PROTEOLYTIC DISSECTION OF RAT BRAIN HEXOKINASE FUNCTION

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1984

ABSTRACT

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Greater than 80% of the hexokinase activity in rat brain homogenates is particulate in nature, being specifically associated with the outer mitochondrial membrane. The binding of hexokinase to the membrane is thought to play a role in the regulation of the enzyme's catalytic activity. It has also been suggested that hexokinase maintains a preferential access to the nucleotide substrate, ATP, when bound to the mitochondrial membrane.

Factors promoting the transformation of the membrane bindable hexokinase to nonbindable enzyme forms were investigated and found to be proteolytic in nature. Bindable and nonbindable hexokinase species were isolated by high performance liquid chromatography (HPLC), characterized, and shown to be identical in all properties examined

with the exception of a minor difference in primary structure at the NH_2-terminus of the protein chain. A highly hydrophobic NH_2-terminal peptide present in the bindable hexokinase is absent in the nonbindable enzyme forms.

Further investigations of brain hexokinase protein structure were performed by limited proteolysis with trypsin. Cleavage at two principal sites in the polypeptide chain resulted in the generation of three major digestion products of molecular weights 10, 40 and 50K, which could only be dissociated under denaturing conditions. When compared under nondenaturing conditions, native and tryptically digested hexokinase were found to be nearly identical with respect to molecular weight, catalytic properties, and interactions with the mitochondrial membrane. The isolated tryptic digestion products were characterized and a map illustrating the relationship of the fragments to each other was constructed.

Artifacts resulting from endoprotease contaminants in carboxypeptidase preparations were encountered during the course of this work and are also described here.

To my parents

for giving me the complete freedom

and ability to pursue my own goals.

ACKNOWLEDGMENTS

First, I would like to thank John Wilson for his confidence, patience, unending enthusiasm and my stipend. Many thanks to the members of my guidance committee for their contributions in taking a genuine interest in my progress. I would also like to thank Alan Smith. I could have done it without him, but it would have taken a lot longer. Special thanks to Julie Doll, an island of normalcy in a sea of psychosis, not to mention a remarkable typist. Thanks to Steve Pittler for building my strength with his excellent backhand and my ego with his weak second serve. And finally my gratitude goes out to all those people who share a childlike fascination for nature, particularly Dwight Needels, who knows the real reason why bubbles get larger as they rise to the surface of a glass of beer.

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LIST OF ABBREVIATIONS

BSA	bovine serum albumin
DAB	3,3'-diaminobenzidine
DEAE	diethylaminoethyl
Glc	glucose
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IgG	immunoglobin G
PMSF	phenylmethylsulfonyl fluoride
OPA	o-phthalaldehyde
SDS	sodium dodecyl sulfate
TPCK	L-1-tosylamide 2-phenylethyl chloromethyl ketone

INTRODUCTION

The first step in glycolysis, the phosphorylation of glucose to glucose-6-phosphate, is catalyzed by hexokinase. The phosphorylation of glucose, and the phosphofructokinase catalyzed phosphorylation of fructose-1-phosphate, are recognized as the major control points of glycolysis in the brain (79). It is generally accepted that under normal physiological conditions the principal energy source for the adult brain is glucose (3,111) of which more than 90% is metabolized via the glycolytic pathway (80). In view of these findings, it is not surprising that among mammalian tissues, brain contains the highest hexokinase activity (68).

Brain hexokinase has been termed an 'ambiquitous' enzyme because of its noteworthy capacity to reversibly associate with the outer membrane of mitochondria (141). The effects of metabolites on the soluble-particulate distribution of hexokinase (98,105), along with observed differences in kinetic properties of the bound and solubilized enzyme (123), suggest that the membrane binding process may be a feature important to the regulation of glucose phosphorylation. In vitro, the addition of low levels of glucose-6-phosphate solubilize hexokinase from the mitochondrial membrane while inorganic phosphate tends to reverse this effect (98,105). Compared to the membrane bound hexokinase, the solubilized enzyme exhibits a K_m for ATP at least two fold higher and a K_i for glucose-6-phosphate which is some five fold

lower (123).

The low levels of glucose-6-phosphate and high levels of inorganic phosphate that would occur during periods of glycolytic stress would tend to promote the membrane binding of hexokinase which in turn, could result in an elevation of the hexokinase activity.

It has also been proposed that the enzyme's intimate association with the mitochondria may provide it with a preferential access to ATP (40,41). A hexokinase binding protein has been purified from the outer mitochondrial membrane (28) and more recently this protein has shown to be identical to mitochondrial porin (29,77), a polypeptide responsible for pore-forming activity in mitochondria (103). Of further interest to this issue is the recent evidence that glycerol kinase binds, in a competitive manner with respect to hexokinase, to this same mitochondrial membrane protein (29).

Considering the possible metabolic significance of the hexokinase membrane interaction, and possible analogous situations for other enzymes (92), it is of interest to investigate the features of the hexokinase molecule which are essential to the binding process. The existence of a hexokinase form identical to the solubilized mitochondrial enzyme, with the exception of its inability to associate with the mitochondria, provides an excellent opportunity for such an investigation.

The first chapter of this dissertation outlines the factors that contribute to the generation of nonbindable hexokinase forms. Also described here is a chromatographic technique employed for the isolation of bindable and nonbindable hexokinase forms and the results of characterization studies which have led to identification of the

critical distinction between these two molecules. In the course of this work, artifacts associated with the use of carboxypeptidase in COOH-terminal sequencing studies were revealed. These problems are reported here for the benefit of those wishing to pursue similar investigations.

The proteolytic dissection of enzymes into discrete domains can provide insight into the nature of the structural and functional entities that make up the intact molecule. In an effort to further define the region of the hexokinase molecule important for membrane binding, I performed partial tryptic digestion of hexokinase and isolated the major digestion products. The second chapter of this thesis deals with the characterization of the proteolyzed hexokinase, the assignment of locations for the tryptic fragments within the intact molecule, and the identification of a 10,000 molecular weight digestion product containing the membrane binding domain. A technique, involving two dimensional peptide mapping and immunoblotting, for the localization of antigenic determinants within the hexokinase molecule is also presented.

Literature Survey

Hexokinase Isozymes

Three major isozymes of low K_m hexokinase, designated types I, II and III, in order of increasing electrophoretic mobility, have been identified in a variety of animal cells (39,44,62) and a fourth isozyme, high K_m "glucokinase", is the predominant form present in liver tissue (126,129). The low K_m enzyme forms are similar with respect to molecular weight, pH optimum and nucleotide specificity but can be distinguished on the basis of their electrophoretic and chromatographic properties and certain kinetic parameters (44). The relative proportion of the isozymes to each other show variations from tissue to tissue (44,62). The liver contains all four isozymes, skeletal muscle predominantly type II, fat pad, heart, and intestine approximately equal amounts of types I and II, while minor amounts of type III are found in kidney and intestine (44,62). Greater than 90% of the hexokinase activity in brain tissue is type I (140).

Subcellular location of hexokinase

Forty years ago, Utter et al. (124), reported a correlation between the loss of hexokinase activity in the supernatants of rat brain homogenates and the degree of centrifugation. Crane and Sols (15) further investigated the particulate nature of hexokinase and noted the sedimentation of the enzyme to be in the same range of centrifugal force as required to sediment mitochondria. Johnson (17), studying the intracellular distribution of all glycolytic enzymes in rat brain found hexokinase to be the only enzyme to co-sediment with

the mitochondrial marker, succinic dehydrogenase. It is now commonly accepted that greater than 80% of the hexokinase activity of rat brain homogenates is associated with the mitochondria (98,133,134). With the use of immunohistochemical techniques (17) and procedures for the preparation of inner and outer mitochondrial membranes (18), Craven et al. concluded that rabbit brain hexokinase was located on the outer mitochondrial membrane. Kao-Jen and Wilson (59), in an electron micrographic study of rat cerebellar cortex observed increased staining at mitochondrial profiles of both neurons and astrocytes.

More than 50% of the total hexokinase activity in brain homogenates is present as a 'latent' form, i.e. the activity is detectable only after treatment which results in the disruption of membranes (6,63,132). Crane and Sols (15) reported a sharp increase in hexokinase activity, which was not accompanied by solubilization of the enzyme, when a brain particulate fraction was treated with lipase and deoxycholate. Wilson (135) showed that the latent hexokinase activity co-sediments with vesicles containing entrapped cytoplasmic enzymes but the hexokinase released from ruptured vesicles appears to remain bound to the mitochondria. The results with the latent form of the enzyme indicate a synaptosomal location, although tissues such as lung (108), presumably devoid of nerve terminals, contain significant levels of latent type I hexokinase. The explanation for latency in tissues other than brain remains to be elucidated.

The association of hexokinase with mitochondria is not exclusive to brain tissue; heart muscle (47), adipose tissue (114), lymphocytes and platelets (101), ascites tumor cells (104), fetal rat liver (53), the protozoan, tetrahymena (102) and plant cells (107) all exhibit this

phenomenon to varying degrees, although usually considerably less than that observed in brain.

Nature of binding

Early attempts at the purification of particulate hexokinase employed relatively harsh procedures for solubilization of the enzyme from the membrane (89,109,120). Purification of mitochondrial hexokinase to homogeneity by Schwartz and Basford (109) required the use of chymotrypsin, deoxycholate and Triton X-100 to solubilize the enzyme from the mitochondrial sediment. In 1965, Rose and Warms (104), studying particulate hexokinase of ascites tumor, reported the selective solubilization of the enzyme with low levels of glucose-6-phosphate. This discovery led to similar investigations involving glucose-6-phosphate solubilization of particulate hexokinase from brain (85,132), lung (108), heart (47) and blood cells (101).

Rose and Warms (105) demonstrated the reversible nature of the hexokinase-mitochondria interaction, representing a simple equilibrium: $ME \rightleftharpoons M + E$, where M is the mitochondrial membrane, E is the free enzyme and ME the associated complex. These authors demonstrated the rebinding of glucose-6-phosphate solubilized hexokinase to liver, brain, and tumor mitochondria in Mg^{2+} dependent fashion. Examining the effects of salt, pH, nucleotides and Mg^{2+}, they concluded that electrostatic forces were of primary importance to the binding process, although hydrophobic interactions may also be involved. Felgner and Wilson (27) confirmed these conclusions in conducting a study on the effects of neutral salts on the association of rat brain hexokinase with the mitochondrial membrane. They reported that low concentrations

of neutral salts enhanced the binding interactions while higher concentrations have a disruptive effect and that the solubilization by salts is more effective at alkaline pH.

A model was proposed featuring repulsive electrostatic forces acting between the negatively charged components of the hexokinase molecule and the anionic groups present on the surface of the mitochondrial membrane (27). The efficacy of divalent cations such as Mg^{2+}, known to greatly enhance the hexokinase-membrane association *in vitro*, is thought to result from the ability of the ion to serve as an electrostatic bridge between the two anionic species (27). Attractive electrostatic forces operating within the hydrophobic region of the membrane, where hexokinase is thought to interact with the binding protein, are considered as a second component of the membrane binding interaction (27). Felgner *et al.* (28) more recently isolated a 31,000 molecular weight protein from the outer membrane of liver mitochondria which can be incorporated into lipid vesicles and bind hexokinase in a glucose-6-phosphate sensitive fashion.

Based on lack of a correlation between the binding of hexoses and the ability of the respective hexose-6-phosphate to inhibit hexokinase, Crane and Sols (16) proposed a separate, regulatory binding site for glucose-6-phosphate. Further studies have demonstrated that the binding of glucose-6-phosphate results in dramatic conformational changes of the enzyme (13,23,99). Wilson (138) maintains that these conformational changes are involved in the mode of inhibition of hexokinase by glucose-6-phosphate. Hexoses which enhance the inhibitory effect of glucose-6-phosphate also enhance glucose-6-phosphate mediated solubilization of hexokinase from the

mitochondrial membrane (140) suggesting that glucose-6-phosphate induced conformational changes may be involved in the mechanism of solubilization. Wilson (141) has proposed a model depicting ligand-induced conformational changes resulting in a retraction of the N-terminal region of hexokinase from the binding protein.

Significance of hexokinase membrane binding

Hexokinase is known to be a key regulator of glycolysis in the brain (3,34,79,80). As the membrane bound hexokinase has been shown to be kinetically more active than the soluble form (61,67,78,87,123), the reversible association of the enzyme with the membrane is considered to be a feature important to its regulation (123,132,133). Thus, the interaction of hexokinase with the membrane may contribute to the regulation of the glycolytic rate. The observation that the levels of glucose-6-phosphate required to induce solubilization of hexokinase *in vitro* was comparable to the concentration required to cause inhibition of the enzyme by this agent, led Rose and Warms (105) to speculate on the physiological significance of the hexokinase-mitochondria interaction. The levels of the hexokinase metabolites, glucose-6-phosphate and ATP, may act *in vivo* to control the intracellular distribution of the enzyme and therefore adjust its catalytic activity according to the needs of the cell. Evidence supporting this view is found in the work of Hochman and Sacktor (51) who reported that physiological concentrations of ATP, generated by the mitochondria during coupled respiration, resulted in a two fold increase in solubilization of hexokinase. Moreover, Salatora and Singh (108) have investigated the reversible dissociation of

mitochondrial hexokinase from lung tissue and note that estimated intracellular concentrations of glucose-6-phosphate and P_i (which reverses the solubilizing effect of glucose-6-phosphate (133)) are such as to significantly affect the intracellular distribution of the enzyme.

The results of *in vivo* studies examining the distribution of bound and soluble hexokinase also suggest a physiological role for the association of the enzyme with the mitochondrial membrane (141). Knull *et al.* (65) reported a significant redistribution of hexokinase in chick cerebellum from the cytosolic to the particulate state following brief induction of ischemia.

Maintaining chicks on high galactose diets, which is known to alter the cerebral levels of several glycolytic metabolites, resulted in a similar redistribution of brain hexokinase (65). In a later study (66), these same authors administered insulin to male chicks and observed a diminution of glucose and glucose-6-phosphate, which was accompanied by an increase in particulate hexokinase activity and a decrease in the soluble fraction. These effects were rapidly reversed by the administration of glucose (66). Dirks *et al.* (21) reported a reduction in soluble hexokinase activity in cortical tissue following aglycemic perfusion of rat brain.

The hexokinase reaction has been implicated as a key factor involved in the production of the glycolytic depression resulting from anasthesia (4,46,69). Bielicki and Krieglstein (5) studied the distribution of hexokinase in brain with respect to the thiopental effect and suggest that the phosphorylation of glucose may be suppressed due to the shift of hexokinase activity from the

mitochondrial to the soluble form. Using a lipophilic drug which is not an anesthetic, Hofeler and Krieglstein (52) could achieve solubilization of hexokinase from the mitochondria only at concentrations of the drug lytic to the membrane, demonstrating that the solubilizing effect of thiopental is not solely a consequence of its lipophilic character.

Variation in the levels of enzymes in different cell types may reflect the differences in metabolic capacities of the cells (55,116). Wilson and Felgner (137) found that the capacity of the cell type to utilize glycogen, as determined by the level of phosphoglucomutase activity, was inversely related to its content of mitochondrial hexokinase. This tailoring of a cell's enzymatic makeup is also apparent in the work of Bustamante and Pedersen (10) with hexokinase in hepatoma cell lines. In the highly glycolytic hepatoma cell line H-91, 50% of the hexokinase activity is associated with the mitochondria (10). These authors (10) conclude that the intracellular distribution of hexokinase may be an expression of the metabolic state of the cancer cell.

It has also been proposed that the binding of hexokinase to the mitochondria provides the enzyme with preferential access to intramitochondrially generated ATP (40,41,56,127). Gots and Bessman (40) report that the bound hexokinase exhibits a kinetic advantage for its utilization of intramitochondrially generated ATP while the exogenously added nucleotide is relatively unavailable to the enzyme. Inui and Ishibashi (56), working with rat brain mitochondrial fraction, demonstrated a reduction in glucose-6-phosphate formation upon addition

of the ATP translocase inhibitor, atractyloside, while the hexokinase activity was left unimpaired.

It has recently been proposed that the hexokinase binding protein (28) is identical to the mitochondrial pore-forming protein (29,77). This pore-forming protein has been shown to be essential to the permeability of the outer membrane for ADP (103). It has also been suggested that exchange of ATP and ADP across the mitochondrial membrane can occur only through these pores (29). The demonstration of hexokinase binding to the mitochondrial pore-forming protein is consistent with the view that the association of the enzyme with the membrane provides it with a preferential access to ATP.

One further physiological role regarding the binding of hexokinase to the mitochondrial membrane has been advanced by Rose and Warms (106). Examining the type II mitochondrial hexokinase in ascites tumor cells they report that the association with the membrane promotes enzyme stability, i.e. temperature sensitive oxidations of sulfhydryl groups in hexokinase, usually observed in the absence of glucose, are significantly retarded.

Nonbindable Type I Hexokinase

Approximately 80% of the hexokinase activity in rat brain homogenates is associated with the particulate fraction (15,57). The enzyme remaining in the soluble fraction has been characterized and appears identical to the mitochondrial hexokinase as determined by starch gel electrophoresis (94,132), chromatography on DEAE cellulose (7,120), sucrose gradient sedimentation (134,136) and cross reactivity with antisera raised against purified mitochondrial hexokinase

(17,95,131). Although the crude soluble enzyme and the purified mitochondrial form have previously been reported to differ in kinetic parameters (2,81) and relative activity at pH 6.5 (12,81), Needels and Wilson (91) have more recently purified the soluble hexokinase to homogeneity and show that these distinctions disappear when comparing the purified enzyme forms. These authors (91) conclude that the relative inability of the cytoplasmic hexokinase to rebind to the mitochondria is an artifact probably resulting from proteolytic degradation of the enzyme during purification. The presence of a distinct, nonbindable cytoplasmic form of hexokinase existing *in vivo* in brain now appears doubtful.

To a variable degree, brain mitochondrial hexokinase loses its capacity to rebind to the mitochondria during the purification procedure (19,25). Felgner and Wilson (25) detected the presence of an artifactually created nonbindable hexokinase with DEAE cellulose column chromatography, and Polakis and Wilson (97) later resolved the bindable and nonbindable forms with the use of ion exchange high performance liquid chromatography. Kurokawa *et al.* (73) have been able to obtain purified rat brain hexokinase without loss of binding ability by solubilizing the enzyme from the mitochondria at pH 9.0 and purifying it in the presence of the protease inhibitor, PMSF.

When purified bindable hexokinase is subjected to mild digestion with chymotrypsin, the enzyme loses its capacity to rebind to mitochondria but suffers no loss of catalytic activity (24,71,105). The purified bindable enzyme has been shown to adsorb to Phenyl-Sepharose while the chymotrypsin generated, nonbindable enzyme will not (73), suggesting the presence of an exposed hydrophobic region on the

bindable hexokinase molecule which is necessary for its interaction with the mitochondrial membrane.

Although the hexokinase binding protein is present in liver mitochondria, only a small fraction of the total hexokinase activity is localized in the particulate fraction of liver homogenates (41). Rose and Warms (101) suggest that the action of liver cathepsins on hexokinase may be responsible for its negligible ability to bind to mitochondria in this tissue.

Subtypes of Type I hexokinase

Type I hexokinase accounts for more than 80% of the glucose phosphorylating activity in mature red blood cells (115). Two forms of the type I isozyme have been detected in rabbit red blood cells by starch gel electrophoresis (60) and disc gel electrophoresis (35) and have more recently been purified and characterized by Stocchi et al. (115). The enzyme form eluting with the lowest salt concentration, during DEAE cellulose chromatography, termed type Ia, corresponds to the type I isozyme found in brain and skeletal muscle, while a second form, Type Ib, is chromatographically distinct from any previously reported hexokinase isozyme (115). The rabbit reticulocyte hexokinase type Ib possesses a higher K_m for glucose (32) and decays at a faster rate than the type Ia form during red cell aging (84).

At least 3 forms, types Ia, b, and c, of which only the Ia form co-chromatographs with liver type I hexokinase have been identified in human adult erythrocytes (117). Rijksen et al. (100) report the presence of four hexokinase I subtypes in human erythrocytes, with the Ia form displaying a marked difference, compared to the other forms,

with respect to regulation by phosphate. In a later study involving the examination of hexokinase from a variety of human blood cells, Rijksen et al. (101) found, although not consistently, that solubilized mitochondrial type I hexokinase from platelets existed as two electrophoretically distinct forms. Posttranslocational modifications are considered as a probable explanation for multiple hexokinase forms in red blood cells (32).

Using phosphocellulose column chromatography, Easterby and O'Brien (22) have obtained two forms of pig heart type I hexokinase, designated Ia and Ib. These two subtypes were later characterized (128) and, aside from their chromatographic distinction, were shown to be similar in all respects, leading the authors to suggest that the Ia form may be generated proteolytically from the Ib subtype. Two subforms of Type I hexokinase, peak I and peak II, have also been identified in human spleen tissue extracts (49). The two enzyme forms are nearly identical, although peak I may possess a lower net surface charge based on its chromatographic behavior on DEAE cellulose.

Different molecular weight species of brain hexokinase have been reported (19,119) but have more recently been shown to be artifactually generated (134,136) as the appearance of the high molecular weight species is dependent upon the conditions employed during analysis.

Proteolytic dissection of enzymes

Globular proteins consisting of single polypeptide chains are made up of one of more structural domains (110,130) In some multidomain proteins, these globular regions of density, as they appear by X-ray diffraction analysis, are only loosely connected by single chain strands (110). When isolated from the multidomain protein structure,

single domain structures can be stable themselves and retain the characteristics of a globular protein (110). In some cases, individual subregions excised from the polypeptide chain have been shown to exhibit functions such as catalytic activity and ligand binding (37,122). These functional domains are often obtained as products resulting from the limited proteolysis of globular proteins.

Different proteins sharing a common domain responsible for carrying out a specific function have been identified. For example, three different b type cytochrome containing proteins have been proteolytically cleaved to yield a cytochrome domain separate from a second, unshared functional domain (37,58,113). In the case of flavocytochrome b_2, cleavage in the region of a protease sensitive bridge results in the dissociation of a cytochrome from the lactate dehydrogenase activity of the enzyme (37). Cytochrome b_5 can be proteolytically separated from the oxidase activity of sulfite oxidase (58) or from the hydrophobic membrane binding region of microsomal cytochrome b_5 (113). Based on these observations, Johnson and Rajagopalan (58) have postulated the presence of a common primordial gene coding for a b type cytochrome which has fused with either a gene coding for the membrane binding domain or for one of the enzyme activities mentioned above.

