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FUNCTIONAL ORGANIZATION AND EVOLUTION OF MAMMALIAN HEXOKINASES

By

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ABSTRACT

FUNCTIONAL ORGANIZATION AND EVOLUTION OF MAMMALIAN HEXOKINASES

By

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It has been previously demonstrated that the catalytic function of mammalian hexokinase I is localized in the C-terminal half, while the N-terminal half is devoid of any catalytic activity. It was speculated that the active site in the N-terminal half of hexokinase I has evolved into a regulatory site. Discovery of a second Glc-6-P binding site in the C-terminal half of hexokinase I raised the debate whether the functional regulatory Glc-6-P site is located in the N- or C- terminal half. By using site-directed mutagenesis and constructing chimeric hexokinases, the function of each half of mammalian hexokinases can be examined and location of the functional Glc-6-P site may be elucidated.

In certain cases, several regulatory functions of mammalian hexokinases are associated with the N-terminal halves, e.g. phosphate antagonism of inhibition by AnG-6-P (1,5-anhydroglucitol 6-phosphate), a glucose 6-phosphate analogue, correlates with the N-terminal half of hexokinase I when the C-terminal half is substituted with that of hexokinase II, and higher sensitivity to phosphate inhibition correlates with the N-terminal half of hexokinase II when the C-terminal half is substituted with that of hexokinase I.

Sensitivity to AnG-6-P, however, does not correlate with either half. In fact, diverse sensitivities are observed in chimeric hexokinases NICII, NIIICII, NIICI, and NIIICI, which is perhaps due to the interaction between the two halves.

The unique glucose inhibition associated with hexokinase III correlates with the C-terminal half. Glucose inhibition also leads to a lag before attainment of the steady state.

Organization of catalytic function in hexokinase I and III are similar; the functional active sites are located in their C-terminal halves. However, hexokinase II has two functional active sites, one site in each half with similar specific activities. Because of the dual active sites, hexokinase II functionally most resembles and is probably the closest descendant of the ancient product which has arisen from gene duplication and fusion of a 50 kDa glucose 6-phosphate sensitive hexokinase.

A new model is proposed for catalysis by the mammalian type II hexokinase.

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LITERATURE REVIEW

The reaction catalyzed by hexokinases

Hexokinase (ATP: D-hexose 6-phosphotransferase (EC 2.7.1.1) is the first enzyme in glycolysis, which catalyzes the following reaction:

$$Glc + ATP.Mg^{2+} \text{ -------> } Glc\text{-}6\text{-}P + ADP.Mg^{2+}$$

This reaction is the first reaction after glucose being transported into cytoplasmic space, and is, in essence, phosphorylation of glucose at the expense of high energy ATP (ΔG = -7.3 kcal/mole hydrolyzed at pH 7). The reaction forms a lower energy phosphoglucose, Glc-6-P (ΔG = -3.3 kcal/mole hydrolyzed at pH 7); therefore, the ΔG for this reaction is -4.0 kcal/mole at pH 7 (1). This reaction not only activates glucose for further metabolism but because of the large negative free energy change, the reaction is thermodynamically favored to the right and essentially irreversible (2).

The product of this reaction, Glc-6-P, is a common substrate for phosphohexoisomerase, phosphoglucomutase, and Glc-6-P dehydrogenase, which introduce Glc-6-P into glycolysis, glycogen synthesis, and the pentose phosphate pathway, respectively. Thus, hexokinase plays an important role in admitting glucose into metabolism.

As the name implies, hexokinases are able to phosphorylate a variety of hexoses (3), e.g. mannose, 2-deoxyglucose, glucosamine and, to a lesser extent, fructose and galactose. All of these hexoses are phosphorylated at the 6-hydroxyl position, forming hexose 6-phosphate.

Evolution of mammalian hexokinases

There are four distinct hexokinase isozymes found in mammalian tissues (3-5), designated as type I, II, III, and IV hexokinase. Type I, II, and III isozymes are monomeric with molecular weight of approximately 100 kDa, whereas type IV isozyme, better known as glucokinase, is only 50 kDa.

Because the molecular weight of mammalian hexokinase I, II, and III is twice that of yeast hexokinase (50 kDa), it has been suggested first by Easterby (6) and Colowick (7) that mammalian 100 kDa hexokinases arose by duplication and fusion of a gene encoding an ancestral 50 kDa hexokinase similar to present-day yeast hexokinase. If this hypothesis is true, the original ancestral 100 kDa hexokinase should have two identical halves with one active site in each half (6,7). Easterby and Colowick further postulated that this original 100 kDa hexokinase in turn gave rise to modern hexokinase I, II, and III.

By comparing amino acid sequences deduced from cDNAs of various sources, hexokinase I, II, and III are indeed very similar (5,8-10). In addition, they exhibit internal 50 kDa repetitions which are similar to the sequence encoding 50 kDa yeast hexokinase (5,8-10). Furthermore, several residues involved in glucose binding or catalysis are well conserved in each repetition as well as in yeast hexokinase (5,8-10), supporting the theory that modern mammalian 100 kDa hexokinases are derived from a common ancestral hexokinase

which itself was a product of gene duplication and fusion (5,11,12).

One important piece of evidence supporting this gene duplication and fusion theory is by Printz et al. (13) and Kogure et al. (14) in which they found that the splicing sites and the exon sizes of hexokinase II gene showed a direct repetition between the N- and C- terminal halves. Interestingly, this intron/exon pattern is also observed in glucokinase gene (13,14), and therefore they suggested that an ancestral gene encoding a 50 kDa hexokinase similar to glucokinase underwent gene duplication and fusion to form the hexokinase II gene.

However, instead of glucokinase being the precursor of hexokinase II, Griffin et al. (12) suggested an alternative view in which the glucokinase gene arose by resplitting of the hexokinase II gene and thus it retained the intron/exon pattern of the hexokinase II gene. In fact, the latter view is perhaps a better model, because it is better supported by the evolution scheme. In lampreys (*Agnatha*; jawless fish), only one hexokinase isozyme is present, which is about 90 kDa in size (15), while type I, II, III isozymes but not glucokinase are present in further evolved *Osteichthys* (bony fish). Glucokinase is not present until the emergence of *Amphibia* and higher animals (except for *Aves*; no glucokinase is found in *Aves*) (15,16). These findings would suggest that the gene duplication and fusion event of hexokinase occurred

by the time when primitive fish emerged, while glucokinase did not arise until the emergence of *Amphibia*. Furthermore, Griffin et al. (12) found that glucokinase has a closer sequence similarity to the mammalian 100 kDa hexokinases than to yeast hexokinase. Therefore, the glucokinase gene seems more likely to be a product of the splitting of the hexokinase II gene, not the precursor.

Properties of mammalian hexokinases

Mammalian hexokinases I, II, and III are sometimes referred as low K_m hexokinases (4,5), because they have low K_m, i.e. high affinity, for glucose (K_m: 10-150 μM). Mammalian low K_m hexokinases are sensitive to inhibition by the product, Glc-6-P, with K_i being about 10-100 μM. Type IV isozyme, on the other hand, has lower affinity for glucose with K_m being about 5-10 mM, and unlike low K_m hexokinases, it is not sensitive to inhibition by physiological concentrations of Glc-6-P (3,5).

While hexokinase I is the dominant isozyme found in most tissues, hexokinase II is mostly found in those insulin sensitive organs and tissues, e.g. muscles, liver, and adipose tissues, due to, partially at least, the up-regulation of hexokinase II by insulin (13,17). Because of this tissue distribution and hormonal regulation along with other kinetic properties, it has been speculated that the hexokinase I is responsible for introducing glucose into glycolysis, while

hexokinase II is responsible for converting glucose into glycogen in insulin sensitive organs and tissues (4,18) (also see Kinetics of hexokinases).

Intracellular localization of hexokinases is not homogeneous (17), which is believed to bear functional significance (5). A large portion of hexokinase I and II was found to co-sediment with mitochondria (17). Later, hexokinase I and II were shown to be bound to the mitochondrial membrane (4,5,17) by interaction of the N-terminal hydrophobic sequences and, presumably, mitochondrial membrane lipids (19) and by interaction between the negative charge on hexokinase surface and presumably mitochondrial membrane phospholipids, bridged by divalent cations (20).

In the case of brain hexokinase I, Glc-6-P is able to cause dissociation of hexokinase I from mitochondria to various extent depending on species; the degree of dissociation ranges from 90% in rats to 20% in human, with guinea pig and bovine in between (21). Whether hexokinase II has the same properties is not known. It is noteworthy that there persists a portion of bound hexokinase resistant to Glc-6-P solubilization, which can be, however, solubilized by 0.5 M KSCN. Comparison of the Glc-6-P soluble and KSCN-solubilized hexokinases showed no distinguishable difference, which led to the postulation by Kabir and Wilson (21) that there are two subtypes of hexokinase-binding-sites on the mitochondrial surface. That is, solubilities of the

hexokinase bound on mitochondria are determined by the nature of hexokinase-binding-sites rather than by different subtypes of hexokinase or mitochondria.

The functional significance of intracellular localization of hexokinase I on mitochondria is suggested by BeltrandelRio and Wilson (22) as the feedback link between oxidative phosphorylation and glycolysis. Because of the close proximity, mitochondria-bound hexokinase I utilizes ATP primarily generated from mitochondria to phosphorylate glucose (22). In this scenario, when the energy charge state in the cell becomes low and mitochondrial oxidative phosphorylation is stimulated, the increased flux of ATP from mitochondria would elevate the flux of glucose phosphorylation (by hexokinase I) which in turn supports the need for carbon sources in glycolysis and Krebs cycle.

Hexokinase III is found in many tissues but in much lower amount than other hexokinase isozymes (23). Intracellular localization of hexokinase III was thought to be in the cytoplasmic space, but recently hexokinase III was found to be localized at the nuclear periphery. The association of hexokinase III to the nuclear periphery is apparently rather weak, and disrupted when tissue is homogenized (23). The role of hexokinase III and its intracellular localization in cell physiology is not clear.

Glucokinase is mainly found in liver (24, 25) and in pancreas (25). Because its K_m for glucose is in the range of

circulating blood glucose levels, fluctuation of blood glucose can greatly influence the rate of glucose phosphorylation catalyzed by glucokinase. During a postmeal high blood glucose state, the high K_m for glucose makes glucokinase ideal to convert excess blood glucose into glycogen in liver or to serve as a sensor governing the release of insulin in the pancreatic ß-islets.

Glucokinase was thought to be a cytosolic enzyme; however, Miwa et al. (26) have demonstrated that glucokinase is present in the nucleus as well as in the cytosol. The physiological significance of this intracellular localization of glucokinase is not known.

Kinetics of hexokinases

The hexokinase reaction proceeds by a ternary complex mechanism, i.e. hexokinase-glucose-ATP.Mg^{2+} must be formed before the catalysis can occur (4). Whether the substrate binding follows a sequential order or random order has been the subject of a long debate (4,5). Fromm and his coworkers reported that the binding order is a rapid equilibrium random mechanism (18,27,28); that is, either glucose or ATP.Mg^{2+} can bind to hexokinase first, followed by the binding of the other substrate. However, most other investigators reported that substrate binding follows a sequential order in which glucose binds first (4,5,7). To prove the latter view and to avoid the inherent ambiguity of traditional product inhibition

kinetics, Gregoriou et al. (29) demonstrated, with the flux ratio method, that the substrate binding is a sequential ordered mechanism with glucose binding to the enzyme first. Later Ganson and Fromm (30) rebutted with results using the equilibrium isotope exchange method, and reported that the reaction operates by a random mechanism but with a preferred pathway, in which two thirds of the reaction proceeds with glucose binding first and ATP.Mg^{2+} later. Since the mid 1980, the argument about the kinetic mechanism of hexokinases seems to have subsided, and to this date, there is no clear and decisive report on whether hexokinases follow a sequential order or a random order in the substrate binding.

Glc-6-P is a competitive inhibitor vs. ATP.Mg^{2+} (3-5,7), indicating Glc-6-P and ATP.Mg^{2+} are mutually exclusive when binding to hexokinase. Whether Glc-6-P binds at a distinct allosteric site or a site overlapping with the ATP.Mg^{2+} is another ongoing debate (see Allosteric inhibition of hexokinase and Functional organization of hexokinases in this chapter).

Most investigators have reported that Glc-6-P is a noncompetitive inhibitor vs. glucose (3-5,31,32). However, their double reciprocal (Lineweaver-Burk) plots showed almost parallel lines (31,32), which is characteristic of uncompetitive inhibition. In fact, Fromm and Zewe (33) reported that the inhibition is uncompetitive. This near parallel pattern, or near uncompetitive inhibition, is

probably due to the synergistic effect of glucose and Glc-6-P binding (34,35), in which the converging point of the Lineweaver-Burk plot becomes distant from the origin (36). Thus, this unusual "mixed type" inhibition pattern closely resembles uncompetitive inhibition.

One other unique characteristic of hexokinase I is that inorganic phosphate can antagonize Glc-6-P inhibition at low mM concentrations (11,27,28,37,38). This unique characteristic is believed to play a significant role under certain physiological conditions in the brain (11). In brain, hexokinase I is considerably inhibited by its product, Glc-6-P, under normal conditions. When an energy demanding circumstance arises, the elevated phosphate concentration, resulted from hydrolysis of ATP, serves to relieve hexokinase I from Glc-6-P inhibition (11) and thus hexokinase I introduces more glucose into glycolysis and the Krebs cycle to meet the energy demand. At higher concentrations (>5 mM), however, phosphate starts to exhibit its inhibitory effect on hexokinase I (4,5,38).

In the case of hexokinase II, antagonism of Glc-6-P inhibition by inorganic phosphate is not seen and phosphate is solely an inhibitor with K_i being about 3 mM (18,38). This inhibitory effect of inorganic phosphate would suggest that during muscle exercise, elevated phosphate concentrations, again resulting from ATP hydrolysis, could inhibit hexokinase II and at the same time activate glycogen phosphorylase and

phosphofructokinase (18). As a result, myoglycogen is funneled into glycolysis to generate more ATP for muscle contractions. Such a mechanism indicates that the source of carbon fuel for muscle contraction is myoglycogen not free glucose; therefore, hexokinase II is responsible for converting glucose to myoglycogen rather than introducing glucose into glycolysis.

Compared to hexokinase I and II, hexokinase III has lower K_m for glucose (10-30 μM) and higher K_i for Glc-6-P (approx. 100 μM) (3-5), and uniquely, hexokinase III exhibits substrate inhibition by glucose in the mid to high mM range (3,39). The physiological significance of these kinetic properties is not clear.

Allosteric inhibition of hexokinases

By using a series of analogues, Sols and Crane (40,41) discovered that brain hexokinase I has different specificities for the hexose moiety of substrates and inhibitors, and therefore suggested that the inhibitor occupies a distinct allosteric site different from the active site where substrates bind. For example, both mannose and 2-deoxyglucose are good substrates for hexokinase I, but their phosphorylated counterparts, mannose-6-P and 2-deoxyglucose-6-P are poor inhibitors. This hypothesis was further tested by Sols (42) and Ureta et al. (43) with the reverse reaction catalyzed by hexokinase I, in which Glc-6-P serves both as a substrate and

as an inhibitor. The reverse reaction showed substrate inhibition when Glc-6-P was used as the substrate, whereas 2-deoxyglucose-6-P showed no substrate inhibition due to its poor ability to inhibit hexokinase I. Furthermore, yeast hexokinase, which is insensitive to Glc-6-P inhibition, showed no substrate inhibition when Glc-6-P was used as the substrate in the reverse reaction. These experiments (43) suggested that Glc-6-P exerts it inhibitory effect on hexokinase I by binding to a distinct allosteric site rather than the active site.

The separate allosteric site in hexokinase I is also evident from the fact that Glc-6-P is not a competitive inhibitor vs. glucose (3). If Glc-6-P inhibits hexokinase I by binding to the active site, it should have been a competitive inhibitor vs. glucose, since both molecules cannot occupy the same active site at the same time. The fact that Glc-6-P is not a competitive inhibitor vs. glucose and that both Glc-6-P and glucose can bind to hexokinase I simultaneously (34,35) confirms the discrete allosteric and active sites on hexokinase I. In fact, Glc-6-P and glucose showed synergism when they bind to hexokinase I (34,35); that is, binding of either ligand will promote the binding of the other ligand. Thus, Glc-6-P must bind at a discrete allosteric site, which does not overlap with the active site where glucose binds.

Structure and ligand binding

Although there is no mammalian 100 kDa hexokinase crystal structure available, a significantly related hexokinase from yeast (50 kDa) has been crystallized and structures determined at 2.1 Å and 3.5 Å resolution (44-47). Since mammalian 100 kDa hexokinases are believed to arise from duplication and fusion of a gene encoding a 50 kDa hexokinase similar to the yeast hexokinase (5-14), these 50 kDa yeast hexokinase crystal structures have been used for modeling mammalian 100 kDa hexokinases (48,49) as well as 50 kDa glucokinase (50-51).

These two yeast hexokinase structures represent two different states during hexokinase catalysis: one is in the "open" conformation (resolution at 2.1 Å), which represents the structure before the glucose-induced cleft closure; the other is in the "closed" conformation (resolution at 3.5 Å) with a glucose molecule trapped at the active site. The enzyme consists of two lobes surrounding the active site, a structural feature also found in many other kinases (45). When glucose binds to the active site between the two lobes of the enzyme, the smaller lobe of the enzyme closes down and bends 12° relative to the large lobe, forming the "closed" conformation (45-47). The enclosure of glucose in the active pocket effectively shields most of the glucose molecule from the solvent environment, exposing only the 6-hydroxyl group for accepting the phosphate transfer from ATP.

From these structures, it is observed that Ser 158, Asn

210, Asp 211, Gln 269, and Gln 302 are involved in hydrogen bonding to the 1-, 3-, 4-, and 6- hydroxyl groups on the "trapped" glucose molecule (46,47). Interestingly, these hydroxyl groups have been reported by Crane and Sols (40,41) to be critical for hexose specificity in brain hexokinase I. Such observation is probably not coincidence, but rather it reinforces the concept that mammalian hexokinases arose from an ancestral hexokinase similar to present-day yeast hexokinase.

Since the active site of hexokinase I is located in the C-terminal half (11,49,53-56), one may expect that alterations in one of these corresponding amino acid residues in the C-terminal half may affect glucose binding of hexokinase I. Later mutageneses studies (49,53-55) were consistent in general with this hypothesis; alterations in one of those corresponding residues (Asp 657, Gln 708, Gln 742) in the C-terminal half of hexokinase I hindered the glucose binding affinity and thus catalytic activity of hexokinase I, while alterations in those corresponding residues in the N-terminal half have no such effect.

One rather surprising observation made by Arora et al. (50) was that mutation of S603A in the C-terminal half, as one may expect, decreased the catalytic activity of hexokinase I, but the binding affinity for glucose was surprisingly <u>increased</u>. Although Harrison (47) suggested that the 3-hydroxyl group of glucose forms hydrogen bond with the oxygen

on the carbonyl group, not the hydroxyl group on the side chain, of corresponding Serine 158 in yeast hexokinase, how the substitution of Ser to Ala would actually increase the glucose binding affinity remains to be examined further.

Functional organization of hexokinases

Easterby (6) and Colowick (7) first hypothesized that the allosteric site of hexokinase I evolved from one of the two active sites, leaving the other active site intact for catalysis. This hypothesis was later supported by ligand-protected limited proteolysis of rat brain hexokinase I (11,56). White and Wilson demonstrated that Glc-6-P was able to protect the N-terminal half of hexokinase I from trypsin attack under mild denaturing conditions, while the glucose analogue, N-acetyl-glucosamine, along with ATP were able to protect the C-terminal half under the same conditions. According to the hypothesis, one may expect that N- and C-terminal halves of hexokinase I isolated under such conditions will retain their specialized function; that is, the N-terminal half will show no catalytic activity, but C-terminal half will retain full catalytic activity with no sensitivity to Glc-6-P inhibition.

Surprisingly however, the C-terminal half prepared under such conditions not only possesses full catalytic activity of hexokinase I, but is also sensitive Glc-6-P inhibition, which implies that the C-terminal half possesses its own Glc-6-P

binding site (11). Upon further examination, the N-terminal half of hexokinase I was found to have its own glucose binding site as well as a Glc-6-P binding site. In other words, both N- and C-terminal halves have a glucose binding site and a Glc-6-P binding site; therefore, White and Wilson (11) suggested that the ancestral 50 kDa hexokinase must have acquired an additional allosteric site before the gene duplication and fusion event occurred. Indeed, such 50 kDa Glc-6-P sensitive hexokinases have been found in silkworm (57), in the parasite, *Schistosoma mansoni* (58), in starfish (11,59) and other marine species. Hence the allosteric site in hexokinase I did not evolve from an active site but rather was shaped by nature before the gene duplication and fusion event occurred.

Although there are potentially two active sites and two allosteric sites present in each 100 kDa hexokinase, Chou and Wilson (60) found that there is only one glucose binding (active) site and one Glc-6-P binding (allosteric) site detectable by Scatchard analysis in each 100 kDa hexokinase I. Similar observations have also been reported by Ellison et al. (34). Since Scatchard analysis measures the functional binding sites, White and Wilson (11) suggested that the "active site" in the N-terminal half and the "allosteric site" in C-terminal half of hexokinase I are latent and inaccessible to substrate or inhibitor, and therefore they are not detectable by Scatchard analysis.

Since the catalytic activity has been shown to be associated with the C-terminal half of hexokinase I by several laboratories (11,49,53-56), it is obvious that the functional active site of the intact hexokinase I is located in the C-terminal.

The location of the functional Glc-6-P binding site, whether it is located in the N- or C-terminal half of hexokinase I, remains in dispute (11,49,53-56). However, since Glc-6-P can protect the N-terminal half of hexokinase I from trypsin digestion (11,56) or sulfhydryl modification (61) at the μM levels, which are comparable to the K_i value of Glc-6-P, White and Wilson (11) suggested that the functional Glc-6-P binding site is located in the N-terminal half.

Several other investigators (49,62,63) have suggested that the functional Glc-6-P binding site is located near the active site in the C-terminal half rather than in the N-terminal half. Arora et al. (49) reported that the functional Glc-6-P site is the one in the C-terminal half because 1) truncated 50 kDa C-terminal half of hexokinase I is sensitive to Glc-6-P inhibition, and 2) mutation of residues in the N-terminal half corresponding to those responsible for glucose binding in the C-terminal half of hexokinase I has no effect on the K_i of Glc-6-P.

Nevertheless, these arguments are not flawless. First, glucose and Glc-6-P binding sites in both N- and C-terminal halves are accessible in the isolated 50 kDa truncated forms.

In the intact 100 kDa form, however, only one glucose site and one Glc-6-P site are accessible in hexokinase I (34,60). The fact that Glc-6-P inhibits the 50 kDa C-terminal half does not necessarily mean this Glc-6-P site in the C-terminal half is the regulatory site in the 100 kDa form. If their argument is correct that Glc-6-P binds at the C-terminal half of hexokinase I, then why is the N-terminal half protected by Glc-6-P from proteolysis? If Glc-6-P can bind at both the N- and C-terminal halves, then why does Scatchard analysis indicate there is only one Glc-6-P binding site in the entire hexokinase I?

Second, though Solheim and Fromm (62) and Jarori et al. (63) have suggested that Glc-6-P binds very close to the active site (glucose binding site), the fact that Glc-6-P and glucose can bind to hexokinase I simultaneously (34,35) and that Glc-6-P is not a competitive inhibitor vs. glucose even with the isolated 50 kDa C-terminal half (11), suggests that there is no overlap between the Glc-6-P binding site and the glucose binding site. Therefore, it is very unlikely that both glucose and Glc-6-P share several residues for binding to the enzyme. That is, residues responsible for glucose binding are not necessarily responsible for Glc-6-P binding. Thus, mutation at residues responsible for glucose binding in the N-terminal half of hexokinase I may have little, if any, effect on Glc-6-P binding (49), and mutation of such residues does not provide information about the location of the functional

allosteric site.

Jarori et al.(63) reported that glucose and Glc-6-P are 11 Å and 8 Å, respectively, away from a Mn^{2+} binding site, which means that glucose and Glc-6-P may be as close as 3 Å and as far as 19 Å from each other when bound to hexokinase I. Considering that the diameter of a glucose is no more than 6 Å (distance between the oxygen atoms on carbon 2 and 6), and a typical hydrogen bond is in the range of 2.6 - 3.1 Å (distance between the donor atom and the acceptor atom), the minimal 3 Å distance between glucose and Glc-6-P still represents a relatively large distance, and such distance would seem to preclude the possibility that glucose and Glc-6-P share the same residues for binding (see the previous paragraph). The actual distance between glucose and Glc-6-P is not known, but likely to be more than 3 Å. Based on the result of Jarori et al.(63), whether Glc-6-P binds close to or far from glucose is a matter of individual belief and perception.

