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Mapping of glucose and glucose-6-phosphate binding sites on bovine brain hexokinase

A ^{1}H- and ^{31}P-NMR investigation

Gotam K. JARORI, Sujata B. IYER, Sitapati R. KASTURI and Umakant W. KENKARE

Tata Institute of Fundamental Research, Bombay, India

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Inhibition of bovine brain hexokinase by its product, glucose 6-phosphate, is considered to be a major regulatory step in controlling the glycolytic flux in the brain. Investigations on the molecular basis of this regulation, i.e. allosteric or product inhibition, have led to various proposals. Here, we attempt to resolve this issue by ascertaining the location of the binding sites for glucose and glucose 6-phosphate on the enzyme with respect to a divalent-cation-binding site characterized previously [Jarori, G. K., Kasturi, S. R. & Kenkare, U. W. (1981) *Arch. Biochem. Biophys. 211*, 258–268]. The paramagnetic effect of enzyme-bound Mn(II) on the spin-lattice relaxation rates (T_1^{-1}) of ligand nuclei (^{1}H and ^{31}P) in E · Mn(II) · Glc and E · Mn(II) · Glc6*P* complexes have been measured. The paramagnetic effect of Mn(II) on the proton relaxation rates of C1-H_α, C1-H_β and C2-H_β of glucose in the E · Mn(II) · Glc complex was measured at 270 MHz and 500 MHz. The temperature dependence of these rates was also studied in the range of 5–30 °C at 500 MHz. The ligand nuclear relaxation rates in E · Mn(II) · Glc are field-dependent and the Arrhenius plot yields an activation energy (ΔE) of 16.7–20.9 kJ/mol. Similar measurements have also been carried out on C1-H_α, C1-H_β and C6-^{31}P at 270 MHz (^{1}H) and 202.5 MHz (^{31}P) for the E · Mn(II) · Glc6*P* complex. The temperature dependence of ^{31}P relaxation rates in this complex was measured in the range 5–30 °C, which yielded ΔE = 9.2 kJ/mol. The electron-nuclear dipolar correlation time (τ_c), determined from the field-dependent measurements of proton relaxation rates in the E · Mn(II) · Glc complex, is 0.22–1.27 ns. The distances determined between Mn(II) and C1-H of glucose and glucose 6-phosphate are ≈ 1.1 nm and ≈ 0.8 nm, respectively. These data, considered together with our recent results [Mehta, A., Jarori, G. K. & Kenkare, U. W. (1988) *J. Biol. Chem. 263*, 15492–15498], suggest that glucose and glucose 6-phosphate may bind to very nearly the same region of the enzyme.

The structure of the binary Glc6*P* · Mn(II) complex has also been determined. The phosphoryl group of the sugar phosphate forms a first co-ordination complex with the cation. However, on the enzyme, the phosphoryl group is located at a distance of ≈ 0.5–0.6 nm from the cation.

The step catalysed by hexokinase is accepted as a major control point in cerebral glycolysis in brain [1]. Activity of this enzyme is strongly inhibited by glucose 6-phosphate (Glc6*P*) and this inhibition can be relieved by P_i [2]. This has been an attractive model for regulation of glycolysis in the brain as simultaneous de-inhibition of hexokinase and stimulation of phosphofructokinase by P_i would lead to increased glycolytic flux. An understanding of the molecular mechanism of brain hexokinase regulation requires knowledge of the nature of Glc6*P* binding to the enzyme. On the basis of the non-competitive nature of Glc6*P* inhibition with respect to glucose [3] and observation of differences in molecular specificities of sugar substrate and inhibitor [4], an allosteric inhibitory site, spatially distinct from the catalytic site for Glc6*P*, was proposed. However, several kinetic studies by other workers [5, 6] and the observation of a reverse hexokinase reaction [7], strongly support the binding of Glc6*P* at the product site.

