PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

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Phil. Trans. R. Soc. Lond. B 1981 **293**, 43-52
doi: 10.1098/rstb.1981.0058

Phil. Trans. R. Soc. Lond. B **293**, 43–52 (1981)
Printed in Great Britain

Structural dynamics of yeast hexokinase during catalysis

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The binding of the substrate glucose to yeast hexokinase results in a substantial enzyme conformational change that is essential for catalysis and may be important for the enzyme's specificity, as well as the control of its activity. From high-resolution crystal structures of the monomeric enzyme crystallized both in the presence and in the absence of glucose, we find that glucose binds into the deep cleft that separates the molecule into two lobes and causes these two lobes to move together and close off the cleft. The structure of the hexokinase crystallized in the presence of xylose and ADP is being determined at low resolution. In this crystal form, the enzyme was thought to be in the conformation of the ternary complex. However, a low-resolution structure of this crystal form shows clearly that the enzyme is in the 'open' form and is not a ternary complex. Crystals of the A isozyme with glucose and ADP may be. Further, chemically sequenced tryptic peptides are being incorporated into the model obtained by crystallographic refinement at 2.1 Å resolution. Completion of the sequence and the structure of the ternary complex should allow a detailed description of the enzymatic mechanism of this kinase and the role of substrate-induced conformational changes in catalysis and control.

It has been said that trying to understand how an enzyme works from X-ray crystallography is like trying to understand how a race horse runs on the basis of a single photograph. Clearly the way to solve this problem with either a horse or an enzyme is to take a series of pictures and make a cine film. Thus, one must crystallize and solve the structure of an enzyme trapped in the various steps of catalysis if one is to understand the structural dynamics of enzyme action.

Yeast hexokinase can exist as an active monomer ($M_r = 50000$) or an active dimer, which in the course of catalysis forms binary complexes with sugar or ATP substrates and then forms ternary complexes with both sugar and nucleotide substrates (figure 1). Phosphoryl transfer occurs only when both substrates are bound to the enzyme. Towards our goal of determining the structure of hexokinase at each step in the reaction, we have solved and refined at 2.1 and 3.5 Å respectively the structure of the native monomeric enzyme (E) crystallized without sugar (Fletterick *et al.* 1975; Anderson *et al.* 1978*a*) and the monomeric enzyme crystallized in the presence of glucose substrate (E′·Gluc) (Bennett & Steitz 1978, 1980). Furthermore, we have solved the structure of a hexokinase dimer (E_2) at 3.5 Å resolution (Steitz *et al.* 1976, 1977). Complexes of native enzyme, E, with either sugar or nucleotide substrates have been examined by diffusing these ligands into the crystals of the enzyme grown in the absence of these ligands. The binary complexes so formed show only minor conformational changes and thus correspond to forms E·gluc (Anderson *et al.* 1978*b*) and E·ATP (Shoham & Steitz 1980) shown in figure 1. The crystal structure that has not as yet been determined is the structure of hexokinase complexed with appropriate analogues of both substrates forming a ternary complex that may be functionally active.

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HEXOKINASE STRUCTURE WITH AND WITHOUT GLUCOSE

The structure of the B isozyme crystallized without glucose has been refined at 2.1 Å resolution to a crystallographic *R* factor of 0.25 (Anderson *et al.* 1978*a*). Since the amino acid sequence has not been chemically determined, and some side chains are disordered in our electron density maps, about 300 of the 3260 atoms other than hydrogen are missing in the current model. Figure 2 shows a stereo space-filling model drawn with the use of these refined coordinates. The shape and size of the deep cleft dividing this enzyme into two lobes are clearly portrayed.

