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MOLECULAR BASIS FOR THE INTERACTION OF RAT BRAIN HEXOKINASE WITH MITOCHONDRIA

By

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ABSTRACT

MOLECULAR BASIS FOR THE INTERACTION OF RAT BRAIN HEXOKINASE WITH MITOCHONDRIA

by

Guochun Xie

Rat brain hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) has the ability to bind rapidly and reversibly to the outer mitochondrial membrane. The bound enzyme has a lower K_m for its substrate ATP and higher K_i for inhibition by its product Glc-6-P. In addition, it has been shown that the bound enzyme has preferential access to mitochondrially-generated ATP. The binding involves both electrostatic and hydrophobic interactions. Certain metabolites such as Glc-6-P and P_i affect the distribution of hexokinase between the bound and soluble form. Based on these observations, it has been suggested the reversible binding plays an important role in the regulation of hexokinase and glycolysis, and thereby the energy metabolism in the brain.

This project was undertaken to study the structural aspects of the membrane-bound rat brain hexokinase. To detect regions of the hexokinase molecule which might be inserted into the lipid bilayer, a hydrophobic and

photoactivatable reagent, 3-(trifluoromethyl)-3-(m-[^{125}I]iodophenyl)diazirine ([^{125}I]TID), was used to label rat brain hexokinase bound to mitochondria. This highly hydrophobic reagent preferentially partitions into a hydrophobic environment such as lipid bilayers and upon photolysis labels molecules within the lipid core. Photolysis resulted in labeling of hexokinase. Proteolytic digestion of the labeled enzyme reveals that most of the label is attached to the N-terminal 11 amino acid residues, which confirms the postulation that the hydrophobic N-terminal sequence of the enzyme is inserted into the membrane.

A chemical crosslinking method was employed in an attempt to identify molecules in the outer mitochondrial membrane which interact with hexokinase. Specifically, rat brain hexokinase was derivatized with a photosensitive and cleavable cross-linking agent, sulfosuccinimidyl 2-(m-azido-o-nitrobenzamido)-ethyl-1,3'-dithiopropionate (SAND). The derivatized enzyme was then bound to rat liver mitochondria. Photolysis resulted in the formation of a 460 kDa crosslinked complex. Cleavage of this complex generates only hexokinase monomer. It is thus concluded that rat brain hexokinase, when bound to mitochondria, forms a tetramer. No evidence of dimeric or trimeric structures was seen even when only a small fraction of the available binding sites on the mitochondrial membrane were occupied.

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Xie, G., and Wilson, J. E., Rat Brain Hexokinase: The Hydrophobic N-Terminus of the Mitochondrially-Bound Enzyme Is Inserted in the Lipid Bilayer, Arch. Biochem. Biophys., 267, 803-810, 1988.

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To my parents and wife

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ABBREVIATIONS

ANB-NOS	N-5-azido-2-nitrobenzoyloxysuccinimide
Glc-6-P	glucose 6-phosphate
HEPES	N-2-hydroxyethylpiperazineethanesulfonic acid
SAND	sulfosuccinimidyl-2-(m-azido-o-nitrobenzamido)-ethyl-1,3'-dithiopropionate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis.
[^{125}I]TID	3-(trifluoromethyl)-3-(m-[^{125}I]iodophenyl)diazirine
TPCK	L-1-tosylamide-2-phenylethylchloromethyl ketone.

INTRODUCTION

Hexokinase catalyzes the first reaction in glycolysis, the phosphorylation of glucose to Glc-6-P using $MgATP^{--}$ as the phosphoryl donor. This reaction is generally accepted as one of the principal regulating steps in glycolysis (1).

Rat brain hexokinase is a single polypeptide enzyme of 100 kDa (2). Limited trypsin digestion cleaves this enzyme into three fragments of 10 kDa, 50 kDa, and 40 kDa, listed in order of their sequence from N- to C-terminus of the enzyme (3). Each fragment was suggested to represent a domain which was associated with a specific function. The 10 kDa domain has been shown to be important in the binding of the enzyme to mitochondria (4, 5). The 40 kDa is associated with the catalytic function of the enzyme (6, 7) and the 50 kDa has been suggested to be the regulatory domain (7). Recent studies (8, 9, 10, 11), however, suggested that rat brain hexokinase consists of two homologous halves instead of three domains. Both halves contain binding sites for hexoses, nucleotides, and Glc-6-P (8, 10, 11). These results are consistent with the proposal that mammalian hexokinase evolved from the same ancestral gene coding for a 50 kDa enzyme such as yeast hexokinase (12-18).

The association of hexokinase with mitochondria has generally been observed in tissues exhibiting high glycolytic rate such as brain (19), blood platelets (20), lymphocytes (20), and highly glycolytic regions in the kidney (21). In brain, under normal conditions, virtually all the energy is supplied by metabolism of glucose *via* glycolysis and subsequent oxidation by the mitochondrial tricarboxylic acid cycle and electron transport and oxidative phosphorylation (22). Up to 80% or more of the hexokinase in brain homogenates is found to be associated with the mitochondria (23). The binding of hexokinase is regulated by cellular metabolites such as Glc-6-P and inorganic phosphate (24-26). Glc-6-P solubilizes the enzyme from the mitochondria while inorganic phosphate antagonizes the effect of Glc-6-P. Kinetic studies showed that the bound enzyme is more active than the soluble enzyme, i.e. low K_m for ATP and high K_i for Glc-6-P (25, 26). It has been proposed that the soluble-bound hexokinase distribution may be involved in the regulation of glycolysis (19, 25, 27).

Binding of rat brain hexokinase to mitochondria appears to involve both electrostatic (28, 29) and hydrophobic forces (4, 29, 30). Hexokinase binding protein (HBP), is at least partly responsible for binding hexokinase (31). HBP was isolated from rat liver mitochondria (31) and was found to be identical to mitochondrial porin (32, 33). Limited chymotrypsin digestion, which preferentially cleaves within

the 11 hydrophobic residues at the N-terminus of the enzyme, has no effect on catalytic activity (4). However, the cleavage(s) render(s) the enzyme nonbindable. It was suggested that this hydrophobic segment might be inserted into the lipid bilayer of outer mitochondrial membrane although no direct evidence was available to support this. The work in Chapter II provides that evidence. Moreover, chemical crosslinking studies described in Chapter III have led to the totally unexpected finding that bound enzyme exists in a tetrameric form; though the significance of this is not yet clear, there is reason to believe that the tetramerization may have physiologically important consequences.

This thesis consists of four chapters. Chapter I is a literature review on studies on mammalian hexokinase and mitochondrial porin. Chapter II and Chapter III are adapted from the publications in Arch. Biochem. Biophys. (Chapter II, 267, 803-810, 1988; Chapter III, 276, 285-293, 1990). Chapter IV is a brief summary and perspective.

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Chapter I

LITERATURE REVIEW

LITERATURE REVIEW

I. Mammalian Hexokinase

A. Hexokinase Isozymes

There are four hexokinase isozymes, Type I, II, III, and IV in order of increasing electrophoretic mobility toward the anode of a starch gel at pH 8.4 (1). Type I is found in every tissue studied (2) although it is the predominant isozyme in tissues which are critically dependent on glucose metabolism such as brain (3), blood platelets (4), lymphocytes (4), and highly glycolytic regions in the kidney (5). Brain contains virtually only type I isozyme. Type II is the major form in insulin-sensitive tissues such as muscle (6-9). Type III has not been shown to be dominant in any tissue (2). Type IV, which is also referred to as glucokinase, seems to exist only in liver (10, 11) and pancreatic islet cells (12). In contrast to Type I, II, and III, glucokinase has a much higher Km (10^{-2} M) for glucose than hexokinase I-III (10^{-4} to 10^{-6} M) (1, 13-16). Its size (50,000) (17-19) is only half of that for hexokinase I-III (100,000) (20-27).

B. Association of Hexokinase with Subcellular Structures

Crane and Sols were the first to suggest the association of hexokinase with brain mitochondria (28). Further study by Johnson confirmed that in brain homogenates, hexokinase, but not other glycolytic enzymes, was associated with mitochondria (29). Later studies revealed particulate hexokinase in other tissues such as human blood cells (4), heart (30-32), and skeletal muscle (33). In most cases, hexokinase was found to be associated with mitochondria (29, 32-37). In lymphocytes, 78% of the total hexokinase activity is membrane-bound (presumably mitochondria) and 88% in platelets (4). In an similar study of rat kidney, it was observed the specific activity of hexokinase in mitochondrial fractions of the three major tissue zones, papilla, medulla, and cortex, correlates with the rate of glycolysis in these tissue zones (5). Papilla has a much higher rate of aerobic glycolysis than the medulla or cortex (38). The specific activity of hexokinase in the papillary mitochondrial fraction was significantly greater than in the papillary cytoplasmic fraction (5). The specific activity of hexokinase in mitochondrial fractions, however, was not significantly different from that in cytoplasmic fractions in the medulla or cortex (5).

The binding of other hexokinase isozymes has not been

studied as extensively as Type I hexokinase. Type II has been shown to be associated with both mitochondria and sarcoplasmic reticulum (30, 39-41). Reid and Masters suggested that HK II may redistribute between the mitochondria and the sarcoplasmic reticulum depending on whether the muscle is mainly involved in glycolysis or glycogen synthesis (41). Binding of type III isozyme to subcellular structure in normal tissues has not been reported although one report showed type III has a mitochondrial location in tumor cells (42). Glucokinase has not been found to be associated with mitochondria in the liver (43) but it has been suggested to be associated with mitochondria in normal (44) and tumoral (44, 45) islet cells.

II. Rat Brain Hexokinase

A. Structure of Rat Brain Hexokinase

Rat brain hexokinase contains a single polypeptide with a molecular weight of about 100,000 (21). Limited trypsin digestion under nondenaturing conditions cleaves this enzyme into three fragments, with estimated molecular masses of 10 kDa, 40 kDa, and 50 kDa (46) based on their mobility in SDS-PAGE. The N-terminal 10 kDa region has been shown to be important in the binding of hexokinase to mitochondria (47,

48). The C-terminal 40 kDa domain is involved in the catalysis (49, 50).

The middle 50 kDa domain was proposed to be the regulatory domain where Glc-6-P binds allosterically and regulate the enzyme's activity (51). The proposal of the existence of an allosteric site for Glc-6-P by several investigators (28, 51-54) has been argued by Fromm and his colleagues (55-59).

There have been some reports indicating the existence of an allosteric site for Glc-6-P (28, 51-54). Weil-Malherbe and Bone (52) reported that inhibition of rat brain hexokinase by Glc-6-P was independent of the concentration of either glucose or ATP. They concluded that Glc-6-P occupies a site different from that which reacts with either glucose or ATP. Studies by Crane and Sols (28) showed that Glc-6-P inhibits hexokinase in a noncompetitive manner relative to either glucose or ATP. In addition, they also noted the apparent specific inhibition of hexokinase by Glc-6-P. For example, glucose, mannose, and 2-deoxyglucose were all rather good substrates of hexokinase, however, only Glc-6-P was a potent inhibitor of the enzyme. Based on these results, Crane and Sols (28) also suggested the existence of a third specific binding site for Glc-6-P in addition to binding sites for glucose and ATP. Lazo et al. (53) studied

the binding of Glc-6-P to rat brain hexokinase by Paulus ultrafiltration method. Their results indicated two Glc-6-P binding sites on the enzyme, a lower affinity product site, and a high affinity allosteric site. In the study of the reverse reaction of rat brain hexokinase, Ureta et al. (54) found their results are fully consistent with an allosteric site for Glc-6-P.

In contrast, Fromm and his colleagues suggested that the inhibitory Glc-6-P binding site is partially overlapping with the catalytic site rather than a separate site (55-59). For example, Fromm and Zewe (55) found Glc-6-P inhibition was noncompetitive relative to glucose but competitive relative to ATP. They indicated it was unnecessary to postulate a second site on the enzyme; instead, they suggested that the phosphate of Glc-6-P interacts primarily with the γ phosphate subsite of the ATP pocket on brain hexokinase. The competitive inhibition of brain hexokinase by Glc-6-P relative to ATP was also shown by Ellison et al. (56).

In addition, difference spectroscopic investigation on the interaction of hexokinase with glucose and Glc-6-P led Mehta et al. to conclude that an allosteric site for Glc-6-P occurs on the enzyme only in the presence of glucose, i.e., hexokinase has no preexisting allosteric site for Glc-6-P

(60).

Recent work by White and Wilson supports existence of an allosteric binding site for Glc-6-P in the 50 kDa domain (61-63). When hexokinase is denatured with 0.6 M guanidine hydrochloride, it becomes highly susceptible to proteolysis by trypsin. In addition, trypsin digestion of the enzyme produces two new fragments of 48 kDa and 52 kDa. The susceptibility to trypsin is affected by ligands. Glc-6-P selectively protects the N-terminal half of the enzyme (52 kDa) from trypsin digestion. N-acetylglucosamine, a glucose analog, protects only the C-terminal half of the enzyme from trypsin digestion. These observations indicate that the C-terminal half contains a binding site for N-acetylglucosamine and the N-terminal half contains a binding site for Glc-6-P (61). Further binding studies (62, 63) showed that both fragments (48 kDa and 52 kDa) have binding sites for hexoses, nucleotides and Glc-6-P. The binding site for Glc-6-P in the N-terminal half has all the characteristics for an allosteric effector site in terms of affinity for Glc-6-P, specificity, and synergistic interactions with the hexose binding site in the C-terminal half of the molecule.

Dimerization has been reported for hexokinase from bovine brain (64), pig heart (65), human heart (66), and

human erythrocytes (67). In pig heart, for example, hexokinase was shown to exist in an equilibrium of monomer and dimer (65). In the absence of ligands, 91% of the enzyme exists as monomer and 9% as dimer. In the presence of 1 mM Glc-6-P, 51% of the enzyme was in the dimer form and 49% of the enzyme was in the monomer form. The dimerization process is reversible upon removal of Glc-6-P. Inorganic phosphate can antagonize the Glc-6-P dependent dimerization.

B. Binding of Rat Brain Hexokinase to Mitochondria

1. Forces of Binding

The binding of hexokinase to mitochondria is thought to involve both electrostatic (68, 69) and hydrophobic (47, 70) forces.

a. Electrostatic Forces

Based on the fact that high ionic strength causes extensive solubilization of membrane bound hexokinase, Teichgraber and Biesold (68) suggested the involvement of electrostatic forces in the binding of hexokinase to the mitochondrial membrane. Felgner and Wilson (69) found that at low ionic strengths (<~0.02 M), neutral salts enhance binding while at high ionic strength they cause solubilization. Through the study of effects of neutral

salts and pH on the binding of hexokinase to mitochondria, they proposed that there are two kinds of electrostatic interactions between the enzyme and outer mitochondrial membrane: a repulsive electrostatic interaction between negative charges on both enzyme and membrane, and an attractive electrostatic interaction within a hydrophobic milieu between oppositely charged groups on the two interacting entities. At low ionic strength, the salts enhance hexokinase binding by shielding the negative charges on the enzyme and the membrane, while at high salt concentration, the attractive forces were disrupted and thus cause solubilization of the enzyme (69). Divalent cations are very effective in promoting binding of hexokinase to mitochondria, even in the presence of Glc-6-P which weakens the binding (2, 69, 71). A model has been proposed recently by Wilson (71) for the interaction of rat brain hexokinase with mitochondria. In this model, a hydrophobic N-terminal segment inserts into the membrane and divalent cations bridge the negatively charged groups on enzyme and membrane. The bound enzyme can exist in two conformations. In the absence of Glc-6-P, both hydrophobic and electrostatic interactions are involved and the enzyme exists in its active conformation capable of interacting with both membrane and its substrates. In the presence of Glc-6-P, the enzyme lost its ability to bind nucleotides and its interactions with the membrane is maintained only by

electrostatic forces. The absence of divalent cations in the presence of Glc-6-P resulted in complete release of the enzyme into soluble forms.

A carboxyl residue located within a hydrophobic region of the receptor complex (pore) has been proposed to play a critical role in binding of hexokinase to hepatoma mitochondria based on the following experiment (72). N,N'-dicyclohexylcarbodiimide (DCCD) is known to interact with carboxyl groups located within a hydrophobic milieu. [C^{14}]DCCD at low dosage (2 nmol of DCCD/mg of mitochondrial protein) selectively labeled porin together with two other proteins in mitochondria. The treated mitochondria from either rat liver or AS-30D hepatoma lost more than 90% of the original hexokinase binding ability. Treatment of mitochondria with water-soluble carbodiimides had no effect on hexokinase binding (72).