Allosteric regulation of enzyme activity results from the interaction of a ligand at a site which is distinct from the catalytic site (138). Limited proteolysis has been used to dissociate the catalytic and regulatory regions of some enzymes suggesting the presence of separate domain structures responsible for these two functions (42,122). Tucker et al. (122) have shown that limited

tryptic treatment of brain cyclic nucleotide phosphodiesterase results in the inability of the enzyme to bind calmodulin, and thus a failure of the enzyme to be regulated by this molecule. On the other hand the intact enzyme exhibits a 100 fold activation of catalytic activity when associated with calmodulin. These authors (122) propose the existence of separate calmodulin binding and catalytic domains in cyclic nucleotide phosphodiesterase.

In a similar study with phosphofructokinase, Gottschalk et al. (42) reported the proteolytic removal of the sites required for the binding of catalytic substrates while the degraded enzyme remained functionally normal with respect to the binding of several regulatory ligands. The results (42) suggested the presence of a discrete domain essential to the catalytic activity of the enzyme but structurally distinct from the allosteric regulatory sites.

MATERIALS AND METHODS

Materials

PMSF, DAB, acrylamide, TPCK-treated trypsin, chymotrypsin, and carboxypeptidases A, Y and B were purchased from Sigma Chemical Co. (St. Louis, MO), Staphylococcal aureus V8 protease from Miles Research Laboratories (Elkhart, IN), dansyl chloride, o-phthalaldehyde and polyamide thin layer sheets from Pierce (Rockford, IL), agarose powder, catalog No. 1620100 and SDS from BioRad Laboratories (Richmond, CA), acetonitrile from J.T. Baker Co. (Philadelphia, PA), immunochemical reagents from Cappel Laboratories (West Chester, PA). Gel Bond and Isogel agarose from FMC Corp. (Rockland, ME), trifluoroacetic acid from Mallinckrodt, Inc. (St. Louis, MO), and Ampholine ampholytes from LKB Corp. (Stockholm, Sweden).

Methods

Purification of Rat Brain Hexokinase - Rat brain hexokinase was prepared by a slight modification of the Chou and Wilson (11) procedure. Thioglycerol (10 mM), 10 mM Glc was used in place of 0.25 M sucrose as the medium for the initial homogenization and for subsequent washing of the crude mitochondrial fraction; the pH of the original homogenate as well as that of the mitochondrial suspensions was adjusted to 8.2 with NaOH. After chromatography on DEAE-cellulose according to Chou and Wilson (11), the enzyme was further purified by affinity chromatography on Blue Dextran Sepharose with elution by

Glc-6-P, as described by Needels and Wilson (91). The purified enzyme is stored at -20°C in 0.1 M sodium phosphate, pH 7.0, 0.1M glucose, 0.01M thioglycerol and 0.5 mM EDTA. This medium is referred to as 'storge buffer' throughout this thesis.

Hexokinase assay.

Hexokinase activity was determined spectrophotometrically as described in (11).

Protein determinations.

Protein determinations for native hexokinase or hexokinase tryptic fragments eluted from SDS acrylamide gels were carried out using the procedure of Hess et al. (48).

Binding assays.

Binding assays were performed with rat liver mitochondria according to Polakis and Wilson (97).

Separation of bindable and nonbindable hexokinase forms by HPLC.

Separation of hexokinase forms by ion exchange HPLC followed the procedure of Polakis and Wilson (97).

Limited tryptic digestion of rat brain hexokinase.

Purified hexokinase (~1 mg/ml) in 'storage buffer' was treated with trypsin (0.2 mg/ml) at room temperature for 1 hour unless specified otherwise. Digestions were terminated by the addition of PMSF to 1 mM followed by incubation on ice for 15 minutes.

Preparative electrophoretic isolation of hexokinase tryptic digestion products.

Typically, 1 mg of hexokinase digested with trypsin (0.1 mg/ml) was electrophoresed on SDS gels as described below. Protein bands, visualized by immersing the gel in cold 0.2M KCL (83), were excised

with a razor blade and transferred to test tubes containing 15 ml of 0.125 M Tris-glycine, 0.1 % SDS, pH 6.8. The gel slices were homogenized in the buffer and the slurry was boiled at 100°C for 10 minutes to safeguard against subsequent proteolytic degradation.[1] After standing overnight at room temperature, the gel homogenates were centrifuged at 40,000 x g for forty minutes and the supernatants filtered through Whatman 1 paper. The filtrates were lyophilized to dryness, redissolved in 3 ml of doubly-distilled H_2O, dialyzed exhaustively vs. doubly-distilled H_2O, and once again lyophilized.

Two dimensional peptide mapping in SDS polyacrylamide gels.

Peptide mapping in SDS gels were performed essentially as described by Cleveland (14). Approximately 50 µg of purified hexokinase was digested with trypsin (0.1 mg/ml) for 1 hour at room temperature and prepared for SDS gel electrophoresis as described below. The tryptic fragments were first separated by electrophoresis in 6-20% gradient SDS tube gels (dimension 10 cm X 0.5 cm I.D.) identical in composition to the slab gel system described below. After electrophoresis at 2 mA/tube for 3.5 hours, the gels were removed from the glass tubes and equilibrated for 15 minutes in stacking gel acrylamide solution, minus the ammonium persulfate. The entire tube gel was inserted into the slab gel apparatus containing a preformed 6-20% gradient separating gel and a 5% stacking gel. The tube gel, resting on the upper surface of the stacking gel, was overlayed with fresh stacking gel solution, such that after polymerization, the new stacking gel interface was flush with the upper surface of the tube gel. After the addition of the upper reservoir buffer, 300 µl of an S. aureus V8 protease solution (5 µg/ml in 0.125 M Tris HCl, pH 6.8, 10%

glycerol, 0.1% SDS and 0.0005% bromphenol blue) was layered evenly onto the stacking gel surface and electrophoresis was carried out at 40 V, constant voltage, until the tracking dye reached the separating gel interface. The power was turned off for 30 minutes and then resumed at 100 V, constant voltage, until the tracking dye reached the bottom of the separating gel. Gels were stained with Coomassie Blue as described in (142).

Binding and solubilization studies.

Purified rat brain hexokinase, 0.1 units, either native or digested with trypsin, was added to 50 µl of outer membranes (15 mg/ml, 0.25 M sucrose) prepared from rat liver mitochondria (112), along with 0.25 M sucrose to a final volume of 100 µl. After the addition of $MgCl_2$ to 3 mM the samples were incubated on ice for 15 minutes and then centrifuged at 40,000 x g for 15 minutes. Supernatants, and pellets resuspended in 0.2 ml of 0.25 M sucrose made 0.5% in Triton X-100, were assayed for hexokinase activity. Fraction bindable is expressed as units in pellet over total units recovered.

Fraction solubilized by glucose-6-phosphate was determined by following the protocol for binding described above with the exception that 0.5% Triton X-100 was replaced with 1.2 mM glucose-6-phosphate. The resuspended pellets were incubated for 30 minutes at room temperature, centrifuged at 40,000 x g for 15 minutes and hexokinase activity in pellets and supernatants determined. Fraction solubilized was calculated as activity in supernatant over total activity recovered.

Sucrose density gradient centrifugation.

Linear, 5-20% sucrose density gradients were prepared in 0.01 M sodium phosphate, pH 7.0, 0.02 M glucose and centrifugation was performed as described in (11).

NH_2-terminal identification.

Identification of the amino terminal amino acids of native hexokinase or its tryptic fragments eluted from SDS polyacrylamide gels was done using a modification (26) of the technique described by Gray (43) except as suggested by Tapuhi et al. (118), sodium carbonate was replaced by lithium carbonate in order to minimize decomposition of the dansylated amino acids.

Analysis of amino acids.

Analysis of free amino acids was carried out essentially according to the procedure of Hill et al. (50). Amino acids were derivatized by orthophthaldialdelyde (OPA) and the fluorescent products were separated by reverse phase HPLC.

Preparation of saturated borate buffer solution - Doubly distilled H_2O was preheated to 60°C and boric acid was added to saturation. The solution was filtered through Whatman 1 filter paper and pH adjusted to 9.5 with 1N NaOH after cooling to room temperature.

Preparation of OPA derivatizing solution. - To 5 mg of OPA dissolved in 0.45 ml of methanol, 50 µl of saturated borate buffer, pH 9.5, and 5 µl of ethanethiol were added and the solution was mixed. The solution could be used immediately but was routinely discarded after a 48 hr period.

Derivatization of the standard amino acid mixture - To 25 µl of methanol and 10 µl of borate buffer, 10 µl of diluted standard amino acid mixture (25 nmoles of each amino acid/ml doubly-distilled H_2O)

were added and the solution was mixed. Following a 5 µl addition of the OPA derivatizing solution the mixture was briefly agitated and allowed to react for 1 min and filtered through a 0.25 µM regenerated cellulose membrane. (To avoid sample contamination the membranes must first be thoroughly washed with methanol: borate buffer mixture, 25:10.) Immediately following filtration, 35 µl of the sample was injected onto the HPLC column.

Chromatography of OPA amino acids - Chromatography was performed on a Beckman-Altex Model 332 liquid chromatography system employing two Model 110 A pumps, a Model 420 microprocessor controller, and a Model 210 sample injector valve equipped with a 50 µl sample loop. All separations were carried out on a 7.5 cm x 4.6 mm I.D. Ultrapore RPSC column (Beckman, Berkeley, CA) and the OPA derivatives were detected with an Aminco Fluorocolorimeter equipped with an 18 µl quartz continuous flow cell (SLM instruments, Urbana, IL). Reaction adducts were monitored at an excitation wavelength of 370 nM with a 415 nM cut-off filter with the fluorometer connected to a Kipp and Zonen Model BD-40 recorder. All quantitations were made by measuring peak heights.

Gradient elution of OPA amino acids - Operating conditions: flow rate: 1.5 ml/min, solvent A, 0.025 M sodium phosphate pH 7.2; Solvent B, acetonitrile; gradient program; 7% B for 2 min. from time of sample injection, linear increase to 11% B in 13 min., isocratic elution for 4 min. at 11%B, linear increase to 21% B in 8 min., linear increase to 30% B in 10 min., linear increase to 60% B in 5 min., isocratic elution at 60% B for 5 min., linear decrease to 7% B in 5 min., isocratic hold at 7% B for 10 min, after which time a second sample could be injected

and the program repeated. The elution profile for the OPA-derivatives of 15 amino acids is shown in Figure 1.

Isoelectric focusing in Agarose Gels.

All isoelectric focusing was performed on an LKB 2117 Multiphor apparatus.

Native conditions, pH 5-8. - After cooling 15 ml of a 1% Agarose solution to approximately 60°C, ampholytes and Triton X-100 (this detergent enhances the nitro blue tetrazolium coupled activity stain (93)) were added to 1% each and the solution was thoroughly mixed. The gel solution was poured onto a 10 x 12.5 cm piece of Gel Bond applied to a 1.5 mM thick glass plate. The Gel Bond was surrounded by strips of modeling clay to prevent the solution from running off the edges. After cooling at room temperature for 5 minutes the gel slab was transferred to a humidified chamber and cured at 4°C for at least 1 hour prior to use.

The cooling platform of the Multiphor apparatus was preadjusted to 4°C with the use of a circulating antifreeze bath. A thin film of H_2O was sandwiched between the platform surface and glass plate containing the gel and the gel was briefly blotted with a piece of Whatman 1 filter paper to remove excess moisture. Paper electrode wicks, soaked in either 1M NaOH or 1M H_3PO_4 were positioned at the cathode and anode ends of the gel, respectively. Samples were applied to pieces of Whatman 1 filter paper (0.5 x 1.0 cm) which were placed on the gel surface approximately 1 cm from the cathode wick.

Power was applied at 400 V, constant voltage, for 15 minutes, turned off in order to remove the sample filter paper, and then resumed at 1000 V, constant voltage, for another 30 minutes. The gel was

Figure 1. Elution profile of OPA amino acid standards. Derivatization of the amino acid standard mixture (Amino Acid Standard H, Pierce Chem. Co., Rockford, IL) with OPA and their separation by reversed phase HPLC was carried out as described in Methods. Each peak represents 175 pmols of the indicated amino acid derivative. The elution gradient (broken line) indicates the percent CH_3CN (percent solvent B) at the corresponding time (minutes) following sample injection.

FLUORESCENCE (ARBITRARY UNITS)
2
4
6
8
asp
glu
ser
his
gly
thr
arg
ala
tyr
val
met
ile
leu
phe
lys
10
20
30
40
MINUTES
PERCENT SOLVENT B
0
20
40
60

Figure 1

removed from the glass plate, and after cutting away the ends containing the electrode wicks, transferred to a dish for staining.

Gels were stained for hexokinase activity as described in (91). Gels to be stained for protein were first equilibrated with 10% trichloroacetic acid and then washed for 12 hours in 3 changes (250 ml ea.) of 0.15 M NaCl. Protein bands were visualized by staining with Coomassie Blue (142).

Isoelectric focusing in denaturing agarose gels - The procedure for native gels described above is followed with the exception of including 7 M urea in the gel solution and the 1% pH 5-8 Ampholyte concentration was replaced with a mixture of 0.75% pH 5-8 and 0.5% pH 3.5-10 Ampholytes. These gels will only solidify at 4°C after several hours.

Determination of pI - Immediately following focusing, a length wise strip (i.e. from cathode to anode) of the agarose gel was sectioned horizontally into 0.5 cm slices and the gel pieces broken up and equilibrated in 1 ml each of doubly-distilled H_2O. The pH of each fraction was determined and a plot of distance (cm) from cathode vs pH was used to estimate pI values of focused proteins.

Peptide mapping in SDS gels.

Peptide mapping by limited proteolysis in SDS gels essentially followed the procedure of Cleveland (14). Purified hexokinase, either the bindable or nonbindable form, at a concentration of 1 mg/ml in 0.1 sodium phosphate buffer, pH 7.0, was mixed at a ratio of 1:1 with 0.125 M Tris-HCl, pH 6.8, 1% SDS, 30% glycerol, and 0.0005% bromphenol blue. The samples were boiled at 100°C for 2 min, and after being equilibrated to 37°C the indicated amount of protease was added. Proteases most commonly employed were Subtilisin, S. aureus V8

protease, and chymotrypsin, all prepared in 0.125 M Tris-HCl, pH 6.8. A 30 min. incubation at 37°C was followed by the addition of SDS and mercaptoethanol to final concentrations of 2% and 5%, respectively, and the digestions were terminated by boiling at 100°C for 2 min. After cooling, samples were loaded directly into the wells of an SDS-gel and electrophoresis was performed as described below.

Protease assay.

The protease assay was carried out as described in (8). Slab gels, consisting of 1% agarose and 0.033% powdered milk in 10 mM sodium phosphate, pH 7.0, 0.15 M NaCl, were formed on microscope slides and wells, 0.25 cm in diameter, were cut into the center of each gel. Approximately 15 µl of enzyme solution was added to the well and the slab was incubated for 24 hours at room temperature in a humidified chamber. After the incubation period the gels were fixed in 5% acetic acid and then stained with Coomassie Blue (142).

Electrophoresis

All SDS polyacrylamide slab gel electrophoresis was carried out using a BioRad 220 or a Protean 16 cm apparatus. Stock solutions included: 34.5% acrylamide + 0.62% N,N'-methylbisacrylamide, 1 M Tris-HCl, pH 6.8 and pH 8.8, 20% SDS, and 10% ammonium persulfate. Separating gels, formed as 6-20% linear acrylamide gradients, contained a final concentration of 0.37 M Tris-HCl, pH 8.8. Stacking gels were 5% acrylamide in 0.125 M Tris-HCl, pH 6.8. Running buffer (25 mM Tris and 192 mM glycine, pH 8.3) and gels contained 0.1% SDS.

Samples, in 1% SDS, 5% mercaptoethanol, 0.125 M Tris-HCl, pH 6.8, 10% glycerol and 0.0005% bromphenol blue, were boiled at 100°C for 2 minutes just prior to loading. Power was applied at 80V, constant

voltage, until the tracking dye reached the bottom edge of the separating gel. Gels were stained for protein with Coomassie Blue (142).

Molecular weights were estimated from log molecular weight vs mobility plots carried out on the following molecular weight markers purchased from Bethesda Research Laboratories (Bethesda, MD): cytochrome C, 12,300; β-lactoglobin, 18,400; α-chymotrypsin, 25,700; ovalbumin, 43,000; bovine serum albumin, 68,000; phosphorylase B, 92,500, myosin, 200,000.

Native gel electrophoresis was performed as described in (91).

Carboxypeptidase digestions of hexokinase.

Purified hexokinase was treated with carboxypeptidase at room temperature and at the specified times, 10 µl aliquots were removed from the digestion mixture and added to 25 µl of methanol and 10 µl of saturated sodium borate buffer, pH 9.5. The protein precipitates under these conditions, terminating the digestion. The samples were kept on ice until immediately prior to derivatization with o-phthaldialdehyde. Analysis follows exactly the procedure described above for free amino acids.

Carboxypeptidase Y was dissolved in doubly-distilled H_2O and stored frozen as a 10 mg/ml solution.

Carboxypeptidase A suspension was centrifuged and the protein pellet dissolved in 2 M LiCl. A 20 mg/ml stock solution of the enzyme was prepared fresh prior to each use.

Carboxypeptidase B, supplied as a 2 mg/ml solution, was diluted 50 fold with 0.1 M sodium phosphate, pH 7.0 and reconcentrated using an

Amicon Centricon 30 microconcentrator to 2 mg/ml in order to remove contaminating amino acids.

Exhaustive carboxypeptidase digestion - Peptides were treated with carboxypeptidase Y (0.1 mg/ml) and the digestion was terminated at 0.5 and 1.5 hours. The pmol levels of amino acids detected at these two times were identical.

Preparation of detergent solubilized brain protease.

Crude mitochondria, obtained from 90 g of rat brain were prepared, and hexokinase activity solubilized by glucose-6-phosphate, as described in (11). After ultracentrifugation the pellet was resuspended in 200 ml of 0.25% Triton X-100 in 0.25 M sucrose and incubated at room temperature for 30 min. The suspension was ultracentrifuged at 105,000 x g for 3 hours and the supernatant was collected and concentrated to approximately 20 ml. The solution was made 0.01 M in sodium phosphate, pH 7.0 and 20% in glycerol. Proteolytic activity, determined by the ability to destroy hexokinase binding ability, was not stable under these conditions and completely diminished over a period of several days at 4°C.

Isolation of peptides by reversed phase HPLC.

All separations were performed on a Beckman Model 332 HPLC system (described above) equipped with an Altex Ultrasphere ODS reverse phase column (4.6 mm I.D. x 25 cm) and a Beckman Model 160 U.V. detector with a fixed wavelength of 214 nm. Solvent A, 0.1% trifluoroacetic acid, Solvent B, 0.1% trifluoracetic acid in CH_3CN, flow rate, 1.0 ml/min. The column was equilibrated at 10% B in A and immediately following sample injection solvent B was linearly increased to 60% over a period

of 40 minutes. Peaks were collected in 2ml volumes and the solvents were evaporated under reduced pressure.

Acid hydrolysis of peptides.

Peptides were dissolved in 50 µl each of constant boiling 6N HCl, and transferred to hydrolysis vials. After sealing the vials under reduced pressure, hydrolysis was carried out at 110°C for 18 hrs.

Immunoblotting Procedures - All gels used for immunoblotting experiments were prepared in the Model 50 "minigel" apparatus obtained from Aquebogue Machine Shop (Aquebogue, NY). Immunoblots were prepared after either 1-dimensional SDS gel electrophoresis or 2-dimensional electrophoresis, with SDS gel electrophoresis in the second dimension, had been performed by adaptations (to the minigel apparatus) of methods described above. Gels were electroblotted essentially as described by Towbin et al. (121) using an apparatus constructed and generously provided to us by Dr. Larry Eng. The buffer used for electroblotting was 25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3, recommended by Towbin et al. (121); where indicated, 0.02% SDS was added to this buffer. Blotting was performed at 4° for 20 hrs at approximately 5 V/cm (current approximately 150 ma).

Two monoclonal antibodies were used in the experiments described here. Procedures used in raising and characterizing these antibodies have been described in detail elsewhere (30). The antibody designated 5A was of the IgM class, and has been shown to react only with denatured hexokinase (30). The monoclonal antibody designated 21 is of the IgG_1 class and reacts readily with the native enzyme but poorly with denatured hexokinase (unpublished observations), as determined by previously described methods (30). For this reason, immunoblots to be

stained with antibody 21 were prepared without addition of SDS to the blotting buffer; apparently sufficient renaturation occurs during the transfer to nitrocellulose to permit detection with this antibody (it was much less effective with blots prepared in the presence of SDS). Transfer from the gel to the nitrocellulose was not complete when electroblotting was done in the absence of SDS; in this case, the residual protein in the gel could be stained with Coomassie Blue (142), facilitating direct correlation with the species stained by monoclonal antibody 21 on the immunoblots. With monoclonal antibody 5A, transfer was performed with SDS added to the buffer to maintain the denatured form of the enzyme recognized by 5A. Under these conditions, transfer was complete and species reactive with antibody 5A were identified by comparison with duplicate gels stained for protein with Coomassie Blue (142).

Immunoreactive species were detected by the following protocol. After blotting, the nitrocellulose sheets were washed for 10 min in 20 mM Tris Cl, 0.5 M NaCl, pH 7.5 (Tris Buffered Saline, TBS) then gently agitated for 1 hr in a blocking solution of 3% (w/v) gelatin in TBS. The blots were then incubated for 2 hr with gentle agitation in medium harvested from cultures producing the indicated antibody, 5A or 21, and to which 0.05% (v/v) Tween 20 had been added. The blots were washed thoroughly in TBS supplemented with 0.05% Tween 20 (TTBS); three washes (2 for 15 min, and a final 10 min wash), with gentle agitation in 75 ml of TTBS for each wash, were done. The washed blots were incubated for 1 hr with gentle agitation in a 1:10,000 dilution of horseradish peroxidase-conjugated antimouse immunoglobulins, diluted in TBS containing 1% (w/v) gelatin. After thorough washing in TTBS as above,

the blots were incubated in the dark in a solution of TBS to which DAB (0.5 mg/ml), $CoCl_2$ (0.02%, w/v), and H_2O_2 (0.02%, w/v) were added (54). Color development was rapid, being complete in 1-5 min. The stained blots were washed thoroughly in water and stored in the dark until photographed.

[1]If the slurries were not promptly boiled, a high degree of heterogeneity was noted when the samples were subjected to re-electrophoresis.

