounts of exogenously supplied

Figure 8. Rate of Glc phosphorylation as a function of ATP concentration, with generation of ATP by oxidative phosphorylation. Oxidative phosphorylation was initiated by addition of 0.32 mM ADP. At various times thereafter, total ATP present was determined as described in the text. In parallel reactions, the rate of Glc phosphorylation immediately before (□) and after (o) addition of KCN was determined as a function of ATP present. Also shown is the rate of Glc phosphorylation (Δ) supported by the addition of exogenous ATP, and with oxidative phosphorylation precluded by omission of ADP from the reaction medium. The inset shows the latter values, along with additional rates determined with still higher concentrations of ATP, plotted in double reciprocal format; the K_m, determined by the EZ fit program (30), was 0.29 mM for this experiment.

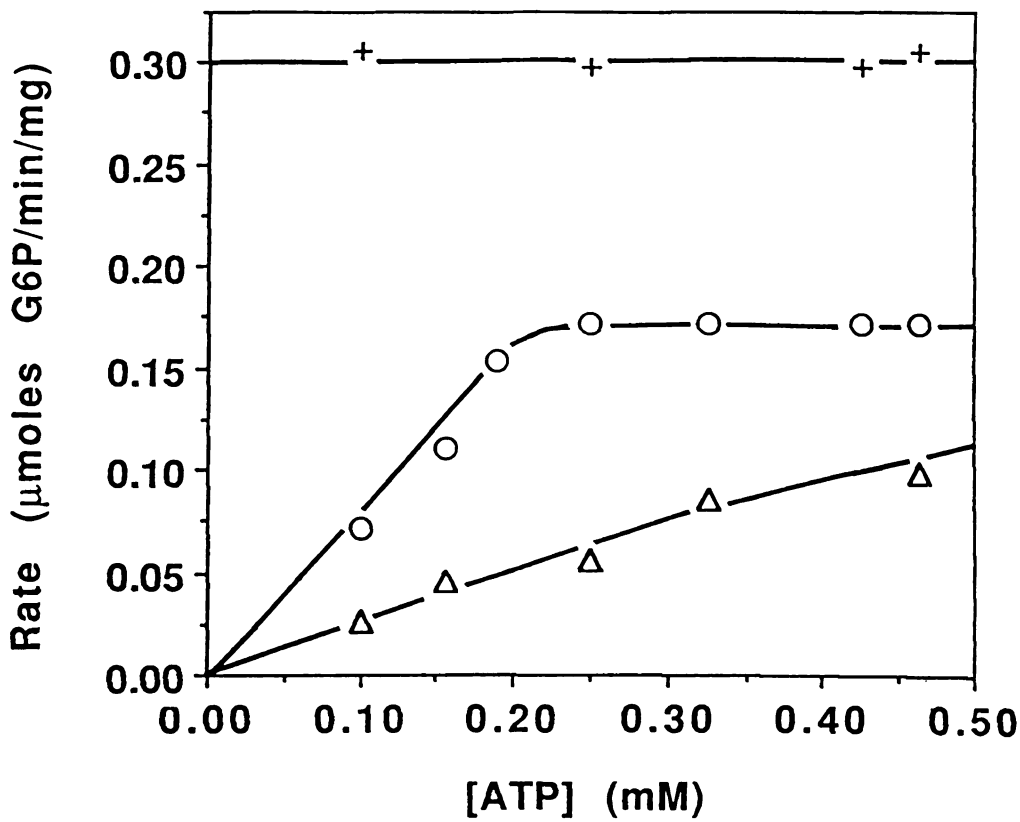

Rate (μmoles G6P/min/mg)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0.00 0.05 0.10 0.15 0.20 0.25 0.30
ATP (mM)
1/V
30
20
10
-4 0 4 8 12 16 20
1/S

substrate; this, together with the plateauing of this postinhibitor rate at higher ATP levels (longer reaction times), suggested continued reliance on an intramitochondrial ATP source during the period immediately after the addition of the inhibitor.

Although slow accumulation of ATP, generated by oxidative phosphorylation, to levels above approximately 0.2 mM did not result in increased rates of Glc phosphorylation, acute increase in ATP by supplementation with exogenous ATP did result in increased hexokinase activity (Fig. 9). The abscissa in Fig. 9 again indicates ATP levels generated by oxidative phosphorylation (in this case, initiated by addition of 0.54 mM ADP and with extension of the experimental period to allow accumulation of ATP levels still higher than those shown in Fig. 8) or added exogenously. The rate of Glc phosphorylation, using ATP generated by oxidative phosphorylation, was determined (open circles) as a function of total ATP present (recall that this is linearly related to the time after initiation of oxidative phosphorylation); as previously noted, this rate became maximal at approximately 0.2 mM ATP. In parallel reactions, endogenous ATP levels were supplemented by addition of ATP; such acute increases in total ATP levels were accompanied by immediate increases in rate of Glc phosphorylation, with maximal rates (i. e., at saturating ATP) being approximately twice the maximal rate attained with endogenously generated ATP (Fig. 9) consistent with results shown in Fig. 8.

Also shown in Fig. 9 are the rates attained in the absence of oxidative phosphorylation, using endogenous ATP as substrate. In this experiment, oxidative phosphorylation was prevented by

Figure 9. Response of mitochondrially bound hexokinase to slow increases in ATP generated by oxidative phosphorylation, and to acute increases in exogenously added ATP. This experiment is similar to that shown in Fig. 8. Oxidative phosphorylation was initiated by addition of 0.54 mM ADP, and total ATP present (shown on abscissa) at various times thereafter was determined as described under Materials and Methods. In parallel reactions, the rate of Glc phosphorylation was determined, both before (o) and after (+) total ATP were acutely raised to saturating level, 4.6 mM, by addition of an appropriate amount of ATP. Also shown are the rates of Glc phosphorylation (Δ) with exogenously added ATP, and with oxidative phosphorylation precluded by omission of pyruvate and malate from the reaction medium.

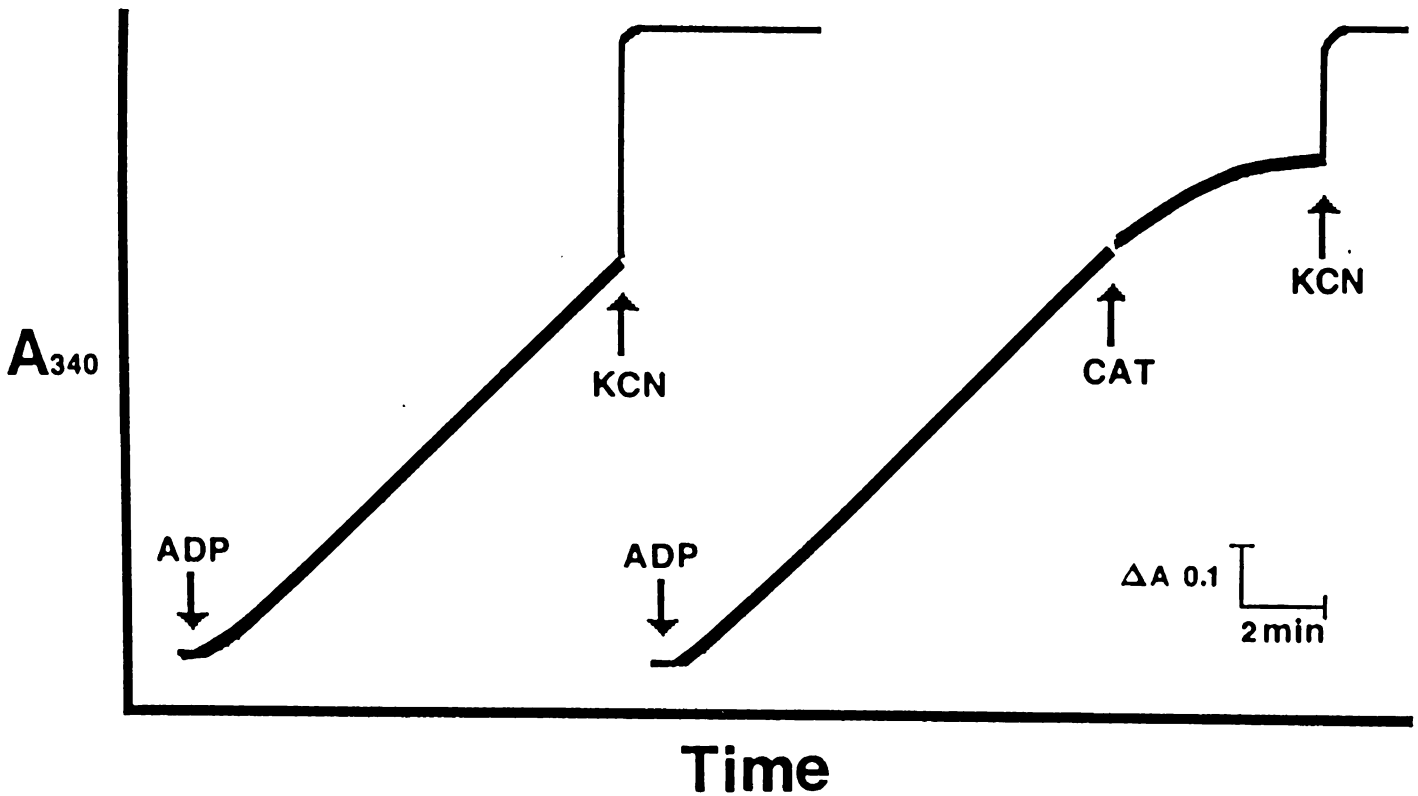

Rate (μmoles G6P/min/mg)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.00
0.10
0.20
0.30
0.40
0.50
[ATP] (mM)

deletion of the substrates, pyruvate and malate, rather than by deletion of ADP. Hence, ADP was present in the reaction medium as it was, of course, during oxidative phosphorylation. Since ADP is an inhibitor of brain hexokinase although a weak one with $K_i \geq 1$ mM (31,32), the rates seen with exogenous ATP were diminished from those seen in the absence of ADP (e. g., as in the experiment with results shown in Fig. 8), and the difference between the rates of Glc phosphorylation supported by exogenous and endogenously generated ATP is even more striking.

Intramitochondrial compartments of ATP. Intramitochondrial compartmentation of ATP was investigated in experiments in which excess yeast hexokinase was present throughout the reaction period. As noted above, this enzyme effectively scavenges extramitochondrial ATP. It thus follows that increased rates of Glc phosphorylation by excess yeast enzyme reflect the release of extramitochondrial ATP from intramitochondrial sources.

Figure 10 shows the results of experiments in which ATP was being generated by oxidative phosphorylation, with the inhibitors, 5 mM KCN or CAT (0.075 mg/mg mitochondrial protein), added during the steady state period. Either of these inhibitors is expected to effectively prevent further formation of ATP (confirmed by results note below), and both KCN (Fig. 2) and CAT (not shown) blocked mitochondrial respiration within seconds after their addition. Addition of KCN resulted in an immediate rapid burst of Glc phosphorylation (Fig. 10), indicating that this inhibitor induced a rapid release of intramitochondrial ATP.

Figure 10. Release of intramitochondrial compartments. ATP generation by oxidative phosphorylation was initiated by addition of ADP. At various times thereafter (approximately 10-12 min in the examples shown in this figure), inhibitors (CAT, KCN, oligomycin, or sodium azide) were added to block further formation of ATP. Release of ATP from intramitochondrial compartments was followed by coupling it to NADPH formation with excess yeast hexokinase and Glc-6-P dehydrogenase present in the reaction medium. No absorbance changes were detected in the absence of NADP or coupling enzymes, i.e., the observed changes were indeed due to NADPH formation, not turbidimetric changes resulting from possible effects of inhibitors on mitochondrial structure.


A340
ADP
KCN
ADP
CAT
KCN
ΔA 0.1
2 min
Time

In contrast, addition of CAT had no immediate effect on the rate of Glc phosphorylation, which continued briefly at the preinhibitor rate then gradually ceased. Addition of KCN after CAT again provoked an immediate burst of ATP release, with the total ATP released by KCN alone being equal to that released by CAT and KCN. These results indicate the existence of two distinct intramitochondrial compartments of ATP, both being disrupted virtually immediately upon addition of KCN. In contrast, addition of CAT results in slow depletion of the ATP in one of these compartments but not the other.

Other inhibitors examined were oligomycin (0.075 μg/ml) and sodium azide (1 mM); these gave results indistinguishable form those with CAT. The dissimilarity in results with cyanide and azide are perplexing since these inhibitors are generally considered similar in their effects on mitochondrial processed, with both inhibiting cytochrome oxidase (27); this obviously suggests that the effect of cyanide on release of intramitochondrial ATP is due to something other than inhibition of the cytochrome oxidase reaction.

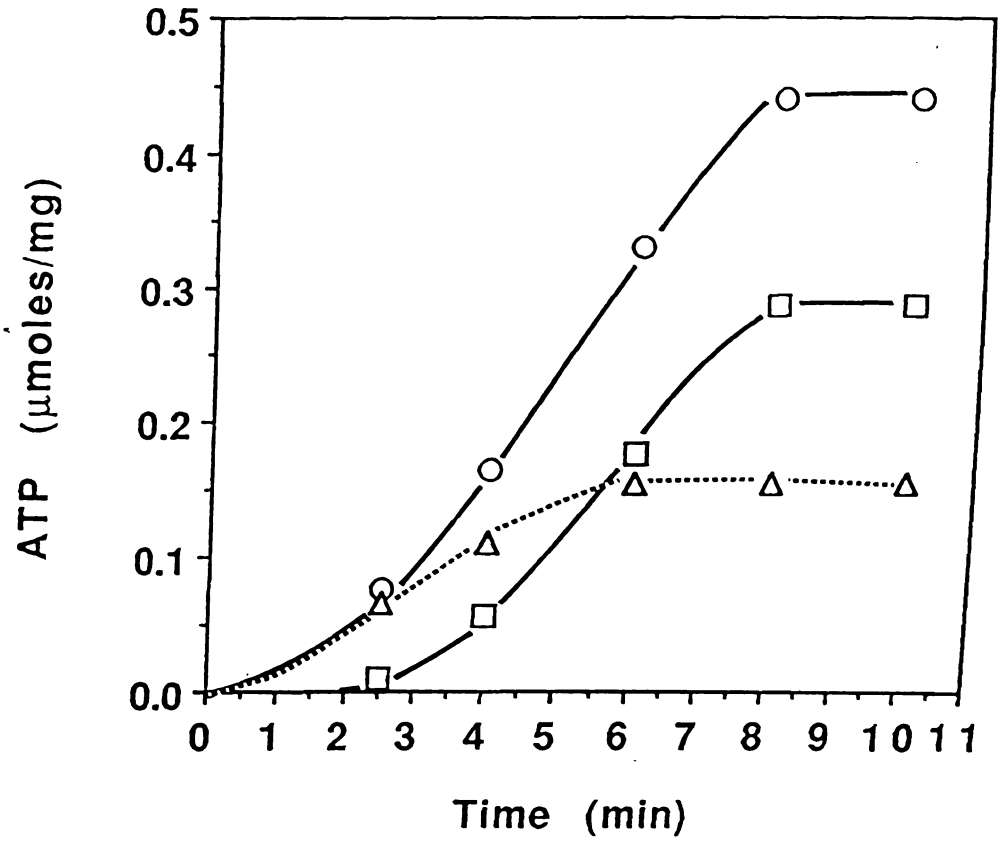
It should be noted that the total absence of detectable Glc phosphorylation after consumption of ATP corresponding to these intramitochondrial compartments (Fig. 10) confirms that ATP production has indeed been blocked. It is, however, puzzling that some detectable phosphorylation rate is not seen after CAT addition. Clearly there is residual ATP in an intramitochondrial cyanide-sensitive compartment. Either utilization by mitochondrially bound hexokinase or slow diffusion from the compartment and utilization by the exogenous yeast enzyme might have been expected. It would

appear that inhibition by CAT (or oligomycin or azide) effectively "locks" the ATP in this intramitochondrial compartment.

The time course for filling of the two compartments is shown in Fig. 11. This was determined in experiments analogous to those shown in Fig. 10, adding KCN or CAT followed by KCN, at various times after initiation of oxidative phosphorylation (with excess yeast hexokinase present throughout). The CAT-sensitive compartment fills initially, followed by filling of the second compartment, with ATP in the solely cyanide-sensitive compartment being 2-3 times that sequestered in the CAT-sensitive compartments. The amount of ATP in both CAT and KCN-sensitive, determined when steady state levels had been attained, exhibited the expected linear dependence on the amount of mitochondria present in the reaction mixture (results not shown).

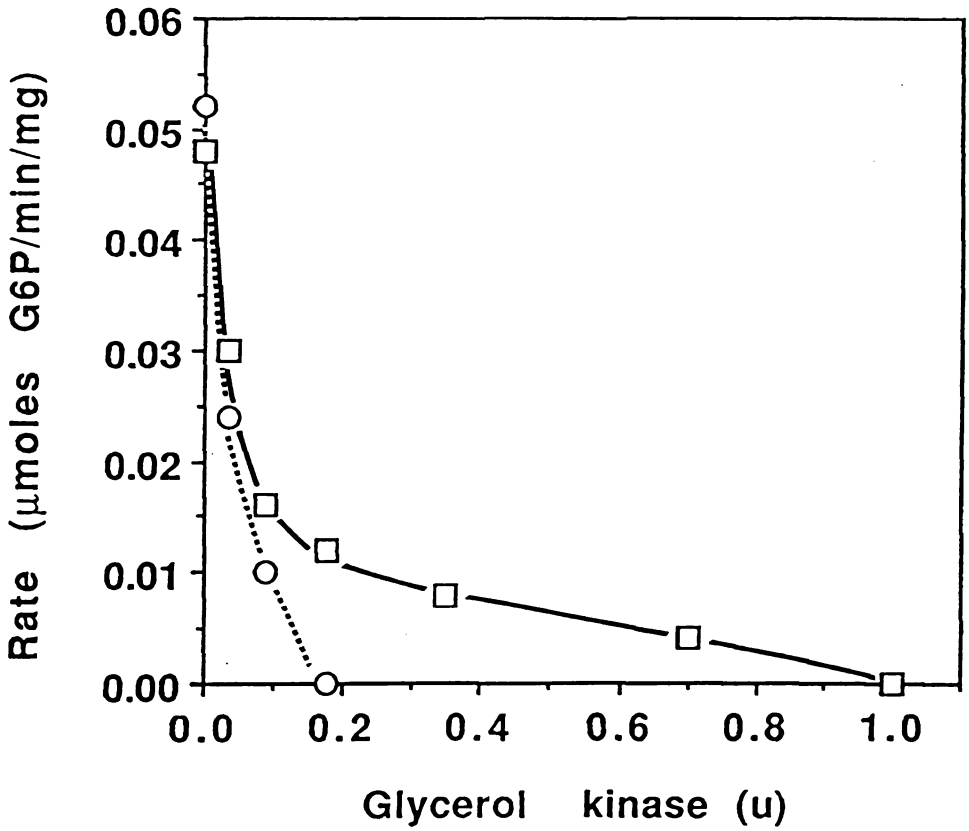
Competition between mitochondrially bound hexokinase and glycerol kinase for extramitochondrial or intramitochondrially generated ATP. The proposition that mitochondrially bound hexokinase has privileged access to intramitochondrial compartments of ATP leads to the expectation that hexokinase should compete effectively for this substrate when challenged by extramitochondrial kinases, to which intramitochondrial ATP should be inaccessible. We have examined this situation, using yeast glycerol kinase as competitor. Although mammalian glycerol kinase has been reported to bind to mitochondria (33, 34), we have not detected any binding of the yeast enzyme to isolated brain mitochondria.

ure 11. Time course for filling of intramitochondrial
npartments of ATP. The basic protocol of this experiment is
cribed in the legend to Fig. 10. Absorbance increases seen after
lition of KCN (o), or CAT (Δ) followed by a subsequent addition of
N (□) are shown as a function of time after initiation of ATP
mation by oxidative phosphorylation.


0.5
0.4
0.3
0.2
0.1
0.0
ATP (µmoles/mg)
0 1 2 3 4 5 6 7 8 9 10 11
Time (min)

Mitochondrial ATP production by oxidative phosphorylation was initiated by addition of ADP, as described under Materials and Methods except that 5 mM glycerol and increasing amounts of yeast glycerol kinase were included in the reaction mixtures. Steady state rates of Glc phosphorylation by mitochondrially bound hexokinase were determined. Parallel experiments were performed in which Glc phosphorylation was dependent on exogenous ATP, with the concentration of ATP adjusted so that the observed rate of Glc phosphorylation in the absence of glycerol kinase approximated that in the oxidative phosphorylation-dependent system. Although it was evident that glycerol kinase did effectively compete for the ATP being utilized by the mitochondrially bound hexokinase, much higher levels of glycerol kinase were required to completely suppress Glc phosphorylation with intramitochondrially generated ATP than was the case with the exogenously supplied substrate (Fig 12). Moreover, with exogenous ATP, the inhibition with glycerol kinase showed an essentially linear dependence on added enzyme. In contrast, competition for intramitochondrially generated ATP had a distinct biphasic character, suggesting that the mitochondrially bound hexokinase had access to two compartments of ATP, with glycerol kinase competing very effectively for one of these but less effectively for the other.

Figure 12. Inhibition of hexokinase activity by competition with yeast glycerol kinase for substrate ATP. Steady state rates of Glc phosphorylation, supported by oxidative phosphorylation, were determined in the presence of increasing amounts of exogenous glycerol kinase (□). Similar measurements were made in the absence of oxidative phosphorylation, using exogenously supplied ATP as substrate (○). The amount of ATP added was such that, *in the absence of glycerol kinase,* virtually identical rates were obtained with either exogenously added or endogenously generated ATP. This varied slightly depending upon the activity of the particular mitochondrial preparation; for the experiment shown, 0.12 mM was used.


0.06
0.05
0.04
0.03
0.02
0.01
0.00
Rate (µmoles G6P/min/mg)
0.0
0.2
0.4
0.6
0.8
1.0
Glycerol kinase (u)

Discussion

During active oxidative phosphorylation and at the ATP levels greater than approximately 0.2 mM, mitochondrially bound hexokinase is unresponsive to gradual increases in ATP, even though it does respond to acute increases in ATP produced by addition of exogenous substrate. The mechanism by which this remarkable behavior might be produced, remains quite unclear, though it must surely be related to coupling of hexokinase to intramitochondrial compartments of ATP.

The primary dependence of mitochondrially bound hexokinase on intramitochondrial ATP as a substrate, and lack of responsiveness to gradual changes in extramitochondrial ATP levels, could have considerable physiological significance. Cytoplasmic concentrations of ATP are estimated to be in the range of 2 mM (35, 36), and change relatively slowly even during such extreme states as convulsion (36). The present study suggests that, under these conditions, hexokinase activity would be insensitive to modest changes in cytoplasmic ATP levels, but would remain closely linked to intramitochondrial ATP sources expected to reflect mitochondrial energy status. It is probable that this is an important factor in the overall regulation of cerebral energy metabolism which is, under normal conditions, critically dependent on maintaining appropriate balance between mitochondrial oxidative activity and introduction of Glc into glycolysis via the hexokinase reaction.

Compared with oxidative phosphorylation, adenylate kinase is not an effective source of ATP for mitochondrially bound hexokinase of brain. In this respect, brain mitochondria and their bound hexokinase differ markedly from mitochondria derived form rapidly proliferating, highly glycolytic undifferentiated tumors (18, 22, 23), in which adenylate kinase can provide a substantial fraction- perhaps 50 %- of the ATP utilized by bound hexokinase. However, hexokinase bound to mitochondria from a differentiated tumor cell line (23) is similar to that of brain mitochondria in exhibiting a major dependence on ATP generated by oxidative phosphorylation. It thus seems that effective coupling between mitochondrial oxidative phosphorylation and Glc phosphorylation may be characteristic of differentiated tissues, with loss of this linkage between oxidative and glycolytic metabolism accounting for excessive glycolysis characteristic of undifferentiated tumors (8).

Two distinct intramitochondrial compartments of ATP were detected in the work described here. Although the present study provides no firm basis for associating these compartments with any specific intramitochondrial space, it seems reasonable to consider whether they might correspond to the compartments envisaged by Brdiczka (37). One of these is thought to be specifically associated with "contact sites" (points at which inner and outer mitochondrial membranes are closely apposed). There is evidence that hexokinase is preferentially bound at contact sites in mitochondria from brain (38, 39) and other tissues (40), and it is not difficult to imagine that it might have some special access to ATP sequestered in this region. It is further postulated (37) that this compartment is

preferentially supplied with ATP from oxidative phosphorylation, which would be consistent with the effective use of ATP from this source by hexokinase bound at the contact sites. Presumably the ADP formed by the hexokinase bound in these regions might also be effectively recycled back to the oxidative phosphorylation apparatus, as suggested by the present results and previous work (19).

The other compartment of ATP envisaged by Brdiczka (37) lies in the intermembranal space located between contact sites. This space is also thought to contain the adenylate kinase, which could therefore generate ATP in this second compartment (though an additional contribution could come from oxidative phosphorylation, with ATP entering this compartment through the ATP/ADP translocase located in inner membrane regions not involved in contact sites). Since hexokinase is suggested to bind preferentially (but not exclusively) to contact sites, while ATP in this second compartment would presumably exit through pores located in noncontact regions, the relative ineffectiveness of adenylate kinase in providing substrate ATP might be expected.

Parry and Pedersen (41) have recently suggested that hexokinase is not associated with brain mitochondria *per se,* but rather with a "microsomal" contaminant in the mitochondrial preparations. While the results presented here do not directly address the mode of binding of hexokinase, they surely demonstrate that binding results in intimate interaction between hexokinase and intramitochondrial processes. It seems difficult to reconcile this

with binding of hexokinase to a nonmitochondrial "contaminant" in the mitochondrial preparation.

