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Biophysical investigations on the active site of brain hexokinase

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Abstract. Replacement of Mg (II), the natural activator of brain hexokinase (EC 2.7.1.1) by paramagnetic Mn (II) without affecting the physiological properties of the enzyme, has rendered brain hexokinase accessible to investigations by magnetic resonance methods. Based on such studies, a site on the enzyme, where Mn (II) binds directly with high affinity has been identified and characterized in detail. Use of *β, γ*-bidentate Cr (III) ATP as an exchange-inert analogue for Mn (II) ATP has shown that Mn (II) binding directly to the enzyme has no catalytic role but another Mn (II) ion binding simultaneously and independently to the enzyme through the nucleotide bridge participates in enzyme function. However, using this direct binding Mn (II) ion and a covalently bound spin label as paramagnetic probes a beginning has been made in mapping the ligand binding sites of the enzyme. Ultra-violet difference spectroscopy has revealed the presence of at least two glucose 6-phosphate locations on the enzyme one of which presumably is the high affinity regulatory site modulated by substrate glucose. Elution behaviour of the enzyme on a phosphocellulose column suggests that glucose induces a specific phosphate site on the enzyme to which the phosphate bearing regulatory ligands of the enzyme may bind.

Keywords. Hexokinase; brain; active site; magnetic resonance studies; enzyme ligand interactions; ligand site mapping.

Introduction

Bovine brain hexokinase (EC 2.7.1.1), an enzyme catalyzing the phosphorylation of glucose, requires Mg (II) as an activating cation for its function. It is a key enzyme in the control of energy metabolism in the brain and derives its regulatory properties principally from the inhibitory action of its reaction product, glucose 6-phosphate (Glc-6-P), deinhibitory action of substrate, ATP and effector, P_i. Inspite of voluminous work using kinetic and thermodynamic methods to study the interaction of this enzyme with its substrates and effectors, it is still unclear whether the regulatory action of these ligands occurs at its active site or allosteric sites (Colowick, 1973; Purich *et al.*, 1973). The answer to this question and the related one of the mechanism of action of this enzyme, lies in the determination of conformations of substrates, regulators and metal ions interacting with this enzyme and their relative location on the enzyme surface.

In recent years we have attempted to resolve these issues using a combination of spectroscopic and chromatographic methods. We have been able to replace Mg (II) by

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Abbreviations used: Glc-6-P, Glucose 6-phosphate; P_i, inorganic phosphate; PRR, proton relaxation rate; PRE, proton relaxation rate enhancement; NEM, N-ethyl morpholine; DTNB, 5, 5'-dithiobis-(2-nitro-benzoic acid).

paramagnetic Mn (II) as the activating cation without any deleterious effects on the physiological properties of this enzyme (Jarori *et al.*,1981,1984). This substitution has enabled us to use magnetic resonance methodology, especially relaxation and spin-labelling techniques (Mildvan and Cohn, 1970; Mildvan and Gupta, 1978; Mildvan *et al.*, 1980; Dwek, 1973) to address ourselves to the problem of the disposition of the various ligand binding sites on the enzyme. In this paper we summarize and review our progress to date.

Materials and methods

Chemicals

Sources of chemicals used in this work have been mentioned elsewhere (Jarori *et al.*, 1981,1984). Preparation of β, γ-bidentate Cr (III) ATP has been described by Jarori *et al.* (1984).

Preparation of hexokinase

Type 1 hexokinase was purified from bovine brain mitochondria (Redkar and Kenkare, 1972) with one modification. EDTA was omitted from the second DEAE-cellulose chromatography. In some experiments, the enzyme was prepared by the procedure of Swarup and Kenkare (1980). Methods for the assay of hexokinase activity and the determination of protein concentration have been described previously (Redkar and Kenkare, 1972). The specific activity of the enzyme used in these experiments varied between 50–80 units per mg protein. A unit is defined as the amount of enzyme which converts one μ mol of substrate into product in 1 min. at 30°C.

Magnetic resonance experiments

Spin-lattice relaxation times (T_1) of solvent water protons were measured as reported earlier (Jarori *et al.*, 1981, 1984). Determination of free Mn (II) was carried out using ESR by measuring the amplitude of the third line towards the high field (Jarori *et al.*, 1981). High resolution proton NMR spectra of glucose and Glc-6-P and the relaxation rates of their protons were obtained using a WH-270 Brüker high resolution Fourier Transform (FT) NMR spectrometer located at Bangalore, India, and AM-500 high resolution FT NMR spectrometer located at the Tata Institute of Fundamental Research, Bombay. Proton T_1 of glucose and Glc-6-P were measured using the inversion recovery method. Other experimental details and methods for the preparation of enzyme samples for NMR experiments have been published (Jarori *et al.*, 1981, 1984).