CHAPTER I

STRUCTURAL FEATURES ESSENTIAL TO THE MEMBRANE BINDING OF HEXOKINASE

A correlation between the relative amounts of the multiple forms of rat brain hexokinase, as detected by isoelectric focusing and ion exchange chromatography, and the ability of the enzyme to bind to the outer mitochondrial membrane was established. Processes resulting in the generation of the nonbindable form from the bindable hexokinase were studied, including the investigation of a brain protease capable of catalyzing the transformation. The bindable and nonbindable hexokinase forms separated by ion exchange HPLC were compared and certain structural features essential to the membrane binding process were elucidated. During the course of this investigation technical difficulties associated with the use of carboxypeptidases were detected and are described here.

RESULTS

Agarose gel isoelectric focusing of rat brain hexokinase.

Purified rat brain hexokinase exhibits a deficiency, although widely variable, in its ability to rebind to the mitochondrial membrane (19,25). With the use of DEAE cellulose column chromatography, Felgner and Wilson (25) were able to detect the presence of a distinct, nonbindable hexokinase species. The early elution of the nonbindable enzyme from the anion exchange matrix suggested an elevated net

positive charge, relative to the bindable form, and led to the analysis of hexokinase by isoelectric focusing. Crude, glucose-6-phosphate solubilized, or purified hexokinase, was subjected to isoelectric focusing in agarose slab gels, pH range 5-8, and the gels were stained for enzymatic activity. The ability of each of these hexokinase samples to rebind to the mitochondria was also examined (Figure 2). It is apparent that a charge heterogeneity exists in the purified enzyme preparation and that the relative abundance of the pI 6.35 form correlates well with membrane binding ability. The analysis of individual fractions obtained during DEAE cellulose chromatography demonstrates, as already reported by Felgner and Wilson (25), the early elution of the nonbindable enzyme form (Figure 3). These same fractions also exhibit an increased proportion of the pI 6.45 isoelectric form (Figure 3). Minor isoelectric forms were sometimes observed at pI values below 6.35, e.g. fraction 32, Figure 3. These low pI forms are considered membrane bindable, as fractions containing substantial amounts of them did not exhibit any deficiency in binding ability.

Factors contributing to the loss of hexokinase binding ability.

Prolonged storage. It was noticed that with storage over several days, the crude glucose-6-phosphate solubilized hexokinase, contained in 0.25 M sucrose solution, underwent a transformation to the nonbindable enzyme form. Individual components of the buffer mixture used for the purification of hexokinase, were added to aliquots of freshly solubilized enzyme to test their effectiveness at promoting or retarding the bindable to nonbindable transition. Following several days storage at 4°C, the samples containing thioglycerol, and to a

Figure 2. Agarose gel isoelectrofocusing of rat brain hexokinase. Approximately 8 milliunits of either crude, glucose-6-phosphate solubilized (lane 1) or purified hexokinase taken from various preparations, (lanes 2-4) were applied to each lane. Binding assays (% Bindable), and pI determinations were carried out as described in methods. The gel was stained for hexokinase activity.

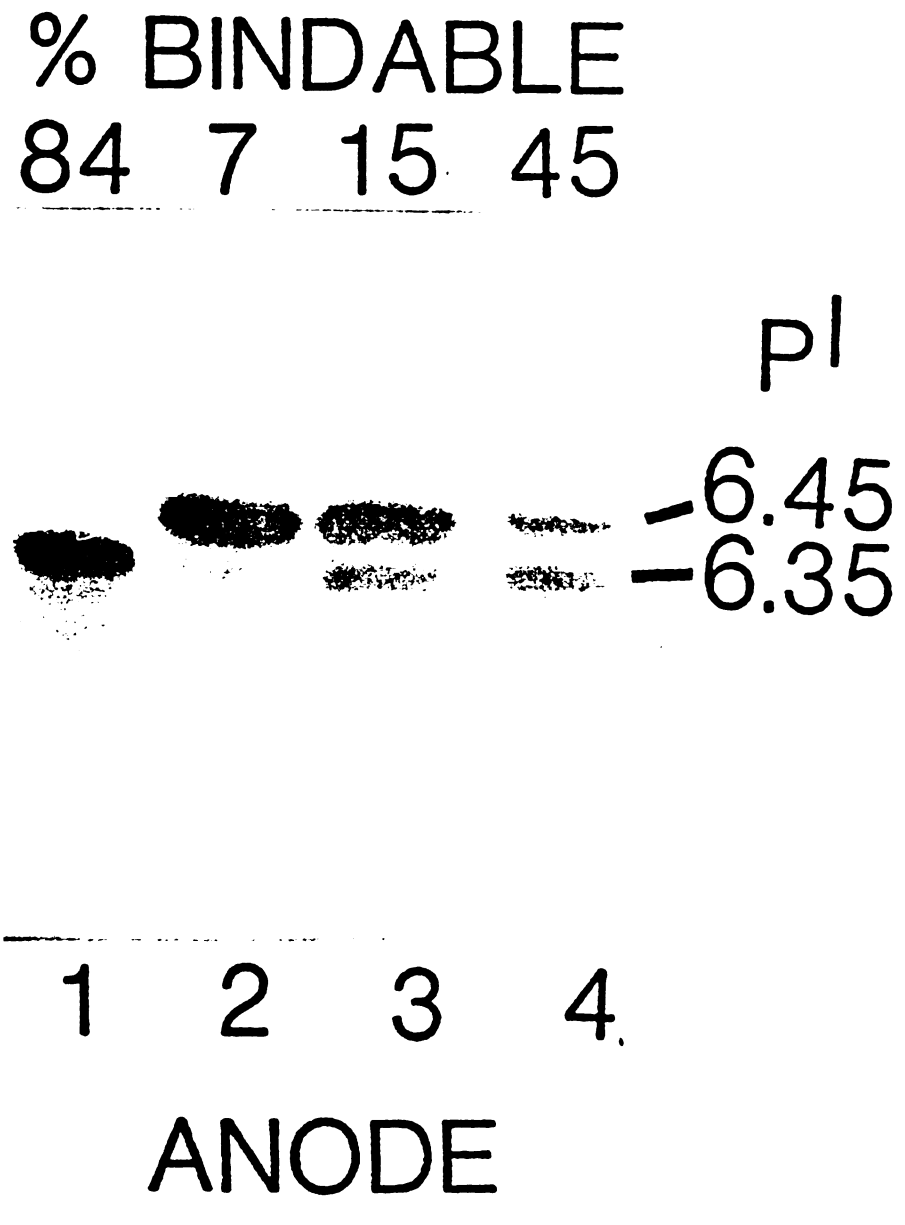

Figure 2

Figure 3. Isoelectric focusing and binding ability of hexokinase chromatographed on DEAE cellulose. Aliquots of hexokinase obtained from individual fractions eluted from a DEAE cellulose column were dialyzed against 0.25 M sucrose and subjected to agarose gel isoelectric focusing (upper insert, values correspond to fraction numbers). Binding assays were also performed and the resulting percentages (closed circles) are plotted on the chromatographic profile along with hexokinase activity (open circles). Chromatography was performed as in (11).

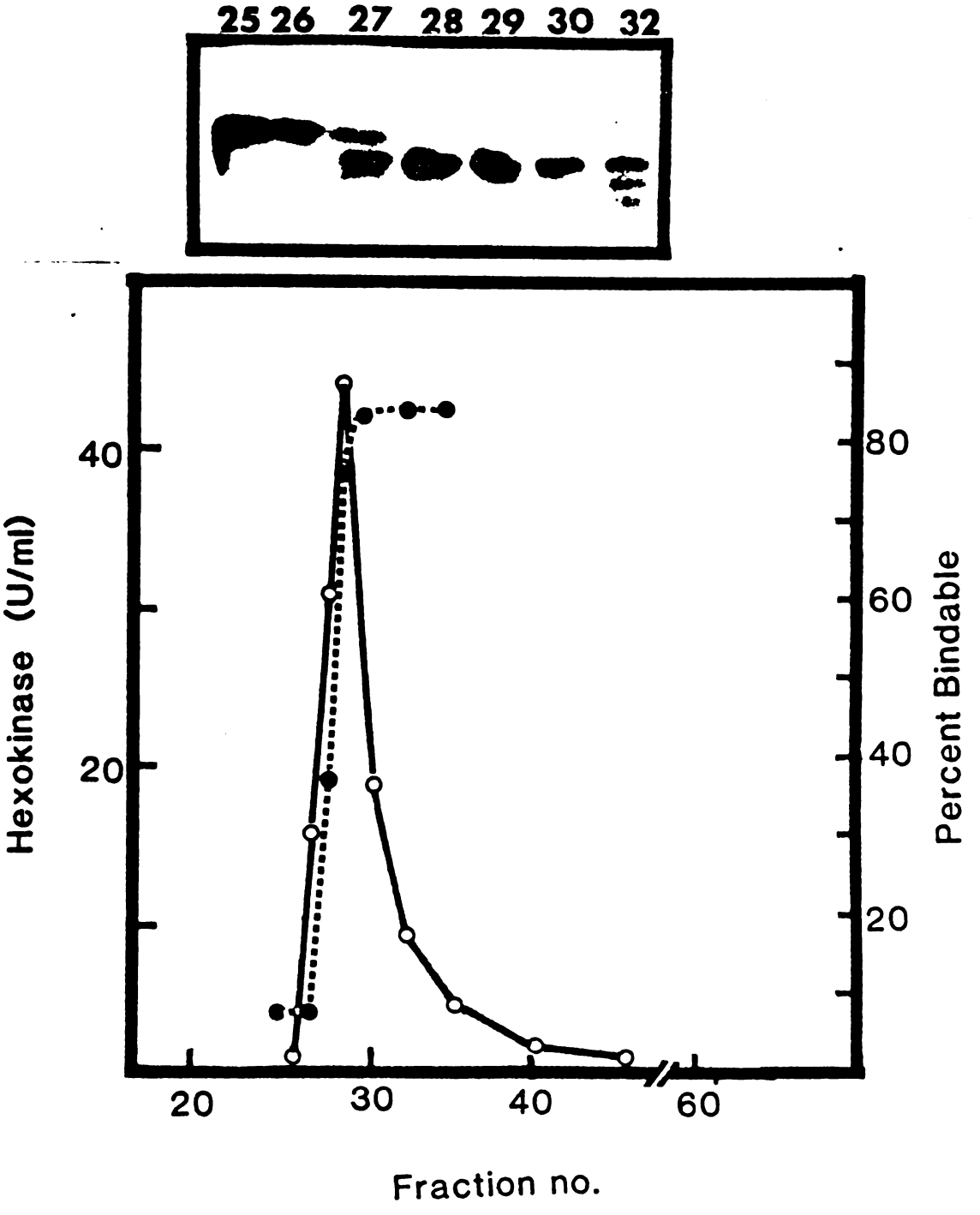
25 26 27 28 29 30 32
Hexokinase (U/ml)
40
20
80
60
40
20
Percent Bindable
20
30
40
60
Fraction no.

Figure 3

lesser extent, EDTA, appeared less altered than the remaining aliquots (Figure 4). The metal chelating properties of thioglycerol are apparently responsible for the efficacy of this agent, as micromolar amounts of the Zn^{2+} chelating protease inhibitor, 1,10 phenanthroline, was subsequently found to be equally effective in preventing the appearance of the nonbindable enzyme.

Limited Proteolysis. Rose and Warms (105) had previously reported that very low levels of chymotrypsin could effectively eliminate the membrane binding ability of ascites tumor hexokinase without a concomitant loss of catalytic activity or apparent reduction in molecular weight. Incubation of bindable rat brain hexokinase with this protease produces similar results which are accompanied by a shift in isoelectric point to the pI 6.45 form (Figure 5). Under these digestion conditions, the membrane binding ability dropped from 81 to 6 percent while 93 percent of the catalytic activity was retained. Rose and Warms (105) also detected an iodoacetate sensitive factor in liver mitochondria preparations which produced consequences similar to those seen with chymotryptic treatment of hexokinase. When rat brain hexokinase is incubated with increasing amounts of liver mitochondria suspension, in the absence of $MgCl_2$, a progressive loss of binding capacity is observed (Figure 6). This binding loss was also concomitant with the isoelectric point alteration (not shown).

Detection of a Brain Protease Capable of Converting the Bindable Hexokinase to the Nonbindable Form. The implication that limited proteolysis was responsible for the loss of hexokinase binding ability prompted the examination of brain homogenates for a protease capable of catalyzing the generation of the nonbindable enzyme form. When Triton

Figure 4. Effects of prolonged storage on the binding ability and isoelectric point of hexokinase. Freshly prepared, crude, glucose-6-phosphate solubilized hexokinase, in 0.25 M sucrose, was maintained at at 4°C with no addition, (NA, ●) or in the presence of 0.5 mM EDTA, (E, ▲), 10 mM glucose (G, ○), 10 mM sodium phosphate, pH 7.0, (P, ■), or 10 mM thioglycerol (TG, □). Hexokinase assays (u/ml, upper graph), binding assays (% Activity Bindable, lower graph), and isoelectric focusing (IEF) were performed at the specified times. For comparison, activity and binding of enzyme incubated in 0.25 M sucrose at room temperature is also shown (△).

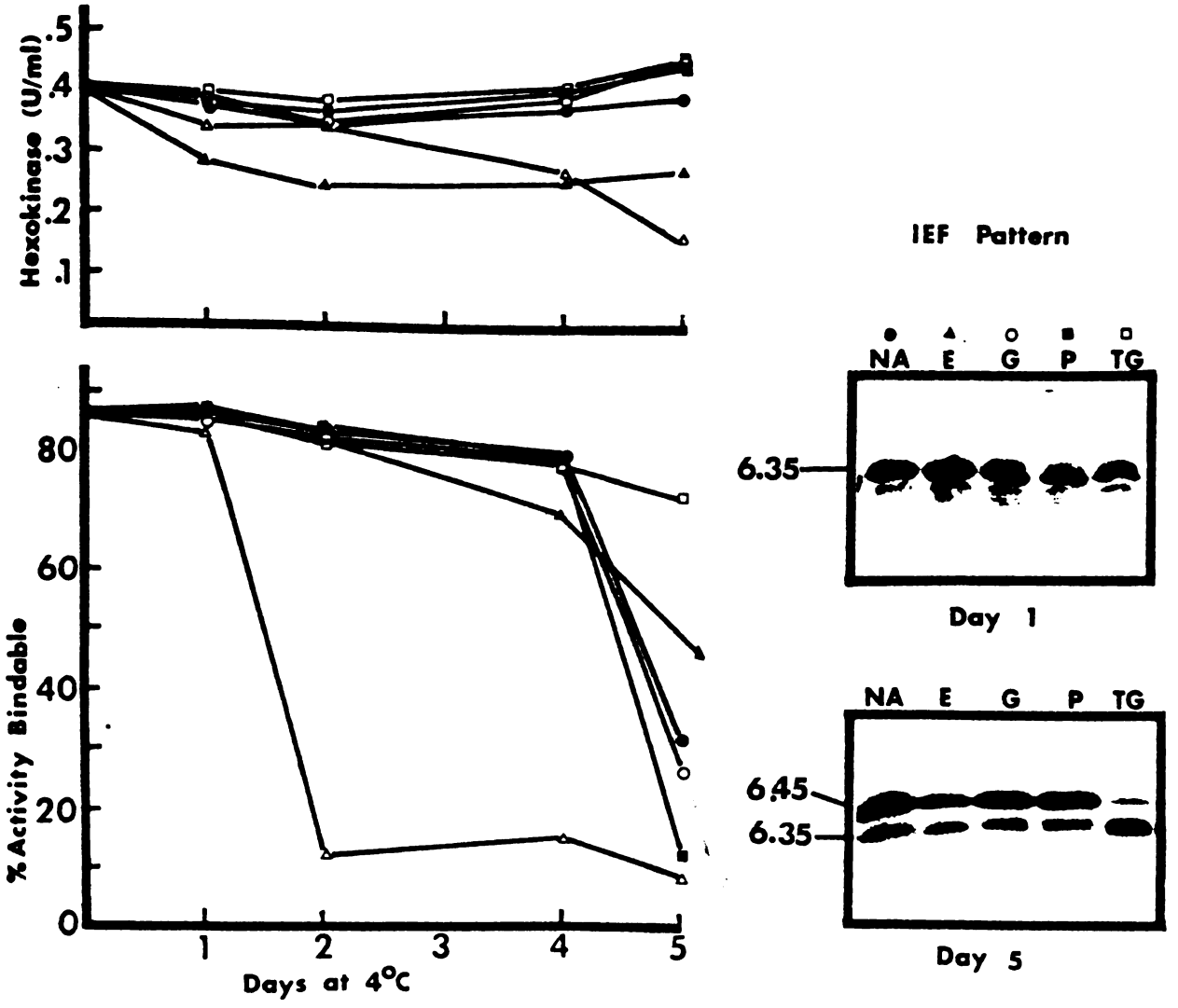
Hexokinase (U/ml)
.5
.4
.3
.2
.1
% Activity Bindable
80
60
40
20
0
1
2
3
4
5
Days at 4°C
IEF Pattern
NA
E
G
P
TG
6.35
Day 1
NA
E
G
P
TG
6.45
6.35
Day 5

Figure 4

Figure 5. Limited chymotryptic treatment of bindable hexokinase. Purified bindable hexokinase, approximately 1 mg/ml in storage buffer, was treated with chymotrypsin (protease:protein, 1:100) at room temperature for 1 hour. The digested (CT) and native (N) hexokinase were subjected to agarose gel isoelectric focusing (IEF) and SDS polyacrylamide gel electrophoresis (SDS-PAGE).

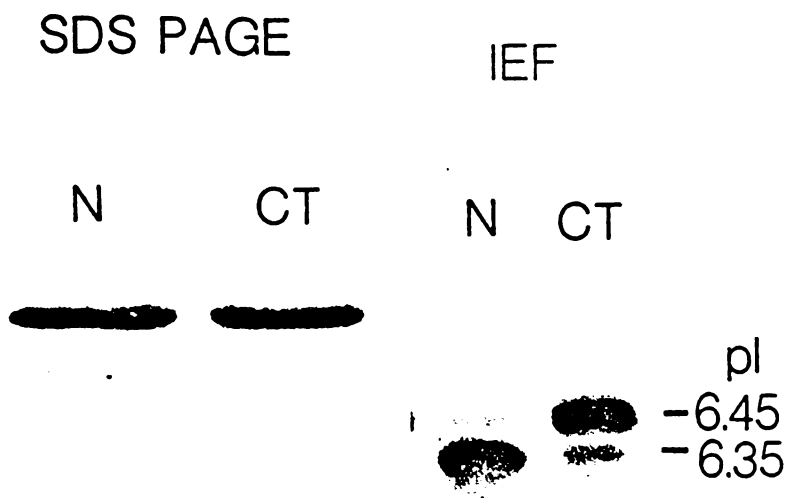

Figure 5

Figure 6. Incubation of bindable hexokinase with liver mitochondria suspension. Approximately 200 milliunits of crude, glucose-6-phosphate solubilized hexokinase (4 units/ml in 0.25 M sucrose) was incubated at room temperature for 2 hours in the presence of the indicated amounts of liver mitochondria protein (ug). Liver mitochondria were removed by centrifugation and the hexokinase was assayed for binding ability. Results are expressed as percent of total activity recovered in supernatant (broken line) and pellet (solid line).

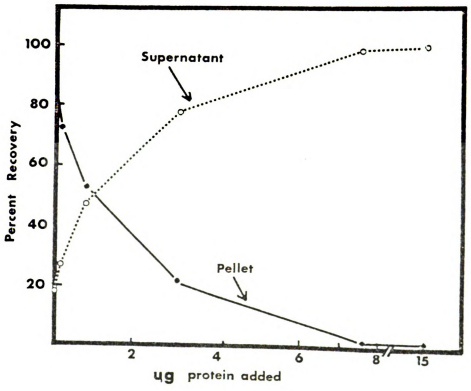
100
80
60
40
20
Percent Recovery
Supernatant
Pellet
2
4
6
8
15
ug protein added

Figure 6

Figure 7. Effect of pH and protease inhibitors on detergent solubilized brain protease. A) Crude, glucose-6-phosphate solubilized hexokinase (0.5 u/ml in .1M sodium phosphate) was incubated at room temperature for 2 hours in the presence of detergent solubilized brain protease (protease: hexokinase, 1:5, v/v) at the indicated pH values, or at pH 7.2 in the absence of protease (NA). Agarose gel isoelectric focusing was performed as described in Methods and gels were stained for hexokinase activity. B) Agarose gel isoelectric focusing was performed on purified bindable hexokinase (lane 1) pretreated at room temperature for 2 hours with detergent solubilized brain acid protease (protease: hexokinse, 1:1, v/v) in the absence (lane 2) or presence of the following inhibitors; 2 mM iodoacetate (lane 4), 2 mM PMSF (lane 5), 10 μM pepstatin A (lane 6), 2 mM 1,10 phenanthroline (lane 7). Protease solution boiled for 10 min. at 100°C prior to addition to hexokinase was also ineffective (lane 3). The gels were stained for hexokinase activity. All hexokinase samples were 25 units/ml in 'storage buffer' adjusted to pH 6.5. Detergent solubilized brain protease was prepared as described in Methods.

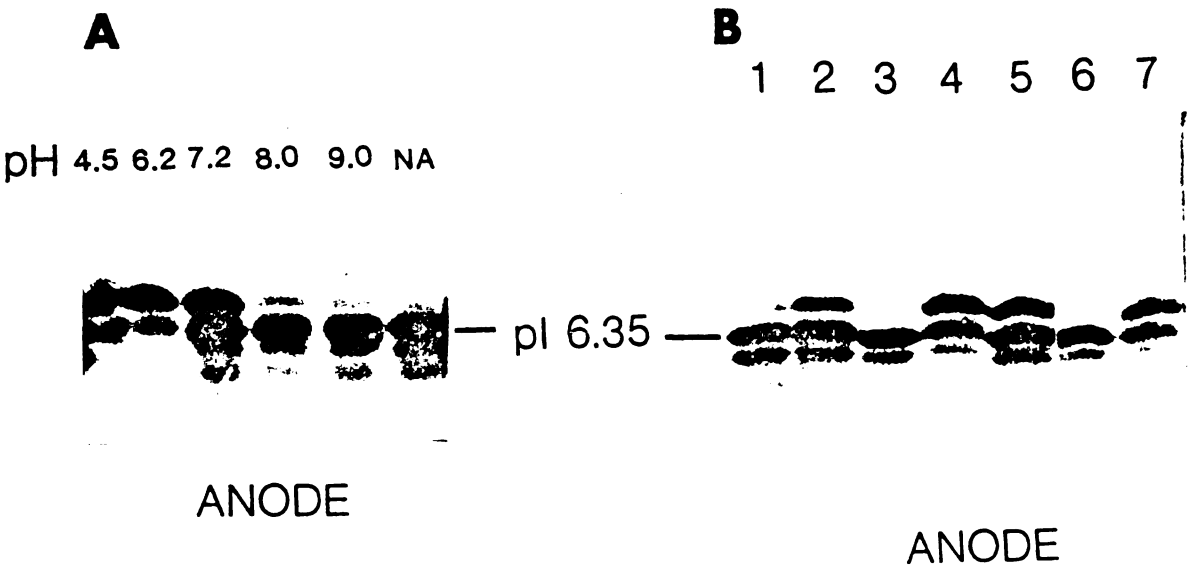

Figure 7

Figure 8. Sucrose density gradient sedimentation of detergent solubilized brain protease. Detergent solubilized brain protease, was subjected to centrifugation in 5-20% sucrose gradients. The gradient was fractionated and 10 µl aliquots removed from each fraction were added to 10 µl aliquots of bindable hexokinase (5 units/ml) in storage buffer, pH 6.2. Following incubation at room temperature for 2 hours the hexokinase was assayed for binding ability as described in Methods. Protease activity is expressed as percent binding lost (O) As molecular weight standards, alcohol dehydrogenase (150 K), hexokinase (98K) and ovalbumin (43 K), 33 µg each, were centrifuged in a parallel gradient and absorbance at 280 nM (●) was determined for each fraction.