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Chapter III

Coordinated Regulation of Cerebral Glycolytic and Oxidative Metabolism, Mediated by Mitochondrially Bound Hexokinase Dependent on Intramitochondrially Generated ATP

Abstract

Hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) of rat brain mitochondria is associated with membrane regions thought to correspond to contact sites (regions of close interaction of the inner and outer mitochondrial membranes). Two intramitochondrial compartments of ATP also appear to be located at contact sites, and are dependent on oxidative phosphorylation for their generation. *Neither* of these compartments was associated with the intermembranal space containing adenylate kinase, nor was there detectable intramitochondrial compartmentation of ATP generated by the adenylate kinase reaction. Formation of these compartments was not dependent on the presence of bound hexokinase since equivalent amounts of compartmented ATP were found in mitochondria from which a major portion of the hexokinase had been removed by treatment with Glc-6-P. During active oxidative phosphorylation, mitochondrially bound hexokinase is totally dependent upon intramitochondrially compartmented ATP as a substrate. Both the levels of ATP in the intramitochondrial compartments *and* the rate of glucose phosphorylation by mitochondrially bound hexokinase were shown to be correlated with the rate of oxidative phosphorylation. This dependence of hexokinase on intramitochondrial ATP levels that reflect the status of mitochondrial oxidative metabolism provides a

mechanism by which hexokinase can serve as a mediator, coordinating the rate at which glucose is introduced into the glycolytic pathway with terminal oxidative stages of metabolism and avoiding the accumulation of lactate which has been associated with toxic effects on the brain.

Introduction

Under normal circumstances, glucose represents virtually the sole substrate for a rather intense energy metabolism required to support cerebral function (1). Glucose is metabolized almost exclusively *via* the aerobic glycolytic pathway, and hence interactions between cytoplasmic glycolytic metabolism and mitochondrial oxidative phosphorylation are important in ensuring coordinated metabolic function. Hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) appears to be an important link between these two processes (2).

Hexokinase catalyzes the phosphorylation of Glc, the initial step in glycolysis, and regulation of hexokinase activity is a major factor governing the cerebral glycolytic rate (3,4). Hexokinase binds specifically to the outer mitochondrial membrane, apparently through interactions with porin, the protein that forms the pores through which metabolites - including ADP and ATP - enter and exit the mitochondrion (5-8). This physical association provides a topological basis for intimate interaction between the ATP-requiring, ADP-generating hexokinase reaction and intramitochondrial ADP-requiring, ATP-generating processes. Several investigators (9-18) have reported that mitochondrially bound hexokinase has "privileged access" to intramitochondrially generated ATP, using it in preference to ATP furnished from the extramitochondrial environment, with ADP generated in the hexokinase reaction

being effectively redirected back to intramitochondrial phosphorylation processes.

In a recent study (18), we observed that, during active oxidative phosphorylation, the hexokinase of rat brain mitochondria was totally dependent on intramitochondrial ATP as a substrate, and unresponsive to gradual changes in extramitochondrial ATP concentrations even though the latter were well below saturating levels (i.e., $\leq K_m$ for ATP). Since intramitochondrial ATP concentrations can be expected to reflect the status of mitochondrial oxidative metabolism, *this dependency of mitochondrially bound hexokinase on intramitochondrial ATP offers a mechanism by which coordination could be maintained between initiation of glycolytic metabolism and the terminal stages of aerobic glycolysis*, i.e., the rate at which Glc was introduced into the pathway would be coordinated with the status of mitochondrial oxidative metabolism. This could be critical in avoiding glycolytic metabolism in excess of oxidative capacity, with resulting increase in cerebral lactate which has been associated with toxic effects (19)[1].

In the previous study (18), two compartments of intramitochondrial ATP, present during active oxidative phosphorylation, could be distinguished based on their selective release after addition of inhibitors. Addition of KCN, an inhibitor of electron transfer (20), caused an immediate and complete release of the ATP in these intramitochondrial compartments. In contrast, addition of carboxyatractyloside (CAT), an inhibitor of the ATP/ADP translocase (21), caused release of only about 30% of that ATP; this

was referred to as the "CAT-sensitive compartment" (18). Subsequent addition of KCN resulted in release of the remaining ATP from what was, for convenience, referred to as the "KCN-sensitive" compartment (even though, as noted, *both* compartments were sensitive to release by KCN).

The location of these compartments within the mitochondrial structure could not be determined from the previous results (18). However, it was suggested that these might correspond to the compartments envisaged by Brdiczka (22), one being associated with "contact sites" (23) - regions in which the inner and outer mitochondrial membranes are closely apposed, perhaps even "semi-fused" (24), and to which hexokinase has been reported to bind preferentially (22,25,26) - while the other was associated with the intermembranal space between contact sites, a compartment marked by the presence of adenylate kinase activity (21,27). In the present study, we directed specific attention to the question of the intramitochondrial location of these compartments, and have further examined their role in coupling mitochondrially bound hexokinase activity to intramitochondrial ATP formation *via* either oxidative phosphorylation or the adenylate kinase reaction.

Materials and Methods

Materials. Glc-6-P dehydrogenase was purchased from Boehringer Mannheim (Indianapolis, IN), and digitonin was a product of Merck (Darmstadt, Germany). Bongkrekic acid (BKA) was a gift of Dr. Klaas Nicolay, University of Utrecht. Ficoll 400, homovanillic acid, P_1,P_5-di(adenosine-5')pentaphosphate (A_2P_5), rotenone, tyramine, and other biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO). The BCA Protein Assay Reagent and the BSA standard were from Pierce Chemical Co. (Rockford, IL). All other chemicals were of reagent grade and obtained from various commercial sources.

Assays. Adenylate kinase (28), fumarase (29), hexokinase (30), monoamine oxidase (31), and rotenone insensitive NADPH- and NADH-cytochrome c reductases (32) were assayed as previously described.

Brain mitochondria. Brains were obtained from adult (150-250 g) Sprague-Dawley rats of either sex. Mitochondria were isolated as in the previous study (18). Mitochondria isolated from four brains were suspended in 1.0 ml of "isolation medium" (33), which contained 0.25 M sucrose, 0.1 mM EGTA, and 5 mM Mops, adjusted to pH 7.4 with KOH; the mitochondrial suspensions contained 8.5 ±1.0 mg protein and 3.1 ± 0.3 units of hexokinase activity per ml (mean ± SD for 20 preparations).

Digitonin treatment of brain mitochondria. The digitonin stock solution (5 mg/ml) was made in "incubation medium" (33), prepared by diluting 30 ml 0.5 M KCl, 10 ml 1 mM EGTA, 10 ml 0.1 M Tris-Cl, pH 7.4 , and 5 ml 0.1 M Tris-phosphate, pH 7.2, to a total volume of 100 ml, with final pH adjusted to 7.4 with KOH. The fractionation was done in the same medium. Unless indicated otherwise, 0.1 ml of mitochondrial suspension was added to 0.4 ml of incubation medium containing the indicated amount of digitonin, incubated for two minutes at room temperature, and then centrifuged for 2 minutes in a microcentrifuge. Both supernatants and resuspended pellets were assayed for marker enzymes; recovery of total activity was always greater than 90%. Pellets were resuspended in 0.5 ml of incubation medium and made 0.5% (v/v) in Triton X-100, except when assaying the rotenone insensitive NADH-cytochrome c reductase, which was inhibited by detergent treatment.

Measurement of ATP production by mitochondria, ATP utilization by mitochondrially bound hexokinase, and release of ATP from intramitochondrial compartments. The procedures used were as described previously (18). Briefly, reactions were carried out in incubation medium supplemented with 5 mM glucose, 5 mM $MgCl_2$, 5 mM pyruvate, 2.5 mM malate, 0.63 mM NADP, and 1 unit of glucose-6-phosphate dehydrogenase in a final volume of 1 ml. The reaction also contained mitochondrial suspension (approx. 0.08 mg mitochondrial protein) with 0.30 mM ADP, unless indicated otherwise, added to initiate ATP formation. Selective formation of ATP *via* either the adenylate kinase reaction or oxidative phosphorylation was obtained

by inclusion of either 5 mM KCN or 0.1 mM A_2P_5, respectively, in the reaction; KCN is an inhibitor of electron transport (20) and A_2P_5 is an inhibitor of adenylate kinase (34). These concentrations were shown to completely block ATP formation by the susceptible path. Alternatively, when inclusion of KCN was undesirable (see below), selective formation of ATP *via* the adenylate kinase reaction was attained by deletion of phosphate from the incubation medium, with corresponding increase in the Tris-Cl concentration.

As in the previous study (18), rates of ATP production and utilization, and release of ATP from intramitochondrial compartments, were monitored spectrophotometrically (at 340 nm) by coupling the hexokinase and Glc-6-P dehydrogenase reactions. To facilitate understanding of how the several parameters of interest were determined, representative reaction progress curves are shown in Fig. 1. The *total* rate of ATP production (Fig. 1, left) was measured in the presence of excess (3 units) yeast hexokinase; under these conditions, there is negligible accumulation of extramitochondrial ATP (18) and hence the rate of NADPH formation is equivalent to the rate of ATP production, which is calculated from the region labeled "A" in Fig. 1. The rate of ATP *utilization* by mitochondrially bound hexokinase was measured in the same way but in the absence of yeast hexokinase (Fig. 1, right); addition of ADP initiates an extended transient phase, during which intramitochondrial compartments of ATP are filled (18), followed by a steady state rate of ATP utilization by the mitochondrially bound hexokinase (region labeled "B").

Figure 1. Measurement of total rate of ATP production, release of ATP from intramitochondrial compartments, and rate of ATP utilization by mitochondrially bound hexokinase. These are representative tracings which illustrate how various parameters of interest in these experiments were determined. See text for specific comments.

Release of ATP from the intramitochondrial compartments was also monitored in the presence of excess yeast hexokinase, assuring effective scavenging of the ATP as it became available in the extramitochondrial space. This is discussed further below, but it is appropriate in the present context to illustrate the nature of the results observed (Fig. 1, left and center). Addition of agents such as KCN, BKA, or CAT at various times after initiation of ATP formation resulted in a burst of NADPH formation, corresponding to release of ATP from intramitochondrial compartments, followed by cessation of further increase in A_{340} due to the inhibition of additional ATP production; the amount of ATP in the intramitochondrial compartment was calculated from the increase in A_{340} that occurred subsequent to addition of the releasing agent (A values indicated in Fig. 1). That these agents *did* completely inhibit further oxidative phosphorylation was confirmed by the immediate cessation of oxygen uptake, determined polarographically, after their addition (results not shown, but see Fig. 2 in ref. 18).

Elution of mitochondrially bound hexokinase by treatment with Glc-6-P. The product of the hexokinase reaction, Glc-6-P, induces release of the mitochondrially bound enzyme (2). The mitochondrial suspension was diluted 1:5 in isolation medium. The suspension was made 1 mM in Glc-6-P, incubated for 5 min at room temperature, then centrifuged for 2 minutes in a microcentrifuge. The supernatant was carefully removed and the mitochondrial pellet, now extensively

depleted of bound hexokinase, was resuspended in isolation medium to restore the original mitochondrial protein concentration. Hexokinase activity was assayed in both supernatant and pellet.

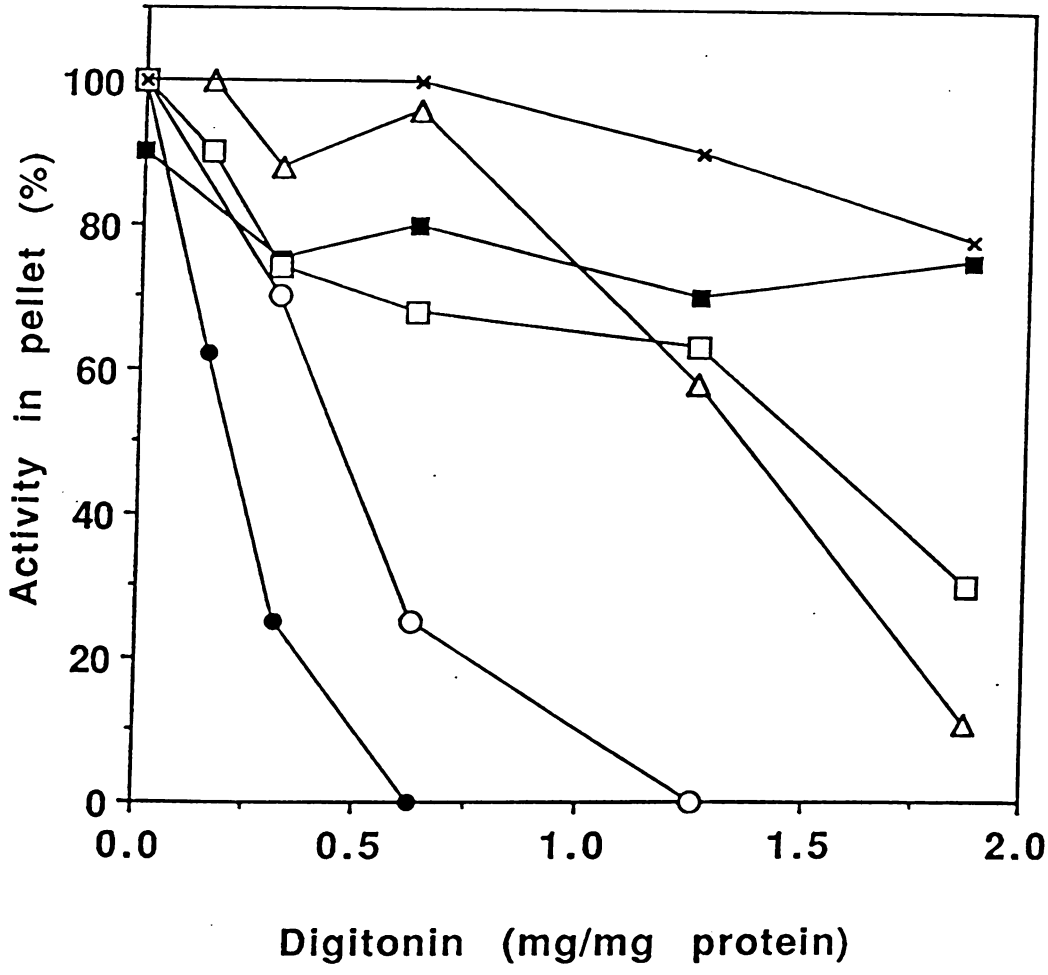
Rebinding of hexokinase to mitochondria. Hexokinase solubilized with Glc-6-P was rebound to mitochondria that had previously been depleted of bound hexokinase by treatment with Glc-6-P. The solubilized enzyme and depleted mitochondria were incubated for 15 minutes at room temperature in the presence of 5 mM $MgCl_2$, followed by centrifugation for 2 minutes in the microcentrifuge. The pellet was resuspended in fresh isolation medium to restore the original mitochondrial protein concentration.

Results

Digitonin fractionation. Digitonin treatment has proven to be a useful method for selective disruption of the outer mitochondrial membrane, and has been applied in fractionation studies using mitochondria from several different tissues (26,27,35-39), including rat brain (26,39). Since the fractionation pattern observed depends somewhat on the exact experimental conditions (26,38,39), results for a typical experiment under our conditions are shown in Fig. 2. The overall pattern is similar to that seen in previous studies, with rupture of the outer mitochondrial membrane - as indicated by release of the intermembranal space marker, adenylate kinase - occurring at digitonin concentrations well below those required to cause extensive dissociation of the membrane, reflected by release of monoamine oxidase. The inner mitochondrial membrane is relatively resistant to digitonin, with disruption evidenced by release of the matrix enzyme, fumarase, seen only at rather high concentrations of digitonin.

As found by Parry and Pedersen (39), release of monoamine oxidase and rotenone insensitive NADH-cytochrome c reductase, which are associated with the outer mitochondrial membrane (27), occurred with similar but not identical dependency on concentration of digitonin. Compared to the latter two enzymes, NADPH-cytochrome c reductase was considerably *more* susceptible

Figure 2. Digitonin fractionation of rat brain mitochondria. Digitonin treatment was performed as described in Methods, and activities of adenylate kinase (●), rotenone insensitive NADPH-cytochrome c reductase (O), rotenone insensitive NADH-cytochrome c reductase (□), monoamine oxidase (Δ), hexokinase (■), and fumarase (x) were determined in both supernatant and pellet. Results are presented as percentage of the total activity that remained in particulate form after treatment of the mitochondria with the indicated concentration of digitonin.


Activity in pellet (%)
100
80
60
40
20
0
0.0
0.5
1.0
1.5
2.0
Digitonin (mg/mg protein)

to release by digitonin, in contrast to the results found by Parry and Pedersen (39). The latter authors took NADPH-cytochrome c reductase to be a marker for microsomal contaminants in the brain mitochondrial preparation; however, there is evidence to indicate that significant NADPH-cytochrome c reductase activity is intrinsic to the outer mitochondrial membrane (40,41). Clearly the lack of uniformity in the release of these enzymes suggests their location in outer mitochondrial membrane domains differing in susceptibility to disruption by digitonin.

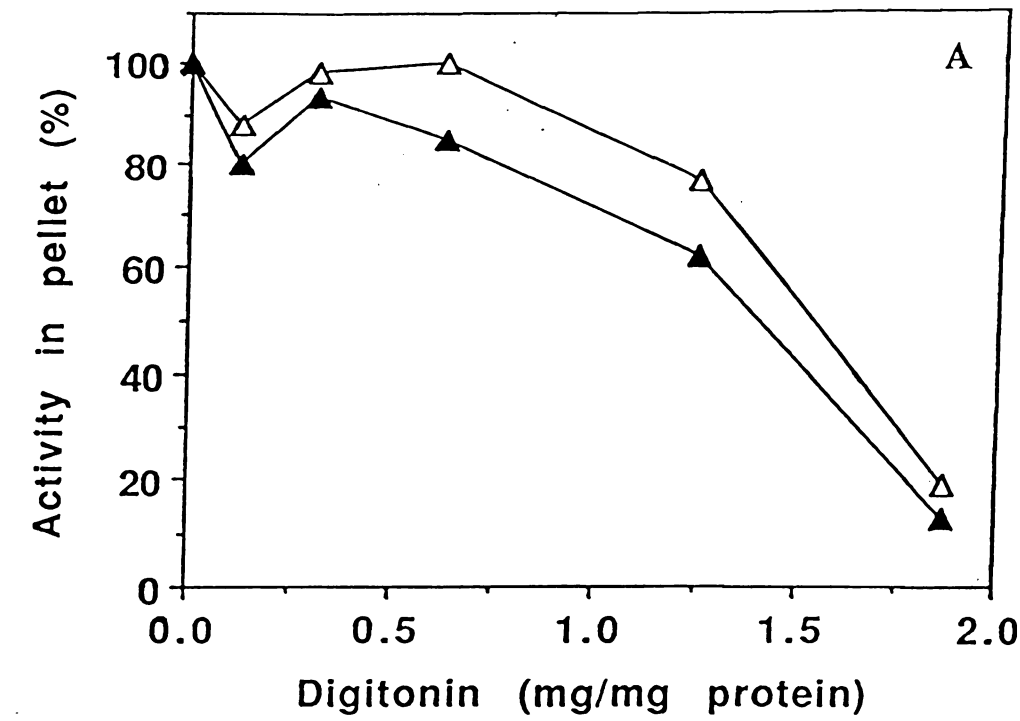
A high percentage of the hexokinase (about 70-80%), remained particulate even at digitonin concentrations of 2 mg/mg mitochondrial protein. This resistance of brain mitochondrial hexokinase to digitonin was previously observed by other workers (26,39). Since the membrane-disrupting action of digitonin is attributed to complexation of this agent with membrane cholesterol (42), the resistance of mitochondrial hexokinase from brain to solubilization with digitonin has been attributed (26) to association of this enzyme with cholesterol-deficient domains within the outer mitochondrial membrane. The latter are thought to be identified with contact sites (22,25,26,43).

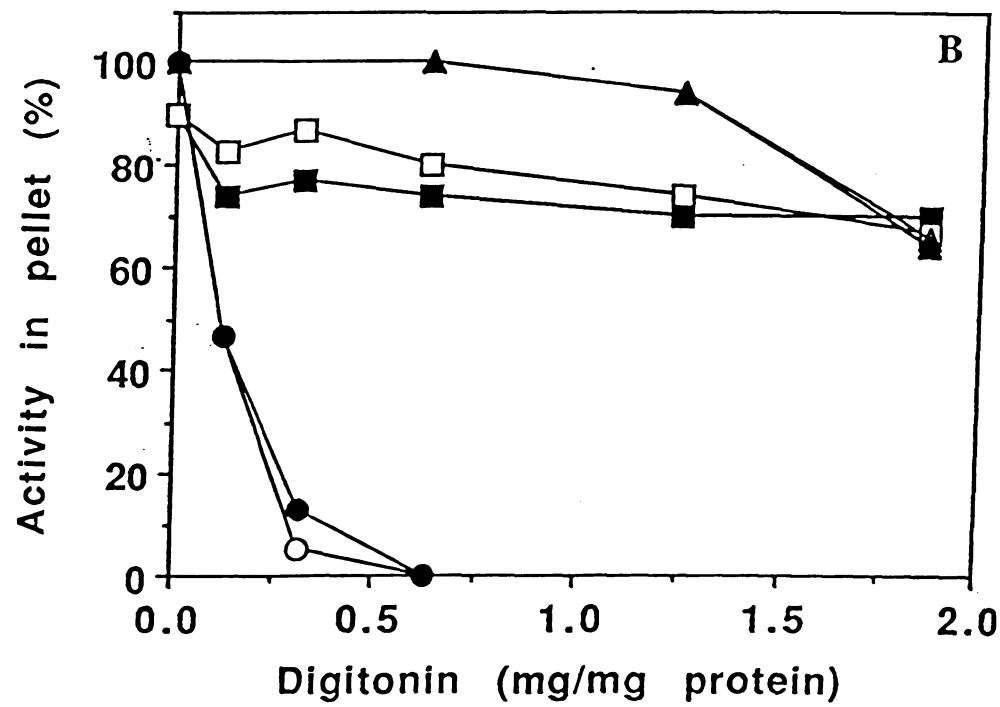
It has been proposed that contact sites are dynamic structures, varying in number with metabolic status of the mitochondrion, with increased numbers of contact sites during periods of active oxidative phosphorylation (25,43). Kottke *et al.* (25) reported a marginal increase in the resistance of mitochondrially bound hexokinase to solubilization by digitonin when the treatment was performed during active oxidative

phosphorylation, consistent with their view that contact sites were increased under these conditions.

We have not been able to confirm these results. Fig. 3 shows representative results obtained when digitonin treatment was performed under phosphorylating and nonphosphorylating conditions. In both cases, fractionation was done as described in Methods with the following exceptions: 1) in the phosphorylating group, the mitochondria were suspended in incubation medium supplemented with 5 mM pyruvate, 2.5 mM malate, and 1.2 mM ADP; polarographic measurement of respiration in duplicate samples confirmed that active oxidative phosphorylation was maintained throughout the two minute period of treatment with digitonin; 2) in the nonphosphorylating group, the Tris-phosphate in the incubation medium was replaced with a compensatory increase in Tris-Cl, i.e., oxidative phosphorylation was precluded by the absence of P_i; polarographic measurements with duplicate samples confirmed the absence of significant oxygen uptake throughout the period of treatment with digitonin. Although a slight increase in resistance of monoamine oxidase to solubilization by digitonin did appear to be associated with active oxidative phosphorylation (Fig. 3 A), no reproducible difference was seen in the effect of digitonin on release of adenylate kinase, hexokinase, or fumarase from phosphorylating and nonphosphorylating mitochondria (Fig. 3 B). The fractionation pattern for these enzymes was also unaffected when digitonin treatment was performed with phosphate present in the medium but with electron transport inhibited by KCN, or with the

Figure 3. Digitonin fractionation of rat brain mitochondria under phosphorylating and nonphosphorylating conditions. Mitochondria were treated with the indicated concentrations of digitonin under conditions that permitted (open symbols) or did not permit (closed symbols) oxidative phosphorylation. As in Fig. 2, results are expressed as percentage of total activity that remained in particulate form after digitonin treatment. Panel A, monoamine oxidase. Panel B, adenylate kinase (○,●), hexokinase (□,■), and fumarase (△,▲).


A
Activity in pellet (%)
100
80
60
40
20
0
0.0
0.5
1.0
1.5
2.0
Digitonin (mg/mg protein)


B
Activity in pellet (%)
100
80
60
40
20
0
0.0
0.5
1.0
1.5
2.0
Digitonin (mg/mg protein)

uncoupler, 2,4-dinitrophenol, present (results not shown). Thus, these hexokinase-rich digitonin resistant regions, presumably contact sites, are relatively stable structures, and not rapidly responsive to altered metabolic status of the mitochondrion. Neupert and his coworkers (44,45) previously reached this same conclusion based on their study of contact sites in mitochondria from *Neurospora*.

