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Brain Hexokinase Has No Preexisting Allosteric Site for Glucose 6-Phosphate*

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Difference spectroscopic investigations on the interaction of brain hexokinase with glucose and glucose 6-phosphate (Glc-6-P) show that the binary complexes *E*-glucose and *E*-Glc-6-P give very similar UV difference spectra. However, the spectrum of the ternary *E*-glucose-Glc-6-P complex differs markedly from the spectra of the binary complexes, but resembles that produced by the *E*-glucose-P_i complex.

Direct binding studies of the interaction of Glc-6-P with brain hexokinase detect only a single high-affinity binding site for Glc-6-P (K_D = 2.8 μM). In the ternary *E*-glucose-Glc-6-P complex, Glc-6-P has a much higher affinity for the enzyme (K_D = 0.9 μM) and a single binding site. Ribose 5-phosphate displaces Glc-6-P from *E*-glucose-Glc-6-P only, but not from *E*-Glc-6-P complex. It also fails to displace glucose from *E*-glucose and *E*-glucose-Glc-6-P complexes. Scatchard plots of the binding of glucose to brain hexokinase reveal only a single binding site but show distinct evidence of positive cooperativity, which is abolished by Glc-6-P and P_i. These ligands, as well as ribose 5-phosphate, substantially increase the binding affinity of glucose for the enzyme.

The spectral evidence, as well as the interactive nature of the sites binding glucose and phosphate-bearing ligands, lead us to conclude that an allosteric site for Glc-6-P of physiological relevance occurs on the enzyme only in the presence of glucose, as a common locus where Glc-6-P, P_i, and ribose 5-phosphate bind. In the absence of glucose, Glc-6-P binds to the enzyme at its active site with high affinity. We also discuss the possibility that, in the absence of glucose, Glc-6-P may still bind to the allosteric site, but with very low affinity, as has been observed in studies on the reverse hexokinase reaction.

Brain hexokinase, the first enzyme of the glycolytic pathway, activates glucose for the degradation process by catalyzing its phosphorylation using ATP as the phosphoryl donor. It is a crucial enzyme in the regulation of energy metabolism in the brain, and owes its control properties to inhibition by its reaction product, Glc-6-P[1] and de-inhibition by substrate ATP and effector P_i. The enzyme is a monomer of 98 kDa. (1–3).

In spite of extensive work carried out on this enzyme, an unanswered question to date is whether the regulatory action of these ligands occurs at the acitve site or at allosteric site(s) (4–7). One suggestion has Glc-6-P inhibiting enzyme activity by binding to a "high-affinity" allosteric site (K_D = 2.5 μM) in addition to the "low-affinity" product site, implying that two molecules of Glc-6-P can interact with the enzyme (6). In another viewpoint, Glc-6-P inhibits enzyme activity by binding tightly to the product site (7).

In our laboratory, an observation made during the chromatography of the enzyme on phosphocellulose suggested a role for glucose in the interaction of these phosphate-bearing ligands with brain hexokinase. Briefly, the enzyme in Tris-HCl buffer (pH 7.7) containing 10 mM glucose could be bound to the phosphocellulose matrix and then eluted by the phosphate-bearing ligands at concentrations comparable to their K_D values. The presence of glucose in the buffer during chromatography was crucial. In its absence the enzyme could not be eluted from the column (8, 9).

These results suggested a possible role for glucose in the interaction of the phosphate-bearing ligands with brain hexokinase, a view also implied earlier (10). With a view to clarifying the role of glucose in this interaction, we have carried out equilibrium binding and difference spectroscopic studies with the enzyme. The evidence suggests that an allosteric site for Glc-6-P of physiological significance does not preexist on the enzyme, but requires its prior interaction with glucose. Furthermore, in the absence of glucose, Glc-6-P binds to the product site.

MATERIALS AND METHODS

Chemicals—D-[U-^{14}C]Glucose (specific activity, 255 and 292 mCi/mmol) and $^{32}P_i$ as phosphoric acid (carrier-free) were obtained from the Isotope Division, Bhabha Atomic Research Centre, Trombay, Bombay, India. D-[U-^{14}C]Glucose 6-phosphate (specific activity, 282 mCi/mmol), was obtained from Amersham, United Kingdom.

N-Ethyl morpholine, ribose 5-phosphate, and 2-mercaptoethanol were obtained from Sigma. Ammonium sulfate (enzyme grade) was from Schwarz-Mann. Glucose 6-phosphate dehydrogenase, NADP, ATP, and Glc-6-P were obtained from Boehringer Mannheim, Federal Republic of Germany. All other chemicals were Analytical or Guaranteed Reagent quality obtained from British Drug Houses or Merck.

