Magnetic resonance studies on the interaction of metal-ion and nucleotide ligands with brain hexokinase

Gotam K. JARORI, Alka MEHTA, Sitapati R. KASTURI and Umakant W. KENKARE

Tata Institute of Fundamental Research, Bombay

Our previous studies have shown that one manganous ion binds tightly to bovine brain hexokinase, with a $K_d = 25 \pm 4$ μM. The characteristic proton relaxation rate (PRR) enhancement of this binary complex (ε_b) is 3.5 at 9 MHz and 23 °C [Jarori, G. K. Kasturi, S. R., and Kenkare, U. W. (1981) *Arch. Biochem. Biophys. 211*, 258 − 268]. On the basis of PRR enhancement patterns, observed on the addition of nucleotides ATP and ADP to this E · Mn binary complex, we now show the formation of a nucleotide-bridge ternary complex, enzyme · nucleotide · Mn. Addition of glucose 6-phosphate to enzyme · ATP · Mn, results in a competitive displacement of ATP Mn from the enzyme. However, a quaternary complex E · ADP · Mn · Glc-6-*P* appears to be formed when both the products are present.

β, γ-Bidentate Cr(III)ATP has been used to elucidate the role of direct binding of Mn(II) in catalysis, and the stoichiometry of metal-ion interaction with the enzyme in the presence of nucleotide. Bidentate Cr(III)ATP serves as a substrate for brain hexokinase without any additional requirement for a divalent cation. However, electron-spin resonance studies on the binding of Mn(II) to the enzyme in the presence of Cr(III)ATP suggest that, in the presence of nucleotide, two metal ions interact with hexokinase, one binding directly to the enzyme and the second interacting via the nucleotide bridge. It is this latter one which participates in catalysis.

Experiments carried out with hexokinase spin-labeled with 3-(2-iodo-acetamido)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl clearly showed that the direct-binding Mn site on the enzyme is distinctly located from its ATP Mn binding site.

Bovine brain hexokinase catalyzes the transfer of the γ-phosphoryl group of ATP to the sixth hydroxyl group of glucose, resulting in the formation of Glc-6-*P* and ADP. The enzyme shows an absolute requirement for a divalent cation, which is Mg(II) *in vivo* [1]. Our earlier studies have shown that Mg(II) can be replaced by Mn(II) and that the enzyme has one tight binding site for Mn(II) [2]. Kinetic studies have shown that $MgATP^{2-}$ is the true substrate for hexokinase [3]. However, the mode of co-ordination of metal-ion · nucleotide complex with brain hexokinase is not understood. The first objective of our study was to investigate the nature of enzyme, nucleotide and metal ion co-cordination using a proton relaxation enhancement (PRE) method [4,5]. Since ATP itself has a high affinity for divalent cations and since hexokinase forms a fairly tight E · Mn binary complex [2], the second objective of our study was to investigate the possible existence of a quaternary Mn · E · ATP · Mn complex using principally magnetic resonance methods.

MATERIALS AND METHODS

Chemicals

ATP, ADP, NADP, Glc-6-*P* and Glc-6-*P* dehydrogenase were obtained from Boehringer-Mannheim (FRG). Manganous chloride (0.1 M) solution was supplied by Sigma Chemical Company (St. Louis, Missouri). Bovine serum albumin was obtained from Nutritional Biochemicals Corporation (USA). Dowex-50× −2H$^+$ was from Bio-Rad (USA). [U-^{14}C]Glucose was supplied by Bhabha Atomic Research Centre (Bombay, India). The spin label 3-(2-iodoacetamido)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl was obtained from Syva Research Chemicals (California, USA).

Preparation of hexokinase

Type I hexokinase was purified to apparent homogeneity from bovine brain mitochondria as described by Redkar and Kenkare [6] with one modification. EDTA was eliminated in the second DEAE-cellulose chromatography. Protein concentration in purified preparations was determined by the method of Lowry et al. [7] using crystalline bovine serum albumin as standard. For all magnetic resonance experiments, samples were prepared as described earlier [2]. The enzyme used in these experiments was at least 95 % pure and had a specific activity of approximately 60 units/mg protein.

Kinetic studies

Hexokinase was assayed spectrophotometrically by the method described earlier [6]. In the experiments where Cr(III)ATP was used as an inhibitor versus MgATP, the reaction was started by the addition of hexokinase and was monitored for 1 − 2 min only. This precaution was taken to avoid hydrolysis of Cr(III)ATP at the pH 8.0 of the assay mixture.

Abbreviations. PRR, proton relaxation rate; Glc-6-*P*, glucose 6-phosphate.

Enzymes, Hexokinase (EC 2.7.1.1); creatine kinase (EC 2.7.3.2); phosphofructokinase (EC 2.7.1.11); arginine kinase (EC 2.7.3.3); glucose-6-phosphate dehydrogenase (EC 1.1.1.49).

The substrate activity of β,γ-bidentate Cr(III)ATP for brain hexokinase was monitored using Dowex-50 assay[8]. Reaction mixture, containing [U-^{14}C]glucose, Cr(III)ATP and enzyme, was incubated for 10 min. The reaction was terminated by the addition of 20 µl perchloric acid and 2 drops of CCl$_4$ to the vortexing mixture. The solution was then diluted to 1.0 ml and centrifuged (10000 × g). The supernatant was adsorbed on a 25 × 0.5-cm column of Dowex-50 × $-2H^+$ at 4 °C. The column was washed with 75 ml 10 mM HCl followed by 75 ml 1 M HCl. The 5.0-ml fractions were assayed for ^{14}C and the relative amounts of unreacted substrate and product were determined.