In order to ascertain the nature of Glc6*P*-induced inhibition of hexokinase activity (allosteric or product), we decided to map the glucose- and Glc6*P*-binding sites on the enzyme using the paramagnetic relaxation probe method [8–10]. The paramagnetic reference point used in this study was the high-affinity Mn(II)-binding site on the enzyme characterized earlier [11]. The metal ion bound at this site was later shown to be non-essential for catalysis [12]. A nitroxyl spin-label was also introduced on one of the non-essential –SH groups on the enzyme. This spin-label was located about 2.0 nm away from the high-affinity cation-binding site on the enzyme [13]. No measurable paramagnetic effect of the spin-label on the spin-lattice relaxation rates of ligand protons was observable in the complexes of glucose and Glc6*P* with spin-labelled enzyme (Jarori, G. K., Mehta, A. & Kenkare, U. W., unpublished experiments). Thus the spin-label probe was found to be unsuitable for mapping sugar-binding sites. In the following report we present our results on the mapping of

Correspondence to G. K. Jarori, Tata Institute of Fundamental Research, Homi Bhabha Road, Colaba, Bombay 400 005, India

Note. Preliminary data on the mapping of the glucose-binding site formed part of an invited poster presented at the XI International Conference on Magnetic Resonance in Biological Systems (Goa, India, Sept. 17–23, 1984) and appeared as part of an invited article published in *Magnetic Resonance in Biology and Medicine* (Govil, Khetrapal and Saran, eds) pp. 163–181, McGraw-Hill Publishing Company Ltd, New Delhi, India.

Enzymes. Hexokinase (EC 2.7.1.1); glucose-6-phosphate dehydrogenase (EC 1.1.1.49).

glucose- and Glc6*P*-binding sites using enzyme-bound Mn(II) as a relaxation probe.

EXPERIMENTAL PROCEDURES

Materials

ATP, 0.1 M $MnCl_2$ solution in 0.15 M NaCl and Glc6*P* were purchased from Sigma Chemical Company. NADP and Glc6*P* dehydrogenase were obtained from Boehringer-Mannheim (FRG). Bovine serum albumin was obtained from Nutritional Biochemical Corporation (USA). Chelex-100 resin was supplied by Bio-Rad (Richmond, CA, USA) and D_2O (99.8%) was obtained from Stholer Isotope Chemical Company. All other chemicals used were of analytical reagent grade.

Enzyme preparation

Hexokinase type-I was prepared from bovine mitochondria by the method of Redkar and Kenkare [14] with one modification: EDTA was eliminated during the second DEAE-cellulose column chromatography. Protein concentration was determined by the method of Lowry et al. [15], using crystalline bovine serum albumin as a standard. Enzyme activity was assayed by the Glc6*P*-dehydrogenase-coupled assay [14]. The specific activity of the enzyme used in these experiments was about 60 – 65 units/mg protein. The molarity of enzyme solution was determined by taking 98 kDa as the molecular mass of the enzyme [14].

For NMR measurements, enzyme and ligand solutions were passed through Chelex-100 which was previously washed with acid and equilibrated to pH 8.0 with 10 mM Tris/HCl prepared in deionized water. For ^{1}H-NMR, final enzyme samples were in 10 mM Tris/HCl pH 8.0 containing 5 mM 2-mercaptoethanol. These samples were dialyzed three times against 5 vol. 10 mM Tris/HCl pH 8 in D_2O. The samples were then lyophilized and dissolved in D_2O containing 5 mM 2-mercaptoethanol. Lyophilized enzyme samples usually had a final specific activity of about 55 units/mg. For ^{31}P-NMR experiments, enzyme samples were in 50 mM Tris/HCl pH 8.0; 15 – 20% D_2O was added to the samples for field/frequency lock. D_2O used for this purpose was passed through a 2-ml Chelex-100 column.

NMR measurements

^{1}H-NMR measurements at 270 MHz and 500 MHz were made on Bruker WH-270 (located at Bangalore) and AM-500 (located at Bombay). ^{31}P-NMR measurements at 202.5 MHz were made on the AM-500 which is equipped with a 10-mm multinuclear probe, a variable temperature controller and an Aspect-3000 computer. Spin-lattice relaxation time (T_1) measurements were made using a standard inversion recovery sequence (180-τ-90, 5T_1). Proton chemical shifts have been measured relative to sodium 3-trimethylsilyl-(2,2,3,3-$^2H_4$)propionate which was used as an internal reference. The solvent resonance was suppressed using the gated-decoupling technique to avoid dynamic range problem. All samples for ^{1}H-NMR were placed in 5-mm sample tubes and measurements were made at 20 °C with a spinning rate of 20 Hz. ^{31}P-NMR chemical shifts reported here are with respect to $\approx$ 85% H_3PO_4 placed in a capillary as an external reference. The errors quoted for relaxation rates are based on standard deviations between measurements made on independent samples.