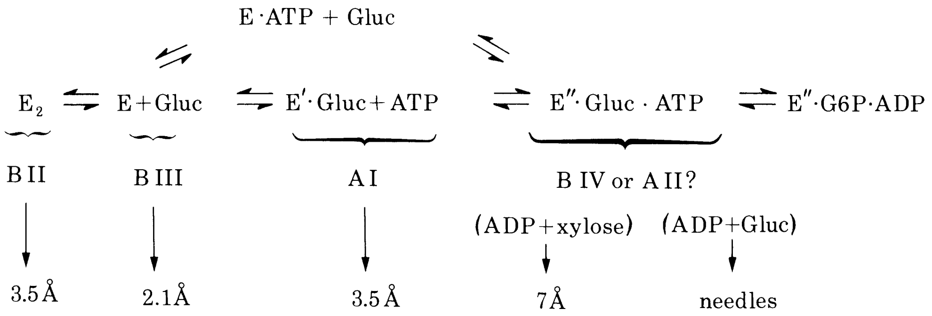

FIGURE 1. Steps in the reaction pathway of the yeast hexokinase monomer, E, which can form an enzymatically active dimer E_2. The preferred pathway is first the addition of glucose to form E′·glucose. E′ and E″ represent conformational alterations induced by ligand binding. The crystals that have been studied and their correspondence to the reaction pathway are indicated.

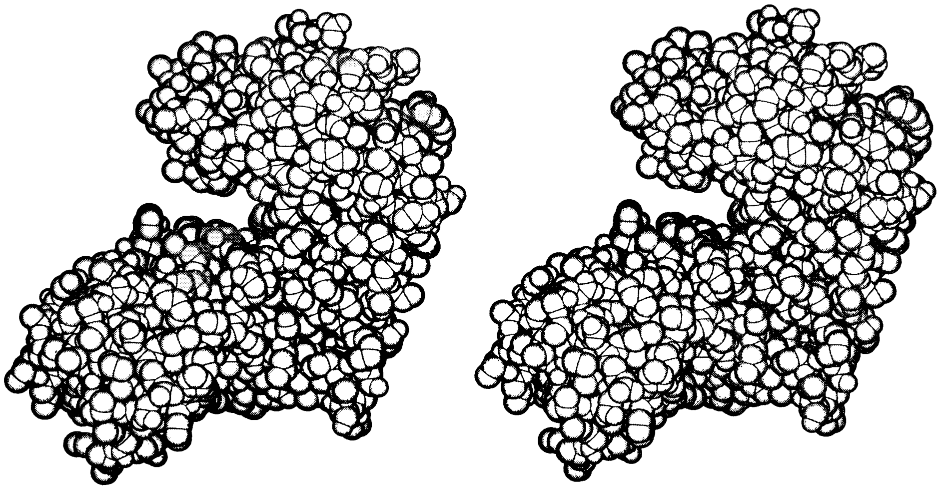

FIGURE 2. A stereo drawing of a space-filling model of yeast hexokinase B in its unliganded, native conformation. The most prominent feature of this drawing is the cleft that divides the molecule into two lobes and is the binding site for glucose.

The structure of the A isozyme crystallized as a complex with glucose has been independently solved at 4.5 Å resolution and refined at 3.5 Å resolution by Bennett & Steitz (1978, 1980). Comparison of these two structures shows a striking difference in the protein conformation; one lobe, constituting about 40% of the molecule, rotates by 12° relative to the other lobe, resulting in atomic movements of the polypeptide backbone of as much as 8 Å (Bennett & Steitz 1978, 1980).

Several lines of evidence establish that this conformational change is produced by the binding of glucose and is not the result of an isozyme difference or crystal forces. Most conclusive is the fact that the radius of gyration of the B isozyme in solution, measured by X-ray scattering, is reduced by 0.95 ± 0.24 Å when glucose is bound (McDonald *et al.* 1979). This value is accurately predicted by the difference of 0.9 Å in the radius of gyration calculated between the refined coordinates of the native B isozyme and the A isozyme–glucose complex. Also, the disintegration of crystals of the A isozyme when glucose is removed, and of B isozyme crystals when high concentrations of glucose are introduced suggest that glucose produces the change. In both crystal forms it appears that the sugar-induced conformational change is sufficiently large to disrupt the crystal lattice if allowed to go to completion.