Solheim and Fromm (62) determined the K_m of Glc-6-P in the reverse reaction of hexokinase I to be about 25 μM. Because of this strong affinity, Fromm insisted that this is the high affinity site for Glc-6-P and it represents the inhibitory nature of Glc-6-P, i.e. Glc-6-P functions as an inhibitor by binding to the active site with a high affinity. However, this speculation conflicts with one important fact. The fact that Glc-6-P is not a competitive inhibitor vs.

glucose and both molecules can bind to hexokinase I simultaneously precludes the possibility that the inhibitory Glc-6-P binds at the active site (see Allosteric inhibition of hexokinases).

Solheim and Fromm (62) then proposed a model in which the Glc-6-P binding site is located next to the active site. In this model, the phosphate moiety of Glc-6-P occupies the space of the γ-phosphate of ATP, which explains the competitive inhibition nature of Glc-6-P vs. ATP. The glucose moiety of Glc-6-P, however, occupies a distinct site from the glucose binding site, which explains why glucose and Glc-6-P can bind to hexokinase simultaneously. They proposed that when Glc-6-P is formed after the catalysis, the phosphate moiety remains in place but the glucose moiety dissociates and binds to a new site. Note that in this model, the site for binding to the glucose moiety of Glc-6-P is distinct from the active site (glucose binding site).

However, Fromm's explanation for how Glc-6-P binds to its new site after catalysis is not well supported by structural observations from Steitz (45). Glucose has a strong affinity for hexokinase with K_m of about 50 μM and the ability to induce a conformational change in hexokinase I (35,45-47). Based on the crystal structure of yeast hexokinase, Steitz speculated that because of the glucose induced conformation change, the dissociation of Glc-6-P is perhaps the rate limiting step in hexokinase catalysis (45), where cleft

opening is the limiting factor to Glc-6-P dissociation. In such case, it would be hard to conceptualize how the phosphate moiety remained anchored to the enzyme, while the glucose moiety would dissociate from the enzyme and bind to a different site.

HYPOTHESIS AND OBJECTIVE

To find out whether the location of the regulatory Glc-6-P site is in the N- or the C-terminal half of hexokinase I, chimeric hexokinases constructed with the N- and C-terminal halves from type I, II, and III isozymes were employed. The hypothesis of this study is that Glc-6-P binds to the N-terminal half of hexokinase I, and therefore, the regulatory function should be associated with the N-terminal of hexokinases.

This work was initiated to test such hypothesis. However, during the course of this study, it became evident that the functional organization in hexokinase II is quite different from those in hexokinase I and III (64,65). This observation has an impact on the current evolution scheme proposed by White and Wilson (11) and Griffin et al. (12); subsequently, the evolution scheme of mammalian hexokinases was revised to accommodate such observation (64).

In addition to constructing chimeric hexokinases from hexokinase I and II, modification of hexokinase III cDNA was needed. The cDNA of hexokinase III has been previously subcloned as two separate pieces with several undesirable restriction sites in the open reading frame (65); therefore, assembly and extensive site-directed mutageneses were needed to bring the hexokinase III cDNA into a form that is compatible with type I and II cDNAs (65). Completion of this task would not only allow construction of chimeric hexokinases

with type III isozyme, but also provide an opportunity to examine its kinetic properties. Among those hexokinase III properties, glucose inhibition is unique and particularly interesting. Thus, it is of great interest to determine the half/domain that is responsible for this glucose inhibition as well as the overall functional organization in hexokinase III.

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Chapter II

Functional Organization of Mammalian Hexokinases: Characterization of Chimeric Hexokinases Constructed from the N- and C- Terminal Domains of the Rat Type I and Type II Isozymes

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ABSTRACT

Chimeric hexokinases consisting of either the N-terminal half of Type I hexokinase fused with the C-terminal half of the Type II isozyme (NICII) or the inverse pair (NIICI), along with the parental isozymes, were expressed in COS-1 cells. The thermal stability of the chimeras was intermediate between that of the highly labile Type II isozyme and the relatively stable Type I hexokinase. In their K_ms for substrates, Glc and ATP, the chimeric enzymes were similar to the parental isozyme from which the C-terminal half was derived. Although the Type I and Type II isozymes were similar in their sensitivity to inhibition (competitive *vs*. ATP) by the Glc-6-P analogs, 1,5-anhydroglucitol 6-phosphate (AnGlc-6-P) and Glc-1,6-bisphosphate, the chimeric enzymes differed markedly, with the NIICI chimera being much more sensitive and the NICII chimera much less sensitive than either parental form to these inhibitors. In contrast, the response of the chimeras to P_i, either as an antagonist of inhibition by AnGlc-6-P or, at higher concentrations, as an inhibitor, was correlated with the origin of the N-terminal domain. The results are consistent with the view that catalytic function is associated with the C-terminal domain of the Type I isozyme, with regulatory function - inhibition by Glc-6-P and its analogs and antagonism of this inhibition by P_i - being mediated by the N-terminal domain.

Mammalian tissues contain three isozymes of hexokinase (ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1), generally designated as Types I-III, which exhibit marked similarity in some of their properties (reviewed in ref. 1). These include existence as monomeric enzymes with molecular weights of approximately 100,000, and sensitivity to inhibition by physiological levels of the product, Glc-6-P^1. The availability of amino acid sequences for the Type I, II and III isozymes, deduced from the nucleotide sequence of cDNAs cloned from various sources (2-8), has revealed extensive sequence similarity among the mammalian isozymes, confirming that they are all closely related members of a family which also includes other kinases (9). Moreover, the sequences of these isozymes exhibit internal repetition, i.e., the sequence of the N-terminal half is similar to that of the C-terminal half, and further, these sequences show similarity to that of various 50 kDa hexokinases that exist in organisms such as yeast (1,10,11). These results are consistent with the suggestion (12-15) that the 100 kDa mammalian enzymes evolved by duplication and fusion of a gene encoding an ancestral 50 kDa hexokinase.

The extensive similarity in amino acid sequences of the N- and C-terminal halves of the 100 kDa mammalian hexokinases clearly implies a corresponding similarity in secondary and tertiary structure (16). Moreover, a number of residues which, based on earlier work with the homologous yeast hexokinase

(17), are known to be of catalytic importance are conserved in *both* N- and C-terminal halves of all mammalian hexokinases that have been sequenced thus far (1-8). Despite this striking similarity in sequence and, presumably, structure, there is clear evidence that, at least for the Type I isozyme, the N- and C-terminal halves of the enzyme are *functionally* distinct. Thus, White and Wilson (15) demonstrated that the N- and C-terminal halves of the rat Type I isozyme could be isolated after limited proteolysis, with complete retention of catalytic activity in the C-terminal half while the isolated N-terminal half was devoid of detectable activity. Consistent with this, site-directed mutation of catalytically important residues in the C-terminal half of the rat (18) or murine (19,20) Type I isozyme result in drastic reductions of catalytic function, while mutation of analogous residues in the N-terminal halves have no effect on catalysis (18,20). Finally, the C-terminal halves of Type I hexokinase, expressed in *E. coli* using the cloned cDNAs for the murine (20) or human (21,22) isozymes, exhibit catalytic activity while the corresponding expressed N-terminal half does not (20).

While catalytic function has unequivocally been associated with the C-terminal half of the Type I isozyme, assignment of regulatory function to a particular structural region has been much less straightforward and remains controversial. This has been extensively reviewed elsewhere (1) but basic elements of the debate are summarized here.

White and Wilson (23,24) demonstrated the presence of a Glc-6-P binding site in the N-terminal half; the affinity (K_d ≈ 10 μM) of this site for Glc-6-P was virtually identical to the kinetically determined K_i, and the binding of various hexose 6-phosphates to this site, with resulting effect on conformation of the N-terminal half, was correlated with their inhibitory effectiveness. Hutny and Wilson (25) showed that binding of Glc-6-P preferentially protected sulfhydryl groups in the N-terminal half of the enzyme from reaction with a sulfhydryl-selective reagent, again with effective concentrations in the low μM range. Previous binding studies (26-28) had shown a single site (per 100 kDa molecule) for binding of Glc-6-P with a K_d in the low μM range. Thus, the N-terminal site was proposed as the regulatory site. According to this view, inhibition is considered to result from conformational changes, induced by the binding of Glc-6-P to the N-terminal half, which preclude binding of ATP at the catalytic site in the C-terminal half (24,25).

The situation was complicated by the finding that the isolated (by limited proteolysis) (15) or expressed (from cloned cDNAs) (20-22) C-terminal half of the enzyme retained sensitivity to inhibition by Glc-6-P, leading to suggestions (20,22) that the functional regulatory site is in the C-terminal half, with the role of the Glc-6-P binding site in the N-terminal half unspecified. However, based on this suggestion, one would expect *two* binding sites (per 100 kDa

molecule) for Glc-6-P, which conflicts with the observed stoichiometry (26-28). An alternative view (24), consistent with the stoichiometry, is that the Glc-6-P binding site in the C-terminal half of the molecule is latent in the intact 100 kDa enzyme, with the N-terminal site serving regulatory function.

Whether the functional organization proposed (15, 23-25) for the Type I isozyme, i.e., catalytic C-terminal half and a regulatory N-terminal half, is parallelled in the other isozymes of mammalian hexokinase remains to be determined. In the present study, we take one approach toward addressing this question. We have prepared constructs that permit expression of chimeric hexokinase molecules, comprised of the N-terminal half of Type I hexokinase and the C-terminal half of the Type II isozyme, and *vice versa*. We ask whether regulatory characteristics of the chimeric enzymes can be correlated with those of the parental enzyme from which the N-terminal half of the chimera was derived. For the Type I and Type II isozymes, a major distinguishing characteristic is their response to P_i. With Type I hexokinase, low concentrations of P_i antagonize the binding of Glc-6-P and the inhibition resulting from binding of this ligand (26,27,29). At higher concentrations, P_i itself becomes inhibitory, competitive with ATP (27). As noted by Ellison *et al.* (27), this bimodal action of P_i implies the existence of two distinct sites for binding of this ligand. It has been suggested that the high affinity site is located in

the N-terminal half of the molecule (24,25), with competition between P_i and the 6-phosphate group of Glc-6-P for a common anion binding site accounting for the mutually exclusive binding of these ligands (26,27) and thereby, reversal of the inhibition by Glc-6-P (26,27,29). The second site, with lower affinity and accounting for the inhibition of Type I hexokinase at elevated concentrations of P_i, is suggested to be located in the C-terminal half (24,25). In contrast to this dual action on the Type I isozyme, P_i does not antagonize inhibition of Type II hexokinase by Glc-6-P, but is solely inhibitory, again competitive with ATP, at concentrations throughout the mM range (30).

MATERIALS AND METHODS

Materials. Restriction enzymes, T4 DNA ligase, and Glc-6-P dehydrogenase were products of Boehringer Mannheim (Indianapolis, IN). BCA Protein Assay Reagent and bovine serum albumin standard were purchased from Pierce Chemical Co. (Rockford, IL) and all other biochemicals from Sigma Chemical Co. (St. Louis, MO). Isoelectric focusing was performed on pH 3-10 gels obtained from Serva (Paramus, NJ) and cellulose acetate electrophoresis on Titan III plates from Helena Laboratories (Beaumont, TX).

Construction of vectors and expression of chimeric hexokinases in COS-1 cells. Procedures for restriction

digestion, ligation of DNA and transformation of *E. coli* (DH5-α) were essentially as described by Sambrook *et al.* (31).

The previously described (3) 3.6 kb full length cDNA for rat Type I (HKI 1.4-7) was cloned into the *Eco* RI site of pUC18. An *Nco* I site (Fig. 1) is conveniently located near the center of the coding region (cleavage at nucleotide 1452), while a *Pst* I site (cleavage at nucleotide 3156) is located approximately 300 bp past the termination codon. Thus, digestion with *Eco* RI and *Nco* I gave a fragment of about 1.4 kb containing the coding sequence for the N-terminal half of the molecule, and digestion with *Nco* I and *Pst* I gave a 1.7 kb fragment containing the coding sequence for the C-terminal half of the enzyme.

As previously described (6), the nucleotide sequence encoding the rat Type II isozyme was contained in two overlapping clones, 12-1.3C coding for the N-terminal half of the molecule and RG2B coding for the C-terminal half. A full length cDNA for the rat Type II isozyme was generously provided by Dr. Annette Thelen. It had been constructed by ligation of complementary fragments from 12-1.3C and RG2B. In addition, a thymidine corresponding to nucleotide 1559 in the full length cDNA had been mutated to cytosine to yield an *Nco* I site at a position equivalent to that in the cDNA encoding the Type I isozyme (Fig. 1). Thus, digestion with *Eco* RI-*Nco* I gave a fragment of about 1.5 kb containing the coding sequence for the N-terminal half of Type II hexokinase, while

Type I	450	Gly Lys Gly Ala Ala Met Val Thr Ala
Type I	1439	GGC AAG GGG GCC GCC ATG GTG ACG GCA
Type II	1545	GGC AAG GGG GCT GCT ATG GTG ACG GCG
Type II	450	Gly Lys Gly Ala Ala Met Val Thr Ala

Figure 1. Nucleotide and deduced amino acid sequences surrounding the centrally located *Nco* I sites in cDNAs for rat Type I and Type II hexokinases. *Nco* I cleaves after the cytosine residue that is underlined in the nucleotide sequence for the Type I isozyme. The thymidine residue that was mutated to cytosine to generate an *Nco* I site in the Type II cDNA is shown in bold.

digestion with *Nco* I-*Pst* I gave a 1.5 kb fragment encoding the C-terminal half of the Type II isozyme.

At both the nucleotide and amino acid levels, the sequences of the Type I and II isozymes were virtually identical in the region surrounding the central (and sole) *Nco* I site (Fig. 1). Chimeric cDNAs were constructed after digestion of plasmids containing cDNAs for the Type I and Type II isozymes with *Nco* I-*Pst* I, excising the regions corresponding to the C-terminal halves of the parental isozymes. The *Nco* I-*Pst* I fragments were gel isolated, and religated back into the complementary larger fragment (vector plus cDNA region encoding N-terminal half of the molecule) from the *Nco* I-*Pst* I digestions. The chimeric cDNAs were cloned in pUC18, then subcloned into the vector pSVT7 (32) for expression in COS-1 cells.

COS-1 cells were cultured as previously described (18), and transfected (10 μg plasmid DNA per 100 mm culture dish) following the procedure of DeWitt *et al.* (33) with addition of a 90 sec treatment with dimethylsulfoxide at the end of transfection (34). Control cultures were untransfected or were transfected with the pSVT7 vector alone (sham transfected); both gave equivalent results. Cells were harvested 2-3 days after transfection, suspended in 1 ml (per 100 mm dish) of ice-cold BTGE buffer (50 mM Bicine, 10 mM thioglycerol, 10 mM Glc, 0.5 mM EDTA, pH 8.2) and sonicated for 10 sec. Sonicates were centrifuged at 800 x g for 10 min at 4° C. Hexokinase

activity in the supernatants was determined immediately, and supernatants stored at -80° C for further use. Where necessary (e.g., for isoelectric focusing or cellulose acetate electrophoresis), samples were concentrated using a Centricon-30 device (Amicon Corp., Beverly, MA).

Preparation of brain extract containing Type I hexokinase. After decapitation of a Sprague-Dawley rat (either sex, approx. 200 g) under CO_2 anesthesia, the brain was rapidly removed and homogenized in BTGE buffer (3 ml per g brain) using a Teflon-glass homogenizer (A.H. Thomas Co., Philadelphia, PA). The homogenate was centrifuged at 4° C and 3000 x g for 5 min, and the fluffy white layer (mostly myelin) removed. The remainder of the homogenate (with pellet resuspended in the supernatant) was made 2 mM in Glc-6-P and incubated for 30 min at room temperature to promote release of the mitochondrially bound hexokinase (35). After centrifugation at 164,000 x g for 1 hr, the solubilized hexokinase was recovered in the supernatant.

Determination of hexokinase activity and protein. Hexokinase activity was assayed as described previously (35). Determination of K_ms for Glc and ATP were done under the same conditions, except that the specified substrate was varied over an appropriate concentration range. Prior to determination of the K_m values and heat stability, cell extracts were chromatographed on spin columns of Sephadex G-25 (fine) equilibrated with HET buffer (50 mM Hepes, 0.5 mM EDTA,

10 mM thioglycerol, pH 7.5); absence of Glc in the eluted enzyme was confirmed by enzymatic assay. Inhibition by the Glc-6-P analog, AnGlc-6-P, was determined as specified by Baijal and Wilson (18); AnGlc-6-P is not a substrate for Glc-6-P dehydrogenase and thus the convenience of the standard coupled spectrophotometric assay could be retained. Inhibition by Glc-1,6-bisphosphate was studied in a similar manner, maintaining the concentration of Mg^{++} at 0.5 mM excess over the varied ATP concentration. All kinetic data were analyzed using the EZ Fit program of Perrella (36).

Protein was determined with the BCA Protein Assay system from Pierce Chemical Co., using bovine serum albumin as standard. Samples were pretreated with excess iodoacetamide (37) to avoid interference by thioglycerol present in the BTGE buffer.

Electrophoresis and isoelectric focusing. SDS acrylamide gel electrophoresis and immunoblotting procedures were as described by Smith and Wilson (38). Electrophoresis under nondenaturing conditions was performed on Titan III cellulose acetate plates equilibrated with TBPE buffer (180 mM Tris base, 100 mM boric acid, 20 mM Na_2HPO_4, 2 mM EDTA). Extracts of transfected cells were concentrated to approximately 2 units hexokinase/ml; extracts from sham transfected cells were concentrated to a comparable extent on a volume basis but, of course, the hexokinase activity was much lower. Samples were loaded using a Zip Zone sample applicator (Helena

Laboratories, Beaumont, TX); the exact amount of sample loaded cannot be controlled with this applicator, but was estimated to be approximately 1 μl. Electrophoresis in TBPE buffer was performed at 175 volts for 30 min at 4° C. Hexokinase activity was detected by covering the plate with a warm (approx. 45° C) solution containing 1% agar, 45 mM TrisCl (pH 9.5), 7.4 mM $MgCl_2$, 3.7 mM Glc, 0.02 mg/ml phenazine methosulfate, 0.25 mg/ml nitro blue tetrazolium, 0.7 mg/ml NADP, and 3.2 units/ml Glc-6-P dehydrogenase; the last four components were added immediately before use. When the agar had solidified, the plate was incubated in the dark at room temperature until the bands of activity were evident; staining was stopped by removing the agar layer and placing the plates in 10% acetic acid.

Isoelectric focusing was performed on pH 3-10 gradient gels, with an anode buffer containing 3.3 g aspartic acid and 3.7 g glutamic acid per l and a cathode buffer containing 4 g arginine and 4 g lysine per l. Samples (5 μl) contained approximately 0.01 units of activity. Focusing was conducted for 2 hrs with gradual increase of power: 1 watt for 30 min, 5 watts for 60 min, then 10 watts for 30 min. Hexokinase activity was detected by incubation of the focusing gels in a solution as used for staining of cellulose acetate plates (see above) except that agar was deleted and 1% (v/v) Triton X-100 was added. Staining was stopped by placing the gel in 10% acetic acid.

Calculation of isoelectric points. Isoelectric points of the Type I and Type II isozymes, and their N- and C-terminal halves, were calculated from the amino acid sequences (3,6) using the Isoelectric routine included in the GCG Sequence Analysis Software Package (Genetics Computer Group, Inc., Madison, WI)

RESULTS

We will refer to chimeric hexokinases by symbolism that identifies the origin of the N- and C-terminal halves. Thus, NICII refers to the chimeric enzyme comprised of the N-terminal half (residues 1-454) of the Type I isozyme and the C-terminal half (residues 455-917) of Type II hexokinase, and NIICI represents the chimera comprised of residues 1-454 from Type II hexokinase and residues 455-918 from Type I hexokinase.

Verification of chimeric cDNA constructs. The chimeric constructs, along with the parental cDNAs, were examined by restriction analysis (Fig. 2). Digestion with *Eco* RI and *Nco* I gave fragments of the size expected for regions encoding the N-terminal halves of the Type I and II isozymes, 1.4 kb and 1.5 kb, respectively. Digestion with *Nco* I and *Pst* I gave fragments of 1.7 kb and 1.5 kb, corresponding to the coding regions for the C-terminal halves of the Type I and Type II hexokinase, respectively. In all cases, the chimeric cDNAs

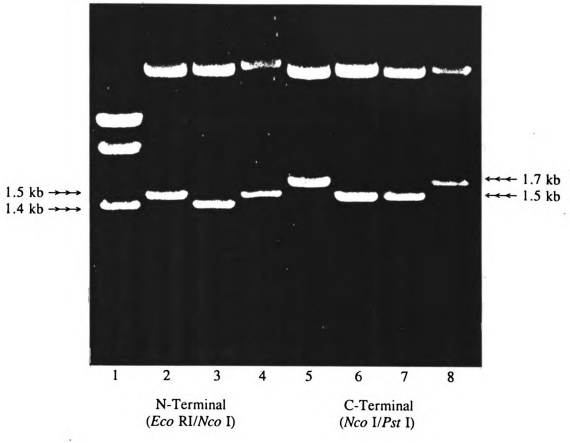

Figure 2. Restriction analysis of parental cDNAs and chimeric constructs. Fragments of interest are indicated at left and right of figure. Lanes 1-4, products of digestion with *Eco* RI and *Nco* I, which release 1.4 kb and 1.5 kb fragments encoding the N-terminal halves of the Type I and Type II isozymes, respectively. Lane 1, plasmid containing cDNA for Type I isozyme; Lane 2, plasmid containing cDNA for Type II isozyme; Lane 3, plasmid containing cDNA for NICII chimera; Lane 4, plasmid containing cDNA for NIICI chimera. Lanes 5-8 correspond to Lanes 1-4, but with digestion by *Nco* I and *Pst* I to release 1.7 kb and 1.5 kb fragments encoding the C-terminal halves of the Type I and Type II isozymes, respectively.

gave fragments of the size expected from the parental origins of the N- and C-terminal halves.

Expression of chimeric hexokinases in COS-1 cells. Extracts of COS-1 cells that had been transfected with pSVT7 constructs containing cDNAs for the Type I or Type II isozymes or for chimeric hexokinases all showed hexokinase activities that were well above that seen in extracts from sham transfected controls (Table I). Although the variation between experiments was considerable, the specific activities in extracts from cells transfected with cDNA for Type II hexokinase or for the NIICI chimera were consistently somewhat higher than those for cells transfected with cDNA for Type I hexokinase or the NICII chimera; by two-way ANOVA and Bonferroni's t-test, the Type II and NIICI values differed from the others with $p<0.01$. This could not be attributed to expression of higher levels of Type II hexokinase or the NIICI chimera since all forms were expressed at similar levels based on intensity of staining with Coomassie Blue or on Western blots (see below). Thus, it would appear that the presence of the N-terminal half of the Type II isozyme somehow leads to increased specific activity. One obvious possibility is that the N-terminal half of the Type II isozyme, unlike that of Type I hexokinase (15,18,19-22), possesses intrinsic catalytic activity. This is currently being investigated but, if true, both halves must be quite similar in kinetic properties (39) since no departure from simple Michaelis-Menten behavior for

Table I. Hexokinase Activity in Extracts of COS-1 Cells Transfected with cDNAs for Type I or Type II Hexokinase or for Chimeric Hexokinases.

cDNA Used for Transfection	Hexokinase Activity[a] (units/mg protein)
Sham Control	0.08 ± 0.03
Type I	0.98 ± 0.21
Type II	1.48 ± 0.35
NICII	0.63 ± 0.21
NIICI	1.50 ± 0.69

[a]Mean ± SD for 11 different transfections.

the Type II isozyme has been observed in previous studies (40) or in the present work (results presented below). The observation that the NIICI chimera had K_ms for Glc and ATP (see below) that were intermediate between those of the parental Type I and Type II isozymes is consistent with this possibility (39).

All extracts from transfected cells contained a prominent component with apparent molecular weight of approximately 100,000 (Fig. 3). No intense band was seen in this region in control extracts from untransfected or sham transfected COS-1 cells, and thus these components can reasonably be assumed to correspond to the expressed hexokinases (supported by immunoblotting results, presented below). The mobility of the Type I isozyme expressed in COS-1 cells was indistinguishable from that of the Type I isozyme isolated from rat brain (35). Although the Type I and Type II isozymes are virtually identical in size - 918 residues with calculated M_r of 102,317 for Type I (3) and 917 residues and calculated M_r of 102,555 for Type II (6) - the expressed Type II isozyme consistently migrated slightly faster than did the Type I isozyme; this same result has been seen with the Type II isozyme isolated from rat skeletal muscle (A.P. Thelen and J.E. Wilson, unpublished observation). The slight difference in mobility of these isozymes on SDS gels presumably results from charge differences which provide the basis for resolution of the isozymes by electrophoresis under nondenaturing conditions

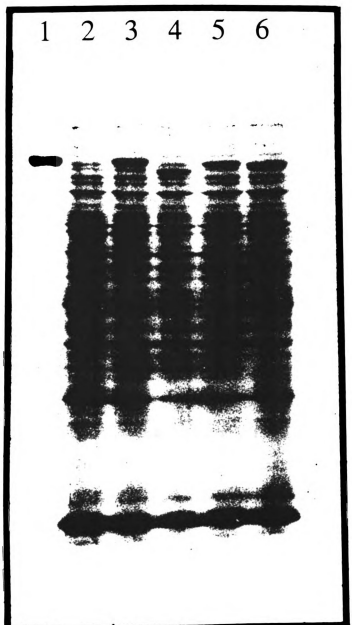

Figure 3. SDS gel electrophoretic analysis of COS-1 cell extracts. Lane 1, Type I hexokinase purified from rat brain (35); Lane 2, extract from sham transfected COS-1 cells; Lanes 3-6, extracts from COS-1 cells transfected with cDNAs for Type I, Type II, NICII, and NIICI hexokinases, respectively.

