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

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

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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) · Glc6P complexes have been measured. The paramagnetic effect of Mn(II) on the proton relaxation rates of C1-Ha, C1-H, and C2-H 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 ($\Delta E$) of 16.7–20.9 kJ/mol. Similar measurements have also been carried out on C1-Ha, C1-H, and C6-$^{31}$P at 270 MHz ($^1$H) and 202.5 MHz ($^{31}$P) for the E · Mn(II) · Glc6P complex. The temperature dependence of $^{31}$P relaxation rates in this complex was measured in the range 5–30°C, which yielded $\Delta E = 9.2$ kJ/mol. The electron-nuclear dipolar correlation time ($\tau_D$), 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 $\approx 1.1$ nm and $\approx 0.8$ 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 Glc6P · 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 $\approx 0.5–0.6$ 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 (Glc6P) and this inhibition can be relieved by Pi [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 Pi would lead to increased glycolytic flux. An understanding of the molecular mechanism of brain hexokinase regulation requires knowledge of the nature of Glc6P binding to the enzyme. On the basis of the non-competitive nature of Glc6P 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 Glc6P, 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 Glc6P at the product site.

In order to ascertain the nature of Glc6P-induced inhibition of hexokinase activity (allosteric or product), we decided to map the glucose- and Glc6P-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 Glc6P 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

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Enzymes. Hexokinase (EC 2.7.1.1); glucose-6-phosphate dehydrogenase (EC 1.1.1.49).
glucose- and Glc6P-binding sites using enzyme-bound Mn(II) as a relaxation probe.

EXPERIMENTAL PROCEDURES

Materials

ATP, 0.1 M MnCl₂ solution in 0.15 M NaCl and Glc6P were purchased from Sigma Chemical Company. NADP and Glc6P 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 D₂O (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 Glc6P-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 'H-NMR, final enzyme solutions were in 50 mM Tris/HCl against 2-mercaptoethanol. These samples were dialyzed three times against 2-mercaptoethanol. Lyophilized enzyme samples usually had molecular mass of the enzyme [14].

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NMR measurements

'1H-NMR measurements at 270 MHz and 500 MHz were made on Bruker WH-270 (located at Bangalore) and AM-500 (located at Bombay). 31P-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 a 2-ml Chelex-100 column. Spin-lattice relaxation time (T₁) measurements were made using a standard inversion recovery sequence (180°-τ-90°, 5T₁). Proton chemical shifts have been measured relative to sodium 3-trimethylsilyl-(2,2,3,3-²H₄)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 '1H-NMR were placed in 5-mm sample tubes and measurements were made at 20°C with a spinning rate of 20 Hz. 31P-NMR chemical shifts reported here are with respect to 85% H₃PO₄ 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 (T₁⁻¹) of a ligand nucleus [16–19] in a macromolecule \cdot cation \cdot ligand complex has been reviewed extensively [8, 9, 20–22]. The effect of a paramagnetic ion on the spin-lattice relaxation rate (T₁⁻¹) of a ligand nucleus in a macromolecular complex is given by

\[ T_{1M}^{-1} = p(T_{1M} + \tau_{M}) \]

where \( T_{1M}^{-1} \) is the relaxation rate of the observed nucleus in the paramagnetic complex; \( T_{1M} \) 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 \( T_{1M}^{-1} \) will not yield structural information. However, if \( \tau_{M} \gg T_{1M} \), the distance-dependent relaxation rate \( T_{1M}^{-1} \) can be obtained from \( T_{1M}^{-1} \). \( T_{1M}^{-1} \) is related to the distance \( r \) between the cation and the ligand nucleus by

\[ T_{1M}^{-1} = \frac{C}{r^6} \cdot f(\tau_{c}), \]

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

\[ f(\tau_{c}) = 3 \tau_{c}/(1 + \omega_{l}^2 \tau_{c}^2), \]

where \( \tau_{c} \) 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 Eqs. (1–3), it can easily be shown that

\[ pT_{1p} = \tau_{M} + (r/C)\cdot(1/3\tau_{c} + (r/C)\cdot\omega_{l}^2 \tau_{c}) \]

so that a plot of \( pT_{1p} \) (normalized relaxation time) vs \( \omega_{l}^2 \) will be a straight line with \( (r/C)\cdot(1/3\tau_{c} + \tau_{M}) \) as the y-axis intercept and \( (r/C)\cdot\omega_{l}^2 \tau_{c} \) as the slope. The square root of the ratio of slope to intercept then would yield the value of \( \tau_{c} \), if \( T_{1M} > \tau_{M} \). In the case where \( T_{1M} > \tau_{M} \) and a precise value of \( \tau_{c} \) cannot be evaluated, the y-axis intercept of a plot of \( \omega_{l}^2 \) vs \( pT_{1p} \) will give \( \tau_{M} + (r/C)\cdot(1/3\tau_{c}) \). 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 '1H-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 \( \alpha \) and \( \beta \) anomers. Integration of the area under the resonances of the three protons, C₁-H₁, C₂-H₂, and C₂-H₃ at 4.65 ppm indicate that about 34% of glucose is present as the \( \alpha \)-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-H₁, C₁-H₂ and C₂-H₃ (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
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 \( \cdot \) Mn(II) \( \cdot \) 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. \([E] \ll [\text{glucose}] \gg K_d \cdot \text{Glc}\) 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 \((E \cdot \text{Mn(II)} \cdot \text{Glc})\) is