Theoretical basis for distance determination

The theory for the effect of a paramagnetic cation on the spin-lattice relaxation rate (T_1^{-1}) of a ligand nucleus [16 – 19] in a macromolecule · cation · ligand complex has been reviewed extensively [8, 9, 20 – 22]. The effect of a paramagnetic ion on the spin-lattice relaxation rate (T_{1p}^{-1}) of a ligand nucleus in a macromolecular complex is given by

$$T_{1p}^{-1} = p/(T_{1M} + \tau_M), \tag{1}$$

where T_{1M}^{-1} is the relaxation rate of the observed nucleus in the paramagnetic complex; τ_M is the life-time of the complex and p is the mole fraction of the ligand in the macromolecular paramagnetic complex. Clearly, if $\tau_M \gg T_{1M}$ (i.e. slow exchange), measuring T_{1p}^{-1} will not yield structural information. However, if $\tau_M \gg T_{1M}$, the distance-dependent relaxation rate (T_{1M}^{-1}) can be obtained from T_{1p}^{-1}. T_{1M}^{-1} is related to the distance r, between the cation and the ligand nucleus by

$$T_{1M}^{-1} = (C/r)^6 \cdot f(\tau_c), \tag{2}$$

in which C is a constant whose value depends on the nature of paramagnetic ion and the relaxing nucleus. For Mn(II)-^{1}H and Mn(II)-^{31}P, the values of C are 81.2 and 60.1 nm $s^{-1/3}$ respectively [10]. For Mn(II) complexes, at the magnetic field used in present experiments [23]:

$$f(\tau_c) = 3\,\tau_c/(1 + \omega_I^2\tau_c^2), \tag{3}$$

where τ_c is the correlation time for the electron nuclear dipolar interaction.

In the equations above, the contribution of hyperfine coupling between the cation and the ligand nucleus is neglected [24, 25] and the assumptions implicit in this equation can be found in earlier reviews [9, 18, 20 – 22]. These assumptions are quite justifiable for Mn(II) [8].

From Eqns (1 – 3), it can easily be shown that

$$pT_{1p} = \tau_M + (r/C)^6 \cdot 1/3\tau_c + (r/C)^6 \cdot \omega_I^2\tau_C/3, \tag{4}$$

so that a plot of pT_{1p} (normalized relaxation time) vs ω_I^2 will be a straight line with $(r/C)^6/3\tau_c + \tau_M$ as the y-axis intercept and $(r/C)^6\tau_c/3$ as the slope. The square root of the ratio of slope to intercept then would yield the value of τ_c, if $T_{1M} \gg \tau_M$. In the case where $T_{1M} > \tau_M$ and a precise value of τ_M cannot be evaluated, the y-axis intercept of a plot of ω_I^2 vs pT_{1p} will give $\tau_M + (r/C)^6 \cdot 1/3\ \tau_c$. In such a case, the ratio of slope to intercept will yield a lower limit of electron-nuclear dipolar correlation time.

RESULTS

Fig. 1a shows ^{1}H-NMR spectrum of glucose at 500 MHz. Tentative assignments for various resonances have been made earlier [26, 27]. In solution, glucose exists as a mixture of α and β anomers. Integration of the area under the resonances assigned to α-anomeric proton at 5.24 ppm (C1-H_α) and β-anomeric proton (C1-H_β) at 4.65 ppm indicate that about 34% of glucose is present as the α-anomer. This is in agreement with earlier studies [28]. This ratio of two anomers remains unchanged in the presence of brain hexokinase. Spin-lattice relaxation rates (T_1^{-1}) of the three protons C1-H_α, C1-H_β and C2-H_β (3.22 ppm) have been measured in the presence and absence of the enzyme and the paramagnetic cation, Mn(II).