(41) or ion exchange chromatography (42) and which, despite the presence of SDS, influence the electrophoretic migration on SDS gels. The chimeric hexokinases (Fig. 3, Lanes 5 and 6) migrated to positions intermediate between those of the parental isozymes.

The identity of the chimeric hexokinases was confirmed by Western blots (Fig. 4) probed with monoclonal antibodies 21 and 5A, previously shown (43) to recognize segmental epitopes located in the N- and C-terminal halves, respectively, of the Type I isozyme. With both antibodies, extracts from COS-1 cells transfected with the Type I cDNA showed an immunoreactive band migrating identically to authentic Type I hexokinase purified from rat brain (35), while extracts from sham transfected cells showed no such band. Extracts from cells transfected with the cDNA for Type II hexokinase showed no detectable reactivity with monoclonal antibody 21; with prolonged staining, faint reactivity with monoclonal antibody 5A could be detected but this was clearly much less intense than with the Type I isozyme. The chimeric hexokinases (Fig. 4, Lanes 5 and 6) displayed the expected immunoreactivity. Thus, NICII (Lane 5) was intensely stained with monoclonal antibody 21 but only weakly detected with monoclonal antibody 5A; conversely, NIICI (Lane 6) was intensely reactive with monoclonal antibody 5A but showed no detectable reactivity with monoclonal antibody 21. The immunoreactive bands seen with extracts from cells transfected with the chimeric cDNAs

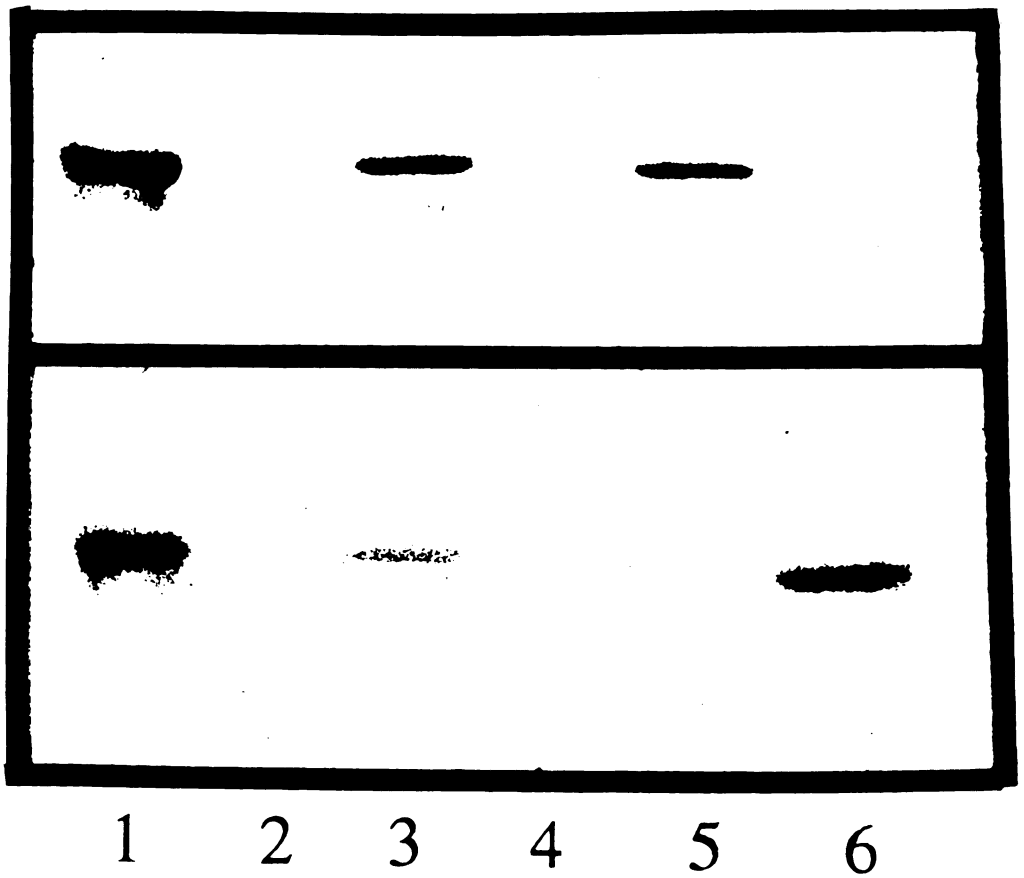

Figure 4. Western blot analysis of COS-1 cell extracts. Lane 1, Type I hexokinase purified from rat brain (35); Lane 2, sham tranfected COS-1 cells; Lanes 3-6, extracts from COS-1 cells transfected with cDNAs for Type I, Type II, NICII, and NIICI hexokinases, respectively. Top panel, blot probed with monoclonal antibody 21, which recognizes an epitope located in the N-terminal half of Type I hexokinase (43). Bottom panel, blot probed with monoclonal antibody 5A, which recognizes an epitope located in the C-terminal half of Type I hexokinase (43). Weak reactivity of antibody 5A with the Type II isozyme is detectable; this antibody also recognizes another component of lower molecular weight, present in extracts from both sham transfected (Lane 2) and transfected (Lanes 3-6) cells.

were, as on the Coomassie Blue stained gel (Fig. 3), located at positions intermediate between the parental Type I and Type II isozymes. A minor immunoreactive species, with M_r of approximately 90,000, was detected with monoclonal antibody 21 in extracts from cells transfected with the Type I and NICII cDNAs; this is likely a result of proteolysis by endogenous proteases that cleave within the N-terminal domain, as previously shown to occur in brain homogenates (35).

Isoelectric focusing (Fig. 5) and electrophoresis under nondenaturing conditions (Fig. 6) revealed a single band of hexokinase activity in extracts from COS-1 cells transfected with either the parental or chimeric cDNAs; endogenous activity in extracts from sham transfected cells was too low to be detected in these procedures. The isoelectric point and electrophoretic mobility of the expressed Type I hexokinase was identical to that of authentic Type I hexokinase from rat brain, while the Type II isozyme exhibited a more acidic pI and greater electrophoretic mobility consistent with its previously described electrophoretic and ion exchange chromatographic behavior (41,42). The chimeric hexokinases exhibited pIs and electrophoretic mobilities intermediate between those of the parental isozymes. It is interesting to note that the charge properties of both the chimeras and parental enzymes appear to be dominated by the N-terminal half, i.e., NICII was more similar to the Type I isozyme, while NIICI was more similar to the Type II isozyme. This is

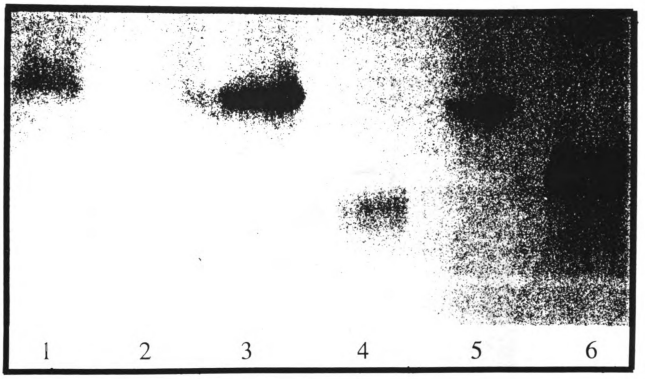

Figure 5. Analysis of COS-1 cell extracts by isoelectric focusing. Lane 1, Type I hexokinase in extract from rat brain; Lane 2, extract from sham transfected COS-1 cells; Lanes 3-6, extracts from cells transfected with cDNAs for Type I, Type II, NICII, and NIICI hexokinases, respectively. After focusing, the gel was stained for hexokinase activity.

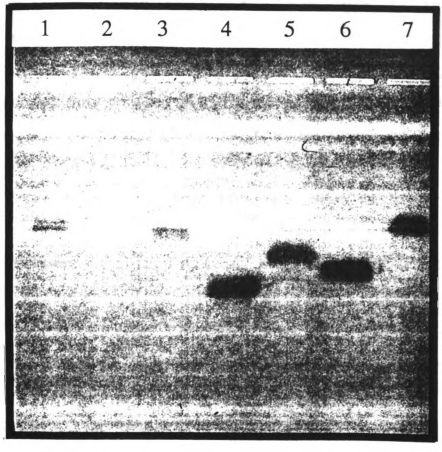

Figure 6. Analysis of COS-1 cell extracts by electrophoresis under nondenaturing conditions. Lanes 1 and 7, Type I hexokinase in extract from rat brain; Lane 2, extract from sham transfected COS-1 cells; Lanes 3-6, extracts from cells transfected with cDNAs for Type I, Type II, NICII, and NIICI hexokinases, respectively. After electrophoresis, the cellulose acetate plate was stained for hexokinase activity.

consistent with pIs calculated from the amino acid sequences, which are 6.4 and 5.7 for the N-terminal halves of the Type I and Type II isozymes, respectively, while the C-terminal halves are considerably less different in calculated pIs, being 6.7 and 6.3 for the Type I and Type II isozymes, respectively.

Heat stability of Type I, Type II, and chimeric hexokinases. Grossbard and Schimke (40) first noted the marked difference in heat stability of the isozymes of mammalian hexokinase, with the Type II isozyme being much more labile than the Type I isozyme. This was also seen with the Type I and Type II isozymes expressed in COS-1 cells (Fig. 7). Both chimeric enzymes exhibited similar thermal stability that was intermediate between that of the parental isozymes. It is notable that the chimeric enzymes were not more labile than the Type II isozyme. Thermal stability is reasonably taken as at least a gross measure of overall structural integrity; by this criterion, creation of the chimeric proteins has not led to marked structural destabilization. Structural integrity is also implied by the preservation of parental-like kinetic properties in the chimeric enzymes.

Kinetic and regulatory properties of Type I, Type II, and chimeric hexokinases. The K_ms of the Type I and Type II isozymes, and their chimeras, for Glc and ATP are shown in Table II. The results are quite similar to those reported by Grossbard and Schimke (40) and others (14) for the rat Type I

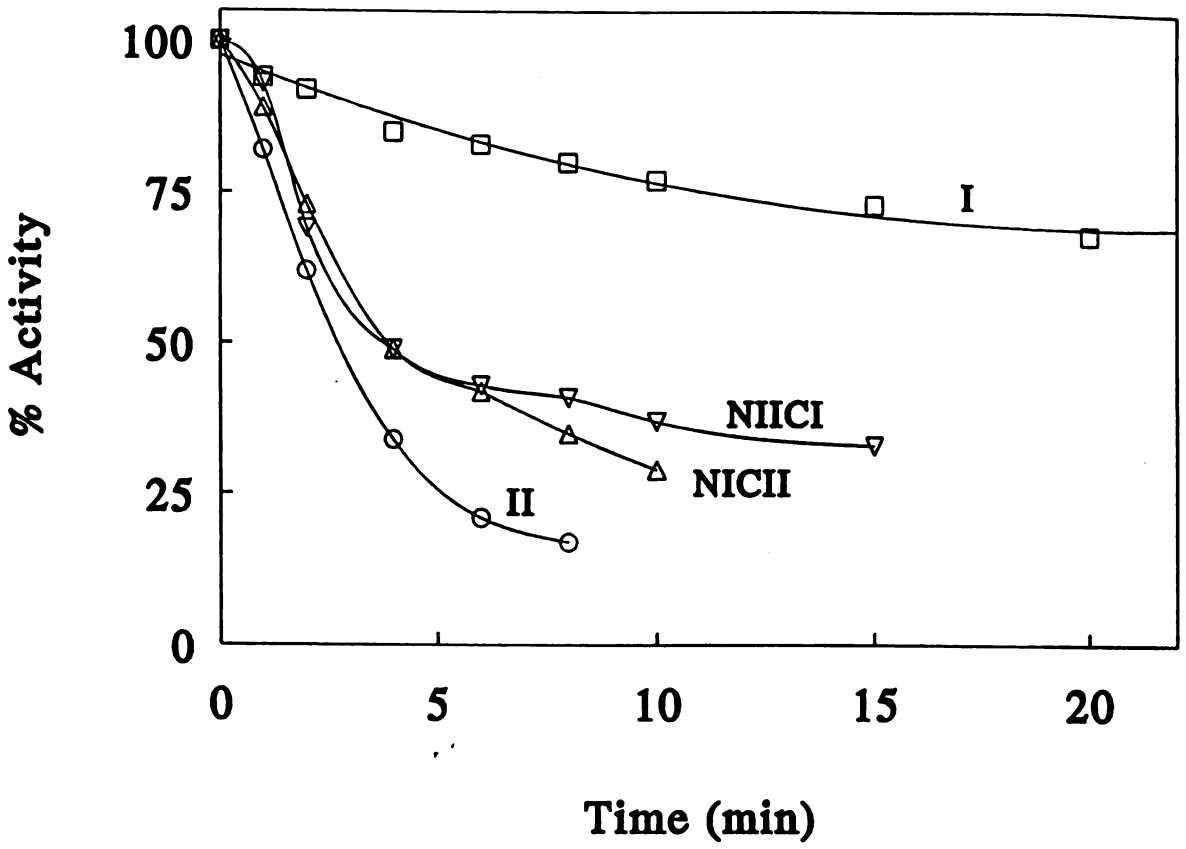

Figure 7. Heat stability of Type I, Type II, and chimeric hexokinases. Hexokinase activity in extracts of COS-1 cells transfected with cDNAs for Type I (□), Type II (○), NICII (△), and NIICI (▽) hexokinases was determined as a function of time at 40° C. Values shown are the average from two experiments (each with extracts from a different transfection) which gave similar results; average deviation between experiments was ± 4% from the values shown.

Table II. Kinetic Properties of Type I and Type II Hexokinase and the Chimeric Hexokinases.

Isozyme	K_m for Glucose(μM)[a]	K_m for ATP (mM)[a]	K_i for AnGlc-6-P (μM)[b]
Type I	65 ± 4	0.52 ± 0.04	17 ± 1
Type II	142 ± 1	0.77 ± 0.07	22 ± 4
NICII	140 ± 17	1.01 ± 0.15	72 ± 20
NIICI	82 ± 13	0.64 ± 0.06	6 ± 1

[a]Mean ± SD for 3 determinations, each with an extract from a different transfection.
[b]Mean ± SD for 4 determinations, each with an extract from a different transfection.

and Type II isozymes, and indicate somewhat higher apparent affinity (lower K_m) of the Type I isozyme for both substrates. Kinetically, the chimeras resembled the parental isozyme from which the C-terminal half was derived, i.e., NIICI had a lower K_m for both substrates than did NICII.

As previously found for Glc-6-P itself (14,40) as well as its analog, AnGlc-6-P (44,45), the Type I and Type II isozymes were quite similar in their sensitivity to inhibition by AnGlc-6-P (Table II). In contrast to the parental isozymes, the chimeric enzymes differed markedly in their sensitivity to this inhibitor, with NICII having a K_i approximately 10-fold greater than that seen with NIICI. For all forms, inhibition by AnGlc-6-P was competitive *vs*. ATP, as found in earlier studies with the parental isozymes (44,45).

In contrast to the similarity in sensitivity of the Type I and Type II isozymes to inhibition by AnGlc-6-P (44, and Table II), Rose *et al*. (44) had found that the Type II isozyme was considerably more sensitive to inhibition by Glc-1,6-bisphosphate. This was confirmed in the present study (Fig. 8). As in the case of inhibition by AnGlc-6-P, the chimeric enzymes differed much more markedly than did the parental isozymes in their sensitivity to inhibition by Glc-1,6-bisphosphate, with the NIICI chimera again being much more susceptible than was the NICII chimeric hexokinase (Fig. 8). In agreement with Rose *et al*. (44), inhibition was found to be competitive *vs*. ATP (data not shown); K_i values determined (one

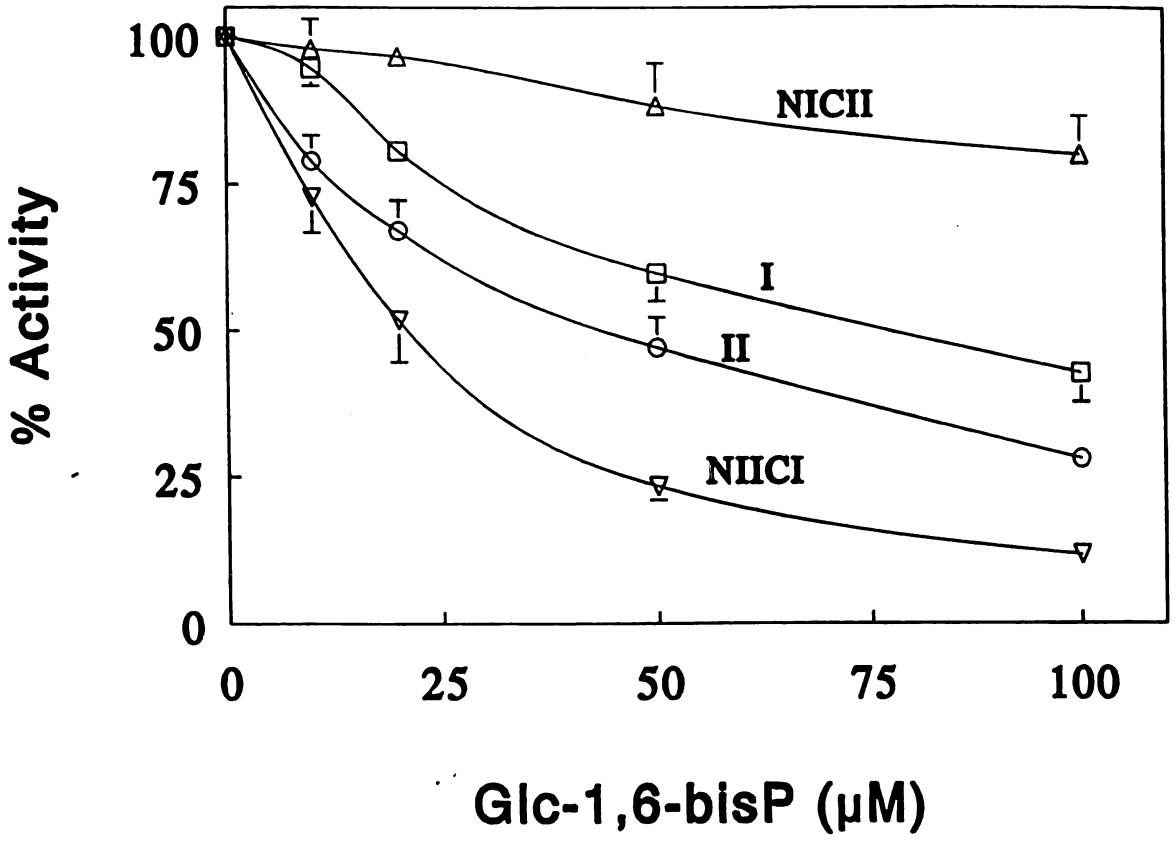

Figure 8. Inhibition of Type I, Type II, and chimeric hexokinases by Glc-1,6-bisphosphate. Activity was determined as a function of increasing Glc-1,6-bisphosphate concentration, with initial ATP concentration in each assay equal to the K_m value (Table II) for the respective hexokinase. (□), Type I; (○), Type II; (△), NICII; (▽), NIICI. Values shown are mean ± SD from three experiments, each with extracts from a different transfection; where no error bars are seen, they are obscured by the data point symbol.

experiment) for the Type I, Type II, NICII, and NIICI hexokinases were 40, 11, 143, and 4 μM, respectively.

Although P_i is an inhibitor, competitive *vs*. ATP, for both Type I and Type II hexokinase, the Type I isozyme was reported to be much less sensitive to this inhibitor, with a K_i of 35 mM (27), well above the K_i of 2.7 mM found for the Type II isozyme (30). We have confirmed this marked difference in sensitivity to P_i with the Type I and Type II isozymes expressed in COS-1 cells (Fig. 9). The chimeric enzymes were indistinguishable from the parental isozyme from which the N-terminal half was derived, i.e., NICII responded as the Type I isozyme, and NIICI as the Type II isozyme (Fig. 9).

This same correlation with the origin of the N-terminal half was seen in the response of the chimeric enzymes to P_i as an antagonist of inhibition by the Glc-6-P analog, AnGlc-6-P (Fig. 10). The Type I isozyme, expressed in COS-1 cells, responded as previously reported for the Type I isozyme from brain (26,27,29), i.e., low concentrations of P_i reversed the inhibition while at higher concentrations, inhibition by P_i itself became evident.

The NICII chimera was virtually identical to the Type I isozyme in this response. In contrast, P_i did not reverse inhibition of the Type II isozyme, either from rat skeletal muscle (30) or expressed in COS-1 cells (Fig. 10), and the NIICI chimeric hexokinase was indistinguishable from the Type II isozyme in this lack of response to P_i as an antagonist of

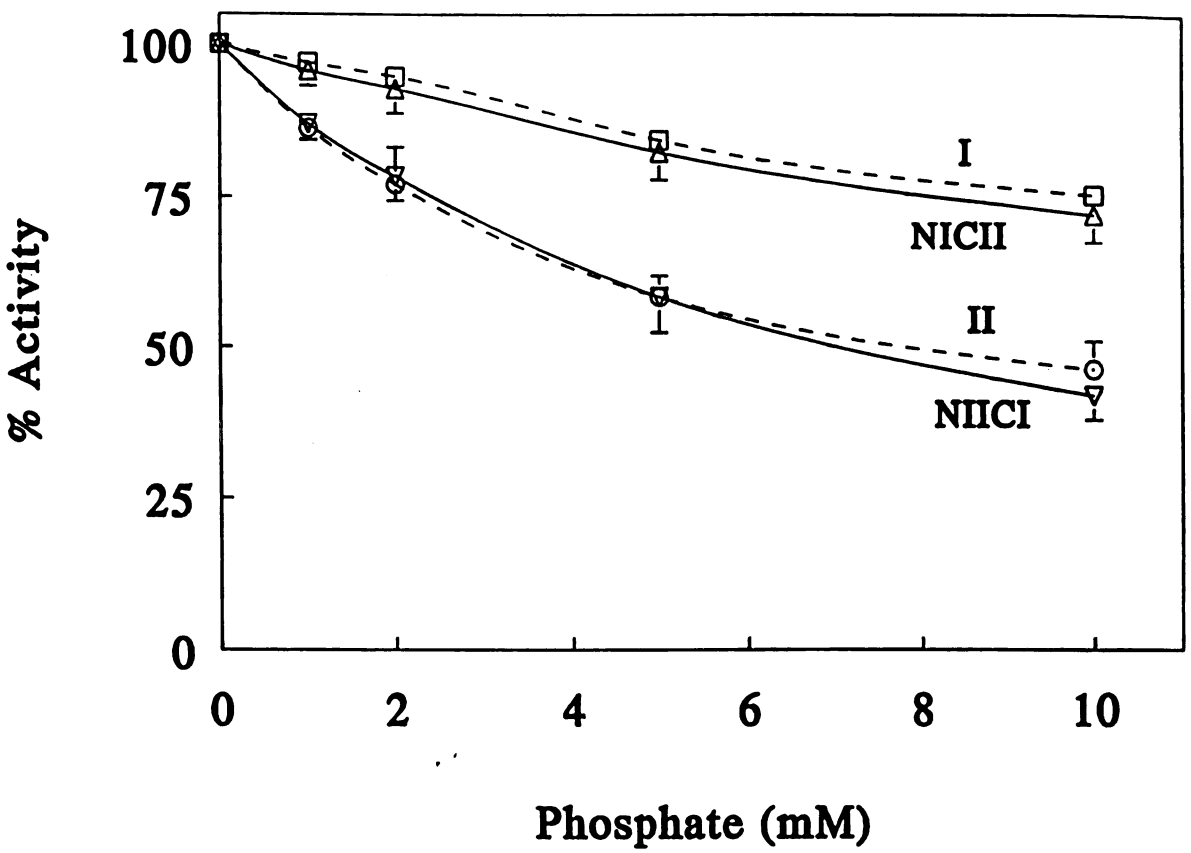

Figure 9. Inhibition of Type I, Type II, and chimeric hexokinases by P_i. Activity was determined as a function of increasing P_i concentration, with initial ATP concentration in each assay equal to the K_m value (Table II) for the respective hexokinase. (□), Type I; (○), Type II; (△), NICII; (▽), NIICI; where no error bars are seen, they are obscured by the data point symbol.