Phospholipids have also been suggested to affect the interaction between hexokinase and mitochondria (73). Repeated washing of brain mitochondria gradually decreases the proportion of bound hexokinase which can be later solubilized by Glc-6-P. Addition of phospholipids removed during washing enhances solubilization of hexokinase by Glc-6-P. Pure lysophospholipids and phospholipids (anionic at pH 7) also enhance the solubilization. These phospholipids have

no solubilization effects themselves. The mechanism for the enhancement of solubilization of hexokinase by lysophospholipids, or acidic phospholipids was not clear. However, the authors suggested that the negative charges of lysophospholipids and acidic phospholipids (anionic at pH 7) could increase the repulsive forces between membrane and hexokinase and thus enhance solubilization. It was also suggested that the binding of hexokinase might be governed by variations in the lipid composition in the membrane (73).

b. Hydrophobic Forces

The importance of hydrophobic interaction in the binding has been demonstrated by several investigators (47, 70). Chymotrypsin preferentially cleaves hexokinase within the 9 amino acid residues located at the N-terminus of the enzyme (47). The digested enzyme showed no difference in its apparent molecular weight on SDS polyacrylamide gel compared with the native enzyme (47, 70). However, it lost the ability to bind either to outer mitochondrial membrane (47) or to a Phenyl-Sepharose (70). It was suggested that mitochondria-bindable hexokinase has a hydrophobic region on its surface which is involved in the binding of hexokinase to mitochondria. Sequence analysis of fragments generated with chymotrypsin reveals a very hydrophobic N-terminal sequence (47). The existence of a hydrophobic segment at the N-terminus of the enzyme has been further confirmed by the

deduced amino acid sequence of the enzyme from its cDNA sequence (74).

2. Hexokinase Binding Protein (HBP) in the Binding

Brain hexokinase binds only to outer mitochondrial membrane, but not to inner mitochondrial membrane, or to microsomal membranes, or to plasma membrane of human erythrocytes (75). This specificity of binding was taken to indicate the presence of a specific hexokinase binding factor in outer mitochondrial membrane.

a. Purification

Hexokinase binding protein was first purified from outer mitochondrial membrane of rat liver by Felgner et al. (75). The method involves repetitive solubilization of the outer mitochondrial membrane with octyl-ß-D-glucopyranoside. The solubilized HBP is preferentially reconstituted into membranous vesicles when the detergent was removed by dialysis. HBP, when reconstituted into lipid vesicles, confers the ability to bind hexokinase specifically and in a Glc-6-P sensitive manner as the binding of hexokinase to outer mitochondrial membrane.

b. Identity with mitochondrial porin

It has been showed that bound hexokinase could directly exchange ATP inside mitochondria for ADP (76). Passage of

ATP/ADP through the outer mitochondrial membrane is possible only through the pore. Discovery of HBP led to the suspicion that HBP may be the mitochondrial porin(s) which form(s) the aqueous pore on the outer mitochondrial membrane. Further experiments (77, 78) demonstrated that HBP and mitochondrial porin from rat liver are indeed identical based on the following criteria. HBP and mitochondrial porin (see below) purified with different methods confer the same ability to bind hexokinase. Both have the same apparent molecular weight (30,000) on SDS-PAGE. Both show identical peptide patterns when subjected to the digestion with either *Staphylococcus aureus* V8 protease or chymotrypsin. Both show identical isoelectric points. They both form aqueous pores permeable to small molecules. In addition, HBP is reactive with the anti-porin antibody.

III. Mitochondrial Porin

Mitochondrial porin was named according to the fact that it forms pores in outer mitochondrial membrane similar to those formed by bacterial porin in bacterial outer membranes. It exists only in the outer mitochondrial membrane in rat liver mitochondria (79), and this is presumably the same with mitochondria from other tissues. The aqueous pore is permeable to low molecular weight water-soluble molecules (80-82). The permeability has been

measured by conductance across a lipid bilayer (83-87), liposome swelling (87), and leakage of radioactivity from lipid vesicles loaded with radioactively labeled molecules during reconstitution (88).

Gellerfors and Linden reported that all of the peptides of the outer mitochondrial membrane are translated on cytoplasmic ribosomes (89). Mitochondrial preporin (porin before being inserted into outer mitochondrial membrane) is synthesized with the same size as the mature porin and inserted into outer mitochondrial membrane (90-92).

A. Structure of Mitochondrial Porin

The pore formed by porin is slightly anion-selective and subject to voltage regulation (80, 87, 93-97). It is also called voltage-dependent anion channel (VDAC) because of its voltage dependency. Modification of porin with succinic anhydride virtually eliminates its voltage dependence and changes the channel from anion-selective to cation-selective (94-96). Since succinic anhydride converts amino groups into carboxyl groups, it was suggested that the amino groups are responsible for porin's voltage dependence and anion selectivity. Recent work by Mirzabekov and Ermishkin suggested both negative and positive charges are involved in determining the voltage dependency of porin

(98). Modification of mitochondrial porin with water-soluble carbodiimide in the presence of ethylenediamine resulted in conversion of negative charges of the carboxyl groups of the protein into positive charges. This modification has little effect on the channel-forming ability of porin and the open-state conductance of the channel. However, the modification significantly enhances the voltage dependence of the channel and increases the anion selectivity of the channel (98). In addition to the effects of voltage, a soluble protein has been suggested to regulate the permeability of porin by inducing channel closure (99).

1. Primary Structure

The rat liver mitochondrial porin has a polarity index (100) of 47.8% (101). The polarity index for porin from *Neurospora* mitochondria and yeast mitochondria is 45.5% (84) and 46.3% (98) respectively. The polarity index for mitochondrial porin is relatively high for a membrane protein. Capaldi and Vanderkooi (100) studied 13 membrane proteins which need detergents or organic solvents to be solubilized. Eleven of them have a polarity of less than 43.5%. Only two of them have a polarity greater than 43.5%, microsomal cytochrome b_5 (51.8%) and calsequestrin from sarcoplasmic reticulum (53.6%).

The amino acid sequences of mitochondrial porin from yeast (102, 103) and *Neurospora* (104) have been deduced from their cDNA clones. However, no amino acid sequence for rat liver porin is available up to the present time.

2. Secondary Structure

Based on the amino acid sequence of yeast mitochondrial porin, a ß-barrel structure has been proposed (103, 105). Analysis of the amino acid sequence of yeast mitochondrial porin reveals that the protein appears to have no segments which could form either a hydrophobic or amphipathic α-helix with enough length to span the membrane but many segments of the protein have alternating hydrophilic and hydrophobic residues which are long enough to span across the membrane as ß-strands. Based on these analyses, a ß-barrel structure was proposed for yeast porin by dividing the protein into 19 transmembrane ß segments. The dimension of the ß-barrel structure has a pore dimension comparable to that determined by electron microscopy (106) and biophysical analysis (107).

3. Oligomeric Structure

Mitochondrial porin forms an aqueous pore on the outer mitochondrial membrane. The diameter of the pore has not been well-established. Single channel conductance measurement estimated that the diameter of the pore is 1.7

nm in yeast (83), 2 nm in rat liver (85) and 2 nm in *Neurospora crassa* (83, 84). This size of the pore was calculated with the following equation (83-85):

$$\Lambda = \sigma \pi r^2/l$$

Λ is the conductance measured, σ is the specific conductance of the salt solution (depending on the kind of salt and its concentration), r is the radius of the pore, and l is the length of the pore (the thickness of outer mitochondrial membrane). The equation assumes the pores are filled with the same external solution and assumes a cylindrical pore with a length of 6 nm (83), or 7 nm (84), or 7.5 nm (85). X-ray diffraction and electron microscopy studies shows a inner diameter of about 2.5 nm and an outer diameter of about 5 nm (82).

Using sucrose gradient centrifugation of porin-Triton X-100 complex, Linden and Gellerfors (108) determined the Stokes radius (5.4 nm), the sedimentation coefficient (2.6 S), and the partial specific volume (0.908 cm^3/g) for porin-Triton X-100 complex. Based on these parameters, a molecular weight of about 170,000 was calculated for porin-Triton X-100 complex. When the amount of Triton X-100 was subtracted from the complex (1.8 g Triton X-100/g porin), the molecular weight for the protein is about 60,000 which corresponds to

a dimer form.

4. Supramolecular Organization: Mitochondrial Contact Sites

Mitochondrial contact sites are regions where inner mitochondrial membrane and outer mitochondrial membrane are apposed (109-113). They have been proposed to be the molecular basis of direct channeling of ATP/ADP through the pore (109, 110, 112, 113). A fraction of mitochondrial membrane that contains both inner and outer mitochondrial membrane marker enzymes has been prepared by Ohlendieck et al. (110). The ratio of hexokinase to monoamine oxidase in this fraction is approximately 5-times higher than the pure outer mitochondrial membrane and the ratio of hexokinase to porin is approximately 3-times higher. Subfractionation of rat brain nonsynaptic mitochondria by digitonin revealed two populations of porin (114). One population appears to form contact sites with the inner mitochondrial membrane and binds most of the hexokinase. It was suggested that the porin-hexokinase complex is located in a cholesterol-free membrane domain together with inner membrane components (114).

Tumor cells usually have a very high glycolytic rate compared with normal cells (115-118). This has been linked to the lack of mitochondrial contact sites in the poorly

differentiated and highly glycolytic tumor cells (112). In these tumor cells, the percentage of ATP from oxidative phosphorylation utilized by membrane bound hexokinase is decreased (119, 120).

Nelson and Kabir reported that in Zajdela hepatoma cells, adenylate kinase, which is located in the intermembrane space of the mitochondria, can supply as much as 50% of the ATP from externally added ADP (119). They argued that oxidative phosphorylation is not the preferential source of ATP for hexokinase bound to mitochondria in hepatoma. They also suggested the lack of direct channeling of ATP/ADP through bound hexokinase.

Lack of contact sites has been reported for undifferentiated but not for differentiated HT29 adenocarcinoma cells (120). HT 29 cells, when grown in the presence of glucose, are poorly differentiated and highly glycolytic. On the other hand, HT 29 cells are 'enterocyte-like' differentiated and less glycolytic in the absence of glucose (121). Ultrastructural studies of mitochondria showed that HT29 cells grown in the absence of glucose possess many contacts between inner and outer mitochondrial membrane (122). In these cells, almost all the ATP used by bound hexokinase originates from oxidative phosphorylation. However, HT29 cells grown in the presence of glucose lack

these contact sites and adenylate kinase contributes up to 55% of the total ATP used by bound hexokinase (120).

B. Function of Mitochondrial Porin

1. Pathway for Water Soluble Molecules

The role of mitochondrial porin in forming an aqueous pore in the outer mitochondrial membrane has been well established (82, 123-126). The pore serves as the entry/exit pathway for water-soluble molecules such as ATP and ADP.

2. Binding of Cytosolic Enzymes

In addition to hexokinase, glycerol kinase has also been found to bind to the outer mitochondrial membrane in a competitive manner with hexokinase (127). Mitochondrial contact sites are also enriched in glutathione transferase (110), creatine kinase (111-113), and diphosphate kinase (113). However, the significance of the binding of glycerol kinase, glutathione transferase, creatine kinase, and diphosphate kinase to outer mitochondrial membrane is not clear.

IV. Significance of Binding of Hexokinase in Regulation of Energy Metabolism

Based on the kinetic differences between bound and

soluble hexokinase, the ability of certain cellular metabolites to affect the distribution of the enzyme between the soluble and bound form, it has been proposed this binding-solubilization is involved in the regulation of hexokinase and glycolysis (3, 71). In addition, binding of hexokinase to the pore presents a physical advantage for capturing ATP which traverses the pore and returning ADP through the pore for oxidative phosphorylation.

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Chapter II

RAT BRAIN HEXOKINASE: THE HYDROPHOBIC N-TERMINUS OF THE MITOCHONDRIALLY-BOUND ENZYME IS INSERTED IN THE LIPID BILAYER

ABSTRACT

Mitochondrially-bound rat brain hexokinase was labeled with the photoactivatable reagent, 3-(trifluoromethyl)-3-(m-[^{125}I]iodophenyl)diazirine. This highly hydrophobic reagent is strongly partitioned into the hydrophobic environment of the membrane core, and thus selectively labels segments of a protein that penetrate this region of the membrane. Labeling of hexokinase was shown to be restricted to the N-terminal region of the molecule. Approximately 80% of the radiolabel was removed by treatment of the enzyme with chymotrypsin, which preferentially cleaves a hydrophobic 9-residue sequence at the extreme N-terminus of the enzyme, and it is considered likely that the remaining 20% was associated with two additional hydrophobic residues, immediately adjacent to this segment but not susceptible to cleavage by chymotrypsin. Labeling of the enzyme was shown to be dependent on maintenance of the association with the membrane. These results are consistent with a model in which binding of hexokinase involves insertion of an 11-residue hydrophobic N-terminal "tail", possibly existing in α-helical secondary structure, into the hydrophobic core of the membrane.

INTRODUCTION

Brain hexokinase (ATP:D-hexose 6-phosphotransferase; EC 2.7.1.1) binds specifically, rapidly, and reversibly to the outer mitochondrial membrane; this interaction is influenced by levels of Glc-6-P and P_i, metabolites closely associated with glycolytic metabolism, and has been postulated to be involved in regulation of hexokinase activity <u>in vivo</u> (1). Briefly, the latter view is based on the observation that binding of the enzyme results in a substantial decrease in its susceptibility to inhibition by the product, Glc-6-P, which is generally considered to be a major regulatory influence <u>in vivo</u>. Furthermore, hexokinase appears to interact with a specific protein of the outer mitochondrial membrane (2), originally called the "hexokinase binding protein" (HBP) but subsequently shown to be identical to the pore-forming protein of the outer mitochondrial membrane (3,4); this location of mitochondrially-bound hexokinase at the pore through which adenine nucleotides traverse the outer mitochondrial membrane may lead to development of a microcompartment within which intramitochondrial oxidative phosphorylation is linked to extramitochondrial glycolysis (1). Thus, definition of the molecular basis for this

interaction of hexokinase and the outer mitochondrial membrane is directly relevant to our understanding of the physiological function and regulation of this enzyme catalyzing the initial step of glycolytic metabolism.

Binding of hexokinase to the outer mitochondrial membrane is thought to involve both electrostatic and hydrophobic forces (5,6). More specifically, and with respect to the latter, the ability of the enzyme to bind to mitochondria has been shown to be critically dependent on a hydrophobic sequence at the N-terminus of the enzyme (6). The involvement of the N-terminal region of the enzyme in this interaction with the outer mitochondrial membrane has also been indicated by the observation that binding of hexokinase to mitochondria is selectively inhibited by monoclonal antibodies recognizing epitopes mapped to this region of the enzyme molecule (6,7). Considering the hydrophobic character of the N-terminal sequence and the role of analogous hydrophobic sequences in other membrane-bound enzymes (e.g.,9,10), it was suggested (6) that this hydrophobic N-terminal "tail" may insert into the hydrophobic core of the membrane, tethering the enzyme at the mitochondrial surface. There was, however, no direct evidence to indicate that the N-terminus of the enzyme is directly inserted into the mitochondrial outer membrane, and it was conceivable that this segment might play a more indirect role in the binding. In the present work, this

proposal has been tested directly using 3-(trifluoromethyl)-3-(m-[^{125}I] iodophenyl)diazirine ([^{125}I]TID), a photoactivatable labeling reagent that partitions into the hydrophobic lipid core of membranes (10) and which has been found quite useful in identifying peptide segments that interact with this region of the membrane (e.g.,10-15).

MATERIALS AND METHODS

Materials

Glc, Glc-6-P,thioglycerol, acrylamide, chymotrypsin and TPCK-treated trypsin were purchased from Sigma Chemical Co. (St. Louis,MO). Affi-Gel Blue (100-200 mesh) was the product of Bio-Rad Laboratories (Richmond, CA), and [^{125}I]TID was from Amersham Corp. (Arlington Heights, IL).

Methods

Assay of Hexokinase Activity and Protein.

Hexokinase activity was determined spectrophotometrically, coupling the hexokinase reaction to NADPH production in the presence of added Glc-6-P dehydrogenase under conditions described previously (16). Protein was determined with the BCA protein assay (Pierce Chemical Co., Rockford, IL), with bovine serum albumin as standard.

Photolabeling of Mitochondrially-Bound Hexokinase by [^{125}I]TID.

Rat brain mitochondria were isolated by the method of Gray and Whittaker (17), resuspended in 0.32M sucrose, and stored at -20°C. Prior to use, the mitochondria were thawed, pelleted by centrifugation for 3 min in an Eppendorf Model 5414 Microfuge, then resuspended in 10 mM HEPES-0.25 M sucrose, pH 7.0, to yield a suspension containing 7.5 mg protein/ml and 4 units hexokinase activity/ml. Subsequent operations were done in subdued light. To one ml of the mitochondrial suspension, 6 μl of [^{125}I]TID (8 μCi/μl, specific activity 10 Ci/mmole) was added, followed by gentle mixing. After incubation, on ice, for 40 min, the mitochondria were again collected by centrifugation as above, washed twice by resuspension in 1 ml 10 mM Glc-10 mM thioglycerol-1 mM $MgCl_2$ followed by centrifugation, and finally resuspended in 2 ml 0.25 M sucrose. Aliquots (0.5 ml) were placed in individual wells of a glass spot plate (Corning No. 7220), and unless otherwise indicated, photolysis was done with 5 consecutive flashes from a xenon photographic flash unit (Vivitar Model 283) from which the plastic filter had been removed; the flash unit was positioned directly above the wells and in contact with the top surface of the plate.