Addition of the enzyme to glucose results in a slight increase in the relaxation rates of all the three protons. Inclusion

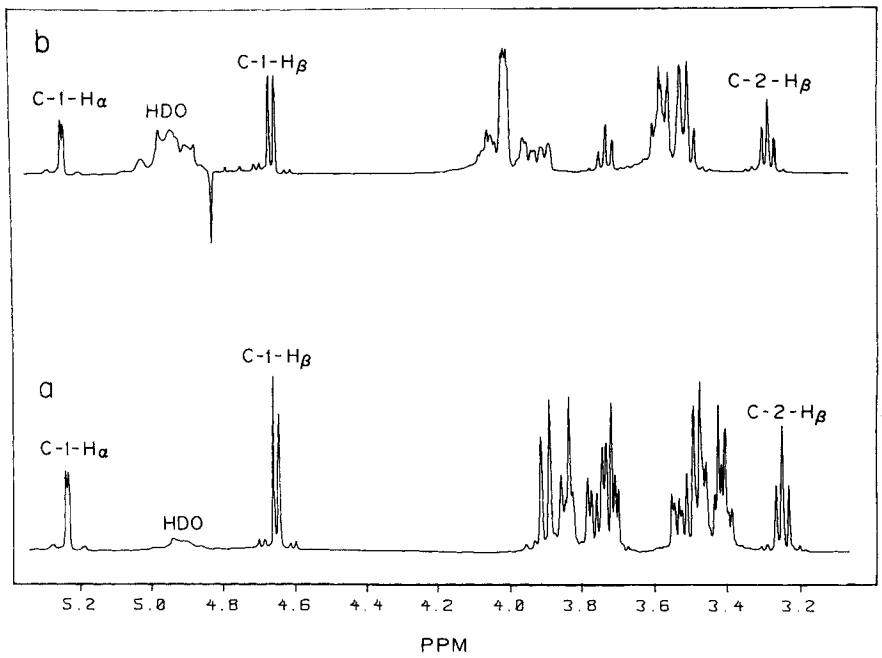

Fig. 1. *500-MHz ^{1}H-NMR spectrum of D-glucose (a) and D-glucose 6-phosphate (b)*

Table 1. *Normalized paramagnetic proton relaxation rates, $(pT_{1p})^{-1}$, for E · Mn(II) · Glc complex*

All the samples were in 10 mM Tris/HCl pH 8.0. Measurements were made at 25 ± 1 °C. The composition of samples: (A) 62–186 μM hexokinase; 10–20 mM Glc and 40–200 μM $MnCl_2$; (B) 91.6–97.3 μM hexokinase; 5 mM Glc and 50–200 μM $MnCl_2$. Concentration of E · Mn(II) · Glc complex was calculated assuming K_d = 25 μM for Mn(II) binding and that the enzyme is fully saturated with glucose

Sample	^{1}H-NMR frequency	$(pT_{1p})^{-1}$ for		
		C1-H_α	C1-H_β	C2-H_β
	MHz	s^{-1}		
A	270	78 ± 23	111 ± 27	65 ± 16
B	500	55 ± 11	55 ± 9	39 ± 9

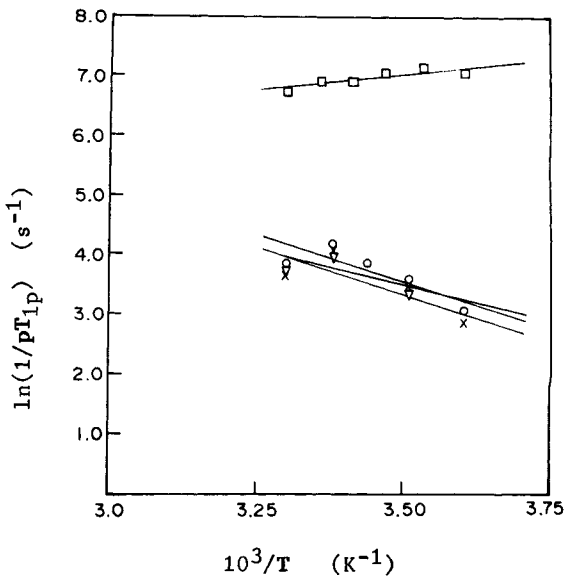

Fig. 2. *Plot of ln(1/pT$_{1p}$) vs 10^3/T for ^{31}P of E · Glc6P · Mn(II) (□) and C1-H_α (○), C1-H_β (×) and C2-H_β (▽) of E · Mn(II) · Glc.* See text for activation energies (*ΔE*). Sample conditions are same as in Tables 1 and 2