Spin-labelling of hexokinase using 3-(2-iodoacetamido)-2,2,5,5-tetramethyl-l-pyrrolidinyloxyl was carried out as described by Jarori *et al.* (1984).

Analysis of NMR data

The interaction of the metal ion (Mn (II) in the present case) with brain hexokinase was characterized in terms of the enhancement factor (ε^*), as defined elsewhere (Jarori *et al.*,

1981, 1984). Water PRR data obtained under different conditions of experiment were used to calculate the binary enhancement ε_b and free and bound fractions of Mn(II) (Jarori *et al.*, 1981, 1984) as discussed in detail by Mildvan and Cohn (1963) and Dwek (1973).

Strategy for the determination of distance between a ligand nucleus and a paramagnetic probe, such as enzyme bound metal ion or spin label has been discussed by Mildvan and Gupta (1978) and by Mildvan *et al.* (1980) and will not be presented here. The method essentially consists of determining the effect of the paramagnetic probe on the spin-lattice relaxation rate of a ligand nucleus exchanging into the enzyme-probe complex. The following equation was used to calculate distances

$$r = C\left[qfT_{1p}\left(\frac{3\tau_C}{1+\omega_I^2\tau_C^2} + \frac{7\tau_C}{1+\omega_S^2\tau_C^2}\right)\right]^{1/6}, \qquad (1)$$

where r is the distance from the paramagnetic probe to the nucleus in the complex, C is a constant with a value of 812 for Mn (II) ion-proton interaction (Mildvan *et al.*, 1980), f is the ratio of the concentrations of the enzyme-probe-ligand complex and of the total ligand in solution, T_{1p} is the spin-lattice relaxation time of the ligand nucleus, τ_C is the correlation time characterizing the dipole-dipole interaction between the electron and proton spins, and ω_I and ω_s are the nuclear and electron resonance frequencies respectively. The validity of eq. (1) is based on the assumption that the outer sphere contribution ($1/T_{O.S.}$) is negligible and that the relaxation time T_{1M} is very much greater than the life time (τ_M) of the enzyme-probe-ligand complex, where T_{1M} is the relaxation time of the ligand nucleus in the enzyme-paramagnetic probe complex.

If the assumptions made in deriving equation (1) are justified, distance can be found after the three unknown parameters of eq. (1) (τ_c, q and fT_{1p}) are experimentally measured. Conversely if r is known from other studies as in the case of Mn–H_2O distance, then q the number of water ligands exchanging into the first co-ordination sphere of Mn (II) bound to the enzyme, can also be determined.

Ultra-violet difference spectroscopy

Ultra-violet difference spectra were recorded on a Cary-17D spectrophotometer. 1·0 cm path length quartz cells were used. The baseline was recorded with both the cells containing the same enzyme solution. The difference spectrum produced by interaction of the ligand was recorded after the addition of saturating concentration of the ligand to the sample cell and an equal volume of buffer to the reference cell. Resulting 0·5 % increase in volume has been neglected. Recorded spectra were corrected for base line deviations. All spectra were recorded at room temperature (21°C).

Phosphocellulose chromatography

For preparation of the phosphocellulose column, cellulose phosphate was washed successively with ethanol, water, 0·1 M HCl, water, 0·1 M NaOH and water. It was then equilibrated with the appropriate buffer.

Results and discussion

Characterization of Mn (II) and nucleotide binding sites of brain hexokinase

The divalant cation Mg (II) is the natural activator of brain hexokinase. This cation being colourless and diamagnetic is not suitable for spectroscopic investigations. Substitution of Mg (II) by Mn(II) has enabled us to employ magnetic resonance methods to study the mode of co-ordination of metal ion-nucleotide complex with this enzyme (Jarori *et al.*, 1981, 1984). Water PRE data has been used to evaluate equilibrium constants and the number of binding sites with respect to enzyme–Mn (II) interaction. Our conclusions have been reinforced by the use of ESR to monitor formation of enzyme–Mn (II) complex. Hydration number of Mn (II) bound to the enzyme has also been determined on the basis of PRE data although the theoretical groundwork for the determination of the hydration number is a matter of controversy (Koenig, 1978; Waysbort and Navon, 1978). The results of our water PRE and ESR studies (Jarori *et al.*, 1981, 1984) are summarized below:

(i) Replacement of Mg (II) reduces the specific activity of hexokinase by about 40 %. However, this replacement has no effect on the affinity of ATP for the enzyme or on the regulatory properties of the enzyme.

(ii) Enzyme has one tight binding site for Mn (II) with K_D = 25 ± 4 μM at pH 8·0, 23°C.