\[
E \cdot \text{Glc} + \text{Mn(II)} \rightleftharpoons E \cdot \text{Glc} \cdot \text{Mn(II)},
\]

with a \(K_d = 25 \pm 4 \mu\text{M}\) 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 \(T_1\) associated processes (4.2–12.6 kJ/mol) [8, 21]. Since the life-time of the enzyme complex \((\tau_M)\) is frequency-indepen-
dent, the observed frequency dependence of \((pT1_p)^{-1}\) suggests that \(T1\) is the dominant contributing process. However, the possibility of some contribution from \(\tau_{m}\) cannot be ruled out. Our previous estimate of a spin-spin relaxation rate \([(pT2_p)^{-1} = 1200 s^{-1}] [41]\) in the E · Mn(II) · Glc complex supports the notion that fast exchange prevails as \((pT1_p)^{-1} \leq (pT2_p)^{-1} < \tau_m^{-1}\). However, it cannot be taken as unequivocal evidence for fast exchange conditions as \((pT2_p)^{-1}\), 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. \(31P, 13C\) etc.) [30].

The frequency dependence 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 lifetime of glucose in E · Mn(II) · Glc will be in the range 0–4 ms.

The frequency dependence of \((pT1_p)^{-1}\) for the glucose protons can be used to determine \(\tau_c\) [31], provided \(\tau_c\) is field-independent and \(\tau_m < T1M\). In cases where a partial contribution from the exchange correlation time is observable (i.e. \(\tau_m < T1M\)), only a lower limit of \(\tau_c\) can be obtained. Further, it is obvious from Eqn (4) that the \(y\)-intercept of the plot of \((pT1_p) vs \omega'\) 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 lifetime of glucose in a macromolecular complex will lie in the range of 0–4 ms. With this range of \(\tau_m\), the estimated value of \(\tau_c\) 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 \(\tau_c\) values calculated, including the partial contribution from the exchange correlation time (\(\tau_m\)) and experimental uncertainties.

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

In the \(^1H\)-NMR spectrum, C1-H\(_a\) and C1-H\(_b\) anomic protons of Glc6P give rise to two doublets at 5.24 and 4.65 ppm (Fig. 1b). In the \(^31P\)-NMR spectrum, two resonances arising from the two anomers are observable. However, \(^31P\) chemical shifts of Glc6P are highly pH-dependent. Since, Glc6P interacts only weakly with Mn(II) \((K_d = 34\) mM) [32], we have measured \(^1H\) and \(^31P\) spin-lattice relaxation rates as a function of Mn(II) concentration. Normalized relaxation rates \([(pT1_p)^{-1}]\) are presented in Table 2. For small-molecular mass complexes, \(\tau_c\) is dominated by the rotational correlation time \(\tau_r\) at high magnetic fields. Using a value of \(\tau_r = 0.1\) ns [31], distances between Mn(II) and various ligand nuclei have been determined. C1-H\(_a\) and C1-H\(_b\) are located at a distance of 0.58 ± 0.02 nm and 0.59 ± 0.02 nm, respectively, from Mn(II) in the Glc6P · Mn(II) complex. The distance of 0.28 ± 0.01 nm between Mn(II) and \(^31P\) suggests the formation of a first co-ordination complex with the cation.

In the presence of hexokinase, the \(^1H\) as well as the \(^31P\) resonances of Glc6P 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 anomic specificity in the binding of Glc6P with the enzyme. Paramagnetic relaxation rates due to Mn(II) were measured at five different cation concentrations. Normalized relaxation rates were computed assuming the following two equilibria:

\[
E \cdot \text{Glc6P} + \text{Mn(II)} \Leftrightarrow E \cdot \text{Mn(II)} \cdot \text{Glc6P} \quad K_d = 25 \pm 4 \mu M
\]

\[
E \cdot \text{Glc6P} + \text{Mn(II)} \Leftrightarrow \text{Glc6P} \cdot \text{Mn(II)} \quad K_d = 34 \mu M
\]