Preparation of β,γ-bidentate Cr(III)ATP

β,γ-Bidentate Cr(III)ATP was prepared by the pH titration at 4 °C of a mixture of Na$_2$H$_2$ATP and CrCl$_3$ up to pH 5.7. The solution was then allowed to warm to room temperature for 30 min. β,γ-bidentate Cr(III)ATP was then purified by adsorption on a Dowex-50 × $-2H^+$ column followed by elution with aniline. The aniline was removed by extraction with ether. The solution was stored at $-20°C$ [9]. This preparation did not have any free ATP. However, the presence of small amounts of tridentate Cr(III)ATP cannot be ruled out.

Magnetic resonance measurements

Spin-lattice relaxation times (T_1) of solvent water protons were measured at 9 MHz using a Spin-Lock model CPS-2 spectrometer (Spin-Lock Ltd, Port Credit, Ontario, Canada) and a box-car averager (Princeton Applied Research Corporation, model 162), using the methods described earlier [2].

In Mn(II) binding assays the concentrations of free Mn(II) were monitored using an X-band (9.5 GHz) Varian E-line ESR spectrometer located at the Regional Sophisticated Instrumentation Centre (Powai, Bombay). 20 – 25 µl samples were placed in constant-diameter glass capillaries for recording the ESR spectrum of free Mn(II). Amplitudes of the first low-field line were used to measure the amount of free Mn(II) [10].

Analysis of NMR data

The enhancement factor ε^* is defined in the usual way as the ratio of the paramagnetic contributions to the relaxation rate in the presence and the absence of the macromolecule, i.e.

$$\varepsilon^* = \frac{1/T_{1p}^*}{1/T_{1p}} = \frac{1/T_1^* - 1/T_{1(0)}^*}{1/T_1 - 1/T_{1(0)}}$$

where asterisks indicate the presence of macromolecule and the $1/T_{1(0)}$ is the relaxation rate in the absence of the paramagnetic ion.

Spin labeling

The spin label used was 3-(2-iodoacetamido)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl. For spin labeling, hexokinase (70 – 80 units/mg) was first dialyzed against 100 mM N-ethylmorpholine acetate buffer pH 8.0 (4 × 100 ml) in order to remove 2-mercaptoethanol. Subsequently it was mixed with spin label in ratios of 1:1 or 1:4 in the presence of 10 mM glucose and 10 mM Glc-6-P. This mixture was allowed to react
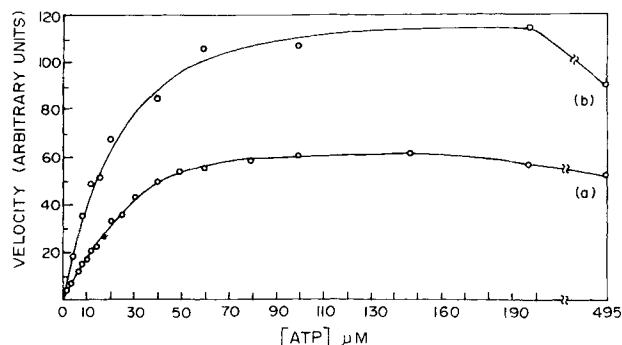

Fig. 1. *Determination of* K$_{mATPM^{2+}}$ *for brain hexokinase at low metal ion concentration.* Hexokinase activity was monitored at 340 nm on a Cary 17D spectrophotometer at room temperature (21 °C). The concentration of ATP was varied from 2.0 µM to 495 µM. Assay solution consisted of 5 mM glucose, 0.5 mM NADP, 1 µg glucose-6-phosphate dehydrogenase, 10 mM N-ethylmorpholine acetate, pH 8.0 and 50 µM MnCl$_2$(a) or 50 µM MgCl$_2$(b). The reaction was started by adding 7 µg brain hexokinase. 1 ml assay mixture was used for each assay

at 4 °C for 10 – 12 h. The enzyme was then precipitated twice with 70% (NH$_4$)$_2$SO$_4$ in 100 mM phosphate buffer pH 8.0. This resulted in complete removal of Glc-6-P, glucose and unreacted spin label. The precipitated enzyme was finally dissolved in 100 mM N-ethylmorpholine acetate buffer pH 8.0 and dialyzed against the same buffer (4 × 100 ml). The spin-labeled enzyme had a specific activity of approximately 60 units/mg protein. For two of the three experiments reported in this paper the enzyme had a stoichiometry of spin labeling of 1:1. In the third experiment the stoichiometry of incorporation of spin label was greater than 1. ESR spectra of spin-labeled enzyme were recorded on samples (≈ 30 µl) filling glass capillaries using an E-line (9.5 GHz) Varian ESR spectrometer.

RESULTS

Kinetic studies with Mn(II)