of Mn(II) along with the enzyme led to a large increase in the glucose proton relaxation rates. This is due to the formation of the enzyme · Mn(II) · Glc complex. The paramagnetic contribution to relaxation rates of the three glucose protons was evaluated by measuring the relaxation rates as a function of Mn(II) ion concentration. Care was taken to limit the Mn(II) concentration so as to permit binding only at the high-affinity site on the enzyme. High concentrations of glucose (i.e. [E] ≪ [glucose] ≫ $K_{d,E\cdot Glc}$) assured complete saturation of enzyme with the ligand. Since there is only one binding site for glucose on the enzyme [29], the only relevant equilibrium which needs to be considered for the evaluation of mole fraction (p) of glucose in the macromolecular paramagnetic complex (E · Mn(II) · Glc) is

$$E \cdot Glc + Mn(II) \rightleftharpoons E \cdot Glc \cdot Mn(II),$$

with a K_d = 25 ± 4 μM for the binding of Mn(II) ion on the enzyme at the tight binding site. Further, since the enzyme does not exhibit any anomeric specificity, it has been assumed that anomeric distribution of the enzyme-bound glucose is similar to that of free glucose [11]. Table 1 summarizes the normalized relaxation rates $[(pT_{1p})^{-1}]$ measured at 270 and 500 MHz.

To evaluate the effect of exchange on relaxation rates, a temperature dependence of $(pT_{1p})^{-1}$ was studied in the range 5–30 °C at 500 MHz. The results are presented as an Arrhenius plot (Fig. 2). The activation energy obtained for the three protons of glucose are in the range 16.7–20.9 kJ/mol. These energies are slightly higher than the range expected for T_{1M}^{-1}-associated processes (4.2–12.6 kJ/mol) [8, 21]. Since the life-time of the enzyme complex (τ_M) is frequency-indepen-

dent, the observed frequency dependence of $(pT_{1p})^{-1}$ suggests that T_{1M}^{-1} is the dominant contributing process. However, the possibility of some contribution from τ_M^{-1} cannot be ruled out. Our previous estimate of a spin-spin relaxation rate $[(pT_{2p})^{-1} = 1200\ s^{-1}]$ [11] in the E · Mn(II) · Glc complex supports the notion that fast exchange prevails as $(pT_{1p})^{-1} \ll (pT_{2p})^{-1} < \tau_M^{-1}$. However, it cannot be taken as unequivocal evidence for fast exchange conditions as $(pT_{2p})^{-1}$, evaluated from line-width measurements, is based on assumptions which are not easy to verify, particularly so in the case of nuclei which have a large chemical shift range (e.g. ^{31}P, ^{13}C etc.) [30]. The turnover rate of hexokinase is about 170 s^{-1}, i.e. it takes about 5–6 ms for a single reaction cycle to be completed. Usually, partial complexes of enzyme substrates have life-times which are much smaller than the single turnover time. It is reasonable to assume that the life-time of glucose in E · Mn(II) · Glc will be in the range 0–4 ms.

The frequency dependence of $(pT_{1p})^{-1}$ for the glucose protons can be used to determine τ_c [31], provided τ_c is field-independent and $\tau_M \ll T_{1M}$. In cases where a partial contribution from the exchange correlation time is observable (i.e. $\tau_M < T_{1M}$), only a lower limit of τ_c can be obtained. Further, it is obvious from Eqn (4) that the y-intercept of the plot of pT_{1p} vs ω_I^2 will always be $> \tau_M$. The lowest value of the y-intercept among the three glucose protons was 5.5 ms, indicating $\tau_M < 5.5$ ms. It may be assumed that the life-time of glucose in a macromolecular complex will lie in the range of 0–4 ms. With this range of τ_M, the estimated value of τ_c is in the range 0.22–1.27 ns. Distances calculated between Mn(II) and various protons of glucose on enzyme are summarized in Table 3. The glucose-binding site is located at about 1.0–1.2 nm from the Mn(II)-binding site on the enzyme. This range represents the outer limits for the distance and is arrived at by taking into account the range of τ_c values calculated, including the partial contribution from the exchange correlation time (τ_M) and experimental uncertainties.