Preparation of Enzyme—Type I hexokinase was purified from bovine brain mitochondria by the method detailed before (11). The purified enzyme was stored at 4 °C in 10 mM phosphate buffer (pH 7.7) containing 10 mM glucose, 1 mM EDTA, and 5 mM 2-mercaptoethanol. Assay of enzyme activity and determination of protein concentration have also been described previously (11). The specific activity of the enzyme used in these experiments was usually between 50 and 80 units/mg of protein.

Removal of Glucose from the Enzyme—Because earlier methods using exhaustive dialysis for removal of glucose failed to free the enzyme completely from this ligand, an alternative procedure was followed. In this experiment, the enzyme was precipitated three times to 70% saturation with ammonium sulfate in 100 mM phosphate

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[1] The abbreviations used are: Glc-6-P, glucose 6-phosphate; Rib-5-P, ribose 5-phosphate; NEM acetate, *N*-ethyl morpholine acetate.

buffer (pH 8.0). This was followed by exhaustive dialysis (four changes of 500 ml of buffer over 24 h). The removal of glucose by this method was confirmed by the glucose 6-phosphate dehydrogenase-linked assay. Glucose contamination detected by this method was ~0.5 μM.

Ligand Binding Experiments—Binding of ligands to the enzyme was studied using the method of Colowick and Womack (12, 13), employing a home-built Lucite dialysis cell. The dimensions of the cell were closer to the smaller one suggested by Brown and Reichard (14) and had a lower chamber volume of 0.4 ml and an upper chamber volume of 1.0 ml. The dialysis membrane was a square, cut from ordinary Spectrapor dialysis tubing. The flow rate from the cell was maintained at 3.5 ml/min and 0.7-ml fractions were collected. 0.6-ml aliquots in Brays solution were counted in an LKB 1219 Rackbeta counter.

All binding experiments were carried out in 100 mM NEM acetate buffer (pH 8.0) containing 5 mM 2-mercaptoethanol and other additions as detailed in figure legends. Diffusion of the charged ligands, D-[U-^{14}C]Glc-6-P and $^{32}P_i$ showed a tendency to be retarded by the dialysis membrane. This problem was overcome by the addition of 0.1 and 0.2 M KCl, respectively, to the buffer during the course of the binding experiment. In final control runs carried out with each labeled ligand, addition of excess unlabeled ligand had no effect on the steady-state concentration of isotope in the effluent. Loss of isotope from the upper chamber during dialysis was <3%. All binding experiments were carried out at 25 ± 2 °C.

Results were plotted according to Scatchard (15) where $\bar{\mu}$ = concentration of bound ligand/total enzyme concentration and [F] is the concentration of free ligand. The intercept (n) on the x-axis is the number of binding sites, and the slope is the reciprocal of the dissociation constant for the complex.

Ultraviolet Difference Spectra—These were recorded on a Cary-17D spectrophotometer. Matched 1.0-cm path-length quartz cuvettes were used. The base line was recorded with both cuvettes containing the same enzyme solution in 10 mM NEM acetate buffer (pH 8.0) and 5 mM 2-mercaptoethanol. In these experiments sugar ligands at the concentrations used did not show any absorbance in the range of wavelength scanned. The difference spectrum produced by interaction of the ligand was recorded after the addition of saturating concentrations of the ligand to the sample cuvette and an equal volume of buffer to the reference cuvette. At the end of each spectral scan, addition of the same concentration of ligand to the reference cuvette restored the original base line. This precaution was taken to make sure that there was no time-dependent baseline shift. Recorded spectra were corrected for base-line deviations. All spectra were recorded at 22 ± 1 °C.

RESULTS

Ultraviolet Difference Spectra Induced by Sugar Ligands—Fig. 1*A* presents the UV difference spectra resulting from the addition of saturating levels of P_i or glucose to the enzyme. Although P_i by itself does not cause any spectral perturbations in the 260–300 nm region, the glucose spectrum is characterized by the presence of several positive and negative peaks in this spectral range.[2] Addition of saturating levels of Glc-6-P induces a difference spectrum (Fig. 1*B*) very similar to that of glucose (Fig. 1*A*). Binding of P_i to the enzyme does not produce any difference spectrum (Fig. 1*A*), but its interaction with the enzyme in the presence of glucose (Fig. 1*C*) gives rise to a spectrum which cannot be accounted for simply as the additive effect of these two ligands. This indicates the formation of an E-glucose-P_i complex where the two ligand sites are mutually interactive. Fig. 1*D* is the difference spectrum produced by the interaction of Glc-6-P with the enzyme in the presence of glucose. This spectrum is clearly different from that produced by the binding of glucose (Fig. 1*A*) or Glc-6-P (Fig. 1*B*) with the enzyme and indicates that both