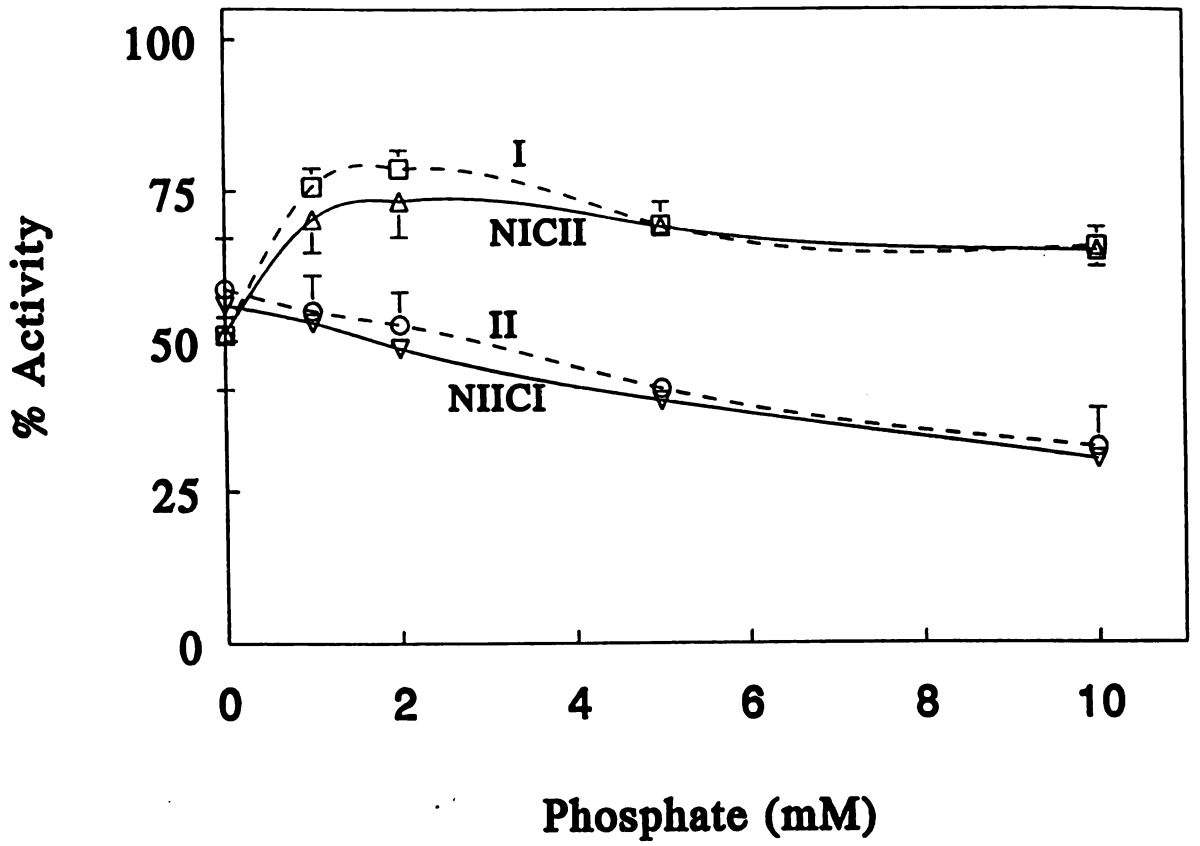

Figure 10. Effectiveness of P_i as an antagonist of inhibition by AnGlc-6-P. Activity was determined as a function of increasing P_i concentration, with initial ATP concentration in each assay equal to the K_m value (Table II) for the respective hexokinase, and AnGlc-6-P concentration sufficient to reduce the activity to approximately 50% of that seen in the absence of AnGlc-6-P. (□), Type I; (○), Type II; (△), NICII; (▽), NIICI. Values shown are mean ± SD from three experiments, each with extracts from a different transfection; where no error bars are seen, they are obscured by the data point symbol.

inhibition by AnGlc-6-P.

DISCUSSION

There are at least two possible interpretations for the observation that chimeric hexokinases can be expressed as highly active species with retention of kinetic characteristics and thermal stabilities that are comparable to the parental isozymes. One would be that the N- and C-terminal halves of these molecules exist as quasi-independent domains. If this were the case, the properties of a particular domain would essentially be independent of the other, e.g., the catalytic activity of the C-terminal domain of Type I hexokinase (15) would be fully expressed in chimeric forms, and its catalytic and regulatory properties would not depend on the identity of the N-terminal domain with which it was fused. The present work demonstrates that the latter prediction is *not* fulfilled. The inhibition by P_i and the effectiveness of P_i as an antagonist of inhibition by AnGlc-6-P are clearly dependent on the origin of the N-terminal domain.

Previous studies provide additional evidence against the view that the N- and C-terminal halves are essentially independent. Thus, if these regions were tethered together by a linking polypeptide sequence but with no significant noncovalent interactions between the halves, one might anticipate the linking polypeptide segment to be readily

susceptible to proteolysis, as is frequently the case with segments linking functionally and structurally independent domains (46). In fact, cleavage within the segment linking the N- and C-terminal halves of Type I hexokinase requires perturbation of the structure with low concentrations of a denaturing agent, guanidine hydrochloride, implying that the linking segment is "buried" in the native structure as a result of close spatial interactions between the N- and C-terminal regions (15). Moreover, if the N- and C-terminal regions were quasi-independent, then one would anticipate that conformational changes induced by the binding of ligands to a particular domain would largely be restricted to that domain. However, there is abundant evidence that binding of various ligands, including hexoses, hexose 6-phosphates, ATP, and P_i, evokes conformational effects *throughout* the molecule, affecting susceptibility to proteolytic attack or chemical modification of sulfhydryl or arginyl residues, thermal stability, and immunoreactivity with monoclonal antibodies recognizing conformational epitopes (25,47-51).

Collectively, these observations provide a solid basis for rejecting the view that the N- and C-terminal halves represent functionally and structurally discrete, quasi-independent entities. On the contrary, they lead to the conclusion that the N- and C-terminal halves of these isozymes are in intimate contact, providing a structural basis for functional interactions between these regions. Thus, an

alternative interpretation for the preservation of catalytic function in the chimeric enzymes would be that interactions between the N- and C-terminal domains have been maintained, without distortion of structural features critical for catalysis. In other words, it seems likely that interactions between the N- and C-terminal halves are similar in the Type I and Type II isozymes. In view of the extensive similarity between the amino acid sequences of these isozymes (and also that of the Type III isozyme) (1), which obviously must include segments involved in interactions between the N- and C-terminal domains, it is perhaps not surprising that this would be the case.

As noted in the introduction to this paper, the location of the functional allosteric site to which the inhibitory Glc-6-P is bound remains in dispute (20,22,23-25). The present work obviously does not resolve this controversy since there is no clear correlation between the relative susceptibility to inhibition by the Glc-6-P analogs, AnGlc-6-P and Glc-1,6-bisphosphate, and the parental origin of either the N- or C-terminal domain of the chimeric hexokinases (but see below). The present study demonstrates that the affinity for these inhibitors is markedly influenced by the identity of the N- and C-terminal domains comprising the 100 kDa enzymes. It thus seems likely that the apparent affinity for inhibitory hexose phosphates is determined by complex interactions involving *both* the N- and C-terminal domains, and depends on specific

aspects of the interdomain interactions that, despite an overall similarity, vary somewhat in both the parental isozymes as well as the chimeras.

Such ambiguity is *not* seen in the response to P_i as an inhibitor and as an antagonist of inhibition by AnGlc-6-P. It is clear that this is correlated with the origin of the N-terminal domain. The results are fully consistent with the proposal (24,25) that the *N-terminal* half of Type I hexokinase is the location of an anion binding site for which P_i and the 6-phosphate of Glc-6-P (or analogs) compete, accounting for both the mutually exclusive binding of these ligands (26,27) and the resulting antagonism of inhibition by the hexose 6-phosphates (with binding of P_i at the N-terminal site *not* being inhibitory), while the C-terminal half includes a site to which P_i binds with lower affinity and with resulting inhibition.

Finally, we note that the present study has revealed that the chimeric hexokinases differ significantly from the parental isozymes in their response to product inhibition, i.e., the marked sensitivity of the NIICI chimera, and relative insensitivity of the NICII chimera, to inhibition by Glc-6-P analogs (and presumably Glc-6-P itself). This offers an intriguing opportunity for examining the allosteric regulation of hexokinase activity *in situ* by transfection of cells with DNA encoding hexokinase forms differing substantially in their sensitivity to product inhibition.

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FOOTNOTES

[1]Abbreviations used: Glc-6-P, glucose 6-phosphate; AnGlc-6-P, 1,5-anhydroglucitol 6-phosphate; SDS, sodium dodecyl sulfate; ANOVA, analysis of variance.

Chapter III

Functional Organization of Mammalian Hexokinases: Both N- and C-Terminal Halves of the Rat Type II Isozyme Possess Catalytic Sites

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ABSTRACT

Previous work has shown that catalytic function is associated exclusively with the C-terminal half of the Type I isozyme of mammalian hexokinase. In contrast, we now demonstrate that *both* halves of the Type II isozyme possess comparable catalytic activities. Mutation of a catalytically important Ser residue to Ala at analogous positions in either the N- or C-terminal halves (S155A or S603A, respectively) of the rat Type II isozyme resulted in approximately 60% reduction in specific activity of the enzyme, with more than 90% reduction in the doubly mutated enzyme (S155A/S603A). Catalytic activity was retained in a chimeric hexokinase comprised of the N-terminal half of Type II hexokinase and catalytically inactive (by site-directed mutation) C-terminal half of the Type I isozyme. The N- and C-terminal catalytic sites of Type II hexokinase are similar in V_{max} and K_m (≈130 μM) for Glc; however, the N-terminal site has a lower (0.45 mM *vs.* 1.1 mM) K_m for ATP, is slightly more sensitive to inhibition by the product analog, 1,5-anhydroglucitol-6-P, and is much more sensitive to inhibition by P_i. It is suggested that the Type II isozyme most closely resembles the 100 kDa hexokinase which resulted from duplication and fusion of a gene encoding an ancestral 50 kDa hexokinase and which was the precursor for the contemporary Type I, Type II, and Type III mammalian isozymes. Subsequent evolutionary changes could then have led to functional differentiation of the N- and C-

terminal halves, as seen with the Type I (and possibly the Type III) isozyme.

Mammalian tissues contain three isozymes of hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1), generally designated as Types I, II, and III. These exist as monomeric species with molecular weights of approximately 100,000 and show similarity in amino acid sequence (reviewed in Ref. 1) consistent with their membership in a closely related family which also includes other kinases (2). Moreover, these isozymes exhibit internal sequence repetition, i.e. there is extensive sequence similarity between the N-terminal and C-terminal halves of the enzymes (1,3,4), and these in turn are similar to sequences of 50 kDa hexokinases such as those found in yeast (5,6) or *Schistosoma mansoni* (7). Such observations have led to the current view that the 100 kDa mammalian isozymes have evolved by duplication and fusion of a gene encoding an ancestral 50 kDa hexokinase, which also gave rise to the 50 kDa hexokinases of contemporary organisms (1).

Site-directed mutagenesis studies (8-12) have identified several amino acid residues as being catalytically important, and all of these are conserved in *both* N- and C-terminal halves of all Type I, II, or III isozymes whose sequences have been determined (1). Despite this, it is clear that the two halves of the Type I isozyme are functionally distinct, with catalytic activity associated solely with the C-terminal half (9-11,13) while the N-terminal half is catalytically inactive and thought to serve a regulatory function (13-15). Whether this same functional organization exists in the Type II and

Type III isozymes has been uncertain.

A recent study (15) of chimeric hexokinases, constructed by interchange of cDNA segments encoding the N- and C-terminal halves of the Type I and Type II isozymes followed by expression in COS1 cells, confirmed that catalytic function *was* associated with the C-terminal half of Type II hexokinase, as with the Type I isozyme. A chimera comprised of the catalytically inactive N-terminal half of Type I hexokinase and the C-terminal half of the Type II isozyme was catalytically active. However, we also noted that higher specific activities were associated with hexokinases possessing the N-terminal half of the Type II isozyme (either the Type II isozyme itself or the chimera produced by combination of the N-terminal half of Type II with the C-terminal half of Type I). As one possible explanation for this observation, we speculated that, unlike the N-terminal half of the Type I isozyme, the N-terminal half of the Type II isozyme might retain intrinsic catalytic activity. The present study has shown this to be the case. In addition to contributing to our understanding of the functional organization of the mammalian isozymes of hexokinase, these findings have implications with respect to the evolutionary relationship between these isozymes.

MATERIALS AND METHODS

Materials. Restriction enzymes, T4 DNA ligase, and Glc-6-P[1] dehydrogenase were purchased from Boehringer Mannheim (Indianapolis, IN) and other biochemicals from Sigma Chemical Co. (St. Louis, MO). Plasmid DNAs were purified using plasmid purification kits obtained from Qiagen, Inc. (Chatsworth, CA). DMEM (high glucose) for culture of COS1 cells was a product of GIBCO BRL (Gaithersburg, MD), and supplemented with fetal bovine serum (2%) and defined bovine calf serum (8%) from HyClone Laboratories (Logan, UT). The BCA Protein Assay Reagent and BSA standard, as well as the Imject Activation Immunogen Conjugation Kit were purchased from Pierce Chemical Co. (Rockford, IL).

Site-directed mutagenesis and construction of vectors for expression in COS1 cells. A previously described (15) full-length cDNA encoding rat Type II hexokinase was used. Sequence encoding the N-terminal half of the enzyme is encoded by an *Eco* RI-*Nco* I fragment, while a complementary *Nco* I-*Pst* I fragment encodes the C-terminal half. This division was convenient for selectively generating site-directed mutations within the N- or C-terminal halves, and then reconstructing full-length coding sequences with any desired mutations.

Site-directed mutagenesis was done using the Altered Sites kit and pSelect vector from Promega (Madison, WI), as previously described in detail (10). An *Nco* I site had been

engineered (10) into the multiple cloning site of the pSelect vector to facilitate manipulations. The *Eco* RI-*Nco* I or *Nco* I-*Pst* I fragments were cloned into the modified pSelect to give vectors containing sequence encoding the N- or C-terminal halves, respectively. Mutations were made at a Ser residue that is conserved in both N- and C-terminal halves of the mammalian hexokinases (1) and which was previously shown to be of critical importance for catalytic function in the Type I isozyme (8,10). Mutated forms of the rat Type II isozyme with Ser converted to Ala in the N-terminal half, the C-terminal half, or both halves are designated as S155A, S603A, or S155A/S603A, respectively. The mutant S155A was made using the oligonucleotide GGTTTCACCTTCGCGTTCCCCTG, where the underlined base corresponds to conversion of the Ser codon (TCG) to the Ala codon (GCG); the analogous mutation in the C-terminal half, S603A, was made using the oligonucleotide GGTTTCACATTCGCCTTCCCTTG, with the underlined base resulting in conversion of the TCC coding for Ser to GCC coding for Ala. Complete coding sequences for the Type II isozyme, with any desired mutations, were reconstructed by ligation of complementary fragments and cloning in pUC18. The construct was then subcloned into pSVT7 for expresssion in COS1 cells as previously described (15). All mutations were confirmed by sequencing, first in the pSelect vector in which the mutation had been made, and then again after transfer of the mutated construct to pSVT7.

Expression of Type II hexokinase and mutants in COS1 cells. The transfection procedure was exactly as described previously. Sham transfected cells were treated identically except that no plasmid DNA was added. Cells were harvested 3 days after transfection and extracts prepared as previously described (15) except that the sonicate was centrifuged at 15,000 x g for 10 min prior to analysis. Protein and hexokinase activity were determined immediately after preparation of the extracts, which were then stored at -80° C for further use.

Determination of hexokinase activity and protein. Hexokinase activity was determined using a spectrophotometric assay in which Glc-6-P formation is coupled to NADPH production, monitored at 340 nm, in the presence of excess Glc-6-P dehydrogenase (16). The K_ms for substrates, Glc and ATP, were determined under the same conditions, except for appropriate variations in the concentration of the relevant substrate. Prior to determination of the K_m for Glc, Glc present in the extraction buffer was removed by chromatography on spin columns of Sephadex G-25 (fine), equilibrated with 50 mM Hepes, 0.5 mM EDTA, 10 mM thioglycerol, pH 7.5; absence of Glc in the eluted enzyme was confirmed by enzymatic assay. All kinetic data conformed to Michaelis-Menten behavior and were analyzed using the EZ Fit program of Perrella (17). Inhibition by the Glc-6-P analog, AnGlc-6-P, and by P_i and its analogs, sulfate and arsenate, was determined as in previous studies

(10,15).

Protein was assayed using the BCA Protein Assay reagent. Samples were pretreated with iodoacetamide (18) to avoid interference from thioglycerol present in the extraction buffer.

Production of a polyclonal antiserum specific for the Type II isozyme of hexokinase. While the isozymes of mammalian hexokinase exhibit extensive similarity throughout virtually all of their amino acid sequences, their C-terminal sequences are unique (1). An octapeptide having the sequence CIREAGQR was synthesized in the Macromolecular Structure, Sequencing & Synthesis Facility, Michigan State University, with purity and identity confirmed by MALDI TOF mass spectrometry. The last seven residues in this peptide correspond to the C-terminal sequence of the rat Type II isozyme of hexokinase, which is quite distinct from that of the Type I or Type III isozymes; the Cys residue at the N-terminus was included to facilitate conjugation to carrier protein for immunization. The latter was done, with keyhole limpet hemocyanin as carrier, using the Imject Conjugation Kit from Pierce Chemical Co. The conjugate was provided to University Laboratory Animal Resources, Michigan State University, for production of antiserum. A rabbit was immunized with 100 μg of the conjugate, emulsified in Hunter's Titer Max, with subsequent booster injections approximately one and two months later. Titer was determined by ELISA, essentially as described previously (19), except

that wells of the microtiter plate were coated with BSA that had been conjugated with the above peptide, again using the conjugation kit from Pierce Chemical Co. Several bleedings showing good titer were combined and used for these experiments.

SDS gel electrophoresis and immunoblotting. Procedures for SDS gel electrophoresis were essentially as described earlier (11,20) except that blotting was done using a Trans-Blot SD Transfer Cell (BioRad Laboratories, Richmond, CA). Immunoblots were analyzed quantitatively (11) using the GDS-2000 gel documentation system and associated software from UVP, Inc. (San Gabriel, CA).

Sequence comparisons. Sequences of the N- and C-terminal halves of the isozymes were compared using the BESTFIT routine of the GCG Sequence Analysis Software Package (Genetics Computer Group, Inc., Madison, WI). Default weighting values (gap penalty = 3.00; gap extension penalty = 0.10) were used. Sequences used in comparisons were: residues 1-475, 1-475, and 1-488 for the N-terminal halves of Types I, II, and III, respectively; residues 476-918, 476-917, and 489-924 for the respective C-terminal halves.

RESULTS

Expression of Type II hexokinase and mutant forms in COS1 cells. Specific activities of hexokinase in extracts from COS1 cells transfected with pSVT7 constructs encoding the wild type

Type II isozyme or mutant forms are shown in Table I. As in the previous study (15), transfection with the cDNA for the wild type isozyme resulted in hexokinase activities that were well above those in sham tranfected cells, i.e., above endogenous levels of hexokinase in COS1 cells. Transfection with cDNAs encoding the singly mutated forms, S155A or S603A, gave activities that were also well above those in sham transfected cells, but lower than that with wild type cDNA. Hexokinase activities in extracts from cells transfected with cDNA encoding the double mutant, S155A/S603A, were markedly reduced but still higher than those of sham transfected cells.

Examination of extracts by SDS gel electrophoresis (Fig. 1) disclosed the expected presence of an additional component in extracts from transfected cells, migrating slightly ahead of the Type I hexokinase (16) used as marker; no other differences were detected between transfected cells and the sham transfected control. It has previously been noted (15) that, despite the close similarity in actual molecular weight of the Type I and Type II isozymes, the Type II isozyme migrates slightly faster on SDS gels. It was not possible to quantitate the relative amounts of these expressed species by densitometric methods due to inadequate resolution from other closely adjacent components. However, visual estimation of the relative staining intensities indicated that the mutant forms (both single and double mutant) were present in amounts somewhat greater than the wild type enzyme. This was supported

Table I. Hexokinase Activity in Extracts of COS1 Cells Transfected with cDNAs Encoding Wild Type and Mutant Forms of Type II Hexokinase

cDNA used for transfection	Hexokinase activity[a] (units/mg protein)
Sham control	0.05 ± 0.02
Wild type	0.76 ± 0.25
S155A	0.52 ± 0.16
S603A	0.51 ± 0.21
S155A/S603A	0.09 ± 0.03

[a] Mean ± SD for 11 different transfections. ANOVA analysis and t-test indicated significant differences ($p<0.01$) between sham control and all other values, between wild type and all mutants, and between the single mutants and the double mutant; there was no significant difference between the S155A and S603A mutants.

Figure 1. SDS-gel electrophoretic analysis of COS1 cell extracts. Lane 1, Type I hexokinase purified from rat brain (16); lane 2, extract from sham-transfected COS1 cells; lanes 3, extract from COS1 cells transfected with cDNA encoding wild type Type II hexokinase; lanes 4-6, extracts from COS1 cells transfected with cDNAs encoding mutants S155A, S603A, and the double mutant S155A/S603A, respectively. The gel was stained with Coomassie Blue. The position of the Type II isozyme, and mutant forms, is indicated by the arrow at the right.

by immunoblotting results (Fig. 2) which indicated more intense staining of bands corresponding to the mutant forms. Since the epitope recognized by the antiserum is the C-terminal sequence common to the wild type and mutant forms, it is reasonable to assume that the immunoreactivity of the various forms is equivalent, and thus that more intense staining on the blot is indicative of higher levels of the particular protein.

Relative specific activities of wild type and mutant forms of Type II hexokinase. Staining intensity of the Type II hexokinase band on immunoblots increased linearly with the amount of extract from cells transfected with the wild type enzyme (Fig. 3). Since it is reasonable to assume that mutant and wild type forms exhibit equivalent immunoreactivity, it is therefore possible to estimate the relative amounts of wild type and mutant proteins based on relative staining intensities. On this basis, the mutants S155A, S603A, and S155A/S603A were expressed at levels 1.5- to 2-fold higher than the wild type enzyme.

Knowing the relative amounts of these enzymes present in the extracts (from immunoblotting), as well as the specific activities of hexokinase present in the extracts (e.g., Table I), it was possible to calculate the relative specific activities of the mutant and wild type forms. The specific activity of the endogenous COS1 enzyme in cell extracts was assumed to be constant and equal to that of the sham

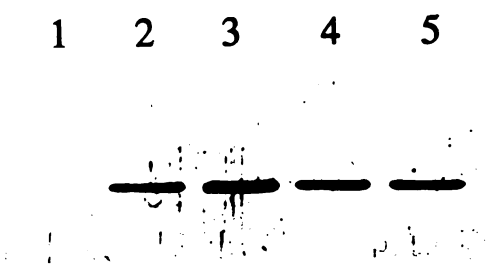

Figure 2. Immunoblot analysis of COS1 cell extracts. Extracts were from: lane 1, sham-transfected cells; lanes 2-5, cells transfected with cDNAs encoding wild type Type II hexokinase, S155A, S603A, or S155A/S603A, respectively. The blot was probed with antiserum raised against a peptide corresponding to sequence at the C-terminus of rat Type II hexokinase.