Glc6P · Mn(II) and E · Mn(II) · Glc6P complexes

In the ^1H-NMR spectrum, C1-H_α and C1-H_β anomeric protons of Glc6*P* give rise to two doublets at 5.24 and 4.65 ppm (Fig. 1b). In the ^{31}P-NMR spectrum, two resonances arising from the two anomers are observable. However, ^{31}P chemical shifts of Glc6*P* are highly pH-dependent. Since, Glc6*P* interacts only weakly with Mn(II) ($K_d \approx 34$ mM) [32], we have measured ^1H and ^{31}P spin-lattice relaxation rates as a function of Mn(II) concentration. Normalized relaxation rates $[(pT_{1p})^{-1}]$ are presented in Table 2. For small-molecular-mass complexes, τ_c is dominated by the rotational correlation time (τ_r) at high magnetic fields. Using a value of $\tau_c = 0.1$ ns [31], distances between Mn(II) and various ligand nuclei have been determined. C1-H_α and C1-H_β are located at a distance of 0.58 ± 0.02 nm and 0.59 ± 0.02 nm, respectively, from Mn(II) in the Glc6*P* · Mn(II) complex. The distance of 0.28 ± 0.01 nm between Mn(II) and ^{31}P suggests the formation of a first co-ordination complex with the cation.

In the presence of hexokinase, the ^1H as well as the ^{31}P resonances of Glc6*P* are broadened, both the anomers exhibiting this broadening. It was not possible to resolve phosphorus resonances of the anomers in the presence of the enzyme. Thus there is no observable anomeric specificity in the binding of Glc6*P* with the enzyme. Paramagnetic relaxation rates due to Mn(II) were measured at five different cation concentrations. Normalized relaxation rates were computed assumming the following two equilibria:

$$E \cdot Glc6P + Mn(II) \rightleftharpoons E \cdot Mn(II) \cdot Glc6P \quad K_d = 25 \pm 4\ \mu M;$$

$$Glc6P + Mn(II) \rightleftharpoons Glc6P \cdot Mn(II) \quad K_d = 34\ mM.$$

In these experiments the Glc6*P* concentration was varied over 3.33–9.5 mM and since the K_d for Glc6*P* with enzyme is < 3 μM [6, 29], all the enzyme will be bound with Glc6*P*. Hence binding of Mn(II) with free enzyme was not considered.

The normalized relaxation rates $[(pT_{1p})^{-1}]$ for C1-H_α, C1-H_β and C6-P in the macromolecular complex are summarized in Table 2. (In determining the mole fraction of E · Mn(II) · Glc6*P*, only one binding site for Glc6*P* on the enzyme has been considered, in agreement with our recent binding studies [29].) In these experiments, the enzyme concentrations used are much less than the ligand concentration. This leads to the formation of two paramagnetic complexes, viz. Glc6*P* · Mn(II) and E · Mn(II) · Glc6*P*, contributing to the observed relaxation rates. It is imperative to ensure that the major contribution to the paramagnetic relaxation rate is made by the complex of interest, which is E · Mn(II) · Glc6*P* in this case. The contribution to the measured paramagnetic relaxation rate arising from the Glc6*P* · Mn(II) complex was $< 10\%$ (in the case of ^{31}P) and $< 20\%$ (in the case of ^1H).

In order to ascertain whether the relaxation rates in the macromolecular complex are affected by the life-time (τ_M) of the complex, $(pT_{1p})^{-1}$ of ^{31}P was measured as a function of temperature in the range 5–30 °C. Results are presented as an Arrhenius plot (Fig. 2) and the activation energy (ΔE) determined from the plot is 9.2 kJ/mol. This value is significantly lower than the ΔE expected for τ_M (usually > 16.7 kJ/mol). Decrease in $(pT_{1p})^{-1}$ with increase in temperature and the low value of ΔE suggest that relaxation rates are dominated by T_{1M}^{-1} and fast exchange conditions prevail (i.e. $T_{1M}^{-1} \ll \tau_M^{-1}$). Since $(pT_{1p})^{-1}$ for C1-H_α and C1-H_β are smaller than