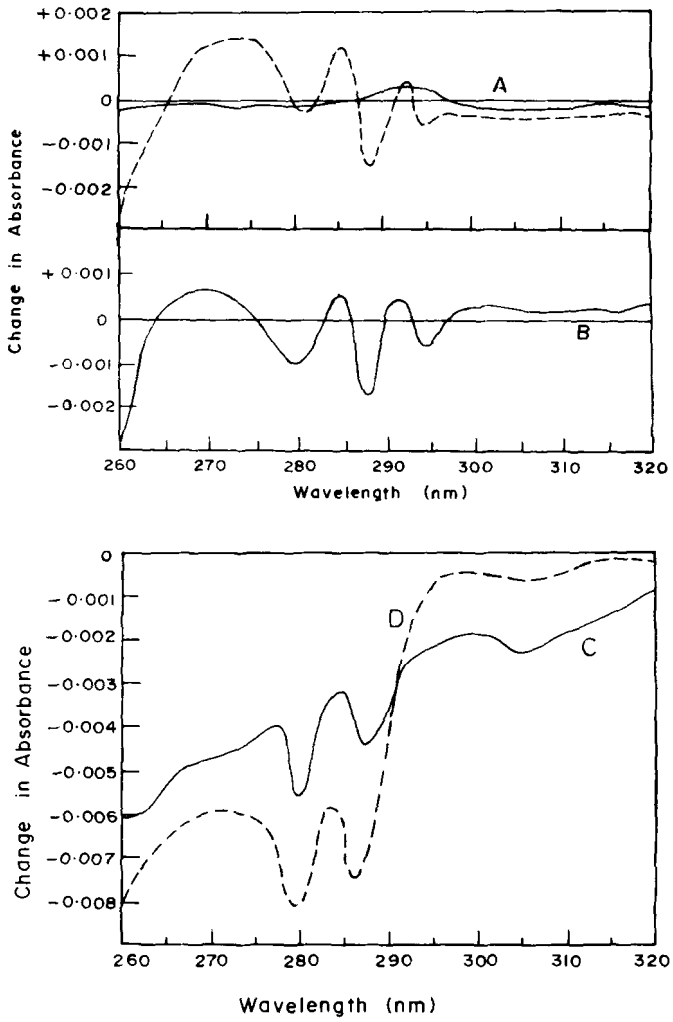

FIG. 1. **Ultraviolet difference spectra of brain hexokinase induced by different ligands.** In *A*, reference cuvette, 11 μM hexokinase; sample cuvette, 11 μM hexokinase + 200 μM glucose are shown (– – –). Reference cuvette, 13 μM hexokinase; sample cuvette, 13 μM hexokinase + 1 mM P_i are indicated (——). *B* is reference cuvette, 11 μM hexokinase; sample cuvette 11 μM hexokinase + 100 μM Glc-6-P. *C* is reference cuvette, 13.5 μM hexokinase + 10 mM glucose; sample cuvette, 13.5 μM hexokinase + 10 mM glucose + 1 mM P_i. *D* shows reference cuvette, 17 μM hexokinase + 10 mM glucose; sample cuvette, 17 μM hexokinase + 10 mM glucose + 100 μM Glc-6-P.

glucose and Glc-6-P can bind simultaneously to the enzyme, a result reported earlier (6, 7, 9, 10, 17). It is interesting to note that, in the presence of glucose, Glc-6-P and P_i induce similar difference spectra (Fig. 1, *C* and *D*).

Binding of Glc-6-P to Brain Hexokinase in the Presence and Absence of Glucose—The number of binding sites (n) and dissociation constants (K_D) of Glc-6-P with respect to the enzyme were determined as described under "Materials and Methods."

In two different experiments where enzyme concentrations were 4.0 and 8.8 μM, and Glc-6-P concentrations were varied from 4 to 104 μM, free and bound ligand concentrations were determined. Scatchard plot analysis of these data gave n = 0.82 ± 0.14 and K_D = 2.8 ± 0.48 μM (Fig. 2*A*). These results clearly indicate that there is a single high-affinity site for Glc-6-P on the enzyme.

Binding of Glc-6-P with hexokinase was also studied in the presence of glucose (2.5 mM). The resultant Scatchard plot (Fig. 2*B*) analysis gave n = 0.89 ± 0.05 and K_D = 0.9 ± 0.05 μM.

[2] Earlier experiments using enzyme from which glucose had been removed by conventional dialysis failed to give glucose-induced difference spectra. However, when the present method for removal of glucose by multiple precipitations was used, difference spectra were obtained, indicating contamination of glucose in earlier samples of the enzyme.