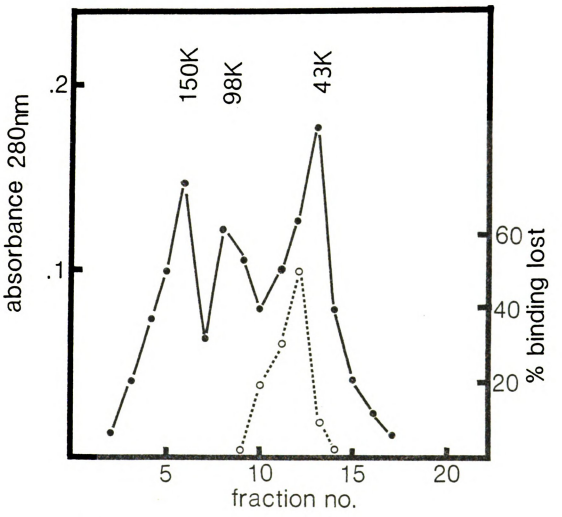
absorbance 280nm
.2
.1
150K
98K
43K
60
40
20
% binding lost
5
10
15
20
fraction no.

Figure 8

Figure 9. Separation of bindable and nonbindable hexokinase forms by HPLC. Chromatography was carried out on a Synchropak AX-300 ion exchange column (4.1 x 250 mm) equilibrated in Solvent A (0.01 M sodium phosphate, pH 7.0, 0.1 M glucose, and 0.01 M thioglycerol). Solvent B is solvent A made 0.35 M in KCL. Flow rate is 1.5 ml/min. After sample injection, the column is eluted with the following gradient program (solid line): 0-10 min, 0% solvent B; 10-25 min, linear increase from 0 to 44% B; 25-45 min, linear increase from 44 to 56% B; 45-60 min, linear increase from 56 to 100% B. Fractions of 1.1 ml were collected and assayed for hexokinase activity (●) and binding ability (○). Left ordinate, hexokinase activity; right ordinate, percent solvent B (solid line) or percent hexokinase bindable (○). A) 25 units of hexokinase previously chromatographed on DEAE cellulose. B) 15 units of crude, freshly glucose-6-phosphate solubilized hexokinase.

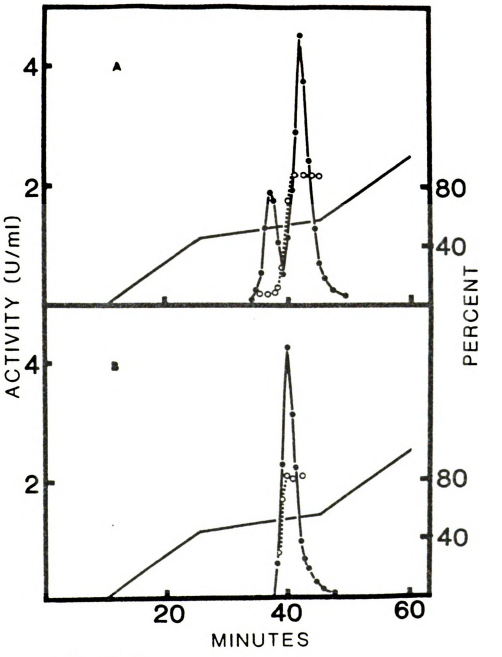

Figure 9

X-100 extracts of brain mitochondria fractions were incubated with a hexokinase preparation consisting predominantly of the pI 6.35 isoelectric form, the pI 6.45 form was rapidly generated. This detergent extractable factor displayed an acid pH dependence (Figure7A) and was shown to be heat labile, and insensitive to the protease inhibitors, iodoacetate, PMSF, or 1,10 phenanthroline, but inhibited by micromolar levels of pepstatin A (Figure 7B). The activty sedimented in sucrose density gradients with an apparent molecular weight of 58,000 (Figure 8). This proteolytic factor was likely responsible for some of the binding loss incurred during the purification of hexokinase, however, inclusion of pepstatin A in all buffers employed, did not totally prevent the phenomenon from occurring. Presumably hexokinse was also being degraded by at least one other protease, insensitive to pepstatin A.

Separation of bindable and nonbindable hexokianse forms by HPLC

As already noted by Felgner and Wilson (25), and presented above in this chapter, the early eluting fractions (from DEAE cellulose columns), of hexokinase activity, contain the nonbindable enzyme. With the use of anion exchange high performance liquid chromatography (HPLC), mixtures of bindable and nonbindable hexokinases can be resolved with good recovery of enzymatic activity (Figure 9B). Unlike DEAE cellulose chromatography, crude preparations of highly bindable hexokinase can be purified by the HPLC method without a substantial loss of the enzymes membrane binding ability (compare Figures 9A to B). Rechromatography of the isolated bindable or nonbindable enzyme peak results in the elution of a single peak with the expected retention time.

Comparison of isolated bindable and nonbindable hexokinase forms. Comparative peptide mapping. Heterogeneity in apparent molecular weight, as determined by SDS gel electrophoresis, could not be observed in hexokinase preparations containing substantial quantities of both the bindable and nonbindable enzyme forms. However, as brain hexokinase is a 98,000 molecular weight monomer (11), the detection of minor molecular weight differences by SDS gel electrophoresis is unlikely. Peptide mapping by limited proteolysis, as described by Cleveland et al. (14), was carried out on the bindable and nonbindable hexokinase with the prospect of detecting size differences in peptide fragments migrating in the low molecular weight region of the SDS gel, where greater separation is achieved. The digestion patterns of the two hexokinase forms, generated by three different proteases, reveal no detectable molecular weight differences in any region of the SDS gel (Figure 10), indicating that the loss of primary structure giving rise to the nonbindable enzyme must be very minor.

Proteolytic inactivation. The modifications resulting in the generation of the nonbindable hexokinase may produce conformational changes in enzyme structure which could be responsible for alterations in net surface charge and the loss of membrane binding capacity. Wilson (140) has previously demonstrated that the binding of glucose or glucose-6-phosphate to hexokinase results in a marked protection of the enzyme against inactivation by proteolysis. The binding of these same ligands has also been shown to dramatically limit the reactivity of sulfhydryl groups in the enzyme (138). Based on these observations, Wilson (138) has proposed that changes in hexokinase conformation result from the binding of ligands such as glucose and

Figure 10. Comparative peptide mapping of bindable and nonbindable hexokinase forms. Peptide mapping in SDS gels followed the procedure of Cleveland et al. (14) as decribed in Methods. Purified hexokinase was digested with S. aureus V-8 protease (4 ug/ml), chymotrypsin (5 µg/ml), or pronase (2 µg/ml) for 0.5 hours at 37°C and 40 µg of each digest, or 20 µg of nondigested hexokinase ("none") was applied to the gel. b, bindable hexokinase; nb, nonbindable hexokinase. BSA (68K), ovalbumin (43K) and cytochrome C (12.3 K) were used as molecular weight standards. The gel was stained for protein with Coomassie Blue (142).

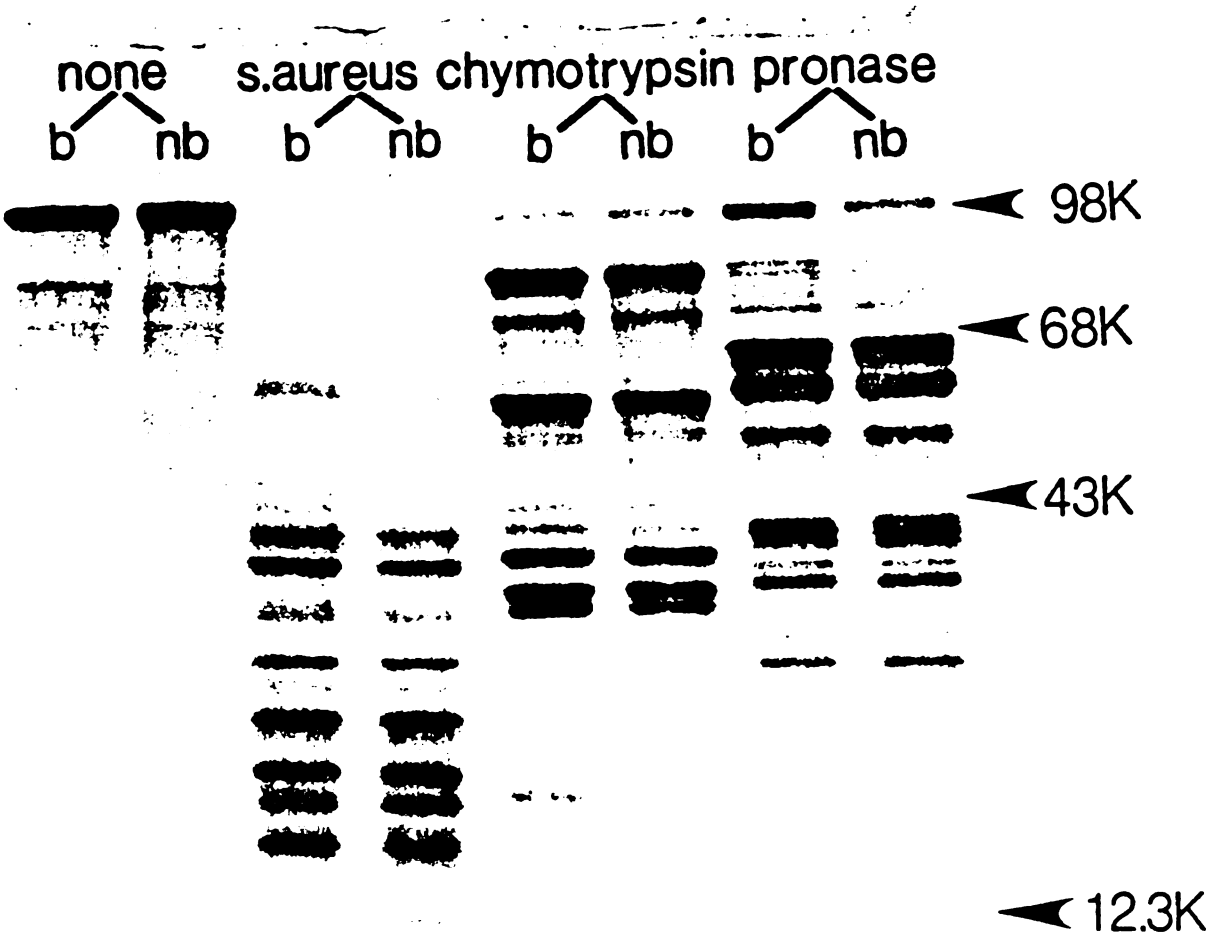
none
s.aureus
chymotrypsin
pronase
b
nb
b
nb
b
nb
b
nb
98K
68K
43K
12.3K

Figure 10

glucose-6-phosphate. These changes in conformation may also be responsible for the observed protection of hexokinase against inactivation by proteolysis (140). Therefore, it may be possible to detect differences in conformational properties of the bindable and nonbindable hexokinase forms by studying their relative rates of inactivation when subjected to proteolysis. To investigate this possibility, I incubated the hexokinase forms with high levels of pronase or chymotrypsin and monitored the loss of enzymatic activity over time. The resulting inactivation curves, generated by either protease, were virtually identical for the two hexokinase forms (figure 11). At least as judged by susceptibility to proteolysis, the hexokinase forms do not differ dramatically in structural properties.

Isoelectric focusing in urea agarose gels. Conformational changes in structure have been reported to cause variations in electrophoretic mobility of proteins (38). To further investigate the possibility of conformationally induced alterations in isoelectric point of the hexokinase molecule, the enzyme forms were subjected to isoelectric focusing in agarose gels made 7M in urea. It is clear that the nonbindable hexokinase form maintains an elevated pI, relative to the bindable enzyme, even under strongly denaturing conditions (Figure 12) suggesting that the differences in net surface charge do not result from differences in conformational properties of the enzyme forms.

NH_2-terminal dansylation. The proteolytic modification of bindable hexokinase to the nonbindable form indicated the loss of a small peptide from one or both termini of the protein chain. When the isolated hexokinase forms were examined using the danyslation technique both forms exhibited NH_2-terminal heterogeneity (Figure 13).

Figure 11. Proteolytic inactivation of bindable and nonbindable hexokinase forms. Either bindable or nonbindable hexokinase, approximately 4.0 units/ml in 0.02 M Hepes, pH 7.5, 0.01 M thioglycerol, and 0.5 mM EDTA was incubated at 23°C in the presence of pronase (0.1 mg/ml) or chymotrypsin (1 mg/ml). Aliquots were removed at the indicated times and hexokinase activity determined. Bindable hexokinase (solid line), nonbindable hexokinase (broken line).

Figure 12. Isoelectric focusing in agarose gels containing 7M urea. Approximately 25 μg of bindable (lane 1), nonbindable (Lane 2) or a mixture of 10 μg each of both hexokinase forms (lane 3) were applied to the gel. Focusing and protein staining were carried out as described in Methods. The pI of denatured hexokinase was not directly determined here, however, based on the focusing position observed with the native isoelectric focusing system, the pI is clearly more basic than that of the nondenatured enzyme.

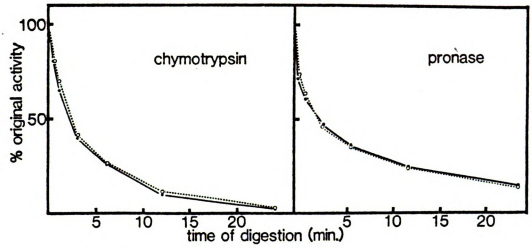

Figure 11

Figure 12

Figure 13. Amino terminal dansylation of bindable and nonbindable hexokinase forms. The hydrolyzed, dansylated bindable (b) or nonbindable (nb) hexokinase was subjected to 2-dimensional chromatography on micropolyamide plates. The location of dansyl tyrosine (tyr) and phenylalanine (phe) are indicated. Intense fluorescence in the lower section of the plate is dansylic acid.

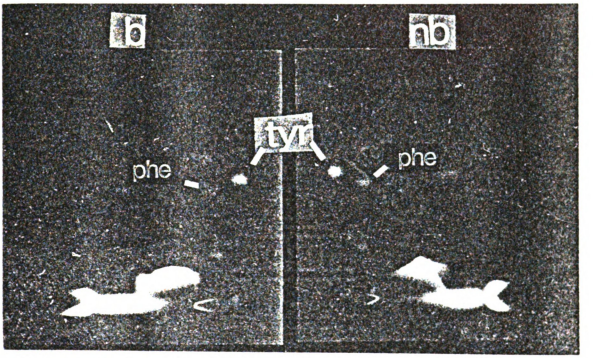

Figure 13

Tyrosine is detected as the predominant NH_2-terminal residue for either the bindable or nonbindable hexokinase, while the dansyl phenylalanine yield appears to be more intense for the nonbindable enzyme. The NH_2-terminal heterogeneity detected here indicates that proteolysis has probably occurred at this end of the hexokinase molecule. Although the two hexokinase forms exhibit differences in the relative levels of phenylalanine, the presence of tyrosine observed for both enzyme forms makes it difficult to clearly distinguish the bindable and nonbindable hexokinases based on these results.

Carboxypeptidase treatment of hexokinase. As a result of unrecognized contaminating endoprotease present in carboxypeptidase Y preparations (see subsequent sections, pg. 66) the results of experiments involving carboxypeptidase Y treatment of hexokinase were initially misleading. However, these data are presented below in order to clarify the nature of the difficulties associated with the use of carboxypeptidase Y. Treatment of bindable hexokinase with low levels of carboxypeptidase Y resulted in a rapid loss of membrane binding capacity without an apparent loss of catalytic activity (Figure 14). The characteristic shift in isoelectric point to the pI 6.45 form accompanied this binding loss while no detectable loss of molecular weight was observed. Treatment with carboxypeptidase A produced these same effects (not shown). Carboxyterminal sequence studies, performed by progressive degradation of hexokinase with carboxypeptidase Y and identification of the liberated amino acids by high performance liquid chromatography, were also carried out. The amino acid release pattern obtained with the bindable enzyme (Figure 15A) led to the proposed COOH-terminal sequence: -ile-ala-leu-ala-tyr. Nonbindable hexokinase obtained from

Figure 14. Carboxypeptidase Y treatment of bindable hexokinase. Bindable hexokinase, approximately 1 mg/ml in 'storage buffer', was treated with carboxypeptidase Y (5 µg/ml) at room temperature. The digestion was terminated at the indicated time intervals and aliquots were assayed for binding ability and enzyme activity. Results are expressed as percentages of original hexokinase activity (O) and binding ability (■) remaining after the given time (HOURS) of digestion.

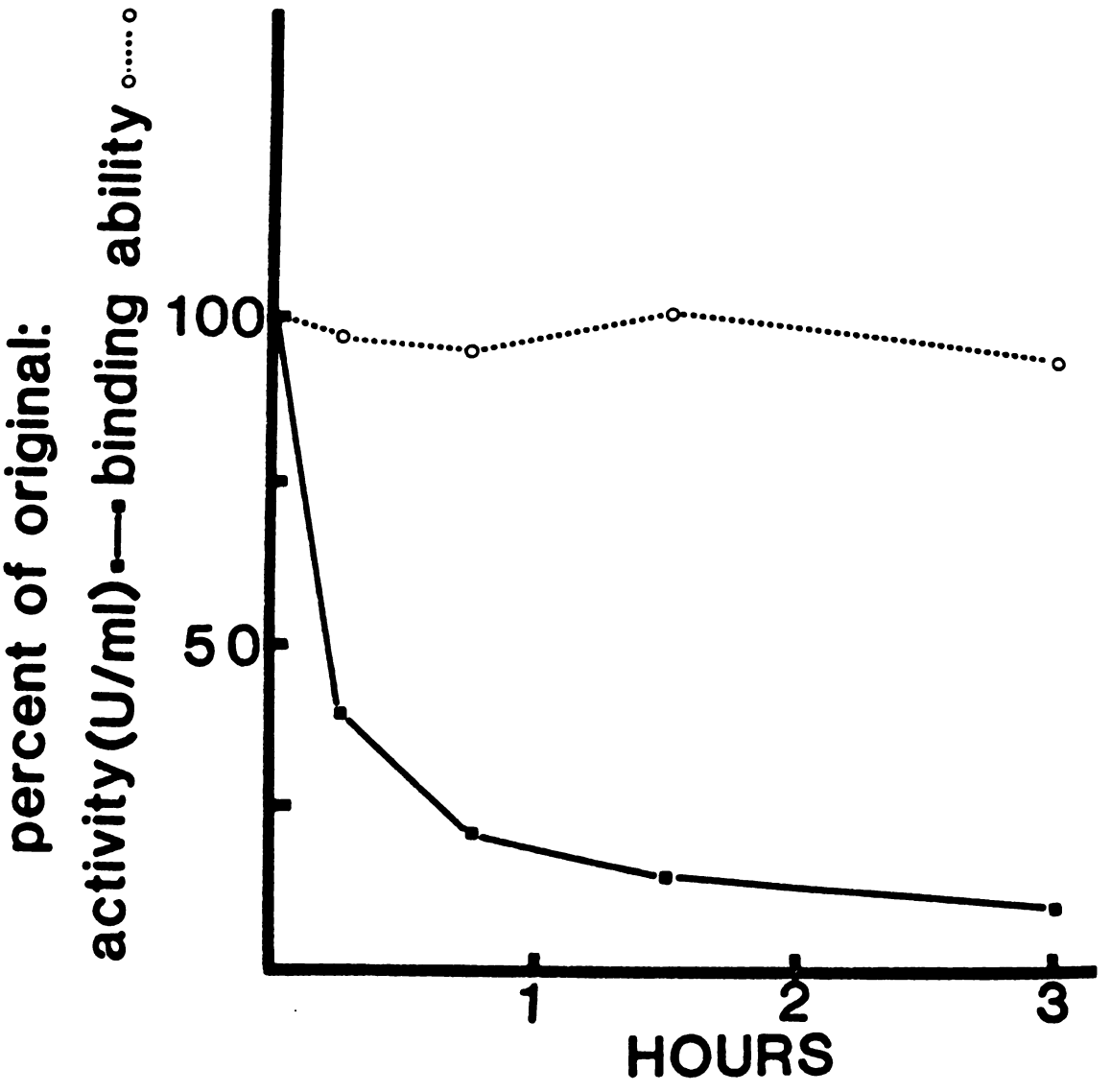
percent of original:
activity(U/ml)—▪ binding ability ○.....○
100
50
1
2
3
HOURS

Figure 14

Figure 15. Carboxypeptidase Y generated amino acid release patterns for bindable and nonbindable hexokinase forms. A) Purified bindable hexokinase, approximately 2.8 mg/ml in 0.01 M sodium phosphate, pH 7.0, 0.02 M glucose was treated with carboxypeptidase Y (10 µg/ml) at room temperature and aliquots were removed at the indicated times and analyzed for free amino acids as described in methods. Approximately 200 pmols represents one stoichiometric equivalent of hexokinase. B) Purified nonbindable hexokinase, approximately 2.0 mg/ml, was treated exactly as described for the bindable enzyme (Fig. 15A). Approximately 140 pmol represents one stoichiometric equivalent of hexokinase.