Table 2. *Normalized paramagnetic relaxation rates (pT_{1p}^{-1}) for ^{31}P and ^1H in Glc6P · Mn(II) and E · Mn(II) · Glc6P complexes*
All samples were in 10 mM Tris/HCl pH 8.0 for ^1H-NMR measurements and 50 mM Tris/HCl pH 8.0 for ^{31}P-NMR. Measurements were made at 202.5 MHz for ^{31}P, 270 MHz for ^1H, and at 25 ± 1 °C. Composition of samples: (A) 3.33 mM Glc6*P* and 3.33–20 μM $MnCl_2$; (B) 10–100 mM Glc6*P* and 50–100 μM $MnCl_2$; (C) 140 μM hexokinase, 9.5 mM Glc6*P* and 50–100 μM $MnCl_2$; (D) 132.7 μM hexokinase, 3.33 mM Glc6*P* and 3.33–20 μM $MnCl_2$. In the samples containing the enzyme (C and D) and Glc6*P*, all the enzyme was assumed to be present as E · Glc6*P* complex ($K_d = 2$ μM). The equilibrium concentration of Glc6*P* · Mn(II) and E · Mn(II) · Glc6*P* in the samples containing Glc6*P*, $MnCl_2$ and enzyme, have been calculated assuming the following equilibria:
$E \cdot Glc6P + Mn(II) \rightleftharpoons E \cdot Mn(II) \cdot Glc6P \quad K_d = 25\ \mu M$
$Glc6P + Mn(II) \rightleftharpoons Glc6P \cdot Mn(II) \quad K_d = 34\ mM$

Sample	$(pT_{1p})^{-1}$		
	^{31}P	^1H	
	C6-P	C1-H_α	C1-H_β
	s^{-1}		
A	27300 ± 4900	–	–
B	–	4470 ± 580	3850 ± 390
C	–	890 ± 35	640 ± 150
D	1180 ± 340	–	–

Table 3. *Distance of Mn(II) from various ligand nuclei in E · Mn(II) · Glc, E · Mn(II) · Glc6P and Glc6P · Mn(II) complexes*
The range of $f(\tau_c)$ values provided here for all enzyme complexes reflects the uncertainty in the determination of τ_c value. The distance range provided for E · Mn(II) · Glc complex also absorbs the uncertainty in the lifetime of the complex. The distances have been computed with $\tau_M = 0-4$ ms (see text for details)

Complex	Nucleus of the ligand	Frequency	$f(\tau_c)$	Distance (r)
		MHz	ns	nm
E · Mn(II) · Glc	C1-H_α	270	0.58 – 0.87	1.07 – 1.21
	C1-H_β	270	0.58 – 0.87	0.97 – 1.14
	C2-H_β	270	0.58 – 0.87	1.11 – 1.25
E · Mn(II) · Glc6*P*	C1-H_α	270	0.58 – 0.87	0.76 – 0.81
	C1-H_β	270	0.58 – 0.87	0.77 – 0.89
	C6-P	202.5	0.61 – 1.07	0.52 – 0.63
Glc6*P* · Mn(II)	C1-H_α	270	0.30	0.58 ± 0.02
	C1-H_β	270	0.30	0.59 ± 0.02
	C6-P	202.5	0.30	0.28 ± 0.01

that of C6-P, it can be concluded that measured relaxation rates in the macromolecular complex are a function of distance between the cation and the relaxing nucleus rather than that of the life-time (τ_M) of the complex. Using $\tau_c = 0.22-1.27$ ns, distances between the cation and various nuclei have been calculated. C1-H_α, C1-H_β and C6-P of Glc6*P* are located at ≈ 0.79, ≈ 0.83 and ≈ 0.58 nm, respectively, from the cation on the enzyme (see Table 3).

Note that in the preceding calculations, the same τ_c value has been used for E · Mn(II) · Glc and E · Mn(II) · Glc6*P* complexes. The value of $\tau_c = 0.22-1.27$ ns is short enough to be attributable to τ_s (electron spin relaxation time) of Mn(II) in the macromolecular complex. The rotational correlation time (τ_r) for this enzyme is about 40 ns. Also, since hexokinase dimerizes in the presence of Glc6*P* [40], τ_r will be even larger (80 ns) and hence not likely to make any significant contribution to τ_c.

DISCUSSION

The molecular mechanism of inhibition of brain hexokinase by Glc6*P* has been controversial ever since 1954 when Crane and Sols [4] made their proposal for a separate site (other than the active site) on the enzyme for Glc6*P*. This proposal was based on the failure of glucose to reverse the inhibition and on the kinetically observed differences in the specificities for the binding of glycosyl portion of glucose and Glc6*P* to the enzyme.