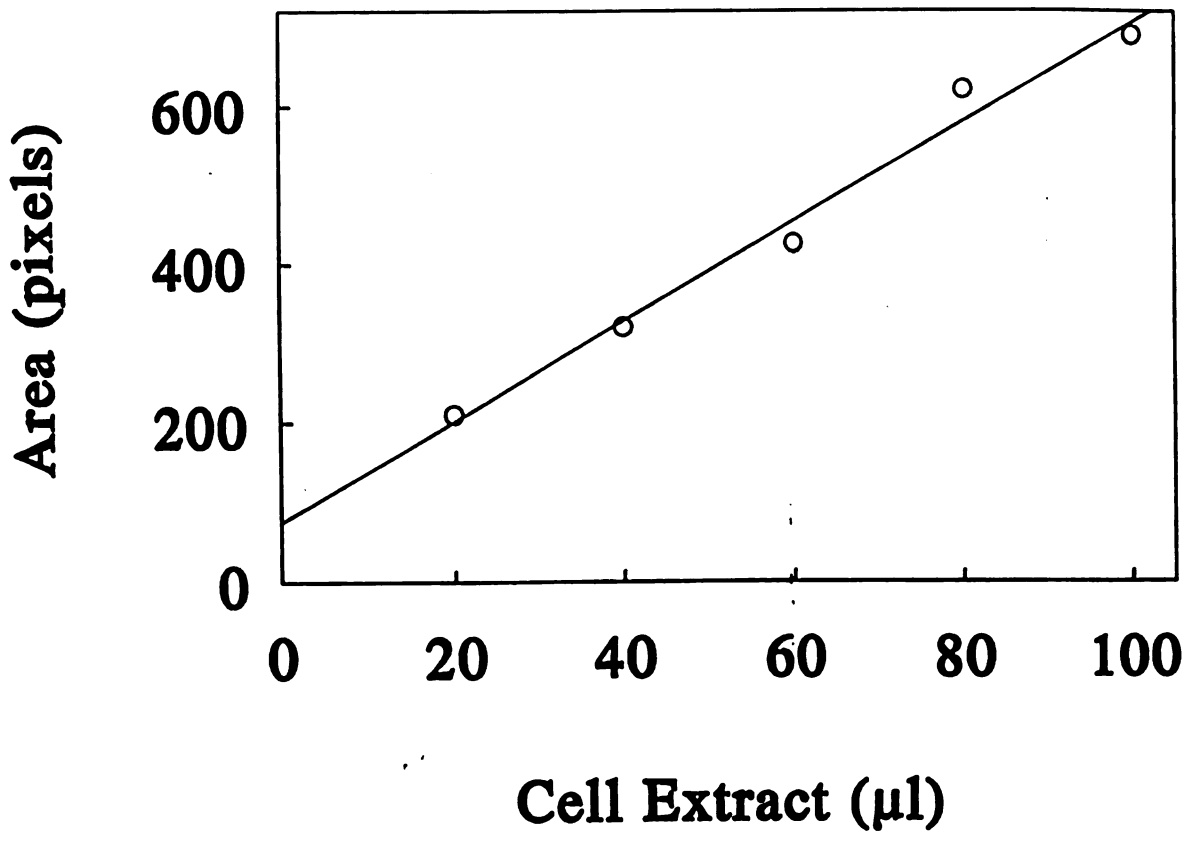

Figure 3. Densitometric analysis of an immunoblot. Increasing amounts of an extract from COS1 cells transfected with cDNA encoding the wild type Type II isozyme were used, and the intensity of the bands on the immunoblot was quantitated as described in Methods. The resulting standard curve was then used to estimate quantities of the mutant forms, S155A, S603A, or S155A/S603A, present in extracts from cells transfected with the respective cDNAs; the latter extracts were included on the same blot used for generating the standard curve.

transfected control; this value was subtracted from the specific activities seen in extracts from cells transfected with wild type or mutant cDNAs. The specific activity of a mutant form, relative to that of the wild type enzyme, could then be calculated as the ratio of the activity present in the "mutant" extract and that present in a "wild type" extract containing an equivalent amount of the expressed isozyme. On this basis, the specific activities of the S155A, S603A, and S155A/S603A mutants were calculated to be 36%, 43%, and 4% of the wild type isozyme, respectively. In another determination, using extracts from a different transfection experiment, the corresponding values were quite similar, being 33%, 40%, and 7%, respectively.

Kinetic parameters and regulatory properties of wild type and mutant forms of Type II hexokinase. The K_ms of the expressed wild type enzyme as well as the S155A and S603A mutants are shown in Table II; the various forms were not distinguishable in their apparent affinity for Glc, but differed significantly in apparent affinity for ATP. The mutant and wild type isozymes did not differ greatly in their sensitivity to inhibition by AnGlc-6-P (Fig. 4), although S155A was slightly less susceptible, and S603A slightly more susceptible, than the wild type Type II isozyme. In contrast, the S155A mutant was markedly less sensitive, and the S603A mutant considerably more sensitive than wild type Type II hexokinase to inhibition by P_i (Fig. 5), previously shown to

Table II. Kinetic Properties of Wild Type and Mutant Forms of Type II Hexokinase

Extract from cells transfected with cDNA for	K_m for Glucose (μM)[a]	ATP (mM)[b]
Wild type	146 ± 22	0.64 ± 0.04
S155A	126 ± 15	1.09 ± 0.20
S603A	143 ± 10	0.45 ± 0.05

[a]Mean ± SD for three determinations, each with an extract from a different transfection.
[b]Mean ± SD for six determinations, each with an extract from a different transfection.

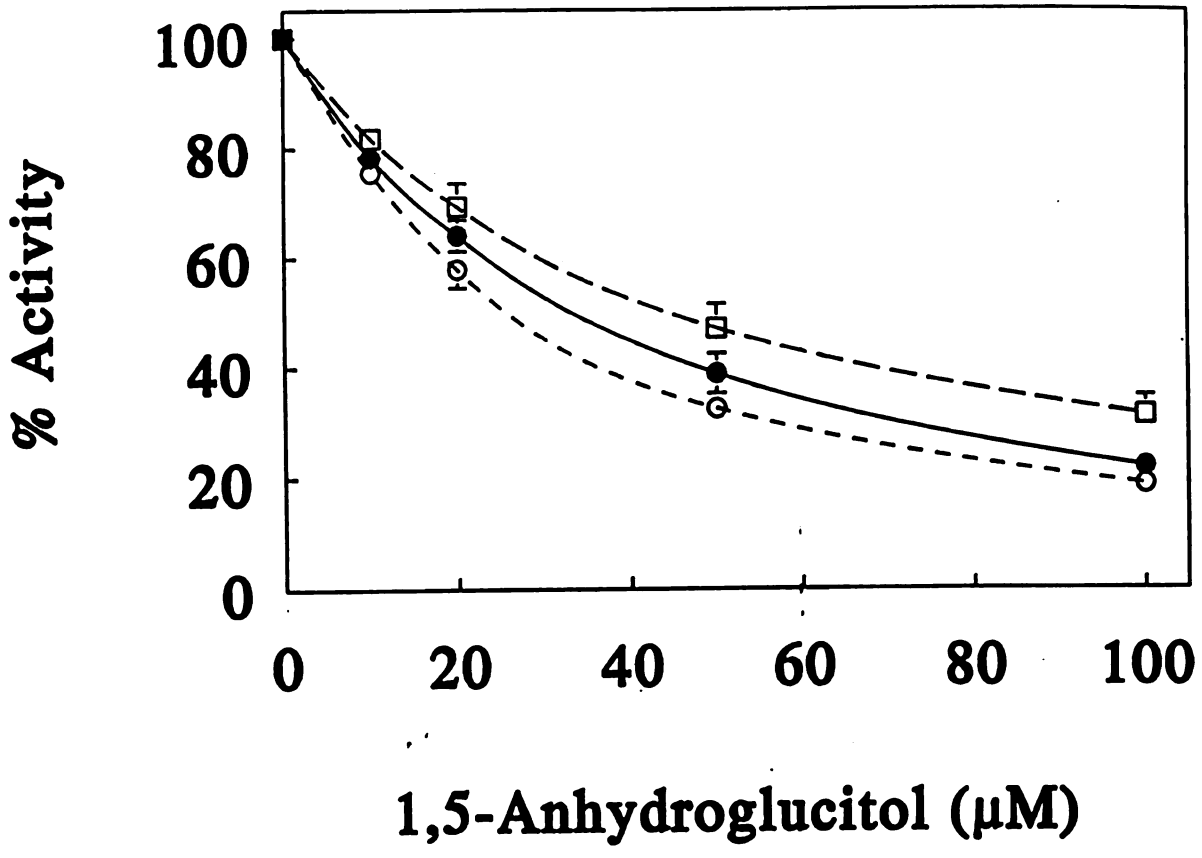

Figure 4. Inhibition of wild type and mutant forms of Type II hexokinase by the Glc-6-P analog, 1,5-anhydroglucitol-6-P. Activity was determined as a function of increasing inhibitor concentrations, with initial ATP concentration in each assay equal to the K_m value (Table II) for the respective enzyme. (●), Wild type Type II hexokinase; (□), S155A; (○), S603A.

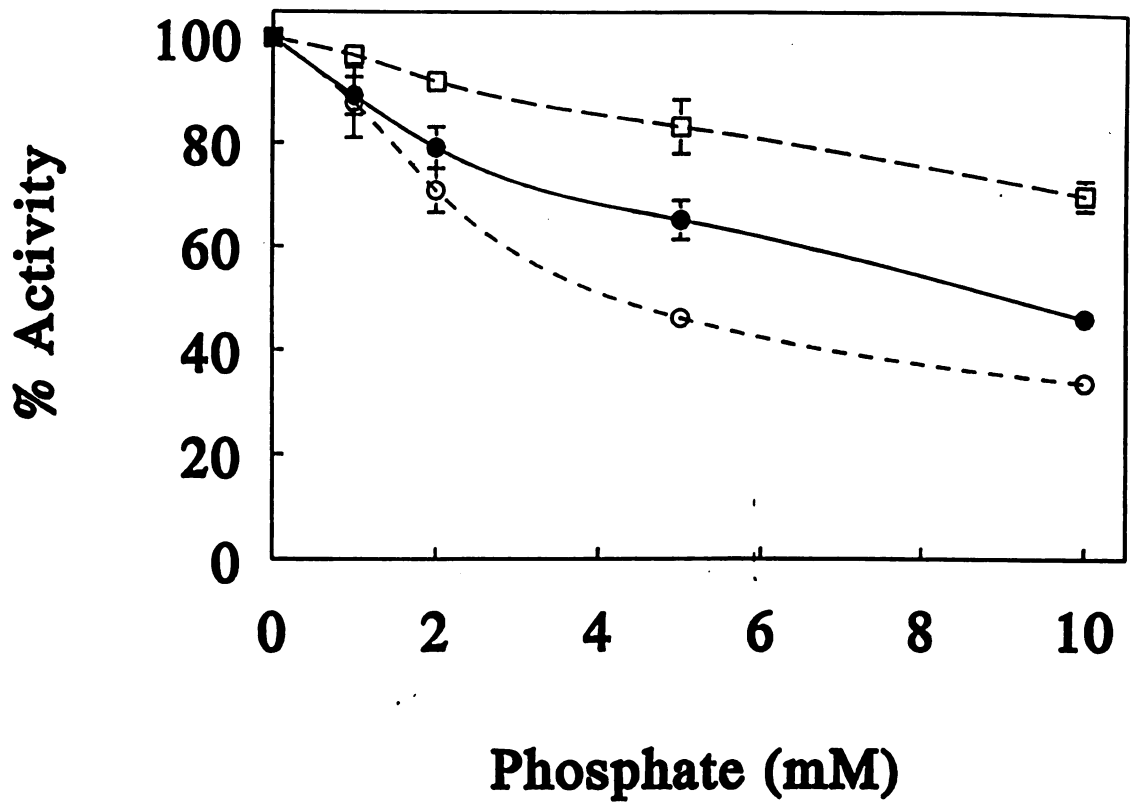

Figure 5. Inhibition of wild type and mutant forms of Type II hexokinase by inorganic phosphate. Activity was determined as a function of increasing phosphate concentrations, with initial ATP concentration in each assay equal to the K_m value (Table II) for the respective enzyme. (●), Wild type Type II hexokinase; (□), S155A; (○), S603A.

inhibit competitively *vs*. ATP (21). Similar results (not shown) were seen for inhibition by the P_i analogs, sulfate and arsenate.

Retention of activity in a chimeric hexokinase, constructed from an inactivated C-terminal (catalytic) domain of Type I and the N-terminal half of Type II hexokinase. It is apparent that the most straightforward interpretation of the above results is that *both* N- and C-terminal halves of Type II hexokinase possess intrinsic, and comparable, catalytic activities. Thus, mutation of a catalytically important Ser residue in either half results in reduction of specific activity to somewhat less than half of wild type, while the double mutation results in almost complete loss of activity. This was further examined by an alternative strategy based on previous work from this laboratory (10,15).

Baijal and Wilson (10) showed that mutation of Ser 603 to Ala resulted in virtually complete loss of catalytic activity in the Type I isozyme. Using the same strategy previously used to prepare chimeric enzymes from complementary halves of the Type I and Type II isozymes (15), a chimera was prepared in which the N-terminal half of Type II was combined with a C-terminal half of Type I, but with Ser 603 changed to Ala in the latter (10). It has previously been shown (10,15) that the wild type Type I isozyme, the Type I mutant S603A, and the NIICI chimera (comprised of the N-terminal half of Type II hexokinase and the C-terminal half of the Type I isozyme) are

all expressed at similar levels under these conditions. This was confirmed in the present study and, based on staining intensity with Coomassie Blue, the mutated chimera NIICI(S603A) was also expressed in amounts comparable to the other forms (results not shown). Activities seen in extracts of COS1 cells transfected with these constructs are shown in Table III; similar results were seen in other experiments employing various subsets of these cDNAs. In confirmation of previous reports, these results demonstrate the devastating effect of the S603A mutation on activity of the Type I isozyme (8,10) and the enhanced activity seen with the NIICI chimera (15), the latter being the observation that led to the present study (see comments in Introduction). Most interesting in the present context is the last entry in Table III, demonstrating retention of substantial catalytic activity in the chimeric enzyme produced from the N-terminal half of Type II and an inactive C-terminal half of Type I hexokinase. This clearly supports assignation of catalytic activity directly to the N-terminal half of the Type II isozyme.

DISCUSSION

The catalytic sites in the N- and C-terminal halves of the Type II isozyme appear to function more-or-less independently - for example, there is no indication of cooperativity in binding of substrates. Moreover, they have

Table III. Hexokinase Activity in Extracts from COS1 Cells Transfected with cDNAs Encoding Wild Type and Chimeric Forms of Hexokinase

cDNA used for transfection	Hexokinase Activity (units/mg protein)
Sham	0.05
Type I	1.14
Mutant Type I(S603A)	0.07
NIICI chimera	1.87
NIICI(S603A) chimera	0.66

comparable V_{max} values, with mutation of analogous catalytically important residues (Ser 155 or Ser 603) in either half having similar effect on specific activity[2]. The two sites do differ significantly in K_m for ATP but not for Glc, with the catalytic site in the N-terminal half having the higher apparent affinity for this nucleotide substrate. In principle, differences in K_m should lead to deviation from monophasic Michaelis-Menten kinetics, but this would not be expected to be detectable with the relatively slight difference in the K_ms of the N- and C-terminal sites for ATP (22). Indeed, simulation of kinetic results that would be expected from two sites functioning with the same V_{max} but with K_ms for ATP of 1.09 and 0.45 mM (Table II) was consistent with monophasic Michaelis-Menten behavior and an apparent K_m of 0.66 mM, virtually identical with the value found for the Type II isozyme (Table II).

Deviation from simple Michaelis-Menten behavior might also be predicted for the single mutants, S155A and S603A, since they were comprised of N- and C-terminal halves with markedly different V_{max} values (22). However, this Ser to Ala mutation results in reduction of V_{max} to approximately 5% of V_{max} for the unmodified enzyme (8,10, and present work). Simulation demonstrates that the kinetics would be dominated by the unmodified site, consistent with the observation of monophasic Michaelis-Menten behavior for the mutant enzymes.

Okazaki *et al*. (23) have previously reported that the C-terminal half of Type II hexokinase was preferentially labeled by the ATP analog, 2′,3′-dialdehyde ATP. These authors took this to indicate that the catalytic site was associated solely with the C-terminal half, as with the Type I isozyme (9-11,13). The present study indicates that an alternative explanation is necessary, perhaps a difference in the effectiveness with which this analog labels the ATP binding sites in the N- and C-terminal halves of Type II hexokinase. This is not unreasonable since the differences in K_m for ATP do, in fact, indicate that the two sites are not identical.

The two sites also differ in their sensitivity to inhibition by P_i, with the N-terminal site having greater affinity for this effector. Type I hexokinase also has two sites for binding of P_i, with the higher affinity site again being associated with the N-terminal half (14,15,24). However, *functionally*, the effects of binding to these N-terminal sites are quite different. With the Type II isozyme, binding results in inhibition. In contrast, with the Type I isozyme, binding of P_i to the high affinity site in the N-terminal half does not lead to inhibition, but rather, antagonizes the binding of Glc-6-P and thereby the inhibition by this product; in effect, binding of P_i at the N-terminal site serves an activating function with the Type I isozyme. It has previously been noted (21,25) that these different responses to the physiologically relevant modulator, P_i, are likely to be a significant factor

in defining distinct metabolic roles for these two isozymes.

As depicted schematically in Fig. 6, the 100 kDa mammalian hexokinases appear to have evolved by duplication and fusion of a gene for an ancestral 50 kDa hexokinase (1,13). This 50 kDa ancestral hexokinase had already acquired sensitivity to inhibition by the product, Glc-6-P, and thus resembled the hexokinase found in present-day organisms such as starfish (13) or the parasitic *S. mansoni* (26). The initial 100 kDa hexokinase produced in this fashion would have possessed two catalytic sites, one in each half and both subject to regulation by the product Glc-6-P, as found for the Type II isozyme. We thus suggest that the Type II isozyme is the more "ancient", most closely resembling the ancestral hexokinase first generated by the gene duplication and fusion event. Subsequent evolutionary changes resulted in differentiation of function in the N- and C-terminal halves, as found for the Type I isozyme. The functional organization of the Type III isozyme remains to be determined.

The N- and C-terminal halves of Type II hexokinase exhibit 60% identity in amino acid sequence. The comparable values for the Type I and Type III isozymes are 51% and 47%. The greater similarity between the halves of the Type II isozyme is also consistent with this isozyme being most closely related to the ancestral 100 kDa product of gene duplication and fusion (for which there would have been, presumably, 100% identity between the halves). The lower value

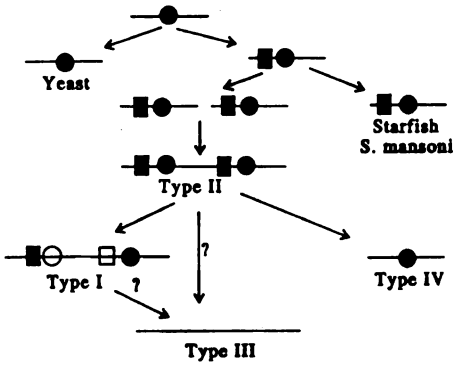

Figure 6. Evolution of the isozymes of mammalian hexokinase. Relative molecular weights (approximately 50,000 or 100,000) are represented by the length of the horizontal lines. Catalytic sites are represented by filled circles, and regulatory sites, at which Glc-6-P is bound with resulting inhibition, are represented by filled squares. Hexokinases are viewed as evolving from an ancestral 50 kDa hexokinase, insensitive to regulation by Glc-6-P, similar to the enzyme found in present-day yeast. Acquisition of a regulatory site resulted in an ancestral 50 kDa, Glc-6-P-sensitive form whose descendants persist in present day organisms such as starfish and *S. mansoni*. Duplication and fusion of the gene encoding this ancestral form gave rise to the 100 kDa, Glc-6-P-sensitive mammalian isozymes. The initial product of this duplication and fusion event would be expected to possess two catalytic sites, each sensitive to regulation by Glc-6-P. As shown in the present study, Type II hexokinase has these characteristics and accordingly, is suggested to most closely resemble this ancestral form. The Type I isozyme evolved by further mutations that resulted in functional differentiation of the two halves, the C-terminal half retaining catalytic function while the N-terminal half took on additional regulatory roles; in the course of the latter process, the Glc binding site in the N-terminal half, and the Glc-6-P binding site in the C-terminal half, of the Type I isozyme became latent (indicated by the open symbols) - see refs. 1, 13, or 24 for more extensive comments about the functional organization of the Type I isozyme. The disposition of catalytic and regulatory functions within the Type III isozyme remains to be determined. Although the mammalian Type IV isozyme ("glucokinase") is a 50 kDa enzyme, like the hexokinases of yeast, starfish, and *S. mansoni*, sequence comparisons indicate that it is much more closely related to the 100 kDa mammalian isozymes. Thus, Type IV hexokinase is suggested (28,31) to have evolved by a resplitting of the gene, with additional mutations resulting in loss of sensitivity to Glc-6-P.

for the Type I isozyme is consistent with further mutations giving rise to the known functional differentiation in this isozyme. Based on the still lower identity between the two halves of Type III hexokinase, we predict that functional differentiation will also be found between the halves of the Type III isozyme (though not necessarily the same as that seen with the Type I isozyme).

A fourth isozyme, Type IV hexokinase, more commonly called glucokinase, is also found in mammalian tissues, principally liver and pancreas but also in selected cells in brain and gut (27). In contrast to Types I-III, the molecular weight of Type IV hexokinase is about 50,000, and it is insensitive to inhibition by product Glc-6-P (1). Based on sequence comparisons (1,2,28), Type IV hexokinase is much more closely related to the 100 kDa mammalian hexokinases than it is to the 50 kDa hexokinases found in yeast or *S. mansoni*; moreover, there is conservation of the intron/exon structure in the genes for Types II and IV hexokinase (29,30), further implying very close relationship between these two species. We thus agree with the suggestions of Ureta (31) and Griffin *et al.* (28) that the Type IV hexokinase most likely arose from a resplitting of the gene encoding a 100 kDa "mammalian" hexokinase (Fig. 6), rather than evolving independently from an ancestral 50 kDa hexokinase that was the precursor for the 100 kDa enzymes (29,30).

After submission of this manuscript, we learned of the

recently published results of Ardehali *et al.* (32), which are in agreement with the present study, i.e., catalytic activity is associated with both N- and C-terminal halves of Type II hexokinase. (We are grateful to Dr. Daryl Granner for providing us with a preprint of this publication.) Ardehali *et al.* (32) also found that, relative to the N-terminal site, the C-terminal site had a somewhat higher K_m for ATP and decreased sensitivity to inhibition by Glc-6-P. However, the magnitude of these differences was considerably greater than observed in the present work, probably owing to differences in kinetic methods used. It is of particular note that Ardehali *et al.* (32) expressed the individual halves of the Type II isozyme in catalytically active form, demonstrating that - as previously shown for the Type I isozyme (9,12,13) - catalytic function does not require interactions with the complementary half.

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FOOTNOTES

[1]Abbreviations used: Glc-6-P, glucose 6-phosphate; AnGlc-6-P, 1,5-anhydroglucitol 6-phosphate; DMEM, Dulbecco's modified Eagle's medium; Hepes, N-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MALDI TOF, matrix assisted laser desorption ionization time of flight; ELISA, enzyme-linked immunosorbant assay; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; ANOVA, analysis of variance.

[2]If both sites had exactly the same V_{max} and were completely independent, one would obviously expect mutations in either half to reduce the specific activity to approximately 50%. This is close to, but not identical with, the 60-70% reduction estimated experimentally. Whether this discrepancy reflects errors in the estimation, or greater complexity in the effect of mutation, or both, is uncertain.

Chapter IV

Functional Organization of Mammalian Hexokinases: Characterization of the Rat Type III Isozyme and Its Chimeric Forms, Constructed with the N- and C-Terminal Halves of the Type I and Type II Isozymes

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ABSTRACT

Previous studies have shown that catalytic function is associated with both halves of the Type II isozyme of mammalian hexokinase, while the Type I isozyme is functionally differentiated into a catalytic C-terminal half and regulatory N-terminal half. The Type III isozyme has now been shown to be similar to the Type I isozyme in its functional organization. Chimeras comprised of the N-terminal half of Type III hexokinase and the C-terminal half of either Type I or Type II hexokinase have activities that can be attributed to the C-terminal half, and are similar in activity to chimeras comprised of the C-terminal half of Type III and the intrinsically inactive N-terminal domain of Type I or the inactivated (by site-directed mutation) N-terminal half of Type II hexokinase. Virtually no activity was seen with chimeras constructed with the N-terminal half of the Type III isozyme and catalytically inactive (by site-directed mutation) C-terminal halves of Type I or Type II hexokinase. Substrate inhibition by Glc is seen only with the Type III isozyme and with chimeric forms containing the C-terminal half of Type III hexokinase and the N-terminal half of Type I or Type II isozyme, the latter inactivated by site-directed mutation; this is attributed to conformational changes induced by binding of Glc to a low affinity site in the N-terminal half, with subsequent effect on catalytic activity of the C-terminal half. These results also provide further insight into the role

of interactions (or lack of interactions) between the N- and C-terminal halves in the inhibition of the Type I-III isozymes by Glc-6-P, its antagonism by low concentrations of P_i, and the inhibition seen at higher concentrations of P_i.

Three isozymes of hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) with molecular weights of approximately 100,000 are found in mammalian tissues (see Ref. 1 for review). The Type I, Type II, and Type III isozymes differ in their tissue distribution, in their tendency to associate with specific subcellular structures (Types I and II with mitochondria, Type III with the nuclear periphery), and in their kinetic and regulatory properties. It is reasonable to presume that these differences adapt the three isozymes for distinct roles in mammalian glucose metabolism.