In order to evaluate the effect of replacement of Mg(II) by Mn(II), kinetic studies were carried out in presence of the latter ion. Mn(II) serves effectively as a cofactor and yields about 50% of the rate found with magnesium. However, K_m values for MgATP and MnATP are very similar. In the higher concentration range of ATP(> 200 µM), free nucleotide accumulates, which probably competes with metal-nucleotide on the enzyme and brings about the observed inhibition (Fig. 1). To clarify the role of Mn(II) at the direct binding site [2] and the metal-nucleotide binding site of hexokinase, kinetic analysis of Mn(II)-activated enzyme was carried out. Initially the kinetics of phosphorylation of glucose were studied under the limiting conditions of constant Mn(II) with variable ATP (Fig. 2B) and of constant ATP with variable Mn(II) (Fig. 2A). The reaction velocity has a simple hyperbolic dependence on MnATP yielding an apparent $K_{m(MnATP)} = 0.03$ mM (Fig. 2B), which is very similar to $K_{m(MgATP)}$ (see Fig. 1) obtained under similar conditions. In addition, it also exhibits a hyperbolic dependence on [Mn(II)]$_f$ (Fig. 2A). The activator constant for Mn(II) derived from this is 8.5 µM, which suggests a twofold tighter binding of Mn(II) with E · ATP complex as compared to ATP alone [11]. Another important feature of Fig. 2A is that in the region of high [Mn(II)]$_f$, the plot shows no upward
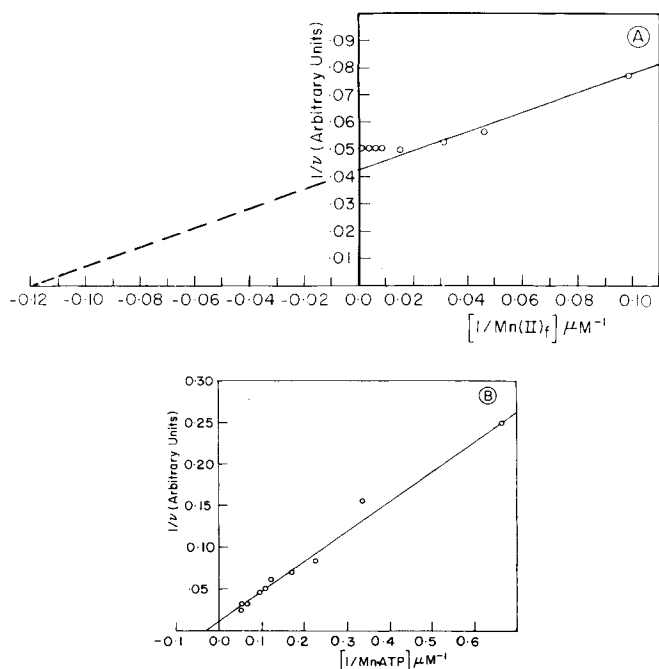
Fig. 2. *Effect of [MnATP] and [Mn(II)]$_f$ on the initial rates of hexokinase reaction.* (A) Double-reciprocal plot of velocity versus [Mn(II)]$_f$ at fixed (99 µM) [ATP]$_t$. [Mn(II)]$_t$ was varied from 50 µM to 400 µM. The calculated kinetic parameter $K_{A\,Mn(II)} = 8.5$ µM. (B) Double-reciprocal plot of velocity versus [MnATP]. [Mn(II)]$_t$ was fixed at 50 µM and ATP was varied from 1.5 µM to 29.5 µM. Calculated kinetic constant $K_{m\,MnATP} = 30$ µM. [Mn(II)]$_f$ and [MnATP] were calculated assuming a metal-nucleotide dissociation constant of 15 µM

or downward curvature. In this range of metal ion concentration, the direct Mn(II) binding site will be approximately 90 % saturated. This indicates that binding of Mn(II) at a direct binding site, previously identified by magnetic resonance methods, does not have any effect on catalytic activity of the enzyme. Activating and inhibitory effects of Mn(II) on hexokinase have been observed albeit at a much higher concentration range [2].

Substrate activity of β,γ-bidentate Cr(III)ATP for brain hexokinase

Substrate activity of β,γ-bidentate Cr(III)ATP, a substitution inert analogue of MnATP, for brain hexokinase was assayed by the method of Dunaway-Mariano and Cleland [8]. 1.0 ml 11.6 µM hexokinase was incubated with 500 µM Mn(II), 1.0 mM Cr(III)ATP and 1 mM [U-^{14}C]glucose (6.12 × 10^6 cpm/mM) in 100 mM N-ethylmorpholine acetate buffer pH 6.5 for 10 min at 25 °C. The reaction was stopped by precipitating the enzyme and the reaction mixture was analyzed as described in Materials and Methods. Fig. 3A shows the elution profile from the Dowex column. The amount of product, i.e. Glc-6-P·Cr(III)ADP, formed was 13.0 µM, indicating that brain hexokinase undergoes a single turnover when bidentate Cr(III)ATP is used as the substrate in the presence of Mn(II). Similar results were obtained in another experiment where Mn(II) was omitted from the reaction mixture (Fig. 3B). It is evident from this experiment that only the metal ion interacting with the enzyme via nucleotide is the one that participates in catalysis. The direct-binding Mn(II) has no apparent function in this respect. However, its parti-
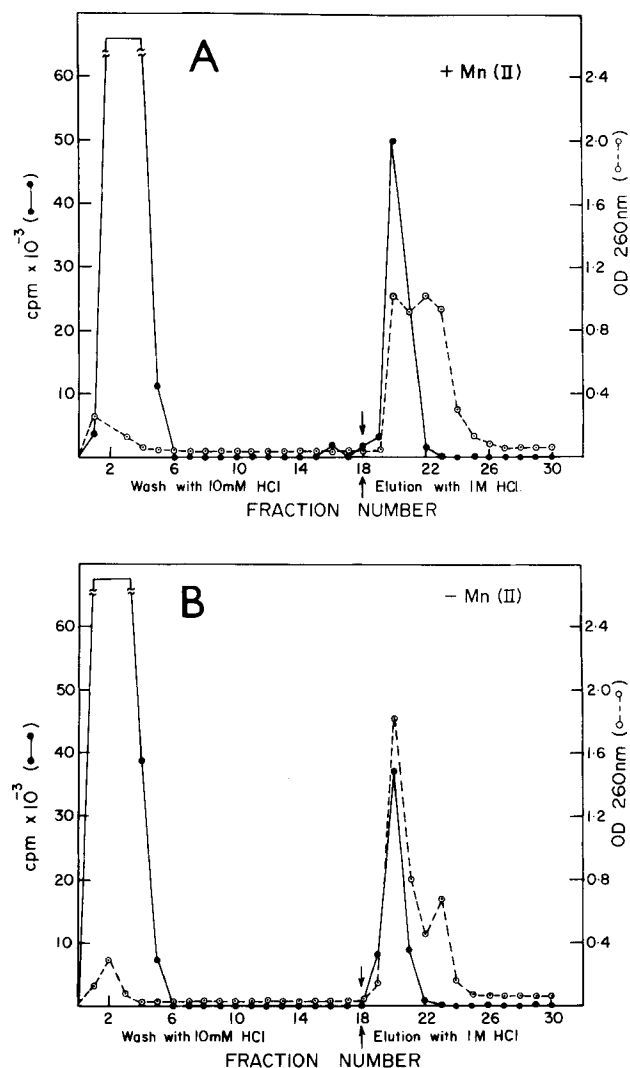