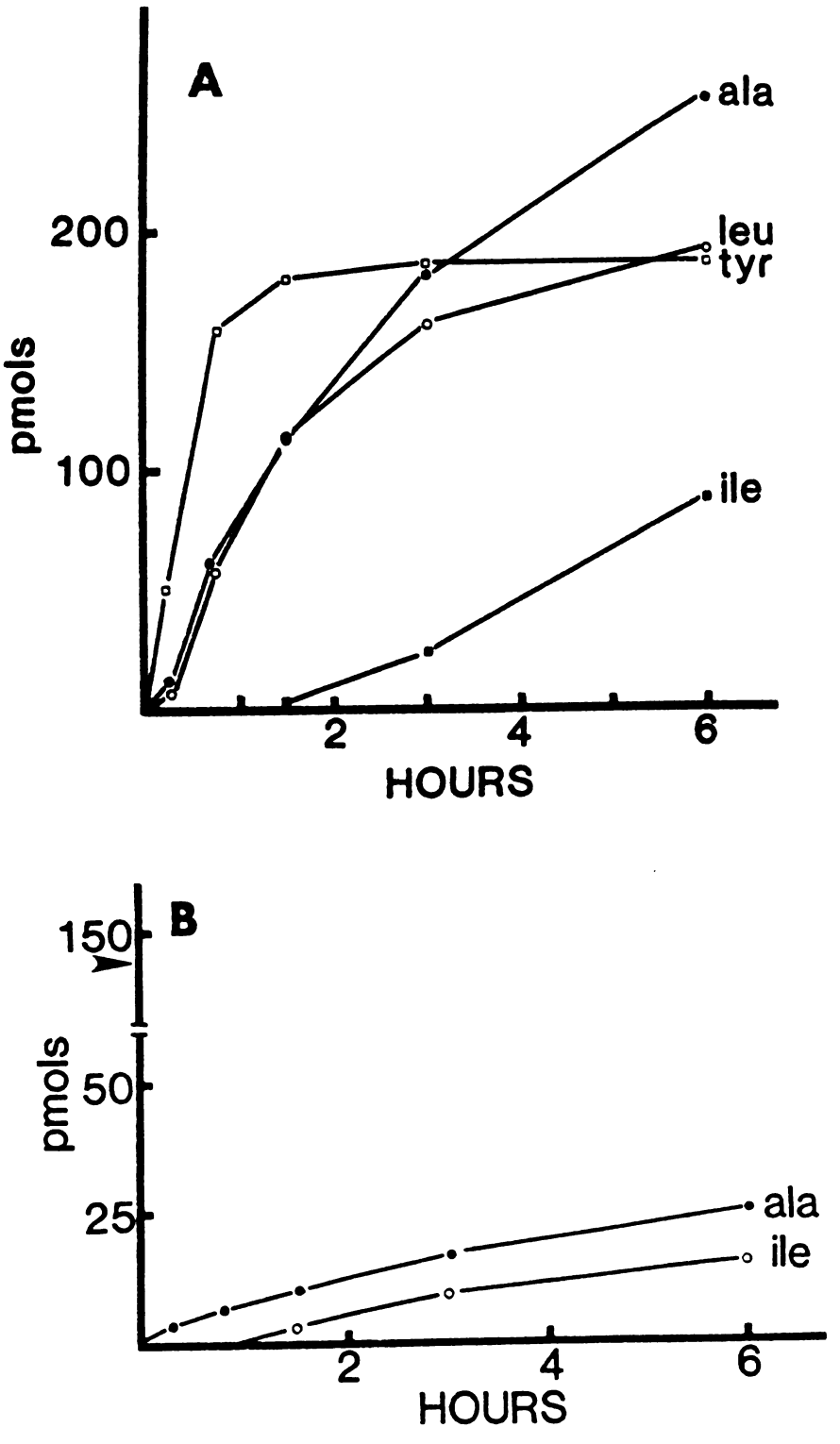
A
ala
leu
tyr
ile
200
100
pmols
2
4
6
HOURS
B
150
50
25
pmols
ala
ile
2
4
6
HOURS

Figure 15

DEAE cellulose purified preparations, or generated by limited chymotryptic treatment of bindable hexokinase, exhibited the release of only minor amounts of alanine and isoleucine when treated with carboxypeptidase Y under conditions identical to those employed with the bindable enzyme (Figure 15B).

Artifacts associated with the use of carboxypeptidases.

Modification of the hexokinase NH_2-terminus. Even though differences in primary structure at the COOH-termini of the bindable and nonbindable hexokinases were suggested by carboxypeptidase Y digestion, these data could not provide a satisfactory explanation for the observed differences in isoelectric point. Under the conditions used to produce the pI alteration in hexokinase, carboxypeptidase Y digestion did not result in the release of a charged amino acid. Moreover, examining the correlation between progressive binding loss and released amino acids showed that binding capacity diminished well ahead of the release of tyrosine (Figure 16), the first amino acid in the sequence proposed above.

Suspicions of a contaminating endoprotease activity in the carboxypeptidase Y preparation were confirmed by NH_2-terminal dansylation experiments performed on the carboxypeptidase Y treated hexokinase. Fluorescence of the dansyl-phenylalanine derivative is greatly enhanced in the carboxypeptidase treated sample (compare N to +CPY, Figure 17). The inclusion of the protease inhibitor pepstatin A, to 10 μM in the incubation medium, prevented the generation of NH_2-terminal phenylalanine (the dansyl pattern could not be distinguished from that shown in N, Figure 17), the loss of binding ability (Table 1), and the release of amino acids resulting from

Figure 16. Loss of hexokinase binding ability vs. release of tyrosine. Purified bindable hexokinase was treated with carboxypeptidase Y essentially as described in the legend of fig. 14. Aliquots were removed at the indicated times and were analyzed for free tyrosine and assayed for binding ability. Percent tyrosine remaining is determined by dividing the amount released at each time point by the total amount released upon exhaustive digestion with carboxypeptidase Y. Percent binding ability remaining is calculated from percent bindable at the indicated times divided by percent bindable at time zero (82% in this case).

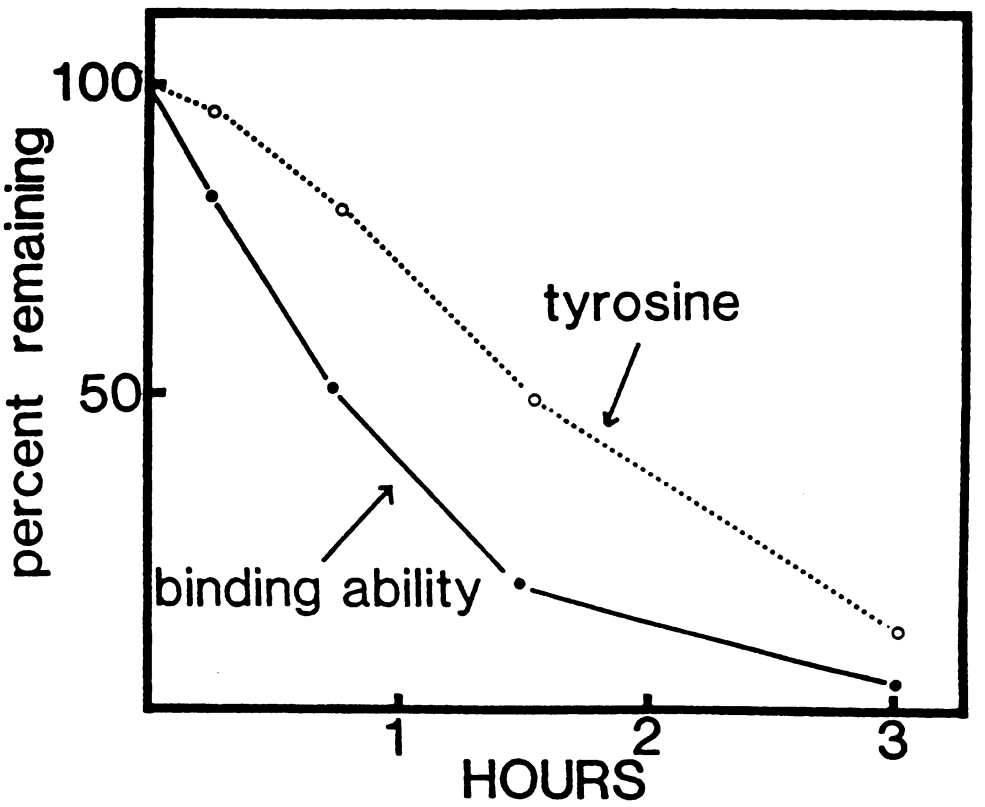
100
50
percent remaining
tyrosine
binding ability
1
2
3
HOURS

Figure 16

Figure 17. Amino-terminal dansylation of carboxypeptidase Y treated bindable hexokinase. Purified bindable hexokinase, native (N) or digested with carboxypeptidase Y (+CPY), (20 μg/ml) for 4 hours at room temperature was subjected to the amino terminal dansylation procedure described in Methods. Tyr, dansyl tyrosine, phe, dansyl phenylalanine.

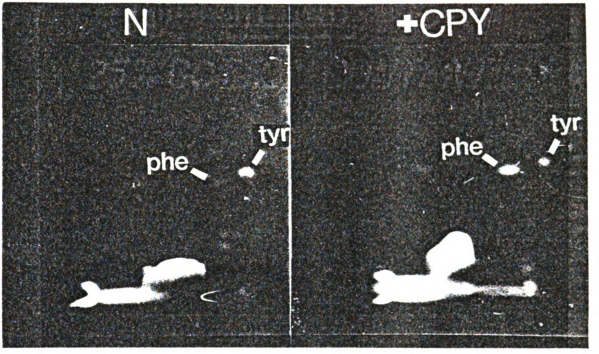

Figure 17

Table 1

Effect of protease inhibitors on carboxypeptidase Y induced loss of hexokinase binding ability.

incubation conditions	% Bindable
No Additions	74
+ CPY (no inhibitors)	10
+ CPY + 10 μM pepstatin A	71
+ CPY + 2 mM, 1,10 phenanthroline	13
+ CPY + 2 mM iodoacetate	10
+ CPY + 2 mM PMSF	11

Hexokinase (HK, 0.5 mg/ml in 'storage buffer') was incubated at room temperature for 2 hours in the absence (no additions) or presence (CPY) of carboxypeptidase Y (50 ug/ml) along with the specified amounts of the indicated protease inhibitors. Carboxypeptidase Y was also preincubated with the inhibitors, at the same concentrations used in the sample, for 15 minutes at room temperature prior to addition to hexokinase. Binding assays (% Bindable) were carried out using liver mitochondria as described in methods.

carboxypeptidase Y treatment of hexokinase. Moreover, the inclusion of a known carboxypeptidase Y inhibitor, PMSF (45), does not prevent the loss of binding ability (Table 1). Iodoacetate or 1,10 phenanthroline also have no effect on the activity resulting in the loss of binding ability (Table 1).

Protease assay with carboxypeptidase Y preparation. In order to substantiate the presence of protease contamination, protease assays were carried out on the carboxypeptidase Y preparation in the presence and absence of pepstatin A. The assay employed utilizes the digestion of casein, immobilized in agarose gel, as an indication of proteolysis. The proteolytic degradation of the milk proteins is observed as a clear ring in the gel resulting from a lack of staining by Coomassie Blue in this area (Figure 18). Only some of this degradation results from the activity of carboxypeptidase Y itself, as the inclusion of PMSF, a potent carboxypeptidase Y inhibitor (45), reduces the level of proteolysis but does not eliminate it entirely. The proteolytic activity in the carboxypeptidase Y preparation can be almost completely eliminated if pepstatin A and PMSF are both included in the gel.

Endoprotease contamination in carboxypeptidase A preparations. As noted above, carboxypeptidase A treatment of hexokinase also resulted in the loss of the enzyme's binding ability and a shift in isoelectric point. Upon examining the effects of various protease inhibitors on the carboxypeptidase A preparation, a PMSF sensitive protease activity responsible for the elimination of hexokinase binding ability, was detected (Table 2). The inclusion of 1,10 phenanthroline, a potent carboxypeptidase A inhibitor (96), in the incubation medium, did not

Figure 18. Determination of endoprotease activity in carboxypeptidase Y preparations. Approximately 10 µl of carboxypeptidase Y solution (5 mg/ml) was added to agarose slab gels containing 0.033% milk powder and either 1 mM PMSF, 10 µM pepstatin A, both inhibitors at these concentrations, or no inhibitor (no addition). The gels were incubated in a humidified chamber overnight and then stained for protein with Coomassie Blue (142). Buffer only (0.1 M sodium phosphate, pH 7.0) was added to the left well of each gel.

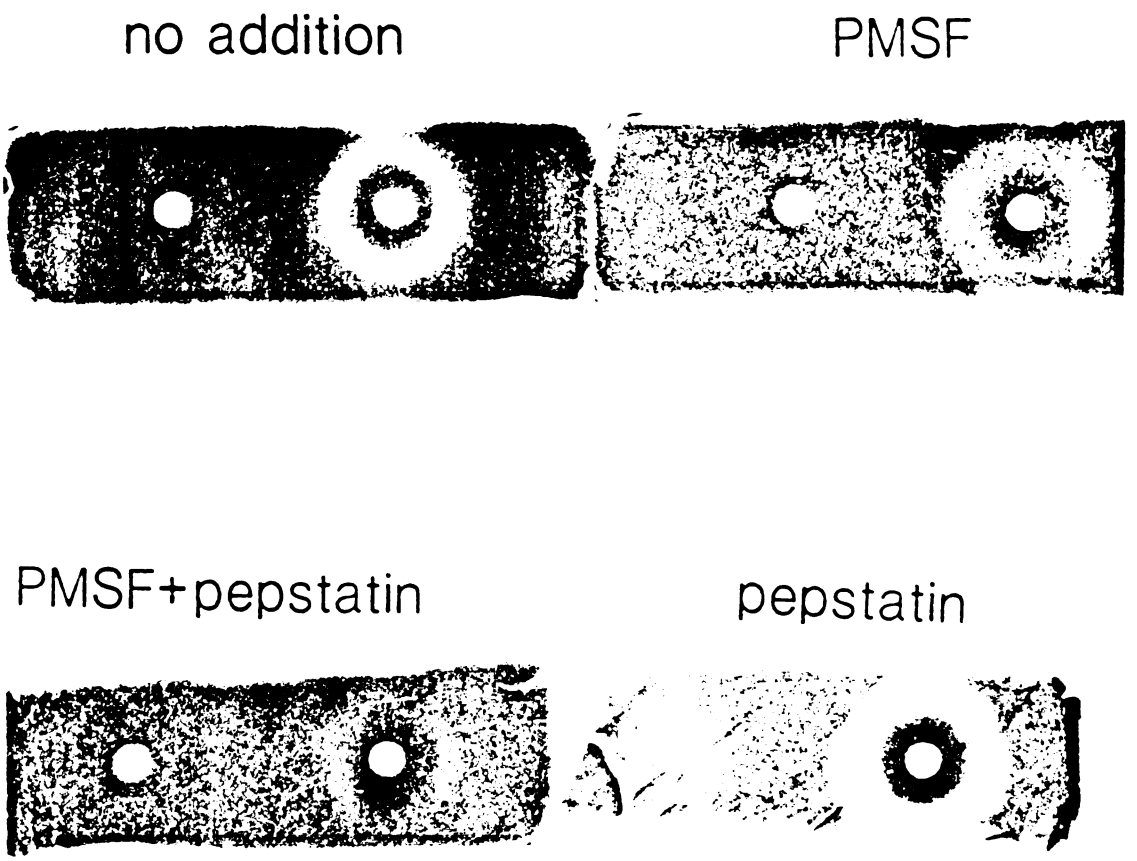

Figure 18

Table 2

Effect of protease inhibitors on carboxypeptidase A induced loss of hexokinase binding ability.

incubation conditions	% Bindable
No Additions	82
+ CPA (no inhibitor)	23
+ CPA + 1 mM, 1,10 phenanthroline	36
+ CPA + 10 μM pepstatin A	29
+ CPA + 1 mM PMSF	74

Hexokinase (HK, 1 mg/ml in storage buffer') was incubated at room temperature for 6 hours in the absence (no additions) or presence (CPA) of carboxypeptidase A (1 mg/ml) along with the specified amounts of the indicated protease inhibitors. Carboxypeptidase A was also preincubated with the inhibitors, at the same concentrations used in the sample, for 15 min at room temperature prior to addition to hexokinase. Binding assays (% bindable) were performed using liver mitochondria as described in Methods.

substantially inhibit the activity affecting hexokinase binding ability.

Edman degradation of bindable and nonbindable hexokinases.

Purified bindable and nonbindable hexokinases were submitted to an outside facility (UM Protein Sequencing Facility, Ann Arbor, MI) for NH_2-terminal sequence analysis. The bindable enzyme form was reported to be NH_2-terminally blocked as no detectable release of any amino acid was observed. The nonbindable enzyme form, however, exhibited the release of thiohydantoin derivatives, but appeared highly heterogeneous as several different amino acids were identified with each cycle.

The data obtained with the nonbindable hexokinase form support the results of the dansylation experiments (pg. 56) which indicated heterogeneity at the NH_2-terminus. The detection of dansyl tyrosine with the bindable hexokinase is inconsistent with the failure to detect any NH_2-terminal residue when Edman degradation is attempted on this enzyme form. However, the dansylation technique is not quantitative and the tyrosine observed for the bindable enzyme using this method may result from the presence of a minor component, bearing NH_2-terminal tyrosine, at a level insufficient for detection by the Edman method.

Identification of carboxy-terminal amino acids by PMSF treated carboxypeptidase A degradation of hexokinase.

When either the bindable on nonbindable hexokinase is treated with carboxypeptidase A, preincubated with PMSF, alanine and isoleucine are liberated (Figure 19). The isoelectric focusing pattern and binding properties of the carboxypeptidase A digested bindable hexokinase form were also examined at the indicated time points (Figure 20). These

Figure 19. Carboxypeptidase A digestion of bindable and nonbindable hexokinase forms. Bindable (b) or nonbindable (nb) hexokinase (1 mg/ml) in 0.1M sodium phosphate pH 8.0, 2 mM PMSF was incubated at room temperature in the presence of carboxypeptidase A (250 or 200 g/ml, respectively) and at the specified times the digestions were terminated and analysis of free amino acids were performed. Carboxypeptidase A was preincubated in 2 mM PMSF for 15 min prior to addition to hexokinase. Approximately 74 and 82 pmols represents one stoichiometric equivalent of bindable and nonbindable hexokinase, respectively. Low levels of glycine, leucine, and phenylanine (< 10 pmols ea.) were also detected for both b and nb but are not shown here.

Figure 20. Isoelectric focusing and binding ability of carboxypeptidase A digested bindable hexokinase. Aliquots were removed from the carboxypeptidase A digestion medium used to generate the amino acid release pattern for bindable hexokinase (shown in figure 19) following 0, 15, 45, 90 and 180 min of digestion, diluted (1:10) into ice cold 0.1 M Hepes, pH 7.5, 0.02 M glucose, 0.01 M thioglycerol, 2 mM PMSF and 2 mM 1,10 phenanthroline. Samples were assayed for binding ability (% bindable, top) and subjected to isoelectric focusing as described in methods. The agarose gel was stained for hexokinase activity.

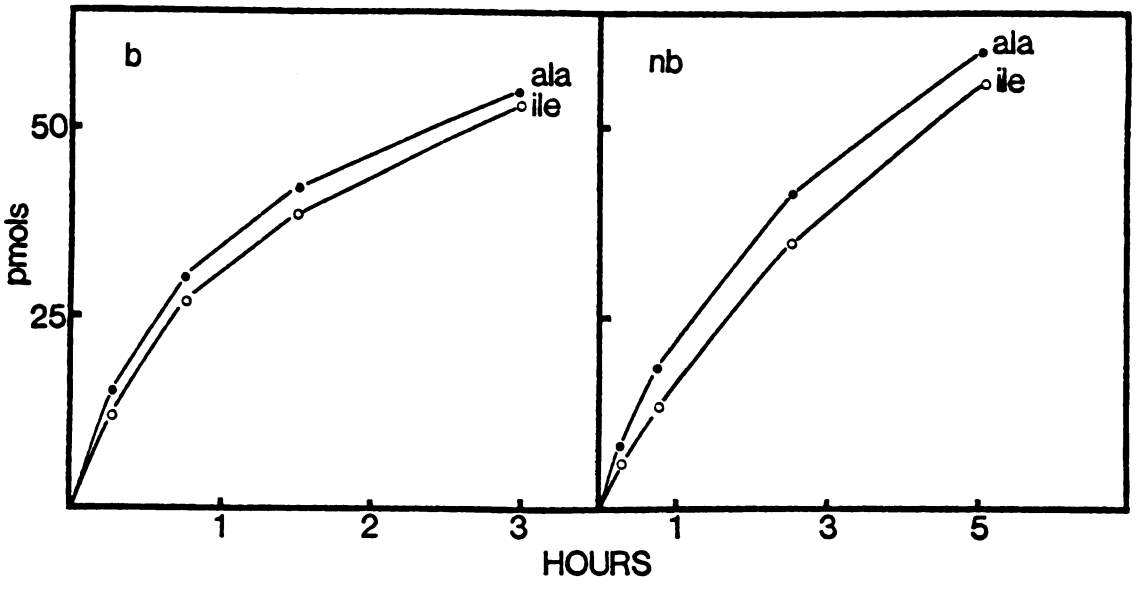

Figure 19

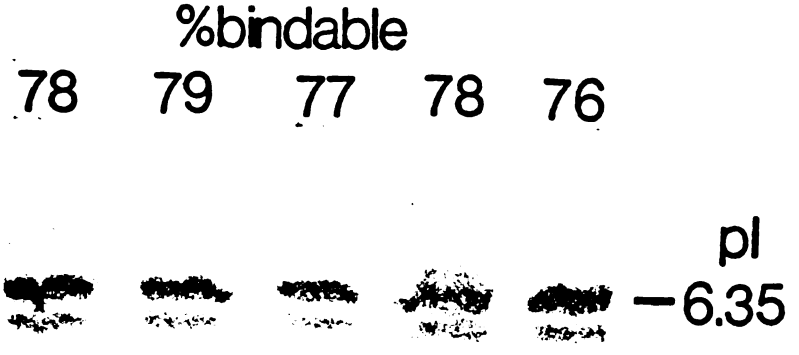

Figure 20

data demonstrate that the alanine and isoleucine are the authentic COOH-terminal amino acids of either the bindable or nonbindable hexokinase and that their removal does not contribute to a loss of binding capacity or alteration in isoelectric point of the bindable enzyme. Further evidence supporting this view is presented in Chapter Two of this thesis (pg. 116).

Characterization of peptides released by limited chymotryptic treatment of bindable hexokinase.