The normalized relaxation rates \([(pT1_p)^{-1}]\) for C1-H\(_a\) and C1-H\(_b\) in the macromolecular complex are summarized in Table 2. In determining the mole fraction of E · Mn(II) · Glc6P, only one binding site for Glc6P 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. Glc6P · Mn(II) and E · Mn(II) · Glc6P, 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) · Glc6P in this case. The contribution to the measured paramagnetic relaxation rate arising from the Glc6P · Mn(II) complex was < 10\% (in the case of \(^31P\)) 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 \(\tau_m\) of the complex, \((pT1_p)^{-1}\) of \(^31P\) 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 \((AE)\) determined from the plot is 92 kJ/mol. This value is significantly lower than the \(AE\) expected for \(\tau_m\) (usually > 16.7 kJ/mol). Decrease in \((pT1_p)^{-1}\) with increase in temperature and the low value of \(AE\) suggest that relaxation rates are dominated by \(T1M\) and fast exchange conditions prevail (i.e. \(T1M \ll \tau_m^{-1}\)). Since \((pT1_p)^{-1}\) for C1-H\(_a\) and C1-H\(_b\) are smaller than...
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 of that of the life-time (\( \tau_M \)) of the complex. Using \( \tau_c = 0.22 - 1.27 \) ns, distances between the cation and various nuclei have been calculated. C1-H, C1-H, and C6-P of Glc6P are located at distances between 0.89 and 0.97 nm, respectively, from the cation on the enzyme (see Table 3).

Note that in the preceding calculations, the same \( \tau_c \) value has been used for E·Mn(II)·Glc and E·Mn(II)·Glc6P complexes. The value of \( \tau_c = 0.22 - 1.27 \) ns is short enough to be attributable to \( \tau_c \) (electron spin relaxation time) of Mn(II) in the macromolecular complex. The rotational correlation time \( \tau_r \) for this enzyme is about 40 ns. Also, since hexokinase dimerizes in the presence of Glc6P [40], \( \tau_r \) will be even larger (80 ns) and hence not likely to make any significant contribution to \( \tau_c \).

### DISCUSSION

The molecular mechanism of inhibition of brain hexokinase by Glc6P 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 Glc6P. 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 portions of glucose and Glc6P 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 Glc6P was also attractive from an evolutionary view point. This model predicts the existence of two binding sites for Glc6P (product and allosteric) per molecule of the enzyme. However, in several binding studies, only one binding site for Glc6P has been detected [6, 29, 35]. Although in one study Lazo et al. [36] provided evidence for two Glc6P-binding sites on the enzyme, the validity of their data is strongly contested [7]. The finding of Fromm and co-workers, that Glc6P inhibition is competitive versus ATP [5], led them to suggest that Glc6P competes with the \( \gamma \)-phosphoryl group of ATP at the nucleotide site on the enzyme. Further, they suggested that the hexose moiety of Glc6P 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 Glc6P: (a) both sugar ligands bind at the same site and hence Glc6P-induced inhibition is a case of product inhibition; (b) glucose binds at the active site but Glc6P occupies the ribosyl-phosphate-binding pocket of the ATP subsite at the active-site domain; (c) glucose and Glc6P bind at topologically distinct sites, as would be expected if the mode of Glc6P 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 Glc6P-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 Glc6P molecule of the enzyme. However, in several binding studies, only one binding site for Glc6P has been detected [6, 29, 35].

<table>
<thead>
<tr>
<th>Complex</th>
<th>Nucleus of the ligand</th>
<th>Frequency ( f(\tau_c) )</th>
<th>Distance ( \langle r \rangle )</th>
</tr>
</thead>
<tbody>
<tr>
<td>E·Mn(II)·Glc</td>
<td>C1-H</td>
<td>0.58 - 0.87</td>
<td>0.97 - 1.14</td>
</tr>
<tr>
<td></td>
<td>C1-H</td>
<td>0.58 - 0.87</td>
<td>1.11 - 1.25</td>
</tr>
<tr>
<td>E·Mn(II)·Glc6P</td>
<td>C1-H</td>
<td>0.58 - 0.87</td>
<td>0.76 - 0.81</td>
</tr>
<tr>
<td></td>
<td>C1-H</td>
<td>0.58 - 0.87</td>
<td>0.77 - 0.89</td>
</tr>
<tr>
<td></td>
<td>C6-P</td>
<td>202.5</td>
<td>0.61 - 1.07</td>
</tr>
<tr>
<td>Glc6P·Mn(II)</td>
<td>C1-H</td>
<td>0.30</td>
<td>0.58 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>C1-H</td>
<td>0.30</td>
<td>0.59 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>C6-P</td>
<td>202.5</td>
<td>0.38 ± 0.01</td>
</tr>
</tbody>
</table>

The work reported by Mehta et al. [29] leads to the conclusion that glucose and Glc6P 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 Glc6P. 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 Glc6P, the more so because this implies two binding sites for Glc6P 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 Glc6P-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 Glc6P 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 Glc6P-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 Glc6P-binding sites from the cation-binding site is small but significant. The possibility that this difference arises due to different conformational states of glucose and Glc6P 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 $(\rho T_1)^{-1}$ for $^{31}$P in E $\cdot$ Mn(II) $\cdot$ Glc6P, one can calculate the minimum dissociation rate constant, $k_{\text{cat}}$, for Glc6P. This value is $1180 \pm 340$ s$^{-1}$. Since the $k_{\text{cat}}$ for formation of Glc6P 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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