Fig. 3. *Assay of substrate activity of β,γ-bidentate Cr(III)ATP.* (A) 1.0 ml of the assay mixture contained 11.6 µM brain hexokinase, 1 mM Cr(III)ATP, 1 mM glucose (6.12 × 10^6 cpm/mM) 5 mM 2-mercaptoethanol, 100 mM N-ethylmorpholine acetate (pH 6.5) and 500 µM Mn(II). The solution was incubated at 25 °C for 10 min, and the product, Cr(III)ADP-Glc-6-P was purified on a Dowex-50× −2H$^+$ Column (25 × 0.5 cm). The column was washed with 75 ml 10 mM HCl and developed with 75 ml 1 M HCl. 5-ml fractions were collected and counted. Radioactivity in the wash (caused by unreacted glucose) was too high to be shown in the graph. (B) Here the assay mixture contained 7.9 µM enzyme and no Mn(II). The rest of the additions were the same, as were the subsequent steps of the assay

cipation in some subtle regulatory process cannot be entirely ruled out at this stage.

Determination of K$_{iapp}$ for β,γ-bidentate Cr(III)ATP

Various isomers of bidentate and tridentate Cr(III)ATP have been found to serve as competitive inhibitors with respect to MgATP for a number of kinases [8]. Kinetic analysis of initial velocity patterns at various concentrations of Cr(III)ATP suggest it to be competitive with respect to Mg·ATP for brain hexokinase (Fig. 4). The Dixon-plot analysis of these results gave an apparent inhibition constant $K_{iapp} = 8$ µM.
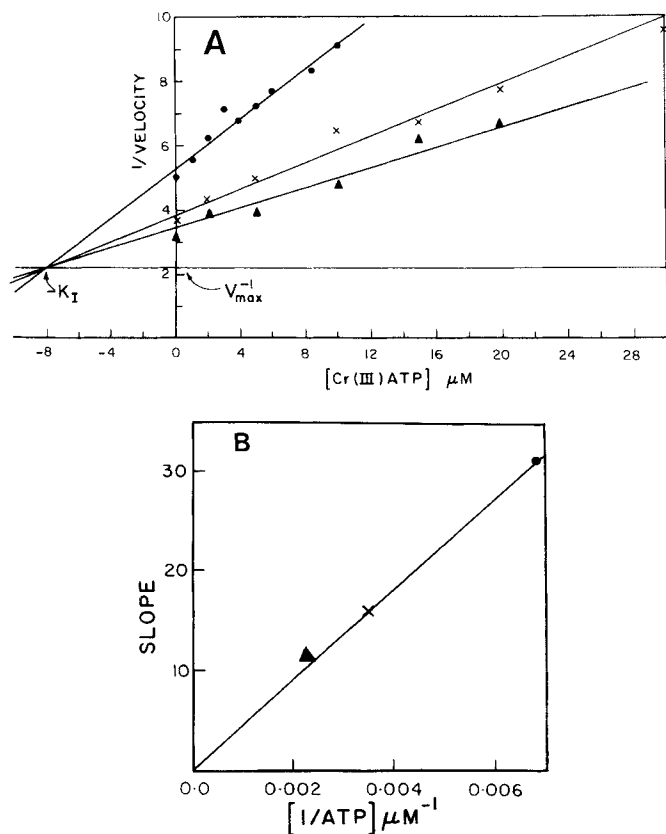
Fig. 4. *Determination of inhibition constant* (K_i) *of Cr(III)ATP for bovine brain hexokinase*. Standard assay mixture was used with ATP concentration 144 μM (—●—); 288 μM (——×——) and 432 μM (—▲—). Various amounts of Cr(III)ATP were added to the assay mixture. Reaction was started by addition of 10 μl 0.6 unit/ml hexokinase. 1.0 ml assay mixture was used for each assay. Change in absorbance at 340 nm was monitored on a Cary-17D spectrophotometer at 23 °C. (A) Dixon plot. (B) Replot of slopes of the Dixon plot versus 1/ATP
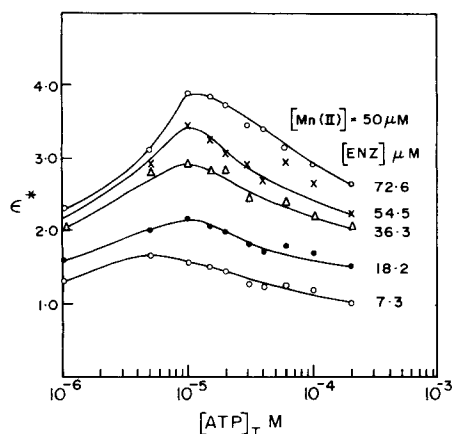

Fig. 5. *PRR titration of hexokinase with ATP in the presence of $MnCl_2$*. Solutions contained 50 μM Mn(II), 5 mM 2-mercaptoethanol, 10 mM N-ethylmorpholine acetate pH 8.0 and 7.2 – 72.6 μM hexokinase. Measurements were made at 9 MHz, 24 °C; solid lines are smooth curves drawn through the experimental points