Solubilization and Purification of Mitochondrial Hexokinase.

Solubilization of the enzyme with Glc-6-P and purification by affinity chromatography on Affi-Gel Blue was

done by adaptations of methods used on the macro scale for purification of the enzyme in this laboratory.

Mitochondria were pelleted by centrifugation in the microfuge, then resuspended at 2.3 mg protein/ml in 10 mM Glc-10 mM thioglycerol-1.2 mM Glc-6-P, pH 8.2. After incubation for 40 min at room temperature, the mitochondria were again pelleted by centrifugation, and the supernatant containing the solubilized hexokinase collected. To each ml of the supernatant, the following were added: 10 μl 1 M sodium phosphate, pH 7.0; 10 μl 1 M Glc; 10 μl 50 mM EDTA, pH 7.0; 0.83 μl thioglycerol.

The solubilized enzyme (about 3-5 ml) was loaded on a 2 ml column of Affi-Gel Blue which had been equilibrated with 50 mM TrisCl-10 mM Glc-20 mM thioglycerol-0.5 mM EDTA-20% (v/v) glycerol, pH 7.0, and the column washed extensively with this buffer, then successively with this same buffer at pH 8.0 and then 9.0. Finally, the enzyme was eluted with 1.5 mM Glc-6-P added to the pH 9 buffer. The enzyme was concentrated in a Centricon 30 device (Amicon Corp., Danvers, MA), diluted approximately 10-fold with 0.1 M sodium phosphate-10 mM Glc-10 mM thioglycerol-0.5 mM EDTA, pH 7.0, and reconcentrated. This operation was repeated twice more, resulting in the exchange of the enzyme from column buffer into the phosphate buffer used for proteolytic digestions.

Proteolytic Digestion of Hexokinase.

Polakis and Wilson (18) have previously shown that limited digestion of hexokinase with trypsin, under the conditions used here, results in generation of three major species: a 10 kDa segment originating from the N-terminus, a 40 kDa segment from the C-terminus, and a 50 kDa fragment representing the intervening region of the molecule; partial cleavage products of 90 kDa (40 plus 50 kDa fragments) and 60 kDa (10 plus 50 kDa fragments) are also observed as transient intermediates during the digestion. The functions associated with these regions of the hexokinase molecule have been defined (6,7,19-21); in the present context, it should be noted that the 10 kDa tryptic fragment includes the hydrophobic N-terminal sequence required for binding to mitochondria (6). In contrast to the internal cleavages seen with trypsin, treatment with chymotrypsin results in preferential cleavage within the hydrophobic N-terminal sequence, rendering the enzyme largely intact but unable to bind to mitochondria (6).

The relatively small amount of hexokinase involved in these experiments made it impractical to determine protein content directly. Therefore, the amount of hexokinase in the preparations was estimated based on hexokinase activity and assuming a specific activity of 64 units/mg for the pure enzyme, the latter value being an average seen in several large scale preparations of the enzyme obtained by a

comparable procedure; based on this estimate, the solutions were adjusted to contain 0.2 mg hexokinase/ml. Trypsin or chymotrypsin (4 μg trypsin or 0.2 μg chymotrypsin per 20 μg hexokinase) were added as stock solutions (2 mg trypsin/ml, or 0.7 mg chymotrypsin/ml) dissolved in 0.001 N HCl, followed by incubation for 1 hr at room temperature. Controls were incubated under the same conditions, after addition of an identical amount of 0.001 N HCl alone. For samples to be analyzed by SDS gel electrophoresis, digestions were stopped by heating for 2 min in a boiling water bath prior to addition of the denaturing SDS solution.

Other Procedures.

SDS polyacrylamide gel electrophoresis was performed and gels stained with Coomassie Blue essentially as described by Polakis and Wilson (18). Autoradiograms of the dried gels were obtained by exposure of Kodak X-Omat AR5 film at -70°C, using DuPont intensifying screens. ^{125}I was counted with a LKB 1271 RIAGAMMA Automatic Gamma Counter. Scanning of stained gels or autoradiograms was done with a GS300 Transmittance/Reflectance Scanning Densitometer from Hoefer Scientific Instruments, San Francisco, CA. The ability of hexokinase to bind to mitochondria, either before or after digestion with chymotrypsin, was assessed using rat liver mitochondria as previously described (18).

RESULTS

Confirmation of the Sequestration of [^{125}I]TID into Mitochondrial Membranes and Determination of Photolysis Conditions.

The highly hydrophobic [^{125}I]TID has previously been shown to strongly partition into the hydrophobic environments provided by membrane interiors or liposomes (10,11), and this was confirmed in the present work. After incubation of brain mitochondria with [^{125}I]TID as described in Methods, more than 95% of the radioactivity was associated with the mitochondrial pellet and was resistant to removal by washing of the organelles. It is likely that even the small amount of radioactivity found in the original supernatant (<5%) or in subsequent washes represents [^{125}I]TID associated with lipids known to be present in such supernatants (22). Thus, in accord with results of previous investigators (10,11), [^{125}I]TID is highly partitioned into mitochondrial membranes[1] and therefore can be considered to selectively label polypeptide segments accessible from this

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[1]Since less than 5% of the [^{125}]TID was present in the vast extramitochondrial aqueous space, it is unlikely that any significant amount is present in intramitochondrial aqueous compartments, the matrix, or intermembrane space.

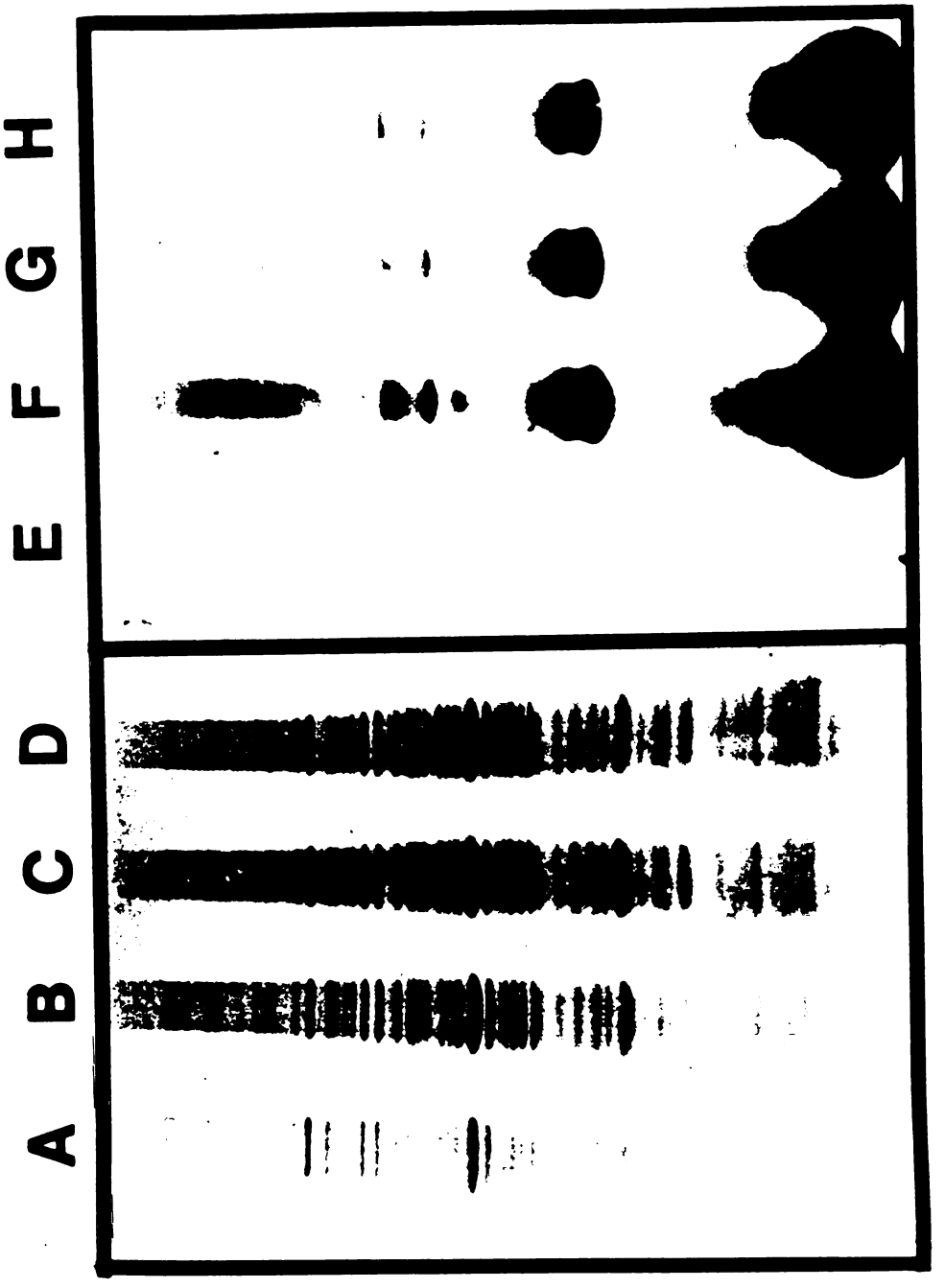
environment. Also consistent with this is the observation that labeling of hexokinase with [^{125}I]TID is dependent on retention of the association between enzyme and mitochondria (see below).

A number of photolysis conditions have been used by previous investigators. Generally, relatively prolonged (several minutes at least) irradiation with rather high intensity sources (xenon arcs,mercury lamps) has been used. This has several potential disadvantages, including considerable heat production. However, as demonstrated by the results shown in Figure 1, photoactivation with a xenon photographic flash unit is at least as effective as irradiation with ultraviolet sources. This has advantages with respect to speed and decreased heat production, and though not directly relevant to the present work, offers the potential for kinetic studies in which photoactivation is restricted to brief controllable periods of time. As stated in Methods, the photographic flash unit was used for the experiments described here.

It should also be noted from the results in Figure 1 that photolabeling of protein and lipid components was strictly dependent on photolysis.

[^{125}I]TID Selectively Labels the N-Terminal Region of Mitochondrially-Bound Hexokinase.

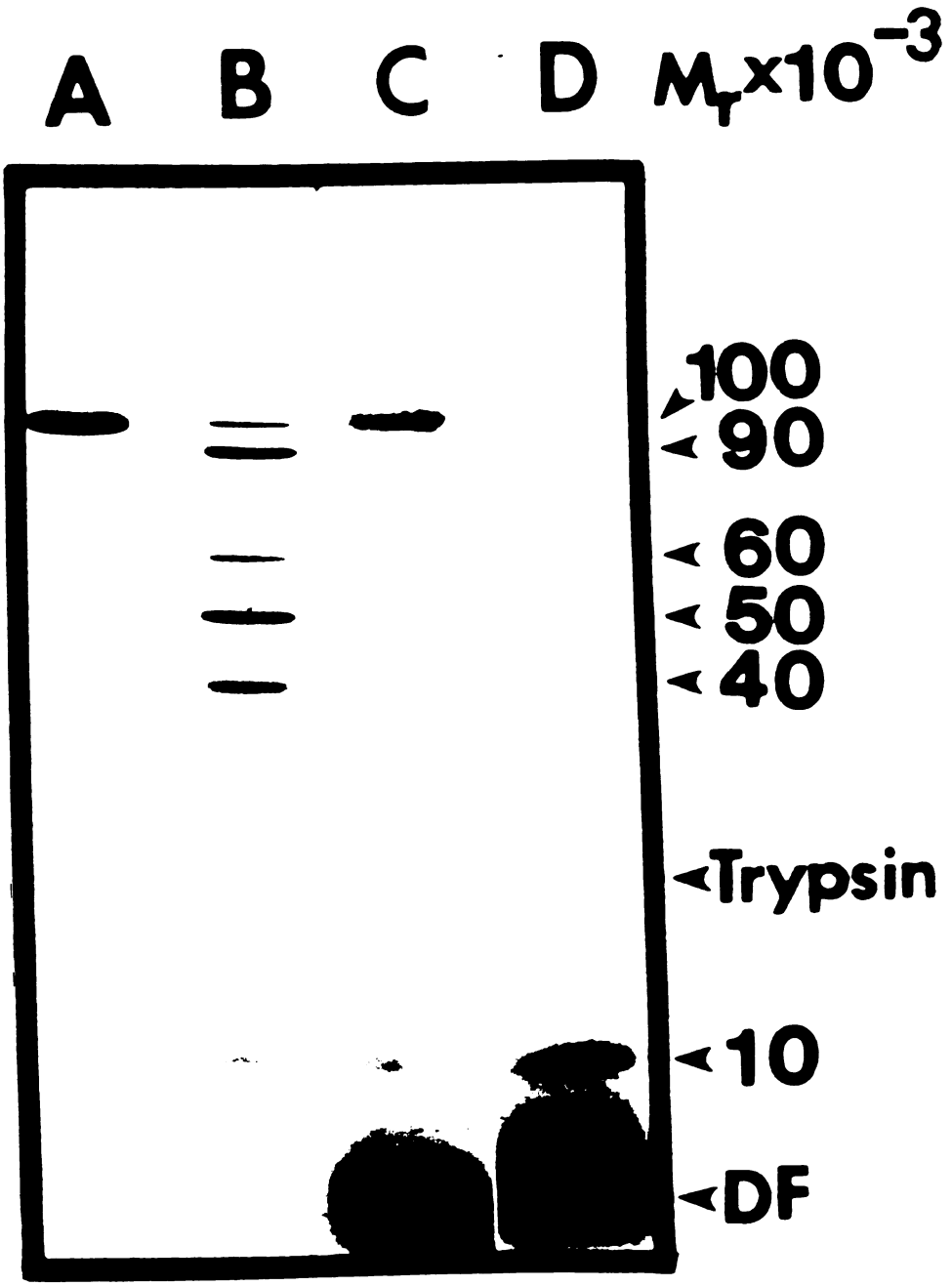
Figure 1. Labeling of Mitochondrial Proteins and Lipids by [^{125}]TID under Different Photolysis Conditions. Rat brain mitochondria were preincubated with [^{125}I]TID as described in Methods, then examined by SDS gel electrophoresis after: A and E, no photolysis; B and F, 5 flashes with a xenon photographic flash unit; C and G, 2 min irradiation with a long wavelength ultraviolet lamp; D and H, 2 min irradiation with a short wavelength ultraviolet lamp. Lanes A-D, Coomassie Blue-stained gel; Lanes E-H, corresponding autoradiogram.


A B C D E F G H

Photolysis of rat brain mitochondria, preloaded with [^{125}I]TID, resulted in labeling of the hexokinase that was subsequently solubilized and purified from the mitochondria (Figure 2, Lanes A and C). A large amount of radioactivity was also associated with a component, or components, migrating with the tracking dye. This almost certainly is due to lipids which are highly labeled with this reagent- Brunner and Semenza (10) found almost 95% of the label to be associated with the lipid fraction of photolyzed erythrocyte membranes- and which can remain tenaciously associated with proteins even after extensive purification. For example, Frielle et al. (12) found that small amounts of highly labeled lipids remained associated with transpeptidase despite repeated precipitations with acetone.

Digestion of the labeled hexokinase with trypsin provided the fragments described above (Figure 2, Lane B) and autoradiography disclosed that the label was almost totally associated with the 10 kDa fragment representing the N-terminal region of the molecule (Figure 2, Lane D). Though not visible in this photograph, close examination of the original autoradiogram indicated that detectable amounts of radioactivity were also associated with the residual (intact) 100 kDa enzyme as well as with the 60 kDa intermediate; both of these species include the 10 kDa fragment within their sequence. A trace amount of a 90 kDa fragment, seen after staining with Coomassie Blue (Figure 2,

Figure 2. **Labeling of Mitochondrially-Bound Hexokinase, and Derived Tryptic Fragments, by** [^{125}I]TID. Rat brain mitochondria containing bound hexokinase were preincubated with [^{125}I]TID and photolyzed, and the labeled enzyme solubilized and purified as described in Methods. Lane A, Coomassie Blue-stained gel after electrophoresis of purified enzyme; Lane C, autoradiogram of preparation shown in Lane A. A duplicate aliquot of the purified enzyme was subjected to limited tryptic digestion (see Methods) prior to SDS gel electrophoresis, giving rise to the cleavage pattern previously described (18); Lane B, Coomassie Blue-stained gel, and Lane D, corresponding autoradiogram. Molecular weights of the various bands are indicated at the right of the figure, as is the position of trypsin. DF, tracking dye front.