(iii) There are about 7–9 weak binding sites for Mn (II) on the enzyme with an average K_D = 1400 ± 440 μM. Most of these sites may be non-specific, but activating and inhibitory sites with their dissociation constants in the millimolar range also lie in this group of binding sites (Jarori *et al.*, 1981)

(iv) Manganous ion in enzyme Mn (II) complex at tight binding site has characteristic binary enhancement ε_b = 3·5 ± 0·4 at 9 MHz, pH 8·0, 23°C.

(v) The average characteristic binary enhancement for weak binding sites is ε_b = 2·3 ± 0·5 at 9 MHz, pH 8·0, 23°C.

(vi) The dissociation constant of Mn (II) at tight binding site is strongly temperature dependent. The K_D decreases with increase in temperature from 5°–30° indicating that the stability of this metal binding site is strongly dependent upon hydrophobic interactions.

(vii) The average number of fast exchanging water molecules from first co-ordination sphere of Mn (II) bound at the tight binding site is 2 at 18°C and about 1 at 30°C. This low hydration number suggests that this tight binding Mn (II) is linked to the enzyme through multiple co-ordination bonds.

Though the activating and inhibitory sites for Mn (II) that we have kinetically identified are presumably among the weakly interacting Mn (II) sites (see figure 1 of Jarori *et al.,* 1981), we did not characterize them further. This was because at the high concentrations of Mn (II) required in such experiments, many non-specific sites would interact with Mn (II) and complicate the analysis. Further experiments described here were confined only to the study of tight binding Mn. (II) site.

Kinetic studies (Ning *et al.*, 1969) have shown that $MgATP^{2-}$ is the true substrate for brain hexokinase. The interaction of nucleotides, ATP and ADP, with the enzyme-Mn (II) binary complex, was studied using the water PRE method (figure 1).

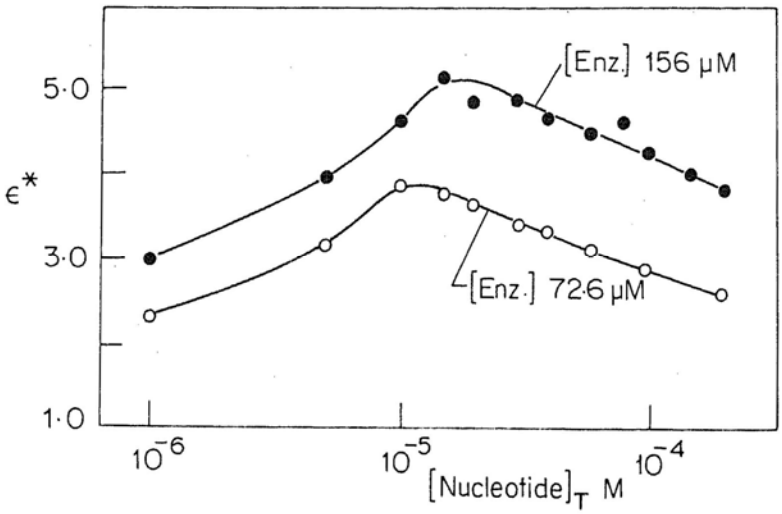

Figure 1. PRE titration of hexokinase with ATP(O) and ADP (●) in the presence of Mn Cl_2. Solutions contained 50 μM Mn Cl_2, 5 mM 2-mercaptoethanol, 10 mM NEM acetate, pH 8·0 and hexokinase as indicated.

When nucleotides ATP or ADP were added to the enzyme Mn (II) binary complex, increased enhancements over that for the binary complex at a given enzyme concentration were observed. As the concentration of the nucleotide is increased the observed enhancement ε^* increases. This initial increase in ε^* can be attributed to the formation of an enzyme-nucleotide-Mn (II) complex. The decrease in ε^* encountered at higher nucleotide concentrations is apparently the result of competition between the free nucleotide and the metal nucleotide complex for the nucleotide site on the enzyme (Jarori *et al.*, 1984).

These results (figure 1) suggested the formation of a nucleotide bridged ternary complex in which Mn (II) is bound to the enzyme through the nucleotide, a pattern characteristic of many other kinases (Mildvan and Cohn, 1970; Buttlaire and Cohn, 1974; Jones *et al.,* 1972). On the other hand, our PRE studies on the interaction of Mn (II) with the enzyme in the absence of the nucleotide, provided evidence for a high affinity site on the enzyme where Mn (II) is bound directly. It was not clear whether in the presence of the nucleotide, the high affinity Mn (II) site gets converted to the nucleotide–Mn (II) site or nucleotide–Mn (II) binds at a separate site. To choose between these two possibilities, the enzyme was titrated with Mn (II) in the presence of Cr (III) ATP. Under these conditions Mn (II) cannot displace Cr (III) from its complex with ATP as Cr (III) ATP is exchange-inert. At various concentrations of enzyme, Mn (II) and Cr (III) ATP, free Mn (II) concentration was monitored using ESR. The results showed good agreement between the measured concentrations of free Mn (II) and those calculated on the basis of Mn (II) binding to hexokinase even with Cr (III) ATP occupying the nucleotide binding site. Mn (II) thus binds to brain hexokinase in the presence of Cr (III) ATP and the stoichiometry of metal ion interaction with the enzyme in the presence of the nucleotide is therefore two (Jarori *et al.*, 1984). Further support to the idea of two metal ions binding to the enzyme in the