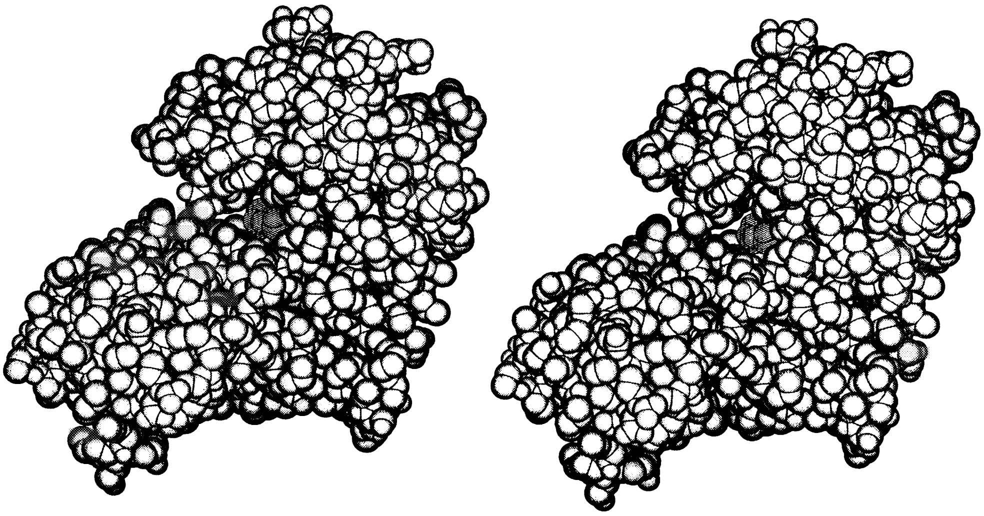

FIGURE 3. A stereo drawing of yeast hexokinase A complexed with glucose. The bound glucose molecule is indicated by a zigzag pattern. The active site cleft is closed by a motion that is essentially a rotation of the upper lobe by 12° relative to the lower lobe.

The structure of the complex between glucose and A isozyme is shown in figure 3. The cleft has closed so that only the 6-hydroxymethyl group of the glucose is accessible to solvent (Bennett & Steitz 1978). Atoms from the smaller lobe come into contact with atoms of the larger lobe and the glucose 6-hydroxyl. Glucose is surrounded by the enzyme in this closed conformation to such an extent that it cannot enter or leave its binding site, which provides an explanation for the observation (Rose *et al.* 1974) that glucose dissociates very slowly (dissociation constant 58 s^{-1}) from its binary complex with hexokinase. Probably, the rate of glucose dissociation is limited by the rate of the enzyme's conformational change from the closed to the open form.

HEXOKINASE·ATP COMPLEX

From a 3.0 Å resolution difference Fourier analysis of an 8-BrAMP complex and model building by using the crystal structure of tripolyphosphatotetraamine cobalt (III) (Merritt *et al.* 1978), a reasonable model for the interaction of Mg-ATP with yeast hexokinase in an initial enzyme–substrate complex has been derived. This provides an approximate view of E·ATP. This approach to determine the mode of ATP binding has been taken because all attempts to bind ATP or ATP analogues with Mg^{2+} or other metals have so far failed with all three of the hexokinase crystal forms that are known at high resolution. In the monomeric hexokinase BIII crystal form, ATP and analogues bind to crystals (in the absence of sugars) but only the adenosine moiety is ordered in the crystal either in 70% saturated ammonium sulphate (Steitz *et al.* 1977) or in 1.4 M citrate (C. M. Anderson & T. A. Steitz, unpublished). Attempts to bind β, γ-imidoATP to crystals of the BII form containing dimer have failed to demonstrate any binding at the active site (Anderson & Steitz 1975), while high concentrations of Cr-ATP disrupt the crystals (C. M. Anderson, R. E. Stenkamp & T. A. Steitz, unpublished). Likewise, diffusion of Cr-ATP into crystals of the hexokinase A complex with glucose results in the disintegration of these crystals.