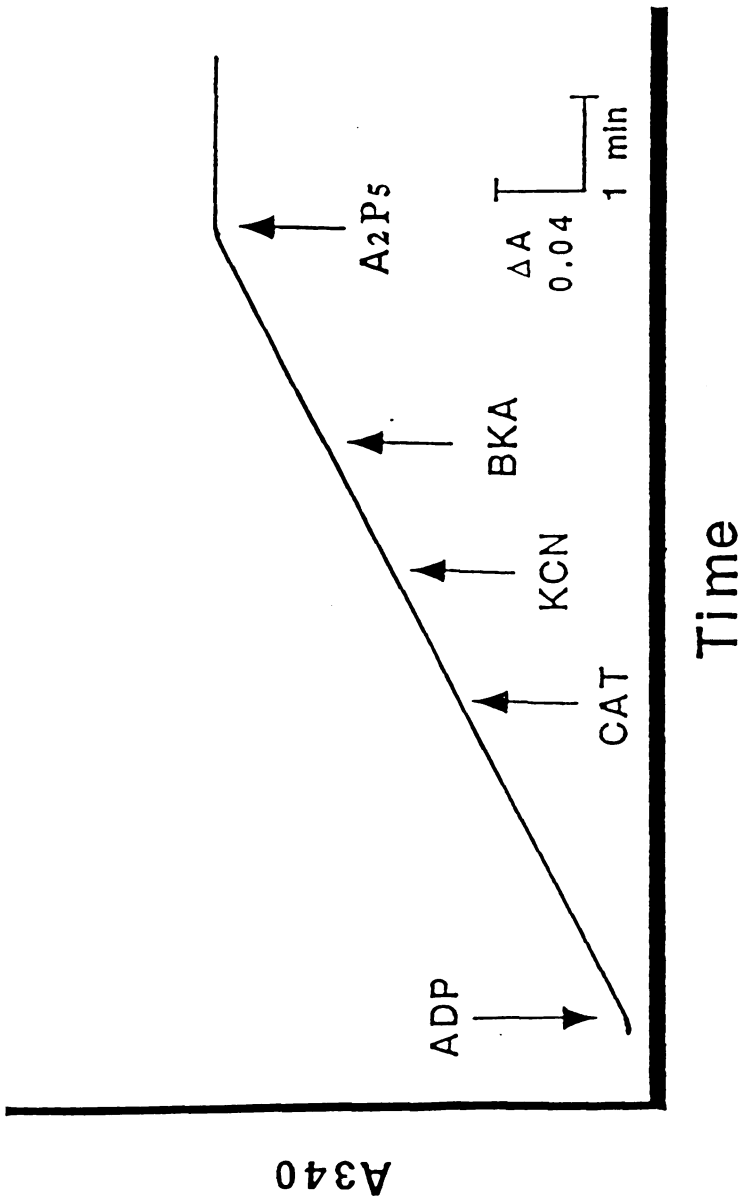
Release of ATP from intramitochondrial compartments with either oxidative phosphorylation or adenylate kinase as the source of ATP. As previously reported (18), two intramitochondrial compartments of ATP, distinguishable based on their selective release with either CAT or KCN (see Fig. 1), were generated during active oxidative phosphorylation. In the present study, we found that the ATP-ADP translocase inhibitor, bongkrekic acid (BKA), was indistinguishable from KCN in causing release of intramitochondrial ATP (Fig. 1, left).

We were interested in whether the presence or absence of mitochondrially bound hexokinase would have any effect on the intramitochondrial compartmentation of ATP. It was conceivable that the enzyme might, in some way, be physically involved in formation of a structure that permitted sequestration of intramitochondrially generated ATP, perhaps forming a compartment to which the enzyme had "privileged access". This was not the case. Treatment of mitochondria with Glc-6-P to reduce mitochondrially bound hexokinase levels had *no* affect on the total rate of ATP production, on the amount of ATP released from the compartments,

or on the pattern in which this release occurred after addition of KCN, BKA, or CAT. For example, in one experiment, the total rate of ATP production with mitochondria containing the normal endogenous level of hexokinase (typically approx. 0.4 units/ mg mitochondrial protein - see Methods) was determined to be 0.22 μmoles ATP/min/mg mitochondrial protein. Addition of CAT evoked release of 0.16 μmoles ATP/ mg mitochondrial protein from the CAT-sensitive compartment, while total compartmented ATP released by KCN was 0.52 μmoles ATP/mg mitochondrial protein. These *same* values were determined using mitochondria that had been treated with Glc-6-P, reducing hexokinase content to 10% of the original endogenous level.

ATP production by adenylate kinase did *not* result in detectable sequestration of ATP in CAT-, KCN-, or BKA-sensitive intramitochondrial compartments (Fig. 4). In this experiment, oxidative phosphorylation was precluded by deletion of inorganic phosphate from the incubation medium. As is evident from comparison of Figs. 1 and 4, and as reported previously (18), the *total* rate of ATP production *via* adenylate kinase was only about 10-20% of the rate of ATP generation by oxidative phosphorylation. Addition of CAT, KCN, or BKA (in any order) had no effect on the rate of ATP production - as expected, since these agents do not affect adenylate kinase activity - nor did it evoke any perceptible release of ATP from intramitochondrial compartments. As expected, addition of the adenylate kinase inhibitor (34), A_2P_5, brought ATP production to an immediate end.

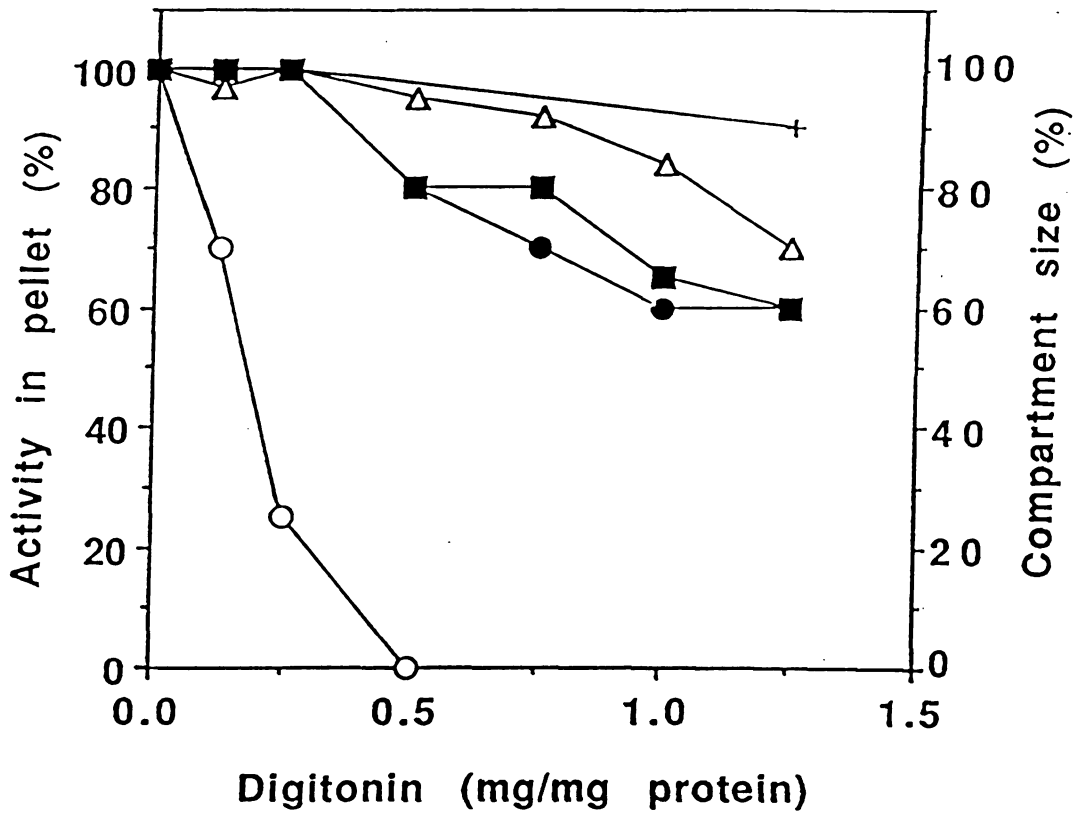
Figure 4. ATP production *via* the adenylate kinase reaction does not generate CAT-sensitive or KCN-sensitive intramitochondrial compartments of ATP. ATP was generated *via* the adenylate kinase reaction, with oxidative phosphorylation precluded by deletion of inorganic phosphate from the incubation medium. Unlike the situation when ATP is generated by oxidative phosphorylation (Fig. 1, left and center), addition of CAT, KCN, or BKA do *not* result in release of ATP from intramitochondrial compartments. Addition of the adenylate kinase inhibitor, A_2P_5, completely inhibits ATP production.


A340
Time
ADP
CAT
KCN
BKA
A2P5
ΔA
0.04
1 min

Effects of digitonin treatment on intramitochondrial compartments of ATP. Mitochondria (0.1 ml) were treated with increasing amounts of digitonin as described in the Methods section, except that the final volume was 1.5 ml rather than the usual 0.5 ml. Increasing the final volume had *no* effect on the release of the various marker enzymes (Fig. 2) but was found to result in better preservation of coupling, for reasons that do not seem evident. The digitonin treated mitochondria were resuspended in 0.1 ml of fresh isolation medium and total ATP production and intramitochondrially compartmented ATP were determined as illustrated in Fig. 1.

Results of a typical experiment are presented in Fig. 5. At the concentrations used, digitonin had *no* effect on the rate of total ATP production, which was 0.22 μmoles ATP/min/mg mitochondrial protein in this experiment; polarographic determination of oxygen consumption confirmed that these same concentrations of digitonin had no effect on oxygen uptake or respiratory control ratios (results not shown). Higher digitonin concentrations did significantly impair oxidative phosphorylation, reflecting damage to the inner mitochondrial membrane as evidenced by significant release of the matrix enzyme, fumarase. This would obviously influence the supply of ATP to intramitochondrial compartments as well as introduce additional potential complications in the interpretation of results; thus, digitonin concentrations in these experiments were restricted to the range shown in Fig. 5.

Figure 5. Effect of digitonin treatment on intramitochondrial ATP compartments. Mitochondria were treated with the indicated concentration of digitonin as described in the text, and release of marker enzymes was determined: adenylate kinase (O), monoamine oxidase (Δ), and fumarase (+). The pelleted mitochondria were then resuspended, and total rate of ATP production and amount of ATP in CAT-sensitive (●) and KCN-sensitive (■) compartments determined as shown in Fig. 1.


Activity in pellet (%)
Compartment size (%)
100
80
60
40
20
0
0.0
0.5
1.0
1.5
Digitonin (mg/mg protein)

The effects of digitonin treatment on release of marker enzymes and on the amount of ATP retained in the CAT-sensitive and KCN-sensitive compartments are compared in Fig. 5. Of major significance is the observation that *neither compartment was disrupted in a manner that correlated with release of adenylate kinase from the intermembranal space.* These results clearly are not in accord with the previous speculation (18) that either the CAT- or KCN-sensitive compartment might correspond to this intramitochondrial region. Similarly, disruption of these compartments did not correlate with disruption of the inner mitochondrial membrane, as reflected by release of fumarase. This is consistent with the previous conclusion (18) that both compartments must lie outside the inner mitochondrial membrane, as judged from their ability to be released in the presence of the translocase inhibitor, CAT; the finding in the present study that another translocase inhibitor, BKA, also does not prevent release from either compartment provides further support for this view.

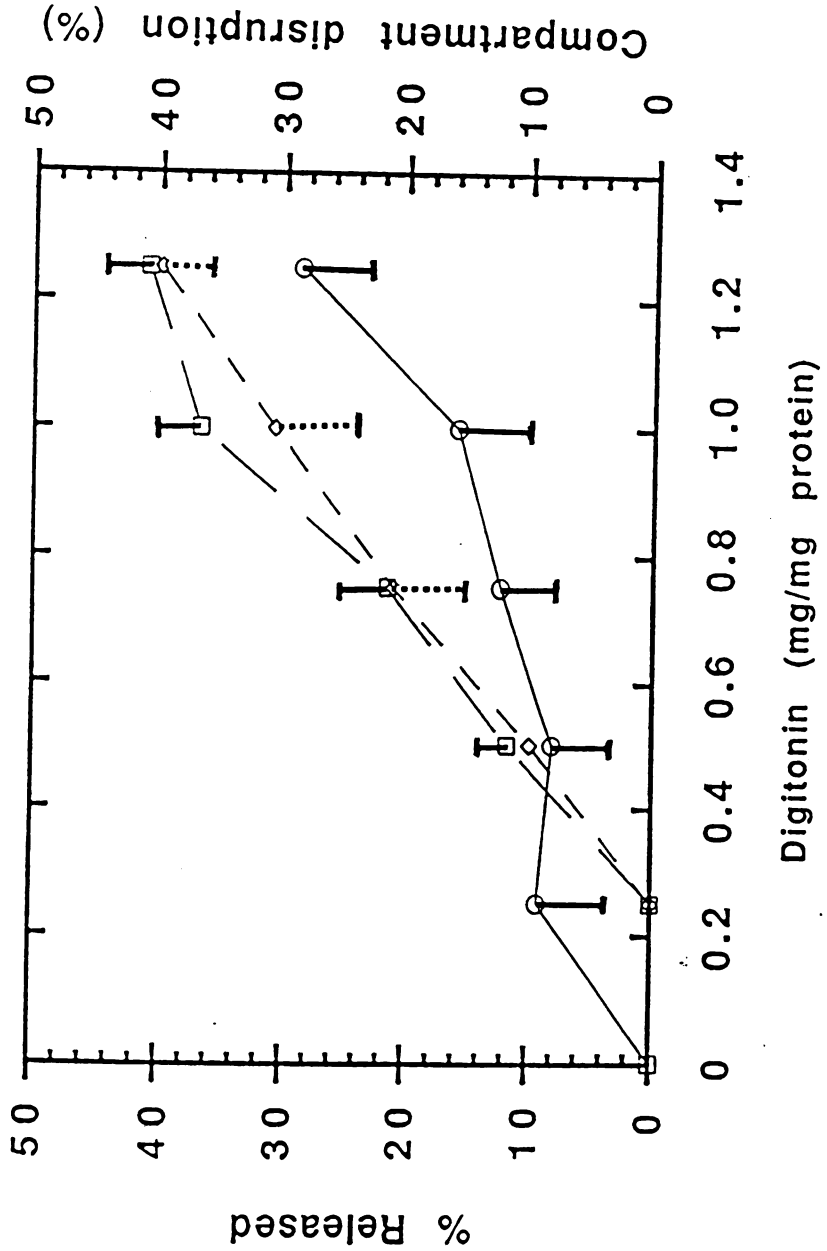
The KCN- and CAT-sensitive compartments were indistinguishable with respect to the degree to which their disruption depended on digitonin concentration. Although, as noted above, disruption of these compartments was distinctly different from the release of adenylate kinase or fumarase, there was consistently a similarity to the pattern of release of monoamine oxidase. Specifically, both the compartments as well as monoamine oxidase were resistant to relatively low levels (< about 0.4 mg digitonin per mg mitochondrial protein), with further increases in digitonin then evoking a progressive increase in release of

monoamine oxidase as well as decrease in compartmented ATP; although the results for the single experiment shown in Fig. 5 might suggest that disruption of the CAT-sensitive compartment was "plateauing" at about 1 mg digitonin/ mg mitochondrial protein, this was not a reproducible observation.

As shown in Fig. 2, increase in digitonin to levels greater than 1.25 mg/ mg mitochondrial protein (maximum level shown in Fig. 5) resulted in correspondingly increased release of monoamine oxidase. It would obviously have been desirable to confirm the suggested correlation between release of monoamine oxidase and disruption of the compartments by showing that further increase in digitonin also resulted in continued decrease in amount of compartmented ATP. This was precluded by the detrimental effect that further increase in digitonin had on oxidative phosphorylation, and hence supply of compartmented ATP, as noted above. Nonetheless, comparison of results from several different experiments (Fig. 6) reinforced the conclusion that release of monoamine oxidase and disruption of the intramitochondrial ATP compartments were quite similar in their dependence on digitonin concentration.

Digitonin treatment has no effect on sensitivity of mitochondrially bound hexokinase to solubilization by Glc-6-P. It is apparent that treatment of the mitochondria with digitonin has a marked effect on the outer mitochondrial membrane. Conceivably, this might alter the membrane environment of mitochondrially bound hexokinase in a way that would affect its susceptibility to

Figure 6. Comparison of release of monoamine oxidase and disruption of intramitochondrial ATP compartments by digitonin. Values presented are the mean ± SD determined in several different experiments of the type shown in Fig. 5. Percentage of total monoamine oxidase activity (O) released at the indicated digitonin concentration (mean ± SD for 8 experiments) is compared with the percentage decrease in levels of ATP in the CAT-sensitive (◇) and KCN-sensitive (□) compartments (mean ± SD for 4 experiments).


Compartment disruption (%)
50
40
30
20
10
0
% Released
50
40
30
20
10
0
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Digitonin (mg/mg protein)

solub

befo

prote

depe

solub

(≥0.1

solub

resul

that

sens

Glc-

direc

Prog

incu

mM)

utiliz

treat

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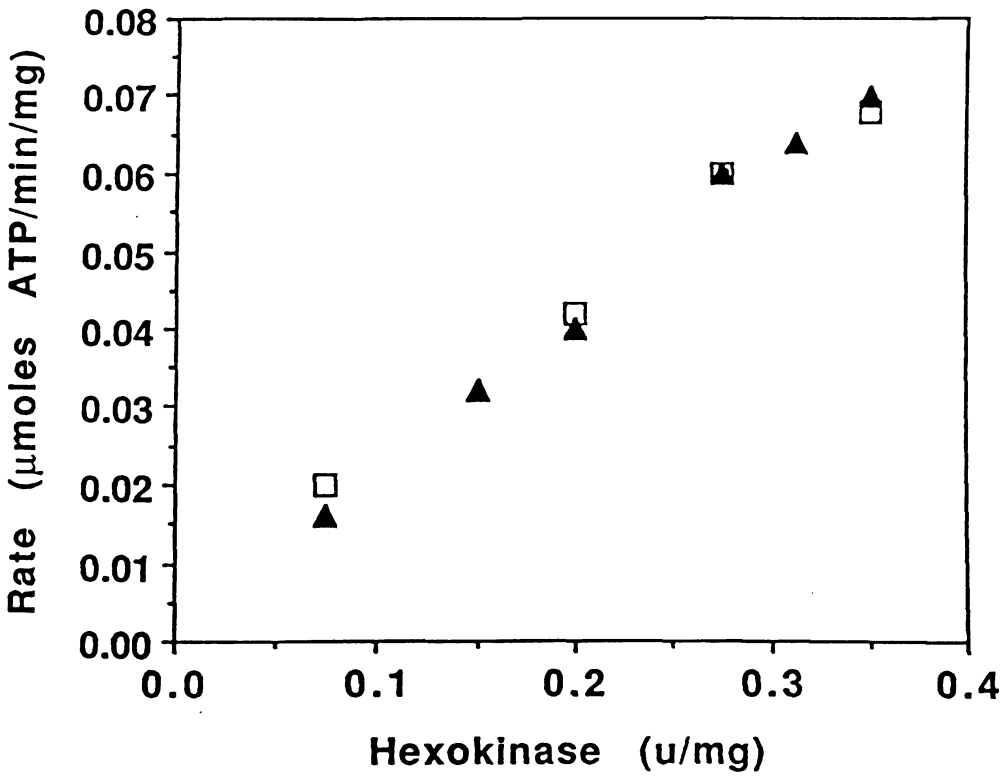
mito

hexo

solubilization by Glc-6-P. However, comparison of mitochondria, before and after treatment with 1.5 mg digitonin/mg mitochondrial protein, showed them to be indistinguishable with respect to the dependence of solubilization on Glc-6-P concentration, the extent of solubilization (85-90% solubilized) at saturating levels of Glc-6-P (≥0.1 mM, under the conditions used here), or the rate at which solubilization occurred in the presence of 1 mM Glc-6-P. These results are in accord with the conclusion of Parry and Pedersen (39) that digitonin treatment of rat brain mitochondria does not alter the sensitivity of mitochondrially bound hexokinase to solubilization by Glc-6-P.

Utilization of ATP produced by oxidative phosphorylation is directly dependent on levels of mitochondrially bound hexokinase. Progressive reduction of mitochondrial hexokinase levels by incubation of mitochondria with increasing concentrations (0-0.04 mM) of Glc-6-P resulted in a correlated decrease in the rate of ATP utilization by the mitochondrially bound hexokinase (Fig. 7); this treatment had *no* effect on the total rate of ATP production which, for the experiment shown in Fig. 7, was 0.22 μmoles ATP/min/mg mitochondrial protein. It is obvious that a total absence of bound hexokinase would necessarily result in a zero rate of utilization, as

Figure 7. Rate of ATP utilization as a function of the levels of mitochondrially bound hexokinase. Utilization of ATP generated by oxidative phosphorylation was determined using mitochondria containing the indicated amount of hexokinase activity (u/mg mitochondrial protein). As described in the text, the latter was manipulated by treatment of mitochondria with increasing concentrations of Glc-6-P, which causes release of mitochondrially bound hexokinase (□), or alternatively, by rebinding Glc-6-P-solubilized hexokinase to mitochondria that had previously been depleted of endogenous hexokinase by incubation with Glc-6-P (▲).


0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Rate (μmoles ATP/min/mg)
0.0
0.1
0.2
0.3
0.4
Hexokinase (u/mg)

defined in Fig. 1. However, it was by no means intuitively obvious that the population of mitochondrially bound hexokinase would be homogeneous, with equivalent access to the intramitochondrial compartments of ATP upon which the bound enzyme has been found to depend (18). The essentially linear dependence of the rate of ATP utilization on the amount of bound hexokinase (Fig. 7) suggests that this is the case.

As also shown in Fig. 7, when the hexokinase levels are restored by rebinding of Glc-6-P-solubilized enzyme to mitochondria that have previously been depleted of endogenously bound hexokinase, the rate of ATP utilization by the rebound enzyme is indistinguishable from that found with an equivalent amount of endogenously bound hexokinase. Thus, rebinding of previously eluted enzyme returns the hexokinase to an environment that provides access to intramitochondrial ATP that is effectively equivalent to seen by the endogenous enzyme.

Correlation of the activity of mitochondrially bound hexokinase and levels of ATP in intramitochondrial compartments with the rate of oxidative phosphorylation. During active oxidative phosphorylation, mitochondrially bound hexokinase has been shown to be dependent on intramitochondrial compartments of ATP, and unresponsive to gradual changes in extramitochondrial ATP (18). That the activity of the mitochondrially bound enzyme would be responsive to changes in intramitochondrial ATP levels seems intuitive, and is consistent with the observation that the duration of the transient phase prior to attainment of steady state (Fig. 1, right)

corresponds to the time required for filling of the intramitochondrial ATP compartments (18). In the present study, we have examined this more directly by manipulating the rate of ATP production *via* oxidative phosphorylation and determining the effect on intramitochondrial ATP levels and hexokinase activity.

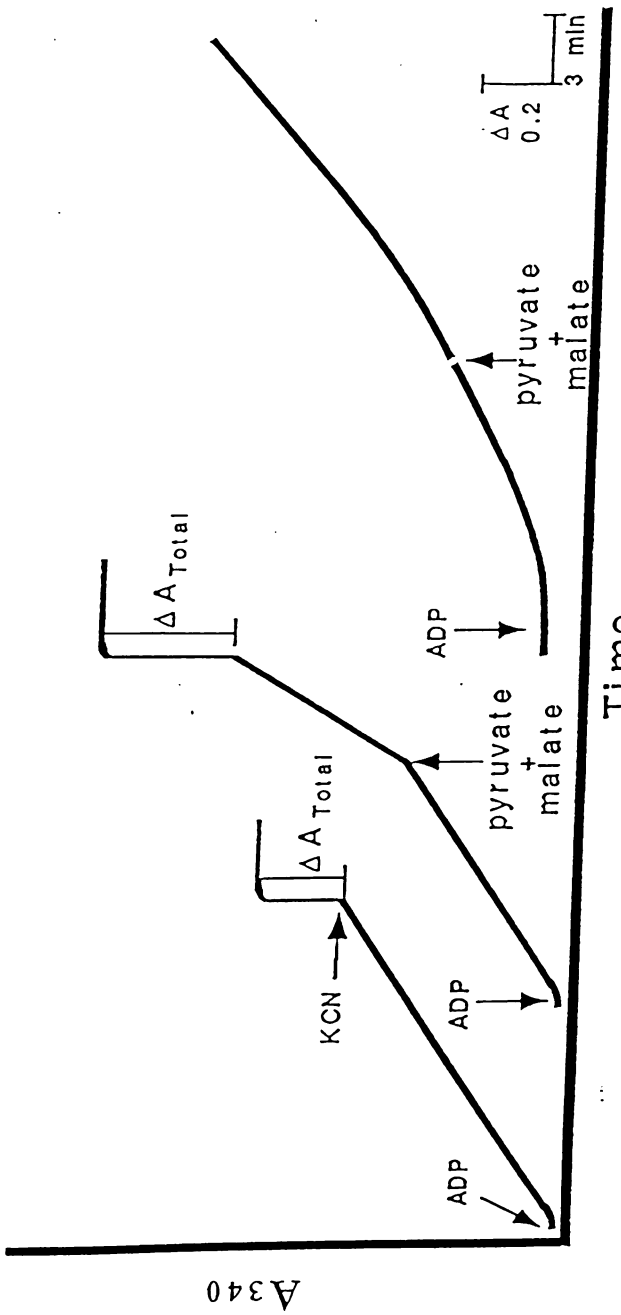
Shown in Fig. 8 is the relationship between the *total* rate of ATP production and the amount of ATP in the intramitochondrial (KCN- plus CAT-sensitive) compartments, as a function of the concentration of pyruvate/malate provided as substrate for oxidative phosphorylation. It is evident that there is a close correlation between ATP production and the level of compartmented ATP *and* that this correlation also extends to the rate of ATP utilization by endogenous hexokinase; thus, *the activity of mitochondrially bound hexokinase is responsive to the rate of oxidative phosphorylation, as reflected in the levels of intramitochondrial ATP serving as substrate for the enzyme.*

The dynamic relationship between oxidative phosphorylation rate, ATP levels in intramitochondrial compartments, and activity of mitochondrially bound hexokinase is confirmed by the results shown in Fig. 9. Subsequent to establishment of a steady state rate of oxidative phosphorylation supported by a subsaturating level of pyruvate/malate, compartmented ATP levels respond to an increased rate of oxidative phosphorylation elicited by an increase in concentration of the oxidative substrates; the *rate of ATP*

Figure 8. Levels of ATP in intramitochondrial compartments as well as the rate of ATP utilization by mitochondrially bound hexokinase are correlated with the total rate of ATP production by oxidative phosphorylation. The total rate of ATP production (O), total ATP in intramitochondrial CAT-sensitive and KCN-sensitive compartments (◇), and rate of ATP utilization by endogenously bound mitochondrial hexokinase (□) were determined as a function of oxidative substrate provided for support of oxidative phosphorylation. Malate was present at 1/2 the concentration of pyruvate shown on the abscissa.