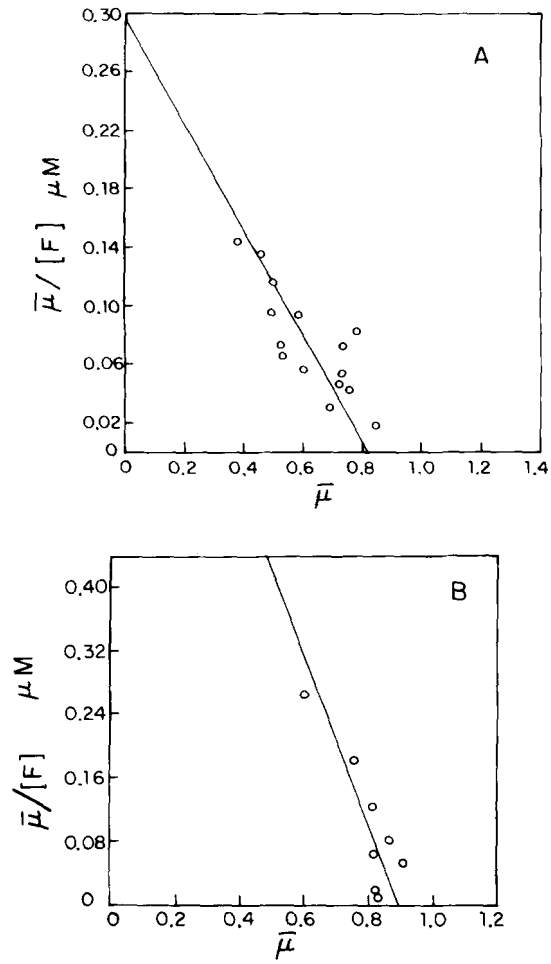

FIG. 2. **Scatchard plots of the binding of glucose 6-phosphate to brain hexokinase in the presence and absence of glucose.** The samples were in 100 mM NEM acetate buffer (pH 8.0) containing 0.1 M KCl and 5 mM 2-mercaptoethanol. Binding was studied at 25 ± 2 °C. In *A*, the results presented are from two different experiments where enzyme concentrations were 4 and 8.8 μM and Glc-6-P concentration was varied from 4 to 104 μM. Least-squares fit gave $n = 0.82 \pm 0.14$ and $K_D = 2.8 \pm 0.48$ μM. For *B*, the sample contained 8.0 μM enzyme and 2.5 mM glucose. Concentration of Glc-6-P was varied from 7 to 200 μM. Least-squares fit analysis gave $n = 0.89 \pm 0.05$ and $K_D = 0.9 \pm 0.05$ μM.

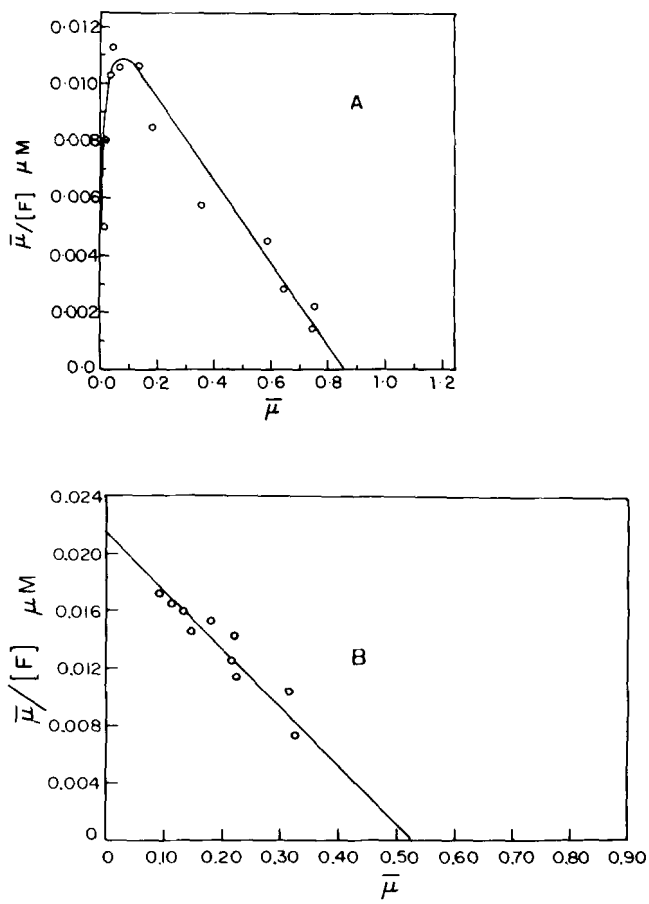

FIG. 3. **Scatchard plots for the binding of glucose to brain hexokinase in the absence and presence of P_i.** The samples were in 100 mM NEM acetate buffer (pH 8.0) containing 5 mM 2-mercaptoethanol. In *A*, enzyme concentration was 120 μM and amounts of glucose varied from 4 to 1000 μM. $n = 0.86 \pm 0.23$ binding sites and $K_D = 72 \pm 4$ μM. The sample in *B* contained 5 mM P_i. The results are presented from two different experiments where enzyme concentrations were 87.2 and 27.5 μM, respectively, and glucose concentration was varied from 8 to 600 μM. Least-squares fit analysis gives $n = 0.51 \pm 0.07$ binding sites with a $K_D = 24 \pm 4$ μM.

