MAGNETIC RESONANCE STUDIES OF THE SPATIAL ARRANGEMENT OF GLUCOSE-6-PHOSPHATE AND CHROMIUM (III)-ADENOSINE DIPHOSPHATE AT THE CATALYTIC SITE OF HEXOKINASE

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ABSTRACT The interaction of CrADP, an exchange-inert paramagnetic analogue of Mg-ADP, with yeast hexokinase has been studied by measuring the effects of CrADP on the longitudinal nuclear relaxation rate ($1/T_1$) of the protons of water and the protons and phosphorus atom of enzyme-bound glucose-6-P. The paramagnetic effect of CrADP on $1/T_1$ of water protons is enhanced upon complexation with the enzyme. Titrations measuring this paramagnetic effect at several enzyme concentrations in the presence of glucose-6-P yielded a characteristic enhancement factor for $1/T_1$ of water protons and the dissociation constant of CrADP from the ternary enzyme·ADPCr·glucose-6-P complex. The latter value (2 mM) is similar to that obtained from kinetic inhibition studies (Danenberg and Cleland [1975]. *Biochemistry.* **14**:28). The presence of glucose-6-P increases the enhancement of the water relaxation rate by enzyme-bound CrADP, suggesting the formation of an enzyme·CrADP·glucose-6-P complex. The existence of such a complex was confirmed by the observation of a paramagnetic effect of enzyme-bound CrADP on the $1/T_1$ of the ^{31}P-nucleus and protons of enzyme-bound glucose-6-P. From the paramagnetic effects of enzyme-bound CrADP on the relaxation rates of the ^{31}P-nucleus and the carbon-bound protons of glucose-6-P in the enzyme·ADPCr·glucose-6-P complex, using the correlation time of ~0.7 ns, determined from the magnetic field-dependence of $1/T_1$ of water protons over the range 24.3–360 MHz, a Cr^{3+} to phosphorus distance of 6.6 ± 0.7 Å and Cr^{3+} to α- and β-anomeric proton distances of 8.9 and 9.7 Å were calculated. These results imply the absence of a direct coordination of the phosphoryl group of glucose-6-P by the nucleotide-bound metal on hexokinase but indicate van der Waals contact between a phosphoryl oxygen of glucose-6-P and the hydration sphere of the nucleotide-bound metal. The distances are consistent with a model that assumes molecular contact between the phosphorus of glucose-6-P and a β-phosphoryl oxygen of ADP suggesting an associative phosphoryl transfer. Because after phosphorylation of ADP, the metal ion is coordinated to the transferred phosphoryl group, the overall migration of the phosphoryl group during the phosphoryl transfer is ~3.6 Å toward the nucleotide-bound metal. Little or no catalysis of phosphoryl transfer from glucose-6-P to α,β-bidentate or β-monodentate CrADP ($\leq$0.05% of the rate found with MgADP) occurred in the presence of hexokinase, as monitored by glucose formation in a coupled assay system using glucose oxidase and peroxidase. The ability of β,γ-bidentate CrATP to act as a substrate (Danenberg and Cleland [1975].

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Biochemistry. **14**:28) and our observation of a lack of turnover in the hexokinase·CrADP·glucose-6-P complex indicate that metal ion coordination of the transferable phosphoryl group precedes phosphoryl transfer and is a requirement of the hexokinase reaction.

INTRODUCTION

Yeast hexokinase catalyzes the transfer of a phosphoryl group from an ATP-Mg^{2+} complex to the 6-hydroxymethyl group of certain hexose sugars in the first reaction of the glycolytic pathway. The three-dimensional structure of the dimeric form of B isoenzyme of hexokinase, of mol wt 102,000, has been solved to 3.5-Å resolution (2, 3). Studies on crystals of the monomeric enzyme at 2.7-Å resolution have revealed a single glucose binding site at the bottom of the deep cleft that separates each subunit into two lobes (3, 4). To date, however, intact crystals with the substrate MgATP bound to the active site have not been obtained (5). Therefore, the spatial arrangement of the substrates at the active site and the precise role of the divalent metal required for hexokinase activity remain incompletely understood at present.

The use of exchange-inert chromium (III)-nucleotide complexes as analogues for MgATP or MgADP has found wide applications in the study of phosphoryl transfer enzymes. The kinetic mechanisms for hexokinase (6–8) and other enzymes (8–16) have been probed using CrATP.[1] These complexes have also been used as active site structural probes (17–19) exploiting the unpaired electrons of Cr^{3+} to determine distances from this paramagnet to the magnetic nuclei of the other substrates and activators by means of nuclear relaxation techniques.

The present work was undertaken to determine distances between the substrates for the reverse reaction of hexokinase, glucose-6-P, and ADP, at the catalytic site of the enzyme. CrADP is used as a paramagnetic probe for the determination of intersubstrate distances using NMR relaxation techniques. The precise location of the transferable phosphoryl group of glucose-6-P relative to the nucleotide-bound metal at the active site is of interest with regards to its implication for the mechanism of enzyme action.

Because bidentate CrADP, the exchange-inert complex of ADP with Cr^{3+}, has been shown to be a competitive inhibitor in the forward reaction with respect to MgATP at the active site of hexokinase (6), and β,γ-bidentate CrATP is a substrate for the forward reaction (6), we also sought to determine whether α,β-bidentate CrADP or β-monodentate CrADP could function as a substrate in the reverse reaction, i.e., the phosphoryl transfer from glucose-6-P to ADP.