A
B
C
D
$M_r \times 10^{-3}$
100
90
60
50
40
Trypsin
10
DF

Lane A), as well as a detectable amount of radioactivity in a 10 kDa band (Figure 2, Lane C) even without addition of trypsin results from the previously noted (6,23) susceptibility of hexokinase to endogenous proteases present in brain homogenates. These fragments remain associated by strong noncovalent forces, with retention of properties characteristic of the intact enzyme (18); the cleaved enzyme apparently copurifies with the intact molecule under these conditions.

[^{125}I]TID Selectively Labels the Hydrophobic N-Terminal Sequence.

The N-terminal sequence of rat brain hexokinase was originally determined by conventional methods of protein chemistry by Polakis and Wilson (6) and has subsequently been confirmed by sequencing of cDNA for the enzyme (24, and D.A.Schwab and J.E.Wilson, manuscript in preparation). Only the first 17 residues are shown here:

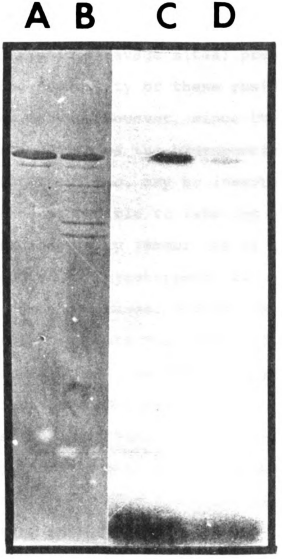
X-met-ile-ala-ala-gln-leu-leu-ala-tyr-tyr-phe-thr-glu-leu-lys-asp-asp

where X represents an unidentified group, probably an acetyl moiety, blocking the N-terminal methionine. The first 11 residues in this sequence obviously constitute a highly hydrophobic segment, and this is followed by a polar region represented by the thr-glu and lys-asp-asp sequences. The

potential significance of this will be considered further in the Discussion; for the present purpose, it is sufficient to note that treatment of the enzyme with chymotrypsin results in preferential cleavage at the positions marked by vertical arrows in the above sequence, and effectively removes the first 9 residues of this sequence (6).

Mitochondrially-bound hexokinase was photolabeled with [^{125}I]TID and the enzyme isolated as described above. Treatment of the enzyme with chymotrypsin resulted in a marked decrease in the ability of the enzyme to bind to mitochondria, 85% being bindable before chymotrypsin treatment compared with 5% after. SDS gel electrophoresis (Figure 3, Lanes A and B) demonstrated that, although some cleavage at internal sites had occurred (generating smaller fragments), the majority of the enzyme- 76% of that in the untreated sample, based on densitometric analysis- retained a mobility indistinguishable from that of the intact enzyme. These results are in accord with the previously described (6) effects of chymotrypsin, noted earlier. In contrast, densitometric analysis of the autoradiograms (Figure 3, Lanes C and D) disclosed that, compared with the untreated sample, only 19% of the radioactivity remained associated with the major component, i.e., with enzyme from which the extreme N-terminal residues have been removed by chymotryptic action (6). Thus, approximately 80% of the

Figure 3. Limited Chymotryptic Digestion of Mitochondrial Hexokinase Labeled with [^{125}]TID. Mitochondrial hexokinase was photochemically labeled with [^{125}I]TID, and solubilized and purified as described in Methods. Duplicate aliquots of the enzyme were examined by SDS gel electrophoresis before (Lanes A and C) or after (Lanes B and D) limited digestion with chymotrypsin (see Methods). A and B, Coomassie Blue-stained gel; C and D, corresponding autoradiogram.


A B C D

total radioactivity is associated with the 9 N-terminal residues removed by chymotrypsin, consistent with the view that it is this segment that is inserted into the TID-containing hydrophobic core of the outer mitochondrial membrane.

As indicated above, chymotrypsin does not cleave residues 10 and 11 (tyr and phe), even though they represent potential chymotryptic cleavage sites; presumably this results from inaccessibility of these residues in the folded structure of the enzyme. However, since these residues represent a continuation of the hydrophobic sequence, it seems likely that they, too, may be inserted into the membrane and hence accessible to labeling by [^{125}I]TID; thus, the remaining radioactivity (about 20% of the original amount), not removed by chymotrypsin, may be incorporated at one or both of these positions. Whether or not that is the case, these results indicate that most, if not all, of the label is restricted to the hydrophobic sequence at the extreme N-terminus of hexokinase.

It may also be noted from the results in Figure 3 (Lanes C and D), that chymotrypsin treatment markedly reduces the amount of labeled lipid associated with the enzyme, suggesting that the hydrophobic N-terminal sequence also serves as the primary location for adsorption of lipids. Nonspecific adsorption of 8-azido-ATP to this region of the molecule has also been observed and attributed to

interactions between this hydrophobic segment and the aromatic adenine ring system (19).

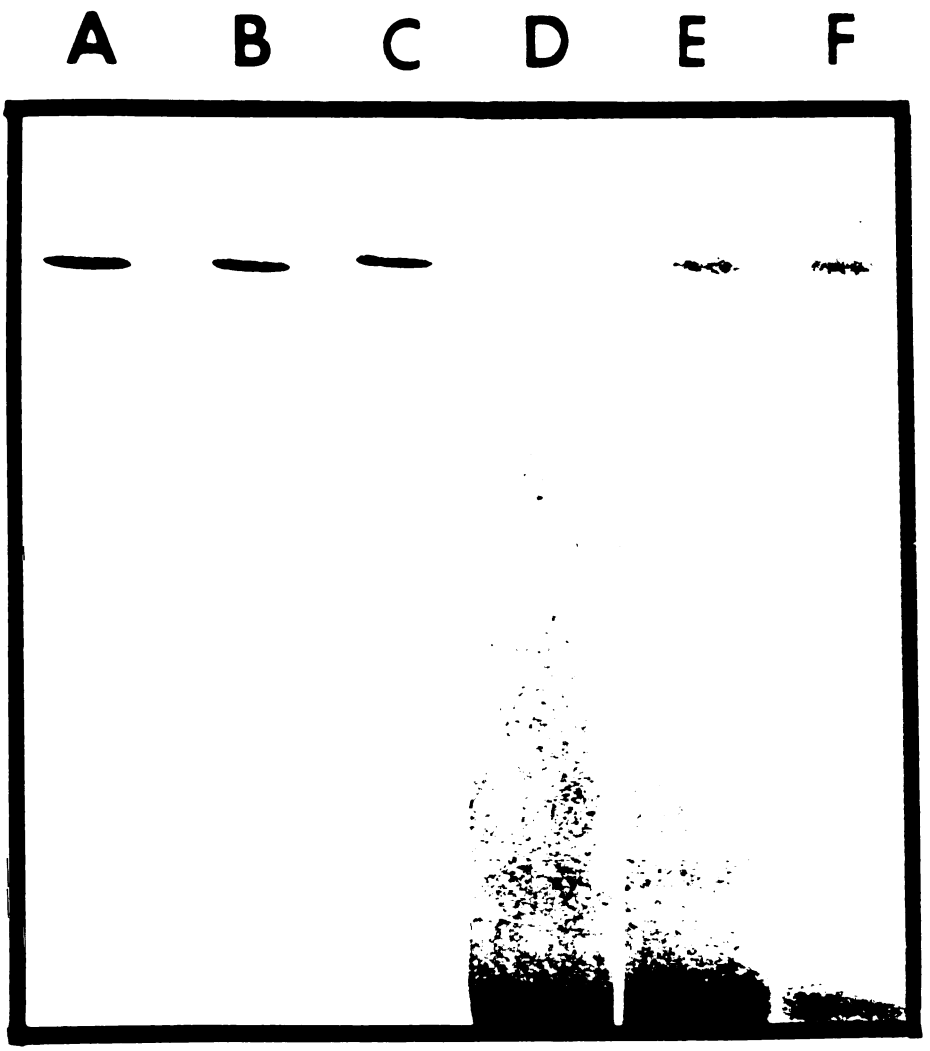
Labeling of Hexokinase Is Dependent on Its Binding to Mitochondria.

Krebs et al. (25) demonstrated that [^{125}I]TID can label calmodulin in aqueous solution, but only in the conformation induced by binding of calcium ion. Frey et al. (26) showed that [^{125}I]TID is an active site directed inhibitor of cytochrome P-450. These results indicate that [^{125}I]TID can label soluble proteins or domains of membrane proteins outside the membrane if there is a suitable hydrophobic site to which [^{125}I]TID can bind. Thus, it was important to verify that labeling of hexokinase by [^{125}]TID was indeed dependent on association of the enzyme with the membrane. The results of the following experiment show that to be the case.

Rat brain mitochondria were preincubated with [^{125}I]TID as described in Methods, then washed twice and resuspended in 10 mM HEPES-10 mM Glc-10 mM thioglycerol, pH 8.2, and divided into three equal aliquots. The following additions were made: Sample 1 received 1.2 mM Glc-6-P, Sample 2 received 1.2 mM Glc-6-P plus 3 mM Mg^{++}, and Sample 3 received 3 mM Mg^{++}. All samples were incubated at room temperature for 40 min, then photolyzed and mitochondria pelleted in the microfuge. Supernatants were assayed for the amount of hexokinase solubilized during the incubation at

room temperature in the presence of added ligands. Incubation with Glc-6-P alone (Sample 1) resulted in solubilization of 52% of the total activity, while less than 3% of the activity was solubilized in the presence of Mg^{++} (Samples 2 and 3); these results are as expected based on previous work showing Glc-6-P to be a potent solubilizing agent, while Mg^{++} counteracts the solubilization by this ligand (1,5). Thus, at the time of photolysis, approximately 50% of the hexokinase was dissociated from the mitochondria in Sample 1, while virtually all of the enzyme maintained its association with the outer mitochondrial membrane in Samples 2 and 3. After photolysis and centrifugation, the mitochondrially-bound enzyme in Samples 2 and 3 was solubilized by resuspension in the indicated buffer containing 1.2 mM Glc-6-P, followed by incubation at room temperature for 40 min; this resulted in solubilization of 48% of the total activity in Sample 2 and 43% in Sample 3, very similar to that solubilized in Sample 1 (52%) when incubated under comparable conditions. The Glc-6-P-solubilized enzyme from each of the samples was then purified by Affi-Gel Blue affinity chromatography, as described in Methods, and the enzymes examined by SDS gel electrophoresis (Figure 4). Although comparable amounts of protein were applied to the gel, as seen after staining with Coomassie Blue (Lanes A-C), the radiolabeling of the enzyme obtained from Sample 1 was markedly decreased compared to

Figure 4. **Labeling of Hexokinase by [^{125}I]TID Is Prevented by Glc-6-P-Induced Release from the Mitochondrial Membrane.** The procedure for this experiment is described in the text. Briefly, mitochondria containing bound hexokinase were preincubated with [^{125}I]TID, then divided into three equal aliquots which were further incubated with Glc-6-P (Lanes A and D), Glc-6-P and $MgCl_2$ (Lanes B and E), or $MgCl_2$ alone (Lanes C and F), prior to photolysis and subsequent purification of the enzyme. In the presence of Glc-6-P alone, approximately 50% of the enzyme had been released from the membrane prior to photolysis, while in the presence of Mg^{++} (with or without Glc-6-P), virtually all of the enzyme maintained its association with the membrane. A-C, Coomassie Blue-stained gel; D-F, corresponding autoradiogram.


A
B
C
D
E
F

that seen with Samples 2 and 3 (Lanes D-F). Based on densitometric analysis, the relative specific radioactivities of the hexokinase in Samples 1, 2, and 3 were approximately 0.25, 1, and 1, respectively.

DISCUSSION

The results presented here indicate that binding of rat brain hexokinase to mitochondria results in insertion of the hydrophobic N-terminal segment into the hydrophobic core of the membrane. Examination of the amino acid sequence in this region of the molecule discloses a sequence of 11 residues that are hydrophobic in character, compatible with such insertion, followed by polar residues that would be incompatible with the hydrophobic environment of the membrane interior and thereby limit further insertion. Since it would be thermodynamically unfavorable for polar elements of peptide bonds to remain isolated in the hydrophobic membrane core, their participation in hydrogen bonding, with resultant secondary structure, can be expected. Application of the Chou-Fasman method (27) to this 11-residue sequence results in a predicted helical potential of P_α=1.17 and ß-sheet potential of P_β=1.19[2]. Frank et al. (28) have argued

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[2]The Chou-Fasman procedure is based on observed secondary structures of globular proteins in an aqueous environment, and its applicability to nonaqueous environments is problematic. Its use here is intended only to indicate that, for hexokinase in aqueous solution, this hydrophobic segment is predicted to show a tendency toward secondary structure formation, a tendency that may be enhanced in the hydrophobic environment of the membrane core (see text).

that a hydrophobic environment would favor helix formation; furthermore, ß-sheet structure obviously implies involvement of multiple segments, while the present results argue strongly for the view that only a limited N-terminal segment of hexokinase is inserted in the membrane. These considerations suggest that this 11-residue sequence may exist in an α-helical structure which penetrates into the hydrophobic core of the outer mitochondrial membrane. Since it is estimated that it would require a helix of approximately 20 residues to span a membrane (29), it is evident that an 11-residue helix at the N-terminus of hexokinase could not completely traverse the membrane, but rather would penetrate into, but not through, the lipid core. Thus it would seem likely that any interactions of this segment with other membrane components are largely restricted to those present in the outer half of the lipid bilayer.

It is interesting that this hydrophobic segment is immediately followed by several residues possessing charged sidechains. Electrostatic forces, possibly with membrane lipid headgroups, have also been implicated in the binding of hexokinase to mitochondria (5). It is of particular interest that negatively charged asp and glu residues are found in this region, and conceivable that they may serve as ligands for divalent cations thought to serve as bridges between negatively charged groups on enzyme and membrane

(1,5).

Clearly there are other factors that determine the binding of hexokinase to the outer mitochondrial membrane. If insertion of the hydrophobic N-terminal segment were the only thing involved, it would be reasonable to expect that any membrane could provide a suitable lipid environment. This contrasts with the marked specificity that the enzyme shows for the outer mitochondrial membrane as a binding site (2). Available evidence would suggest that it is interactions with the pore-forming protein that provide the observed specificity (2-4). Whether these interactions involve the N-terminal segment and occur in the hydrophobic environment of the membrane core, or alternatively, involve other regions of the hexokinase molecule cannot be determined at present. The most straightforward and parsimonious interpretation of currently available information would favor the first alternative. The critical role for the N-terminal hydrophobic sequence has been previously demonstrated (6) and the present work has provided direct evidence for the insertion of this segment into the membrane where it would be in excellent position to interact with the pore-forming protein, which obviously traverses the membrane, including the core region.

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Chapter III

TETRAMERIC STRUCTURE OF MITOCHONDRIALLY-BOUND RAT BRAIN HEXOKINASE: A CROSS-LINKING STUDY

ABSTRACT

Rat brain hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) was derivatized with sulfosuccinimidyl 2-(m-azido-o-nitrobenzamido)-ethyl-1,3'-dithiopropionate (SAND), a photosensitive and cleavable cross-linking agent. The catalytic activity and mitochondrial binding properties of the enzyme were only marginally affected by reaction with SAND. When the derivatized enzyme was bound to liver mitochondria, photolysis resulted in extensive formation of a single cross-linked species with estimated molecular mass 460 kDa. This was determined to contain only hexokinase and thus represents a tetramer of the 116 kDa (apparent molecular mass in gel system used) monomeric enzyme. Although small amounts of tetramer were detected after photolysis of relatively high concentrations of derivatized enzyme in free solution, tetramer formation was greatly enhanced when the enzyme was bound to mitochondria. No evidence of dimeric or trimeric structures was seen even when only a small fraction of the available binding sites on the mitochondrial membrane were occupied. It is thus concluded that tetramer formation is closely linked with binding of the enzyme to the outer

mitochondrial membrane and, more specifically, to the pore structure through which metabolites traverse this membrane. It is speculated that a tetrameric structure surrounding the mitochondrial pores may facilitate interactions between the hexokinase reaction and oxidative phosphorylation, mediated by the adenine nucleotides which are common intermediates in these reactions.

INTRODUCTION

Hexokinase (ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1) catalyzes the initial step in metabolism of glucose. Though inhibition by the product, Glc-6-P, is undoubtedly a major factor in regulation of hexokinase activity (and thereby the cerebral glycolytic rate), reversible interactions between hexokinase and mitochondria have also been suggested to be of regulatory significance (reviewed in 1).