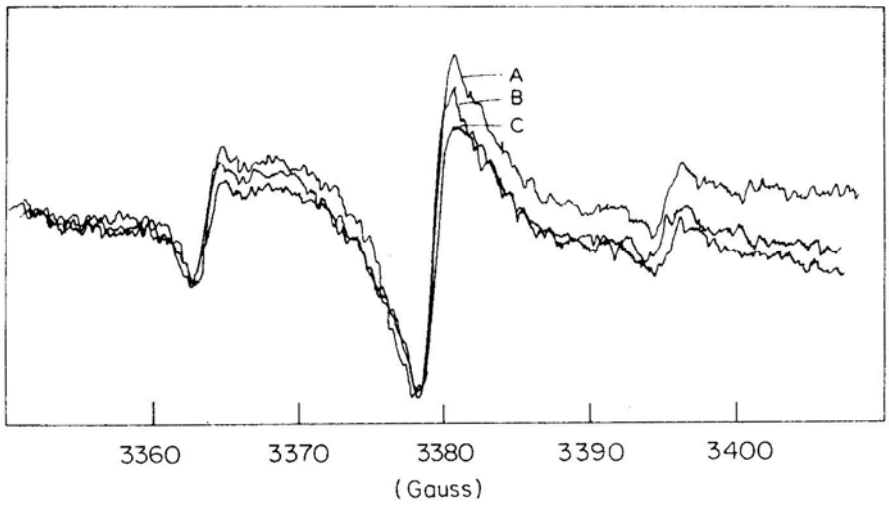

Figure 2. Quenching of ESR signal of spin-labelled hexokinase by Mn (II). Concentration of spin-labelled hexokinase was 67·3 μM. (A), no Mn (II); (B), 50 μM Mn (II); (C), 100 μM Mn (II). Instrument settings were: scan range, 100 G; time constant, 0·50 sec.; modulation amplitude, 2·0 G; receiver gain, $3{\cdot}2 \times 10^4$; microwave power, 12 mW; field setting 3,390 G; scan time, 8 min.; modulation frequency, 100KHz; Microwave frequency, 9·506 GHz, temperature 22°C.

presence of the nucleotide comes from the competitive displacement of ATP-Mn (II) from the enzyme by Glc-6-P resulting in the formation of enzyme-Mn (II)–Glc-6-P complex (Jarori *et al.*, 1984). That these two Mn (II) binding sites are distinctly located on the enzyme is shown by our studies on spin-labelled hexokinase. It was found that Mn (II) bound to the enzyme quenches the ESR signal of the covalently linked nitroxyl radical (figure 2). However binding of ATP-Mn (II) to the enzyme does not have any appreciable effect on the intensity of the ESR signal of the spin-labelled enzyme (Jarori *et al.*, 1984).

Since the divalent cation has an obligatory function in the hexokinase reaction, an obvious question that arose was which one of these two metal ions fulfilled that role. This question was probed using Cr (III) ATP as a phosphoryl donor in the presence of ^{14}C-glucose (Jarori *et al.,* 1984), using the method of Dunaway-Mariano and Cleland (1980). The amount of product formed showed that brain hexokinase underwent approximately a single turnover when Cr (III) ATP is used as the substrate (table 1). A single turnover results since in the catalytic transfer of γ-phosphoryl group of Cr (III) ATP to the 6-hydroxyl group of ^{14}C-glucose, complete transfer does not take place as Cr (III) ATP is an exchange-inert compound. The product obtained (Cr (III) ATP–^{14}C-Glc-6-P) which structurally resembles a transition-state analogue is released very slowly from the enzyme. Addition of 500 μM Mn (II) has no effect on the reaction although it binds at the tight-binding site of hexokinase. Thus it is evident from this experiment, that of the two metal ions interacting with the enzyme in the presence of the nucleotide, it is the one co-ordinated to it *via* the nucleotide that participates in catalysis. The role of the second Mn (II) ion binding directly to the enzyme is not clear

Table 1. Assay of substrate activity of Cr (III) ATP in the presence and absence of Mn (II).