Rate
(μmoles ATP/min/mg)
0.22
0.16
0.11
0.05
0
0
1
2
3
4
5.2
[Pyruvate] (mM)
Compartment size
(μmoles ATP/mg)
0.5
0.4
0.3
0.2
0.1
0

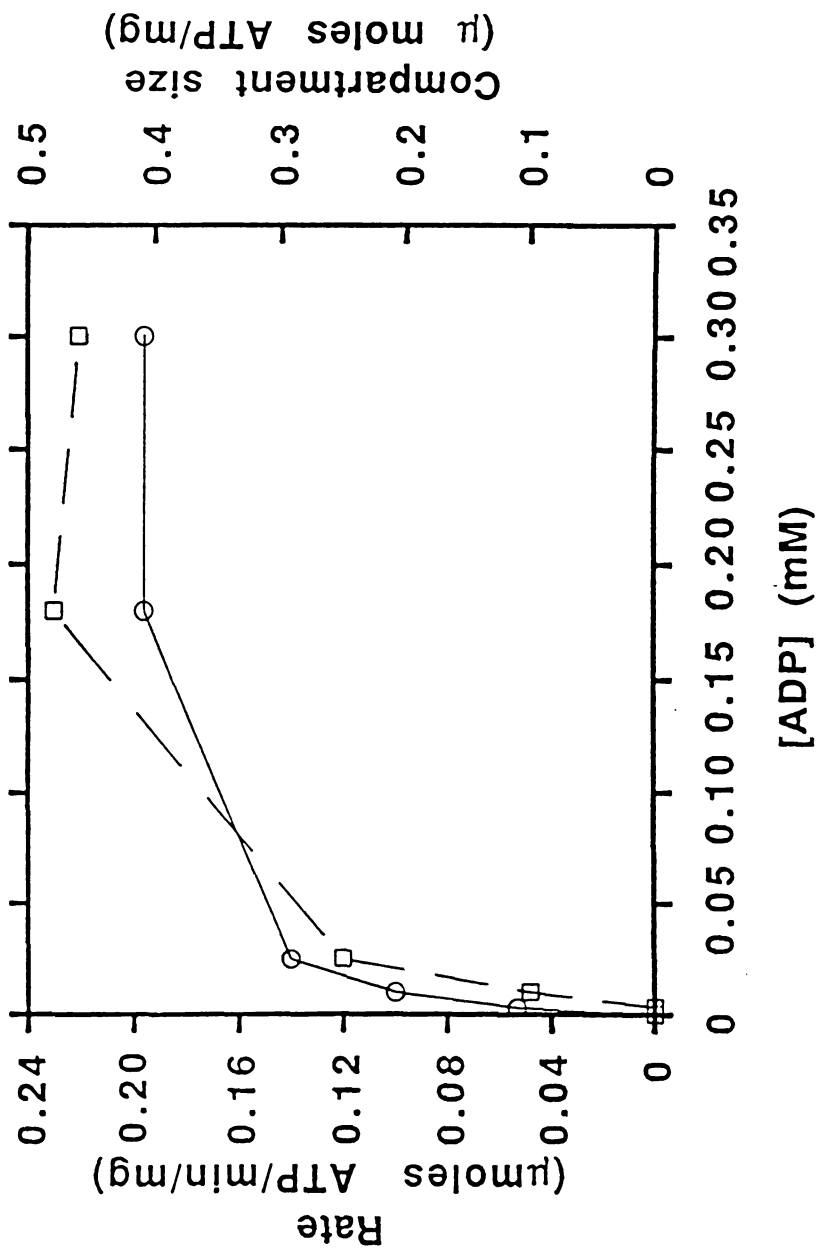
Figure 9. ATP in intramitochondrial compartments and rate of ATP utilization by mitochondrially bound hexokinase are responsive to changes in the rate of ATP production by oxidative phosphorylation. ATP production and intramitochondrially compartmented ATP were determined during oxidative phosphorylation supported by a subsaturating level (0.1 mM, see Fig. 8) of pyruvate/malate (curve at left). Provision of additional substrate (total pyruvate now 3 mM) after establishment of the initial steady state resulted in increase in rate of ATP production and intramitochondrial ATP levels (curve at center). The rate of ATP utilization by endogenous mitochondrial hexokinase was determined in a similar experiment (curve at right). After initiation of ATP formation by addition of ADP to a sample containing 0.1 mM pyruvate, an extended transient period - during which intramitochondrial compartments of ATP are generated (18) - is followed by a steady state rate of glucose phosphorylation; a second addition of substrate (total pyruvate, 3 mM) is followed by a second transient period leading to an increased steady state rate of ATP utilization.


A340
Time
ADP
KCN
ADP
ΔA Total
pyruvate
+
malate
ΔA Total
ADP
pyruvate
+
malate
ΔA
0.2
3 min

utilization by endogenous hexokinase responds in similar fashion to the increase in rate of oxidative phosphorylation and associated increase in level of compartmented ATP. The transient period, between the time at which additional substrates were provided and the time at which an increased steady state rate of glucose phosphorylation was attained, can be attributed to the time required for increasing the level of ATP in intramitochondrial compartments (18).

Results of an alternative approach toward manipulation of the rate of oxidative phosphorylation are shown in Fig. 10. In this case, the rate of oxidative phosphorylation was governed by the level of ADP present in the reaction medium. Again, a correlation between this rate and the levels of ATP in intramitochondrial compartments is apparent. Thus, the correlation of these parameters shown in Figs. 8 and 9 is not uniquely associated with manipulation of oxidative phosphorylation rates by limiting oxidative substrate supply. The steady state rate of ATP utilization by mitochondrially bound hexokinase could not be determined when oxidative phosphorylation rate was governed by manipulation of ADP levels since, in the absence of excess yeast hexokinase (necessarily deleted when measuring activity of the endogenous hexokinase), the ADP was not continuously regenerated and, at the low levels used in these experiments, the supply was rapidly depleted.

Figure 10. Total rate of ATP production and intramitochondrially compartmented ATP levels during oxidative phosphorylation limited by available ADP. The total rate of ATP production (□) and the total ATP in intramitochondrial CAT-sensitive and KCN-sensitive compartments (o) was determined as a function of added ADP; the levels of ADP were maintained at the indicated concentration by regeneration with the excess yeast hexokinase present.


Rate
(μmoles ATP/min/mg)
0.24
0.20
0.16
0.12
0.08
0.04
0
0 0.05 0.10 0.15 0.20 0.25 0.30 0.35
[ADP] (mM)
Compartment size
(μ moles ATP/mg)
0.5
0.4
0.3
0.2
0.1
0

Discussion

It is evident that both the mitochondrially bound hexokinase of rat brain, as well as the intramitochondrial compartments of ATP upon which this enzyme depends during active oxidative phosphorylation (18), are located in digitonin resistant regions of the mitochondrion thought to correspond to contact sites (22,25,26,43). Since contact sites are, by definition, regions of intimate interaction between inner and outer mitochondrial membranes, they are obviously well suited for fostering equally intimate interactions in a metabolic sense, between intramitochondrial ATP generating processes and extramitochondrial kinases - a view extensively promoted by Brdiczka (22,43). While acknowledging that the present study does not provide direct evidence for the identification of these digitonin resistant regions with specific structural elements, we believe that they are reasonably interpreted as being associated with contact sites, and they will be referred to as such in the following discussion.

An earlier suggestion (18) was that either the KCN- or CAT-sensitive compartment might be associated with the intermembranal space marked by the presence of adenylate kinase (27). This clearly is not the case since release of adenylate kinase is virtually complete at concentrations of digitonin that have only

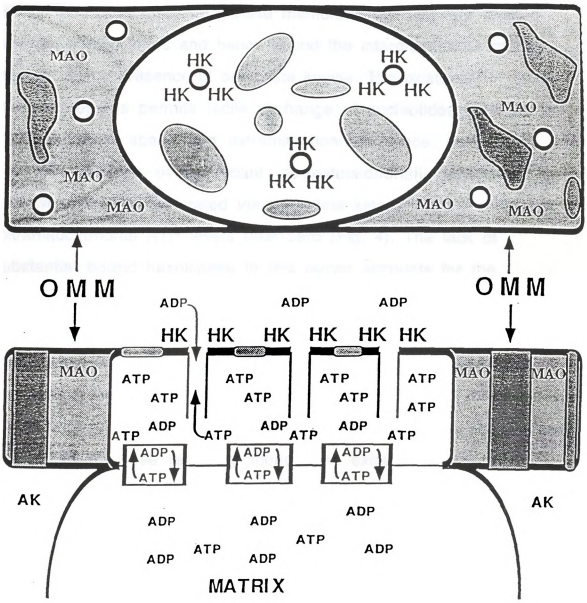
marginal effects on levels of compartmented ATP. Indeed, the present study suggests that there is little if any intramitochondrial compartmentation of ATP generated by the adenylate kinase reaction, consistent with the previous suggestion (18) that there is rapid equilibration of nucleotides between the extramitochondrial space and the intramitochondrial compartment containing adenylate kinase; hence, in the absence of significant extramitochondrial ATP (as was the case in the experimental conditions used here, with excess yeast hexokinase added), negligible ATP would be retained in the intermembranal space associated with adenylate kinase. Moreover, the reliance of mitochondrially bound hexokinase on compartments of ATP, generated by oxidative phosphorylation and discrete from adenylate kinase, is consistent with the observation that ATP generated by adenylate kinase is not a significant substrate for mitochondrial hexokinase during periods of active oxidative phosphorylation (18).

The present study has demonstrated that the existence of the KCN- and CAT-sensitive compartments does not depend on retention of hexokinase bound at the contact sites. However, whether these compartments are associated with different populations of contact sites, or both compartments are present at all contact sites, remains unclear. Similarly, whether one or the other of these compartments is preferentially utilized by mitochondrially bound hexokinase is also uncertain. At present, we have no convenient way to selectively disrupt one of these compartments - they are quite similar in their sensitivity to digitonin, and the only agents found thus far to cause selective release from one of the compartments,

CAT or azide (18), have obvious deleterious effects on oxidative phosphorylation and hence, at least indirectly, upon maintenance of ATP levels in the remaining compartment. It also remains puzzling that two agents generally identified as inhibitors of oxidative phosphorylation, KCN and azide, differ in their ability to cause release of ATP from these compartments -azide being indistinguishable from CAT in causing release of ATP from the CAT-sensitive compartment while KCN causes release from both compartments. This situation was further confounded by the present finding that two inhibitors of the translocase, BKA and CAT, differ in their effect on intramitochondrial compartments of ATP; conceivably, this might be related to their different mode of interaction with the translocase (21) although the relationship does not seem obvious. It is apparent that this aspect of the compartmentation phenomenon requires additional study.

Dorbani *et al.* (26) suggested the existence of three distinct domains within the outer membrane of rat brain mitochondria, differing in their cholesterol content and hence in their susceptibility to disruption with digitonin. Our results are consistent with those of Dorbani *et al.* (26) and we have built upon their suggestion to develop a schematic representation of the interactions between mitochondrially bound hexokinase and intramitochondrial compartments of ATP (Fig. 11). Hexokinase is considered to bind preferentially at pore structures located in cholesterol-poor regions of the outer membrane associated with contact sites (22,25,26). Membrane domains of moderate

Figure 11. Schematic representation illustrating proposed relationships among domains in the outer mitochondrial membrane, hexokinase, and intramitochondrial ATP compartments. In keeping with the suggestion of Dorbani *et al.* (26), the outer mitochondrial membrane is suggested to include domains differing in cholesterol content (indicated by intensity of shading) as judged by susceptibility to disruption with digitonin. Monoamine oxidase (MAO) is associated with regions of intermediate cholesterol content (moderate susceptibility to digitonin), indicated here with light stippling. Hexokinase (HK) is bound preferentially to pores (dark circles) in regions of low cholesterol content (digitonin resistant), indicated here by no stippling, thought to be associated with contact sites. Domains of high cholesterol content (dense stippling) are located in membrane regions between contact sites; facile disruption of these regions by digitonin results in release of adenylate kinase (AK) from the underlying intermembranal space. Intramitochondrial compartments of ATP are viewed to result from conjunction of the inner and outer mitochondrial membranes at contact sites. Hexokinase located at pores in these regions has privileged access to ATP produced by oxidative phosphorylation and introduced into these compartments *via* the ATP/ADP translocase (rectangle) located in the inner mitochondrial membrane.


MAO
HK
OMM
ADP
ATP
AK
MATRIX

cholesterol content contain the major portion of the monoamine oxidase, and also include additional pore structures that have less affinity for hexokinase (26); these membrane domains are located between contact sites and hence bound the intermembranal space marked by the presence of adenylate kinase. The existence of pores in these regions permits facile exchange of nucleotides between this intermembranal space and extramitochondrial space, consistent with the absence of significant intramitochondrially compartmented ATP when ATP is generated *via* adenylate kinase while maintaining extramitochondrial ATP levels near zero (Fig. 4). The lack of substantial bound hexokinase in this region accounts for the relatively poor utilization of ATP generated by adenylate kinase as a substrate for glucose phosphorylation. "Islands" of moderate cholesterol content are also found in the contact site region, accounting for the disruption of intramitochondrial ATP compartments located in this region at digitonin concentrations that are similar to those releasing monoamine oxidase from extra-contact site regions of similar cholesterol content. Interspersed throughout the latter regions are cholesterol-rich domains, preferentially disrupted by digitonin with accompanying release of the adenylate kinase located in the underlying intermembranal space; results in Fig. 2 suggest that rotenone insensitive NADPH-cytochrome c reductase activity may be most closely associated with these domains. Intramitochondrial ATP compartments, to which mitochondrially bound hexokinase has privileged access, are formed in the contact site regions by association of the inner and outer mitochondrial membranes (we have made no attempt to differentiate

between the KCN - and CAT-sensitive compartments), and are linked to intramitochondrial oxidative phosphorylation *via* the ATP/ADP translocase of the inner mitochondrial membrane. Similar representations of these relationships have previously been presented by Brdiczka (e.g., 43), and the major novel feature in the scheme shown in Fig. 11 is a more explicit attempt to relate the various aspects to membrane domains of differing lipid composition.

The brain consumes glucose at a remarkably high rate compared to other organs, with virtually complete oxidation of this carbohydrate to CO_2 *via* aerobic glycolysis (1). As elegantly demonstrated by the studies of Sokoloff and his coworkers (e.g., ref. 46), altered functional activity in various brain regions is characteristically accompanied by corresponding changes in the rate of glucose utilization. Despite what might be considerable variations in the rate of glucose consumption, coordination between glycolysis and mitochondrial oxidative metabolism is maintained such that, under normal conditions, there is no significant production of lactate. We believe that the results presented here and in the previous study (18) provide insight into how such coordination is achieved, preventing formation of toxic lactate (19).

What happens if, for example, increased stimulation of a specific brain region results in increased energy demand, i.e., increased rate of utilization of ATP? Intuitively, it seems apparent that cellular levels of ATP might decrease somewhat. However, even under such extreme conditions as ischemia (3) or convulsion (4), changes in cerebral ATP levels are relatively slow, and precipitous declines in ATP are likely to be associated only with extreme

conditions. Thus, we might anticipate that the increases and decreases in metabolic demand associated with normal cerebral function are accompanied by rather modest changes in cellular ATP level. Since mitochondrially bound hexokinase, which probably represents the major portion of the enzyme in brain (2), is unresponsive to such changes in extramitochondrial ATP (18), these would have no direct effect on the rate of glucose phosphorylation. Moreover, it is apparent that sensitivity of hexokinase to extramitochondrial ATP concentration would produce exactly the *opposite* of the effect that would be desirable, i.e., if hexokinase were responding to extramitochondrial ATP, increased energy demand with associated decrease in cytoplasmic ATP would lead to *decreased* hexokinase activity - hardly a satisfactory response to a situation demanding increased metabolism of glucose! However, the present results suggest a more appropriate metabolic response to this challenge.

Normal cellular levels of ATP are estimated to be in the range of 2.5 mM whereas cytoplasmic ADP levels are in the range of 30 μM (47). Hence, even a very modest change (e.g., 0.05 mM) in the level of ATP would represent a marked change in the concentration of its hydrolysis product, ADP. Moreover, since the cellular levels of ADP are subsaturating for oxidative phosphorylation (see Fig. 10 and ref. 48), one may anticipate changes in the rate of oxidative phosphorylation that are correlated with changes in cytoplasmic ADP concentration. This, in turn, would influence levels of ATP in intramitochondrial compartments (Fig. 10), upon which mitochondrially bound hexokinase is dependent (18), with a resulting

change in the rate of glucose phosphorylation (Figs. 8 and 9) *that is correlated with the change in oxidative phosphorylation and preserves the critical balance between these fundamental metabolic processes.* This provides a mechanism by which glycolytic and oxidative metabolism may be regulated in a coordinated manner in response to altered energy demands - increased or decreased - as reflected in cytoplasmic ADP levels.

The above comments pertain to regulation of glycolytic and oxidative metabolism in response to moderate changes in energy demand associated with normal neurophysiological activity. We have previously suggested that modulation of the levels of mitochondrially bound hexokinase in response to changes in intracellular levels of Glc-6-P might also be of significance in regulation of this enzyme, and thereby, the rate of cerebral glucose metabolism (2). Indeed, there is evidence (reviewed in ref. 2) that alteration in the intracellular distribution of hexokinase does occur in response to severe perturbations such as ischemia. In light of more recent work (18, and the present report), we suggest that modulation of the intracellular distribution of hexokinase may represent a secondary factor in regulation of this enzyme, permitting an appropriate response when confronted with unusual challenges to maintenance of cerebral energy metabolism. For example, characteristic features of cerebral ischemia (3) are increased *anaerobic* glycolysis and decreased levels of Glc-6-P, leading to increased mitochondrial binding of hexokinase (2, and references therein). In this situation, increased binding of the enzyme is not likely to be beneficial in terms of enhancing

privileged access of the enzyme to intramitochondrially generated ATP since oxidative phosphorylation would be severely compromised by the hypoxia/anoxia associated with ischemia. Binding *does*, however, markedly decrease the sensitivity of the enzyme to inhibition by Glc-6-P (2, and references therein), thereby permitting increased introduction of substrate into anaerobic glycolytic metabolism which may serve to sustain cerebral energy demands during limited (19) periods of anoxia/hypoxia.

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Footnote

[1] Increased glycolytic rate with accumulation of lactate does occur under hypoxic/ischemic conditions, frequently with resulting neurological damage attributed, at least in part, to lactate (19). We are *not* considering such abnormal states here. Rather, we refer to the normal situation in which moderate changes in metabolic rate occur in response to altered neurophysiological activity, with maintenance of the balance between glycolytic and oxidative metabolism and negligible production of lactate.

Chapter IV

Interaction of Intramitochondrially Bound Rat Brain Hexokinase with Intramitochondrial Compartments of ATP Generated by Oxidative Phosphorylation and Creatine Kinase

Abstract

Previous work led to the conclusion that, during oxidative phosphorylation, mitochondrially bound hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) from rat brain was dependent on intramitochondrially compartmented ATP as substrate. The present study demonstrated that, *when oxidative phosphorylation was functioning concurrently*, mitochondrial creatine kinase could also generate intramitochondrial ATP serving as substrate for hexokinase. In the absence of concurrent oxidative phosphorylation, the kinetics of glucose phosphorylation with ATP generated by creatine kinase were *not* consistent with supply of ATP from a saturable intramitochondrial compartment as formed during oxidative phosphorylation. Evidence for intramitochondrially compartmented ATP, generated by creatine kinase, was obtained; this was distinct from compartmented ATP generated by oxidative phosphorylation in terms of kinetics of generation of the compartment and its capacity, sensitivity to release by carboxyatractyloside, and sensitivity to disruption by digitonin. That oxidative phosphorylation did induce a dependence on intramitochondrial ATP as a substrate was further indicated by the observation that, although the initial rate of glucose phosphorylation by mitochondrial hexokinase depended on the extramitochondrial concentration of ATP present at the time

oxidative phosphorylation was initiated, a final steady state rate of glucose phosphorylation was attained that was independent of extramitochondrial ATP levels. These and previous results emphasize the probable importance of nucleotide compartmentation in regulation of cerebral glycolytic and oxidative metabolism.

Introduction

Aerobic metabolism of Glc is, under normal circumstances, virtually the sole mechanism for generation of metabolic energy required to support cerebral function (1). Hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) catalyzes the initial step in this pathway, and regulation of hexokinase activity is recognized as an important factor governing the rate of glucose utilization (1).

A major portion of the hexokinase in brain is associated with mitochondria (2, and references therein). More specifically, the enzyme appears to associate with porin, the protein which forms the pores through which metabolites enter or exit the mitochondrion (3-5). Brdiczka and his colleagues (6,7) as well as Dorbani *et al.* (8) have reported that hexokinase is preferentially bound to porin located at contact sites, regions in which the inner and outer mitochondrial membranes are closely apposed and which, presumably due to a low cholesterol content, are resistant to disruption by digitonin. This intimate association of hexokinase with mitochondria may provide a basis for coordination of the initial step of Glc metabolism, catalyzed by hexokinase, and terminal oxidative stages occurring within the mitochondria. This is necessary if glycolytic and oxidative phases of Glc metabolism are to proceed in concert, avoiding production of lactate with resultant toxic effects on the brain (9).

Recent work (10,11) has provided insight into the mechanism by which this coordination might be achieved. It has been shown that, during active oxidative phosphorylation, the hexokinase of rat brain mitochondria is dependent on *intramitochondrial* ATP as a substrate. The rate of Glc phosphorylation by mitochondrially bound hexokinase was directly related to intramitochondrial ATP levels which were, in turn, directly responsive to rates of oxidative phosphorylation as governed by changes in extramitochondrial ADP concentrations within the physiological range. In this manner, the rate at which Glc was introduced into the glycolytic pathway could be correlated with the rate at which oxidation of this substrate was completed by intramitochondrial processes, with both phases of this metabolic pathway responding to altered cellular energy status as reflected by cytoplasmic ADP levels.

Two intramitochondrial compartments of ATP have been identified, based on their selective release with agents such as KCN, carboxyatractyloside (CAT), and bongkrekic acid (10,11); following previous usage (10), these are referred to as the "CAT-sensitive" and "KCN-sensitive" compartments. Based on their resistance to disruption by digitonin (11), both compartments are thought to be associated with contact sites. However, whether the CAT-sensitive and KCN-sensitive compartments are both associated with every contact site, or each with a distinct population of contact sites, remains unknown.

Creatine kinase has also been associated with contact sites in brain mitochondria (6,7,12,13). In the present study, we have examined the relationship between oxidative phosphorylation and

creatine kinase as sources of substrate ATP for mitochondrially bound hexokinase.

Materials and Methods

Materials. ADP, ATP, A_2P_5, creatine, creatine phosphate, Ficoll 400, yeast hexokinase, homovanillic acid, peroxidase, phosphoenolpyruvate, pyruvate kinase-lactate dehydrogenase and tyramine were obtained from Sigma Chemical Co. (St. Louis, MO). Digitonin was a product of Merck (Darmstadt, Germany) and Glc-6-P dehydrogenase was a product of Boehringer Mannheim (Indianapolis, IN). Both the BCA Protein Assay Reagent and the bovine serum albumin standard were purchased from Pierce Chemical Co. (Rockford IL). Other chemicals were of reagent grade and obtained from various commercial sources.

Brain mitochondria. As in our previous studies (10,11), mitochondria were prepared from brains of Sprague-Dawley rats (150-250 g) of either sex, using the method of Lai and Clark (14). Mitochondrial suspensions contained 8.3 ± 0.2 mg protein and 3.3 ± 0.2 units hexokinase activity per milliliter (mean ± SD for 13 preparations). Respiratory rates were determined as described earlier (10); respiratory control ratios, 4.7 ± 0.4 (mean ± SD for 4 preparations), were in good agreement with those of mitochondria used in previous work (10,11).

Assays. Adenylate kinase (15), fumarase (16), hexokinase (17) and monoamine oxidase (18) were assayed by previously described procedures.

Creatine kinase activity in the direction of ATP formation was measured by coupling the latter to NADPH production *via* the hexokinase and Glc-6-P dehydrogenase reactions; NADPH was monitored spectrophotometrically at 340 nm. The assay was done in the "incubation medium" of Lai and Clark (14), prepared as described earlier (10) except that the Tris phosphate was replaced with an equivalent amount of Tris-Cl; the absence of P_i prevented oxidative phosphorylation. For assay purposes, the following additions were also present: 0.1 mM A_2P_5 as an inhibitor of adenylate kinase (19), 2.2 mM ADP, 5 mM Glc, 5 mM $MgCl_2$, 3.3 mM creatine phosphate, 0.5 mg NADP, 3 units yeast hexokinase and 1 unit Glc-6-P dehydrogenase. The final volume was 1 ml.

Creatine kinase activity in the direction of creatine phosphate formation was determined by coupling ADP formation to NADH oxidation, monitored at 340 nm, *via* the pyruvate kinase and lactate dehydrogenase reactions. This assay was also conducted in 1 ml of phosphate free incubation medium (as above) with the following additions: 0.1 mM A_2P_5, 0.8 mM phosphoenolpyruvate, 2.2 mM ATP, 10 mM creatine, 0.13 mM NADH, 5 mM $MgCl_2$, and 5 units of pyruvate kinase and of lactate dehydrogenase. Protein was determined using the BCA method (Pierce Chemical Co., Rockford IL) with bovine serum as standard.