The enzyme binds specifically to the outer mitochondrial membrane (2,3). It is known that a hydrophobic sequence at the N-terminus of the enzyme is critical for binding (4), and that this sequence is inserted into the hydrophobic core of the membrane when the enzyme is bound (5). A protein responsible for the specific interaction with the outer mitochondrial membrane was isolated by Felgner et al. (3) and subsequently found to be identical to the pore-forming protein of the outer mitochondrial membrane (6,7), leading to the view that hexokinase is situated at the pore through which metabolites traverse the outer mitochondrial membrane (1). This provided a topological basis for interpreting the results of several studies (8-10)

which had indicated close interactions between the reaction catalyzed by mitochondrially-bound hexokinase (utilizing ATP, producing ADP) and oxidative phosphorylation (utilizing ADP, producing ATP) associated with the inner mitochondrial membrane. Recent work by Brdiczka and his colleagues (11,12) has suggested that these interactions may occur primarily at the "contact sites" between the inner and outer mitochondrial membranes.

These considerations make it evident that the binding of hexokinase to mitochondria represents an intriguing convergence of cellular structure and metabolism that may be of considerable physiological import. We now report the results of a cross-linking study which has led to a quite unexpected finding: binding of hexokinase is accompanied by formation of an oligomeric structure (tetramer). This has implications with respect to both the physical disposition of the enzyme at the membrane pore structure as well its metabolic interaction with mitochondrial oxidative phosphorylation.

MATERIALS AND METHODS

Materials

SAND was purchased from Pierce (Rockford, IL). Protein molecular weight markers (myosin, 205,000; β-galactosidase, 116,000; phosphorylase b, 97,400; bovine albumin, 66,000; egg albumin, 45,000; carbonic anhydrase, 29,000) were purchased from Sigma Chemical Co. (St. Louis, MO). Thyroglobulin was from Pharmacia LKB Biotechnology Inc. Affi-gel and affinity-purified goat anti-rabbit IgG (H+L) horseradish peroxidase conjugate were the products of Bio-Rad Laboratories (Richmond, CA).

Methods

Rat brain hexokinase was purified according to Wilson (13). Rat liver mitochondria were prepared by differential centrifugation as described by Bustamante et al. (14), suspended in 0.25 M sucrose to give approximately 4 mg mitochondrial protein/ml, and stored at -20°C; the hexokinase binding capacity of seven different preparations of rat liver mitochondria, determined by titration with

increasing amounts of purified rat brain hexokinase, was 1.1 ± 0.2 (av. ± SD) units per mg mitochondrial protein. Affinity-purified rabbit antibodies against rat brain hexokinase were obtained as previously described (15).

Assay of Hexokinase Activity and Protein.

Hexokinase activity was measured spectrophotometrically, coupling the hexokinase reaction to NADPH production in the presence of excess Glc-6-P dehydrogenase (13). Protein was determined with the BCA protein assay (Pierce Chemical Co., Rockford, IL), with bovine serum albumin as standard.

Assay of the Ability of Hexokinase to Bind to Mitochondria.

Hexokinase (0.1 unit) was incubated with excess rat liver mitochondria (0.8 mg protein) in 400 μl 0.25 M sucrose, 3 mM $MgCl_2$. After 20 min on ice, the suspension was centrifuged in a Brinkmann microfuge for 2 minutes. The pellet was dispersed in 400 μl 0.25 M sucrose containing 0.5% Triton X-100. Both the supernatant and the pellet were assayed for hexokinase activity; binding ability is expressed as the activity in the pellet divided by the total activity (supernatant plus pellet).

Preparation of High Molecular Weight Standards by Cross-linking of ß-Galactosidase.

β-galactosidase (5 mg/ml in 0.1 M phosphate, pH 7.0) was incubated with 0.01% (w/w) glutaraldehyde at room temperature for one hour. The reaction was stopped by the addition of 0.1 volume of 1 M lysine, pH 7.0.

Derivatization of Rat Brain Hexokinase with SAND.

SAND is a photoactivatable heterobifunctional cross-linking (spacer arm, 18.5 Å) reagent containing a disulfide group cleavable with reducing agents. SAND reacts with free amino groups (e.g., on surface lysine residues) of proteins under mild conditions via its succinimidyl group. Subsequent photoactivation of the azidophenyl group generates the highly reactive nitrene derivative which reacts avidly and nonspecifically with adjacent molecules, resulting in cross-linking. Subsequent reduction of the disulfide group permits identification of cross-linked species. The following reaction and subsequent spectrophotometric analysis and cross-linking experiments were performed in subdued light.

A 1 mM SAND solution in Buffer A (0.01 M potassium phosphate, 0.01 M Glc, 0.5 mM EDTA, pH 7.5) was added to a solution containing approximately 0.5 mg/ml hexokinase in this same buffer, to give a final concentration of 0.05 mM SAND. After incubation at room temperature for 30 minutes, 0.1 volume of 1 M lysine (pH 7.5) was added and incubation at room temperature continued for another 10 minutes to scavenge unreacted SAND. The sample was then loaded onto a

Sephadex G-25 (fine) column equilibrated with buffer A and eluted with the same buffer. The fractions with hexokinase activity were combined and concentrated to about 40 units/ml using a Centricon 30 device (Amicon Corp., Danvers, MA).

Spectrophotometric Analysis of SAND, Underivatized Hexokinase, and Derivatized Hexokinase.

Hexokinase has a molar extinction coefficient of 5.1 x 10^4 $mole^{-1}$ at 280 nm (16). Since the molar extinction coefficient of SAND was not readily available at the time of the experiment, the molar extinction coefficient of ANB-NOS at 312 nm (17), 9 x 10^3 $mole^{-1}$, was used instead. Both SAND and ANB-NOS have the same 5-azido-2-nitrobenzoyloxy group within their structures, and presumably they also have similar absorption spectra. This assumption was supported by the fact that SAND concentration obtained spectrophotometrically is comparable to that obtained by weight analysis.

Samples were scanned with a Gilford Response spectrophotometer from 240 nm to 480 nm. In the case of SAND, approximately 0.1 mM SAND in buffer A was incubated with 10 mM lysine at room temperature for 30 minutes before the scan. Since its spectrum (240 nm - 480 nm) was not changed by the addition of 10 mM lysine, buffer A was used as the blank in all scans.

SAND and underivatized hexokinase were scanned to

determine the molar extinction coefficients of SAND at 280 nm and hexokinase at 312 nm. The number of SAND residues per derivatized hexokinase molecule was then calculated from the absorption spectrum of derivatized hexokinase using the known and determined molar extinction coefficients of SAND and hexokinase at 280 nm and 312 nm.

Cross-linking of Derivatized Hexokinase Bound to Mitochondria.

Hexokinase that had been derivatized with SAND (0.7 units) was added to 500 μl of a mitochondrial suspension (1.5 mg/ml) in 0.25 M sucrose, 3 mM $MgCl_2$, 10 mM Hepes, pH 7.0. After incubation on ice for 20 min, the mitochondria were sedimented by centrifugation in a microfuge for 2 min. The pellets containing bound hexokinase were washed twice with 0.25 M sucrose, 10 mM HEPES, pH 7.0, and resuspended to their original volume in 0.25 M sucrose.

An aliquot of 200 μl was transferred to a glass spot plate (Corning No. 7220) for photolysis as previously described by Kiehm and Ji (18); this was done with 3-5 flashes using a Xenon electronic flash unit from which the front window had been removed. Control nonphotolyzed samples were maintained in subdued light.

With the exceptions noted below, mitochondria were again pelleted by centrifugation prior to analysis by SDS-PAGE. Virtually all of the hexokinase (>90%) remained

associated with the mitochondria in both photolyzed and nonphotolyzed samples.

SDS-PAGE and Immunoblotting.

SDS-PAGE was performed essentially as described by Polakis and Wilson (19) with some modifications. The pelleted mitochondria were dispersed in sufficient sample buffer (0.063 M Tris, pH 6.8, 2% SDS, 4% glycerol, 0.005% bromophenol blue, 8 mM N-ethylmaleimide) to give a protein concentration of about 3 mg/ml, then heated at 100° C for 1 min. Approximately 150 μg protein/lane was loaded for SDS-PAGE. Gels were run at 60 volts for 15 hours and either stained with Coomassie Blue (20) or transferred to nitrocellulose (21) at a constant current of 150 mA for approximately 20 hours.

Cross-linked species were identified by a second SDS-PAGE after reduction with 4.5% (v/v) 2-mercaptoethanol. Following electrophoresis as described above and staining with Coomassie Blue, the area containing the cross-linked species was cut from the gel. As a control, the corresponding region was also taken from a gel in which the nonphotolyzed sample had been electrophoresed. Proteins were electroeluted using an ISCO electrophoretic concentrator (ISCO, Lincoln, NE) at 3 W for 4-5 hours in 0.021 M Tris, 0.16 M glycine, 0.1% SDS. A 4-fold concentrated version of the SDS gel sample buffer (see above), either with or

without inclusion of 18% (v/v) 2-mercaptoethanol, was added to give final concentrations of sample buffer components as above and 4.5% (v/v) 2-mercaptoethanol. The samples were heated for 1 min at 100°C prior to SDS-PAGE. Gels were either silver stained (22) or electroblotted onto nitrocellulose as described above.

Immunoreactive species on nitrocellulose blots were detected as described previously (23) except that 3% (w/v) nonfat dry milk (24) was used in place of gelatin as blocking agent, and the incubation with the anti-hexokinase antibodies was 2-12 hrs at room temperature (the longer time was for reasons of convenience).

RESULTS

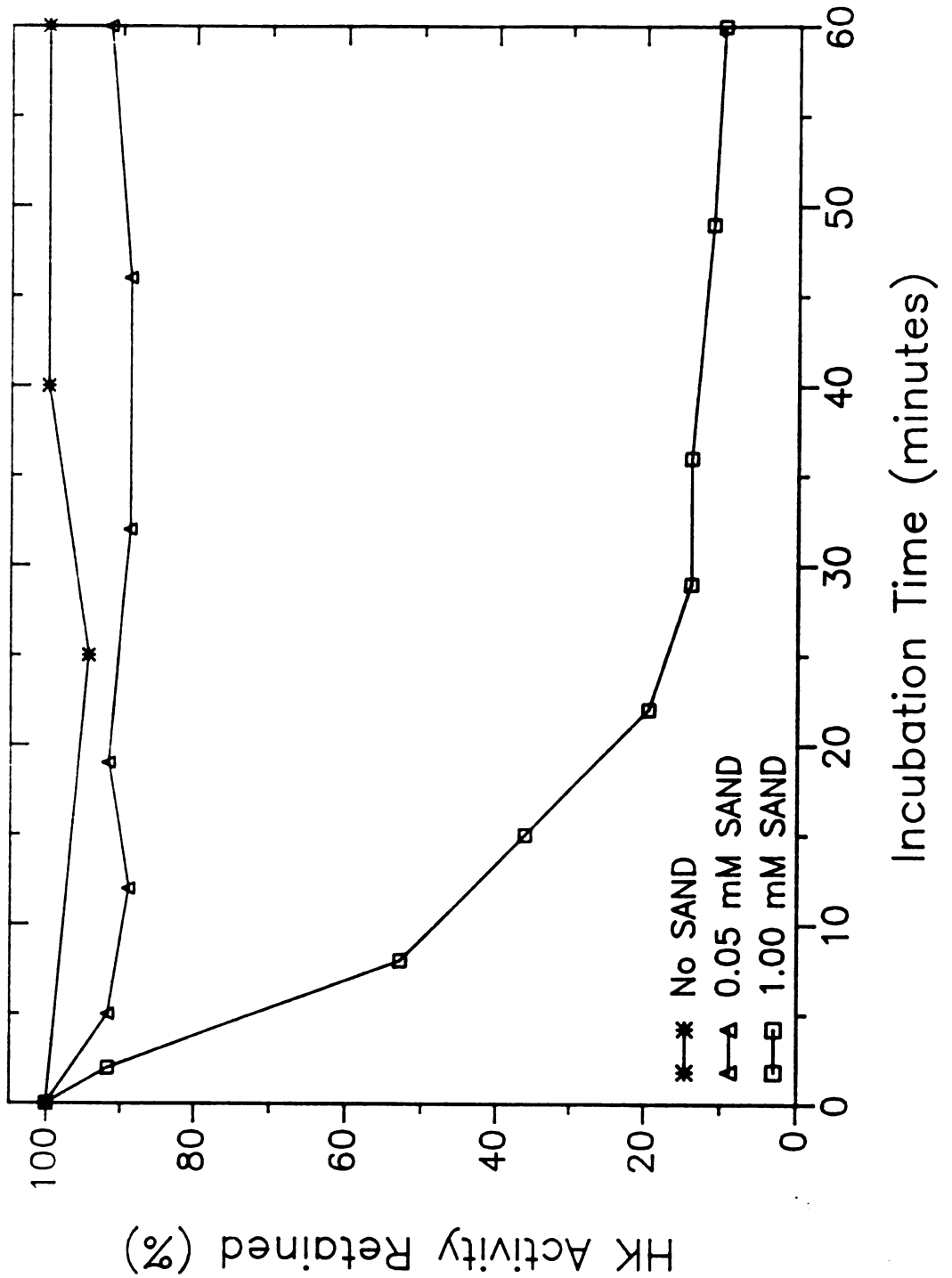
Effects of Derivatization with SAND on the Properties of Hexokinase.

The effects of reaction with 0.05 mM and 1 mM SAND on hexokinase activity are shown in Figure 1. After 1 hr with 0.05 mM SAND (a molar ratio of 10 SAND per hexokinase), more than 90% of the original activity remained while with 1 mM SAND, extensive loss of activity occurred.

As noted above, 0.05 M SAND was used in the standard derivatization procedure. Under the latter conditions, derivatization had only a modest effect on the ability of the enzyme to bind to mitochondria. For example, in one experiment, more than 90% of the total activity was bindable prior to derivatization (some loss of bindability occurs during purification of the enzyme due to limited proteolysis of the hydrophobic N-terminal sequence by endogenous proteases (4)), while after derivatization, 75% was bindable. Similar marginal loss of binding ability was seen in each of two other experiments in which this was monitored.

Mitochondrially-bound hexokinase can be solubilized by Glc-6-P, but much less effectively by other hexose

Figure 1. Effects of SAND on the Activity of Hexokinase. Hexokinase (36 units/ml in buffer A) was incubated at room temperature with 0 mM, 0.05 mM, or 1 mM SAND, and activity monitored as a function of time.


HK Activity Retained (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
Incubation Time (minutes)
No SAND
0.05 mM SAND
1.00 mM SAND

monophosphates, such as Gal-6-P (3). The results in Table I show that derivatized hexokinase retains this characteristic.

These results make it evident that derivatization with 0.05 M SAND does not markedly affect either the catalytic activity or mitochondrial-binding properties of brain hexokinase. Further support for this is the observation that binding of the derivatized enzyme is competitive with that of untreated hexokinase (see below).

Determination of the Number of SAND Residues per Derivatized Hexokinase Molecule.

The molar extinction coefficients of hexokinase at 312 nm and SAND at 280 nm were determined to be 6.5×10^3 $mole^{-1}$ (from Figure 2, Panel A) and 9.8×10^3 $mole^{-1}$ (from Figure 2, Panel B) respectively. The number of SAND residues per derivatized hexokinase molecule was calculated to be 7.5 (from Figure 2, Panel C).

Crosslinking of Mitochondrially-Bound Hexokinase and Analysis of Cross-Linked Species.