Limited digestion of bindable hexokinase with chymotrypsin resulted in total loss of membrane binding ability with no apparent change in molecular weight. Peptides released by chymotryptic cleavage of bindable hexokinase were obtained by ultrafiltration of the protein digest. The filtrate did not contain a significant level of free amino acids but did yield the residues listed in Table 3 when acid hydrolysis or exhaustive degradation with carboxypeptidase Y was carried out. The results of acid hydrolysis suggests an amino acid composition of 3 alanines, 2 leucines, and 1 residue each of tyrosine, leucine, glutamate and methionine. The glutamate presumably results from the deamidation of the glutamine seen in the enzymatic digest. Methionine appears in the acid hydrolysate but not in the enzymatic digest suggesting that this residue may be the blocked NH_2-terminal amino acid of the intact bindable hexokinase molecule.

When the digestion filtrate is subjected to reversed phase HPLC, three major peaks, labeled 1, 2 and 3 in order of elution, are detected at 214 nM (Figure 21). COOH-terminal sequencing of the isolated peaks, carried out by controlled digestion with carboxypeptidase Y, resulted in the amino acid release patterns shown in figure 22. Peak 1

Table 3

Amino acid composition of peptides released by chymotryptic treatment of bindable hexokinase.

	moles/(mole of lowest significant residue)[a]				
	Acid hydrolysis	CPY degradation[b]			
	filtrate[c]	filtrate[c]	peak 1	peak 2	peak 3[d]
alanine	3.10	2.40	-	1.93	2.04
leucine	2.37	1.73	-	1.26	1.96
tyrosine	1.34	1.06	1.00[d]	-	-
glutamine	-	1.00	-	1.00	1.00
glutamate	1.27	-	-	-	-
isoleucine	1.00	1.15	-	1.02	1.08
methionine	1.00	-	-	-	-

[a]Lowest significant residue is the amino acid present at the lowest pmol level and is given the value of 1.00. Amino acids detected at < 20% of this value are not listed.

[b]Exhaustive digestion with carboxypeptidase Y was carried out as described in Methods.

[c]Bindable hexokinase (1 mg/ml in 0.01 M NaPhos., pH 7.0, 0.02 M glucose) was digested with chymotrypsin (5 µg/ml) for 1 hour at room temperature and then subjected to ultrafiltration using an Amicon Centricon 30 microconcentrator. The filtrate was either digested directly with carboxypeptide Y or, following lyophilization, hydrolyzed in 6N HCl as described in Methods.

[d]Reversed phase HPLC, as described in Methods, was carried out on the filtrate and 3 major peaks, labeled 1, 2 and 3 in order of elution from the column, were recovered during chromatography. The peptide residues were dried under vacuum, redissolved in 0.01 M NaPhos., pH 7.0 and degraded with carboxypeptidase Y.

Figure 21. Reversed phase HPLC of peptides released by chymotryptic treatment of bindable hexokinase. Reversed phase HPLC was performed as described in Methods. Peptide filtrate (see legend, Table 3) obtained from the chymotryptic digestion of 300 µg of bindable hexokinase was injected onto the column and gradient elution was started immediately. Peaks 1, 2, and 3 were collected for analysis described in Figure 22 and Table 3. Peaks labelled 'C' were also detected after injection of a control filtrate obtained from a sample containing chymotrypsin only at the same concentration used to digest hexokinase. A major component also detected in the control filtrate is presumably chymotrypsin (CT). All of the peaks listed are single components but appear to elute as doublets due to a void volume present at the column head.

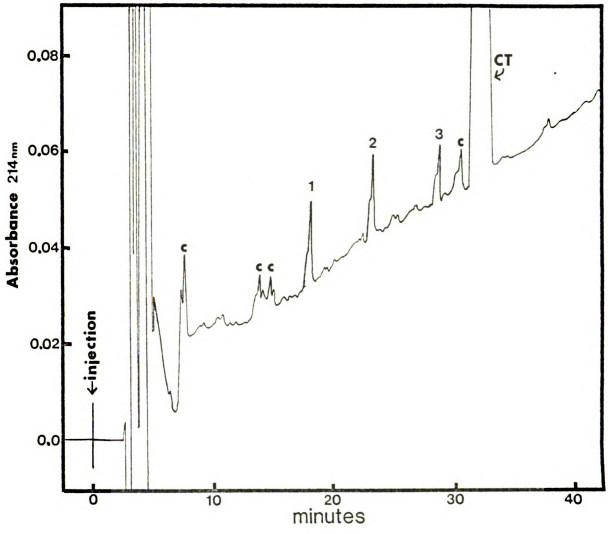
Absorbance 214nm
0.08
0.06
0.04
0.02
0.0
←injection
c
c c
1
2
3
c
CT
0
10
20
30
40
minutes

Figure 21

Figure 22. Carboxypeptidase Y digestion of peptides released by chymotryptic treatment of bindable hexokinase. Peptide filtrate (see legend, Table 3) was subjected to reversed phase HPLC and the isolated peptides were digested with carboxypeptidase Y (5 μg/ml, 0.01 M sodium phosphate, pH 7.0, 10 μM pepstatin A) as described in Methods. Three peptides were recovered during chromatography and are labelled peaks 1,2, and 3 in order of elution from the column.

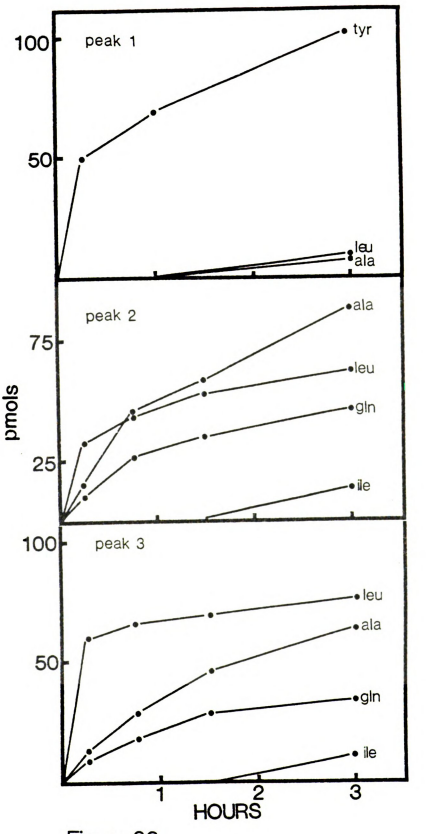
peak 1
100
50
tyr
leu
ala
peak 2
75
25
pmols
ala
leu
gln
ile
peak 3
100
50
leu
ala
gln
ile
1
2
3
HOURS

Figure 22

liberates tyrosine almost exclusively, while at later times, trace levels of alanine and leucine can be detected. As digestion of dipeptides by carboxypeptidase Y is considered to be negligible (45), the release pattern for peak 1 may be indicative of a tripeptide containing COOH-terminal tyrosine. Similar release patterns, both exhibiting COOH-terminal leucine, are generated from carboxypeptidase Y digestion of peaks 2 and 3. Peak 3, however, appears to contain a second leucine residue, apparently adjacent to the first. Upon exhaustive digestion of peaks 2 and 3, glutamine and isoleucine are obtained in approximately equal amounts, being roughly one half that of the alanine level, while the relative yield of leucine differs for the two peptides (Table 3). These data suggest that peaks 2 and 3 are identical with the exception of a single, nonoverlapping leucine residue present at the COOH-terminus of peak 3.

Quantitative amino acid analysis of acid hydrolysates of the isolated peaks has been hindered due to contaminants eluting in the area of the leucine and isoleucine OPA derivatives during chromatography. However, methionine is clearly detected in hydrolysates of peaks 2 and 3 but is absent in peak 1. Based on the data presented above, a possible sequence for the NH_2-terminal region of bindable hexokinase is suggested (Figure 23). The positioning of the HPLC peaks and the sites of chymotryptic cleavage are illustrated.

Ultrafiltrate obtained from chymotryptic digests of nonbindable hexokinase failed to liberate any amino acids when treated with carboxypeptidase Y under exhaustive digestion conditions.

Figure 23. Proposed amino acid sequence and sites of chymotryptic cleavage for the NH_2-terminal region of bindable hexokinase. Peaks 1, 2 and 3 indicate peptides recovered from reversed phase HPLC of chymotryptically digested bindable hexokinase (see fig. 21 and Table 3) and X represents the amino acid adjacent to tyrosine. AcNHmet is used to designate the derivatized NH_2-terminal methionine, although, the actual nature of the blocking group has not been identified.

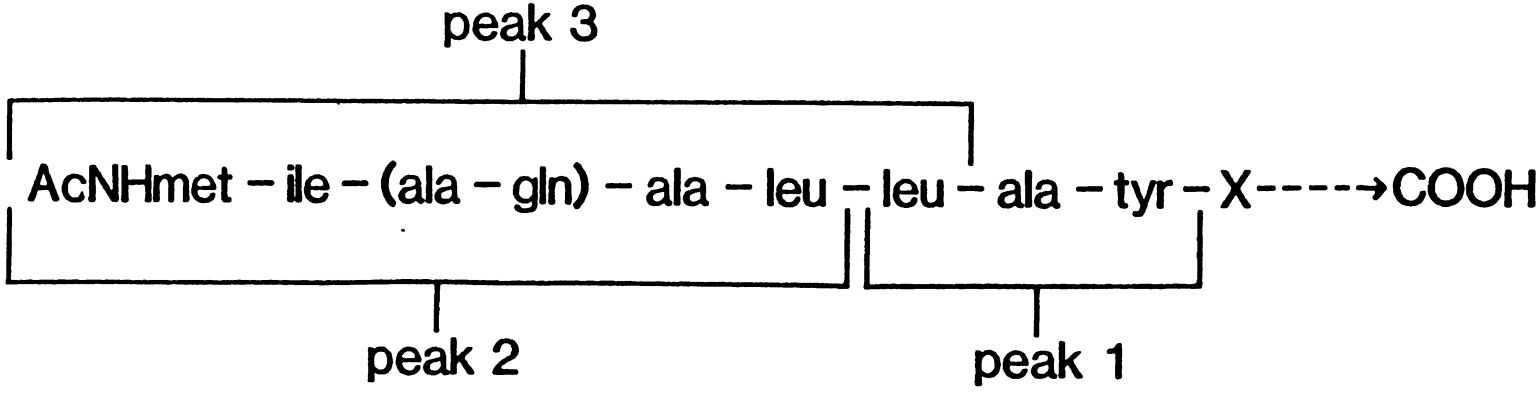
peak 3
AcNHmet – ile – (ala – gln) – ala – leu – leu – ala – tyr – X----→COOH
peak 2
peak 1

Figure 23

Discussion

The presence of nonbindable hexokinase in enzyme preparations derived from glucose-6-phosphate solubilization of rat brain mitochondria is artifactual in nature and results exclusively from proteolytic cleavage at the extreme NH_2-terminal region of the bindable molecule. Proteases, either exogenously added to hexokinase or endogenously present during the purification procedure, promote the generation of the nonbindable enzyme form.

An acid pH dependent, pepstatin A sensitive factor present in the mitochondrial fraction of rat brain homogenates, has been shown to catalyze the bindable to nonbindable hexokinase transformation. Characteristics of the protease indicate a lysosomal origin, making less likely the possibility that it maintains an _in vivo_ function with respect to altering the membrane binding ability of hexokinase. A physiological role for this protease should not be entirely excluded, though, as Mellone _et al._ (86) have proposed a regulatory mechanism for the gluconeogenic pathway, which involves the modification of fructose-1,6-bisphosphatase by a lysosomal protease, and further demonstrate this proteolytic activity with intact lysosomes.

It is evident from the low concentrations of chymotrypsin needed to generate the nonbindable hexokinase form that the NH_2-terminal region of the bindable molecule is extremely sensitive to proteolysis. In some experiments (not shown), incubation at room temperature for periods of 6 hours at pH 7.0, with chymotrypsin to hexokinase ratios as low as 1:10,000, resulted in a loss of greater than 50% of the enzyme's membrane binding ability.

Rat brain mitochondrial hexokinase, as purified by the method of Chou and Wilson (11), exhibits a deficiency in its ability to rebind to

the mitochondrial membrane (19,25). As demonstrated here, storage of the crude glucose-6-phosphate solubilized hexokinase for a period of several days, contributes to the generation of the nonbindable enzyme form. However, chromatography of the enzyme on DEAE cellulose columns dramatically accelerates the binding loss phenomenon. Craven and Basford (19) also reported a marked loss of hexokinase binding ability when the enzyme was chromatographed on this matrix. It is conceivable that the hydrophobic, blocked NH_2-terminal tail of the hexokinase molecule remains highly exposed when the enzyme is associated with this ion exchange resin, thus rendering it susceptible to proteolytic attack. The nature of the proteolysis which occurs during DEAE cellulose chromatography of hexokinase has not been elucidated, nor have attempts to prevent it, by the inclusion of various protease inhibitors in the chromatography buffers, been consistently successful. When ion exchange HPLC is substituted for DEAE cellulose chromatography, the loss of hexokinase binding ability is not observed (97). The reduced time period afforded by HPLC may be an important factor in circumventing the binding loss problem.

The bindable and nonbindable hexokinase forms exhibit a difference in net surface charge, as determined by isoelectric focusing. The elevated pI observed for the nonbindable enzyme, relative to the bindable form, could result from either a decrease in total negative charge or an increase in total positive charge. A decrease in negative charge seems unlikely due to the failure to detect the release of a negatively charged amino acid when bindable hexokinase is proteolyzed to a nonbindable enzyme form. However, the presence of a positively charged free NH_2-terminus on the nonbindable enzyme, as opposed to an

electrically neutral blocked NH_2-terminus on the bindable form, would satisfy the requirements for an increased positive charge. The results of Edman degradation performed on the isolated hexokinase forms indicated a blocked NH_2-terminus for the bindable enzyme only. It is concluded here that the presence of a blocked NH_2-terminus for the bindable enzyme, as opposed to a free NH_2-terminus for the nonbindable enzyme, is responsible for the charge distinction. In some cases, subforms of bindable hexokinase were observed at pI values below that determined for the predominant bindable form i.e., pI 6.35. These minor isoelectric forms must possess an intact NH_2-terminus, shown to be essential for membrane binding, yet may contain some other structural modification, such as a cleaved COOH-terminus or a derivatized functional group, responsible for the lowered isoelectric point.

The presence of contaminating endoproteases in carboxypeptidase Y preparations resulted in the misinterpretation of data initially obtained with the use of this exoprotease. Moreover, the elimination of membrane binding ability, observed with carboxypeptidase A treatment of hexokinase, also proved to be artifactual, resulting from a PMSF sensitive proteolytic contamination present in this carboxypeptidase preparation. Only when hexokinase was incubated at elevated pH, in the presence of PMSF pretreated carboxypeptidase A, could the authentic COOH-terminal residues be identified. The same COOH-terminal amino acids, alanine and isoleucine, were liberated from both the bindable and nonbindable hexokinase forms and from hexokinase made nonbindable by treatment with chymotrypsin. A 40K dal COOH-terminal peptide, generated by tryptic cleavage of the 98K dal hexokinase polypeptide,

also exhibits COOH-terminal alanine and isoleucine (Chaper II, pg. of this thesis).

Some confusion related to the use of carboxypeptidase Y has emerged from studies involving the microsomal membrane binding of NADH cytochrome b_5 reductases. Working with the steer liver enzyme, Kensil et al. (64) present good evidence identifying the NH_2-terminal segment of the molecule as the membrane binding region, while Mihara et al. (88) report a COOH-terminal binding domain for the rabbit liver enzyme. The evidence supporting a COOH-terminal binding region for the rabbit liver enzyme rests largely on the observation that the binding property is eliminated upon treatment of the reductase with carboxypeptidase Y. Kensil et al. (64) suggests that the unrecognized presence of contaminating endoproteases in carboxypeptidase Y preparations may have led to incorrect conclusions for the rabbit liver reductase.

A report has been published describing the presence of a pepstatin A sensitive endoprotease activity in carboxypeptidase Y preparations obtained from Pierce Chemical Co. (75). The protease assays conducted in the course of my work demonstrates the presence of a pepstatin A sensitive protease activity in carboxypeptidase Y preparations obtained from Sigma Chemical Co. At least three different batches of carboxypeptidase Y purchased from Sigma Chemical Co. exhibited the endoprotease contaminant.

The inability to detect substantial NH_2-terminal differences between the bindable and nonbindable hexokinases using the dansylation technique also contributed to the evidence that the COOH-terminus may be critical to the membrane binding process. Tyrosine was identified

as the predominant NH_2-terminal residues for both hexokinase forms; however, upon close inspection of the chromatographic results, relatively higher intensity of the dansyl phenylalanine derivative is observed for the nonbindable enzyme form.

Based on the results of unsuccessful NH_2-terminal sequencing performed by the Edman degradation method, it was concluded that the bindable hexokinase molecule is blocked. The NH_2-terminal tyrosine, observed with dansylation of the bindable enzyme form, may result from some yet unappreciated artifact associated with this method or from the presence of a minor hexokinase subform, containing NH_2-terminal tyrosine.

When NH_2-terminal dansylation is carried out on bindable hexokinase which has been endoproteolytically cleaved in the NH_2-terminal region (Figure 17), a strong dansyl phenylalanine signal is obtained, yet the dansyltyrosine is still observed. The intensity of the dansyl phenylalanine derivative may represent the level of fluorescence expected with near stoichiometric labelling of the hexokinase present, while the persistence of the tyrosine derivative could result from the proteolytic resistance of a minor component exhibiting this NH_2-terminal residue.

The ability of brain hexokinase to associate with the mitochondrial membrane is thought to play a role in regulating the enzymes catalytic activity (141). One component of the binding process involves the interaction of hexokinase with an integral membrane protein buried in the lipid bilayer of the outer mitochondrial membrane (141). It is reasonable to assume that a hydrophobic amino acid sequence, present in the membrane binding region of the hexokinase

molecule, would be required to permit this type of interaction. Here, a portion of the hexokinase primary structure, essential to the membrane binding process, has been characterized and shown to be highly hydrophobic in nature. Kurokawa et al. (73) report that nonbindable rat brain hexokinase, generated by limited chymotryptic treatment of the bindable enzyme, fails to adsorb to Phenyl-Sepharose columns when chromatographed. As the bindable hexokinase binds to this hydrophobic resin, these authors (73) postulate the presence of a hydrophobic peptide, which can be released by chymotrypsin, that is essential for the interaction with Phenyl-Sepahrose, and possibly, membrane binding. This view is consistent with the results presented here.

In summary, the proteolytically induced bindable to nonbindable hexokinase transformation is accompanied by the appearance of a new NH_2-terminal amino acid. Identical amino acids are liberated from the COOH-termini of the bindable, nonbindable, and chymotryptically cleaved hexokinases. The liberation of amino acids by PMSF pretreated carboxypeptidase A degradation of bindable hexokinase, does not result in a loss of membrane binding ability or an alteration in isoelectric point. Highly bindable hydrophobic peptides can be proteolytically released from the NH_2-terminus of bindable, but not from the nonbindable, hexokinase form. Taken together, these points strongly support the view that the extreme NH_2-terminal region of the hexokinase molecule is critical to the enzyme-membrane interaction.

CHAPTER II

PROTEOLYTIC DISSECTION OF BRAIN HEXOKINASE

Purified rat brain hexokinase was subjected to limited proteolytic digestion with trypsin and the native and digested enzymes were compared. The major tryptic digestion products, as separated by SDS gel electrophoresis, were partially characterized and assigned locations within the hexokinase monomer. Based on the interpretation of the digestion pattern, a new method for determining the location of antigenic determinants (epitopes) within the primary sequence was developed and is described here.

Results

Tryptic cleavage pattern of rat brain hexokinase. When purified rat brain hexokinase is treated with trypsin, under nondenaturing conditions, a discrete set of polypeptides, as observed with SDS polyacrylamide gel electrophoresis, is obtained. In Figure 24, the resulting digestion patterns, along with percent of catalytic activity remaining after the indicated time intervals, are shown. At the longer time points it is apparent that the 98 K molecular weight polypeptide is virtually absent, while only a minor percentage of the original catalytic activity is lost. Graphic representation of a similar experiment (Figure 25), illustrating the relative abundance of each of the polypeptides with increasing time of digestion, more clearly

Figure 24. Limited tryptic cleavage of rat brain hexokinase. Purified hexokinase was digested with trypsin, as described in Methods, for the indicated times and aliquots were assayed for enzyme activity (top) and subjected to SDS-gel electrophoresis.

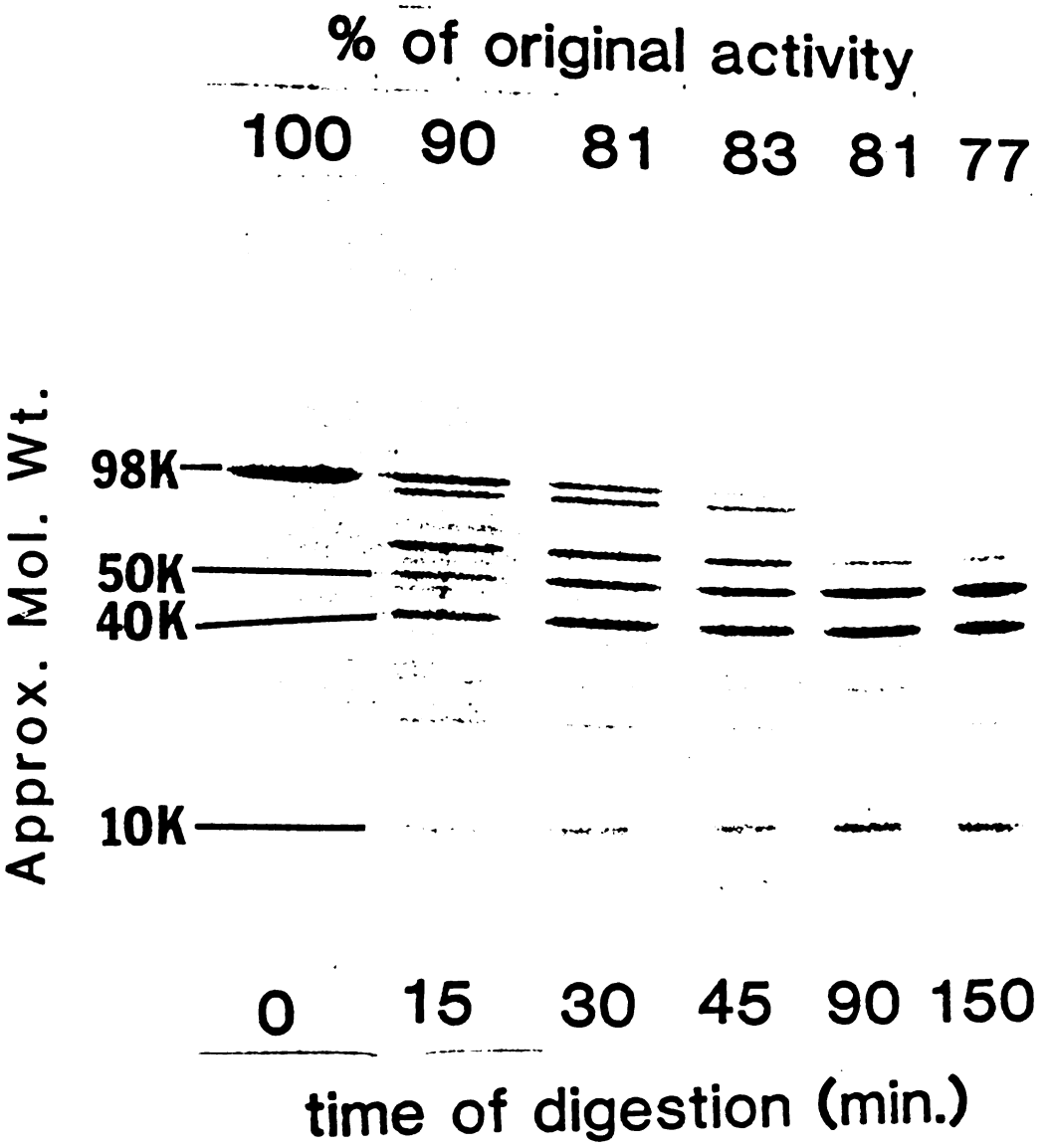
% of original activity
100 90 81 83 81 77
Approx. Mol. Wt.
98K
50K
40K
10K
0 15 30 45 90 150
time of digestion (min.)