Despite their differences, the 100 kDa mammalian isozymes also exhibit notable similarities. For example, all three isozymes are sensitive to inhibition by physiologically relevant levels of the product, Glc-6-P^1, and this is generally considered to be a major factor in regulation of activity *in vivo*. In addition, all three isozymes show striking similarity throughout their amino acid sequence. Moreover, there is internal sequence repetition, i.e., in each isozyme, the sequences of the N-terminal and C-terminal halves are similar, both to each other and to the sequences of 50 kDa hexokinases found in lower eukaryotes such as yeast or *Schistosoma mansoni*. Thus, the 100 kDa mammalian isozymes are thought to have evolved by duplication and fusion of a gene encoding an ancestral 50 kDa hexokinase.

The initial product of a gene duplication and fusion event would be expected to be a 100 kDa enzyme consisting of

two halves that are functionally equivalent. It is now clear that this is the case for the Type II isozyme (2,3). Both the N- and C-terminal halves have been shown to possess catalytic activity that is responsive to inhibition by Glc-6-P. In contrast, the two halves of the Type I isozyme are functionally distinct, with catalytic activity associated solely with the C-terminal half (4-7) while regulatory function, i.e., the site at which the inhibitory Glc-6-P is bound, has been ascribed to the N-terminal half (4,8,9)[2]. The Type II isozyme has been proposed (3) to be the more "ancient", i.e., to most closely resemble the original 100 kDa hexokinase generated by duplication and fusion of the gene encoding the ancestral 50 kDa form. Duplication of the gene encoding this initial 100 kDa form would then give rise to additional 100 kDa isozymes which could undergo further mutations leading to functional differentiation of the N- and C-terminal halves. As noted previously (3), the sequence similarity - 60% identity - between the N- and C-terminal halves of the Type II isozyme is more extensive than the 51% identity in sequences of the two halves of Type I hexokinase. Moreover, when the three isozymes are compared, there is considerably greater conservation of sequence in the C-terminal halves than there is in the N-terminal halves (17). These observations are consistent with the view that functional differentiation of the Type I isozyme resulted from mutations in the N-terminal half, while greater sequence

conservation, with preservation of catalytic function, occurred in the C-terminal half. Based on even less similarity - 47% identity - between the sequences of the N- and C-terminal halves of the Type III isozyme, it was predicted (3) that this isozyme, like the Type I isozyme, would exhibit functional differentiation between its N- and C-terminal halves.

The functional organization of rat Type III hexokinase has been determined in the present study. In addition, chimeric constructs, comprised of the N- or C-terminal half of Type III hexokinase and the complementary half from the Type I or Type II isozyme, have been expressed and characterized. The results provide additional insight into the role of interactions between the N- and C-terminal halves in determining the kinetic and regulatory properties of the mammalian isozymes, Types I-III.

MATERIALS AND METHODS

Materials. Restriction enzymes, T4 DNA ligase, and yeast Glc-6-P dehydrogenase were purchased from Boehringer Mannheim (Indianapolis, IN). NADP was from United States Biochemical (Cleveland, OH), and other biochemicals were products of Sigma Chemical (St. Louis, MO). Site-directed mutations were done using the Altered Sites Kit and pSelect vector from Promega (Madison, WI). For DNA sequencing, the Sequenase Version 2.0

DNA Sequencing Kit from United States Biochemical was used. The pcDNA3 expression vector was from Invitrogen (San Diego, CA). Plasmid DNAs were purified using kits from Qiagen (Chatsworth, CA). Ham's F-12 medium and DMEM, as well as the transfection reagent, lipofectAMINE, were products of Gibco BRL (Gaithersburg, MD). Sera used for cell culture were purchased from HyClone Laboratories (Logan, UT). The BCA Protein Assay Reagent and BSA standard were from Pierce Chemical (Rockford, IL). Antibodies used for immunoblotting - polyclonal (rabbit) antiserum against the Type I (18) and Type II (3) isozymes, monoclonal antibodies 5A and 21 reacting with the Type I isozyme (19), and monoclonal antibody C7C3 specific for the Type III isozyme (20) - have previously been described.

Determination of hexokinase activity and protein. Hexokinase activity and protein were determined exactly as previously described (9,21). Briefly, hexokinase activity was determined by coupling Glc-6-P formation to NADPH production, monitored at 340 nm, in the presence of excess Glc-6-P dehydrogenase. Kinetic parameters were determined under standard assay conditions except for variation in the concentration of the relevant substrate; the K_ms for glucose and ATP determined in this way were actually *apparent* K_ms but the distinction is not critical in this context and, for brevity, we refer to them simply as "K_m". Kinetic data were analyzed using the EZ-Fit program of Perrella (22). Inhibition

by P_i or the Glc-6-P analog, 1,5-AnGlc-6-P, were determined as in our previous studies (3,9); both of these inhibitors are competitive *vs* ATP.

Protein was determined with the BCA Protein Assay Reagent, with BSA as standard; samples were pretreated with excess iodoacetamide (23) to avoid interference from the thioglycerol present in the samples.

Electrophoretic methods and immunoblotting. Procedures for SDS gel electrophoresis and immunoblotting, and for non-denaturing electrophoresis on cellulose acetate plates were as previously described (3,9).

Construction of plasmids for the expression of Type III hexokinase and its chimeric forms. It was convenient to generate separate plasmids with inserts encoding either the N-terminal or C-terminal halves of the isozymes. Construction of pUC18 plasmids containing N- or C-terminal halves of the Type I and Type II isozymes has been described (3,6,7,9); in addition, previous work (3) has provided constructs of the Type II isozyme in which catalytically important (6,24) Ser residues in the N-terminal (Ser155) or C-terminal (Ser603) half were mutated to Ala, resulting in inactivation of the catalytic sites in the respective halves. All of these constructs were designed so that sequences encoding N-terminal halves were cloned as *Eco* RI-*Nco* I fragments, while sequences encoding the C-terminal halves were cloned as *Nco* I-*Pst* I fragments. Complete coding sequences for the 100 kDa isozymes

or chimeras could then be obtained by ligation of the individual halves at the central *Nco* I site. A similar strategy was employed in the present work, but was somewhat more complicated due to the presence of additional *Eco* RI and *Nco* I sites within the coding sequence for the Type III isozyme (25).

The cDNA encoding the rat Type III isozyme had been cloned in pUC18 as two separate *Eco* RI fragments (25), comprised of nucleotides 1-525 and 526-3029 (numbering of nucleotide positions refers to the composite sequence as deposited in GenBank, Accession No. U73859). Plasmids containing these inserts are designated p12.1 and p4.1, respectively.

The insert from p4.1 (nt 526-3029) was subcloned into the *Eco* RI site of pSelect, generating the plasmid pSelect4.1. Elimination of an *Nco* I site (nt 733) and the *Eco* RI site at the 3′ end (nt 3029) and creation of an *Nco* I site at nt 1480 were performed simultaneously, following the protocol suggested by Promega and using oligonucleotides having the sequences ATGTGGTAGCTATGGTGAATG, GTTAGCAAGGGATTCGAGCTC, and GGGGTGTGGCCATGGTGACTG, respectively; the underlined bases indicate the mutations, which were designed to avoid any change in amino acid sequence. Since these mutations resulted in modification of restriction sites, clones were initially selected based on restriction analysis, and mutations subsequently confirmed by DNA sequencing. The resulting

plasmid is designated pSelect4.1m. An *Nco* I- *Xba* I fragment, comprised of nt 1481-3029 from the Type III hexokinase sequence plus about 20 additional bases at the 3′ end that corresponded to sequence between the modified *Eco* RI site and the *Xba* I site in the multiple cloning region of pSelect, represents the sequence encoding the C-terminal half of the enzyme.

Digestion of pSelect4.1m with *Eco* RI and *Nco* I generated a fragment corresponding to nt 526-1480, which was subcloned into a previously modified (6) pSelect vector in which an *Nco* I site had been introduced into the multiple cloning site. After digestion of this vector with *Eco* RI, the *Eco* RI fragment derived from p12.1, corresponding to nt 1-525, was inserted to generate a construct containing the complete coding sequence for the N-terminal half of Type III hexokinase, designated pSelect-NIII. Restriction sites for *Nco* I and *Eco* RI, at nt 250 and 525, respectively, were then eliminated by site-directed mutations using oligonucleotides TTCTGTGTTCTATGGAGCAGG and GCCTCTCTGAGTTCCTGGATG; the underlined bases indicate the mutations, which were without effect on amino acid coding. Mutations were again verified by restriction analysis and sequencing.

The complete cDNA for Type III hexokinase, or for various chimeric constructs, was then generated in pUC18 by combination of the appropriate N- or C-terminal fragments. Identity of the complete constructs was confirmed by

restriction analysis, as in earlier studies (3,9). Following the previous practice (3,9), chimeras are designated based on the origin of their N- and C-terminal halves. For example, NICIII is the chimera containing sequence encoding the N-terminal half of Type I hexokinase and the C-terminal half of the Type III isozyme. Other chimeras studied were NIIICI and NIIICII. In addition, chimeras were constructed with the N- or C-terminal halves of Type II hexokinase, inactivated by the S155A or S603A mutations (3), respectively, or with the C-terminal half of Type I hexokinase inactivated by the S603A mutation (6). The inactivated halves are designated as NII′, CII′, or CI′; the chimeras generated were NII′CIII, NIIICII′, and NIIICI′.

Constructs were then subcloned into pSVT7 (26) or pcDNA3, for expression in COS1 (27) or M+R 42 (28) cells, respectively.

Expression in COS 1 and M+R 42 cells. Initial experiments in the present study were done using COS1 cells, following protocols used in our previous work (3,9). COS1 cells express the T antigen (27), and when transfected with a vector such as pSVT7, containing the SV40 early promoter, relatively high levels of expression generally result. This has obvious advantages and explains the popularity of COS1 cells as an expression system. However, a disadvantage in the use of COS1 cells in the present context is the presence of endogenous hexokinase activity (as would, of course, be true for most

eukaryotic cells). The similarity among mammalian hexokinases makes it difficult to resolve the expressed enzyme from endogenous hexokinase, especially when working on the scale typical of transfection experiments. Although the endogenous activity generally represents only a small fraction of the total activity present (6), it nonetheless may complicate interpretation of results, particularly those based on kinetic methods.

The availability of M+R 42 cells (28), generously provided by Dr. Michael Morgan, prompted us to examine expression of hexokinase in this host. M+R 42 cells are a line derived from Chinese hamster ovary (CHO) cells, and are defective in expression of hexokinase (28). Initial experiments indicated that, not unexpectedly, transfection of M+R 42 cells with the constructs in pSVT7 did not lead to appreciable expression of hexokinase activity. However, transferring the constructs to the pcDNA3 vector, in which expression is driven by the CMV promoter, resulted in levels of expression similar to those seen in COS1 cells.

M+R 42 cells were grown at 37° C and 5% CO_2 in Ham's F-12 medium supplemented with 4 mM sodium pyruvate, 2 mM glutamine, and 10% fetal bovine serum or, in later experiments, Cosmic Calf Serum (HyClone Catalog # A-2169); the cells grew significantly faster with the latter serum. Approximately 10^6 cells were plated per 100 mm tissue culture dish, 18 hrs before transfection. The medium was removed by

aspiration and the cells rinsed with 4 ml PBS. The transfecting DNA (5 μg) was mixed with 20 μl lipofectAMINE in 1 ml of serum-free F-12 medium, and this was added to the plate, followed by an additional 2 ml of F-12 medium (serum-free). After incubation for 4-5 hrs at 37°, the transfection medium was replaced by 10 ml of F-12 medium containing serum, pyruvate, and glutamine (as above), followed by an additional 5 ml of this medium 36-48 hrs later. Three days after transfection, the medium was removed, cells rinsed with PBS, and 1 ml lysis buffer (50 mM Bicine, 10 mM thioglycerol, 10 mM glucose, 0.5 mM EDTA, 0.1% (v/v) Triton X-100, pH 8.2) added. After two freeze (-80°C)/thaw (room temperature) cycles, the samples were microfuged for 10 min at 4° C and supernatants removed for analysis.

Concentration and removal of glucose from cell extracts. Prior to determination of the K_m for Glc or studies of Glc inhibition, glucose was removed from the samples by centrifugation on spin columns of Sephadex G25 (Fine), as previously described (9). When concentration of extracts was necessary (before removal of Glc on spin columns, and for nondenaturing electrophoresis), the extracts were centrifuged at 45,000 rpm for 1 hr in a Beckman Ti50 rotor (4°C) prior to concentration with an Centricon 30 device (Amicon, Beverly, MA).

RESULTS

Expression in COS1 and M+R 42 cells. Hexokinase activities found in extracts of transfected COS1 or M+R 42 cells are compared in Table I. It is evident that, using the pcDNA3 vector, expression in M+R 42 cells resulted in levels of activity comparable to those seen in COS1 cells transfected with the corresponding constructs in pSVT7. However, a striking difference was the absence of significant hexokinase activity in the sham transfected M+R 42 cells, consistent with the reported defect in expression of endogenous hexokinase in these cells (28). The results reported in the remainder of this paper were obtained with isozymes or chimeras expressed in M+R 42 cells.

The identities of various expressed constructs were confirmed by immunoblotting (Fig. 1). Thus, products expressed from constructs containing the C-terminal half of Types I, II, or III hexokinase are reactive with monoclonal antibody 5A, the polyclonal antiserum against the C-terminal peptide of Type II hexokinase, or monoclonal antibody C7C3, respectively. Constructs including the N-terminal half of Type I hexokinase led to expression products reactive with monoclonal antibody 21. Moreover, since the same amount of protein was loaded in each lane of the gel, the similarity in intensity of staining on the immunoblots indicated that the various species were expressed at comparable levels. For example, the similar

Table I. Hexokinase Activity in Extracts of Transfected COS1 and M+R 42 cells

cDNA used for transfection	Hexokinase Activity (units/mg protein) COS1 cells[a]	M+R 42 cells[b]
Sham control	0.04 ± 0.01	<0.001
Type I	0.23 ± 0.06	0.16 ± 0.05
Type II	0.66 ± 0.21	0.37 ± 0.14
Type III	ND[c]	0.12 ± 0.05
NICIII	ND	0.11 ± 0.04
NII'CIII	ND	0.10 ± 0.03
NIIICI	0.16 ± 0.03	0.16 ± 0.03
NIIICII	0.21 ± 0.05	0.12 ± 0.04
NIIICI'	ND	0.008 ± 0.002
NIIICII'	ND	0.007 ± 0.003

[a]Constructs were in pSVT7 vector. Mean ± SD for 4 transfections.
[b]Constructs were in pcDNA3 vector. Mean ± SD for 3 determinations with sham transfected cells or cells transfected with NIIICI' or NIIICII' cDNAs; mean ± SD for 15 transfections with other cDNAs.
[c]Not determined.

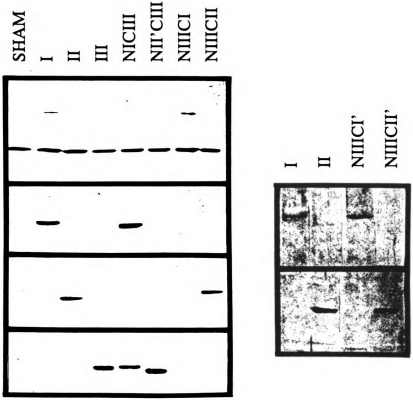

Figure 1. Characterization of expressed hexokinase isozymes and chimeric forms by immunoreactivity. Left panel: replicate immunoblots prepared after SDS gel electrophoresis of extracts (90 μg protein) from sham transfected M+R 42 cells or from cells transfected with the cDNAs indicated at the top of the figure. The blots were probed with, top to bottom: monoclonal antibody 5A, which recognizes an epitope in the C-terminal half of Type I hexokinase (19); monoclonal antibody 21, recognizing an epitope in the N-terminal half of Type I hexokinase (19); polyclonal antiserum recognizing the C-terminal sequence of Type II hexokinase (3); monoclonal antibody C7C3 (20), recognizing an epitope in the C-terminal half of Type III hexokinase. Monoclonal antibody 5A also reacted with an endogenous component (intense bands in *all* lanes in lower part of top panel) found in M+R 42 cells. This is included in the figure as a convenient lane marker; the uniform staining of this component also confirms consistency in loading and blotting of the gel. Right panel: replicate immunoblots prepared after SDS gel electrophoresis of extracts (50 μg protein) from cells transfected with the cDNAs indicated at the top of the figure. The blots were probed with polyclonal antiserum against the Type I (top) or Type II (bottom) isozymes.

intensities seen on the blot probed with monoclonal antibody 5A indicated that the Type I isozyme and the NIIICI chimera were present in similar amount, while the relative staining with monoclonal antibody 21 confirmed that this was also the case with the Type I isozyme and the NICIII chimera. By the same logic, the NICIII chimera was present in amounts comparable to the Type III isozyme itself as well as the NII'CIII chimera, as shown by the similar intensity of staining on the blot probed with monoclonal antibody C7C3. The results shown at the right in Fig. 1 confirm that the NIIICI' and NIIICII' chimeras were expressed at levels comparable to the Type I and Type II isozymes themselves; thus, the extremely low hexokinase activity in extracts from cells transfected with the constructs encoding NIIICI' and NIIICII' (Table I) reflects the inactivity of the chimeric forms, not a failure of expression.

Results of SDS gel electrophoresis (Fig. 2) were also consistent with expression of the various constructs at similar levels, although this was complicated by the presence of substantial amounts of endogenous components which had mobilities in the range of interest.[3] Nonetheless, in some cases, the expressed products could be clearly distinguished (e.g., the Type I isozyme in Lane 3 and the NIIICI chimera in Lane 8 of Fig. 2). In other cases, the expressed products overlapped with an endogenous component of the M+R 42 cells, but their presence could be inferred from increases in

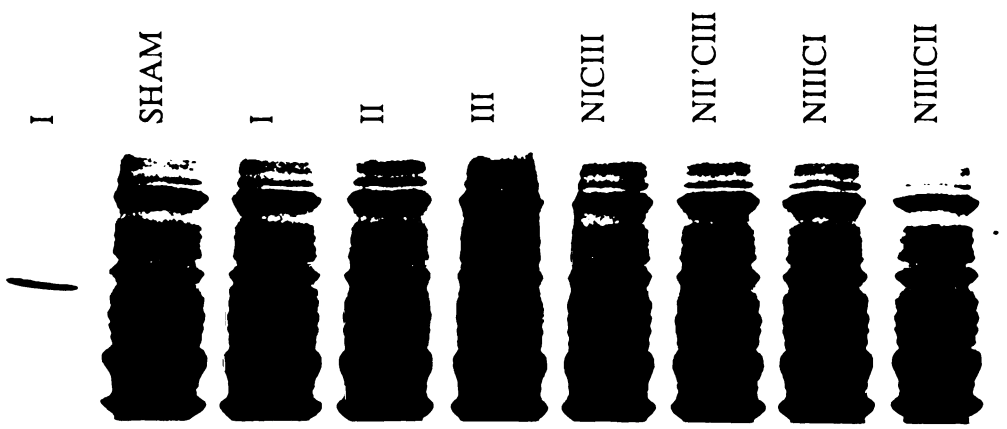

Figure 2. SDS gel electrophoretic analysis of M+R 42 cell extracts. First lane, Type I hexokinase, isolated from rat brain (21). The other lanes were extracts (90 μg protein) from sham transfected cells or cells transfected with the cDNA indicated at the top of the respective lanes. The gel was stained with Coomassie blue.

staining intensity when compared with the corresponding band seen with sham transfected cells. While this is admittedly semi-quantitative, taken together with the blotting results (Fig. 1), these results support the conclusion that the various constructs are expressed at comparable levels in M+R 42 cells, and thus that the activity seen in cell extracts (Table I) reflects the intrinsic activity of the expressed isozyme or chimera.

Nondenaturing electrophoresis. All of the (active) expressed species migrated as single components during electrophoresis under nondenaturing conditions (Fig. 3). As previously noted (9), the mobility of the various chimeric forms is largely determined by the origin of the N-terminal half.

Kinetic properties of the expressed isozymes and chimeras. The K_m values for ATP and Glc are compared in Table II. The K_m values for Glc are identical to those previously reported for the Type I and Type II isozymes expressed in COS1 cells, while the K_m values for ATP were slightly less than those found in the previous study (3). Whether the latter represents experimental variation or reflects a contribution from the endogenous hexokinase present in COS1 cell extracts is not clear. In any case, the results with the expressed Type I, Type II, and Type III isozymes are consistent with earlier work (29) indicating that the apparent affinity for Glc increases in the order of Type II<Type I<Type III. The K_m of

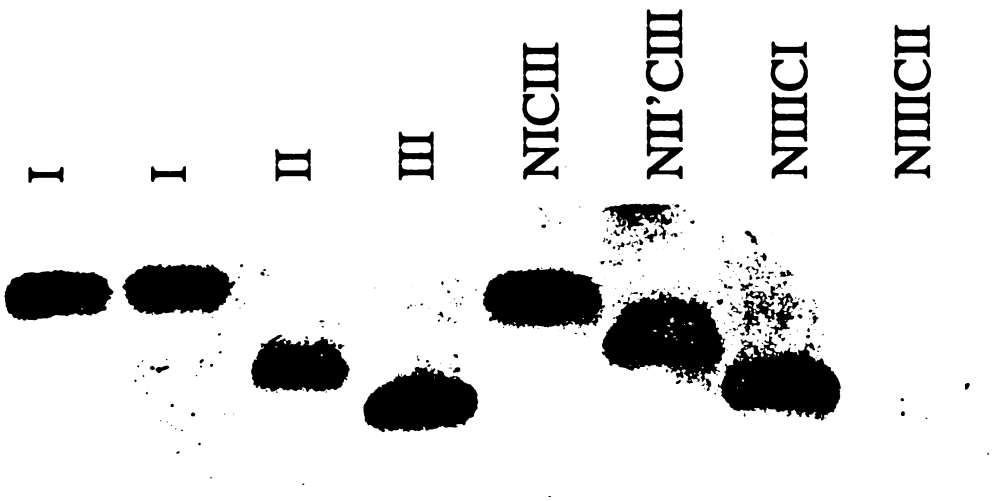

Figure 3. Electrophoresis of expressed isozymes or chimeric forms under nondenaturing conditions. The first lane (at left) contained Type I hexokinase, isolated from rat brain (21). The other lanes received extracts (approx. 5 milliunits of hexokinase activity) from cells transfected with the cDNAs shown at the top of the figure. After electrophoresis, the cellulose acetate plate was stained for hexokinase activity (9). The NIIICII chimera was relatively unstable under the electrophoresis conditions and is barely perceptible in this photograph. However, on the original plate, this was visible as a distinct, though faint, band of activity with a mobility just slightly greater than the NIIICI chimera.

Table II. K_m Values for Glucose and ATP[a]

cDNA expressed	K_m for Glucose (μM)	K_m for ATP (mM)
Type I	61 ± 10	0.38 ± 0.03
Type II	150 ± 18	0.42 ± 0.01
Type III	33 ± 4[b]	0.73 ± 0.08
NICIII	36 ± 4[b]	0.36 ± 0.03
NII'CIII	37 ± 8[b]	0.49 ± 0.06
NIIICI	52 ± 5	0.78 ± 0.08
NIIICII	155 ± 15	0.93 ± 0.16

[a]Mean ± SD for three determinations with samples from different transfections.
[b]These hexokinases exhibit substrate inhibition (see text). All data used for calculation of K_m were obtained at Glc concentrations below those causing inhibition (< 0.5 mM).

the expressed Type III isozyme for ATP was considerably higher than that for the expressed Type I or Type II isozymes; Grossbard and Schimke (29) also found the Type III isozyme to have the highest K_m for ATP.

The Type III isozyme exhibits substrate inhibition as the Glc concentration is increased into the millimolar range (29,30). This was also the case with the expressed Type III isozyme *and* with the expressed chimeric forms, NICIII and NII'CIII (Fig. 4). Substrate inhibition was not seen with the isolated Type I or Type II isozymes (29) nor, in the present study, with the expressed isozymes or NIIICI or NIIICII chimeras (data not shown). Thus, substrate inhibition by Glc is uniquely associated with the C-terminal half of Type III hexokinase.