	Expt. I		Expt. II	
	+ Mn (II) (500 μM)	− Mn (II)	+ Mn (II) (500 μM)	− Mn (II)
Amount of enzyme added (nanomoles)	37	37	11·6	7·9
Radioactivity added as ^{14}C-u-glucose	$2{\cdot}76 \times 10^6$ cpm ($\simeq 5 \times 10^{-7}$ mol)	$2{\cdot}38 \times 10^6$ cpm	$6{\cdot}12 \times 10^6$ cpm ($\simeq 1{\cdot}0 \times 10^{-6}$ mol)	$6{\cdot}16 \times 10^6$ cpm
Radioactivity of eluted peak fractions corresponding to the product (Cr (III) ADP)-(^{14}C-Glc-6-P)	$1{\cdot}365 \times 10^5$ cpm	$1{\cdot}05 \times 10^5$ cpm	$8{\cdot}0 \times 10^4$ cpm	$5{\cdot}5 \times 10^4$ cpm
Amount of product formed (nanomoles)	24·7	22·0	13·0	8·9
Turnover	0·667	0·6	1·1	1·1

1·0 ml reaction mixture contained 1 mM Cr (III) ATP, 1 mM ^{14}C-u-glucose, 5 mM 2-mercaptoethanol, 100 mM NEM acetate buffer pH 6·5 in addition to the hexokinase and Mn (II) as indicated in the table.

to us so far. Meanwhile this Mn (II) at the tight-binding site is being used as a paramagnetic reference point in the mapping of ligand binding sites on the enzyme surface.

Tight-binding Mn (II) as a paramagnetic probe to study enzyme-sugar interactions

In the absence of the metal ion, brain hexokinase has no effect on the line widths of α and β anomeric proton resonances of glucose. Conversely, in the absence of the enzyme, Mn (II) has no effect on the line width. The Mn (II)–hexokinase complex however broadens the C–(l) proton resonances of both α and β anomers. This unequivocally establishes that bovine brain hexokinase does not exhibit any specificity for binding of glucose anomers (Jarori *et al.,* 1981). Glc-6-P, which is a product as well as an inhibitor of hexokinase reaction also showed considerable broadening of the C–(l) proton resonance of α and β anomers upon binding to the enzyme, though in this case Mn (II) had no effect (Jarori *et al.,* 1981). This lack of anomeric specificity for the binding of glucose and Glc-6-P to the enzyme as revealed by magnetic resonance techniques is in agreement with the observations made earlier by others (Sols and Crane, 1954; Salas *et al.,* 1965; Rose *et al.*, 1974).

Interaction of glucose with enzyme-Mn (II) complex results in line broadening of anomeric proton resonances of glucose due to paramagentic effect of Mn (II) on spin-spin relaxation time (T_2) of bound glucose which is in exchange with free glucose molecules. The Swift and Connick (1962) equation was used to work out the spin-spin relaxation rate ($1/T_{2M}$) of glucose anomeric protons in the bound form. Under conditions of fast exchange ($T_{2M} \gg \tau_M$), $1/T_{2M}$ sets the lower limit for the rate of dissociation of glucose from the enzyme which was found to be 1330 ± 170 s^{-1}. The kinetic data shows that the rate of the reverse reaction, *i.e.* the formation of glucose and

ATP from Glc-6-P and ADP, is much slower than the rate of release of glucose (1330 ± 170 s^{-1}) from the enzyme. Thus the release of glucose from the enzyme is not the rate limiting step in the reverse reaction. Interestingly, in yeast hexokinase, the release of glucose from the enzyme in the reverse reaction is rate limiting (Rose *et al.*, 1974).

Tight-binding Mn (II) site as a paramagnetic reference point for mapping of ligand binding sites

Precise location of various regulatory sites of brain hexokinase has become essential for resolving controversies regarding the molecular mechanism of its control. Thus the regulatory function of Glc-6-P, a product inhibitor of the enzyme, is variously described as occurring at an allosteric site (Lazo *et al.,* 1980) or at its active site (Solheim and Fromm, 1981). The sites where ATP and inorganic phosphate bind to reverse the inhibitory action of Glc-6-P is also not known. Results of kinetic studies carried out to settle these issues (Colowick, 1973; Purich *et al.,* 1973) have not been conclusive. Our approach to the solution of these problems is to employ NMR relaxation methods (Mildvan and Cohn, 1970; Mildvan and Gupta, 1978; Mildvan *et al.,* 1980) and spin labelling methods (Mildvan and Weiner, 1969; Jones *et al.,* 1973; Cunningham *et al.*, 1981) to precisely locate the various ligand binding sites of the enzyme using the tightly bound Mn (II) and the covalently linked spin label as paramagnetic reference points. For unequivocal mapping of these sites it is necessary to have more paramagnetic centres such as Cr (III) ATP and covalently or non-covalently attached spin labels.