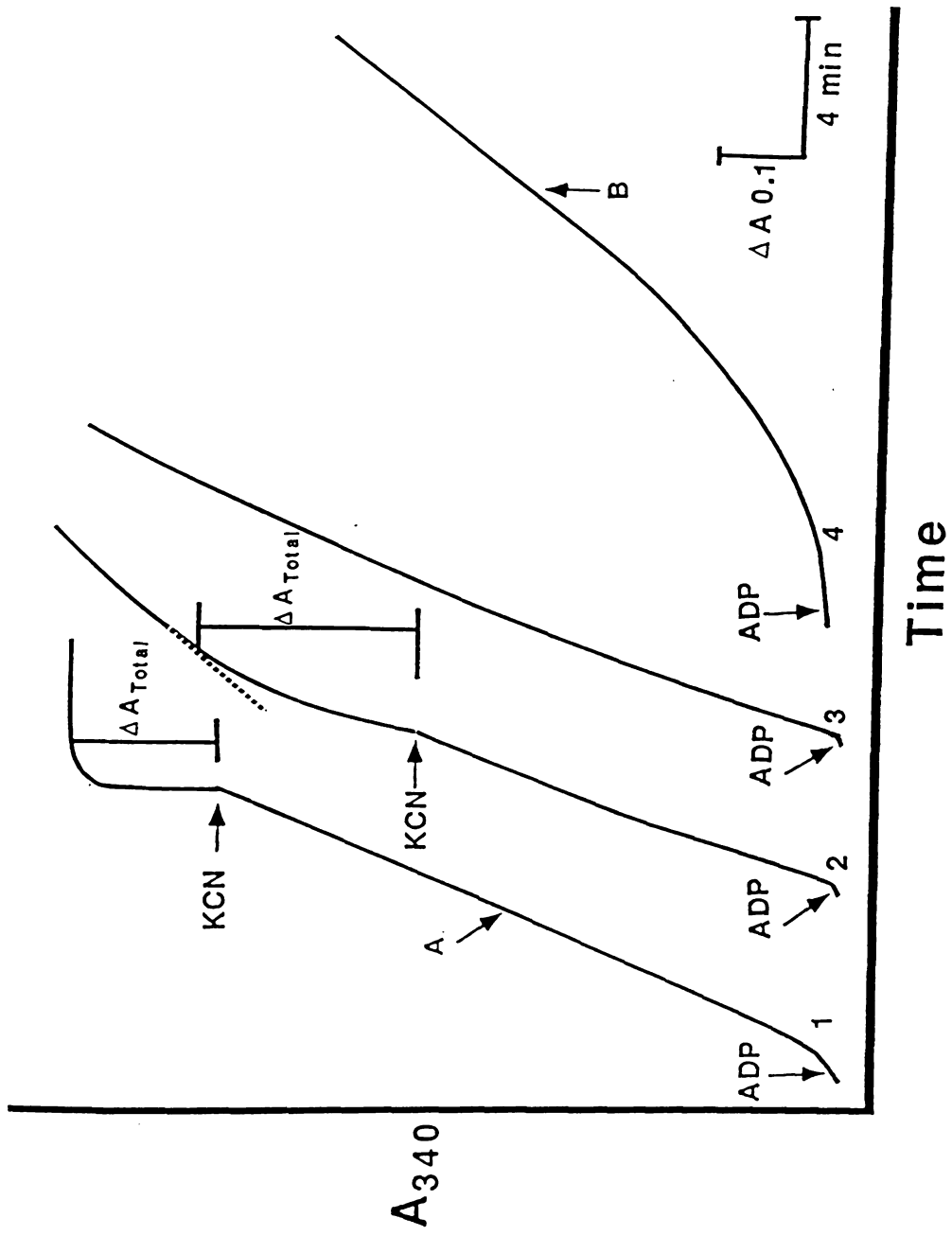
Measurement of ATP production by mitochondria, ATP utilization by mitochondrially bound hexokinase, and release of ATP from intramitochondrial compartments. The procedures used have previously been described in detail (10,11). Briefly, reactions were

carried out at 25 °C. in incubation medium (14) supplemented with 5 mM Glc, 5 mM $MgCl_2$, 5 mM pyruvate, 2.5 mM malate, 0.5 mg NADP, 1 unit of Glc-6-P dehydrogenase, and mitochondria (approximately 0.08 mg mitochondrial protein unless indicated otherwise) in a final volume of 1 ml. Formation of ATP was started by addition of 0.45 mM ADP. Selective formation of ATP *via* oxidative phosphorylation was obtained by inclusion of 0.1 mM A_2P_5 in the reaction. Selective formation of ATP *via* the creatine kinase reaction was attained by deletion of Tris-phosphate from the incubation medium with corresponding increase in the Tris-Cl concentration (as above), and addition of 1.5 mM creatine phosphate and 0.1 mM A_2P_5. This same phosphate-free medium, supplemented with the desired concentration of P_i, was used in experiments in which the rate of ATP production by oxidative phosphorylation was limited by P_i concentration (see below).

Methods for determining the total rate of ATP production, the rate of ATP utilization by endogenously bound mitochondrial hexokinase, and the amount of ATP in intramitochondrial compartments have been described previously (10,11). Since understanding of these parameters is critical to this presentation, we illustrate these basic methods with results presented in Fig. 1.

Total ATP production by oxidative phosphorylation was determined with addition of excess (3 units per ml) yeast hexokinase to the reaction (Fig. 1, Curve 1); under these conditions, the rate of NADPH formation is equivalent to the rate of ATP production since there is negligible accumulation of extramitochondrial ATP (10). After a brief lag, ATP production

Figure 1. Measurement of total rate of ATP production, release of ATP from intramitochondrial compartments, and rate of ATP utilization by mitochondrially bound hexokinase. These representative tracings illustrate how various parameters of interest were determined. Curve 1, total ATP production by oxidative phosphorylation and release of intramitochondrially compartmented ATP by KCN. Curve 2, total ATP production by creatine kinase and release of intramitochondrially compartmented ATP by KCN. Curve 3, total ATP production by creatine kinase without KCN addition. Curve 4, utilization of ATP, generated by oxidative phosphorylation, by endogenous mitochondrially bound hexokinase. See text for specific comments.


A340
Time
ΔA 0.1
4 min
B
A
KCN
KCN
ΔA Total
ΔA Total
ADP
ADP
ADP
ADP
1
2
3
4

enters a steady state from which the rate is calculated (slope of line in region marked "A" in Fig. 1). Subsequent addition of agents such as KCN (as in Fig. 1) or CAT (10) results in a burst of NADPH formation corresponding to release of intramitochondrial ATP, followed by complete cessation of further ATP production, indicating complete inhibition of oxidative phosphorylation; the latter was confirmed by polarographic measurements of oxygen uptake (not shown, but see Fig. 2 in reference (10)). Levels of compartmented ATP are calculated from the absorbance increment following addition of the releasing agent, as indicated in Fig. 1.

The total rate of ATP production by creatine kinase was determined in a similar manner (Fig. 1, Curves 2 and 3), with reaction conditions described above. There was a brief lag after addition of ADP, followed by an apparent steady state rate of ATP production that persisted for only 3-4 minutes and then slowly decreased over the experimental period (Curve 3), e.g., in a representative experiment, the rates of ATP production at 5, 10, and 15 min after ADP addition were 92%, 75%, and 68%, respectively, of the initial steady state rate. Addition of KCN at various times after ADP addition evoked a burst of NADPH formation as intramitochondrial ATP was released (Curve 2). Quantitation of released ATP was more difficult than with ATP produced by oxidative phosphorylation since, in contrast to the latter process, ATP production by creatine kinase was not inhibited by KCN and hence release was followed by continued ATP production. The amount of released ATP was estimated from the absorbance increment between the time of KCN addition and the time at which the rate

again decreased in a manner similar to that observed without KCN addition.

The rate of *utilization* of ATP, generated by oxidative phosphorylation, by mitochondrially bound hexokinase was determined (Fig. 1, Curve 4) in a manner similar to measurement of total ATP production except that yeast hexokinase was not added to the reaction. Hence, NADPH production reflects solely the rate of Glc-6-P formation by endogenous hexokinase activity. After a prolonged transient phase, during which intramitochondrial ATP compartments are filled (10), a steady state rate of Glc-6-P formation is attained (region marked "B" in Fig. 1). Analogous measurements of utilization of ATP generated by creatine kinase are discussed below.

Determination of the ATP concentrations. ATP concentrations were determined enzymatically, coupling the hexokinase reaction to NADPH formation *via* Glc-6-P dehydrogenase. Sample aliquots were added to a cuvette containing hexokinase assay mix (17) (0.04 M Tris-Cl, 6.7 mM $MgCl_2$, 3.3 mM Glc, 10 mM monothioglycerol, pH 8.5) with addition of 5 mM KCN, 0.1 mM A_2P_5, 0.63 mM NADP, and 1 unit of Glc-6-phosphate dehydrogenase in a final volume of 1 ml. After determination of the initial absorbance at 340 nm, 3 units of yeast hexokinase were added and the reaction allowed to go to completion. ATP concentration was calculated from the resulting increment in A_{340}.

Digitonin treatment of brain mitochondria. Fractionation of mitochondria with digitonin was exactly as described earlier (11). Both supernatants and pellets (resuspended in 0.5 ml of incubation medium), were assayed for marker enzymes; recovery of total activity was always greater than 90%.

Results and Discussion

Production of ATP by oxidative phosphorylation or by creatine kinase, and its utilization by mitochondrially bound hexokinase. Steady state rates of total ATP production by oxidative phosphorylation or creatine kinase were similar and strictly additive (Table I). Hence, it is apparent that these processes proceed essentially independently under these conditions.

Utilization of ATP generated by oxidative phosphorylation, creatine kinase, or both processes simultaneously is shown in Fig. 2. Curve B illustrates results obtained when ATP was generated by oxidative phosphorylation alone. As previously noted (10,11), the transient period required for filling of intramitochondrial compartments with ATP was followed by a prolonged steady state rate which persisted throughout the experimental period. The rate of utilization during this steady state (Table I) represented approximately 50% of the total rate of ATP production, in agreement with previous estimates (10).

Similar results were obtained when ATP was produced simultaneously by oxidative phosphorylation and creatine kinase (Fig. 2, Curve A). *Despite the fact that the total rate of ATP production was approximately twice that seen with oxidative phosphorylation alone*, only a modest (about 10%) increase in the steady state rate of ATP utilization was seen (Table I). There

Figure 2. Utilization of ATP produced by oxidative phosphorylation, creatine kinase, or both simultaneously. All reactions contained identical amounts of mitochondria with endogenously bound hexokinase. ATP formation was started by addition of 0.45 mM ADP. Curve A, creatine kinase and oxidative phosphorylation producing ATP simultaneously. After a transient phase of about 6 minutes, a steady state is reached. Curve B, oxidative phosphorylation as the only source of ATP; the steady state rate is about 10% less, and the time required to attain steady state (here about 9 min) somewhat longer than with generation of ATP by both oxidative phosphorylation and creatine kinase (Curve A). Addition of 5-20 mM creatine during steady state does not affect the rate of ATP utilization by hexokinase. Curve C, creatine kinase as the only source of ATP; a slow increase in phosphorylation rate continues throughout the experimental period.

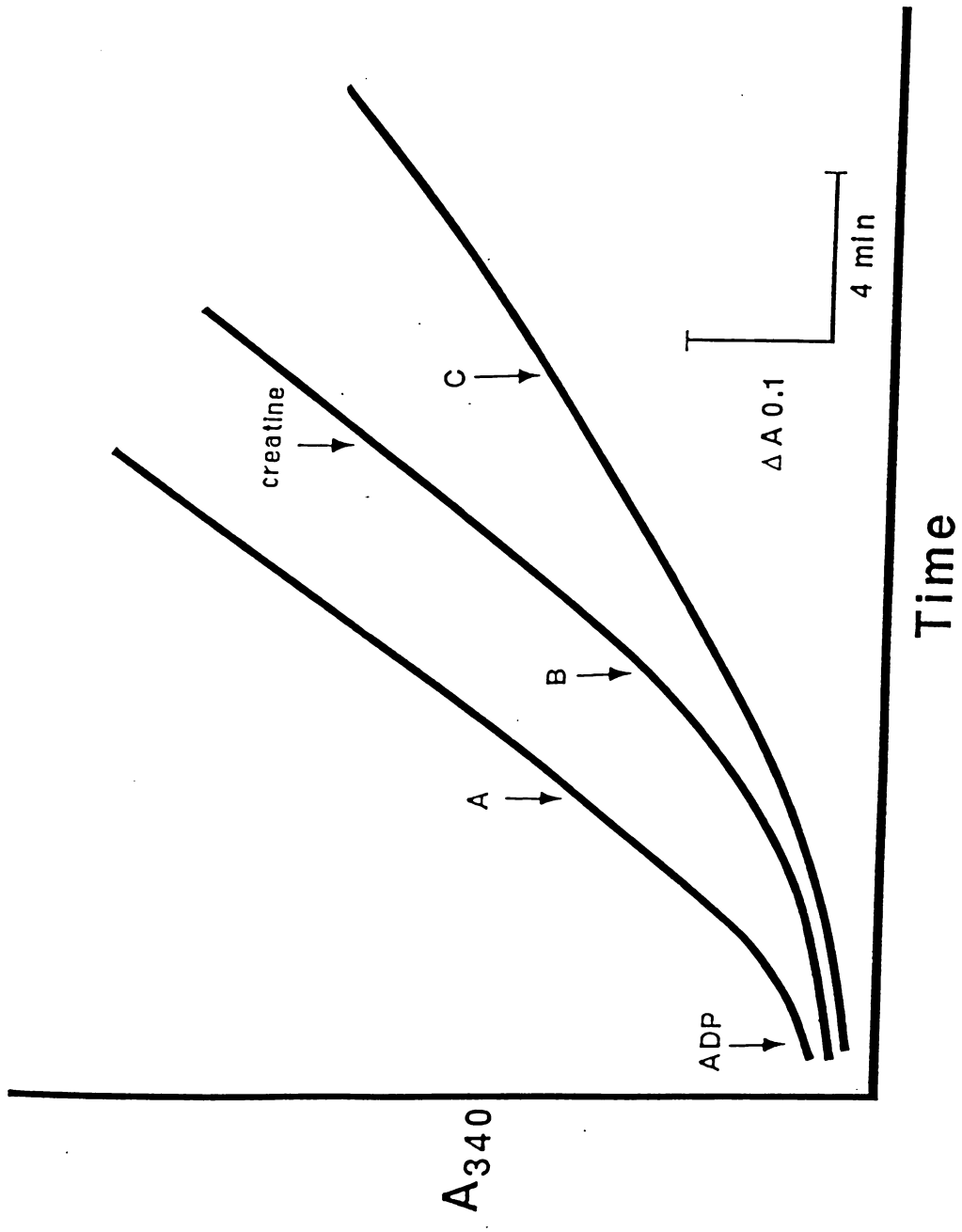

A340
Time
ADP
creatine
A
B
C
ΔA 0.1
4 min

Table I. Production of ATP by Oxidative Phosphorylation or Creatine Kinase and Its Utilization by Mitochondrially Bound Hexokinase

Source of ATP	Total ATP Production[a]	Rate of Utilization[a]
Oxidative Phosphorylation	0.22 ± 0.02	0.12 ± 0.01
Creatine Kinase	0.23 ± 0.03	--[b]
Creatine Kinase plus Oxidative Phosphorylation	0.45 ± 0.05	0.14 ± 0.01

[a] Rates were measured during steady state and are expressed as µmoles ATP (produced or utilized)/min/mg mitochondrial protein. Values given are mean ± SD for six experiments.

[b] A steady state rate of utilization was not attained with ATP generated by creatine kinase alone. See text.

was, however, a significant decrease in the time required for attaining a steady state rate of ATP utilization (Fig. 2), reasonably interpreted as a contribution from creatine kinase to filling of intramitochondrial ATP compartments (10).

With creatine kinase alone as source of ATP, results were quite different (Fig. 2, Curve C). Following addition of ADP, there was a slow but continuing increase in the rate of ATP utilization (tangent to Curve C); it should be noted that this contrasts with the continuing *decrease* in the rate of ATP production by the creatine kinase reaction (Fig.1). These results probably reflect utilization of extramitochondrial ATP; since the rate of ATP production by creatine kinase was always well in excess of the rate of utilization (cf., Figs. 1 and 2), net accumulation occurred in the medium. It has been previously shown that the bound enzyme obeys Michaelis-Menten kinetics with extramitochondrial ATP as substrate, with a K_m of about 0.25 mM (10). Since ATP concentrations attained in these experiments were in the 0-0.5 mM range (i.e., subsaturating), continued increase in rate with increasing extramitochondrial ATP is expected. An alternative possibility would be that substrate ATP was derived, at least partially, from an intramitochondrial compartment generated by creatine kinase; this compartment filled slowly over the time course of these experiments (see below). Assuming that the rate of Glc phosphorylation was correlated with compartment ATP levels, as in the case of compartments generated by oxidative phosphorylation (11), continuing increase in rate of Glc phosphorylation might also be expected. *In either case*, it was clear that Glc phosphorylation supported with ATP generated solely by the

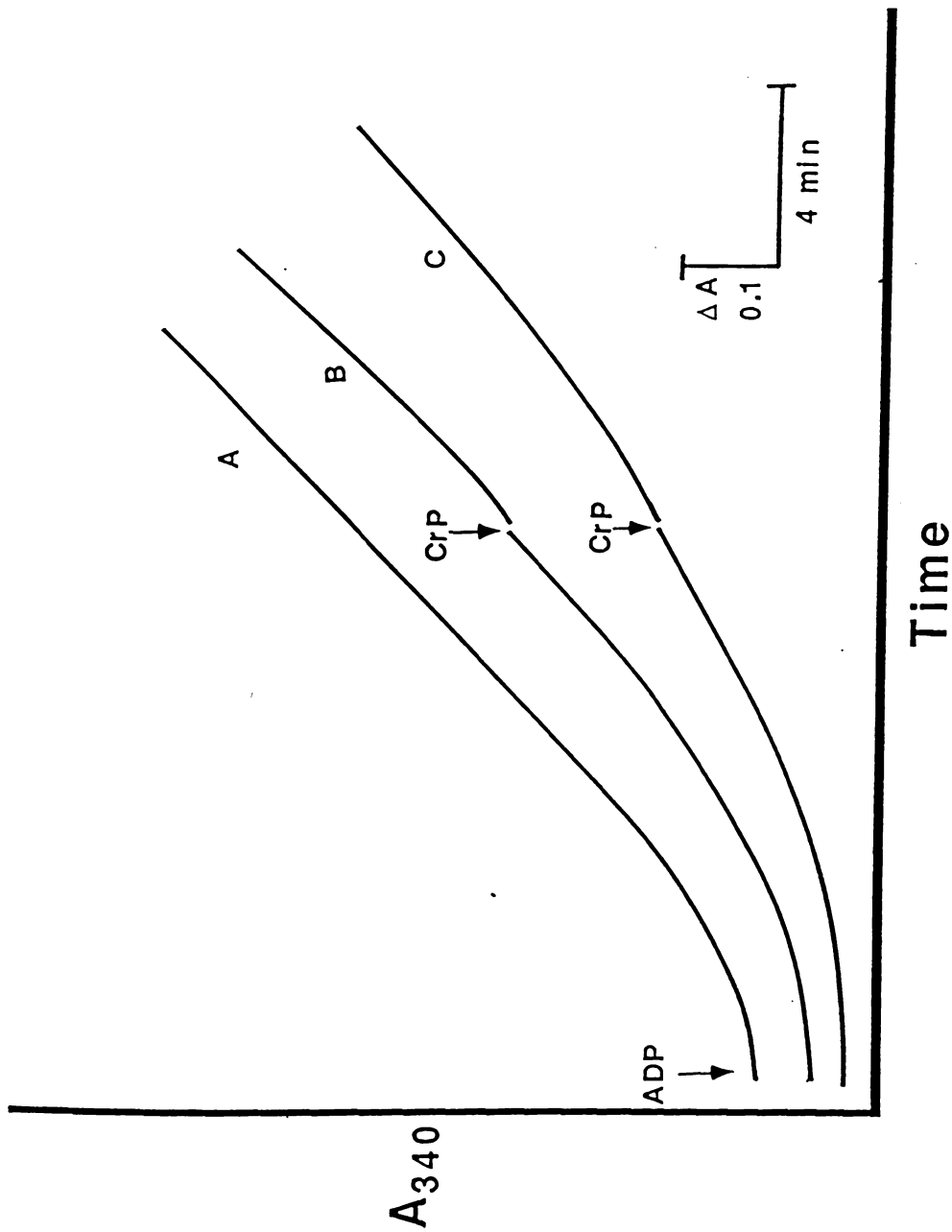
creatine kinase reaction was distinctly different in its kinetics than when oxidative phosphorylation was occurring (Curves A and B).

Despite the fact that *total* ATP production was comparable to that seen with oxidative phosphorylation alone (Table I), it was evident that, throughout the experimental period, ATP produced by creatine kinase was used much less effectively than was ATP produced by oxidative phosphorylation (compare Curves B and C in Fig. 2).

Are creatine kinase, oxidative phosphorylation, and hexokinase linked to a common pool of intramitochondrial ATP ? Addition of up to 20 mM creatine had no effect on the steady state rate of Glc phosphorylation supported with ATP from oxidative phosphorylation (Fig. 2, Curve B). Using extramitochondrial ATP as substrate and the coupled assay system described in Materials and Methods (which maintains ADP concentrations at a low level), creatine phosphate was produced at a rate of 0.38 ± 0.02 μmoles/min/mg mitochondrial protein (mean ± SD for 3 determinations), exceeding the rate of ATP production by oxidative phosphorylation (Table I). Hence, the inability of creatine kinase to divert a significant amount of ATP from the intramitochondrial pool supplying hexokinase with substrate (Fig. 2, Curve B), with resultant decrease in the rate of Glc phosphorylation (11), cannot be attributed simply to a paucity of creatine kinase activity. More likely this reflects the thermodynamic unfavorability of creatine phosphate synthesis with the levels of ADP present in the reaction medium.

The experiment shown in Fig. 3 demonstrates that ATP generated by creatine kinase can enter the compartment(s) supplying hexokinase with substrate. Here, as previously (11), the rate of oxidative phosphorylation and hence the levels of intramitochondrial substrate ATP and the rate of Glc phosphorylation dependent upon these levels, was manipulated by using a subsaturating level of pyruvate/malate as oxidative substrate. The steady state rate of Glc phosphorylation (Curve C) was approximately half that seen with saturating levels of pyruvate/malate (Curve B). Addition of creatine phosphate during the steady state supported with *subsaturating* pyruvate/malate (Curve C) evoked a second transient phase followed by an increased steady state rate that was equal to or slightly less (as in experiment shown in Fig. 3) than the steady state rate seen with oxidative phosphorylation and creatine kinase operating concurrently (Curve A); the latter was only slightly greater than the steady state rate supported solely by oxidative phosphorylation with saturating pyruvate/malate (Curve B). In contrast, addition of creatine phosphate to the reaction occurring with saturating pyruvate/malate (Curve B), had little effect on the rate of Glc phosphorylation.

Figure 3. Phosphorylation of glucose with ATP generated by submaximal levels of pyruvate/malate as substrate, with and without supplementation from the creatine kinase reaction. Curve A, Phosphorylation supported by saturating levels of pyruvate and malate (5 mM and 2.5 mM, respectively), with supplementation from creatine kinase (1.5 mM creatine phosphate present). B, Phosphorylation supported by saturating levels of pyruvate-malate, with addition of creatine phosphate *after* establishment of initial steady state. C, Phosphorylation supported by limiting amounts of oxidative substrates (0.1 mM pyruvate, 0.05 mM malate), with addition of creatine phosphate after establishment of initial steady state.

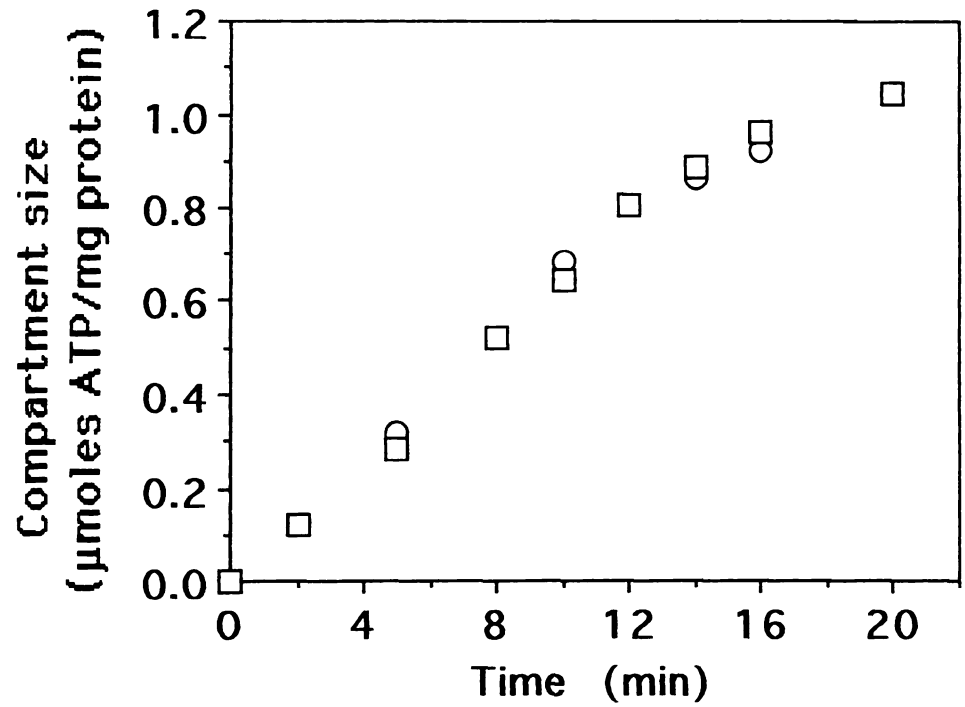

A
B
C
CrP
CrP
ADP
ΔA
0.1
4 min
Time
A340

Hence, whether production of ATP by creatine kinase is initiated before (Table I and Fig. 3, Curve A) or after (Fig. 3, Curve B) attainment of steady state supported by *maximal* rates of oxidative phosphorylation, the result is, at best, a modest increase in the rate of Glc phosphorylation. However, with submaximal levels of oxidative phosphorylation, previously shown (11) to result in diminished intramitochondrial ATP levels and thereby decreased rates of Glc phosphorylation by mitochondrial hexokinase, creatine kinase can supplement the intramitochondrial supply of ATP, with resulting increase in the rate of Glc phosphorylation to maximal or nearly maximal levels. This obviously implies that ATP generated by creatine kinase has access to the intramitochondrial compartment (or compartments) supplying hexokinase with substrate ATP.