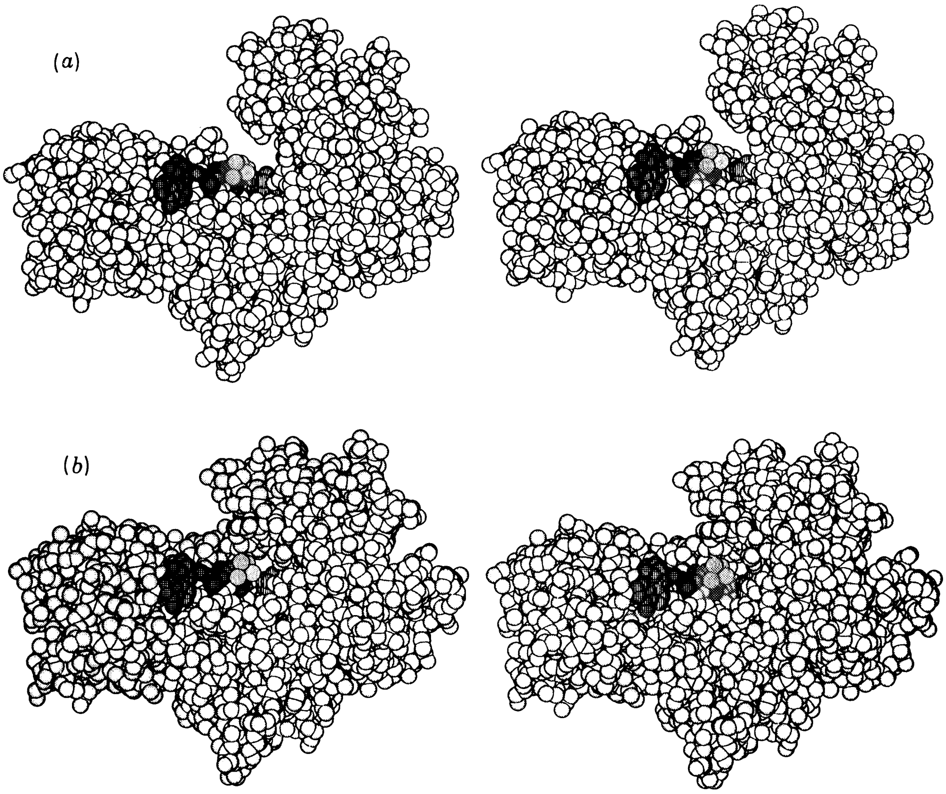

FIGURE 4. Stereo space-filling drawings of hexokinase with both glucose (hatched circles) and cobalt tetraamine ATP bound (cross-hatched circles, ATP; shaded circles, cobalt tetraamine moiety). (*a*) The enzyme in the 'open' form (BIII); (*b*) the enzyme in the 'closed' form (AI).

That the nucleoside binding site described here is the correct active ATP binding site and not a non-specific hydrophobic site is shown by several observations (Steitz *et al.* 1977). While adenosine binds to this site in the same way as AMP, adenine does not bind, demonstrating the importance of the ribose interactions. Also, hexokinase inhibitors, known from studies in solution to be competitive with ATP, bind only to this AMP binding site (Steitz *et al.* 1977).

The most likely explanation for our inability to diffuse ATP into these crystals and produce an ordered complex is that in the presence of both glucose and ATP the enzyme undergoes a substantial conformational change (not accommodated by the crystal lattice) to a structure different from either the open form of the apoenzyme or the closed form of the complex with glucose (Bennett & Steitz 1978). In the absence of glucose and in the apoenzyme form (B III) the phosphates and metal could be disordered because they cannot make the interactions with the small lobe that are possible in the glucose form of the enzyme. This is consistent with the observation that the enzyme's affinity for metal-ATP is about an order of magnitude lower in the absence of glucose (Colowick 1973; Peters & Neet 1978).

It is undoubtedly functionally important that the phosphates of ATP are disordered in the open complex with ATP, since such disorder contributes to reducing the enzyme's inherent ATPase activity. The γ-phosphate of ATP is placed in the correct position for phosphoryl transfer only after the appropriate sugar substrate is bound.