PRR titration with nucleotides

We have previously shown that brain hexokinase enhances the effect of Mn(II) on the longitudinal relaxation rate of water
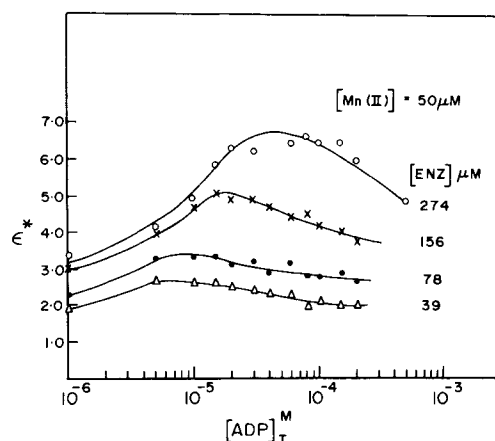

Fig. 6. *PRR titration of hexokinase with ADP in the presence of $MnCl_2$*. Solutions contained 50 μM Mn(II), 5 mM 2-mercaptoethanol, 10 mM N-ethylmorpholine acetate pH 8.0 and 39 – 274 μM hexokinase. Measurements were made at 9 MHz, 24 °C. Solid lines are smooth curves drawn through the experimental points
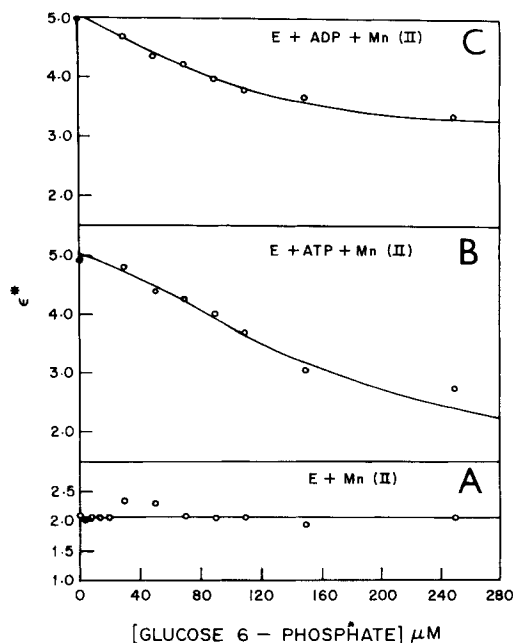

Fig. 7. *PRR titration of hexokinase with Glc-6-P in the presence of Mn(II) and nucleotide ligands (ATP or ADP)*. All solutions contained 50 μM Mn(II), 5 mM 2-mercaptoethanol, 225 μM hexokinase with 10 mM N-ethylmorpholine acetate pH 8.0. (B) and (C) contained, in addition, 20 μM ATP and 20 μM ADP respectively. ε^* were measured at 9 MHz, 23 °C

protons [2]. The characteristic enhancement (ε_b) for enzyme · Mn(II) complex, evaluated from PRR enhancement measurements, was found to be around 3.5 [2]. However, much larger enhancements were observed when nucleotides were added to the enzyme · Mn(II) binary complex.

In Fig. 5 we see the effect of increasing ATP concentration on enhancement behaviour at several fixed enzyme concentrations. Initially, as the nucleotide concentration is increased, the observed enhancement, ε^* increases. At intermediate ATP levels, it passes through a broad maximum and then decreases gradually at high ATP levels. Initial increase in ε^* is indicative of the formation of an enzyme · ATP · Mn(II) ternary complex.

Table 1. *Reverse hexokinase reaction, i.e. the formation of glucose and ATP from glucose 6-phosphate and ADP at 22 °C*

Total volume of the sample was 112 μl. It was incubated for 15 min. The reaction was stopped by adding 10 μl 60 % perchloric acid. These samples were then neutralized with appropriate amount of KOH. Resulting precipitate was removed by centrifugation and supernatant was analyzed on thin-layer chromatography using acetone/H_2O (90:10) solvent system. Specific activity of [^{14}C]glucose 6-phosphate was 10^6 cpm/μmole

Serial no.	Buffer	Sample composition				Glucose formed
		enzyme	Mn(II)	ADP	[^{14}C]Glc-6-*P*	
		μM	mM			μM	
1	0.1 M NEMA pH 8.0	102.6	4.46	3.57	69.6	7.0
2	0.1 M Bistris pH 6.0	86.2	4.46	3.57	69.6	10.4

Table 2. *Comparison of Mn(II) concentrations measured by ESR in the presence of various amounts of enzyme and Cr(III)ATP with the calculated values from Scheme I and Scheme II*

The samples contained 10 mM *N*-ethylmorpholine acetate, pH 8.0, 5 mM 2-mercaptoethanol and appropriate amounts of enzyme, Mn(II) and Cr(III)ATP. 20-μl samples were placed in glass capillaries and Mn(II) ESR spectra were recorded on an E-line Varian spectrometer. Instrumental settings were 0.25 s time constant, 12.5 G modulation amplitude, 100 kHz modulation frequency, 12 mW microwave power, 9.5 GHz microwave frequency and 8 min scan time. All the samples were monitored at 22 °C. Various dissociation constants used to calculate [Mn]$_f$ are E + M $\rightleftharpoons$ EM, $K_d = 25 \pm 4$ μM [2]; E + Cr(III)ATP $\rightleftharpoons$ E · Cr(III)ATP, $K_{i_{app}} = 8$ μM; Cr(III) ATP + Mn(II) $\rightleftharpoons$ Cr(III)ATP Mn; $K_d = 43 \pm 19$ μM (determined by an independent experiment using ESR) and E · M + Cr(III)ATP $\rightleftharpoons$ E · M · Cr(III)ATP; $K_{i_{app}} = 8$ μM

Serial no.	[Enzyme]$_t$	[Cr(III)ATP]	[Mn(II)]$_t$	[Mn(II)]$_f$		
				observed[a]	calculated[b]	
					Scheme I	Scheme II
	μM					
1	82.3	43.5	87.0	35.7	36.5 ± 1.9	41.8 ± 1.8
2	78.8	83.3	83.3	28.3	31.4 ± 2.8	39.1 ± 2.9
3	72.8	154.8	76.9	23.3	20.8 ± 3.7	28.2 ± 3.5

[a] Reproducibility of Mn(II) measurement for a given sample was within 5%.