Photolysis of mitochondrially-bound derivatized hexokinase resulted in the appearance of a major high molecular weight species, easily detected on the Coomassie Blue stained gel (Figure 3); based on comparisons with standards (see below), the M_r of the cross-linked component

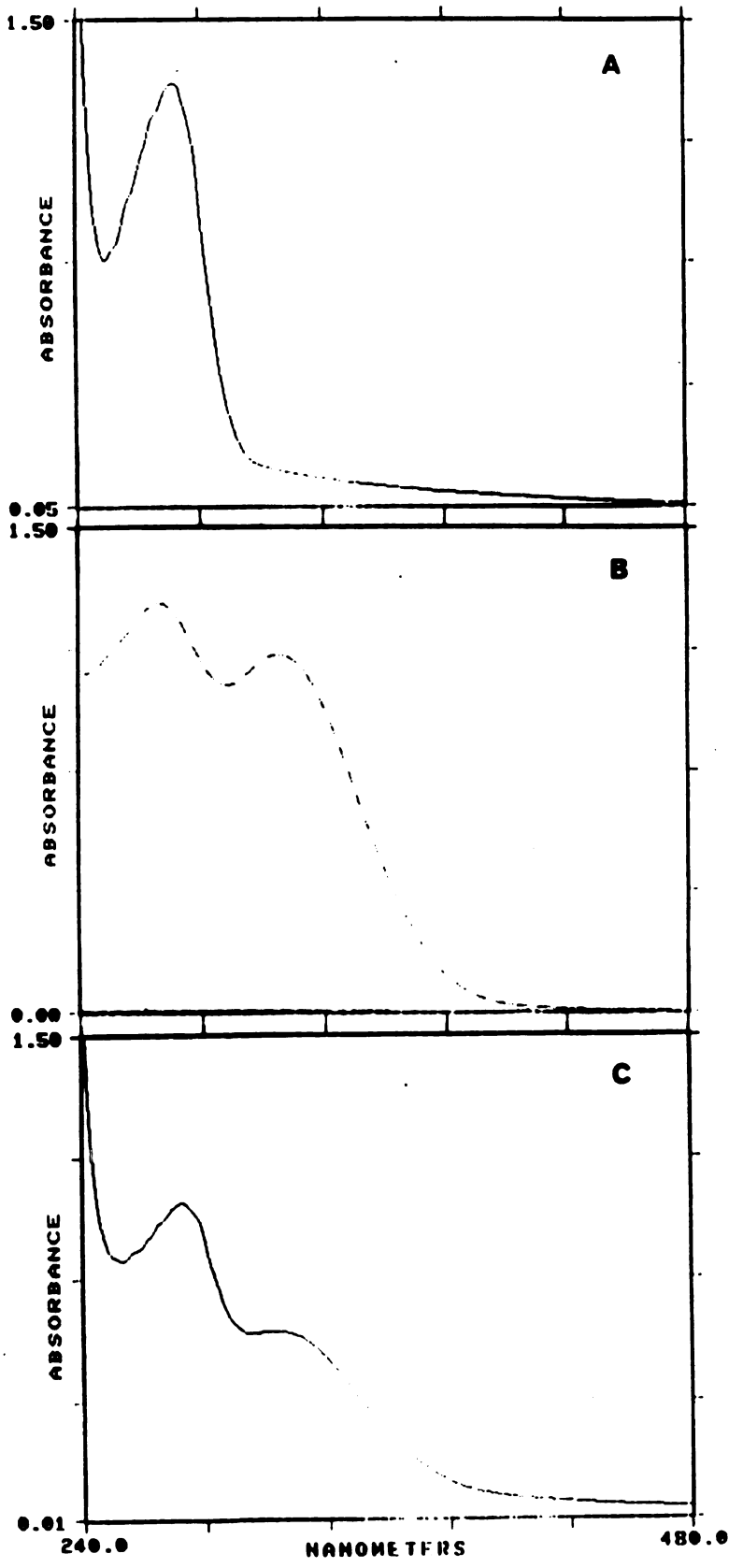
Table I

Solubilization of Mitochondrially-Bound Derivatized and Nonderivatized Hexokinase by Glc-6-P and Gal-6-P[a]

	Hexokinase	
	Nonderivatized	Derivatized with SAND
units bound per mg mitochondrial protein	0.64	0.58
% Solubilized with:		
No ligand	16	23
1 mM Glc-6-P	73	81
1 mM Gal-6-P	23	37

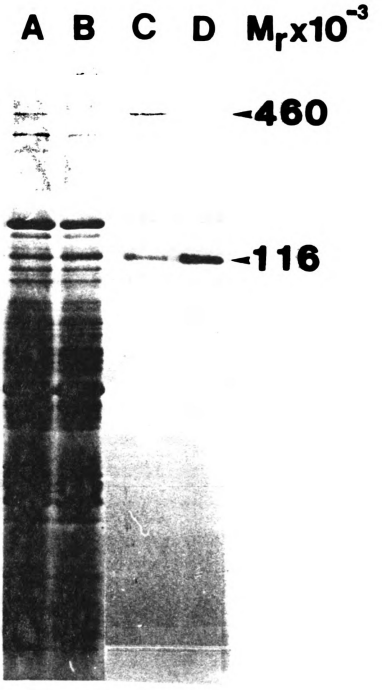
[a]Hexokinase (2 units), either untreated or derivatized with SAND, was incubated for 20 min on ice with rat liver mitochondria (0.57 mg protein) in 2 ml of 0.25 M sucrose, 3 mM $MgCl_2$. The mitochondria, now containing bound hexokinase, were collected by centrifugation and washed twice by resuspending in 0.25 M sucrose and recentrifuging. The washed pellet was resuspended in 1 ml 2 mM sodium phosphate, 2 mM thioglycerol, 2 mM Glc, 0.1 mM EDTA, 0.25 M sucrose, pH 6.6 (3). Bound activity was determined, then replicate aliquots were incubated for 15 min at room temperature with no added ligand, 1 mM Glc-6-P, or 1 mM Gal-6-P. After centrifugation, solubilized activity was determined by assay of supernatant and pellets.

Figure 2. **Absorption Spectra of Underivatized Hexokinase, SAND, and Derivatized Hexokinase. Panel A, underivatized hexokinase in buffer A; Panel B, SAND in buffer A, 10 mM lysine; Panel C, derivatized hexokinase in buffer A.**


1.50
A
ABSORBANCE
0.05
1.50
B
ABSORBANCE
0.00
1.50
C
ABSORBANCE
0.01
240.0
NANOMETERS
480.0

WAVELENGTH

Figure 3. Cross-linking of Mitochondrially-bound Hexokinase. Hexokinase that had been derivatized with SAND was bound to rat liver mitochondria and photolyzed as described in Methods. After SDS-PAGE, the gel shown at the left was stained with Coomassie Blue. Lane A, photolyzed sample; Lane B, nonphotolyzed control. An immunoblot, prepared from a duplicate gel, is shown at the right. Lane C, photolyzed sample; Lane D, nonphotolyzed control. The positions of the cross-linked species, with apparent M_r of 460,000, and of the monomeric hexokinase which migrates as a species of apparent M_r of 116,000 (see text), are marked by the arrows at the right of the figure.

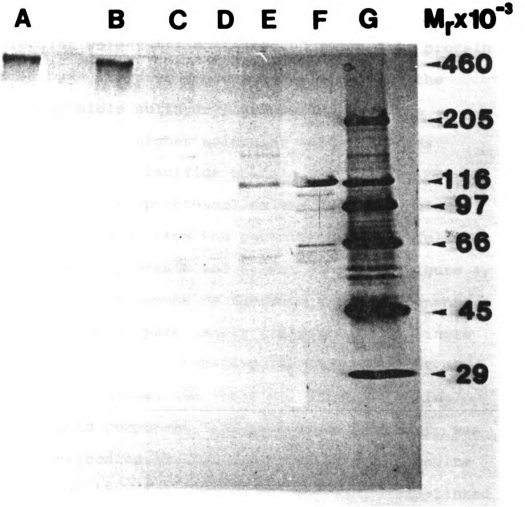

A
B
C
D
$M_r x10^{-3}$
460
116

was estimated as 460,000.

Both the cross-linked species and monomeric hexokinase were detected on immunoblots probed with antibodies against the enzyme (Figure 3). In some cases, including the experiment shown in Figure 3, a second immunoreactive component with apparent molecular weight slightly less than that of the major cross-linked species could also be detected; this was always present in minor amount, and for the purpose of presenting these results, we will consider both of these components as a single cross-linked species. Treatment of the photolyzed sample with 2-mercaptoethanol prior to electrophoresis resulted in disappearance of this component, as detected by either Coomassie Blue or immunoblotting, confirming that the proteins in this component were indeed cross-linked via the cleavable disulfide bridge of SAND.

The cross-linked species, and proteins from the corresponding region of a gel in which the nonphotolyzed sample had been electrophoresed, were recovered by electroelution as described in Methods. A second electrophoresis without treatment with 2-mercaptoethanol confirmed that a single high molecular weight species was present in the electroeluted samples (Figure 4, Lanes A and B). After treatment with 2-mercaptoethanol and a second electrophoresis, three components were detected (by silver staining) in the lane (Lane C) containing the nonphotolyzed

Figure 4. **Analysis of the Cross-Linked Complex by Reduction with 2-Mercaptoethanol Followed by a Second Electrophoresis.** See text for details. Lanes A and B, proteins electroeluted from 460 kDa region of a gel containing nonphotolyzed and photolyzed samples, respectively; no 2-mercaptoethanol added prior to second electrophoresis. Lanes C and E, same samples as in Lanes A and B, but with 2-mercaptoethanol added prior to second electrophoresis. Lane D, 2-mercaptoethanol in sample buffer (i.e., no protein); artifactual bands attributed to 2-mercaptoethanol (25,26) are evident. Lane F, purified rat brain hexokinase, migrating with apparent M_r of 116,000 (see text); minor impurities are also seen in this silver stained gel. Lane G, standard proteins with molecular weights indicated at right.


A
B
C
D
E
F
G
$M_r x 10^{-3}$
460
205
116
97
66
45
29

sample, one with apparent molecular mass of 160 kDa, and a closely spaced doublet in the 60 kDa range. The latter components were also seen in a lane (Lane D) receiving only 2-mercaptoethanol in the sample buffer, and represent artifacts arising from the 2-mercaptoethanol (25,26). The identity of the 160 kDa species is not known but a good possibility may be carbamoyl phosphate synthetase, an abundant protein in the matrix of liver mitochondria with a subunit molecular weight of about 160,000 (27); this protein is influenced by sulfhydryl reagents (27), implying the presence of accessible sulfhydryl groups and thus the possibility of forming higher molecular weight species through intermolecular disulfide bridges.

Addition of 2-mercaptoethanol to the high molecular weight species recovered from the photolyzed sample followed by subsequent electrophoresis and silver staining (Figure 4, Lane E) revealed the presence of the same three components as seen in the nonphotolyzed sample (Lane C) <u>plus</u> a single additional component with a mobility identical to that of authentic rat brain hexokinase (Lane F). Electroblotting confirmed that this component, and only this component, was reactive with antibodies against brain hexokinase (results not shown). It was therefore concluded that the cross-linked species formed upon photolysis of mitochondrially-bound derivatized hexokinase contains hexokinase and <u>only</u> hexokinase.

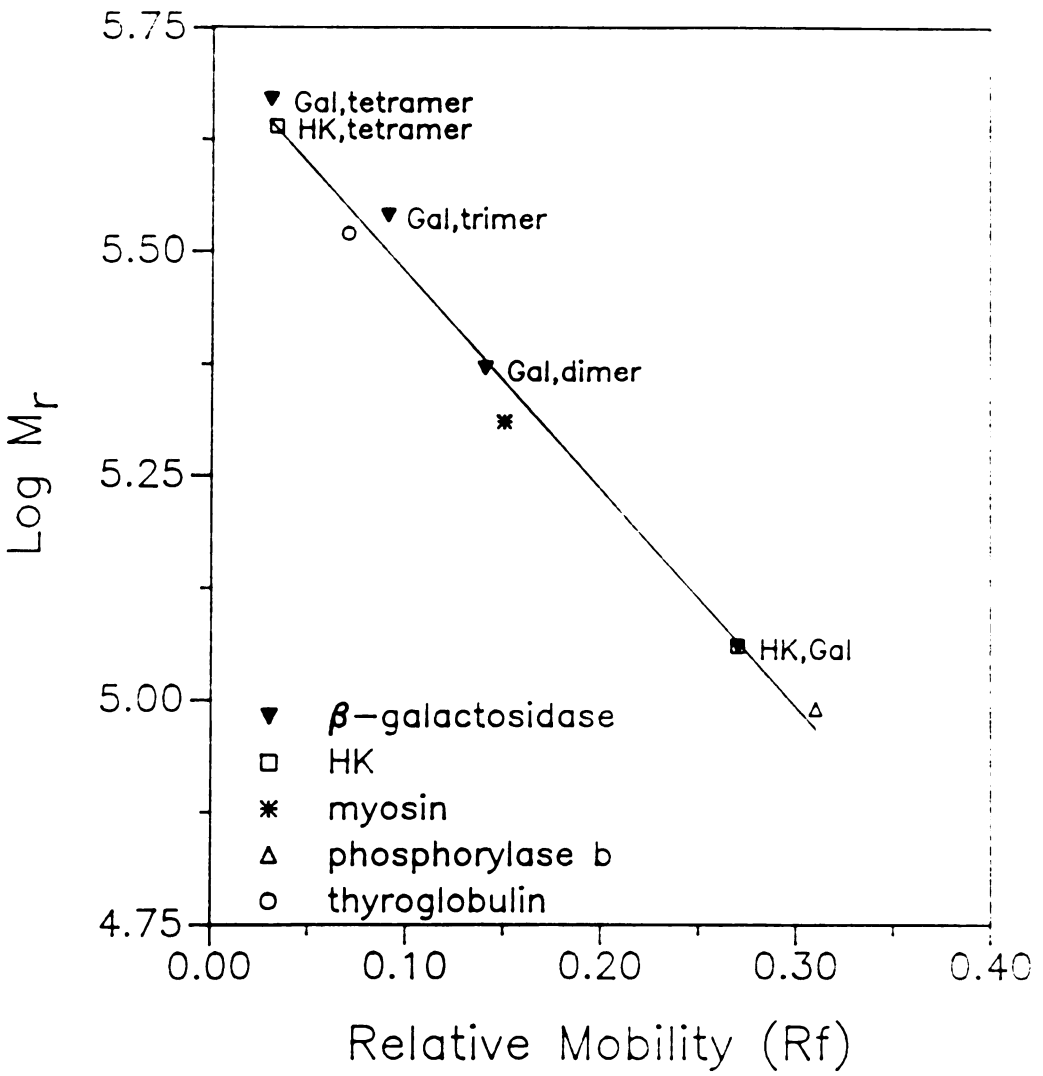
Comparison with various standards (Figure 5) indicated a molecular weight of about 460,000 for the cross-linked species resulting from photolysis of the mitochondrially-bound derivatized enzyme, virtually identical to that of the tetramer produced by cross-linking of ß-galactosidase with glutaraldehyde. Although the Mr of rat brain hexokinase calculated from the known sequence (28) is 102,000, we have previously noted (29) that the monomer migrates with an apparent molecular weight of 116,000, the same as that of ß-galactosidase in this SDS-PAGE system. Thus the cross-linked species is a tetramer of hexokinase.

The 460,000 molecular weight component, plus lower molecular weight species indistinguishable from the dimer and trimer of ß-galactosidase, were seen when hexokinase (approx. 0.5 mg/ml) was cross-linked with glutaraldehyde, as described in Methods for ß-galactosidase (gel not shown, but relative mobilities as in Figure 5). Thus, dimeric and trimeric cross-linked forms of hexokinase should have been detectable if they were formed to a significant extent by photolysis of the mitochondrially-bound derivatized enzyme, but this was not the case (Figs. 3 and 4).

Formation of the Cross-linked Tetramer Is Greatly Enhanced by Retention of Association with the Mitochondria.

Mitochondria with bound derivatized hexokinase were incubated (in subdued light) with or without 1 mM Glc-6-P

Figure 5. Molecular Weight of the Cross-linked Species. The molecular weight of the cross-linked hexokinase was estimated to be 460,000 based on comparison with thyroglobulin, myosin, phosphorylase b, and cross-linked oligomeric species of ß-galactosidase, separated by SDS-PAGE. Since hexokinase was the only protein in the cross-linked species, this corresponded to a tetramer formed by cross-linking of the 116 kDa (apparent molecular mass) monomeric hexokinase.


5.75
5.50
5.25
5.00
4.75
Log M_r
Gal,tetramer
HK,tetramer
Gal,trimer
Gal,dimer
HK,Gal
β−galactosidase
HK
myosin
phosphorylase b
thyroglobulin
0.00
0.10
0.20
0.30
0.40
Relative Mobility (Rf)

prior to photolysis. As noted above, the effect of Glc-6-P is to promote release of the enzyme from the mitochondria, which was confirmed in the present experiment (15% solubilized after incubation without Glc-6-P, and 95% solubilized in its presence). Thus, both samples contained identical amounts of hexokinase and mitochondria, but differed in the distribution of the enzyme between bound and dissociated forms. Subsequent photolysis and analysis[1] by SDS-PAGE and immunoblotting (Figure 6) demonstrated a marked decrease in tetramer formation with the Glc-6-P-treated sample.

An identical result was obtained when hexokinase was solubilized by increasing ionic strength (30), i.e., the amount of cross-linked tetramer was decreased as the enzyme was progressively solubilized by increasing NaCl concentrations (Figure 7). The absence of detectable dimeric or trimeric forms further suggests that interactions between the molecules comprising the tetramer are not affected by changes in ionic strength over a range sufficient to disrupt interactions between enzyme and membrane.

These results demonstrate that formation of the tetramer is correlated with retention of the association with the mitochondrial membrane, and that the effect of

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[1]In this experiment as well as the one with results shown in Fig. 7, the mitochondria were not collected by centrifugation prior to SDS-PAGE. The entire samples were analyzed so that both solubilized and bound hexokinase would be included on the gel.