This dissociation constant for Glc-6-P determined in the presence of glucose is 3-fold smaller than in the absence of glucose. This observation again supports the formation of an *E*-glucose-Glc-6-P complex and suggests synergistic interaction between the two sugar binding sites on the enzyme. Similar observations have been made by earlier workers (6, 9, 10, 17).

Binding of Glucose—Binding of glucose was monitored using [U-^{14}C]glucose. Fig. 3*A* shows the result of a typical binding experiment. In several different experiments where enzyme concentrations were varied from 25 to 125 μM and glucose from 4 to 1000 μM, a downward curvature to the Scatchard plot was always obtained. By extrapolation of the data in the higher concentration range of glucose, we have determined an average $n = 0.67 \pm 0.18$ binding sites and $K_D = 67 \pm 4$ μM. The observed curvature of the Scatchard plot is a characteristic feature of positive cooperativity (18, 19). For bovine brain hexokinase, which is a monomeric enzyme, this is a rather surprising result. The Scatchard plots for the binding of glucose in the presence of 100 μM Glc-6-P (plot not presented) and 5 mM P_i (Fig. 3*B*) are straight lines giving

TABLE I

Dissociation constants and stoichiometry of binding of different ligands with brain hexokinase

Values shown are mean ± S.D.

Serial no.	Ligand	Complex	Binding sites	K_D
			n	μM
1.	Glc-6-P	*E*-Glc-6-P	0.82 ± 0.14	2.8[a] ± 0.48
2.	Glc-6-P	*E*-glucose-Glc-6-P	0.89 ± 0.05	0.9[a] ± 0.05
3.	Glucose	*E*-glucose	0.67 ± 0.18	67.0 ± 4
4.	Glucose	*E*-glucose-P_i	0.51 ± 0.07	24.0 ± 4
5.	Glucose	*E*-glucose-Glc-6-P	0.50 ± 0.03	7.0 ± 0.46
6.	P_i	*E*-P_i	0.63 ± 0.17	197.0[b] ± 55
7.	P_i	*E*-glucose-P_i	0.51 ± 0.11	56.0[b] ± 12
8.	Rib-5-P	*E*-glucose-Rib-5-P	ND[c]	~2500

[a] Binding studies were carried out in the presence of 0.1 M KCl in the buffer.

[b] Binding studies were carried out in the presence of 0.2 M KCl in the buffer.

[c] ND, not determined.

K_D values of 7 ± 0.46 and 24 ± 4 μM, respectively, and about 0.5 binding sites for glucose in the presence of these ligands.

Interaction of P_i with the Enzyme—Inorganic phosphate, which is a regulatory ligand of brain hexokinase, is known to de-inhibit the Glc-6-P inhibited enzyme (21–24). Direct bind-

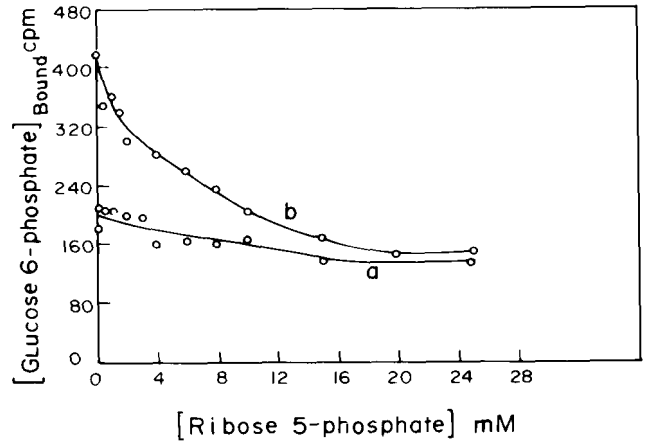

FIG. 4. **Effect of the addition of increasing amounts of Rib-5-P to *E*-[^{14}C]Glc-6-P in the absence and presence of glucose.** The samples were in 100 mM NEM acetate buffer (pH = 8.0) containing 0.1 M KCl and 5 mM 2-mercaptoethanol. *a*, 5.4 μM enzyme and 4.2 μM [^{14}C]Glc-6-P; *b*, 5.2 μM enzyme, 2.5 mM glucose and 4.2 μM [^{14}C]Glc-6-P.