As described earlier, measurement of the paramagnetic effect of a probe, in this case Mn (II), on relaxation rates can under certain conditions provide information about the physical distance between the probe and the relaxing nucleus. As shown in the preceding section, addition of glucose to Mn (II)–hexokinase complex broadens the C–(l) proton resonance of α as well as the β anomer, (H_1^{α} and H_1^{β} resonances) resulting from the electron-nuclear dipolar interaction between the paramagnetic metal ion and the C–(l) proton of the sugar. This result showed that in enzyme-Mn (II)–glucose complex, Mn (II) is close enough to glucose to cause paramagnetic effects on relaxation rates of glucose protons. The assignment of all the proton resonances in D–glucose NMR spectrum is known (Koch and Perlin, 1970). The H_1^{α} H_1^{β} and H_2^{β} (*i.e.* C–(2) proton of β-anomer) resonances of D-glucose are well resolved in the glucose proton spectra at 270 and 500 MHz. Spin-lattice relaxation rate measurements have been made at 270 and 500 MHz on solutions containing enzyme, Mn (II) and glucose at different concentrations. From these measurements, the normalized paramagnetic contributions $1/fT_{1p}$ to the relaxation rates of H_1^{α}, H_1^{β} and H_2^{β} protons have been obtained by subtracting the diamagnetic contributions from the total observed relaxation rates measured in the presence of Mn(II). The normalized relaxation rates, $1/fT_{1p}$ were calculated on the assumption that 38 % of glucose is present as α anomer and 62 % as β anomer (Schray and Benkovic, 1978).

To determine distance r from $1/fT_{1p}$ rates, using eq. (1), one needs to evaluate correlation time τ_C. In addition, it is necessary to establish that the relaxation of the ligand nucleus in the enzyme is not exchange limited and to know the stoichiometry 'q' of the binding of the ligand in enzyme–Mn (II)–ligand complex. τ_C was evaluated from the ratio of $1/fT_{1p}$ at 270 and 500 MHz. Lack of exchange limitation was assumed on

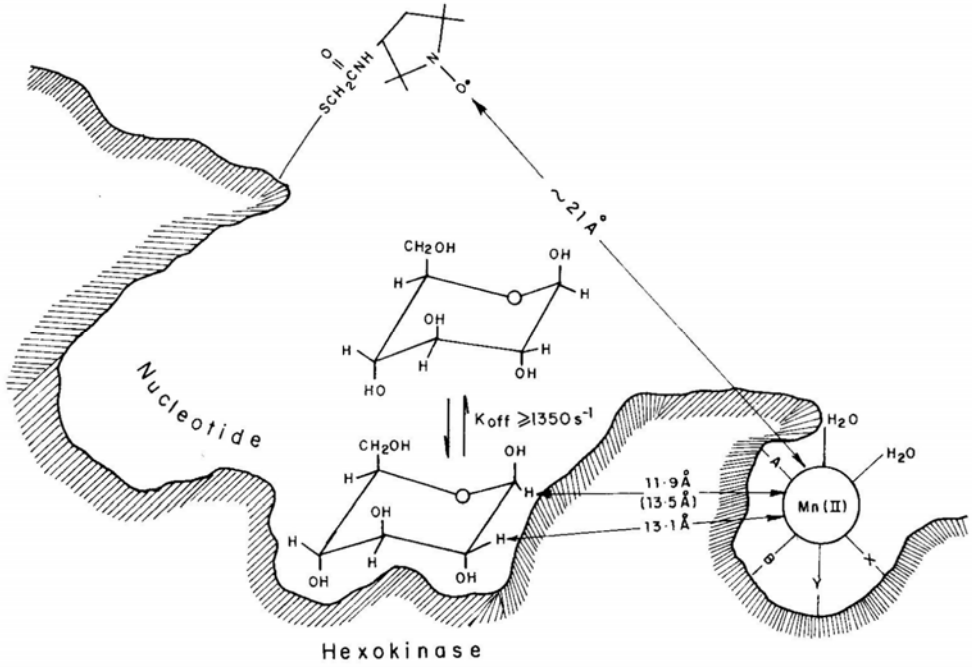

Figure 3. Relative location of D-glucose (β-anomer) and the spin label with respect to the direct binding site of Mn (II). The distance from Mn (II) to the H_1^{α} of glucose is shown in parentheses.

the basis that $1/fT_{1\ p}$ values were an order of magnitude lower than $1/fT_{2\ p}$ values calculated for the anomeric proton (not shown). As only one glucose molecule is known to bind a molecule of brain hexokinase (Ellison *et al.,* 1974) 'q' is equal to one. Eq. (1) was then used to calculate the distances of various protons of glucose from the tight binding Mn (II) ion. This is shown schematically in figure 3.