Figure 24

Figure 25. Kinetic analysis of tryptic digestion of brain hexokinase. Tryptic digestions and SDS-gel electrophoresis were carried out as described in the legend of figure 23. After staining the SDS-gels for protein (142) individual lanes were excised and scanned at 580 nm. Bars represent peak heights (relative absorbance) obtained for each molecular weight polypeptide at the indicated time of digestion (minutes).

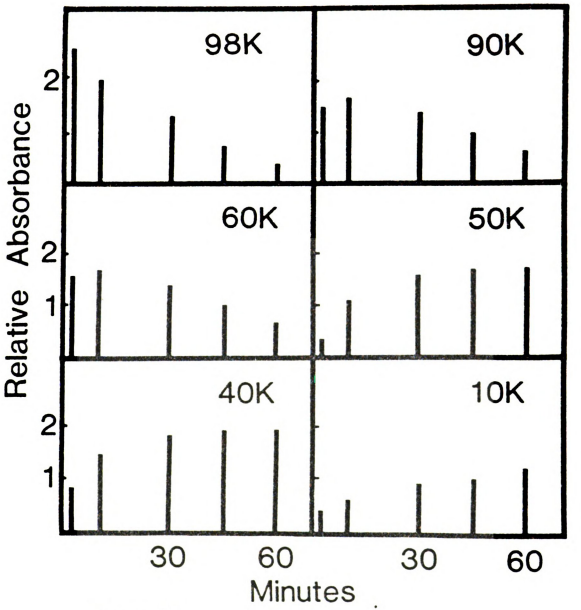
98K
90K
60K
50K
40K
10K
Relative Absorbance
2
2
1
2
1
30
60
30
60
Minutes

Figure 25

demonstrates the progressive and simultaneous accumulation of the 10K, 40K and 50K dal fragments at the expense of the higher molecular weight polypeptides. The 40K and 50K hexokinase fragments exhibit a relative resistance to further degradation, and are considered to be limit digestion products.

Apparent molecular weight of tryptically digested hexokinase under nondenaturing conditions

Several reports have appeared (36,76,125) showing that proteolyzed proteins may retain their native molecular sizes when analyzed under nondenaturing conditions. Although it is clear from the SDS polyacrylamide gel patterns that peptide bond cleavage has occurred, the molecular weight of the proteolyzed hexokinase, as determined by sucrose density gradient sedimentation, appears identical to that of the nondigested molecule (figure 26A). The failure of the hexokinase proteolytic fragments to dissociate spontaneously under native conditions suggests a strong, noncovalent association of the digestion products with each other. In an attempt to dissociate the hexokinase tryptic fragments, without destroying catalytic activity, I carried out sucrose density gradient sedimentation under mildly denaturing conditions. Even when the proteolyzed hexokinase is sedimented in sucrose gradients made 2M in urea, the native molecular weight of 98K is still obtained (figure 26B).

Urea denaturation of tryptically treated hexokinse

As tryptically digested hexokinase remains enzymatically active, it is possible to assess the effects of urea denaturation, relative to the nondigested enzyme, by measuring the decay in catalytic activity as a function of time. When either native or tryptically digested

Figure 26. Sucrose density gradient centrifugation of native and tryptically digested hexokinase. A) Native or tryptically digested hexokinase (4 units) along with 4 units of alcohol dehydrogenase in a total volume of 100 µl, was layered onto a sucrose density gradient and centrifuged as described in Methods. Alcohol dehydrogenase activity (O), hexokinase activity (●). B) The procedure described in the legend for Fig. 26A was followed except that sucrose gradients were made 2 M in urea and 50 µg of lipoyl dehydrogenase [M_r approx. 108,000 (85a)] was included in each sample. Hexokinase activity (●), Lipoyl dehydrogenase (O) was determined fluorometrically using an Aminco Fluorocolormeter (excitation 370 nm, fluorescence > 415 nm), alcohol dehydrogenase activity could not be detected.

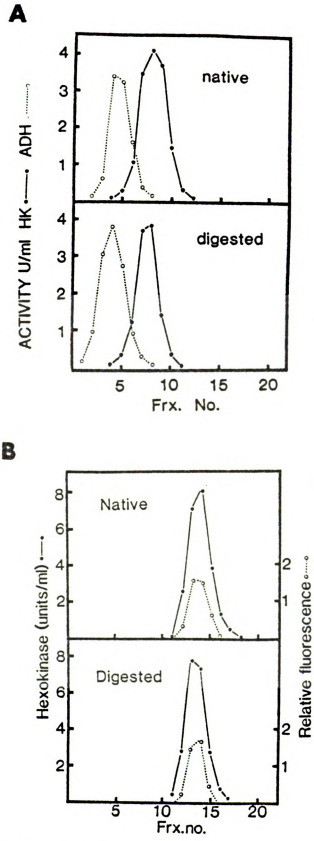
A
native
digested
ACTIVITY U/ml HK •—• ADH ◦···◦
Frx. No.
B
Native
Digested
Hexokinase (units/ml) •—•
Relative fluorescence ◦···◦
Frx.no.

Figure 26

hexokinase solutions are made 5M in urea, a progressive diminution of enzymatic activity is observed (figure 27). As determined by this method, the digested enzyme appears to be more sensitive to denaturation than the intact hexokinase molecule. These results indicate that tryptic cleavage affects the primary structure of hexokinase in a fashion that renders the enzyme more susceptible to inactivation by urea denaturation.

Native gel electrophoresis of digested hexokinase.

When tryptically cleaved hexokinase is subjected to electrophoresis in 2-20% native acrylamide gels, the mobility of the enzyme appears slightly greater than that of the nondigested hexokinase (Figure 28A). Hexokinase activity can be detected in the native system, and the major tryptic fragments can be observed following SDS acrylamide gel electrophoresis in the second dimension (Figure 28B). The reason for the relative increase in electrophoretic mobility of the digested enzyme remains unclear. Although the native gel system is designed to separate molecules on the basis of molecular weight (96), the fragments observed in the SDS gel dimension can account for the complete hexokinase molecule. As the native gel electrophoresis is performed at pH 8.8, hydrolyzed peptide bonds in the cleaved enzyme may add net negative charge to the molecule, and thus contribute to the increased mobility in the gel system. Conformational alterations of the proteolyzed molecule may also account for the increased electrophoretic mobility.

Kinetic evaluation of the proteolyzed hexokinase.

Rat brain hexokinase, in the presence of ATP, will catalyze the phosphorylation of glucose to glucose-6-phosphate, and micromolar

Figure 27. Urea inactivation of native and tryptically digested hexokinase. Purified hexokinase (0.5 mg/ml), either native or tryptically digested, was diluted 20 fold into 5.25 M urea solution pre-equilibrated to 23°C in a circulating water bath. Aliquots were removed at the specified times, diluted 100 fold into hexokinase assay mixture (11) and enzyme activity determined.

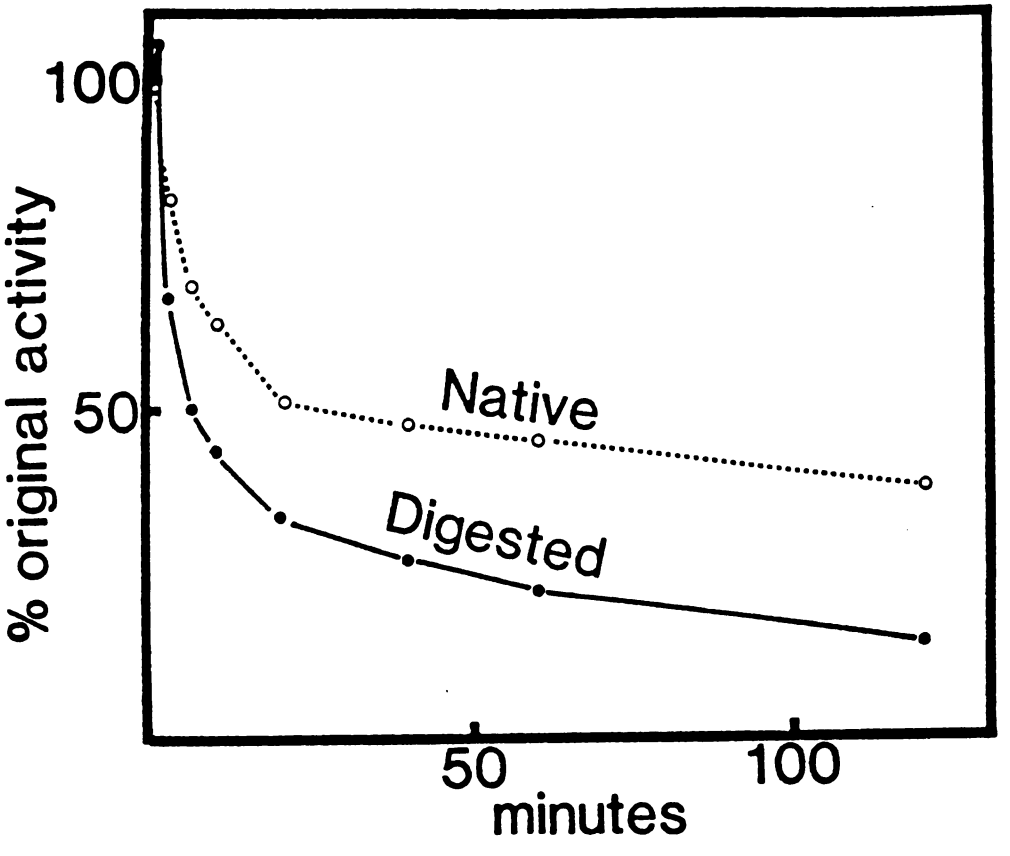

Figure 27

Figure 28. Electrophoresis of native and digested hexokinase under nondenaturing and denaturing conditions. Following electrophoresis of native and tryptically digested hexokinase in a nondenaturing acrylamide gel (a duplicate gel stained for protein is shown in A) the lane containing the digested sample was excised, transferred to an SDS-gel system and electrophoresed under denaturing conditions (B). The major tryptic digestion products are identified by the values in the right margin of B. Both gels shown were stained for protein with Coomassie Blue (142). A nondenaturing gel was also stained for hexokinase activity and produced a pattern identical to that seen in A.

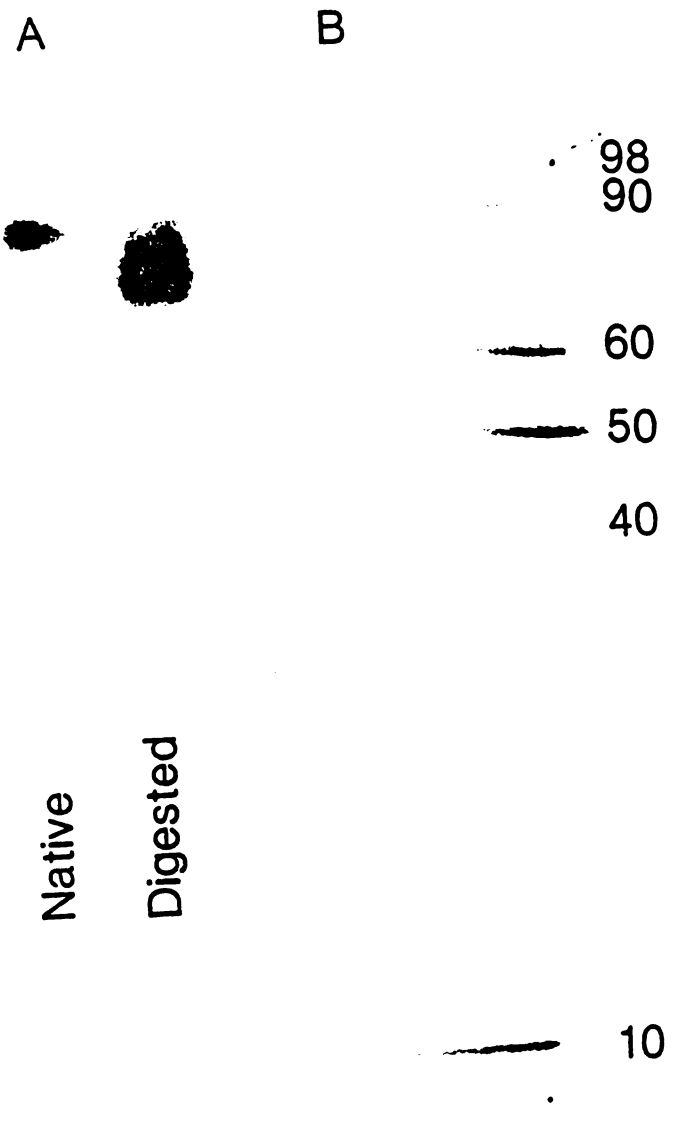

Figure 28

concentrations of the hexose-6-phosphate will inhibit enzyme activity (135). To further examine the functional integrity of the proteolyzed hexokinase molecule, I investigated the effects of limited tryptic digestion of the enzyme on three kinetic parameters. The results of these experiments show that the digested hexokinase exhibits a slight, although consistent, reduction in K_m for ATP, while the K_i for glucose-6-phosphate and the K_m for glucose remained nearly identical to the values determined for the nondigested enzyme (Table 4). There is evidence to support the view that the inhibitory effect of glucose-6-phosphate on hexokinase is evoked allosterically (16) and mediated by dramatic changes in enzyme conformation (138). Assuming this is the case, it is apparent that the catalytic site of tryptically digested hexokinase is still capable of being modulated by actions occurring at a distinct site and requiring conformational changes to produce an effect. In general, the lack of any marked alterations in kinetic parameters of the tryptically cleaved hexokinase, indicates that the proteolyzed enzyme remains highly functional and retains much of its native character.

Membrane binding and glucose-6-phosphate solubilization of tryptically digested hexokinase.

Brain hexokinase has the capacity to reversibly bind to the mitochondria and can be solubilized from the membrane with low levels of glucose-6-phosphate (133). Results presented in Chapter One of this thesis describe the structural features, located at the NH_2-terminus of the hexokinase molecule, that are required for membrane binding. If the tryptically digested hexokinase was still capable of associating with the mitochondrial membrane, it could be concluded that the

Table 4

Effect of Tryptic Digestion on Kinetic Properties

	K_m		K_i
	ATP	Glucose	G6P
Native	0.36 ± 0.10	0.039 ± 0.013	0.014
Digested	0.24 ± 0.08	0.042 ± 0.006	0.019

NH_2-terminal region of the enzyme, as well as other regions which may participate in the membrane binding interaction, were unaffected by the proteolysis. Moreover, glucose-6-phosphate membrane solubilization of hexokinase, as with the already mentioned inhibition of enzymatic activity with this hexose phosphate, is thought to involve substantial conformational changes in the enzyme (141). Membrane bindable hexokinase was subjected to limited tryptic treatment and binding and solubilization studies were performed with the digested and nondigested enzyme. From these experiments it is evident that the proteolyzed hexokinase retains its ability for interacting with the outer mitochondrial membrane in a glucose-6-phosphate sensitive fashion (Table 5). These results substantiate the view that hexokinase remains structurally and functionally intact even though proteolysis has occurred at two major points in the peptide backbone.

Interpretation of the tryptic digestion pattern of hexokinase observed with SDS gel electrophoresis.

Comparative peptide mapping can be used to determine the degree of structural homology between isolated polypeptides (14). In order to assess the relationship of the major tryptic products of hexokinase to each other, I carried out comparative peptide mapping, using the proteolysis-SDS gel electrophoretic technique described by Cleveland (14), on the isolated proteolytic fragments. After tryptically treated hexokinase was subjected to SDS-gel electrophoresis in a polyacrylamide gradient tube gel system, the gel was transferred to a slab gel system, overlayed with S. aureus V8 protease solution, and digestion was allowed to proceed before electrophoresis in the second dimension. The S. aureus V8 protease digestion products of the major tryptic

Table 5

Glucose-6-Phosphate-Sensitive Binding of Native and Tryptically-Cleaved Hexokinase to Outer Mitochondrial Membranes.

Enzyme	First Incubation[a] % Bound	Second Incubation[b] % Solubilized
Native	90	71
Tryptically-Cleaved	78	83

[a]Binding of hexokinase, either before or after digestion with trypsin, to outer mitochondrial membrane was determined as described in Methods.

[b]The pellet containing bound hexokinase (from the first incubation) was further incubated with Glc-6-P and, after centrifugation, the percent of the activity solubilized determined (see Methods).

hexokinase fragments (Figure 29), provide a basis for interpreting the tryptic cleavage pattern. It is clear from the two dimensional gel pattern that the 50K and 60K tryptic fragments are highly related to each other, while the 40K fragment bears no resemblance to these two polypeptides. It is also apparent that the 60K dal fragment is contained completely within the 98K peptide, but not within the 90K piece. The appearance of a 10K dal molecular weight S. aureus V8 protease product in the 98K and 60K lanes, but not in those of the 90K or 50K, indicates that the 98K gives rise to the 90K, and the 60K to the 50K, by loss of this same 10K dal molecular weight peptide. This 10K dal fragment is probably nearly identical to the 10K, tryptically generated fragment which can now be assigned a location at one end of the intact, 98K dal molecular weight, hexokinase monomer. It follows that the 40K tryptic fragment contains the opposite terminus. Thus, the major polypeptide fragments, generated by limited tryptic treatment of hexokinase, can be accounted for if proteolytic cleavage occurs at the two sites, T_1, and T_2, depicted in Figure 30.

Reactivity of a monoclonal antibody raised against hexokinase with the electrophoretically separated tryptic digestion products.

Monoclonal antibodies are normally capable of binding to only a single, specific site on the antigenic molecule. A series of monoclonal antibodies raised against rat brain hexokinase have been characterized, and one of these, designated 5A, reacts avidly with the denatured enzyme (30). Monoclonal antibody 5A was used here to determine which of the tryptically generated hexokinase fragments share a single, common antigenic determinant. An electroblot of the separated tryptic digestion products of hexokinase, was incubated with

Figure 29. Two dimensional peptide mapping of hexokinase. Two dimensional peptide mapping employing trypsin and S. aureus V8 protease was performed as described in Methods. A duplicate SDS-gel (1st dimension) containing the tryptic digestion products was stained for protein (142) and appears at the top of the figure. S. aureus protease digestion products of the major tryptic fragments are indicated by the dark lines. Molecular weight standards appear at the extreme right.

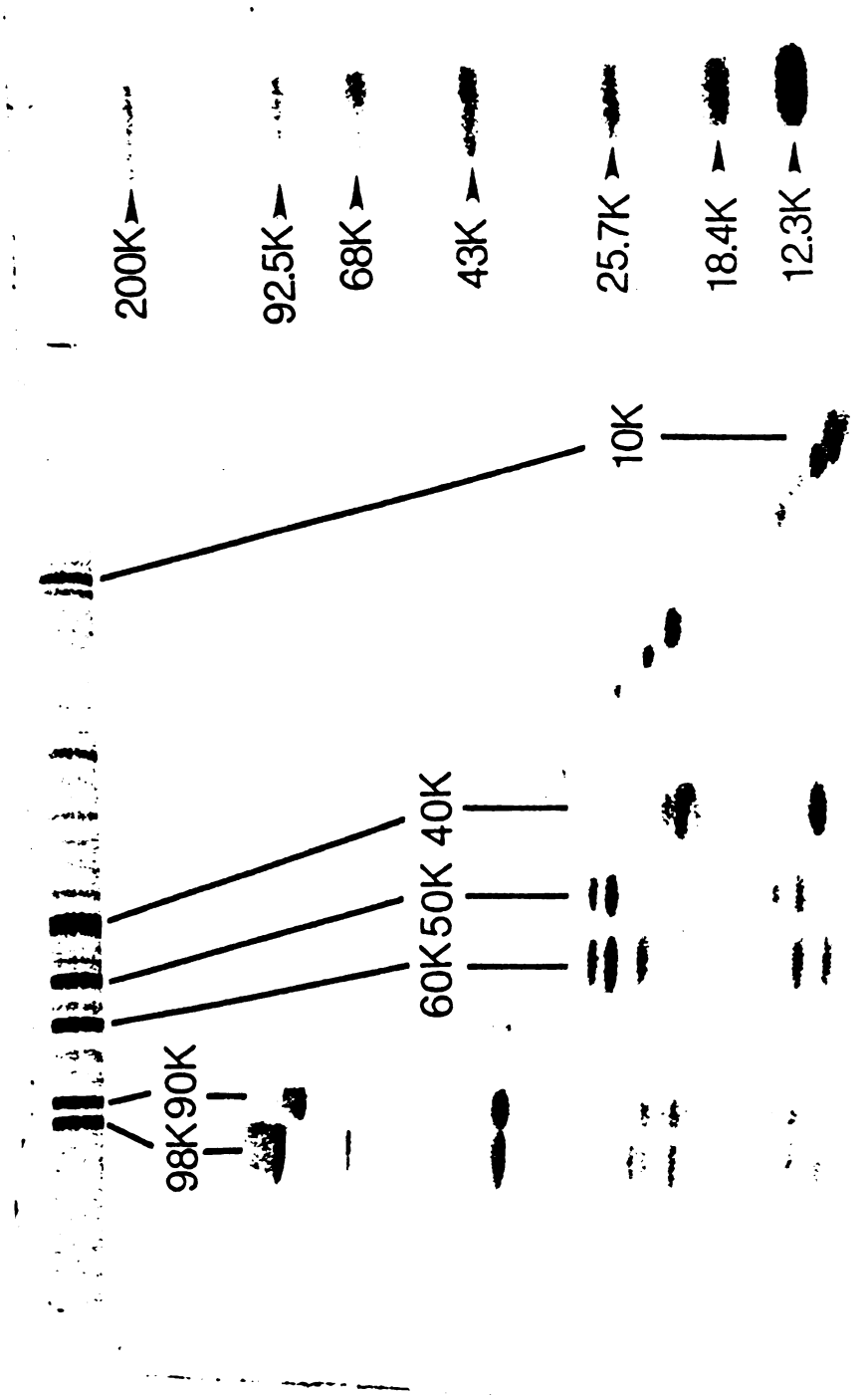

Figure 29

Figure 30. Schematic representation of hexokinase tryptic cleavage pattern. The values shown represent the molecular weights ($X10^{-3}$) of the proteolytic fragments and T_1 and T_2 designate the two major sites of tryptic cleavage. Orientation of the fragments within the 98K hexokinase polypeptide was based on terminal residue identification presented in Table 6.