Figure 6. Formation of the Cross-linked Tetramer Is Greatly Decreased by Glc-6-P-Induced Release of Hexokinase from the Mitochondria. Mitochondria containing bound hexokinase, derivatized with SAND, were preincubated in the absence (Lanes A and B) or presence (Lanes C and D) of 1 mM Glc-6-P prior to photolysis; 15% and 95% of the hexokinase was solubilized in the absence and presence of Glc-6-P, respectively. The entire samples were then analyzed by SDS-PAGE and immunoblotting. Lanes A and C, nonphotolyzed controls; Lanes B and D, photolyzed samples.

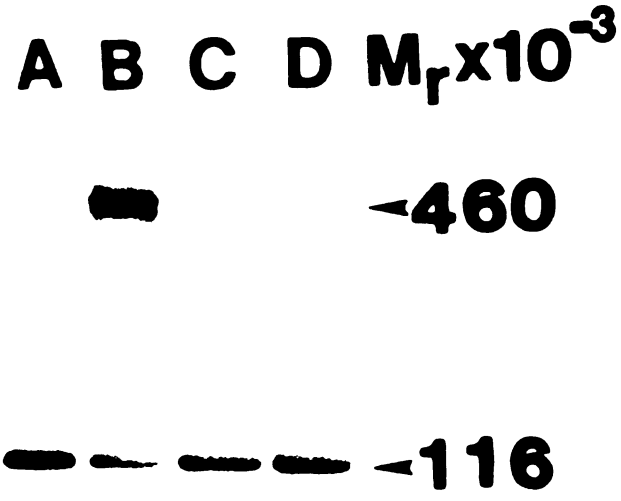
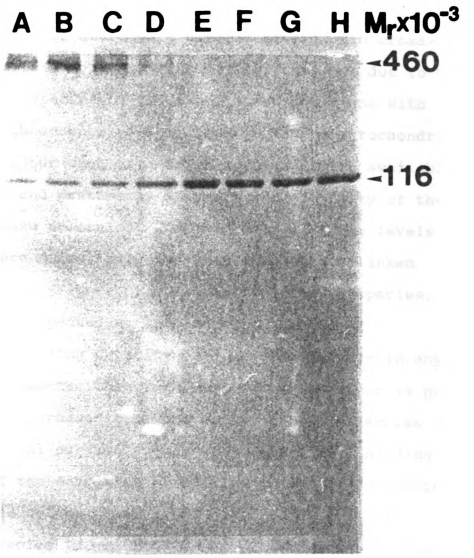

A B C D $M_r x10^{-3}$
-460
-116

Figure 7. **Formation of the Cross-linked Tetramer Is Greatly Decreased When the Association of Hexokinase with the Mitochondria is Disrupted by Increasing Ionic Strength.** Mitochondria containing bound hexokinase, derivatized with SAND, were incubated for 30 min at room temperature with increasing concentrations of NaCl. Aliquots were taken for determination of bound and solubilized activities, and the percent of the total activity retained in bound form is shown in parenthesis after the corresponding salt concentration. The samples were then photolyzed and analyzed by SDS-PAGE and immunoblotting. Lanes A-D, photolyzed samples preincubated with 0 (83% bound), 45 mM (79% bound), 87 mM (59% bound), and 174 mM (25% bound) NaCl, respectively. Lanes E-H, corresponding nonphotolyzed controls.

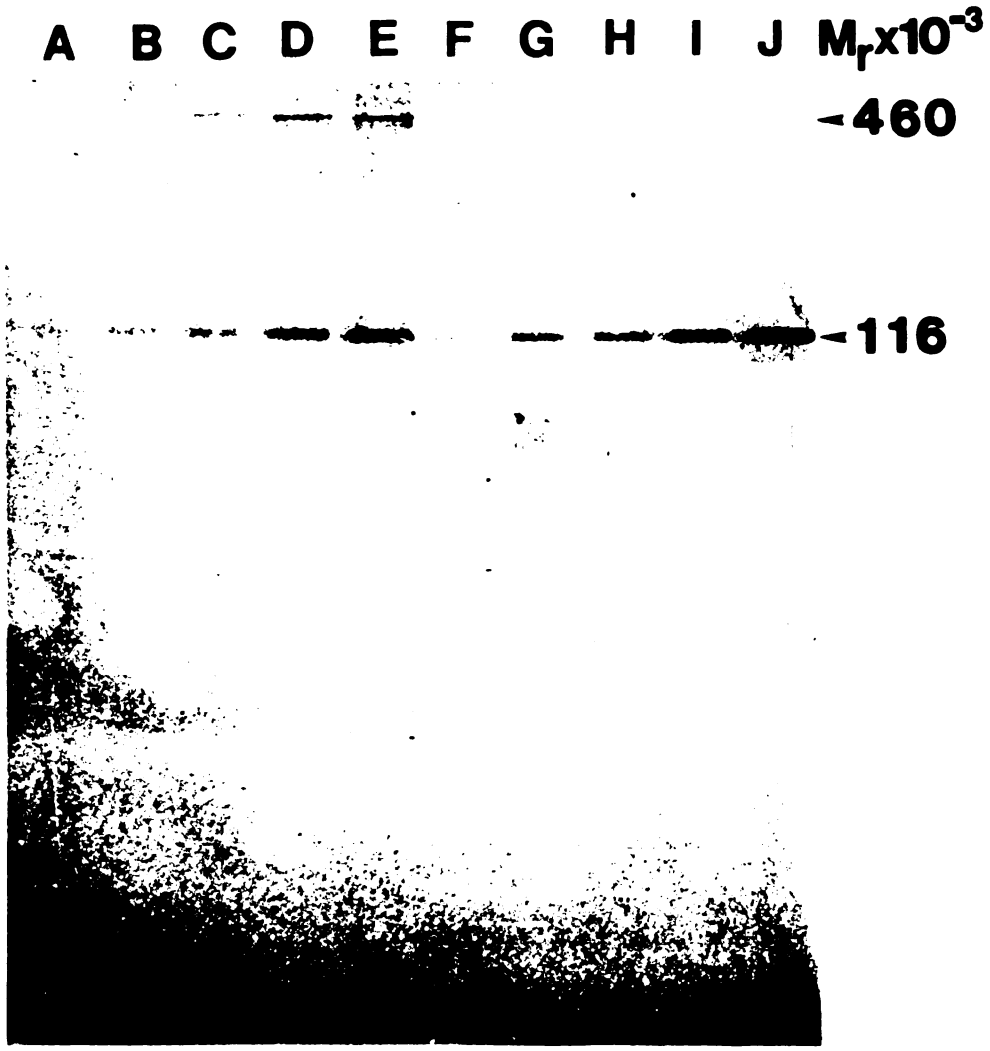

A B C D E F G H $M_r x10^{-3}$
460
116

Glc-6-P on cross-linking was indeed attributable to redistribution of the enzyme between mitochondrially-bound and dissociated forms and not the result of conformational change known to be induced by the binding of Glc-6-P (31).

Only the Tetrameric Cross-linked Species Is Formed.

The absence of detectable dimeric or trimeric cross-linked species was notable. Conceivably, this was due to the fact that the experiments described above were done with relatively high amounts of hexokinase bound to mitochondria, resulting in saturation of a major portion of the available binding sites and presumably in increased proximity of the bound hexokinase molecules. However, even when low levels of hexokinase were bound, only the tetrameric cross-linked species was seen (Figure 8). Dimeric or trimeric species, which might be expected at intermediate levels of saturation of binding capacity have never been seen in any of these experiments. Thus formation of the tetramer is not the result of increased crowding of hexokinase molecules on the mitochondrial surface but is concomitant with binding regardless of the degree of saturation of available binding sites. It also follows that cross-linking to yield the tetrameric species is not the result of random collisions (which would be expected to yield dimeric and trimeric species in even greater proportion), but reflects juxtaposition of four hexokinase molecules at the binding

Figure 8. Only the Tetramer Is Formed Even with Low Amounts of Mitochondrially-Bound Hexokinase. Rat liver mitochondria (0.4 mg protein) were incubated with increasing amounts of derivatized hexokinase to give an increasing degree of saturation of available sites; the binding capacity of the mitochondrial preparation used in this experiment was 1.2 units per mg mitochondrial protein. Lanes A-E, photolyzed samples containing 0.03, 0.08, 0.13, 0.20. and 0.30 units bound hexokinase per mg mitochondrial protein; Lanes F-J, nonphotolyzed controls corresponding to A-E, respectively. After SDS-PAGE, the gel was electroblotted and immunoreactive species detected as described in Methods.


A B C D E F G H I J $M_r x10^{-3}$
460
116

site on the outer mitochondrial membrane.

Underivatized Hexokinase Prevents Binding of the Derivatized Enzyme.

Addition of excess underivatized hexokinase prior to adding the derivatized enzyme to a mitochondrial suspension precluded binding of the latter, as evidenced by prevention of formation of cross-linked species in subsequent photolysis (Figure 9). These results are consistent with competition between derivatized and underivatized enzyme for a common binding site on mitochondria, and exclude the possibility that derivatization with SAND has resulted in anomalous nonspecific binding of the enzyme. In conjunction with the lack of effect on the activity, binding ability, and Glc-6-P-sensitivity of the binding (noted above), these observations generate confidence that the results obtained with the SAND-derivatized enzyme faithfully reflect behavior of the underivatized enzyme.

Formation of Cross-linked Tetramers of Hexokinase in Free Solution.

Small amounts of tetramer, but no dimer or trimer, were detected after photolysis of the derivatized enzyme in free solution (Figure 10). However, this was seen only at very high concentrations of enzyme, much higher than the concentrations (~0.01 mg/ml) that resulted in extensive

Figure 9. Nonderivatized Hexokinase Blocks Binding of Hexokinase Derivatized with SAND. Duplicate aliquots of mitochondria (0.4 mg mitochondrial protein in 280 μl 0.25 M sucrose, 3 mM $MgCl_2$) were prepared. Nonderivatized hexokinase (2.16 units in 60 μl Buffer A) was added to one, while an equal volume of Buffer A alone was added to the other. Derivatized hexokinase (0.37 units in 10 μl Buffer A) was then added to both samples followed by incubation on ice for 20 min. The mitochondria were collected by centrifugation, with subsequent photolysis, SDS-PAGE, and immunoblotting as described in Methods. Lane A, derivatized hexokinase only, photolyzed; Lane C is the nonphotolyzed control for this same sample. Lane B, derivatized hexokinase plus nonderivatized enzyme, photolyzed; Lane D, nonphotolyzed control for this same sample.

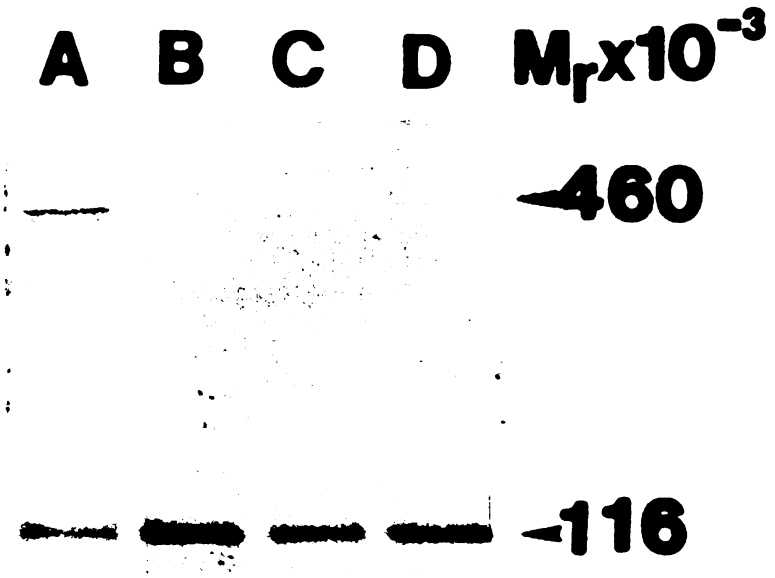
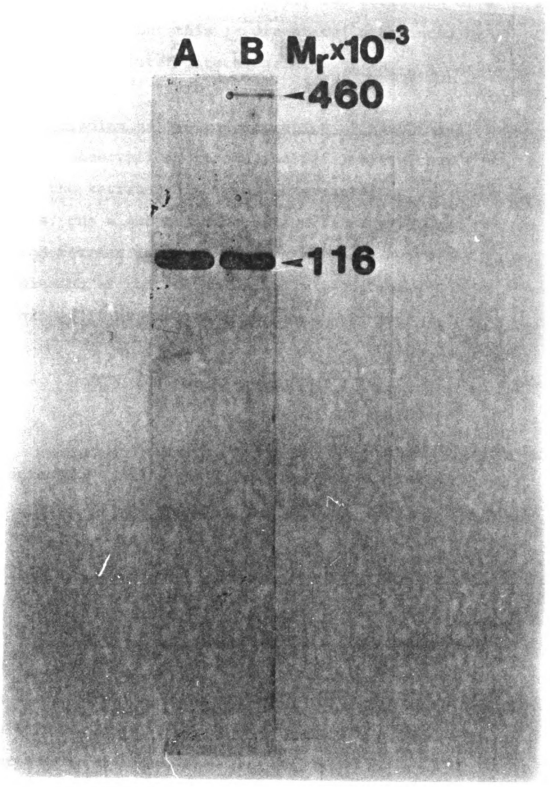

A
B
C
D
$M_r x 10^{-3}$
460
116

Figure 10. Limited Tetramer Formation with Hexokinase in Free Solution. Derivatized hexokinase (0.5 mg/ml) was photolyzed under the same conditions used with the mitochondrially-bound enzyme, and analyzed by SDS-PAGE. The gel was stained with Coomassie Blue. Lane A, nonphotolyzed sample. Lane B, photolyzed sample. When the photolyzed sample was treated with 2-mercaptoethanol prior to electrophoresis, only the monomeric species was detected (not shown).


A
B
$M_r \times 10^{-3}$
460
116

cross-linking of the mitochondrially-bound enzyme. Thus hexokinase has a very limited propensity for formation of a tetrameric structure, but this is dramatically enhanced by association with the outer mitochondrial membrane.

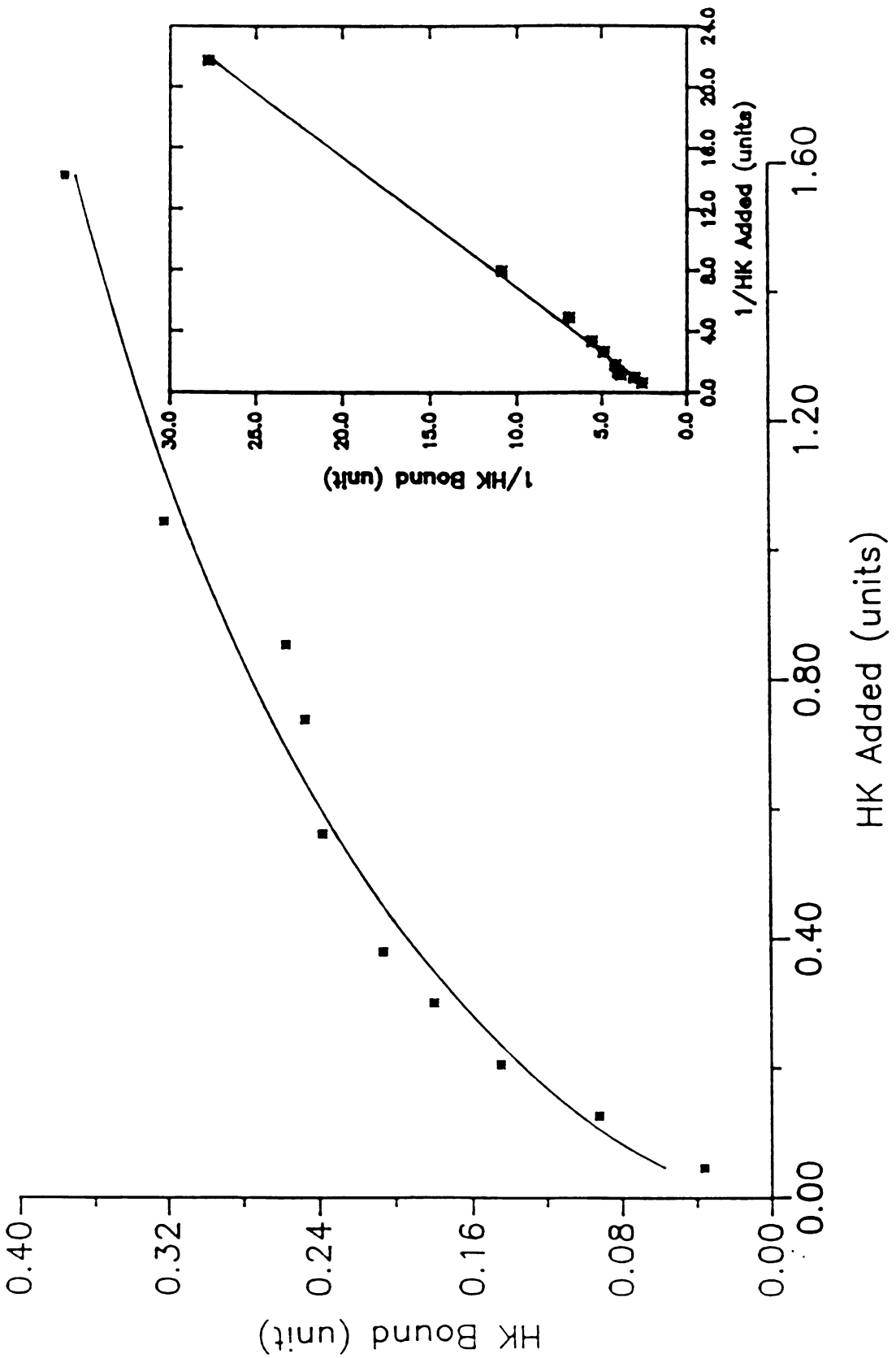
Dependence of Binding on the Concentration of Hexokinase.