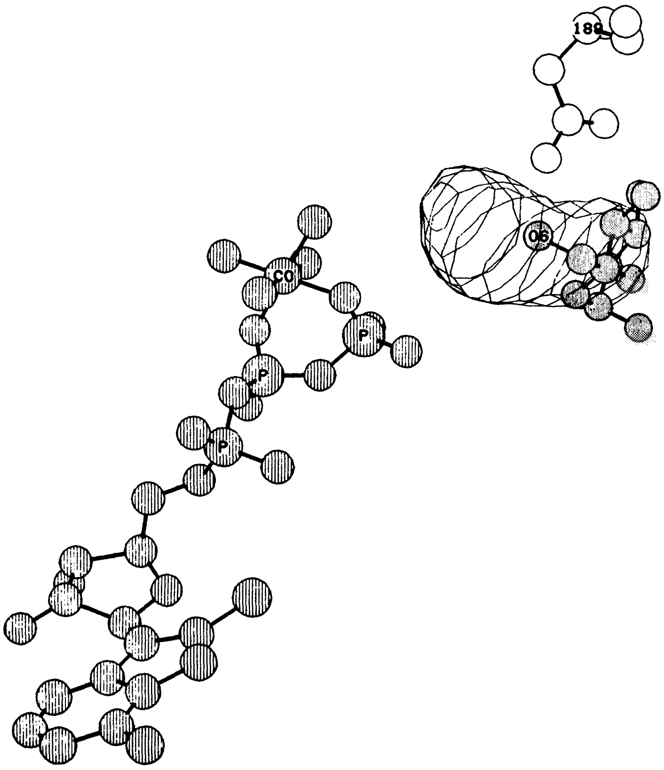

FIGURE 5. A model of bound glucose (shaded circles) superimposed on the difference electron density of a glucose-6-phosphate complex with native hexokinase B III at 5 Å resolution. Cobalt tetraamine 8-BrATP is shown by hatched circles. Asp189 (open circles) is thought to promote the nucleophilic attack of the glucose 6-hydroxyl on the γ-phosphate or ATP.

The model-built Co-ATP is in a completely extended conformation as it is found in complexes with other enzymes (figure 4). The adenine is lying in a shallow slit and against an α-helix in the α-helical domain, similar to the interaction of nucleotide bases with the coat protein of tobacco mosaic virus (Stubbs *et al.* 1977). The adenine is not interacting with the end of a β-sheet as occurs in some other kinases and hydrogenases (Blake & Evans 1974; Bryant *et al.* 1974; Liljas & Rossman 1974). In our current model, the phosphates are interacting with the side chains of Ser393 and Ser212 and perhaps a peptide NH group. None of the phosphates lie at the end of an α-helix as occurs in a number of kinases and dehydrogenases, and no interaction with lysine or arginine groups is currently observed by using the tentative amino acid sequence derived by X-ray diffraction alone (Anderson *et al.* 1978*a*).

It is of considerable interest to examine the ATP complex in both the 'open' structure of the BIII crystals and in the 'closed' structure of the glucose complex to see how the surrounding protein environment changes (figure 4). Since the ATP molecule is located on the surface of the big lobe at the 'mouth' of the big cleft, its surroundings become greatly altered in the closed enzyme conformation (figure 4). Residues 68–72 of the small lobe approach the cobalt ion to within 5 Å. Surprisingly, the three residues 70, 71 and 72 have recently been identified as Gly^{70}-Gly^{71}-Thr^{72} from a chemically sequenced peptide (C. Stachelek, W. Konigsberg, R. Harrison & T. A. Steitz, unpublished). However, at least one peptide NH group is close enough to interact with the ligands of the metal. Interaction between the small lobe and Mg-ATP in the closed complex with glucose but not in the open apoenzyme would explain the synergism in substrate binding to hexokinase, e.g. the sixfold increase in affinity for $Cr(NH_3)_2ATP$ when glucose is bound (Peters & Neet 1978).

In our current model of ATP and glucose bound to the active site, the phosphorus of the γ-phosphate is 5.5–6.0 Å from the 6-hydroxyl of glucose (figure 5). Whether this indicates that the direct phosphoryl transfer to the 6-hydroxyl proceeds by a dissociation mechanism or whether in the 'true' ternary complex the γ-phosphate and 6-hydroxyl are brought closer together remains to be established.

Crystalline hexokinase tertiary complex

Wilkinson & Rose (1981) have shown that neither the crystals of the native monomer (BIII) nor those of the complex with glucose (AI) is capable of catalysing the hexokinase reaction in the crystal. Using isotope trapping methods they have shown, however, that the E′·Gluc structure in the crystal is on the reaction pathway. Presumably, the formation of the ternary complex in the AI crystal results in an altered protein conformation that cannot be accommodated in this crystal form. It is therefore necessary to crystallize a suitable ternary complex from solution.