It is important to contrast these results with those seen when creatine kinase alone is the source of ATP, i.e., when oxidative phosphorylation is *not* occurring (Fig. 2, Curve C). As noted above, under these conditions there is a continuing increase in rate throughout the experimental period. Only in the presence of active oxidative phosphorylation, with or without concurrent ATP production by creatine kinase, are steady state rates of Glc phosphorylation attained. This is consistent with the view (10,11) that oxidative phosphorylation *induces* formation of intramitochondrial compartments of ATP upon which hexokinase becomes solely dependent for substrate; additional support for this conclusion is presented below.

Release of intramitochondrially compartmented ATP produced by creatine kinase. Addition of KCN or CAT results in release of ATP from intramitochondrial compartments receiving ATP from oxidative phosphorylation (10). Similar results were seen with addition of KCN during ATP production by creatine kinase (Fig. 1, Curve 2). The results shown in Fig. 4 indicate that a KCN-sensitive compartment is filled in a time-dependent manner, and that the amount of compartmented ATP is directly proportional to the amount of mitochondria (i.e., when expressed on a per mg mitochondrial protein basis, the amount of compartmented ATP is independent of the amount of mitochondria used in the experiment). The KCN-sensitive compartment resulting from ATP production *via* creatine kinase is, however, different from that formed during oxidative phosphorylation (10) in at least two respects. First, even though, under these conditions, ATP is produced at nearly identical rates by either oxidative phosphorylation or creatine kinase (Table I), the KCN-sensitive compartment supplied by creatine kinase requires approximately 20 min to fill (Fig. 4), in contrast to intramitochondrial compartments filled within 10 min after initiation of ATP formation by oxidative phosphorylation (10). Furthermore, the amount of compartmented ATP with creatine kinase as source, approximately 1 μmole ATP/mg mitochondrial protein (Fig. 4), is more than twice that found in compartments generated by oxidative phosphorylation (10). Hence, despite their similarity in response to KCN, it seems likely that the KCN-sensitive compartments generated by oxidative phosphorylation and by creatine kinase are distinct.

Figure 4. Intramitochondrially compartmented ATP generated by creatine kinase. Intramitochondrially compartmented ATP was determined (see text and Fig. 1) at the indicated times after initiation of ATP formation by creatine kinase. Reactions contained either 0.04 (□) or 0.08 (O) mg mitochondrial protein, and compartmented ATP is expressed on a per mg protein basis.their similarity in response to KCN, it seems likely that the KCN-sensitive compartments generated by oxidative phosphorylation and by creatine kinase are distinct.

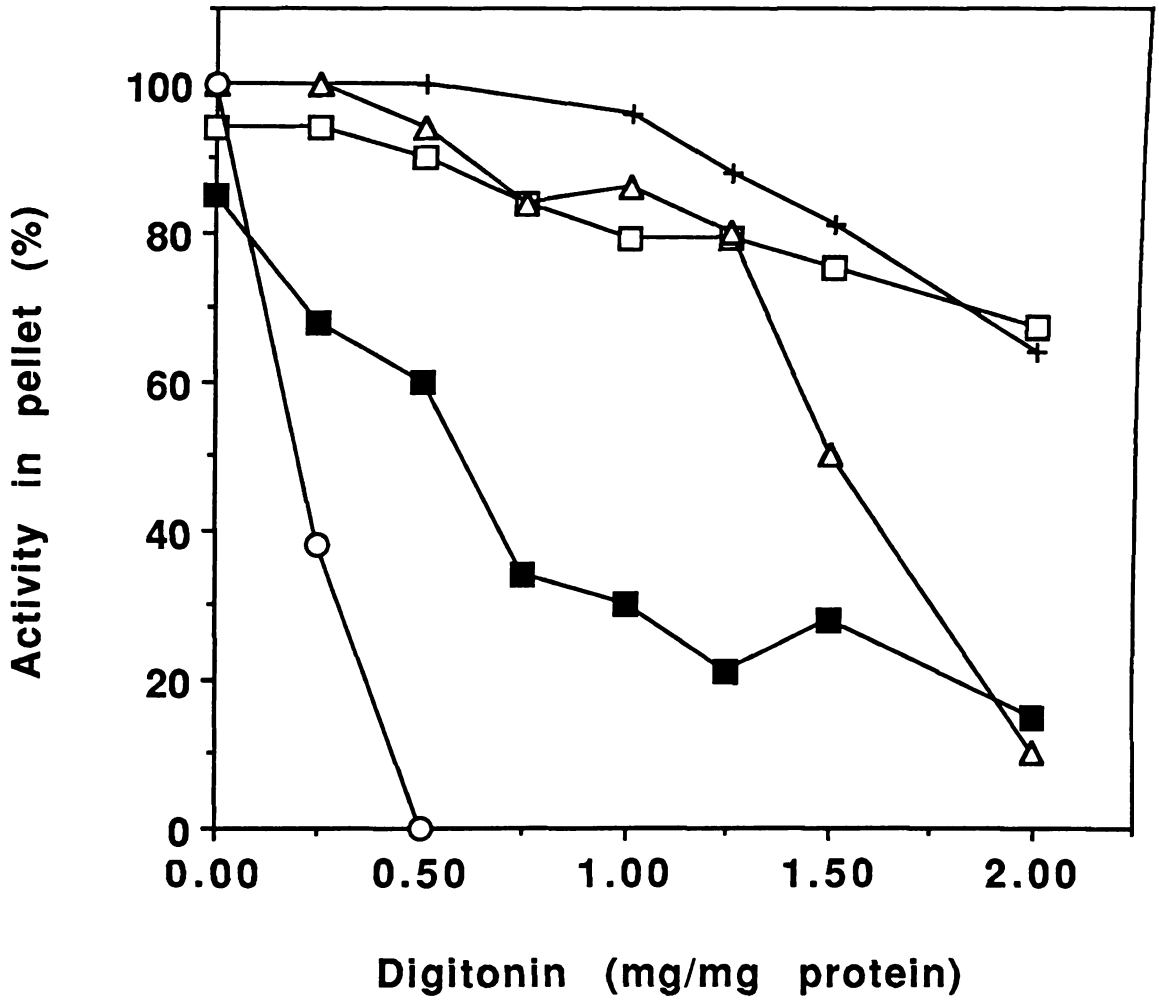

Compartment size
(µmoles ATP/mg protein)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
4
8
12
16
20
Time (min)

We have previously noted (10) that release of intramitochondrially compartmented ATP generated by oxidative phosphorylation was apparently *not* attributable to the action of KCN as an inhibitor of electron transport; this was based on the observation that azide, which is similar to KCN in its effect on electron transport, did *not* cause comparable release of ATP. The present results also support this conclusion, i.e., KCN-induced release of ATP generated by creatine kinase cannot be attributed to inhibitory action since 5 mM KCN had no detectable effect on creatine kinase activity. The mechanism by which KCN evokes release of compartmented ATP remains unknown.

In contrast to results obtained with oxidative phosphorylation as ATP source (10,11), *no* compartmented ATP was detected after addition of CAT to mitochondria generating ATP by the creatine kinase reaction. Thus, it appears that the CAT-sensitive compartment may be uniquely associated with oxidative phosphorylation.

Digitonin fractionation of mitochondria. Results of digitonin fractionation experiments (Fig. 5) were in general agreement with previous work from this (11) and other (6,8,12) laboratories. Rupture of the outer mitochondrial membrane occurred at relatively low digitonin concentrations, causing the release of adenylate kinase, even when most of the monoamine oxidase remained associated with the pellet. The inner mitochondrial membrane is relatively resistant to digitonin, with release of the matrix enzyme, fumarase, being seen only at rather high concentrations of digitonin.

Figure 5. Digitonin fractionation of rat brain mitochondria. Digitonin treatment was performed as described in Materials and Methods, and activities of adenylate kinase (O), creatine kinase (■), hexokinase (□), monoamine oxidase (Δ), and fumarase (+) were determined in both supernatant and pellet. Results are expressed as percentage of the total activity remaining in particulate form after treatment of the mitochondria with the indicated concentration of digitonin.


100
80
60
40
20
0
0.00
0.50
1.00
1.50
2.00
Activity in pellet (%)
Digitonin (mg/mg protein)

Hexokinase is located in digitonin resistant regions, with about 70% of the activity remaining in the pellet even at concentrations high enough to release virtually all of the monoamine oxidase.

The release of creatine kinase appears to be bimodal, with about 70% of the activity being released at relatively low concentrations (less than 0.75 mg digitonin/mg mitochondrial protein); the remaining 30% is more resistant to digitonin, with about 10% of the total activity remaining in the pellet even after treatment with 2 mg digitonin/mg mitochondrial protein. The results of Kottke *et al.* (12) suggest that it is the more digitonin-resistant creatine kinase that is associated with contact sites.

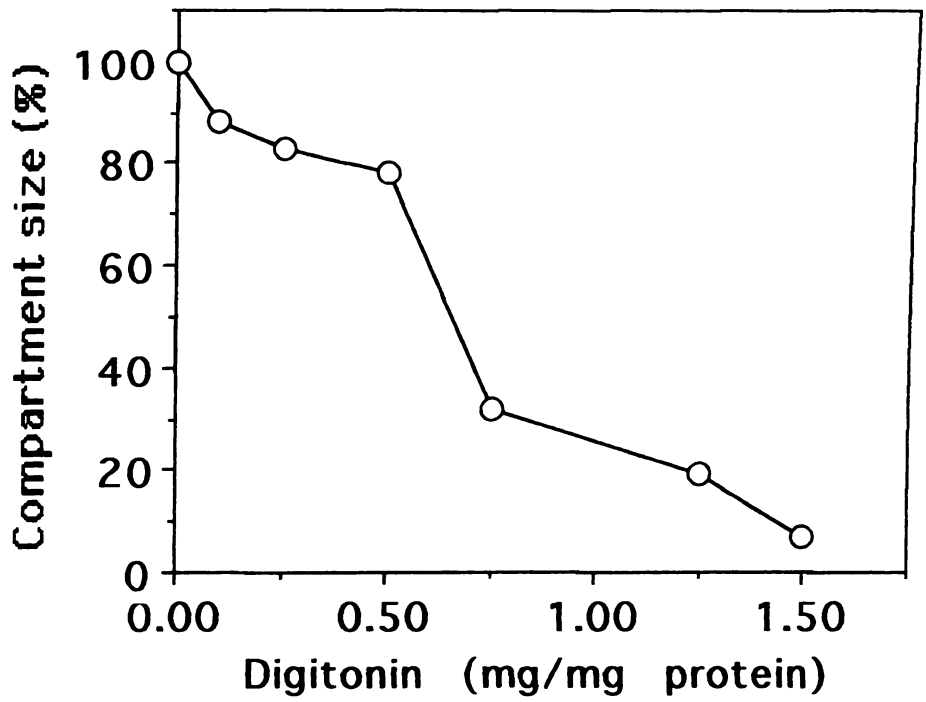
The digitonin sensitivity of intramitochondrially compartmented ATP, generated by the creatine kinase reaction, was also examined (Fig. 6). *If* this compartment were selectively filled by creatine kinase present in digitonin resistant regions (presumably contact sites), it would be expected that removal of creatine kinase from digitonin sensitive regions (Fig. 5) that were not coupled to this compartment would have little effect on the amount of compartmented ATP. This was not observed; the effect of digitonin on compartmented ATP levels was quite similar to its effect on release of creatine kinase itself (Fig. 5). The resistance of the creatine kinase-generated ATP compartment was considerably less than that seen with ATP compartments generated by oxidative phosphorylation (11), e.g., only 30-40% disruption of the latter with 1 mg digitonin/mg mitochondrial protein whereas this same digitonin concentration evoked release of about 80% of the ATP from the creatine kinase-generated compartment (Fig. 6). This is

consistent with the suggestion that these compartments are distinct entities (see above).

It should also be noted that low concentrations of digitonin had relatively little effect on compartmented ATP generated by creatine kinase (Fig. 6), whereas the marker for the intermembranal space, adenylate kinase, is completely released at 0.5 mg digitonin/mg mitochondrial protein (Fig. 5). Thus, little if any of the compartmented ATP generated by creatine kinase appears to be associated with the intermembranal space.

Dependence of mitochondrially bound hexokinase on intramitochondrial ATP is induced by initiation of oxidative phosphorylation. As shown in the previous study (10), when oxidative phosphorylation is the source of ATP for the hexokinase reaction, hexokinase obtains its substrate ATP from intramitochondrial compartments and is oblivious to gradual changes in the concentration of extramitochondrial ATP. The results obtained during the course of the present work are consistent with this. The rate of ATP utilization by mitochondrial hexokinase is nearly the same whether or not ATP generated by oxidative phosphorylation is supplemented with ATP production by creatine kinase, even though the total rate of ATP production is virtually doubled when both processes are functioning concurrently (Table I), with resultant increase in the rate at which extramitochondrial ATP is accumulating. Thus, *during active oxidative phosphorylation*, mitochondrial hexokinase remains unresponsive to metabolically generated increases in extramitochondrial ATP and exhibits a steady

Figure 6. Effect of digitonin on intramitochondrially compartmented ATP generated by creatine kinase. Intramitochondrially compartmented ATP, generated by creatine kinase during an 8 min reaction period, was determined as described in the text and illustrated in Fig. 1. Equivalent amounts of mitochondria, treated with the indicated concentration of digitonin, were compared. Results are expressed as a percentage of the compartmented ATP present in mitochondria not treated with digitonin.


Compartment size (%)
100
80
60
40
20
0
0.00
0.50
1.00
1.50
Digitonin (mg/mg protein)

state rate of Glc phosphorylation governed by levels of intramitochondrial ATP.

Shown in Figs. 7 and 8 are results which provide further evidence for the dependence of mitochondrially bound hexokinase on intramitochondrial ATP reserves during active oxidative phosphorylation. Formation of ATP was initiated by addition of ADP, with increasing concentrations of ATP present at the time of ADP addition, and the rate of Glc phosphorylation by endogenous mitochondrially bound hexokinase was determined as described in Materials and Methods. It is apparent that the initial rate of Glc phosphorylation depends markedly on the concentration of extramitochondrial ATP present at the time of ADP addition. However, after a transient period, a steady state rate of Glc phosphorylation *which is independent of the amount of ATP present initially* is attained.

Initial and final steady state rates calculated from the results shown in Fig. 7 are presented in Fig. 8. Also shown are initial and steady state rates determined in a parallel experiment in which the rate of oxidative phosphorylation, and thereby the intramitochondrial concentration of ATP upon which Glc phosphorylation by mitochondrially bound hexokinase is dependent (11), was decreased by limiting the concentration of P_i available as a substrate for oxidative phosphorylation. The *initial* rate of Glc phosphorylation exhibits an apparent at the time of ADP addition, with an apparent K_m of approximately 0.25 mM in agreement with earlier determinations (10), and does *not* depend on the concentration of P_i present. In contrast, the

Figure 7. Phosphorylation of glucose by mitochondrial hexokinase, with ATP generated by oxidative phosphorylation and with increasing concentrations of extramitochondrial ATP present at the time of initiation of oxidative phosphorylation. Glc-6-P production was monitored (by coupling to NADPH formation as described in Materials and Methods) as a function of time after initiation of oxidative phosphorylation. Also added to the initial reaction medium were various concentrations of ATP; these were 1.1 mM, 0.66 mM, 0.22 mM, and 0 mM for Curves A-D, respectively. steady state rate does not depend on the initial ATP concentration, but *is* dependent on the rate of oxidative phosphorylation, as determined by the concentration of P_i.

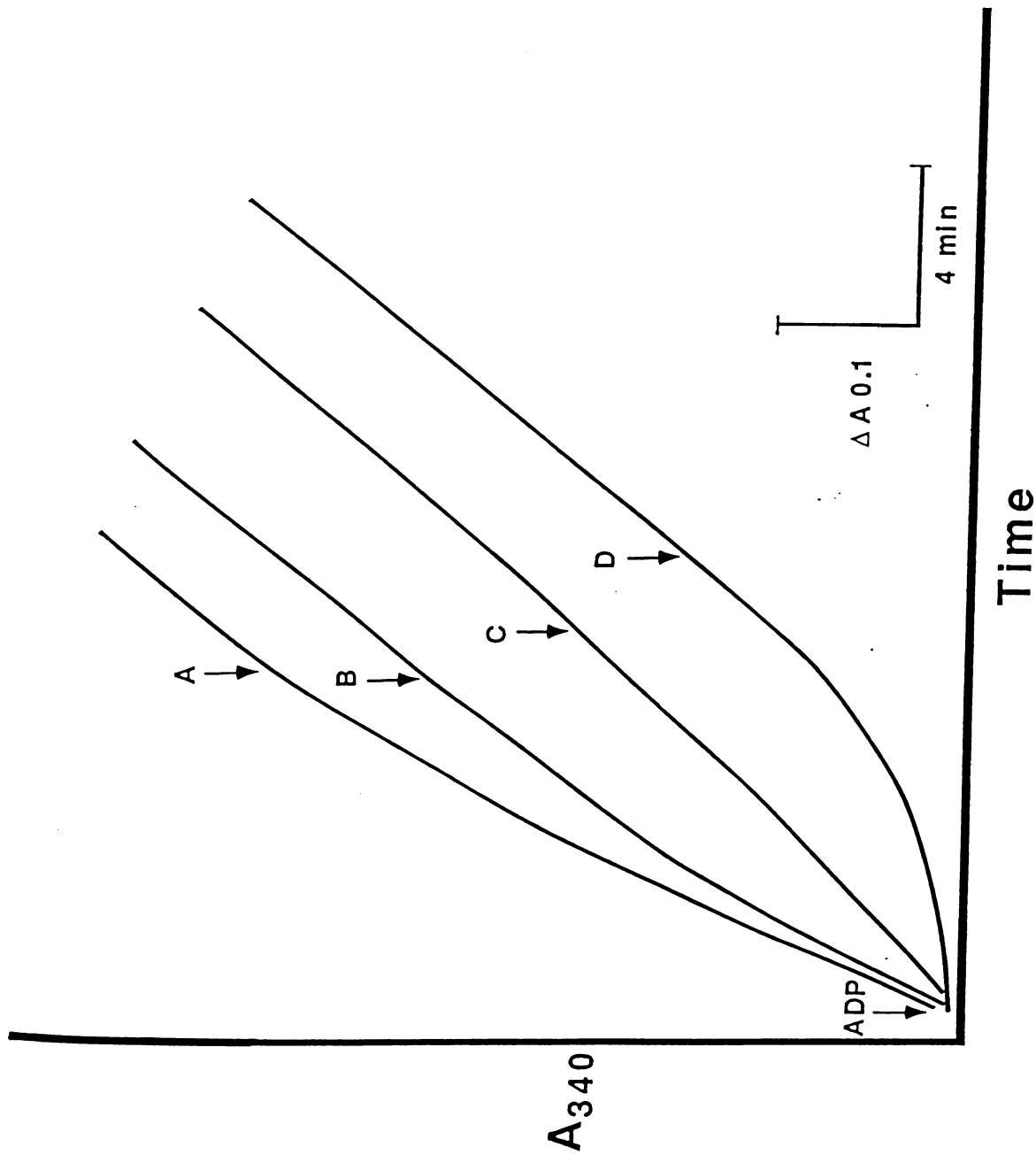
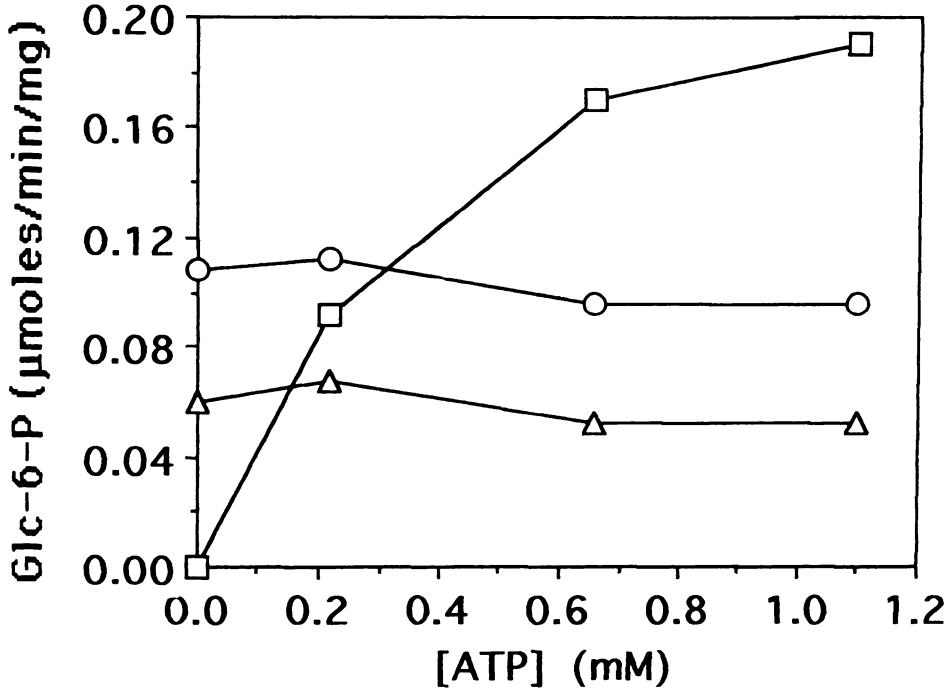

A
B
C
D
ADP
ΔA 0.1
4 min
A340
Time

Figure 8. Initial and steady state rates of glucose phosphorylation, with ATP generated by oxidative phosphorylation in the presence of increasing concentrations of extramitochondrial ATP. The initial (□) and steady state (○) rates of Glc phosphorylation for the experiment shown in Fig. 6 are indicated as a function of the initial ATP concentration. This experiment was done with 3 mM P_i present in the medium, a saturating concentration for support of oxidative phosphorylation. Analogous rate measurements were made using subsaturating (0.6 mM) P_i. The steady state rate of Glc phosphorylation (Δ) was approximately half of that seen with saturating P_i, and was again independent of extramitochondrial ATP concentration; initial rates were indistinguishable from those seen in the presence of 3 mM P_i (■).


0.20
0.16
0.12
0.08
0.04
0.00
Glc-6-P (µmoles/min/mg)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
[ATP] (mM)

Thus initiation of active oxidative phosphorylation induces a state in which mitochondrial hexokinase becomes solely dependent on intramitochondrial ATP as a substrate, and independent of extramitochondrial ATP concentrations even though these are clearly substantial, e.g., for the experiment shown in Curve A of Fig. 7, more than 90% of the added ATP remained at a time when mitochondrially bound hexokinase reverted to total dependence on intramitochondrial reserves, as indicated by attainment of a steady state rate independent of extramitochondrial ATP concentration.

The time required for attainment of steady state (7-9 min) did not depend on the concentration of ATP present initially. Since this corresponds to the time required to fill intramitochondrial compartments with ATP generated by oxidative phosphorylation (10), this implies that the rate of oxidative phosphorylation was itself unaffected by the varying concentrations of ATP present initially. This was confirmed in two ways. First, polarographic measurement of oxygen uptake (results not shown) demonstrated that mitochondrial respiration was unaffected by the presence of ATP concentrations in the range used here (0-2.2 mM). Second, quantitation of total ATP production (Table II) demonstrated that this was not affected by the ATP present at the time oxidative phosphorylation was initiated. For the samples containing no added ATP, measurement of the rate of ATP production as described in Materials and Methods - recall that this is measured in the presence of excess yeast hexokinase, which maintains extramitochondrial

Table II. ATP Production by Oxidative Phosphorylation in the Presence of Increasing Extramitochondrial Concentrations of ATP[a]

Initial ATP[b]	P_i[c]	Final ATP[d]	ATP Utilized[e]	Total ATP Produced[f]
0	0.6	0.13	0.05	0.18
0.22	0.6	0.33	0.06	0.17
0.66	0.6	0.77	0.09	0.20
1.10	0.6	1.19	0.12	0.21
0	3.0	0.23	0.11	0.34
0.22	3.0	0.44	0.14	0.36
0.66	3.0	0.83	0.14	0.31
1.10	3.0	1.29	0.17	0.36

[a]All concentrations are expressed in units of μmoles/ml (mM).

[b]ATP present at the time oxidative phosphorylation was initiated by addition of ADP.

[c]The rate of oxidative phosphorylation was dependent on the concentration of P_i; 0.6 mM P_i gave a rate approximately half that seen with saturating levels (e.g., 3 mM) of P_i.

[d]Determined by assay of ATP levels present 16 min after initiation of oxidative phosphorylation by addition of ADP.

[e]Equivalent to the amount of Glc-6-P formed by hexokinase during the 16 min reaction period, as determined from NADPH produced *via* the coupled Glc-6-P dehydrogenase reaction.

[f]Equal to the sum of the ATP present at the end of the reaction period plus the amount of ATP utilized for phosphorylation of Glc during the reaction period, minus the amount of ATP added initially.

ATP at negligible levels - was also possible. The amounts of ATP that would be formed during the 16 min reaction period were calculated to be 0.17 mM and 0.35 mM in the presence of 0.6 mM and 3 mM P_i, respectively. These are in excellent agreement with the values (Table II) determined for the reaction in the absence of yeast hexokinase, with accumulation of extramitochondrial ATP (10), providing further indication that the latter did not affect the rate of oxidative phosphorylation.