Since the molecular mass of brain hexokinase is twice that of yeast hexokinase, it has been suggested that the brain enzyme might have evolved by duplication and fusion of a gene coding for the 50-kDa polypeptide chain of the yeast enzyme [33] and by subsequent evolution of one of the two catalytic sites into a regulatory one [34]. Thus a proposal for an allosteric site for Glc6*P* was also attractive from an evolutionary view point. This model predicts the existence of two binding sites for Glc6*P* (product and allosteric) per molecule of the enzyme. However, in several binding studies, only one binding site for Glc6*P* has been detected [6, 29, 35]. Although in one study Lazo et al. [36] provided evidence for two Glc6*P*-binding sites on the enzyme, the validity of their data is strongly contested [7]. The finding of Fromm and coworkers, that Glc6*P* inhibition is competitive versus ATP [5], led them to suggest that Glc6*P* competes with the γ-phosphoryl group of ATP at the nucleotide site on the enzyme. Further, they suggested that the hexose moiety of Glc6*P* might occupy an adjacent site within the active-site domain but outside its glucose subsite [6, 37, 38].

There are, therefore, three different possibilities for the relative disposition on hexokinase of binding sites for glucose and Glc6*P*: (a) both sugar ligands bind at the same site and hence Glc6*P*-induced inhibition is a case of product inhibition; (b) glucose binds at the active site but Glc6*P* occupies the ribosyl-phosphate-binding pocket of the ATP subsite at the active-site domain; (c) glucose and Glc6*P* bind at topologically distinct sites, as would be expected if the mode of Glc6*P* inhibition were allosteric.

In this paper, we have attempted to distinguish between these possibilities by adopting a more direct physical approach. Using paramagnetic relaxation measurements, we have localized the glucose- and Glc6*P*-binding sites at about 1.1 nm and 0.8 nm, respectively, from the cation-binding site on the enzyme. This will place the sites occupied by the C1 proton of the substrate and of the inhibitor no more than 1.9 nm apart and no closer than 0.3 nm. Our recent ultraviolet difference spectroscopic and ligand-binding studies [29] support the latter possibility.

The work reported by Mehta et al. [29] leads to the conclusion that glucose and Glc6*P* bind at the same site on the enzyme. However, the present data suggests that, though the two sugars bind at the same region of the enzyme, their binding modes are likely to be different, accounting for the observed 0.3-nm difference in the localization of the C1 proton of glucose and Glc6*P*. It should be noted that since distances have been determined from only one reference point, the ligand-binding site may be located anywhere on the spherical surface with a radius r nm. For unambiguous localization, distances from at least three different reference points will be required [8].

These findings thus rule out the possibility of distinct spatial locations for glucose and Glc6*P*, the more so because this implies two binding sites for Glc6*P* on the enzyme for which there is no satisfactory evidence. Our recent studies [29] and those from other laboratories [6] have clearly shown that there is only one Glc6*P*-binding site on the enzyme of physiological relevance. However, the present data cannot distinguish between the first two possibilities referred to above regarding the precise location of the single Glc6*P* site within the active-site domain of the enzyme.

Caution should also be exercised in using the distance information alone to differentiate among the various proposals made regarding the location of Glc6*P*-binding site. One of the implicit assumptions involved in such an approach is that there is no relative movement of the probe and ligand-binding sites when different sugars bind to the enzyme. This may not hold true for regulatory proteins such as hexokinase which exhibit ligand-induced conformational changes (reviewed in [39]). If the induced conformational changes bring about differential movement of the two sites, rather different distances may be obtained even though the two ligands bind at the same site. The observed difference in distances of the glucose- and Glc6*P*-binding site(s) from the cation-binding site is small but significant. The possibility that this difference arises due to different conformational states of glucose and Glc6*P* bound on the enzyme cannot be ruled out.

The Mn(II) to phosphorus distance of about 0.58 nm suggests a close proximity of the cation-binding site and the active site. However, in our earlier studies [12], it was conclusively shown that it is not essential for catalysis for the cation to be the binding site: it is the nucleotide-bound metal ion which is required for catalysis [12]. The measured distance of 0.58 nm is long enough to exclude the formation of a first-coordination-sphere complex between the phosphoryl group and the metal ion when bound to the enzyme. This supports our previous conclusion that the metal ion at the high-affinity site does not participate in catalysis.

From the value of $(pT_{1p})^{-1}$ for ^{31}P in E · Mn(II) · Glc6*P*, one can calculate the minimum dissociation rate constant, τ_M^{-1}, for Glc6*P*. This value is $> 1180 \pm 340\ s^{-1}$. Since the k_{cat} for formation of Glc6*P* from glucose is about $170\ s^{-1}$, the dissociation of this product from the catalytic site cannot be rate-limiting in the overall reaction cycle.

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