Two crystal forms have been obtained in the presence of sugar and nucleotide substrate analogues. The B isozyme has been crystallized in the presence of 50 mM xylose and 20 mM ADP to give a crystal form called BIV. K. D. Wilkinson and I. A. Rose (personal communication 1980) have obtained crystals of the A isozyme in the presence of ADP and glucose giving a crystal form called AII. The AII crystal form is capable of taking ATP and glucose to products in the crystal, and thus must correspond to the correct ternary complex. Unfortunately, these crystals are to date only very thin needles, completely unsuitable for X-ray analysis. Wilkinson & Rose have not been able to demonstrate that the BIV crystals can take substrates to products; however, these crystals, though small, are large enough for analysis at low resolution.

The BIV crystals are tetragonal space group I4 with $a = b = 142.3$ Å and $c = 58.4$ Å, with one monomer per asymmetric unit; $V_m = 2.8$ $Å^3$ per dalton. These crystals diffract to at least 2.8 Å, as judged from a 'still' photograph. The crystallization conditions included 50% saturated ammonium sulphate and 50 mM sodium acetate, pH 5, in addition to the xylose and ADP. Since these crystals can also be obtained at pH 7.0, the only difference between these crystallization conditions and those for BI crystals is the addition of xylose and ADP, suggesting that these substrates are binding and altering the protein structure.

The structure was independently determined at 7 Å resolution by multiple isomorphous replacement. The four heavy atom derivatives used were methyl mercury chloride, platinum ethylenediamine dichloride, potassium osmate and potassium platinum nitrite. An electron density map was computed at 7 Å resolution with phases corresponding to a figure of merit of 0.8. The general outline and the helical regions of the molecule can be clearly identified in this map. Alpha-carbon backbone chains of the 'open' as well as the 'closed' form of the enzyme were superimposed on this electron density map with the aid of a computer graphics system, as shown in figure 6. The orientation of the molecule relative to the crystal lattice in the BIII form was established by approximate superposition of the 50 Å long helix L with the density followed by rotation of the α-carbon trace about this helix axis until other helices were satisfactorily positioned within the map.