[b] Errors presented here reflect the errors in the determination of $K_d(E · M)$ and $K_d[Cr(III)ATP · Mn]$.

The decrease in ε* at high ATP concentrations reflects the competition between the free nucleotide and the metal-ion · nucleotide complex for binding at the nucleotide site on the enzyme. Similar results were obtained when enzyme · Mn(II) was titrated with ADP (Fig. 6). Brain hexokinase thus binds Mn(II) through the nucleotide bridge, a coordination scheme characteristic of several kinase ternary complexes [4].

Effect of Glc-6-P on PRR enhancement of various enzyme complexes

E · Mn. Addition of Glc-6-*P* to E · Mn complex does not alter the PRR characteristics of the binary complex (Fig. 7A). However, under our experimental conditions Glc-6-*P* is known to interact with E · Mn complex [2].

E · ATP · Mn. Addition of small amounts of ATP to E · Mn results in a large increase in observed enhancement. A solution containing hexokinase (225 μM), Mn(II) (50 μM) and ATP (20 μM), when titrated with Glc-6-*P*, resulted in gradual decrease in observed enhancement (Fig. 7B). On extrapolation to infinite Glc-6-*P* concentration, the ε* approaches a value close to that of E · Mn · Glc-6-*P*. This decrease in observed enhancement on addition of Glc-6-*P* to ternary E · ATP · Mn

complex is attributed to the competitive displacement of ATPMn by Glc-6-*P* from the enzyme. This interpreation is also consistent with earlier kinetic studies [12].

E · ADP · Mn. Addition of the product Glc-6-*P* to the ternary hexokinase · ADP · Mn complex also results in a decrease in PRR enhancement (Fig. 7C). The observed decrease in PRR enhancement may arise as a result of displacement of ADP by Glc-6-*P* for which no kinetic evidence exists. Alternatively, since ADP · Mn and Glc-6-*P* are the products of the hexokinase reaction, there may be a formation of E · ADP · Mn. Glc-6-*P* quaternary complex, which has low characteristic enhancement. In case the latter possibility is true, there will be formation of glucose and ATP because of the reverse reaction. The amount produced will depend upon the equilibrium constant between E · ADP · Mn. Glc-6-*P* and E · ATP · Mn · glucose. In an attempt to detect the formation of ATP and glucose in a mixture of enzyme, ADP, Mn(II) and Glc-6-*P*, we used [U-^{14}C]Glc-6-*P*. Formation of glucose could be detected (Table 1). However, the data indicate that most of the nucleotide will be present as E · ADP · Mn · Glc-6-*P* complex. These results strongly support the suggestion that the observed decrease in PRR enhancement is predominantly due to the formation of a quaternary complex, E · ADP · Mn · Glc-6-*P*.

Interaction of β,γ-bidentate Cr(III)ATP with hexokinase · Mn(II)

To establish whether a complex of the form hexokinase · Mn · Cr(III)ATP containing two cations could be formed, binding of Mn(II) with the enzyme was studied in the presence of Cr(III)ATP. Under these conditions the nucleotide site is blocked by the non-dissociable Cr(III) ion. At different
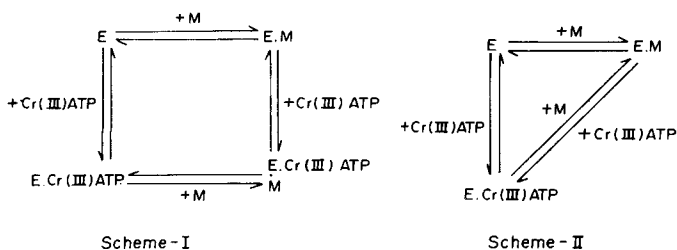
Scheme-I

Scheme-II

concentrations of the enzyme, Mn(II) and Cr(III)ATP, free Mn(II) concentration was determined using ESR (Table 2). These results were analysed assuming either the formation of E · Mn · Cr(III)ATP or E · Cr(III)ATP complex (Schemes I and II). Various coupled equilibria involved were solved using a computer program [13]. The calculated values of $[Mn(II)]_f$, according to Schemes I and II, along with measured values, are presented in Table 2. This analysis was carried out assuming neither co-operativity nor anti-cooperativity between the metal binding site and the nucleotide binding site, i.e. the binding of Mn(II) to enzyme does not affect the affinity of nucleotide · metal-ion for the enzyme. This assumption is supported by the fact that in kinetic studies no curvature was observed in double-reciprocal plots (Fig. 2A). A good agreement between the $[Mn(II)]_f$, calculated according to Scheme I, and the measured values suggests that Mn(II) binds to hexokinase at a tight binding site even when the nucleotide binding site is occupied by Cr(III)ATP.
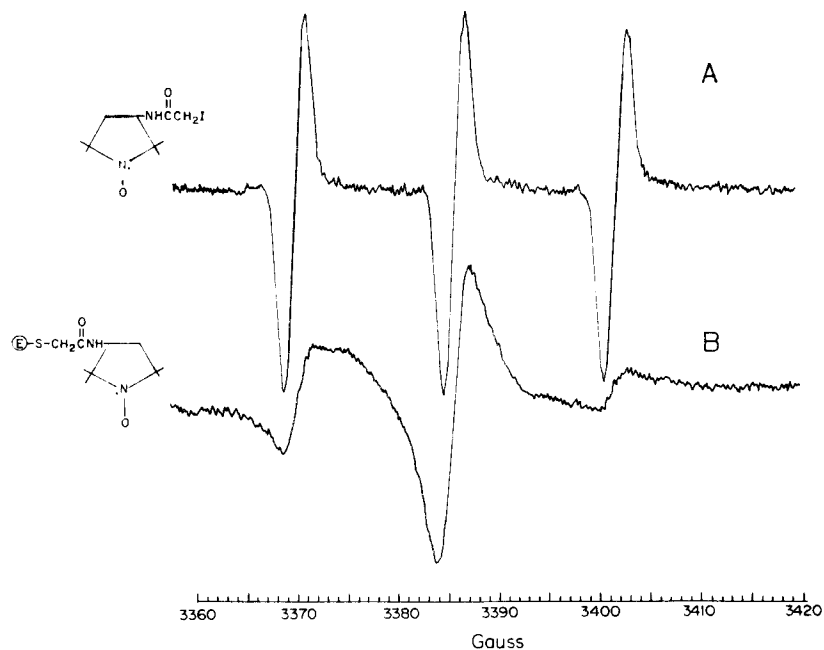