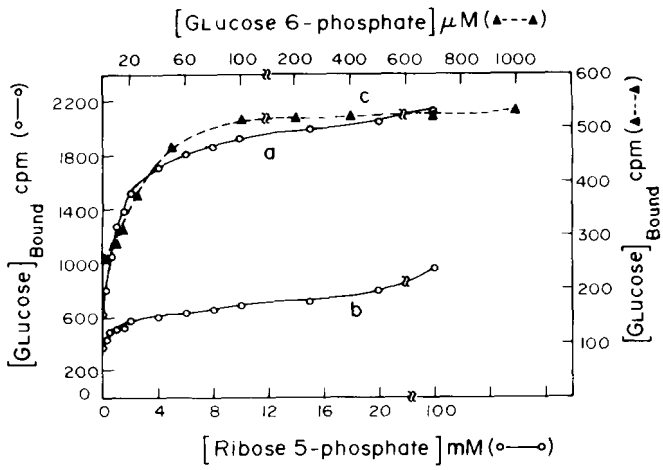

FIG. 5. **Effect of the addition of Rib-5-P or Glc-6-P on the binding of glucose with brain hexokinase.** The samples were in 100 mM NEM acetate buffer (pH 8.0) containing 5 mM 2-mercaptoethanol and *a*, 53 μM hexokinase and 8 μM [^{14}C]glucose; *b*, 16.4 μM hexokinase, 100 μM Glc-6-P and 8 μM [^{14}C]glucose; and *c*, 47 μM enzyme and 6 μM [^{14}C]glucose.

ing of phosphate with the enzyme in the presence and absence of glucose was studied using $^{32}P_i$ (Table I). The enzyme concentrations used in these experiments were 43 and 70 μM, respectively. The P_i concentrations were varied from 10 μM to 5 mM.

Because charged ligands showed a tendency to exhibit nonspecific adsorption on the surface of the dialysis membrane, 0.2 M KCl was included in the buffer. Dissociation constants determined in the presence and the absence of glucose were 56 ± 12 and 197 ± 53 μM, respectively, with a single binding site for this ligand, a result qualitatively similar to that reported earlier (17).

Displacement of [^{14}C]Glc-6-P from-E-[^{14}C]Glc-6-P and E-glucose-[^{14}C]Glc-6-P by Ribose 5-Phosphate—In an attempt to differentiate between the Glc-6-P binding sites in *E*-Glc-6-P and *E*-glucose-Glc-6-P complexes, we employed Rib-5-P to displace Glc-6-P from these complexes. We observed that the pentose phosphate displaces [^{14}C]Glc-6-P only in the presence of glucose (*i.e.* from *E*-glucose-[^{14}C]Glc-6-P) and not in its absence (*i.e.* from *E*-[^{14}C]Glc-6-P) (see Fig. 4). Because glucose and Glc-6-P exhibit synergistic binding, an apparent displacement of Glc-6-P from *E*-glucose-Glc-6-P may also arise if glucose is displaced by Rib-5-P and synergism between the two sites is lost. This possibility was eliminated in an experiment in which the displacement of [^{14}C]glucose was measured in *E*-[^{14}C]glucose and *E*-[^{14}C]glucose-Glc-6-P complexes. The result showed that the addition of Rib-5-P brought about an increase in glucose binding (Fig. 5). The dissociation constant of Rib-5-P in *E*-glucose-Rib-5-P was estimated to be ~2.5 mM (Table I).

It is interesting to note that all the phosphate-bearing ligands (*i.e.* P_i, Glc-6-P, Rib-5-P) have a similar effect on the binding of glucose to the enzyme (Table I).

Rib-5-P was reported to have no inhibitory effect on hexokinase (4). Because we observed competitive displacement of Glc-6-P, we re-investigated the inhibitory properties of Rib-5-P in kinetic experiments (not shown). In these experiments, Rib-5-P was found to inhibit the enzyme, albeit weakly. Assuming inhibition by Rib-5-P to be competitive with respect to ATP, an apparent K_i (~3 mM) was obtained. This is in agreement with the K_D of Rib-5-P in the *E*-glucose-Rib-5-P complex (see Table I).