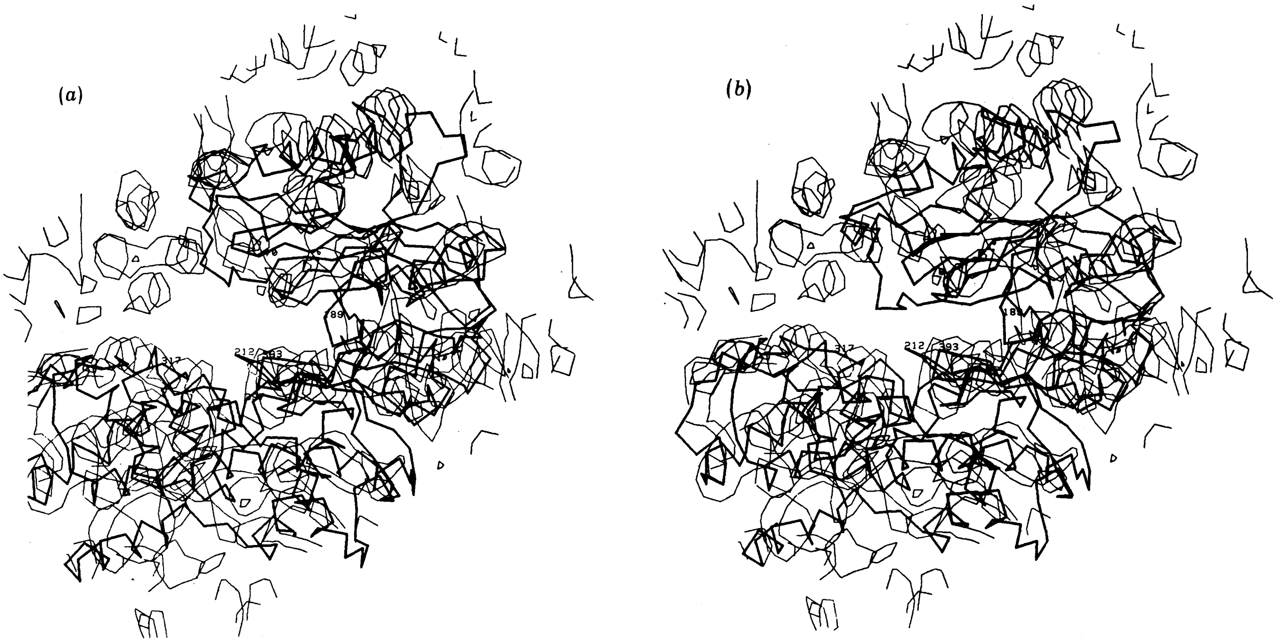

FIGURE 6 (*a*). The α-carbon backbone of the BIII form of hexokinase ('open') superimposed on the 7 Å resolution electron density map of BIV. There is electron density for most major features, suggesting that BIV is in the 'open' conformation. (*b*) The α-carbon backbone of the AI form of hexokinase ('closed') superimposed on the 7 Å resolution electron density map of BIV. The small domain does not fit the density well, suggesting that BIV is 'open'.

BIV is an 'open' conformation, as can be seen in figure 6. At this resolution, 7 Å, the structure is in fact indistinguishable from the 'open' form of the BIII crystal form. Substrate binding studies by the difference Fourier method indicate that the sites for ATP and xylose are the same as the nucleotide and glucose site in BIII.

It is clear that the hexokinase in these crystals is not in the conformation expected for a ternary complex. This would account for the lack of enzymatic activity of these crystals found by Wilkinson & Rose (personal communication, 1980), since the active site region is freely accessible in this crystal to substrates that are diffused in. It is not clear why another crystal form is obtained under identical conditions if the xylose and ADP are not in the crystallization medium.

THE RELATION OF YEAST HEXOKINASE NUCLEOTIDE-BINDING DOMAIN TO OTHER NUCLEOTIDE BINDING DOMAINS

It has often been asked whether the hexokinase nucleotide-binding domain has a structure similar to that of the nucleotide-binding domain of other kinases and dehydrogenases. It is clear that the nucleotide-binding domain of hexokinase looks more like the nucleotide-binding domain of lactate dehydrogenase (LDH) than it looks like haemoglobin. Both nucleotide-binding domains contain a β-pleated sheet flanked on either side by α-helices. Furthermore, the nucleotide binds at the predominantly C-terminal end of the β-sheet. However, half of the hexokinase nucleotide-binding domain has the structural motif of β-β-β, antiparallel β-structure, while the other half, like both halves of the LDH domain, has a β-α-β-α-β motif.

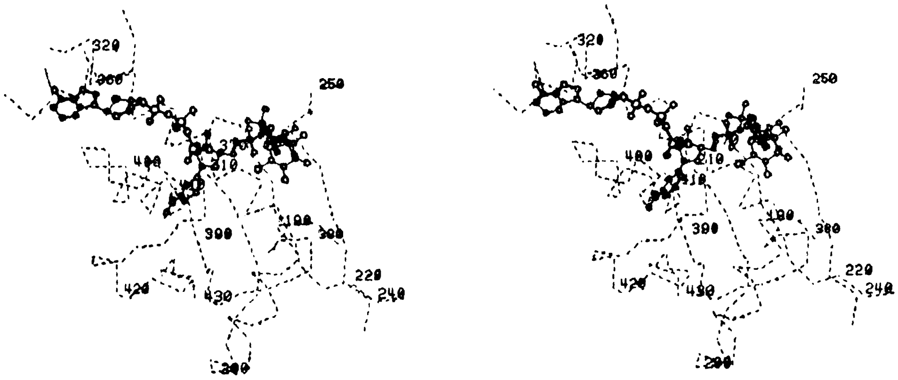

FIGURE 7. A stereo view of the α-carbon backbone of the nucleotide-binding domain of hexokinase with the ATP and glucose substrates superimposed as well as the NAD of LDH, after the nucleotide binding domain of LDH has been aligned. The LDH α-carbon atoms are not shown in this figure.