We also wish to note anomalous kinetic behavior observed with the Type III isozyme and the chimeric forms showing glucose inhibition, i.e., NICIII and NII'CIII. When assayed at noninhibitory Glc concentrations (<0.5 mM), the reactions reached steady state within seconds of mixing the reaction components. However, at inhibitory Glc concentrations, a marked lag was observed before attainment of the steady state rate. The length of the lag was related to the level of inhibition, being on the order of 1-2 min at 2 mM Glc but 3-4 min at 10 mM Glc. No lag was seen at any Glc concentration with the Type I or Type II isozyme, nor with chimeric forms not showing Glc inhibition. This anomalous behavior had not

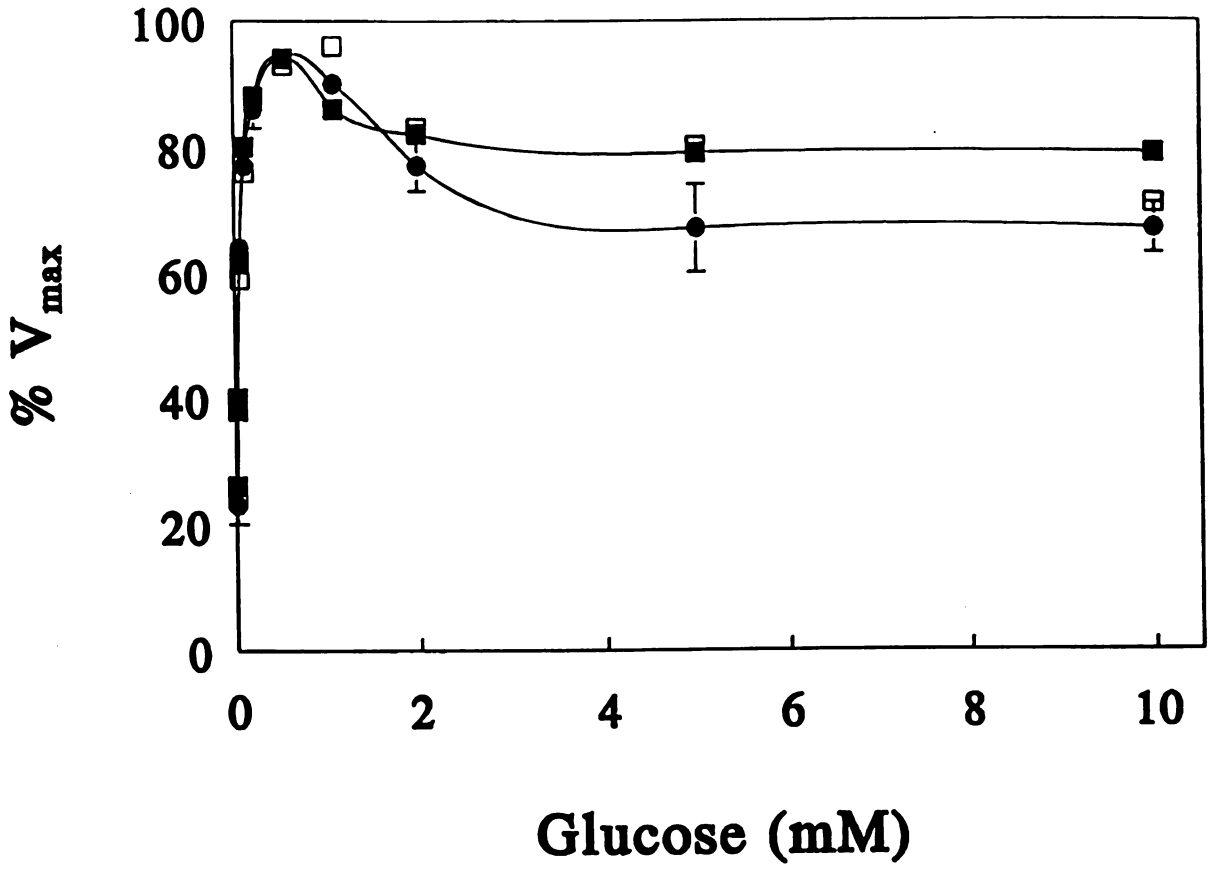

Figure 4. Substrate inhibition of the Type III isozyme and the NICIII and NII'CIII chimeras by glucose. Activity of the Type III isozyme (●), NICIII (□), or NII'CIII (■) is expressed as a percentage of V_{max} values, determined (22) using only data collected at Glc concentrations less than 0.5 mM. Each point is the mean for two experiments. Error bars shown for the Type III isozyme indicate the range of variation; where no error bars are seen, the variations were smaller than the symbols. For clarity, error bars are not shown for NICIII and NII'CIII, but the variation was comparable to that for the Type III isozyme. The curves are purely to guide the eye, and were drawn using the "spline fit" option in SlideWrite Plus (Advanced Graphics Software, Inc.); for clarity, no line has been fitted to the data for NICIII.

been reported in previous studies of the Type III isozyme from rat liver (29) or from rat Novikoff hepatoma cells (30). However, an inquiry to Dr. Tito Ureta elicited the information (T. Ureta, personal communication) that, though not mentioned in the published report, this lag *had* been observed at inhibitory Glc concentrations in the studies with the Type III isozyme from Novikoff hepatoma cells (30) as well as with the Type III from frog liver (31). Moreover, the lag was *not* observed when fructose was used as a substrate for Type III hexokinase from either Novikoff hepatoma cells (30) or frog liver (31); neither of these enzymes shows substrate inhibition by fructose. Thus, the appearance of the lag phase is indeed correlated with the phenomenon of substrate inhibition; moreover, this anomalous kinetic behavior is a general property of the Type III isozyme, whether isolated (30,31) or expressed.

Inhibition by the Glc-6-P analog, 1,5-AnGlc-6-P. When assayed at 3.3 mM Glc, the concentration in the standard assay, the Type I, Type II, and Type III isozymes exhibited similar sensitivity to inhibition by 1,5-AnGlc-6-P (Fig. 5). The chimeric forms, NICIII and NII'CIII, were similar to the parental isozymes in this respect. In contrast, the NIIICI chimera was significantly *more* sensitive, and the NIIICII chimera markedly *less* sensitive than the parental isozymes to inhibition by 1,5-AnGlc-6-P. It is interesting to note that the chimeras constructed with the N-terminal half of Type III

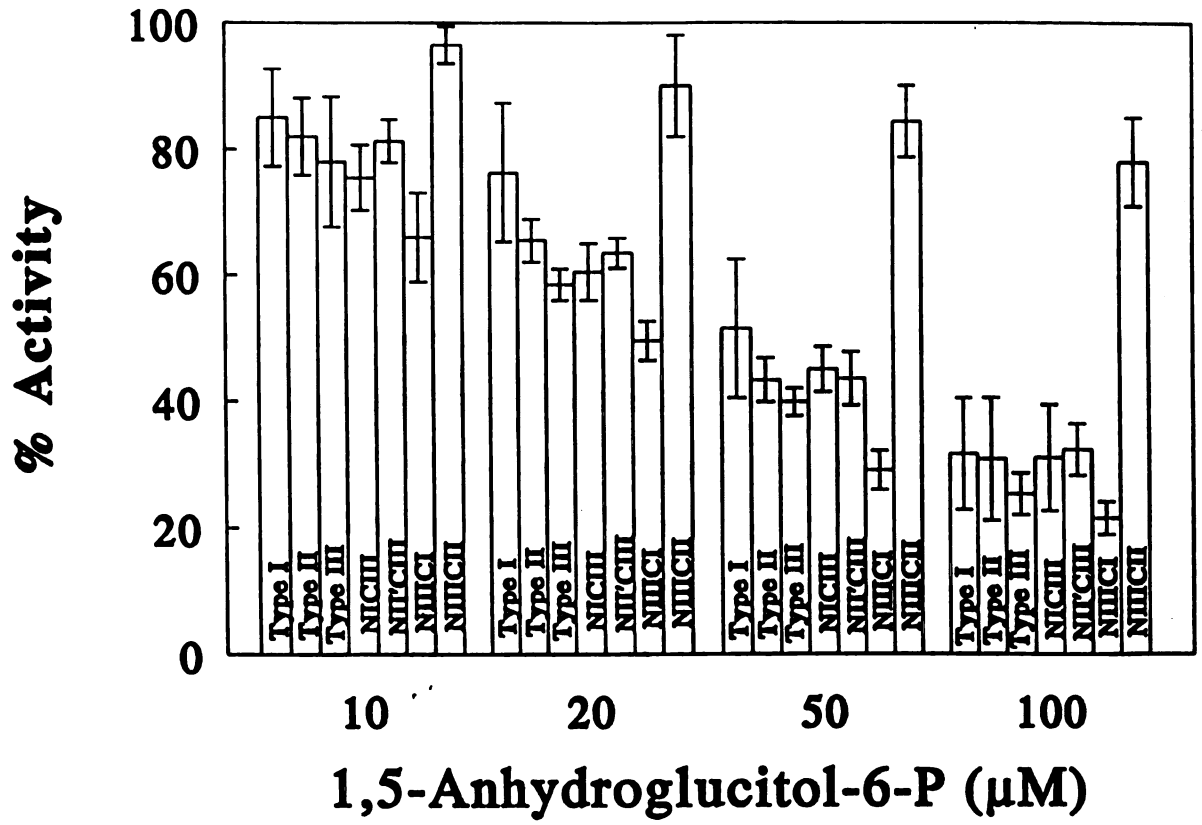

Figure 5. Inhibition by 1,5-AnGlc-6-P at 3.3 mM Glc. Results are expressed as percent of the activity seen in the absence of inhibitor, and are the mean ± SD for 4 determinations, each with extracts from a different transfection.

hexokinase are thus similar to the analogous chimeras constructed with the N-terminal halves of Type I or Type II hexokinase (9), i.e., the NIICI chimera was much *more* sensitive to inhibition, and the NICII chimera much *less* sensitive than the parental isozymes to inhibition by 1,5-AnGlc-6-P. Thus increased sensitivity to this inhibitor is seen with chimeras of the form XCI, and decreased sensitivity to inhibition is found with chimeras of the form XCII, where X is the N-terminal half derived from *either* of the other isozymes.

Since the Type III isozyme as well as the NICIII and NII'CIII chimeras experience significant inhibition at 3.3 mM Glc (Fig. 4), the sensitivity of these forms to inhibition by 1,5-AnGlc-6-P was also determined at a noninhibitory concentration of 0.5 mM Glc. As shown in Fig. 6, this made a substantial difference. Now the Type III isozyme was *less* sensitive than the Type I and Type II isozymes to inhibition by the Glc-6-P analog, consistent with previous reports (29,30) that, when assayed at noninhibitory Glc concentrations, the Type III was the least sensitive to inhibition by Glc-6-P. Although the differences were less striking with the chimeric forms, NICIII and NII'CIII, these too were less sensitive to 1,5-AnGlc-6-P when assayed at the reduced Glc concentration (Fig. 6). It is thus clear that inhibition of the Type III isozyme, or chimeras including the C-terminal half of this isozyme, by Glc-6-P or its analog is

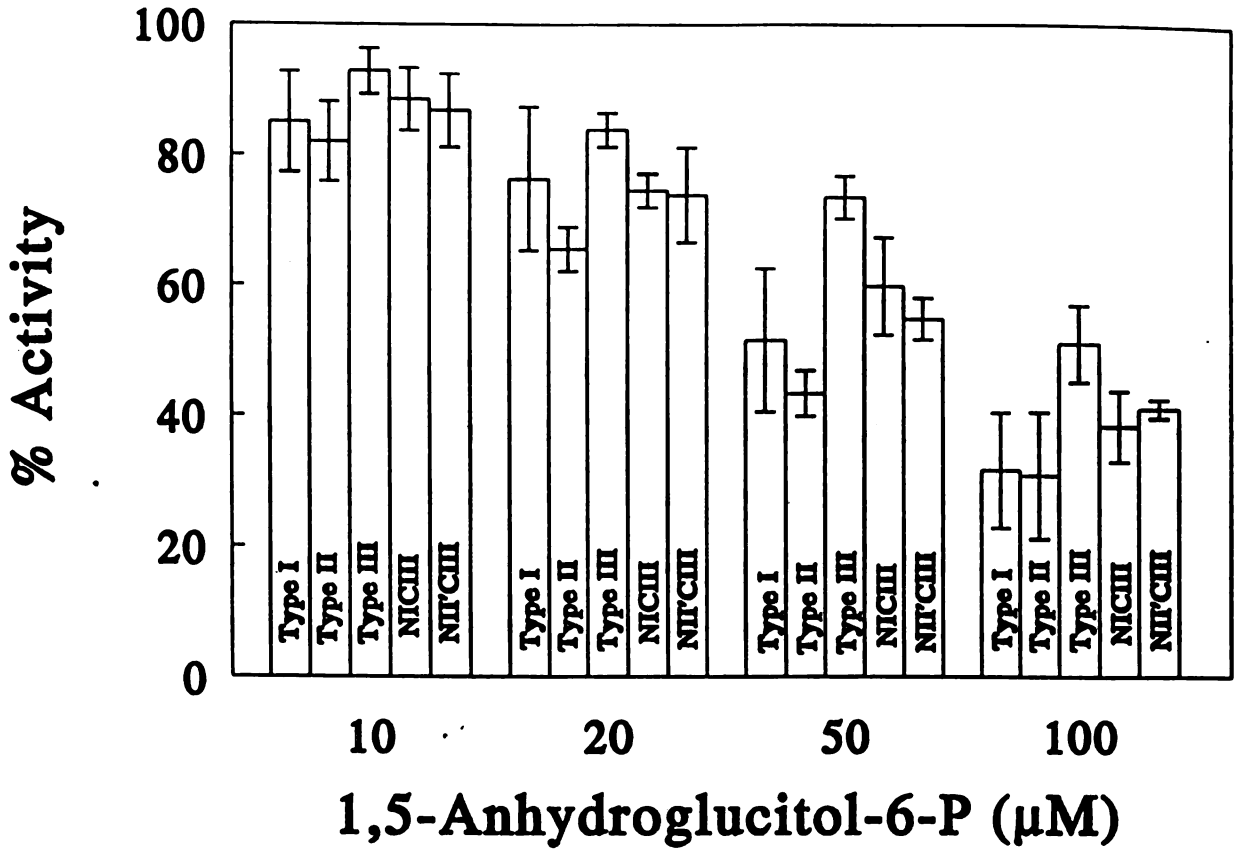

Figure 6. Inhibition of Type III hexokinase and the NICIII and NII'CIII chimeras by 1,5-AnGlc-6-P at 0.5 mM Glc. Results are expressed as percent of the activity seen in the absence of inhibitor, and are the mean ± SD for 4 determinations, each with extracts from a different transfection. For comparison, the results with the Type I and Type II isozymes at 3.3 mM Glc (Fig. 5) have been included.

markedly influenced by the concentration of Glc present, with elevated Glc enhancing inhibition by the 6-phosphate compound. Although Radojković and Ureta (30) did not explicitly comment on this, it is evident from the data in their Fig. 6 that they observed a similar effect with the Type III isozyme from rat Novikoff tumor cells, and Ureta (31) had previously noted that Glc-6-P was a potent inhibitor of the Type III isozyme from frog liver "but only at high glucose levels".

Inhibition of the Type I isozyme, but not Type II or Type III hexokinase, by Glc-6-P or its analogs is antagonized by low concentrations of P_i (1). In the present study, the reversal of inhibition of the expressed Type I isozyme was again confirmed, but this effect was *not* seen with any of the other isozymes or chimeric forms described here (data not shown). For the NICIII chimera, this was examined at both 0.5 mM (noninhibitory) and at 3.3 mM (inhibitory) Glc, but no reversal was seen in either case.

Inhibition by P_i. P_i is also an inhibitor of the Type I, Type II, and Type III isozymes (1,9,12,32). This was confirmed with the expressed isozymes in the present work (Fig. 7). Although the Type I isozyme was considerably less sensitive, all three isozymes showed substantial inhibition by low mM levels of P_i. The chimeric forms were similar to the Type II and Type III isozymes in their sensitivity to this inhibitor, although the NIIICII chimera was marginally less sensitive than the other chimeras.

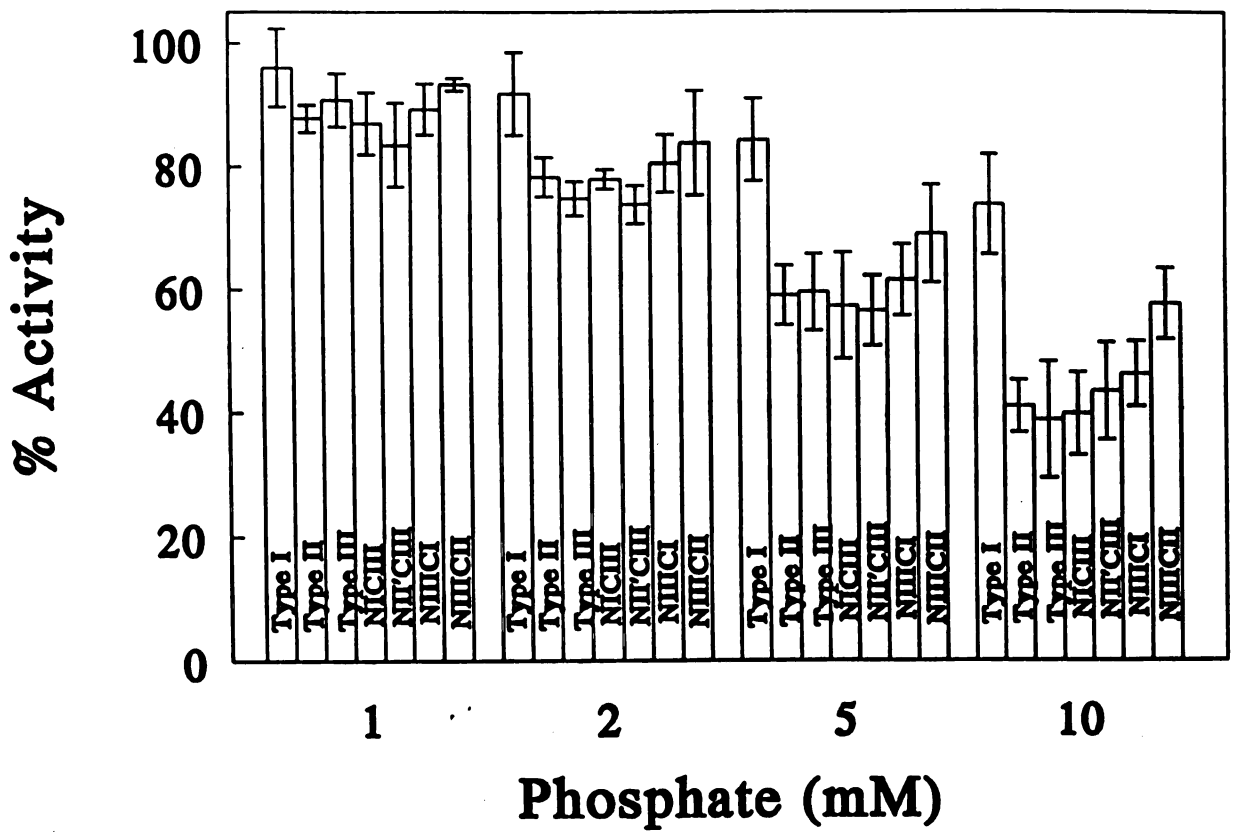

Figure 7. Inhibition by P_i at 3.3 mM Glc. Results are expressed as percent of the activity seen in the absence of inhibitor, and are the mean ± SD for 4 determinations, each with extracts from a different transfection.

The results shown in Fig. 7 were obtained with 3.3 mM Glc in the reaction mixture. Reducing the Glc concentration to a noninhibitory level (0.5 mM) did not affect the inhibition of the Type III isozyme or the NICIII and NII'CIII chimeras by P_i (Fig. 8).

DISCUSSION

The specific activity of hexokinase in extracts of M+R 42 cells expressing the Type III isozyme (Table I) was comparable to that obtained after expression of the Type I isozyme, and was approximately half of the specific activity seen in extracts of cells expressing the Type II isozyme, in which *both* N- and C-terminal halves are known to possess catalytic sites of essentially equal activity (2,3). Moreover, expression of chimeras in which the C-terminal half of Type III hexokinase was fused with N-terminal halves known to be inactive (either the intrinsically inactive NI, or NII', which had been inactivated by site-directed mutation) gave specific activities comparable to those seen with the Type I and Type III isozymes. These results are consistent with the existence of a single catalytic site, present in the C-terminal halves of the Type I (4-7) and Type III isozyme. The intrinsic specific activities of these catalytic sites are similar, to each other and to those located in both N- and C-terminal halves of the Type II isozyme (2,3); this is, of course, not

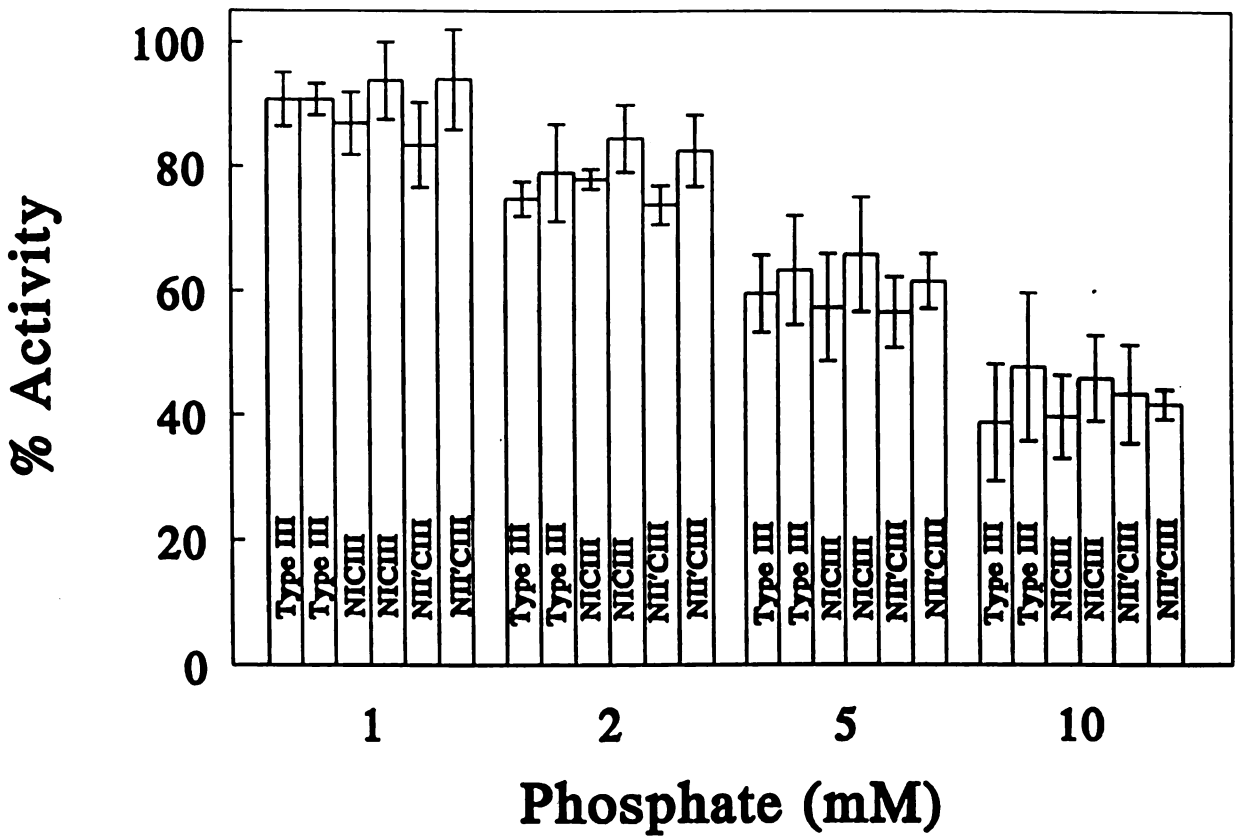

Figure 8. Lack of an effect of Glc concentration on the inhibition of Type III hexokinase and the NICIII and NII'CIII chimeras by P_i. Results are expressed as percent of the activity seen in the absence of inhibitor, and are the mean ± SD for 4 determinations, each with extracts from a different transfection. The results for each form are indicated by a pair of bars; in each pair, the bar at the left indicates results at 3.3 mM Glc (shown in Fig. 7, but included here for comparison) and the bar at the right indicates results with 0.5 mM Glc.

totally unexpected, considering the likely common origin of these isozymes. Furthermore, chimeras in which the N-terminal half of Type III hexokinase was combined with the catalytically active C-terminal halves of Type I or Type II hexokinase were expressed with specific activities comparable to those seen with the Type I and Type III isozymes themselves, indicating the *lack* of significant contribution from NIII to the catalytic activity of these chimeras. Confirming this, chimeras comprised of the N-terminal half of the Type III isozyme and inactivated C-terminal halves from the Type I or Type II isozymes, CI′ and CII′, respectively, were virtually inactive. We conclude that, as with the Type I isozyme (4-7), catalytic function is associated solely with the C-terminal half of the Type III isozyme. This conclusion is consistent with, and extends, the recent study of Palma *et al*. (33); these authors reported that the C-terminal half of human Type III hexokinase, expressed as a discrete species from the truncated cDNA, was catalytically active.