The above observations raised the possibility that formation of the tetramer might be a prerequisite for binding. Thus, one might envisage an equilibrium between monomeric and tetrameric forms in free solution, with selective binding of the tetrameric form effectively shifting this equilibrium toward increased tetramer formation:

$$4\ E \rightleftharpoons E_4 \overset{M}{\rightleftharpoons} M\ E_4$$

where E represents the monomeric form of hexokinase, E_4 the tetramer, and M the mitochondria. If this were the situation, binding might be expected to show higher order dependency on hexokinase concentration (since the "initial step" involves formation of the tetramer). However, titration of mitochondria with increasing concentrations of hexokinase showed apparent first order dependency (Figure 11), with a classical hyperbolic approach to saturation of available binding sites. Similar results have previously been reported for the converse experiment, where fixed

Figure 11. Titration of Rat Liver Mitochondria with Brain Hexokinase. Increasing amounts of rat brain hexokinase in Buffer A were added to rat liver mitochondria (0.38 mg protein) suspended in 0.25 M sucrose containing 3 mM $MgCl_2$; Buffer A was added as necessary to maintain the total addition at 40 μl. After incubation for 20 min on ice, soluble and bound activities were determined as described in Methods. The inset shows the data replotted in double reciprocal format; extrapolation to the intercept gave 1.35 units/mg mitochondrial protein as the binding capacity of this mitochondrial preparation. Lines were determined by regression analysis.


HK Bound (unit)
0.00
0.08
0.16
0.24
0.32
0.40
0.00
0.40
0.80
1.20
1.60
HK Added (units)
1/HK Bound (unit)
30.0
25.0
20.0
15.0
10.0
5.0
0.0
0.0
4.0
8.0
12.0
16.0
20.0
24.0
1/HK Added (units)

amounts of hexokinase were titrated with increasing amounts of mitochondria (32). Although we believe these results are most consistent with the view that hexokinase is bound as a monomeric species, we also frankly acknowledge the inadequacy of the above rapid equilibrium representation. For example, it predicts that isolation of mitochondria containing bound hexokinase and resuspension in a medium lacking hexokinase should result in rapid and extensive dissociation of the enzyme. This does <u>not</u> happen, and addition of Glc-6-P is necessary to facilitate solubilization of the mitochondrially-bound enzyme (2, 3, 13). Obviously much greater understanding is required before an adequate mathematical analysis will be possible.

DISCUSSION

Dimerization of hexokinase from several mammalian tissues (33-36), including brain (33), has been reported, but there have been no reports of formation of higher oligomers, except under conditions of prolonged storage (34). Dimer formation is enhanced in the presence of Glc-6-P (33-36) whereas, as noted above, this ligand decreases binding of the enzyme and associated tetramer formation. These observations make it unlikely that the reported dimerization of the enzyme in solution is related in any direct way to the observed tetramer formation.

Any model in which hexokinase is considered to bind randomly to mitochondrial pores, with subsequent cross-linking dependent on coincidental proximity of the hexokinase-pore complexes at the time of photolysis seems inconsistent with the observed results; particularly at lower levels of occupancy of available binding sites, such a model would predict a predominance of dimers, with decreasing proportions of higher oligomers reflecting the decreasing probability that a random distribution would bring 3, 4, or more molecules into proximity at the time of photolysis. Having discarded such random models, there are

at least two others that might be considered to explain how binding of monomeric hexokinase might lead to formation of tetramers.

First, monomeric hexokinase might bind randomly to pores of the outer mitochondrial membrane and these complexes might diffuse within the plane of the membrane to form a core of four hexokinase molecules, presumably still surrounded by pores interacting with the enzyme. Entropic effects might facilitate formation of tetramers between membrane-bound molecules, enhancing the limited tendency for tetramerization seen with the enzyme in free solution. However, we find it difficult to imagine that such a mechanism would not involve dimers and trimers as significant intermediates. Furthermore, this model attributes only a secondary role to the pore protein in organization of the enzyme clusters, which seems inconsistent with the importance of this protein in both the binding (3, 6, 7) and probable metabolic significance (1, 8-10) of this pore-hexokinase interaction.

An alternative model, which we favor, gives the pore structure itself the central role in organization of the tetramer. In this view, binding is a highly "cooperative" process in which binding of an initial hexokinase dramatically enhances the binding of subsequent hexokinase molecules to yield a tetrameric complex surrounding the

central pore structure[2]. The estimated dimensions of the pore are 2.5 nm inner diameter and 5 nm outer diameter (37). The proposed structure for brain hexokinase is a molecule of two domains, each equivalent to a molecular mass of about 50 kDa (28); assuming globular character, a diameter of about 5 nm would be estimated for a domain of this size. It is readily shown that four hexokinase molecules could be radially oriented about the periphery of a pore with sufficient proximity to permit cross-linking with SAND. Orientation of the C-terminal domain, with which catalytic function is associated (29,38), toward the central pore would facilitate interactions between the hexokinase reaction and oxidative phosphorylation via the adenine nucleotides common to these processes, as discussed in the introduction to this paper. This would also orient the hydrophobic N-terminal segment toward the lipid surrounding the pore, into which it could be inserted (5).

The present study has indicated that tetramer formation is directly correlated with binding of the enzyme to

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[2]It might have been anticipated that this would result in some higher order dependence of binding on hexokinase concentration, analogous to the "sigmoid" relationship between binding of substrate and activity of enzymes showing positive cooperativity, but this was not detected (Fig. 11). However, the concept of cooperativity as applied to sigmoid binding curves is based on rapid equilibrium considerations which, as noted above, do not appear to be simply applicable to the present situation. Hence, by using the term "cooperativity", we mean only to convey the notion that binding of one hexokinase at a pore somehow greatly increases the likelihood that additional hexokinase molecules will be bound at this same pore.

mitochondria. There is no obvious reason why this should not also be the case under in vivo conditions, where association of hexokinase with mitochondria is thought to have important physiological consequences (reviewed in ref. 1). Assuming that tetramer formation serves some useful purpose, it is intriguing to consider what advantage it might confer. We suggest one possibility that evolves from the analysis of Abbott and Nelsestuen (39), which indicates that at a sufficiently high density of membrane-bound enzyme, the kinetics of a reaction reach a "collisional limit" at which every encounter between substrate and the membrane surface is productive. Since the uses of ATP are by no means limited to phosphorylation of Glc, it would be undesirable for hexokinase to capture all of the ATP exiting through the mitochondrial pore, i.e., to be operating at the "collisional limit". However, close packing of a tetrameric arrangement of hexokinase about the central pore might represent an approach toward the collisional limit that would assure that interactions between the initial step of glycolysis and mitochondrial oxidative phosphorylation are adequate to achieve their suggested physiological significance (1).

Finally, we draw attention to the implications of another observation made in this study. The detection of the tetramer as a major species, and the absence of dimers and trimers, attests to the high efficiency with which cross-

linking occurs. Thus it is difficult to simply attribute the presence of residual monomeric enzyme, seen in all of these experiments, to failure of cross-linking; were such failure to occur within a tetrameric structure, "partially" cross-linked dimeric and trimeric species would be expected but were never detected. These results suggest that, while a major fraction of the bound enzyme exists as a tetramer, a substantial amount also exists as a monomer. The molecular basis for this apparent division into monomeric and tetrameric species [i.e., whether it results from binding to different forms of the pore-forming protein, and/or reflects differences in the degree of interaction between the inner and outer mitochondrial membranes (11, 12)] and its potential physiological consequences remain subjects for future studies.

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Chapter IV

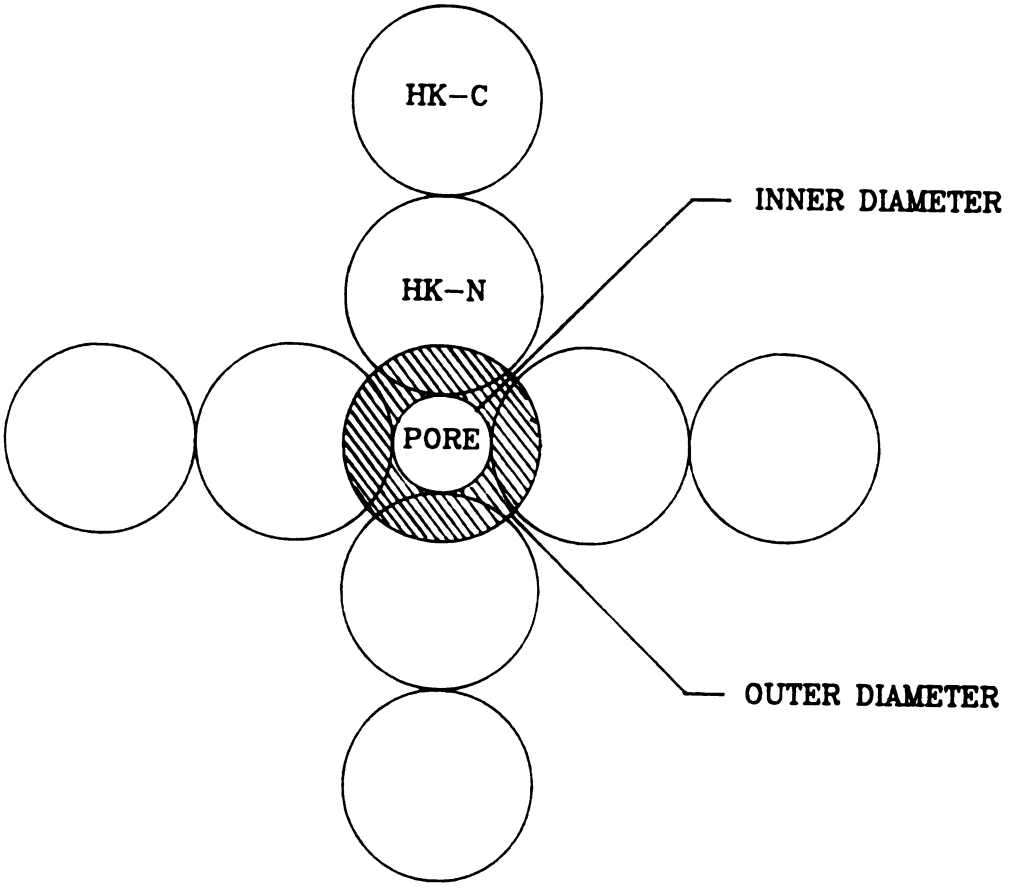
SUMMARY AND PERSPECTIVES

SUMMARY AND PERSPECTIVES

The existence of hexokinase monomer is unlikely the result of incomplete crosslinking because if that were true, hexokinase dimer and trimer should also be detected. The crosslinking experiment was consistent with a model that rat brain hexokinase on outer mitochondrial membrane exists in two populations, either as monomer or as tetramer. Four molecules (instead of one) around a pore could slow down or even block the diffusion of some small molecules and thus create a better chance for hexokinase to capture oxidative ATP.

A schematic model for the arrangement of hexokinase tetramer around mitochondrial pore was shown in Figure 1. The model in intended to show only <u>one</u> of the possible arrangements of hexokinase tetramer around mitochondrial pore. Hexokinase was shown to extend outwards around the pore (parallel to the membrane) in this model although it could extend upwards (perpendicular to the membrane). In the later case, more effective crosslinking may be expected. The pore, as shown in the middle of Figure 1, has a 2.5 nm inner diameter and a 5 nm outer diameter (1). It was assumed in

Figure 1. A Model for the Interaction of Hexokinase with Mitochondrial Pore. HK-N, N-terminal domain of hexokinase; HK-C, C-terminal domain of hexokinase.


HK-C
HK-N
PORE
INNER DIAMETER
OUTER DIAMETER

this model that hexokinase consists of a N-terminal 52 kd domain and a C-terminal 48 kd domain (2), each domain may be approximated as a sphere. The diameters of the 52 kd and 48 kd domains are calculated to be 5 nm and 4.8 nm respectively based on a partial specific volume of 0.74 cm^3/g (3).

It has been reported that mitochondrial creatine kinase binds to the outer side of the inner mitochondrial membrane (4, 5). It was also found in the contact sites formed between inner and outer mitochondrial membrane (4, 5). Mitochondrial creatine kinase (apparent subunit M_r of 42,000) isolated from chicken cardiac muscle exists in two interconvertible forms: octamer and dimer with octamer as the major form (>95%). Electron microscopic analysis revealed uniform square-shaped particles with a central negative-stain-filled cavity in the octamer fraction. The structure of mitochondrial creatine kinase was affected by its substrates (creatine phosphate, creatine), cofactors (ATP/ADP), pH, Mg^{++}, and phosphate. A high cooperativity between dimers to form octamers was also observed (4, 5). Based on these results, a model (4, 5) has been proposed for the interaction of mitochondrial creatine kinase with mitochondrial membrane. In this model, the mitochondrial creatine kinase octamer (exists as a cubic structure with 4-fold symmetry and central cavity or channel) can form complexes with ATP/ADP-translocators in the inner

mitochondrial membrane and pores in the outer mitochondrial membrane. This results in a physical continuous connection of the channel in mitochondrial creatine kinase octamer and the channel in mitochondria outer membrane. The complexes thus formed are most likely transient because the pore in the outer mitochondrial membrane is also the pathway for other small molecules such as ATP/ADP. Alternatively mitochondrial creatine kinase octamer interacts with only a subpopulation of the pore complexes.

The above model, together with the results in Chapter III, raised some very interesting questions. Do all mitochondrially-bound kinases exist in oligomeric form? Does the structure for a particular kinase change under different metabolic states? Further experiments are needed to answer these questions.

It has also been shown that glycerol kinase binds to mitochondrial porin in a competitive manner with hexokinase (6). Similar crosslinking and hydrophobic labeling experiment could be used to study the interaction of glycerol kinase with mitochondria. Before the crosslinking experiment could be carried out, we would need to purify glycerol kinase and raise antibodies to it. It would be interesting to see if these two kinases have the same oligomeric structure.

The existence of two populations of hexokinase or hexokinase binding complex has been suggested from the digitonin subfractionation experiment of rat brain mitochondria. Dorbani et al. (7) reported that at a concentration of 1 mg/mg mitochondrial protein, digitonin releases approximately 61% porin, 36% hexokinase, and 95% monoamine oxidase. Approximately 39% porin and 64% hexokinase remain in the mitochondrial pellet. Further study of hexokinase oligomeric structure after digitonin treatment of mitochondria could provide some information on the difference between the two (or more) populations of porin-hexokinase complex.

In tumor cells (rat hepatoma), at least 50% hexokinase is bound to mitochondria whereas virtually none is bound in normal hepatocytes (8-11). The bound hexokinase has been suggested to play a key role in the high glycolysis of tumor cells (8-10). It has been observed that the glycolytic activity is directly correlated with mitochondrially bound hexokinase (9). Lonidamine (1-(2,4-dichlorobenzyl)-1H-indazole-3-carboxylic acid), an antispermatogenic agent, has been reported to be a highly selective inhibitor of mitochondrially bound hexokinase in tumor cells (12, 13). It is an effective antineoplastic agent in several forms of tumors resistant to standard chemotherapeutic treatment. Standard chemotherapeutic treatment was based on the fact

that rapidly dividing tumor cells are more susceptible to damage by agents that interfere with nucleic acid metabolism than surrounding normal cells. However, there are two drawbacks associated with the use of these agents. The first is that rapidly growing normal cells are also highly susceptible to these agents. The second is that not all tumor cells are rapidly growing. The amino acid composition of AS-30D hepatoma hexokinase has been shown to be different from normal hexokinase from rat brain, rat muscle, and rat liver (13). The discovery of agents which interfere with tumor-specific-enzyme such as hexokinase might provide a more specific approach to treat tumor cells. Comparison of the oligomeric structure might reveal structural differences of bound hexokinase between normal and tumor cells and provide some insight into the uncontrolled growth in tumor cells.

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