Concluding comments. Creatine/creatine phosphate metabolism in brain is surely complex. Mitochondrial and cytoplasmic forms of creatine kinase exist, each presumably serving specific roles in linking energy metabolism to cellular function (13). *In vivo* NMR studies (20) suggest that additional compartmentation of creatine phosphate metabolism may occur at the cellular level, e.g., glial *vs.* neuronal. The present studies obviously do not attempt to address this complexity. They do, however, demonstrate that at least some fraction of mitochondrial creatine kinase is associated with an intramitochondrial compartment, or compartments, upon which mitochondrially bound hexokinase relies for substrate. That this occurs at contact sites is suggested by previous studies on localization of creatine kinase (6,7,12,13), hexokinase (6-8), and intramitochondrial compartments upon which, during active oxidative phosphorylation, hexokinase is dependent (11). Although creatine kinase may introduce ATP into this compartment, it is likely that, under *in vivo* conditions and with physiological levels of

ADP, this creatine kinase functions primarily in the direction of creatine phosphate synthesis (13).

The present study includes additional evidence supporting the previous conclusion (10,11) that oxidative phosphorylation induces formation of a specific compartment (or compartments) which is (are) directly coupled to mitochondrial hexokinase activity. Oxygen consumption and oxidative phosphorylation are characteristic of normal cerebral function - indeed, their cessation is catastrophic. Hence, it follows that dependence of mitochondrial hexokinase on intramitochondrially compartmented ATP is normally the situation in brain. We have previously suggested (11) that the rate of oxidative phosphorylation, compartmented ATP levels, and the rate of Glc phosphorylation by mitochondrial hexokinase are coordinated and responsive to cellular energy status as reflected by levels of ADP. If indeed the creatine kinase reaction were maintained near equilibrium at all times, the substantial cerebral stores of creatine phosphate (21-23) would effectively buffer ADP levels, and the latter could then obviously not be a significant factor in regulation of cerebral glycolytic and oxidative metabolism. While, to a first approximation, the components of the creatine kinase reaction may be maintained near equilibrium (13,23), it is nonetheless clear that substantial changes in ADP levels do occur with altered energy status (21,22). It is likely that compartmentation of nucleotides plays a major role in linking changes in energy demand with appropriate, and coordinated, increases in cerebral glycolytic and oxidative metabolism.

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Chapter V

Crosslinking of Rat Brain Hexokinase Bound to Mitochondria from Rat Brain and Rat liver

Introduction

Hexokinase of rat brain is bound to the outer mitochondrial membrane through interactions with porin, the outer membrane protein that forms the pores through which metabolites enter and exit the mitochondria (1-3). Hexokinase preferentially binds to porin molecules presumably located at the contact sites between the inner and the outer mitochondrial membranes (4,5, Chapter III of this thesis), and obtains substrate ATP from either or both of two intramitochondrial compartments, presumably also located at the contact sites (Chapter III).

Xie and Wilson reported that hexokinase bound to liver mitochondria is present as a monomer and as a tetramer (6), but the physiological implications of these findings are not clear. No studies have been done to determine if hexokinase bound to mitochondria from other tissues is also present in the monomeric and tetrameric forms, and if these two forms bind to distinct regions of the mitochondrial membrane. It is important to determine if one of the two forms (i.e. monomer or tetramer) of hexokinase is preferentially bound to the contact sites; if this were the case, the form that binds to the contact sites would most likely be the more active form.

Also, the contact sites contain less cholesterol than regions of the outer membrane that are not in contact with the inner membrane (4). Cholesterol content of the various regions of the outer

mitochondrial membrane could be a factor that determines the binding of the monomer or tetramer to distinct regions.

In the present study, hexokinase from rat brain was derivatized using SAND, a photoactivatable crosslinking reagent that contains a disulfide group, an azidophenyl group and a succinimidyl group. SAND reacts with free amino groups on the surface of proteins through its succinimidyl group. Subsequent photoactivation of the azidophenyl group results in cross-linking due to the formation of a highly reactive nitrene that reacts nonspecifically and very avidly with adjacent molecules (6).

Derivatized hexokinase was bound to control and digitonin-treated mitochondria to determine if either the tetramer or monomer of hexokinase preferentially binds to the contact sites. Derivatized hexokinase was also bound to cholesterol enriched mitochondria to investigate if the cholesterol content of the membrane affects the relative amount of tetramer and monomer.

Materials and Methods

Materials. Cholesterol and Sephadex G-10-120 were purchased from Sigma Chemical Co. (St. Louis, MO), Sephadex G-25 from Pharmacia, and digitonin from Merck (Darmstadt, Germany). Immobilon-P was purchased from Millipore (Bedford, MA) and nitrocellulose (pore size 0.45 μm) from Schleicher & Schuell (Keene, NH). Goat anti-rabbit Ig G-horseradish peroxidase complex, and protein molecular weight markers (myosin, 205,000; ß-galactosidase, 116,000; phosphorylase b, 97,000; bovine albumin, 66,000 and egg albumin, 45,000) were purchased from Bio-Rad Laboratories (Richmond, CA). SAND was obtained from Pierce (Rockford, IL), and thin layer chromatography plates were obtained from Whatman Chemical Separation Inc. (Clifton, NJ). All other chemicals were of reagent grade and obtained from various commercial sources.

Enzymatic and protein assays. Adenylate kinase (7), fumarase (8), hexokinase (9) and monoamine oxidase (10) were assayed as previously described. Protein was determined using the BCA method of Pierce Chemical Co. (Rockford, IL) with bovine serum albumin as standard.

Mitochondrial isolation and treatment with Glc-6-P. Rat brain mitochondria were isolated using the method of Lai and Clark (11) as

described in appendix A of this thesis. The final suspension contained approximately 8 mg protein and 3.2 units of endogenously bound hexokinase. Before using these mitochondria for the experiments described below, they were treated with Glc-6-P to remove 85-90% of the hexokinase. Mitochondrial suspension was diluted 1 : 5 in "isolation medium" containing 0.25 M sucrose, 0.1 mM EGTA, and 5 mM Mops, pH 7.4. The suspension was made 1 mM in Glc-6-P, incubated for 10 minutes at room temperature and centrifuged at 11,000 r.p.m. using an SS-34 rotor (Ivan Sorvall Inc., Norwalk CN). The pellet was resuspended in fresh isolation medium to restore the initial protein concentration (8 mg/ml) and both pellet and supernatant assayed for hexokinase activity.

Liver mitochondria were isolated using the method of Johnson and Lardy (12) and resuspended in isolation medium to a final concentration of 20 mg/ml.

Cholesterol enrichment of mitochondria. Mitochondria from rat liver and rat brain were enriched in cholesterol using the procedure of Parlo and Coleman (13) with some modifications. Sephadex G-10-120 beads (5 g) were washed with 250 ml of acetone in a round bottom flask, most of the acetone decanted, and the beads were taken to dryness in a rotary evaporator. The washed beads (1 g) were added to 50 ml acetone in which 39 mg of cholesterol had been dissolved, taken to dryness in a rotary evaporator and saved at room temperature in a dark container.

Approximately 2 ml of mitochondrial suspension were used for the procedure. One ml was mixed with 200 mg of cholesterol

enriched Sephadex beads, and 1 ml with 200 mg of plain washed beads as a control. A 30 minute incubation in 1.5 ml microcentrifuge tubes was carried out under constant shaking, using a Labquake (Labindustries, Berkeley, CA.) at approximately 16 cycles per minute. The resulting suspension was layered on about 25 ml of 60 % sucrose and centrifuged at 1,500 r.p.m. for 10 minutes using an HB-4 rotor (Ivan Sorvall Inc. Norwalk, CN). The mitochondria were recovered from the top of the gradient, combined with 25 ml of isolation medium and centrifuged at 15,000 r.p.m. for 10 minutes in an SS-34 rotor. The pellet was resuspended in 0.5 ml of fresh isolation medium and the relative amount of cholesterol of the control and the cholesterol enriched mitochondria was determined by thin layer chromatography.

Thin layer chromatography to determine the relative cholesterol content of mitochondria. Approximately 200 µl of the cholesterol treated mitochondrial suspension, and 200 µl of the mitochondrial suspension treated with plain washed beads were used for this procedure. After determining the fumarase activity in both suspensions, they were centrifuged for 5 minutes in a microcentrifuge and the supernatant discarded. The pellets were resuspended in 1 ml of a 2 : 1 solution of chloroform and methanol, vortexed vigorously and centrifuged for 10 minutes. The supernatant was saved and the procedure repeated. The supernatants from both centrifugations were washed twice with 0.2 ml of buffer containing 10 mM sodium phosphate and 150 mM sodium chloride, pH 7.4 and dried under nitrogen. The resulting material was redissolved in

chloroform (approximately 50 µl), to give lipids from equivalent amounts of mitochondria based on fumarase activity. Samples were spotted on a thin layer chromatography plate (K-5 silica gel) 5 µl on each lane, including a sample containing purified cholesterol used as a marker. The plate was then placed in a beaker that had filter paper around the internal walls with the bottom submerged in a solution containing 85 ml chloroform, 5 ml methanol and 0.8 ml ammonium hydroxide. Enough solution was used to cover the bottom of the beaker; the fluid level was about 1 cm. The filter paper was allowed to absorb this solution until completely wet before the plate was placed in the beaker. The bottom 1 cm of the plate was also submerged in the solution (without touching the spotted samples) and left in it until the solution got to the top of the plate. The plate was allowed to air dry and then stained in a tank containing iodine vapor.

Digitonin treatment of mitochondria. The digitonin stock solution (5 mg/ml) was made in isolation medium. Mitochondrial suspension (100 µl) was added to 0.4 ml of isolation medium containing the indicated amount of digitonin. This was followed by a two minute incubation at room temperature and then centrifuged for 2 minutes in a microcentrifuge. The pellet was resuspended in fresh isolation medium to restore original protein concentration.

Derivatization of rat brain hexokinase with SAND. Hexokinase (0.75 mg) from rat brain purified according to Wilson (9) was chromatographed on a column of Sephadex G-25 equilibrated with

Buffer A containing 10 mM potassium phosphate, 10 mM Glc and 0.5 mM EDTA, pH 7.5. Fractions (0.5 ml) were collected and the hexokinase peak found by measuring the absorbance at 280 nm. The three fractions with the most protein were pooled and concentrated using a Centricon 30 device (Amicon Corp., Danvers, MA) to approximately 1 mg per ml.

The rest of the procedure was done in subdued light to avoid the activation of SAND. SAND (10 µl of a 1 mM solution made in Buffer A) was added to approximately 200µl of the concentrated hexokinase suspension. A 30 minute incubation at room temperature was followed by addition of 0.1 volume of 1 M lysine pH 7.5 to scavenge unreacted SAND. Incubation continued on ice for another 30 minutes.

Binding of hexokinase to rat brain mitochondria. Mitochondria that had been treated with Glc-6-P to remove the endogenous hexokinase, were enriched in cholesterol or treated with digitonin as described above, followed by binding of derivatized hexokinase using the following procedure. Mitochondrial suspension (about 3.2 mg protein) was centrifuged for 5 min in a microcentrifuge and resuspended in 400 µl of Buffer B containing 20 mM Hepes, 250 mM sucrose, 51 mM NaCl and 3 mg/ml BSA. The resulting suspension was incubated on ice for 30 minutes with 5 mM $MgCl_2$ and 20 µg of derivatized hexokinase and then centrifuged for 7 minutes in a microcentrifuge. The pellet was resuspended in 400 µl of 0.25 M sucrose, centrifuged for 5 minutes and the resultant pellet resuspended again in 400 µl of the same solution. The hexokinase

activity of the final suspension was assayed as previously described (9).

Binding of hexokinase to rat liver mitochondria. The procedure for binding hexokinase to liver mitochondria was the same as that used for brain mitochondria, except that mitochondrial suspension containing approximately 6 mg of protein was used.

Crosslinking of derivatized hexokinase bound to mitochondria. The mitochondrial suspension (400 µl in 0.25 M sucrose) containing derivatized hexokinase was divided into two fractions. One fraction (200 µl) was transferred to a glass spot plate (Corning No. 7220) for photolysis as described by Kiehm and Ji (14), and exposed to 5 flashes using a xenon electronic flash from which the front window had been removed. The rest of the suspension was kept in subdued light to be used as the non-photolyzed control for that sample. Both suspensions were centrifuged for 5 minutes and the supernatant discarded. The pellets were processed for SDS-PAGE electrophoresis as described below.

SDS-PAGE and immunoblotting. SDS-PAGE was performed on a linear 6.5-20 % acrylamide gradient. The pelleted mitochondria, resulting from the cross-linking procedure described above, were suspended in 150 µl of sample buffer containing 0.063 M Tris, pH 6.8, 2% SDS, 4% glycerol, 0.005% bromophenol blue and 8 mM NEM. Enough sample was loaded to contain approximately 0.1 unit of

hexokinase activity in all cases. The gel ran at 50 millivolts for about 16 hours, until the dye was at the bottom of the plates.

Protein transfer was done using either a tank system from Hoefer Scientific Instruments (San Francisco, CA) or a Trans-Blot-SD semi-dry gel system from Bio-Rad Laboratories (Richmond, CA). For the transfer done in the tank system, a buffer containing 10 mM sodium bicarbonate, 3 mM sodium carbonate and 25 % methanol in 5 liters was used (15). The protein was transferred using a power supply at 400 milliamps in the constant current mode for 3 hours. The blot was developed using the protocol previously described (16) with some modifications. All incubations were done at room temperature on a shaker at slow speed. Blocking was done with 5 % milk in TBS (20 mM Tris, pH 7.5 and 0.5 M sodium chloride) for 1 hour, the membrane was rinsed three times with TBS to remove the remainder of the milk, and then incubated with rabbit anti-hexokinase serum diluted 1 : 1000 in 1 % gelatin for 2 hours. After incubation with the serum, the membrane was washed with TBS + 0.05 % Tween 20 twice, for 10 minutes each time. This was followed by incubation with a 1 : 2000 dilution of goat anti-rabbit HRP complex for 1 hour, followed by three washes with TBS + 0.05 % Tween 20 for 10 minutes each time. The staining (17) was done with a coloring reaction prepared, just before use, by adding 18 mg NBT, 80 mg NADH, 0.12 ml phenol and 0.04 ml hydrogen peroxide to 60 ml of a 50 mM solution of sodium phosphate pH 7.

For the semi-dry system, the protein was transferred to an Immobilon-P membrane. The membrane was soaked in methanol for about 1 minute and then, the membrane, the filter paper and the gel

were soaked for 5-10 minutes in buffer containing 48 mM Tris, 39 mM glycine, 5 % methanol and 0.0375 % SDS (18). The protein transfer was done in one hour; 15 volts for 15 minutes, 20 volts for 30 minutes and 25 volts for 15 minutes. The limit was set at 0.5 amps.

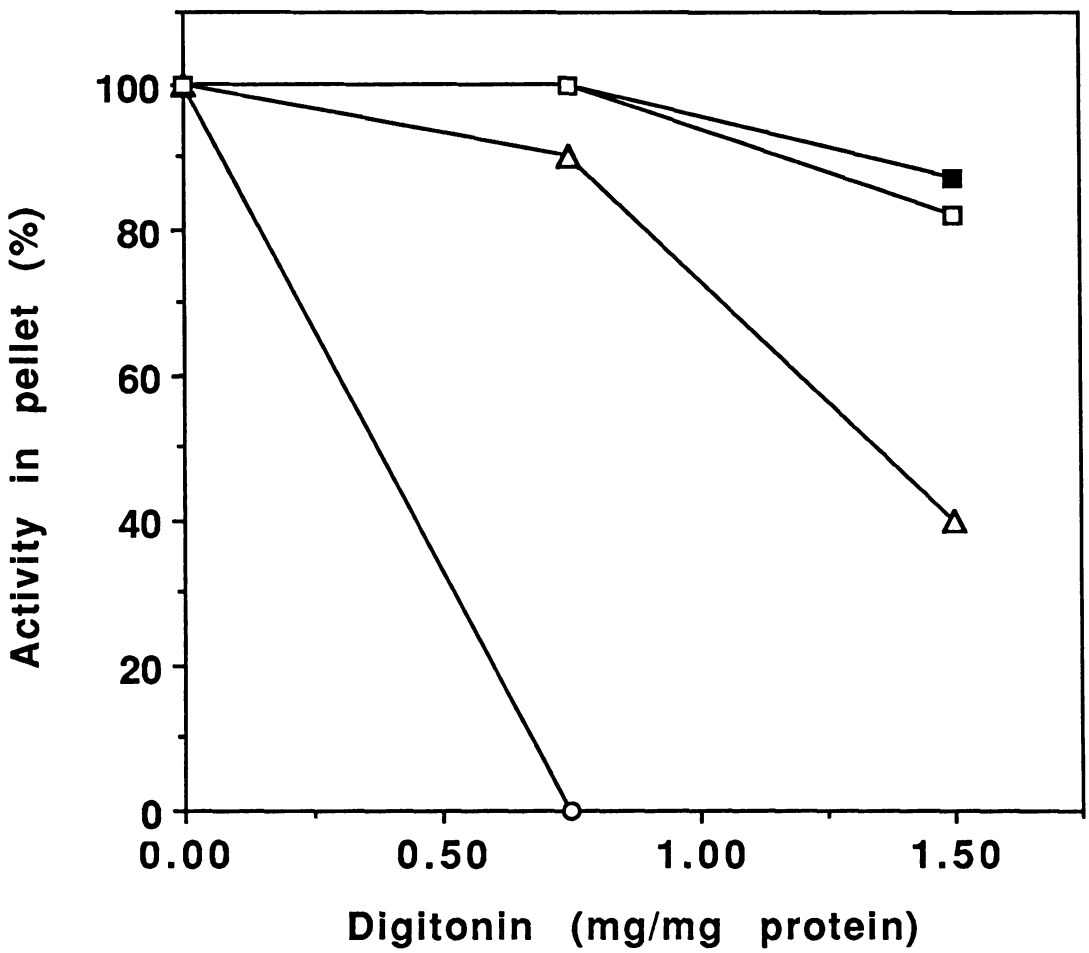
Development of the blot was started by blocking with 2 % gelatin at 37°C for 1 hour; the rest of the procedure, starting with the primary antibody, was as described above.

Results

Binding of derivatized and underivatized hexokinase to liver and brain mitochondria. Approximately 60-70% of the derivatized or underivatized hexokinase added to brain mitochondria remained bound to mitochondria under the conditions used in these experiments. When liver mitochondria were used, the percentage was lower, 40-50 % for both derivatized and underivatized hexokinase.

Digitonin fractionation and binding of derivatized hexokinase to digitonin treated rat brain mitochondria. Mitochondria from which 85-90 % of the endogenous hexokinase had been removed by treating with Glc-6-P, were treated with increasing amounts of digitonin (0, 0.75 and 1.5 mg/mg protein) either before or after the binding of derivatized hexokinase. Figure 1 shows the amount of hexokinase that bound to digitonin treated mitochondria, and the amount of hexokinase that remained in the pellet after digitonin treatment, expressed as a percentage of the hexokinase activity bound to non-treated mitochondria. The hexokinase activity associated with the mitochondria treated with 0.75 mg digitonin/mg protein was the same as the control (100 %) whether the treatment was done before or after the binding of the enzyme. The hexokinase activity associated with mitochondria treated with 1.5 mg digitonin/mg protein was about 80 % of that present in the control

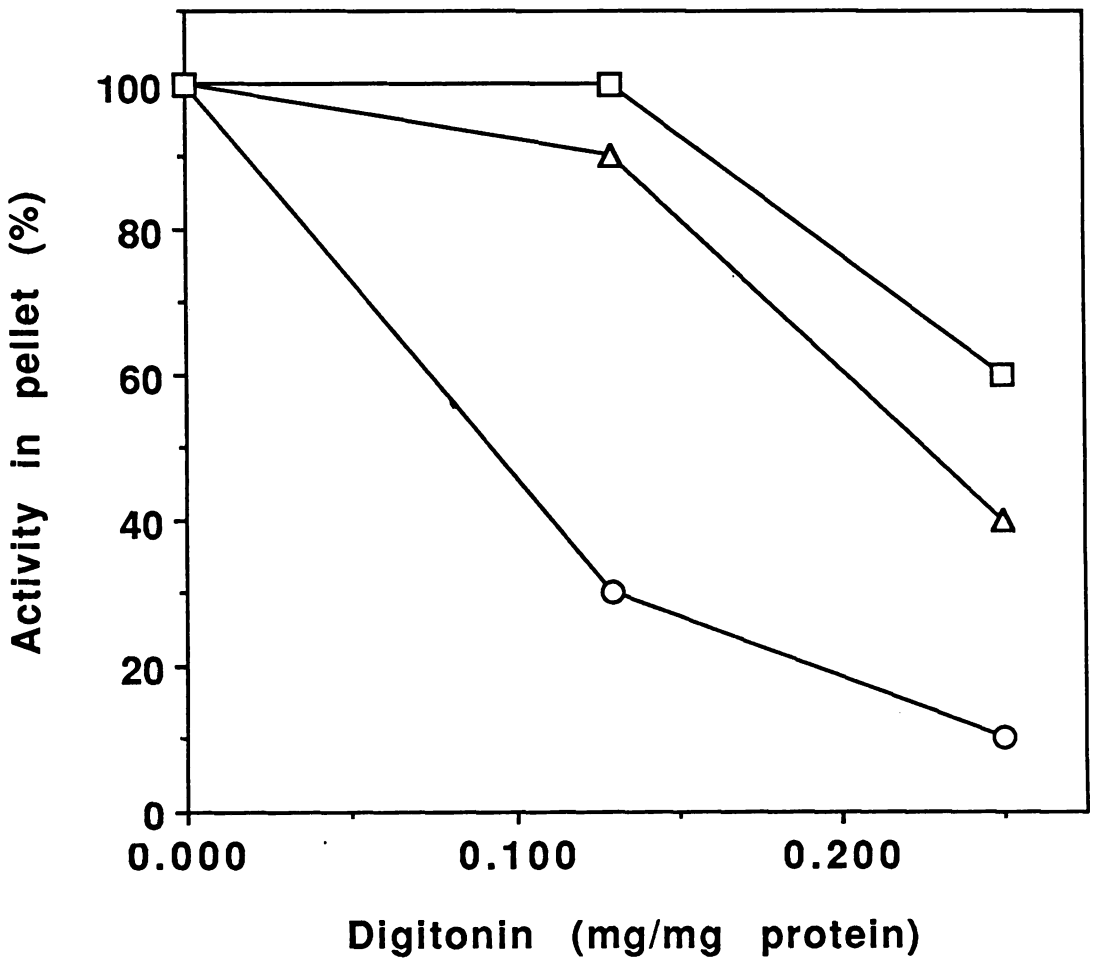
Figure 1. Digitonin fractionation of brain mitochondria. Digitonin treatment as described in Methods. Hexokinase was bound to mitochondria either before (■) or after digitonin treatment (□). The hexokinase activity remaining in the particulate form is presented as a percentage of the hexokinase associated with non-treated mitochondria. Activities of adenylate kinase (O), and monoamine oxidase (Δ) associated with the mitochondrial pellet after treatment with the indicated concentration of digitonin. The results are presented as percentage of the total activity.


100
80
60
40
20
0
0.00
0.50
1.00
1.50
Activity in pellet (%)
Digitonin (mg/mg protein)

mitochondria, also regardless of whether the treatment was done before or after the binding of the enzyme. Figure 1 also shows the percentage of the total adenylate kinase or monoamine oxidase activity remaining in the pellet with no digitonin or after the digitonin treatment. Treatment with 0.75 mg digitonin/mg protein perforates the outer membrane as indicated by the complete release of adenylate kinase, but removes only about 10 % of the monoamine oxidase. Treatment with 1.5 mg/mg protein releases about 60% of the monoamine oxidase.

Digitonin fractionation and binding of derivatized hexokinase to digitonin treated rat liver mitochondria. The mitochondrial suspension was treated with digitonin as described in the Methods section. Figure 2 shows the percentage of the total adenylate kinase or monoamine oxidase activity remaining in the pellet after treating the mitochondria with 0, 0.13 and 0.25 mg digitonin/mg protein. The figure also shows the hexokinase bound to mitochondria treated with digitonin as a percentage of that bound to non-treated mitochondria. Most of the adenylate kinase and about 60 % of the monoamine oxidase were released with 0.25 mg digitonin/ mg protein. The same amount of hexokinase bound to control and mitochondria treated with 0.13 mg digitonin/mg protein, but mitochondria treated with 0.25 mg digitonin/mg protein bound only about 65 % of that bound to the untreated control.

Figure 2. Digitonin fractionation of liver mitochondria. Digitonin treatment as described in Methods. Activities of adenylate kinase (o), monoamine oxidase (Δ) associated with the mitochondrial pellet after treatment with the indicated concentration of digitonin are presented as percentage of the total activity. Hexokinase activity (□) that bound to mitochondria treated with the indicated concentration of digitonin as a percentage of the hexokinase activity that bound to non-treated mitochondria.


100
80
60
40
20
0
Activity in pellet (%)
0.000
0.100
0.200
Digitonin (mg/mg protein)

Crosslinking of hexokinase bound to digitonin treated mitochondria from liver and brain. Monomeric hexokinase, as well as the cross-linked species (tetrameric form) were detected on immunoblots probed with antibodies against the enzyme. Figure 3 shows a Western blot of a representative experiment. The tetrameric form was present in the photolyzed samples of control (non-digitonin treated) mitochondria from liver (lane 2), and the mitochondria treated, before hexokinase binding, with digitonin, 0.13 mg/mg protein (lane 4) and 0.25 mg/mg protein (lane 6). The amount of tetrameric form appeared to decrease as increasing digitonin concentrations were used. However, the bands corresponding to the monomeric form seen in the same lanes, also appeared to decrease in intensity, making it difficult to determine if the decrease in tetrameric form was real or due to a decrease in total hexokinase. The non-photolyzed samples for control mitochondria as well as those treated with 0.13 and 0.25 mg/mg protein are shown in lanes 1, 3, and 5 respectively. As expected, the cross-linked species was not present in the non-photolyzed samples. A band corresponding to purified hexokinase used as a marker can be seen in lane 7; lane 8 contains molecular weight markers.