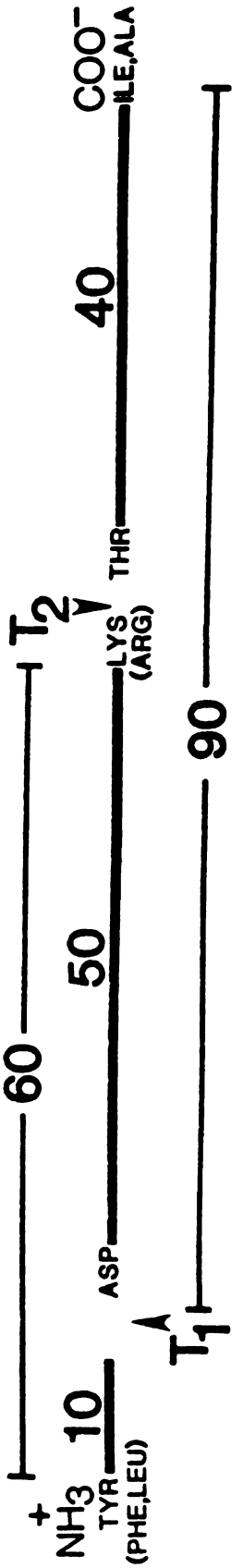

Figure 30

the monoclonal antibody 5A (Figure 31). A second SDS, gel, containing the same samples, was stained for protein (Figure 31). It is evident, from the positive reactions obtained with the immunoblot, that the binding site for the monoclonal antibody 5A resides exclusively in the 98K, 90K and 40K tryptic fragments, while the 50K and 60K digestion products do not exhibit any reactivity. These data provide further evidence that the 40K and 50K tryptic products are structurally distinct, supporting the model depicted in Figure 30.

NH_2 and COOH terminal analysis of the isolated hexokinase tryptic digestion products.

It is clear from the data presented above that tryptic cleavage of the intact 98K dal hexokinase monomer at two sites is responsible for the production of the 10K, 40K and 50K fragments with the intermediate 90K and 60K products, resulting from cleavage at only one of these sites. However, these data do not provide a basis for determining the orientation of the proteolytic products with respect to NH_2 and COOH-terminal positioning. The identification of proteolytic fragments sharing common terminal amino acids would be helpful in establishing such an alignment. Semi-preparative isolation of the major proteolytic products was carried out by SDS-slab gel electrophoresis followed by excision and elution of the polypeptides from the gel slices. Virtually homogeneous samples of the isolated hexokinase digestion products can be obtained in this manner (Figure 32).

The NH_2-terminal amino acids, of the isolated proteolytic fragments, were determined by the danslyation technique as described in Methods (43). Carboxyterminals were degraded enzymatically with either carboxypeptidase A or B, followed by derivatization of the released

Figure 31. Immunoreactivity of hexokinase tryptic fragments with monoclonal antibody 5A. Immunoblotting was carried out as described in Methods. SDS-gel electrophoresis was performed on hexokinase digested with trypsin (0.3 mg/ml) for the indicated times (top). One gel (left) was stained with Coomassie Blue (142) and a duplicate gel was subjected to electroblotting and the nitrocellulose transfer was stained using monoclonal antibody 5A (right). Molecular weight standards (M) appear in the left lane of each gel.

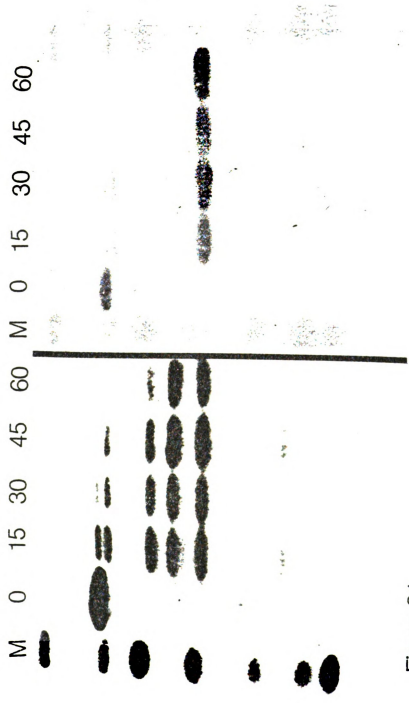

Figure 31

Figure 32. Isolation of brain hexokinase tryptic cleavage fragments. The isolated hexokinase tryptic digestion products were obtained by preparative SDS-gel electrophoresis as described in Methods. The original tryptic digest (lane A) and approximately 5-10 μg each of the isolated polypeptides (lanes B-F, 98K, 90K, 60K, 50K, and 40K, respectively) were subjected to SDS-gel electrophoresis.

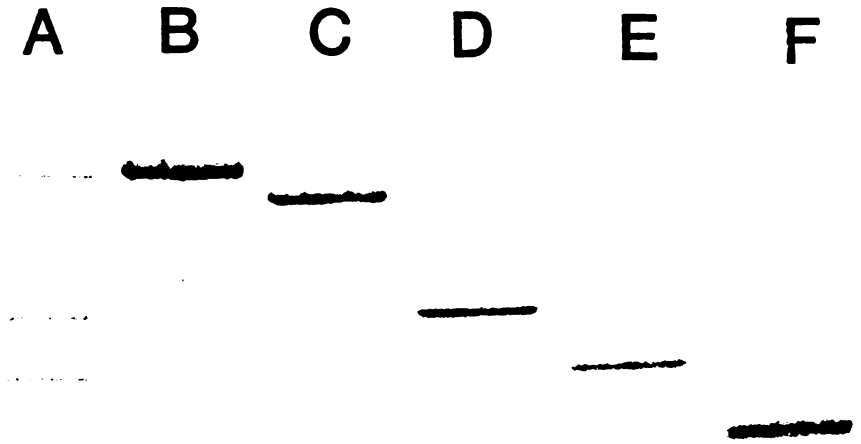

Figure 32

amino acids with orthophthaldialdehyde and analysis by HPLC (50). The results obtained from the NH_2 and COOH terminal identification studies are shown in Table 6. Although the native 98K hexokinase monomer exhibits some heterogeneity at the NH_2-terminus, these same three amino acids are also identified at the NH_2-termini of the 98K and 60K polypeptides eluted from the SDS gel. The appearance of NH_2-terminal aspartate on the 90K and 50K polypeptides, suggests a common NH_2-terminus for these two fragments, resulting from internal cleavage of the 98K hexokinase monomer. The differences observed at the COOH-terminal ends of the 90K and 50K fragments would be expected if their NH_2-termini were identical. The 40K digestion product possesses the original COOH-terminus found on the intact hexokinase molecule. It would follow that the NH_2-terminal residue of the 40K fragment should be distinct from that of any other fragment, and the identification of threonine at this terminus shows this to be the case. Consistent with the internal positioning of the 50K tryptic fragment both the NH_2- and COOH-terminal residues are different from those found on the nondigested hexokinase molecule. Although technical difficulties prevented the isolation of the 10K hexokinase tryptic fragment, by deduction it can be assigned the position at the NH_2-terminal end of the hexokinase monomer. These data are consistent with the view that trypsin cleaves hexokinase primarily at peptide bonds, T_1 and T_2, as depicted in Figure 30.

Localization of S. aureus V8 proteolytic products within the hexokinase molecule.

With the correct interpretation of the tryptic cleavage pattern it becomes possible to assign locations to the S. aureus V8 proteolytic

Table 6

N- and C-Terminal Analysis of Native Hexokinase and Fragments Obtained by Tryptic Cleavage

Polypeptide	C-Terminal Analysis[a]		N-Terminal Analysis[b]
	Carboxypeptidase A	Carboxypeptidase B	
Native (98K)	ala (0.81), ile (0.67)	nd[c]	tyr >> phe, leu[d]
90K	ala (0.83), ile (0.73)	nd	asp
60K	none	lys (0.54), arg (0.25)	tyr >> phe, leu
50K	none	lys (0.48), arg (0.20)	asp
40K	ala (0.80), ile (0.55)	nd	thr

[a]Amino acids released (moles/mole polypeptide) after 1.5 hr digestion with carboxypeptidase A (identical values were found after 3 hr) or after 3 hr digestion with carboxypeptidase B. The assay of Hess et al. (32), with bovine serum albumin as standard, was used to quantitate the amount of polypeptide present.

[b]Dansyl amino acids detected

[c]nd = not determined

[d]In agreement with previous results (22), tyrosine is found as the predominant detectable N-terminal amino acid. Microheterogeneity at the N-terminus is due to limited exoproteolysis (11, and Polakis and Wilson, unpublished observations) which occurs during isolation of the enzyme.

products generated by two dimensional peptide mapping, (Figure 29), within the 98K hexokinase monomer. Presented below is a set of rules which can be applied to at the pattern obtained with the two dimensional peptide mapping technique in order to assign these locations.

S. aureus V8 protease products appearing in the:

- 90K and 98K lanes, but not in the 50K or 40K lanes must overlap the T_2 tryptic site
- 50K and 90K lanes, but not the 98K or 60K lanes, must have tryptic site T_1 as an NH_2-terminus and extend in the COOH terminal direction a distance corresponding to their molecular weights.
- 50K, but not the 90K lane, must have T_2 as a COOH-terminus and extend in the NH_2-terminal direction a distance corresponding to their molecular weights.
- 40K, but not the 90K lane, must have T_2 as an NH_2-terminus and extend in the COOH-terminal direction a distance corresponding to their molecular weights.
- 50K, 90K and 98K lanes must be contained within the body of the 50K fragment.
- 40K, 90K and 98K must be contained within the body of the 40K fragment.

The S. aureus protease generated peptides, labelled in Figure 33, are shown superimposed on a model depicting the points of tryptic cleavage in the hexokinase molecule.

Immunoblotting of the two dimensional peptide map.

Figure 33. Location of peptides obtained by S. aureus V8 protease digestion of the major hexokinase tryptic fragments. The diagram illustrates the hexokinase polypeptide chain (thick solid line) with the principal sites of tryptic cleavage (T_1 and T_2) and the molecular weights ($X10^{-3}$) of the resulting digestion products (10,40,50). The S. aureus protease digestion products (thin solid lines) are indicated by the letter "S" followed by a value corresponding to their molecular weights ($X10^{-3}$). The 'S' peptides are positioned with respect to their locations within the hexokinase polypeptide chain. The locations were determined as described in text.

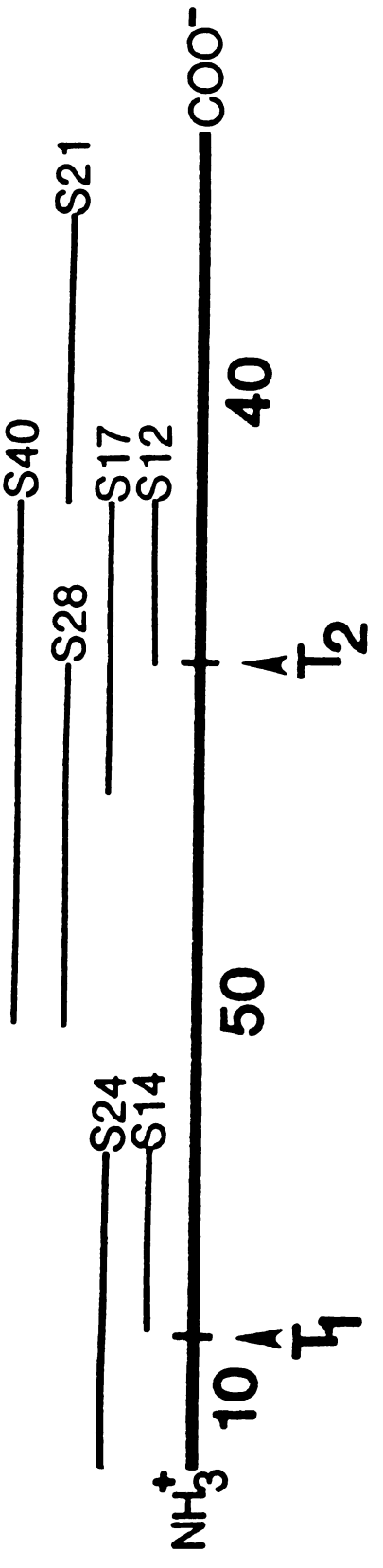

Figure 33

In an attempt to locate the antigenic site for a monoclonal antibody to hexokinase, I carried out immunoblotting experiments with the trypsin - S. aureus V8 protease generated peptide map.

A two dimensional peptide map, as shown in Figure 34A was transferred to nitrocellulose filter paper and incubated with the monoclonal antibody 21. Peptides exhibiting a positive reaction to the antibody can be seen in Figure 34B and their positions in the hexokinase protein chain are shown diagramatically in Figure 33. The S24 peptide of the 98K and 60K lanes, as well as the S14 peptide in the 90K and 50K lanes, show positive reactions to the monoclonal antibody. The site of reactivity for this antibody is within the 50K tryptic fragment and contained within a sequence extending from the tryptic site T_1, 14,000 dal in the COOH-terminal direction.

S. aureus fragments considered distinct from, and nonoverlapping with S14, such as S28 do not show a reaction with the antibody, supporting the assignment of the location for the "S" peptides illustrated in Figure 33.

Discussion

In some cases, the resulting digestion products of proteolyzed enzymes cannot be separated in native systems (36,76), while others have reported spontaneous dissociation of the fragments following proteolysis (37). Tryptically digested brain hexokinase can be considered as an entry in the former category. Electrophoresis of tryptically treated hexokinase in SDS gels, demonstrates that the

Figure 34. Immunoblotting of a two dimensional peptide map using monoclonal antibody 21. Two dimensional peptide mapping with trypsin and S. aureus V8 protease, and electroblotting in the absence of SDS, was carried out as described in methods. A) Residual proteins remaining in the gel after electroblotting were visualized by Coomassie Blue staining (142). A duplicate first dimension gel displaying the hexokinase tryptic fragments appears at the top of the figure. B) Nitrocellulose filter electroblot stained using monoclonal antibody 21. Immunoreactivity is seen in the S24 polypeptide of the 98K and 60K lanes as well as in the 98K, 90K, 60K and 50K tryptic fragments not digested by S. aureus protease, lying along the diagonal. S14 of the 90K and 50K lanes also displays immunoreactivity, but, due to quantitative transfer of this peptide, it cannot be visualized in the protein stained gel. (Refer to figure 29 for clear identification of S14).

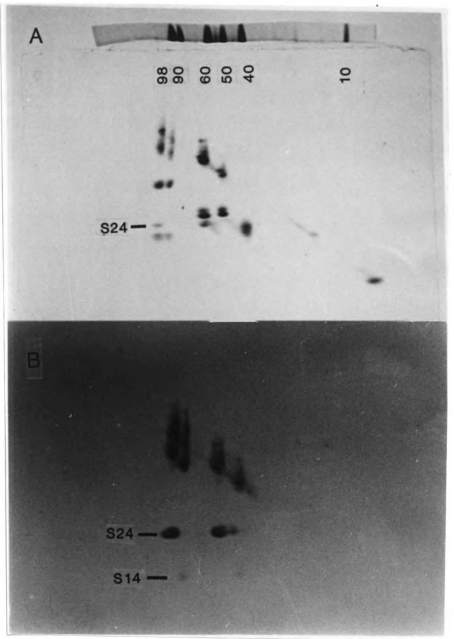

Figure 34

protein chain has been cleaved at two sites to yield three principal polypeptides of molecular weights 10K, 40K and 50K daltons. The noncovalent association of these limit digestion products must be relatively strong as sedimentation in sucrose density gradients, made 2M in urea, failed to alter the molecular size or destroy the catalytic activity of the proteolyzed enzyme. This system exhibits denaturing capacity, as the enzymatic activity of alcohol dehydrogenase, a tetrameric enzyme (58a) also applied to these gradients, could not be recovered after centrifugation. Tryptically digested hexokinase could also be subjected to native gel electrophoresis (Figure 28) or ion exchange chromatography (not shown) without dissociation of the proteolytic fragments or loss of catalytic activity.

The binding of brain hexokinase to the outer mitochondrial membrane is thought to involve electrostatic interactions at the surface of the membrane (27) and an interaction, presumably involving the NH_2-terminus of the enzyme (141), with the binding protein buried in the hydrophobic region of the lipid bilayer (30). The ability of the tryptically digested hexokinase to associate with the mitochondria, to a degree comparable to that of the native enzyme, indicates that the structural features required for these binding interactions remain intact and operational.

Investigations of other functional properties of the proteolyzed hexokinase did not reveal any dramatic alterations, in spite of the cleavage at two sites in the peptide backbone. Surprisingly, even the allosterism and conformational alterations associated with glucose-6-phosphate binding to hexokinase were unaffected by the proteolysis.

The inability to achieve isolation of the 40K and 50K tryptic digestion products of hexokinase, under nondenaturing conditions, hindered the attempts to assign catalytic properties to either of these fragments. Experiments involving urea denaturation show that the inactivation of the proteolyzed hexokinase occurs at a more rapid rate than that observed for the native enzyme. These results indicate that the interaction of the fragments may be required in stabilizing the domain carrying out catalytic functions or that more than one of the digestion products participates in forming the active site of the enzyme. However, similar experiments with the digested enzyme, using lower concentrations of urea, resulted in a rapid, early decay of catalytic activity followed by a stabilization at a reduced level for later times (not shown). One interpretation of these results includes a possible early dissociation of the tryptic digestion products with a subsequent retention of submaximal catalytic activity in one of the separated fragments.

Comparative peptide mapping and immunoblotting experiments with the electrophoretically separated hexokinase fragments clearly demonstrates the lack of homology between the major limit digestion products, i.e. the 40K and 50K polypeptides. The 40K and 50K dal hexokinase digestion products, taken together with the 10K dal fragment, can account for the complete primary structure of the 98K hexokinase monomer. Evidence that the NH_2- and COOH-terminal residues of the 60K dal and 40K dal fragments, respectively, are identical to the NH_2- and COOH-terminal amino acids of the intact hexokinase molecule, argues against the possible release of small peptides from the termini of the enzyme during tryptic digestion.

However, the appearance of both lysine and arginine at the COOH-terminal of either the 50K or 60K dal fragments indicates that proteolysis occurs at more than one site in this region and that peptides may be lost from the newly generated termini. The contribution of these peptides to total primary structure of the enzyme must be small, though, as the limit digestion products can be arithmetically summed to yield the approximate molecular weight of native hexokinase molecule. Although the NH_2-terminus of the native, membrane bindable hexokinase appears to be blocked (Chap. 1, pg. 76) the consistent identification of the NH_2-terminal tyrosine, possibly resulting from a minor population of nonbindable molecules, serves as a reliable marker for this end of the protein chain. The identification of tyrosine residues at the NH_2-termini of either the 98K or 60K polypeptides, and its virtual absence at this end of the 90K and 50K fragments (the 90K and 50K dal fragments exhibit NH_2-terminal aspartate only), establishes the 10K dal tryptic fragment as the NH_2-terminal polypeptide of the hexokinase molecule.

Carboxypeptidase A is known to be very ineffective at hydrolyzing basic amino acids from the COOH-termini of proteins (96), so it is not surprising that the liberation of free amino acids from the 50K and 60K fragments could not be achieved with this enzyme. Carboxypeptidase B, however, is specific for basic residues (31) and was shown to be very effective at hydrolyzing lysine, and to a lesser extent, arginine, from the 50K or 60K polypeptides. The presence of these amino acids is taken as evidence for tryptic cleavage of hexokinase at this site.

As mentioned in Chapter I of this thesis, attempts to carry out COOH-terminal sequencing using carboxypeptidase Y resulted in

endogenous proteolysis at the NH_2-terminal region of hexokinase with subsequent COOH-terminal degradation of the released peptide. However, the alanine and isoleucine liberated during carboxypeptidase A digestion of native hexokinase must originate from the COOH-terminus of the enzyme, as the 40K and 90K tryptic products, which lack the NH_2-terminus of the native hexokinase, also undergo release of these same two residues upon carboxypeptidase A treatment.

Amino terminal domains of glutamyl transpeptidase (33) and NADH cytochrome b_5 reductase (64) are apparently responsible for the membrane binding properties of these enzymes. Rat brain hexokinase can now be added to list of enzymes possessing membrane binding structures at the NH_2-terminal region of the protein chain. Further evidence, consistent with this view, has been obtained with immunoblotting experiments involving the tryptic digestion products and a monoclonal antibody to hexokinase. The monoclonal antibody, 3C, has been shown to interefere with the enzyme's ability to associate with the mitochondrial membrane (30). This same monoclonal antibody also exhibits a positive reaction with the nitrocellulose immobilized, 10K dal, hexokinase tryptic fragment (personal communication, John Wilson and Alan Smith).

The identification of an antibody binding site, to within a 14,000 molecular weight region on the 100K dal polypeptide map, demonstrates the potential usefulness of the two dimensional peptide mapping technique. Recently, an epitope mapping technique employing size exclusion high performance liquid chromatography (20), has been used to determine the relative proximity of different antibody binding sites on nondenatured proteins. Combined with the two dimensional peptide

mapping-immunoblotting technique, epitope mapping may be useful in ascertaining whether or not remote regions of denatured enzyme lie in close proximity to each other in the nondenatured, folded protein chain. Other methods, such as the radiolabelling of functional groups or covalent binding of active site ligands to enzymes, could be combined with the two dimensional peptide mapping technique, to reveal with good accuracy, the positions of functional and structural components in the molecule.

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