In order to plant another paramagnetic locus on the enzyme for mapping of ligand binding sites, we have also covalently modified hexokinase with a spin label 3-(2-iodoacetamido)-2,2,5,5-tetramethyl-l-pyrrolidinyloxyl. DTNB titration of the sulphydryl groups of the labelled and unlabelled enzyme indicates that the spin label is incorporated at a fast reacting sulphydryl residue of the enzyme with a stoichiometry of one. Enzyme retained full catalytic activity and also the same affinity for Mn (II) indicating that the spin labelled enzyme had retained its native conformation. The electron spin resonance spectrum of the labelled enzyme at pH 8·0 indicates that the label has moderate mobility with a rotational correlation time (τ_r) of 4·2 ± 2·1 ns which is much shorter than the τ_r of the enzyme which is approximately 25 ns. Splitting between low field minima and the high field maxima remains unaltered indicating easy accessibility of the label to the solvent. Addition of Mg (II) (500 μM) has no effect on the spectral characteristics of the enzyme-bound spin label. However addition of Mn (II) produces a reduction in the peak height of the central line without any appreciable line broadening. Saturation of the spin labelled enzyme with Mn (II)

quenches the central ESR line by about 23 % (figure 2). Using this maximum reduction in height of the central ESR line and a value of 2·2 ns for electron spin-lattice relaxation time of enzyme bound Mn (II) (Jarori *et al.*, 1981), the distance between tight-binding Mn (II) ion and nitroxyl group on the labelled enzyme has been estimated to be about 21 A (Mehta, 1983,1984). This distance should be considered as approximate since the spin label is not as rigidly immobilized on the enzyme as required by Leigh's theory (Leigh, 1970) used for calculation of the distance.

Figure 3 shows in a schematic way, the location of glucose and spin labelled sites with respect to tight binding Mn (II) ion.

UV difference spectroscopic evidence for more than one binding site for Glc-6-P on the enzyme and for the existence of an enzyme-glucose-Glc-6-P complex

Our UV difference spectroscopic studies have provided us with an additional insight into the nature of interaction of sugar ligands with brain hexokinase. Figure 4 shows that the nature of the difference spectrum induced in brain hexokinase by 100 μM Glc-6-P is quite different from that induced by 2·5 mM Glc-6-P. Since the ratio between the induced difference at any two wavelengths such as 250 nm and 285 nm is quite different at these two concentrations of Glc-6-P, it follows that Glc-6-P interacts

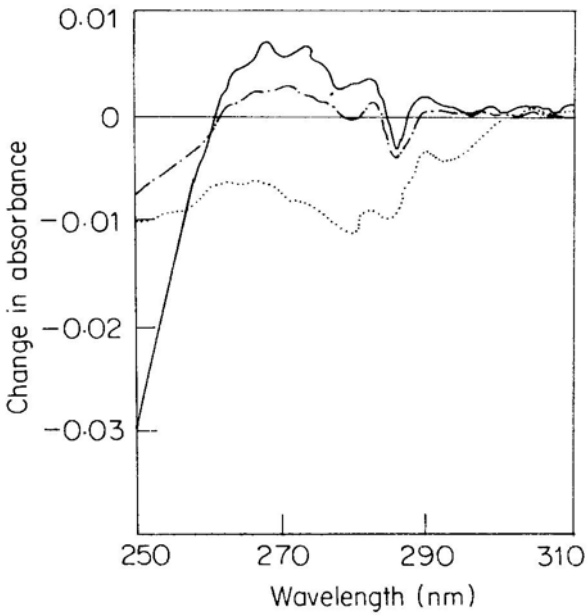

Figure 4. Glc-6-P induced difference spectra of brain hexokinase. Difference spectra were obtained in 10 mM NEM acetate buffer, pH 8·0, containing 5 mM 2-mercaptoethanol; temperature 22°C; 1 cm light path quartz cell; sample volume, 1 ml; scan rate, 6 nm/min.; band width, $\leqslant$ 1 nm.–·–·–, 17·2 μM hexokinase and 100 μM Glc-6-P; ——15·7 μM hexokinase and 2·5 mM Glc-6-P;……18·7 μM hexokinase and 10 mM glucose were present in both the cells. 100 μM Glc-6-P was added to the sample cell.

with the enzyme at more than one site as suggested by Lazo *et al.* (1980) on the basis of their binding studies.

Though glucose by itself does not cause any change in the UV absorption spectrum of the enzyme, together with 100 μM Glc-6-P it induces a difference spectrum quite different from the one induced by 100 μM Glc-6-P alone (figure 4). This signifies the formation of an abortive ternary complex, enzyme-glucose-Glc-6-P and indicates interaction between glucose and Glc-6-P sites on the enzyme, supporting earlier suggestion that binding of Glc-6-P to the enzyme is glucose dependent (Redkar and Kenkare, 1975; Ellison *et al.,* 1975; Lazo *et al.,* 1980). Lazo *et al* (1980) have suggested that the enzyme has high affinity ($K_D \approx 2{\cdot}5$ μM) and low affinity ($K_D \approx 250$ μM) sites for Glc-6-P. if one accepts this suggestion, then it follows that it is the high affinity site which is modulated by glucose.