The similarity in the tertiary structure of parts of hexokinase and the nucleotide-binding domains of lactate dehydrogenase reported by Rossman & Argos (1977) has been re-evaluated with refined protein coordinates and an improved model for the binding of ATP to hexokinase. Superposition of similar regions of the two proteins brings the active site ATP of hexokinase into rough spatial proximity with the binding site for the dinucleotide cofactor of LDH, but the interactions of the two ligands with their respective binding sites are quite different. In fact, the direction of elongation of the extended ATP is perpendicular to the direction of elongation of the extended dinucleotide. Furthermore, the intersubunit ATP site of dimeric hexokinase is not close to the sites predicted by superposition of the two proteins (figure 7), in contrast to the predictions of Rossmann & Argos (1977). Neither the improved comparison of tertiary structures nor the comparison of nucleotide-binding sites supports the idea that these two proteins have evolved from a common precursor.

Comparisons of the nucleotide-binding sites of hexokinase and lactate dehydrogenase were complicated by the observation that two alignments of the relevant domains could be made that gave approximately the same number of equivalent residues and alignment statistics. The existence of such ambiguities suggests that present procedures for comparison of protein tertiary structures produce less reliable quantitative indications of similarity than is generally supposed, at least in instances in which the similarity is as limited as that between these two proteins.

Table 1 lists the distances between the ATP and NAD molecules after the α-carbon backbones of the corresponding nucleotide-binding domains have been superimposed by a least-squares procedure. There are two transformations, 1 and 2, relating the α-carbon atoms of the hexokinase and LDH nucleotide-binding domains that are statistically equivalent in terms of their degree of structural correspondence. In both of these transformations, however, the transferred adenine of LDH lies more than 17 Å from the adenine of ATP in hexokinase. The nicotinamide is in fact somewhat closer, 7 Å in one transformation and 12.5 Å in another. In neither case is the correspondence good. The intersubunit ATP site is even much further away from where it would be predicted to occur if it were in any way related to the nucleotide-binding site of LDH.

Table 1. Distance between observed and predicted nucleotide-binding sites

	hexokinase	ATP	Distance (ATP–NAD)†/Å				
transform	isozyme	site	Ad–Ad	Ad–Ni	Gl–Ra	closest	approach
1	B	A	20.2	12.5	2.9	P_γ–R_N	2.2
2	B	A	17.3	7.1	9.1	P_α–Ni	1.5
3	B	I	26.4	16.4	—	R_A–Ni	15.0

† Distances are between centroids for the groups cited. ATP coordinates were mapped to the LDH coordinate system with the indicated transformation for these calculations; where necessary, I-site ATP coordinates were first converted to the appropriate hexokinase coordinate system by using transformations described by Bennett & Steitz (1980). Abbreviations used: Ad, adenine ring of ATP or NAD; Ni, nicotinamide ring of NAD; Gl, glucose; P_α, P_γ, α, and γ phosphorus atoms of ATP; P_N, phosphorus atom in the nicotinamide mononucleotidyl moiety of NAD; R_N, R_A, ribosyl group attached to the nicotinamide ring of NAD or the adenine ring of either substrate, respectively.

Prospects for future studies of hexokinase

Two major structural experiments remain to be completed. First, the chemically determined amino acid sequence needs to be finished and incorporated into the hexokinase model. The model containing the correct sequence needs to be further refined at 2 Å resolution in the BIII crystal form and at 2.8 Å resolution in the AI crystal form. We are collaborating with W. Konigsberg at Yale on both the sequencing of tryptic peptides and the sequencing of the gene for hexokinase that has recently been cloned in Botstein's laboratory. Secondly, crystallographically suitable crystals of the hexokinase ternary complex with sugar and nucleotide substrate analogues need to be obtained and the structure solved. The best candidates for the ternary complex crystals to date are the AII crystals, known to take substrates to products. We hope that conditions for obtaining larger crystals can be found.

M. Shoham is a Fellow in Cancer Research supported by grant no. DRG-330 FT of the Damon Runyon – Walter Winchell Cancer Fund.

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