The K_ms of the chimeric forms for Glc are correlated with the origin of the C-terminal half (Table II). This implies that, at least with respect to binding of Glc at the catalytic site, the N-terminal half of the molecule has essentially no effect. In contrast, the Type III isozyme itself, as well as chimeric forms in which the catalytically inactive NIII is combined with catalytic C-terminal halves of Type I or Type II hexokinase, have K_ms for ATP that are similar and

significantly higher than those for the Type I or Type II isozymes or for the NICIII and NII'CIII chimeras. This correlation between the presence of an N-terminal half from Type III hexokinase and a relatively high K_m for ATP implies the existence of substantial interactions between the N- and C-terminal halves, with the N-terminal half from Type III evoking an increased K_m for ATP in the catalytic C-terminal half, regardless of its source. Other results, discussed below, provide further evidence for a significant role of the N-terminal half in regulation of catalytic function intrinsic to the C-terminal half of Type III hexokinase, as previously deduced for the Type I isozyme (4,8,10,15).

The expressed C-terminal half of Type III hexokinase, in contrast to the Type III isozyme itself, did *not* show substrate inhibition by Glc, leading Palma *et al.* (33) to propose that substrate inhibition might result from binding of Glc to the N-terminal half of the intact 100 kDa isozyme. We concur with this suggestion. Both N- and C-terminal halves of the Type I and Type II isozymes contain binding sites for Glc (2-4,15), although, in the intact 100 kDa Type I isozyme, the N-terminal site is thought to be latent (15). Based on the extensive similarity between these isozymes, the presence of a Glc binding site in the N-terminus of Type III hexokinase can reasonably be inferred. Binding of Glc or Glc-6-P to sites in the C- or N-terminal halves, respectively, has been shown to induce marked conformational changes involving *both* halves

of mammalian Type I isozyme (1,4,8,15). Thus, it is clear that extensive interactions between the halves can occur, with resulting effect on structure and function. While Glc binds with high affinity to the catalytic site in the C-terminal half of Type III hexokinase, conformational changes induced by binding of Glc to a lower affinity site in the N-terminal half could well exert a negative influence on catalytic function of the C-terminal half.

Based on the above logic, and since the Type III isozyme is unique in exhibiting inhibition by substrate Glc, it had been anticipated that Glc inhibition would be correlated with the presence of NIII in chimeric forms. In fact, that was *not* the case. The NIIICI and NIIICII did not show substrate inhibition, while the NICIII and NII'CIII chimeras did. Clearly, substrate inhibition was correlated with the C-terminal half of Type III hexokinase. *Our interpretation of these results is that the C-terminal half of Type III is uniquely receptive to an inhibitory conformational "signal" induced by binding of Glc to a site in the N-terminal half.* The N-terminal halves of any of the three isozymes are capable of generating that signal. All residues known to be involved in binding of Glc are conserved in the N-terminal[4] halves of all three isozymes (1). It is not unreasonable to expect that Glc is bound in a similar manner to, and with similar effect on conformation of, the N-terminal halves of all three isozymes. Glc inhibition is not seen with the Type II isozyme

because the Glc binding sites in both N- and C-terminal halves are catalytically functional (2,3), i.e., binding to the N-terminal half is productive, not inhibitory. Glc inhibition is not seen with the Type I isozyme because the Glc binding site in the N-terminal half is latent (15). A corollary of this proposal is that, in contrast to the situation in the intact Type I isozyme, the Glc binding site in the N-terminal half of the NICIII chimera is *not* latent, presumably due to structural differences between the native Type I and the NICIII chimera.

The above analysis leads to the view that the functional organization of Type III hexokinase is the same as that of the Type I isozyme (4,6,8,10,15), i.e., catalytic function is associated with the C-terminal half and regulatory function with the N-terminal half. We believe this extends to inhibition by Glc-6-P and its analogs. Thus, we suggest that, at low Glc concentrations, these 6-phosphates bind with relatively low affinity to the N-terminal half of Type III hexokinase, inducing a conformational change that results in loss of the ability to bind ATP at the catalytic site in the C-terminus (as inferred from the competitive nature of the inhibition, implying mutually exclusive binding). In contrast to the situation with the Type I isozyme (15), Glc *can* bind to a site in the N-terminal half of the Type III isozyme. It has previously been shown (4,12,15) that Glc and Glc-6-P (and their analogs) bind synergistically to both the (isolated) N-

and C-terminal halves of Type I hexokinase, and the results presented here demonstrate similar synergism with the Type III isozyme, i.e., inhibition by 1,5-AnGlc-6-P is enhanced in the presence of elevated (inhibitory) Glc levels. Thus, in the case of the Type III isozyme, binding of Glc to the lower affinity site in the N-terminal half of the molecule would enhance binding of Glc-6-P to the regulatory site in the N-terminal half, resulting in increased sensitivity to this inhibitor. This is precisely what is seen with the Type III isozyme (30,31, and present work). Furthermore, this enhancement of inhibition is also seen with the NICIII and NII'CIII chimeras which, as noted above, also exhibit inhibition by the substrate, Glc. It is thus apparent that both substrate inhibition as well as Glc-induced enhancement of inhibition by Glc-6-P and its analogs can be explained by the unique ability of the C-terminal half of Type III hexokinase to respond to conformational signals induced by binding of regulatory ligands to the N-terminal half of the molecule.

It is not within the scope of the present report to provide an explanation for the anomalous kinetic results - the marked lag prior to attainment of steady state in the presence of inhibitory concentrations of Glc - seen with the Type III isozyme and the NICIII and NII'CIII chimeras. It does seem likely that this might involve a slow conformational response to the binding of substrate Glc when the "inhibitor" site has

previously been occupied by a second Glc. At noninhibitory Glc concentrations, no lag was seen with either of the other inhibitors, P_i or 1,5-AnGlc-6-P.

If inhibition of Type I hexokinase resulted from Glc-6-P or its analogs binding directly to the catalytic C-terminal half, as some have proposed (5,14,16), it might be expected that the response of chimeric forms to this inhibitor would be more-or-less independent of the origin of the N-terminal half; after all, other kinetic parameters, the K_ms for Glc and ATP, are only modestly affected, if at all, by the origin of the N-terminal half (3,9, and present study). However, the present work as well as our previous study (9) clearly demonstrates that this is not the case. The markedly increased sensitivity of chimeras of the form XCI and decreased sensitivity of XCII chimeras, X being the N-terminal half derived from *either* of the other isozymes, certainly suggests that inhibition by Glc-6-P is not simply determined by the intrinsic sensitivity of the catalytic C-terminal half. These results are, however, consistent with the view that inhibition involves interactions between the halves, with CI being more receptive and CII less receptive to conformational signals induced by binding of Glc-6-P to a heterologous N-terminal half.

As noted above, inhibition of Type I hexokinase by Glc-6-P or its analogs is antagonized by low concentrations of P_i. This has previously been attributed to competition of Glc-6-P (or its analogs) and P_i for a common binding site in the N-

terminal half of Type I hexokinase (15); consistent with this, P_i also antagonized inhibition of the NICII chimera by 1,5-AnGlc-6-P (9). Although similar antagonism between P_i and 1,5-AnGlc-6-P might have been expected with the NICIII chimera, this was not observed. However, this is readily explained by the marked sensitivity of the Type III isozyme to inhibition by P_i. With Type III hexokinase, as with the Type I isozyme (4,8,9,15), inhibition may be attributed to binding of P_i to the catalytic C-terminal half. Thus, sensitivity to P_i is independent of the nature of the N-terminal half, with the Type III isozyme and the NICIII and NII'CIII chimeras being equivalent in their response to this inhibitor, and sensitivity to P_i is also unaffected by binding of Glc (at higher concentrations) to the inhibitory site in the N-terminal half of these forms. Therefore, even though P_i may in fact be antagonizing the binding of Glc-6-P to the N-terminal half of Type III hexokinase or the NICIII chimera, any resulting increase in activity would be more than offset by the direct, and rather potent, inhibitory effect of P_i on catalytic activity of the C-terminal half. In contrast, the C-terminal halves of the Type I (15) and Type II (3) isozymes have relatively low affinities for P_i. Thus, with Type I hexokinase as well as the NICII chimera, significant antagonism resulting from binding of P_i to a high affinity site in the N-terminal half, in competition with the inhibitory hexose 6-phosphate, can be seen before being masked by

inhibition resulting from further binding of P_i to the low affinity site in the C-terminal half.

The Type III isozyme is, like the Type I isozyme (4,6,8-10,15), comprised of functionally distinct halves, catalytic C-terminal and regulatory N-terminal. Thus, as previously suggested (3), these isozymes likely represent the result of duplication and further evolution of a gene encoding an ancestral 100 kDa hexokinase, to which the present-day Type II isozyme remains most similar. The more complex regulatory features seen with the Type I (e.g., P_i antagonism of inhibition by Glc-6-P) and Type III (e.g., substrate inhibition by Glc) isozymes arise from close functional interactions between the two halves. In contrast, the N- and C-terminal halves of Type II hexokinase seem to function more-or-less independently (2,3). It is interesting that the major evolutionary events leading to Type I and Type III isozymes apparently rested not so much on modification of affinities or specificities of existing ligand binding sites, or development of new ones, but rather, on development of functional interactions between these sites with resulting acquisition of unique catalytic and regulatory properties.

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FOOTNOTES

[1]Abbreviations used: Glc-6-P, glucose 6-phosphate; DMEM, Dulbecco's modified Eagle medium; BSA, bovine serum albumin; 1,5-AnGlc-6-P, 1,5-anhydroglucitol 6-phosphate; PBS, phosphate buffered saline (10 mM sodium phosphate, 154 mM NaCl, pH 7.0); CMV, human cytomegalovirus.

[2]The proposal that regulatory function is associated with the N-terminal half of Type I hexokinase was initially based on the demonstration that the N-terminal domain contained a functional Glc-6-P binding site (10). Ligand binding studies (11-13) had indicated the presence of a single Glc-6-P binding site, and a single Glc binding site, in the intact 100 kDa enzyme. Thus it was reasonable to associate the binding of Glc-6-P, and hence regulatory function, with the site in the N-terminal half (10). This proposal has subsequently been supported by additional evidence (8,9). However, the situation was complicated with the demonstration that the C-terminal half, obtained either by limited proteolysis (4) or expressed from the cDNA (5,14), *retained* sensitivity to inhibition by Glc-6-P, demonstrating the presence of an intrinsic regulatory site in the C-terminal half. A further complication was the demonstration (4,15) that, *when isolated*, both N- and C-terminal halves possessed binding sites for Glc as well as for Glc-6-P. To reconcile these findings with the stoichiometry of *one* Glc site and *one* Glc-6-P site per molecule of the intact 100 kDa enzyme (11-13), White and Wilson (15) proposed that, in the intact enzyme, the Glc site in the N-terminal half as well as the Glc-6-P site in the C-terminal half were "latent", presumably masked by structural constraints present in the 100 kDa enzyme; when these constraints were removed by separation of the halves, both sites were revealed. Alternative explanations certainly could be proposed but to be acceptable, these must account for the existence of a functional Glc-6-P binding site in the N-terminal half of Type I hexokinase (10), and for the discrepancy between the existence of binding sites for Glc-6-P and Glc in *both* N- and C-terminal halves, when isolated, and the observed 1:1 stoichiometry for binding of these ligands to the intact 100 kDa enzyme. Simple assertions that it is the C-terminal site that is responsible for inhibition of the intact enzyme by Glc-6-P (5,14,16) are insufficient.

[3]In this respect, COS1 cells possess an advantage since these cells have relatively low levels of components running in this region of the gel, and thus bands corresponding to the expressed hexokinase isozymes, or chimeras, are readily detectable (3,6,7,9).

[4]Although mutation of Ser155 to Ala, as in NII′, or the analogous Ser603 to Ala mutation in catalytic C-terminal half, as in CI′ or CII′, results in drastic reduction of catalytic activity (3,5,6,34), it does not abolish and may actually enhance (34) binding of Glc.

Chapter V

CONCLUSION

Functional organization of mammalian 100 kDa hexokinases. The objective of this study was to define the functional organization in mammalian 100 kDa hexokinases. In certain cases, several unique properties were found to be associated with either the N- or C-terminal half of these enzymes. For example, phosphate antagonism of inhibition by AnG-6-P correlated with the N-terminal half of hexokinase I (1) when the C-terminal half was substituted with that of hexokinase II in the NICII chimera, and higher sensitivity to phosphate inhibition correlated with the N-terminal half of hexokinase II (1) when the C-terminal half was substituted with that of hexokinase I in the NIICI chimera. However, there are also results that did not correlate with either half, e.g. sensitivity to inhibition by the Glc-6-P analogue, AnG-6-P (1,2). These properties are, presumably, due to the interaction between the two halves.

The domain organization for catalytic function is not uniform among all three mammalian hexokinases; hexokinase II has one active site in each half of the protein (2), while hexokinase I and III have only one active site in the C-terminal halves (3,4). Because of the dual active sites and greater conservation of protein sequence between the N- and C-terminal halves (2), hexokinase II functionally most resembles the ancient hexokinase that has arisen from gene duplication and fusion, which should have one active site in each of the two identical halves (2). On the other hand, hexokinase I and

III have only one functional active site, in their C-terminal half, along with less conservation of amino acid sequence between the N- and C-terminal halves; they have apparently evolved further from hexokinase II (2,4).

Since the N-terminal halves of hexokinase I and III do not possess catalytic function, a favored explanation is that these N-terminal halves may have evolved into regulatory domains (1-4). This speculation is consistent with the fact that truncated 50 kDa C-terminal half of hexokinase I shows no phosphate antagonism of Glc-6-P inhibition, while this property is observed in the 100 kDa intact hexokinase I (3). Although hexokinase I lost catalytic function in the N-terminal half, the trade-off was the gain of a unique regulatory function, providing hexokinase I with additional regulatory sophistication (3).

A new model for mammalian 100 kDa hexokinases. The hypothesis that duplication and fusion of a gene encoding an ancestral 50 kDa hexokinase led to modern mammalian 100 kDa hexokinases has become a well established and accepted model (2). Because the duplication and fusion event occurred after the formation of a discrete Glc-6-P site (3), physically, there should be one Glc-6-P site and one glucose site in each half of the 100 kDa mammalian hexokinase, i.e. two Glc-6-P sites and two glucose sites on the entire molecule. However, two separate laboratories (5,6) have reported that only one glucose and one Glc-6-P site were detected on each 100 kDa

hexokinase I molecule. To explain this discrepancy, White and Wilson (3) proposed a latent site model in which the glucose site in the N-terminal half and the Glc-6-P site in the C-terminal half were latent, presumably because of structural impediment resulting from fusion of the two halves.

This latent site model, however, does not conform to the dual active sites in hexokinase II (2), since the glucose site in the N-terminal half of hexokinase II is obviously not latent (it is catalytically active; therefore it must be accessible to glucose). In addition, computer modelling of mammalian hexokinases, based on the crystal structure of 50 kDa yeast hexokinase, does not exhibit obvious structural impediment of the glucose binding site in the N-terminal half (7). Thus, a revision of this model is needed.

One other model favored by Fromm (7), Pedersen (8), and Magnani (9) is that the functional Glc-6-P site is located in the C-terminal half of hexokinase I, where Glc-6-P competes directly against substrate ATP (mutually exclusive). However, this model cannot explain why Glc-6-P is able to protect the N-terminal half of hexokinase I from trypsin proteolysis (3) or sulfhydryl modification (10), and therefore this model requires revision to accommodate such observations.

Developed with the concept that hexokinase II is the closest descendant of the initial gene duplication and fusion product, the new model is based on the assumption that N- and C-terminal halves of hexokinases are similar structurally and

functionally. Known functional differentiations in the N-terminal halves of hexokinase I and III can be explained with derivations of this model. The following are descriptions of the model:

1. The major assumptions of this model are that all three mammalian hexokinases are similar in three-dimensional structure, and that closure of the clefts (glucose binding sites) in both N- and C-terminal halves occur in a concerted manner because of the structural connection. The former assumption is based on the fact that all three 100 kDa mammalian hexokinases are very similar in protein sequence and they have a common ancestor (2, Chapter 1 of this dissertation). The latter assumption is essentially identical to the symmetry model (11) first proposed by Monod, Wyman, and Changeux (12) where subunits of a protein can exist in two different conformations, but all subunits in a protein molecule adopt the same conformation. If one of the subunits changes conformation, all subunits change conformation simultaneously. In other words, this is an extreme case of co-operative interaction where the coefficient of co-operativity is equal to the number of subunits. As a result of this concerted conformational change in N- and C-terminal halves, one molecule of glucose, when it binds to either half, is sufficient to cause the closure of both N- and C-terminal halves of hexokinase II, and thus prevents the binding of another glucose at the other empty active site. Because

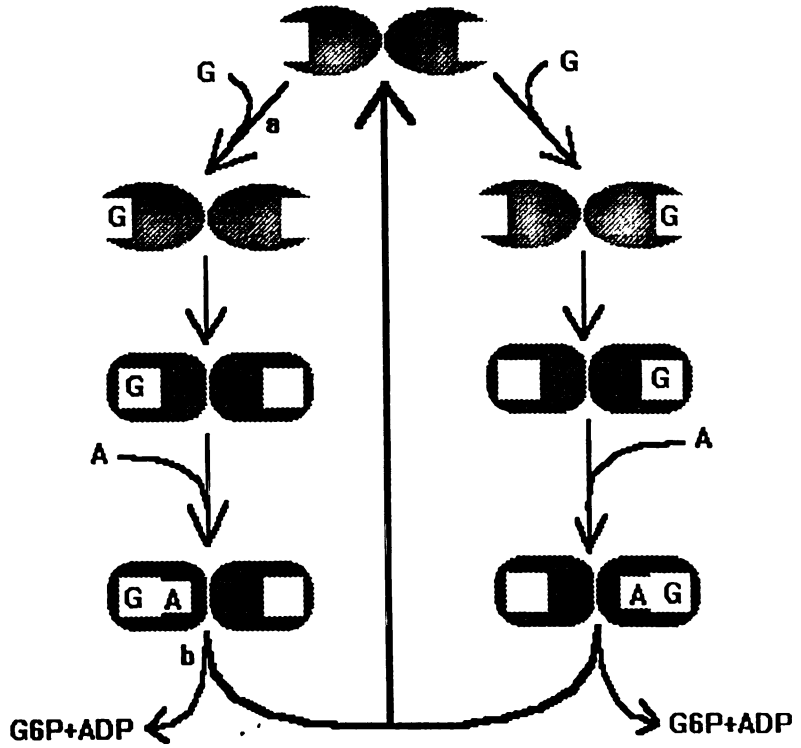
probability of two glucose molecules binding to the two active sites on the same hexokinase molecule simultaneously is very small, the enzyme on average binds one glucose at a given time (Fig. 1).

This symmetry model would predict that despite the presence of two active sites (glucose binding sites) in hexokinase II, there will be only one detectable (accessible) glucose binding site on every hexokinase II molecule. This prediction is supported by the observation made by Connolly and Trayer (13) that reaction with one molecule of glucose analogue, N-bromoacetyl-glucosamine, is sufficient to cause complete loss of catalytic activity in hexokinase II, despite hexokinase II having two separate active sites. The hypothetical mechanism is also supported by the observation made in hexokinase I that despite the presence of two glucose sites, demonstrable in the isolated halves, only one site is detectable by binding assays (5,6).

Under the symmetry model, the ligand will bind to the highest affinity site if the subunits have different affinities for this ligand (see next two paragraphs). In the case of glucose binding sites in hexokinase II, the N- and C-terminal halves appear to have similar affinities for glucose (2,14).

2. A similar mechanism is also true for Glc-6-P as an inhibitory ligand. Glc-6-P binds to one of the two Glc-6-P sites closing both Glc-6-P sites and exerts inhibitory effect

Figure 1. Proposed Symmetry Model for Catalysis by Mammalian Type II Hexokinase.



G: Glucose; A:ATP

a: Glucose analogues, N-acetyl glucosamine and N-bromoacetyl glucosamine, cannot bind to hexokinase I at this step.
b: The N-terminal halves of hexokinase I and III cannot catalyze the reaction at this step.

on both active sites. This hypothesis is based on a more limited evidence from hexokinase I, that only one Glc-6-P site is detectable on the entire 100 kDa molecule (5,6). Whether hexokinase II and III are similar in this respect is yet to be verified. Nonetheless, if this hypothesis is true, a single Glc-6-P molecule should be sufficient to affect both active sites in hexokinase II, which is supported by the fact that the Glc-6-P analogue, AnG-6-P, is a competitive inhibitor to substrate ATP (1), suggesting that both active sites can be affected by this inhibitor.

It is noteworthy that the binding affinity for Glc-6-P may not be identical in the N- and C-terminal halves. It has been suggested that the N-terminal half of hexokinase II has higher affinity for Glc-6-P than the C-terminal half (14). In the presence of Glc-6-P, one molecule of Glc-6-P supposedly binds to the high affinity site in the N-terminal half, which is then sufficient to cause conformational change and lead to inhibition at all active sites. Since binding of one Glc-6-P molecule closes both high and low affinity Glc-6-P sites, only the high affinity site will be detectable by binding assays (5,6), which is why the K_i of Glc-6-P for intact hexokinase II is similar to the K_i for the higher affinity site in the N-terminal half (14).

Because of limited structural information about the Glc-6-P binding site on hexokinases, there is no way of

distinguishing if both Glc-6-P sites are equally accessible to the ligand (symmetry model) or the site in the C-terminal half is actually latent as proposed by White and Wilson (3). Therefore, the latent model remains a viable alternative for explaining the observation that only one Glc-6-P site is detectable by the binding assays (5,6)

3. Because of amino acid substitutions in the N-terminal half of hexokinase I, the active site in the N-terminal half of hexokinase I has become inactive. However, glucose is still able to bind to this site and causes closure of the clefts (4). A similar scenario may also be true for the N-terminal half of hexokinase III, since the N-terminal half of hexokinase III possesses no catalytic activity but appears to retain its ability to bind glucose, which results in substrate glucose inhibition (4). Because of inactivity in the N-terminal half of hexokinase I, 50 % of the cleft closure, induced by the binding of substrates at the N-terminal half, results in no catalysis. Meanwhile, every cleft closure in hexokinase II results in catalysis regardless of which site the catalysis actually occurs; therefore, the specific activity of hexokinase II is about twice that of hexokinase I, e.g. the specific activity of purified hexokinase I is about 60 U/mg protein (3,6,15,16), while the specific activity of purified hexokinase II is about 100 U/mg protein (17).

4. Residue substitutions in the N-terminal half of hexokinase I result in glucose analogues, N-acetyl glucosamine

and N-(bromoacetyl)-D-glucosamine being unable to fit into the glucose binding site in the N-terminal half of hexokinase I. The former analogue is unable to protect the N-terminal half of hexokinase I from proteolytic attack (3), even when the N-terminal half is in the 50 kDa truncated form (3), while the latter analogue is unable to label (cross-link to) the N-terminal half of intact hexokinase I (18). Nonetheless, both analogues are able to bind at the active site in the C-terminal half of hexokinase I, which explains why N-acetyl glucosamine competes with glucose at the active site and selectively protects the C-terminal half from proteolysis (3), while N-(bromoacetyl)-D-glucosamine is able to affinity label the C-terminal half of hexokinase I (18).

FUTURE RESEARCH

The presence of two glucose sites and two Glc-6-P sites on each hexokinase I (3) and the indication of only one detectable glucose site and one Glc-6-P site per hexokinase I molecule by binding assays (5,6) have not been adequately explained on the structural basis. Whether this stoichiometry remains true in hexokinase II and III should be the focus of future research. Although Connolly and Trayer (13) had demonstrated that one bromoacetyl-glucosamine per hexokinase II molecule is sufficient to completely inhibit the hexokinase activity, suggesting one detectable glucose site per 100 kDa molecule, it is yet to be determined if the 1:1 stoichiometry remains true with glucose as the ligand. If hexokinase II is determined to have only one detectable glucose site despite the fact of two functional active (glucose) sites, it would imply that hexokinase II operates under the symmetry model as proposed in this chapter.

It is obvious that the N-terminal halves of hexokinase I and III do not possess catalytic function. However, according to the gene duplication and fusion scheme, these halves should have once been catalytically active, e.g. the N-terminal half of modern hexokinase II still remains active. Like other catalytically active halves in mammalian hexokinases, all critical residues that are known to be involved in glucose binding or catalysis are present in these two inactive halves (19). Therefore, a logical question is what caused the loss

of catalytic function in these two halves? The answer to this question will not only explain what happened in the evolution, but also provide some insight into what other amino acids are important for catalytic function in mammalian hexokinases.

Arora et al. (20) reported that mutation S603A in hexokinase I, as one may have expected, drastically hindered the catalytic activity. Surprisingly, however, this mutation increased the binding affinity for glucose. Although Harrison (21) suggested that the 3-hydroxyl group of glucose forms hydrogen bond with the oxygen on the carbonyl group, not the hydroxyl group on the side chain, of corresponding Ser 158 in yeast hexokinase, it remains unanswered how the mutation can affect the catalytic activity and at the same time increase the affinity for glucose. A careful reexamination of this increased affinity for glucose is needed.

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