Chromatography of brain hexokinase on a phosphocellulose column: Its significance to the problem of ligand binding sites

In our laboratory, routine purification of brain hexokinase involves as a final step cellulose phosphate chromatography at pH 7·7 (Swarup, 1979; Swarup and Kenkare, 1980). Since at this pH, the matrix as well as the enzyme are negatively charged, chromatography of the enzyme on the phosphocellulose column cannot be considered as ion-exchange chromatography. Rather, since the enzyme can be specifically eluted with as low a concentration as 13 μM Glc-6-P or 2 mM ATP, it is proper to consider it as affinity chromatography (Swarup, 1979; Mehta, 1984).

During the course of our experiments to investigate the affinity nature of phosphocellulose chromatography, it became obvious to us that binding of hexokinase to the phosphocellulose column and its elution there from has a bearing on the problem of regulatory sites of brain hexokinase that bind phosphate bearing ligands. These experiments (Mehta, 1984) are briefly discussed below.

Chromatographic behaviour of brain hexokinase on a phosphocellulose column was investigated using a pure preparation. The columns were routinely equilibrated with 10 mM Tris-HCl buffer, pH 7·7, containing 10 mM glucose, 1 mM EDTA and 5 mM 2-mercaptoethanol (Buffer A). After loading the enzyme, the column was washed with two column volumes of the equilibrating buffer. No leakage from the column was observed. The enzyme failed to bind to the column in the presence of 50 μM Glc-6-P or 2 mM P_i. The bound hexokinase could be eluted from the column using the phosphate bearing ligands of brain hexokinase. The actual concentrations of the ligands required for elution was determined by using gradients and are shown in table 2. These ligand concentrations are low enough to rule out ionic strength as the basis of their eluting power. On the other hand, these concentrations are generally within an order of magnitude of their K_D values with respect to the enzyme and also approximately proportional to their K_D values. This leads one to conclude that ligands Glc-6-P, ATP, ADP, AMP and P_i function as affinity eluants in the chromatography of hexokinase on cellulose phosphate. This conclusion is strengthened by the observation that in the absence of phosphate-bearing ligands, 200 mM KCl is required for elution of hexokinase from the phosphocellulose column (table 2).

It soon became clear that the presence of glucose in the regenerating and eluting

Table 2. Concentration of ligands required for elution of brain hexokinase from a phosphocellulose column.

Eluting ligand	Eluting concentration In presence of glucose	Eluting concentration In absence of glucose	K_D^e of ligand with respect to the enzyme
Glc-6-P	13 μM	$\gg$ 100 μM	2·5 μM[a]
ATP	2·4 mM	$\gg$ 20 mM	0·22 mM[b]
ADP	4.5 mM	$\gg$ 20 mM	0·5 mM[c]
AMP	4 mM	$\gg$ 20 mM	16 mM[d]
P_i	8 mM	$\gg$ 20 mM	0·8 mM[e]
KCl	250 mM	500 mM	

[a] Data of Lazo *et al.* (1980).
[b] Data of Ellison *et al.* (1974).
[c] Data of Redkar and Kenkare (1975).
[d] Data of Ning *et al.* (1969).
[e] K_D values for P_i differ widely depending on the method.

buffers is essential for phosphocellulose to function as affinity matrix When glucose was omitted from buffer A, Glc-6-P, ATP or P_i could not elute the enzyme effectively even at concentrations five fold higher than those required when glucose is present in the regenerating buffer (table 2). One may therefore conclude that glucose induces a specific phosphate site on the enzyme through which it binds to phosphocellulose. A corollary to this conclusion is that there is a region on the enzyme to which all the phosphate bearing ligands of the enzyme bind. It may be argued that these ligands bind at different sites but elute the enzyme by inducing conformational changes. It is however difficult to accept this argument, because excepting Glc-6-P, none of the other ligands have been shown to induce recognizable conformational changes. Also, it is easier to assume that glucose induces a single site on the enzyme to which all the ligands bind rather than different sites for different ligands. A common site for all the phosphate bearing ligands can explain the relief of the Glc-6-P induced inhibition of the enzyme by ATP and P_i (Colowick, 1973; Purich *et al.,* 1973). If one assumes as suggested in a previous section that glucose modulates a regulatory site for Glc-6-P on the enzyme, then the present results can also explain how P_i reverses Glc-6-P induced inhibition without having any effect on the catalytic activity of the enzyme. It also explains how P_i stimulates reverse hexokinase reaction though it has no effect on the reaction in the forward direction (Solheim and Fromm, 1983). The results presented here perhaps provides a new way of looking at the regulation of brain hexokinase by suggesting that all the phosphate bearing effector molecules bind at the same site induced by glucose.

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