Fig. 8. *ESR spectra (A)* 10 μM *free spin label and (B)* 140 μM *spin-labeled hexokinase, both in 100 mM* N-*ethylmorpholine acetate buffer, pH 8.0, 19 °C*

Table 3. *Effect of Mn(II) and ATPMn on the height of the central ESR line of the hexokinase bound spin label*
ESR recording conditions were: scan range, 100 G; receiver gain, 3.2 × 10⁴; field set, 3390 G; scan time, 8 min; modulation frequency, 100 kHz; temperature 19 ± 1 °C; microwave frequency, 9.5 GHz. In addition to the above conditions: time constant = 0.25 min (serial no. 1), 0.5 min (2, 3); modulation amplitude = 2.0 G (1), 4.0 G (2, 3); microwave power = 20 mW (1), 12 mW (2, 3)

Serial no.	Concentration of spin-labeled enzyme	Signal height of the central ESR line in the presence of ligands		
		no addition	Mn(II)	ATP · Mn(II)
	μM			
1	66	20.5	15.00 (200 μM[b])	18 (250 μM[c]; 200 μM[b])
2	44	30	24 (100 μM[b])	27 (500 μM[c]; 500 μM[b])
3[a]	48	39	23.5 (100 μM[b])	36 (600 μM[c]; 500 μM[b])

[a] Stoichiometry of incorporation of spin label by enzyme in this sample was greater than 1. In the other two experiments the stoichiometry of spin labeling was 1:1.
[b] Numbers in parentheses represent total concentration of Mn(II).
[c] Numbers in parentheses represent total concentration of ATP.

Interaction of Mn(II) and MnATP with spin-labeled enzyme

Fig. 8B shows the ESR spectrum of the spin-labeled enzyme, which had a stoichiometry of spin labeling of 1:1. The shape of the ESR spectrum is characteristic of moderately immobilized label, with spectral lines broadened and much reduced in height. Binding of Mn(II) leads to a reduction in the amplitude of the central ESR spectral line without any appreciable broadening [14]. However, binding of ATPMn {prepared under conditions where there is no free Mn(II) present i.e. [ATP] > [Mn(II)] and [ATPMn] $\geq K_d$(ATPMn)} does not have any significant effect on the resonance line amplitude (Table 3). The reduction in signal height arises as a result of dipolar interaction between the two electron spins, i.e. the Mn(II) and the nitroxyl spin label, on the same macromolecule [15]. This observation would indicate that the ATPMn binding site on the enzyme is at a locus different from the direct Mn(II) binding site on the enzyme.

DISCUSSION

The data presented in Fig. 1 and 2 show that Mn(II) can replace Mg(II) as a cofactor for the hexokinase reaction. This replacement, however, reduces the activity of the enzyme by about 50% though the affinity of the enzyme for ATPMn remains the same as for ATPMg. The large PRR enhancement observed in the presence of nucleotide (ATP or ADP), compared to that produced by the enzyme · manganese system, places brain hexokinase in the category of creatine kinase [4] arginine kinase [16] and phosphofructokinase [17]. Enhancement pattern alone suggests that ATPMn/ADPMn binds to the enzyme to form a ternary complex via a substrate bridge. Binding of ATP(ADP) to enzyme in the absence of metal ion [18] suggests a direct interaction of nucleotide with the enzyme, which in turn supports the idea of a substrate-bridge ternary complex. Enzyme bridge or metal bridge schemes for the ternary complex are very unlikely on the basis of enhancement data. However, the results do not preclude some kind of Mn(II) · enzyme interaction once the metal ion is bound to the nucleotide, giving a complex of the type $E \overset{S}{\underset{M}{\diagup \!\!\!|}}$ [16,17]. This possibility also finds support from the fact that binding affinity of the enzyme for ATPMg is higher than for free ATP [19]. But the fact that Cr(III)ATP binds at the ATPMn site and serves as a substrate for hexokinase, rules out the possibility of interaction of nucleotide-bound metal ion with the enzyme $(E \overset{S}{\underset{M}{\diagup \!\!\!|}})$, as such an interaction is unlikely between the Cr(III) atom and the enzyme since Cr(III)ATP exchanges ligands very slowly.

Displacement of ATPMn from the enzyme by Glc-6-*P* is consistent with earlier kinetic studies [20] and supports the view that the γ-phosphoryl group of ATP and the phosphoryl group of Glc-6-*P* interact at the same subsite on the enzyme [18,20,21]. Addition of Glc-6-*P* to enzyme · ADP · Mn also causes a decrease in enhancement (Fig. 7C) similar to that observed when Glc-6-*P* is added to enzyme · ATP · Mn (Fig. 7B). However, in view of the observed reverse reaction (Table 1), this decrease has been attributed to the formation of a quaternary complex. Decrease in PRR enhancement in the quaternary complex, as compared to the ternary complex, implies that the active site becomes less and less accessible to

solvent water molecules as successive binding sites are occupied [22,23].