The results of a similar experiment, in this case with brain mitochondria, are shown in Figure 4. Again, the tetrameric form was present only in the photolyzed samples shown in lanes 2 (control mitochondria), 4 (mitochondria treated with 0.75 mg digitonin/mg protein) and 6 (mitochondria treated with 1.5 mg digitonin/mg protein).

Figure 3. Derivatized hexokinase bound to digitonin treated liver mitochondria. Hexokinase was derivatized, bound to control and digitonin treated liver mitochondria, and crosslinked as described in Methods. Lane 1, derivatized hexokinase bound to control mitochondria, non-photolyzed control; lane 2, is the same sample, photolyzed. Lane 3, derivatized hexokinase bound to mitochondria treated with 0.13 mg digitonin/mg protein, non-photolyzed; lane 4, same sample, photolyzed. Lane 5, derivatized hexokinase bound to mitochondria treated with 0.25 mg digitonin/mg protein, non-photolyzed; lane 6, same sample, photolyzed. Lane 7, purified underivatized hexokinase. Lane 8, protein markers. The numbers to the right of the figure are the molecular weights in kDa.

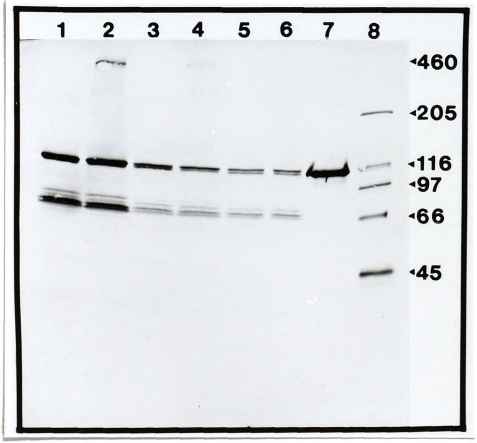
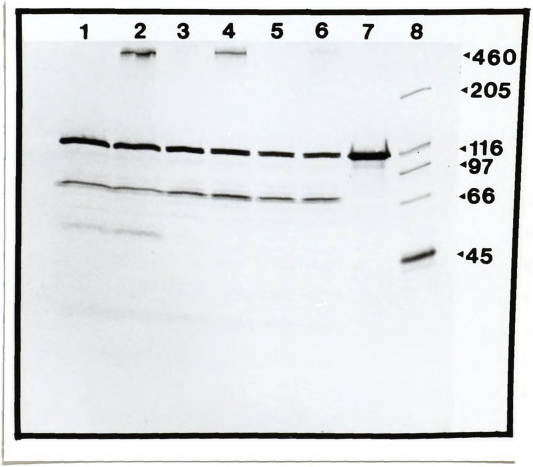

1
2
3
4
5
6
7
8
460
205
116
97
66
45

Figure 4. Derivatized hexokinase bound to digitonin treated brain mitochondria. Hexokinase was derivatized, bound to brain mitochondria that had been treated with digitonin, and crosslinked as described in the Methods. Lane 1, derivatized hexokinase bound to control mitochondria, non-photolyzed sample; lane 2, same sample, photolyzed. Lane 3, derivatized hexokinase bound to mitochondria treated with 0.75 mg digitonin/mg protein, non-photolyzed; lane 4, same sample, photolyzed. Lane 5, derivatized hexokinase bound to mitochondria treated with 1.5 mg digitonin/mg protein, non-photolyzed; lane 6, same sample, photolyzed. Lane 7, underivatized purified hexokinase. Lane 8, molecular weight markers. The numbers to the right of the figure are the molecular weights in kDa.


1
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4
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6
7
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460
205
116
97
66
45

The intensity of the bands corresponding to the tetrameric form decreased as the amount of digitonin used increased. The bands corresponding to the monomeric form of hexokinase also appear to decrease in intensity in the digitonin treated samples. Lanes 1, 3 and 5 show the non-photolyzed controls for the samples in lanes 2, 4 and 6 respectively. The band corresponding to purified hexokinase used as a marker is seen in lane 7 and lane 8 contains the molecular weight markers.

Figure 5 shows another western blot, in this case using brain mitochondria to which derivatized hexokinase was bound before digitonin treatment with 0, 0.75 and 1.5 mg/mg protein. Only the photolyzed samples contain the tetramer, as seen in lanes 2, 4 and 6 for control, 0.75 and 1.5 mg digitonin/mg protein respectively. The decrease in intensity of the bands corresponding to the tetrameric form is not as marked as that seen in Figures 3 and 4, and there is no change in intensity of the bands corresponding to the monomeric form. Non-photolyzed controls are shown in lanes 1, 3 and 5 as in the previous figure, line 7 contains only loading buffer, and a band corresponding to purified hexokinase is shown in lane 8.

Cholesterol enriched mitochondria. Thin layer chromatography was used to determine the relative cholesterol content of control and cholesterol enriched mitochondria as described in the Methods section. Figure 6 shows the results of a typical experiment, where lane 1 contains purified cholesterol, lane 2 contains lipids from control mitochondria and lane 3 contains lipids from cholesterol enriched mitochondria. As seen in the figure, there is significantly

Figure 5. Derivatized hexokinase bound to mitochondria, followed by digitonin treatment. Hexokinase was derivatized, bound to mitochondria from brain, followed by digitonin treatment and crosslinking as described in Methods. Lane 1, derivatized hexokinase bound to mitochondria (no digitonin treatment), non-photolyzed; lane 2, same sample, photolyzed. Lane 3, derivatized hexokinase bound to mitochondria and then treated with 0.75 mg digitonin/mg protein; lane 4, same sample, photolyzed. Lane 5, derivatized hexokinase bound to mitochondria and then treated with 1.5 mg digitonin/mg protein, non-photolyzed; lane 6, same sample, photolyzed. Lane 7 loading buffer only. Lane 8, underivatized purified hexokinase. The numbers to the right of the figure are the molecular weights in kDa.

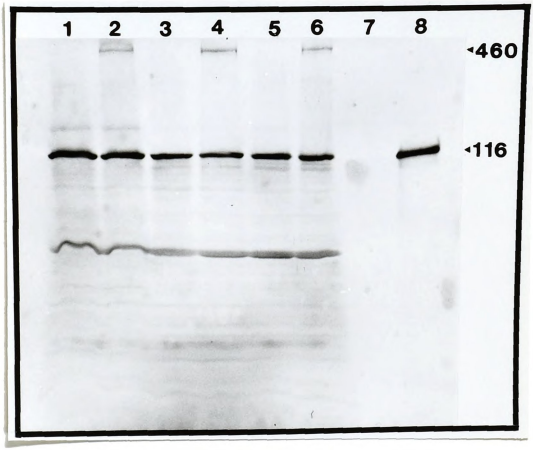
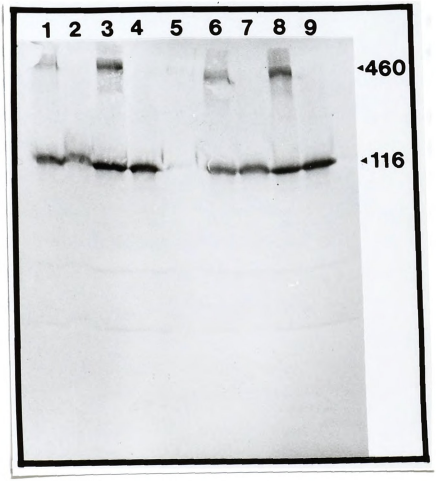

1
2
3
4
5
6
7
8
460
116

Figure 6. Thin layer chromatography to determine the relative cholesterol content of control and cholesterol enriched mitochondria. Mitochondria were enriched with cholesterol as described in the Methods. Lane 1, purified cholesterol. Lane 2, lipids from control mitochondria. Lane 3, Lipids from cholesterol enriched mitochondria.

more cholesterol in the enriched mitochondria than in the control. The actual amount of cholesterol was not determined.

Crosslinking of derivatized hexokinase bound to cholesterol enriched mitochondria. Derivatized hexokinase was bound to mitochondria and cross-linked as described in the Methods section. The Western blots showed distorted bands in the lanes containing cholesterol enriched mitochondria from both rat brain and rat liver. Figure 7 shows a blot typical of these experiments using liver mitochondria. The tetrameric form was present only in the photolyzed samples, shown in lanes 1 and 3 for cholesterol enriched and control samples respectively. The non-photolyzed controls are shown in lanes 2 (cholesterol treated) and 4 (control), and lane 5 contained only loading buffer. Lanes 6-9 contain duplicates of the samples in lanes 1-4, but the final pellet was resuspended in 300 µl instead of 150 µl of sample buffer before loading on the gel. The amount of protein loaded was equivalent for all samples. As seen in the figure, the bands corresponding to the tetrameric and monomeric forms of hexokinase bound to cholesterol treated mitochondria appear diffuse and higher than those from hexokinase bound to control mitochondria. This was seen to a lesser or greater extent in all the experiments, even when the final pellet was resuspended in acetone and centrifuged to try to remove some of the cholesterol before the resuspension in sample buffer.

Figure 7. Derivatized hexokinase bound to cholesterol enriched mitochondria. Hexokinase was derivatized, bound to control and cholesterol enriched mitochondria and crosslinked as described in Methods. Lanes 1-4 contain samples that were resuspended in 150 µl of sample buffer before loading onto the gel (see Methods). Lane 1, derivatized hexokinase bound to cholesterol enriched mitochondria; lane 2, same sample, non-photolyzed control. Lane 3, derivatized hexokinase bound to control mitochondria, photolyzed. Lane 4, same sample, non-photolyzed. Lane 5 loading buffer only. Lanes 6-9 contain duplicates of the samples in lanes 1-4, but they were resuspended in 300 µl of loading buffer before loading onto the gel. Equivalent protein concentrations were loaded on each lane. Lane 6, derivatized hexokinase bound to cholesterol enriched mitochondria, photolyzed; lane 7, same sample, non-photolyzed. Lane 8, derivatized hexokinase bound to control mitochondria, photolyzed sample; lane 9, same sample, non-photolyzed.


1 2 3 4 5 6 7 8 9
460
116

Discussion

The results presented in this study show that hexokinase bound to brain mitochondria is, as in mitochondria from liver, present in monomeric and tetrameric forms. The results of the experiments done with digitonin treated mitochondria to try to localize the tetrameric or the monomeric forms to the contact sites were inconclusive. In several experiments like those described above, the decrease in tetrameric form with increasing digitonin was consistent. However, in some cases, the more or less significant decrease in monomer made it difficult to determine if the decrease in tetramer was real, or if it was because there was less total hexokinase in the digitonin treated samples. As described in the Methods section, the same hexokinase activity was loaded in each lane, although the total volume loaded was greater for the digitonin treated mitochondria since they contained less hexokinase (Figures 1 and 2).

If the samples contained the same amount of hexokinase activity, but less protein was detected on the immunoblots, an explanation considered was that hexokinase was activated upon binding to the contact sites, giving the same amount of activity with less total protein. This idea was based on reports by Weiler *et al.* (19) that there is activation of hexokinase upon binding to the mitochondria. However, no evidence of activation upon binding was seen with either control or digitonin treated mitochondria under the conditions used in these experiments.

Another possibility considered was uneven protein transfer with the tank system. To try to correct this, the semi-dry gel apparatus was used for the protein transfer. Even though with this method, significantly less protein remained on the gel after transfer, the total hexokinase present in the digitonin treated samples appeared to decrease. A careful quantitation of the amount of monomer and tetramer present in control and digitonin treated mitochondria needs to be done to confirm these findings. This could not be done with the Western blots because of incomplete protein transfer from the gel to the membrane, as well as the variability from experiment to experiment.

The results of the experiments with cholesterol enriched mitochondria were also inconclusive due to what appear to be technical problems. However, if the tetrameric form is preferentially bound to the regions of the outer mitochondrial membrane that are richer in cholesterol, an increase in tetrameric form would be expected in cholesterol enriched mitochondria. This was not the case. The band corresponding to the tetrameric hexokinase bound to cholesterol enriched mitochondria, was consistently less intense than that of hexokinase bound to control mitochondria.

The presence of tetrameric and monomeric forms of mitochondrially bound hexokinase does not seem to be due to a random process, since no dimers or trimers have been found in liver or brain mitochondria. As mentioned above, the physiological implications of the existence of these two forms are not clear; determining if the tetrameric and monomeric forms of hexokinase

bind distinct regions of the outer mitochondrial membrane might help understand their function.

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Chapter VI

Summary and Perspectives

The purpose of the present study was to characterize the interactions between mitochondria and to determinehexokinase and the importance of these interactions in the role of hexokinase as a regulator of glucose metabolism in rat brain.

Hexokinase is a major factor in the regulation of glycolysis in brain (1,2) where it is bound to the outer mitochondrial membrane through interactions with porin, the outer membrane protein that forms the pores through which metabolites enter and exit the mitochondria (3-5). Evidence suggests that most of the hexokinase is bound to the contact sites between the inner and the outer mitochondrial membranes (6,7, Chapter III of this thesis) where it obtains substrate ATP from one or two intramitochondrial compartments (Chapter III). Binding to the mitochondria gives the enzyme kinetic advantages (8), and Glc-6-P promotes the release of the enzyme from the mitochondrial membrane (9) and acts as a negative allosteric effector (10,11).

There is a model for the regulation of hexokinase based on the effects of Glc-6-P on the enzyme (8,12). In this model, Glc-6-P and inorganic phosphate (which antagonizes the effects of Glc-6-P) vary in response to metabolic changes thus affecting binding of hexokinase to mitochondria and its activity. When the energy needs of the cell have been met, down regulation of the glycolytic pathway, through inhibition of phosphofructokinase, causes an increase in Glc-6-P concentration. This inhibits hexokinase and promotes its release from the mitochondria (9) with a subsequent decrease in the introduction of Glc into glycolysis. On the other

hand, when ATP is rapidly being hydrolyzed and the flow through the glycolytic pathway increases, Glc-6-P concentration decreases with the concomitant increase in hexokinase activity. This mechanism is probably not fast or sensitive enough to respond to small variations in the rate of ATP production and utilization during normal activities, however, it could come into play during extreme conditions like ischemia or convulsions, that cause an increase in Glc utilization (8). Moreover, unpublished work by Kabir and Wilson has shown that hexokinase of mitochondria from brain of different species responds differently to solubilization by Glc-6-P. In mitochondria from rat brain, 85-90 % of the hexokinase is solubilized by Glc-6-P. This percentage decreases to about 60 % in brain from Guinea pig, to 40 % in bovine brain and to15 % in human brain. Hexokinases from those species have the same molecular weight and pI, and no differences in the K_m for ATP or K_i for Glc-6-P have been found.

A regulatory mechanism, not based on solubilization of hexokinase by Glc-6-P, was proposed in this thesis and could play a role in the regulation of glucose metabolism in brain of higher animals in which a high percentage of the hexokinase is not solubilized by Glc-6-P. In this model, hexokinase functions as a link between glycolysis and oxidative phosphorylation, leading to a perfect balance between the introduction of Glc and the carbons needed for ATP production by intramitochondrial oxidative processes.

The methods used in the present work can be used to determine if compartmentation like that seen in rat brain exists in higher animals. It would be important to measure ATP production by adenylate kinase, creatine kinase and oxidative phosphorylation, as well as the contribution of these ATP sources to the ATP utilized by mitochondrial hexokinase.

As mentioned previously, there appear to be two pools of hexokinase in mitochondria from pig, cow and human brain. One that can be solubilized by Glc-6-P and one that cannot. Determining if hexokinase from one of these pools (or both) is coupled to oxidative phosphorylation might provide further insight into the role of hexokinase (and Glc-6-P) in the regulation of energy metabolism in brain.

Although the word compartment has been used repeatedly in this thesis, it should be noted that the existence of a space at the contact sites has never been observed on electron microscopy. There is a possibility that these compartments are indeed spaces that cannot be seen after the samples have been processed for electron microscopy or that, instead of a physical space, ATP is bound to a protein. One logical candidate would be the ATP/ADP translocase, and this could explain why the inhibitors of this protein cause release of the ATP. However, a simple calculation based on the amount of translocase (13) and the ATP present in the compartments determined that the compartmentalized ATP : translocase ratio is in the order of 100, making it an unlikely candidate. Although ATP could be bound to other proteins, this binding would render it

unavailable (or at least not as easily available) to hexokinase. However, the structural features of the sequestration of ATP were not investigated during the course of this work.

Undoubtedly more studies are needed to investigate the structure and location of these hypothesized compartments and the location of hexokinase on the mitochondrial membrane. Even though some evidence was presented in this thesis which suggests that the compartments and most of the hexokinase are located at the contact sites, the major focus of this project was to study the physiological significance of the hexokinase/mitochondrion interactions; this work did not directly address the structure and location of these compartments or the location of hexokinase on the outer membrane. Electron microscopy combined with immunolabeling techniques and digitonin fractionation of mitochondria could be useful in to determining the location of hexokinase on the mitochondrial membrane.

The inhibitors of both oxidative phosphorylation and the ATP/ADP translocase release the compartmentalized ATP. Finding the mechanism by which this occurs could be very important in understanding the nature of these compartments. Two patterns of ATP release from the compartments filled by oxidative phosphorylation were observed (see Fig. 1 Chapter III of this thesis). ATP release was very fast and complete with the addition of bongkrekic acid or KCN. There was a slower and incomplete release (only 30 %) with the addition of sodium azide and CAT. These results appear to indicate that the known effects of these inhibitors

(i.e. inhibition of cytochrome oxidase by KCN and azide (14), and inhibition of the ATP/ADP translocase by CAT and BKA (15)) are probably not involved in the release of ATP since members of both groups of inhibitors show the same pattern of ATP release.

A mechanism was proposed, based on evidence presented in this thesis, for the synchronization of glycolysis and oxidative metabolism in rat brain to ensure that the right amount of Glc enters the glycolytic pathway for the level of oxidative metabolism at any given time. This mechanism appears to be able to respond to normal variations in ATP production due to changes in the activity levels of brain cells thus avoiding a glycolytic rate in excess of oxidative capacity that could lead to the accumulation of lactate.

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Appendix

Mitochondrial Isolation and Methods Used for the Measurement of the Parameters Used in this Thesis

MITOCHONDRIAL ISOLATION

Reference: Lai, J. C. K., and Clark, J. B. (1979) *in* Methods of Enzymology, (Fleischer, S., and Packer, L. Eds.) Vol. 55, pp. 51-60, Academic Press, New York.

Solutions

Isolation medium

250 mM sucrose.

0.1 mM EGTA.

5 mM Mops pH 7.4. (A 100 mM stock solution of Mops was made and the pH adjusted with KOH).

After mixing all the components, the pH of the final solution was adjusted to 7.4 with KOH.

Ficoll 6 % medium

240 mM mannitol.

60 mM sucrose.

0.1 mM EGTA.

5 mM Mops pH 7.4.

Ficoll 6 % (w/v).

Ficoll 400-DL was obtained from Sigma Chemical Co. (St Louis, MO) and a stock solution (30-40 % w/v) was made by slowly adding the Ficoll to warm double distilled water until completely dissolved. The density of the Ficoll was determined and the exact percentage (w/v) of the stock solution was calculated with the use of a Ficoll density/ w/v chart. To make the 6 % Ficoll medium, the other reagents were mixed with water and enough Ficoll stock solution to

give a final concentration of 6 %. The pH was adjusted to 7.4 with KOH.

Incubation medium

0.1 mM EGTA (1 mM stock solution).

150 mM KCl (500 mM stock solution).

5 mM Tris-Phosphate, pH 7.2.

10 mM Tris-Cl pH 7.4.

The Tris-phosphate and Tris-Cl solutions, were made by adjusting the pH of 100 ml of a 100 mM Tris solution with phosphoric or hydrochloric acid respectively.

The incubation medium was made by combining 5 ml Tris-phosphate, 10 ml Tris-Cl, 10 ml 1 mM EGTA, 30 ml 0.5 M KCl and 45 ml of double distilled water. The pH was adjusted to 7.4 with KOH.

Phosphate-free medium.

Made in the same way as the incubation medium, but deleting the Tris-phosphate and adding 15 ml of Tris-chloride instead of 10 ml.

Isolation Procedure

Three or four brains can be used with the exact same isolation procedure except for the volume of the final suspension, which is 0.7 ml with 3 brains and 1 ml with four brains. The yield is very similar in both cases, protein concentration approximately 8 mg/ml and hexokinase activity approximately 3.2 u/ml.

Brains are obtained from Sprague-Dawley rats (150-250g) of either sex. Rats are put in a desiccator containing dry ice for about 30 seconds (until they are not moving but still alive) before decapitation. Brains are obtained by opening the skull immediately after decapitation, and the cerebellum is removed. The forebrains are put in about 40 ml of ice cold isolation medium and minced finely with scissors. After all the brains have been obtained, the isolation medium is changed two or three times to remove the blood. After the last change, only 20 ml of fresh medium are added.

Homogenization is done with a Teflon-glass homogenizer (size C, Thomas Scientific, Swedesboro, NJ), moving the pestle up and down 10-12 times until the homogenate is smooth. The volume should be adjusted to 30 ml before starting the centrifugation protocol.

Centrifugation is performed at 4°C using the SS-34 rotor (Ivan Sorvall Inc. Norwalk, CN). The homogenate is centrifuged for 3 minutes at 4,000 r.p.m., the supernatant (about 15-20 ml) carefully removed with a 10 ml pipette and transferred to another tube. The pellet is discarded and the supernatant centrifuged at 11,500 r.p.m. for 8 min. The resulting supernatant is decanted sharply to remove the loose fluffy layer on top of the pellet. A kim wipe can be used to remove the fluffy matter from the sides of the tube. The pellet is resuspended in 6 ml of 3 % Ficoll, prepared by diluting the 6 % Ficoll medium with water, and layered on top of 25 ml of the 6 % medium. Centrifuge at 11,000 r.p.m. for 30 minutes. The resultant pellet is gently resuspended in 6 ml of isolation medium with a Pasteur pipette and centrifuged one last time at 11,500 r.p.m. for 10

minutes. Using 12 ml plastic tubes (with adapters) instead of the 50 ml tubes for the last centrifugation facilitates the resuspension of the final pellet. The final resuspension is in either 0.7 ml (3 brains) or 1 ml (4 brains) of isolation medium using a 1 ml Pipetman.

METHODS FOR THE MEASUREMENTS OF PARAMETERS USED IN THIS THESIS

Solutions

ADP (30 mM, pH 7.0 with KOH).

ATP (220 mM, pH 7.0 with NaOH).

A_2P_5 (10 mM).

CAT (3 mg/ml in water).

Glc (1 M).

Glc-6-PDH (100 u/ml in 0.02 M Tris-0.2 % BSA, pH 7.5).

KCN (1 M).

$MgCl_2$ (1 M).

NADP (50 mg/ml in 0.1 M NaH_2PO_4, pH 7.0). When using phosphate-free medium, make NADP in water.

Phosphocreatine (100 mM).

Pyruvate-malate (0.9 M-0.45 M).

Yeast hexokinase (Sigma H-5500).

Mitochondria

Mitochondria (final suspension as isolated, approximately 8 mg/ml).

General Procedure

The "Basic Reaction" is as follows: reactions are carried out in 940 µl of incubation medium and contain mitochondrial suspension (10 µl), Glc-6-P dehydrogenase (10 µl), NADP (10 µl), $MgCl_2$ (5 µl), Glc (5 µl), and ADP (10µl). NADPH formation is followed at 340 nm. ADP is the last reagent added and mitochondria the second last. No specific order of addition was followed for the rest of the reagents.

ATP Production

This is the total ATP production rate and it is measured in the presence of excess yeast hexokinase to use all the ATP as it becomes available in the extramitochondrial space. Rates of ATP production by oxidative phosphorylation, adenylate kinase or creatine kinase can be measured.

Oxidative Phosphorylation. These rates are measured using the basic reaction, supplemented with 5 µl of pyruvate-malate, 10 µl of A_2P_5 (adenylate kinase inhibitor) and 1µl of yeast hexokinase. The rate gets to a steady state very quickly and remains there for several minutes.

Adenylate Kinase. Use the basic reaction supplemented with 1 µl yeast hexokinase and 5 µl of KCN from the beginning of the reaction. The addition of KCN is not necessary if the reactions are carried out in phosphate-free medium instead of incubation medium.

Creatine Kinase. Use the basic reaction supplemented with 1 µl of yeast hexokinase, 10 µl of A_2P_5, 15 µl of phosphocreatine and phosphate-free medium instead of incubation medium. The rate only stays at steady state for 3-4 minutes. Take initial velocity.

ATP Utilization

This is the rate of ATP utilization by mitochondrially bound hexokinase, so these reactions do not contain yeast hexokinase. ATP utilization rates can be measured with one or more of the 3 ATP sources.

Oxidative Phosphorylation. Same as ATP production measurement reaction, but in the absence of yeast hexokinase. The reaction does not reach steady state for about 8 to 10 minutes, but it remains at steady state for more than 20 minutes. The ATP utilization rate should be taken at steady state.

Adenylate Kinase. Same as ATP production measurement reaction, but in the absence of yeast hexokinase.

Creatine Kinase. Same as ATP production measurement reaction, but in the absence of yeast hexokinase.

Mitochondrial Respiration

The reactions are carried out in incubation medium, containing 50-100 µl of mitochondrial suspension. The addition of 10 µl of

pyruvate-malate causes a slight increase in the oxygen consumption rate. The rate of oxygen consumption increases rapidly after the addition of ADP, followed by a return to the rate observed in the presence of pyruvate-malate only, when the ADP has been consumed. Addition of more ADP increases oxygen consumption again.

The oxygen content of the incubation medium at 25 °C equilibrated against air was assumed to be 480 nmoles/ml based on the calculations by Reynafarje *et al.* ((1985) *Anal. Biochem.* **145,** 406-418), for a similar solution. The chamber of the oxygen electrode has a volume of 1.8 ml, so the total is 860 nmoles O.