DISCUSSION

The strong similarity in the difference spectra produced by the interaction of glucose or Glc-6-P with the enzyme allows us to conclude that Glc-6-P by itself binds to the catalytic site of the enzyme. That this is a single high-affinity site (K_D = 2.8 ± 0.48 μM) is evident from our binding data. Lazo *et al.* (6), contradicting earlier results of Chou and Wilson (25) and Ellison *et al.* (17), proposed that there are two binding sites for Glc-6-P on the enzyme, a high-affinity regulatory site (K_D = 2.5 μM) and a low-affinity catalytic one. Solheim and Fromm (7) have pointed out several flaws in the data of Lazo *et al.* (6) which cast some doubts on their conclusion. More serious, Ureta *et al.* (26), using an enzyme preparation similar to that used by Lazo *et al.* (6), have concluded, on the basis of their kinetic data, that there cannot be a major difference in the relative affinities of Glc-6-P for regulatory and catalytic sites. These authors have obtained a K_i value of 200 μM for the inhibitory site on brain hexokinase, as compared with a value of 2.5 μM determined by Lazo *et al.* (6). This difference of 2 orders of magnitude is difficult to explain. Again, if there are two sites for Glc-6-P of similar affinity, then neither Chou and Wilson (25) nor Ellison *et al.* (17) should have failed to detect them in their binding experiments. Our results are in agreement with the view of Solheim and Fromm (7) that, in the concentration range in which it functions as a regulator, Glc-6-P binds to the active site of the enzyme with high affinity. However, this does not mean that Glc-6-P necessarily performs its regulatory function at the active site, for these binding experiments were carried out in the absence of glucose, which would make a difference as shown below. Our conclusion that Glc-6-P binds to the active site of brain hexokinase receives strong support from the results of our experiments on the mapping of sugar ligand binding sites on the enzyme using magnetic resonance methods. In these experiments, the distances determined from the reference Mn(II) at the tight-binding site of the enzyme (27) to the different protons of glucose in the *E*-Mn(II)-glucose complex (28) are close to the values obtained for the corresponding protons of Glc-6-P in the *E*-Mn(II)-Glc-6-P complex.[3]

The data of Solheim and Fromm (7) and of Sols and his

[3] G.K. Jarori, S.R. Kasturi, and U.W. Kenkare, manuscript in preparation (1988).

co-workers (5, 26) provide evidence for the existence of a relatively low-affinity second Glc-6-P binding site on brain hexokinase. These data are suggestive of a low-affinity Glc-6-P inhibition rather than high-affinity Glc-6-P inhibition at high Glc-6-P concentrations. Earlier, Jarori (29) and Kenkare *et al.* (9) presented spectroscopic data to show that, at high concentrations of Glc-6-P (~2.5 mM) the UV difference spectrum is changed, implying that more than one molecule of Glc-6-P interacts with the enzyme. However, such a weak binding second site cannot have any regulatory function in the context of known facts about hexokinase regulation *in vitro*.

The binding and difference spectroscopic data presented in this paper provide strong confirmation of earlier observations regarding the effect of glucose on the binding of Glc-6-P to the enzyme, and the formation of *E*-glucose-Glc-6-P complex (6, 7, 9, 10, 17). In the presence of glucose, the dissociation constant of Glc-6-P for the enzyme shows a 3-fold decrease, and, more importantly, only one binding site is observed. Earlier, Ellison *et al.* (17) had also observed only one binding site for Glc-6-P on the enzyme and a reduction of its K_D from 0.50 to 0.12 μM in the presence of glucose.

It is instructive to compare the difference spectral data for brain and yeast hexokinase in the presence of glucose and Glc-6-P, in terms of the regulatory properties of these enzymes. Roustan *et al.* (30) have shown that glucose and Glc-6-P induce the same spectral effects in yeast hexokinase, concluding that they both bind to the active site of the enzyme. In brain hexokinase, too, we have observed similar effects and drawn the same conclusion. Similarity between the two enzymes, however, ends here. For Roustan *et al.* (30) also found that glucose and Glc-6-P together do not produce any additive spectral effects, as expected from competitive binding. It is to be noted that yeast hexokinase is not inhibited or regulated by Glc-6-P. On the other hand, brain hexokinase, which is so regulated, behaves differently in an identical experiment. In the presence of both ligands it gives a difference spectrum quite unlike that induced by each ligand separately (Fig. 1*D*). Because the regulatory behavior of Glc-6-P in the forward hexokinase reaction is observed only in the presence of glucose, it follows that, in the ternary *E*-glucose-Glc-6-P complex represented by this difference spectrum (Fig. 1*D*), Glc-6-P is bound in the regulatory or allosteric mode. The very high affinity ($K_D = 0.9 \pm 0.05$ μM) with which Glc-6-P binds to the enzyme in this complex is consistent with this hypothesis. Yeast hexokinase is not subject to regulation by Glc-6-P and does not need a site different from its product site for binding Glc-6-P, and hence does not show a change in its difference spectrum when Glc-6-P binds to it in the presence of glucose.

The displacement of Glc-6-P by Rib-5-P from the ternary *E*-glucose-Glc-6-P complex but not from the binary *E*-Glc-6-P complex (Fig. 4) clearly reflects the distinct locations of Glc-6-P in its regulatory and product configurations. It calls to mind the proposal made by Fromm and his co-workers (7, 32) that Glc-6-P, after its formation as a product from glucose, may still bind within the active site domain, but not at the glucose subsite. This difference in the locations of the two binding sites is reflected in the different specificities for the binding of the glycosyl portions of glucose and Glc-6-P as observed by Crane and Sols (4). As expected from its competition with Glc-6-P when bound in the regulatory mode in *E*-glucose-Glc-6-P complex, Rib-5-P caused significant inhibition of the enzyme by apparently displacing ATP from the active site ($K_i \sim 3$ mM). This inhibition by Rib-5-P runs counter to the data of Crane and Sols (4), who failed to observe any inhibition by Rib-5-P.