Hexokinase is somewhat unusual among kinases in that it forms a tight E · Mn binary complex. Possibility of binding of two metal ions in the presence of ATP(ADP) is indicated by the displacement of ATPMn from the enzyme by Glc-6-*P*, resulting in the formation of E · Mn · Glc-6-*P*. Further, support for the formation of a bimetallic complex, in which one metal ion is directly bound to the enzyme and another coordinated to phosphoryl groups of ATP, comes from the fact that Mn(II) can interact with E · Cr(III)ATP complex. The two metal binding sites appear to be of a non-interactive type. A similar conclusion has been drawn from our studies on the spin-labeled enzyme. We have observed that binding of Mn(II) to enzyme reduces the intensity of the ESR signal of the covalently linked nitroxyl radical. However, binding of ATPMn (where ATP > Mn(II) $\geq$ enzyme) does not have any significant effect on ESR signal intensity. This again supports the idea that the two metal ions interact at two spatially different sites on the enzyme surface.

Various isomers of Cr(III)ATP have proved to be very useful in probing the active conformers of nucleotide · Mn(II), which serve as a substrate for various kinases [24]. The preparation of Cr(III)ATP, used in our experiments, is a racemic mixture of various isomers of bidentate Cr(III)ATP. Possibility of presence of small amounts of tridentate Cr(III)ATP in this preparation cannot be ruled out. At this stage no attempt was made to further resolve the various isomers of bidentate Cr(III)ATP. As in the case of yeast hexokinase [8] and other mammalian hexokinases [25], it acts as a substrate for brain hexokinase (type 1) and the product Cr(III)ADP · Glc-6-*P* is released slowly. This observation clearly suggests that the metal ion interacting via nucleotide substrate is the only one required for catalysis. The second metal ion, binding directly to the enzyme [2], does not appear to play any role in enzyme activity. However, the metal ion at this site can be used as a paramagnetic reference point to map the sugar binding sites. Studies in this direction are currently under way in our laboratory.

REFERENCES

1. Purich, D. L., Fromm, H. J. & Rudolph, F. B. (1973) in *Advances in Enzymology* (Meister, A., ed.) vol. 39, pp. 249—326, Interscience, New York.
2. Jarori, G. K., Kasturi, S. R. & Kenkare, U. W. (1981) *Arch. Biochem. Biophys. 211*, 258—268.
3. Ning, J., Purich, D. L. & Fromm, H. J. (1969) *J. Biol. Chem. 244*, 3840—3846.
4. Mildvan, A. S. & Cohn, M. (1970) in *Advances in Enzymology* (Meister, A., ed.) vol. 33, pp. 1—70, Interscience, New York.
5. Mildvan, A. S. (1979) in *Advances in Enzymology* (Meister, A., ed.) vol. 49, pp. 103—126, Interscience, New York.
6. Redkar, V. D. & Kenkare, U. W. (1972) *J. Biol. Chem. 247*, 7576—7584.
7. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265—275.
8. Dunaway-Mariano, D. & Cleland, W. W. (1980) *Biochemistry 19*, 1506—1515.
9. Dunaway-Mariano, D. & Cleland, W. W. (1980) *Biochemistry 19*, 1496—1505.

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10. Cohn, M. & Townsend, J. (1954) *Nature (Lond.)* *173*, 1090 – 1091.
11. Cohn, M. & Leigh, J. S. (1962) *Nature (Lond.)* *193*, 1037 – 1040.
12. Colowick, S. P. (1973) *The Enzymes* 3rd edn (Boyer, P. D., ed.) vol. 9, pp. 1 – 48, Academic Press, New York.
13. Reed, G. H., Cohn, M. & O'Sullivan, W. J. (1970) *J. Biol. Chem.* *245*, 6547 – 6552.
14. Mehta, A., Jarori, G. K. & Kenkare, U. W. (1983) *Bull. Magnet. Resonance 5*, 252.
15. Leigh, J. S. (1970) *J. Chem. Phys. 52*, 2608 – 2612.
16. Buttlaire, D. H. & Cohn, M. (1974) *J. Biol. Chem. 249*, 5733 – 5740.
17. Jones, R., Dwek, R. A. & Walker, I. O. (1972) *Eur. J. Biochem. 28*, 74 – 82.
18. Redkar, V. D. & Kenkare, U. W. (1975) *Biochemistry 14*, 4704 – 4712.
19. Wilson, J. E. (1978) *Arch. Biochem. Biophys. 185*, 88 – 99.
20. Fromm, H. J. & Zewe, V. (1962) *J. Biol. Chem. 237*, 1661 – 1667.
21. Ellison, W. R., Lueck, J. D. & Fromm, H. J. (1975) *J. Biol. Chem. 250*, 1864 – 1871.
22. James, T. L., Reuben, J. & Cohn, M. (1973) *J. Biol. Chem. 248*, 6443 – 6449.
23. Reed, G. H., Diefenbach, H. & Cohn, M. (1972) *J. Biol. Chem. 247*, 3066 – 3072.
24. Cleland, W. W. & Mildvan, A. S. (1979) in *Advances in Inorganic Biochemistry* (Eichhorn, G. L. & Marzelli, L. G., ed.) vol. 1, pp. 163 – 191, Holland Inc.
25. Darby, M. K. & Trayer, I. P. (1983) *Eur. J. Biochem. 129*, 555 – 560.

G. K. Jarori, A. Mehta, S. R. Kasturi and U. W. Kenkare,
Tata Institute of Fundamental Research, Homi Bhabha Road, Colaba, Bombay, India 400 005