The close similarity of the difference spectrum produced by the *E*-glucose-Glc-6-P complex with that of *E*-glucose-P_i is strongly suggestive of a common locus for P_i and the phosphate moiety of Glc-6-P in its allosteric configuration. The stimulation of glucose binding caused by all the phosphate-bearing ligands such as Glc-6-P, Rib-5-P (Fig. 5), and P_i (Table I) and increase in the affinity of Glc-6-P (Fig. 2*B*) and P_i (Table I) in the presence of glucose shows that, in the ternary complexes of these ligands, the phosphate binding subsite is the one that interacts with the glucose binding site. The apparent positive cooperativity exhibited by glucose binding to the enzyme (whatever the reason) is also abolished by the phosphate ligands Glc-6-P and P_i, implying binding at a common locus.

Considering all the data available, we conclude as follows: (*a*) In the range of concentrations in which Glc-6-P functions as a substrate or regulator, it can bind strongly either to the catalytic site or to the allosteric site[4] (in the presence of glucose) but not to both with equal affinity at the same time. (*b*) In the forward reaction in the presence of glucose, it binds at the allosteric site. In the reverse reaction it binds mainly at the catalytic site. (*c*) Increased binding of Glc-6-P observed in the presence of glucose is due to its binding with higher affinity at the allosteric site. (*d*) The high-affinity allosteric site for Glc-6-P observed in the presence of glucose becomes a weak binding site for this ligand in the absence of glucose. We can now explain the high-affinity binding of Glc-6-P to brain hexokinase in the reverse reaction as shown by Solheim and Fromm (7). The weak binding of Glc-6-P to the second site observed by Solheim and Fromm (7) as well as Ureta *et al.* (26) in the reverse hexokinase reaction (wherein glucose was removed from the reaction mixture) must obviously be the allosteric site, which in the absence of glucose binds Glc-6-P very weakly ($K_D \geq 200$ μM). Our experiments suggest a common locus for Glc-6-P and P_i which can explain their antagonism. The stimulation by P_i of the reverse hexokinase reaction (31) can also be explained by the competitive binding of Glc-6-P and P_i at the allosteric site.

In our model, the potent inhibition by Glc-6-P of the forward hexokinase reaction must predominantly be at the allosteric site, which is the only site binding Glc-6-P under these conditions. Depending on the relative concentrations of glucose and Glc-6-P, some product inhibition by Glc-6-P can also be envisaged. The mechanism of inhibition is likely to be the displacement of ATP from the active site by a conformational change induced by Glc-6-P interaction at the allosteric site. Because P_i has no effect on the forward hexokinase reaction in the absence of Glc-6-P, we assume that the γ-phosphate site of ATP is not identical to the phosphate subsite to which either Glc-6-P or P_i binds. A close proximity of these phosphate sites, however, is not ruled out.

The apparent cooperativity in glucose binding to brain hexokinase, which is a monomeric enzyme (Fig. 3*A*), is a puzzling result and we have no obvious way to explain it. The only reasonable explanation we can offer is based on our previous study (20) that, at high concentrations, a fraction of brain hexokinase exists as a dimer. It is possible that the observed cooperativity in the binding of glucose might arise from cooperative interactions between glucose binding sites of the dimer. Dimerization is also induced by Glc-6-P, which is known to bring about large conformational changes in the

[4] We have used the term "allosteric site" in a broad sense to include the binding of Glc-6-P not only to a site topologically distinct from the active site, but also to any locus within the active site domain, but outside its glucose subsite.

enzyme (20). However, a straight-line Scatchard plot is obtained for glucose binding in the presence of Glc-6-P. The two subunits of the Glc-6-P-induced dimer thus appear to be incompetent where cooperative interactions between their glucose binding sites is concerned. The dimerization induced in brain hexokinase by Glc-6-P is also reversed in the presence of P_i (20), which stabilizes the monomeric state. This may thus explain the straight line Scatchard plot obtained for glucose in the presence of P_i (Fig. 3*B*).

Reduction in the number of binding sites for glucose in the presence of Glc-6-P or P_i is also an intriguing result considering the fact that both these ligands are involved in inhibition and de-inhibition, respectively, of the enzyme. Although one may be tempted to call it half-of-the-sites binding, such a conclusion is unwarranted on the basis of our limited data. A proper interpretation of this finding would require an adequate and tested hypothesis to explain the apparent positive cooperativity in glucose binding which is abolished in the presence of P_i (Fig. 3*B*) or Glc-6-P.

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