EXPERIMENTAL PROCEDURE

Materials

Yeast hexokinase (type C-300), a mixture of A and B isoenzymes, was obtained from Sigma Chemical Co. (St. Louis, Mo.) as a crystalline suspension in ammonium sulfate solution. The enzyme was separated from ammonium sulfate by gel-filtration through Sephadex G-25 (medium), and the fractions containing active protein were pooled and concentrated in collodion bags by vacuum dialysis at 4°C. The hexokinase employed in magnetic resonance experiments showed a specific activity ranging from 350 to

[1] *The abbreviations used in this paper are:* CrADP, chromium (III) complex of ADP; CrATP, chromium (III) complex of ATP; Pipes, piperazine-N,N'-bis (2-ethane sulfonic acid); and NMR, nuclear magnetic resonance.

550 units/milligram at 25°C pH 8 based on a spectrophotometric assay for the formation of NADPH in a coupled assay system using glucose-6-phosphate dehydrogenase.

Glucose-6-P, ADP, and NADP were obtained from Sigma Chemical Co. and glucose-6-phosphate dehydrogenase and glucose oxidase from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). o-Dianisidine·HCl was recrystallized from doubly distilled water before use.

α,β-Bidentate CrADP was synthesized by using the procedure of DePamphilis and Cleland (8). Acidic unbuffered solutions of the complex were frozen and stored at −20 or −70°C until required for use. CrADP in D_2O was obtained by readsorbing the complex on Dowex 50-X2-H^+, followed by washing with D_2O. The complex was eluted with 0.3 M aniline in D_2O, and the aniline was extracted with ether. It should be pointed out that CrADP is not stable to lyophilization.

β-Monodentate CrADP was also prepared by the procedure of DePamphilis and Cleland (8). In the final step, the pH of the solution was adjusted in the cold to pH 2.8 with saturated $KHCO_3$, and the solution was immediately frozen. Instability of this complex at higher pH precluded NMR studies using this complex with hexokinase. The conversion of β-monodentate to α,β-bidentate CrADP was followed optically at 430 nm and the half-time of the first order reaction at pH 6.5 was estimated to be 5 min.

Enzymatic Assays

The possibility of β-monodentate and α,β-bidentate CrADP functioning as substrates in the reverse reaction of hexokinase was tested by assaying for the formation of free glucose. In addition to o-dianisidine in 100 mM K^+ Pipes buffer, pH 6.5, the assay mixture contained hexokinase (≤0.25 mg/milliliter), glucose oxidase (10 U/milliliter), and horseradish peroxidase (10 U/milliliter) and was equilibrated with 95% oxygen. Enzymic conversion of glucose-6-P to glucose was initiated by addition of MgADP in the control experiment and the rate of o-dianisidine oxidation followed optically at 436 nm. The CrADP complexes were added as unbuffered solutions (pH ~ 3) and their rates measured immediately (≤2 min) at the higher pH of the assay (~6.5) for comparison with the Mg^{2+}-controls.

NMR Studies

The longitudinal nuclear relaxation rates of water protons were measured at 24.3, 100, and 360 MHz by using a NMR specialties PS 60W spin echo spectrometer, a Varian XL-100-FT spectrometer (Varian Associates, Instruments Div., Palo Alto, Calif.), and a Bruker WH-360 spectrometer (Bruker Instruments, Inc., Billerca, Mass..), respectively. Titrations of solutions of α,β-bidentate CrADP with varying concentrations of enzyme were carried out measuring the longitudinal relaxation rate of water protons at 24.3 MHz. Longitudinal $(T_1)^{-1}$ and transverse $(T_2)^{-1}$ relaxation rates of the glucose-6-P nuclei were measured on the Varian XL-100-FT system (Varian Associates) at 100 MHz for protons and at 40.5 MHz for ^{31}P. The inversion-recovery technique for T_1 determinations and the Carr-Purcell pulse technique for T_2 determinations were employed. 20% D_2O was present in the samples for ^{31}P NMR experiments to permit heteronuclear field-frequency locking on deuterium, and the ^{31}P spectra were proton-noise decoupled. Transfer of the enzyme to D_2O for the ^1H NMR experiments was accomplished by repeated (3 X) lyophilization and reconstitution of the sample with 99.8% D_2O. The hexokinase and glucose-6-P solutions were eluted through a 1-cm Chelex column (Bio-Rad Laboratories, Richmond, Calif.) before any NMR experiments to remove paramagnetic metal impurities. The ^1H NMR experiments were performed at pH 6.4 (pH meter reading uncorrected for deuterium isotope effects), while for ^{31}P NMR experiments, the uncorrected sample pH was 6.7. All samples contained 100 mM K^+-Pipes buffer and 100 mM KCl.

THEORY

Water Proton Relaxation Titrations

The observed enhancement factor, ϵ_{obs}^{Cr}, for the paramagnetic contribution of CrADP to the longitudinal relaxation rate of water protons is defined as:

$$\epsilon_{obs}^{Cr} = [(T_{1p})^{-1}/(T_{1p}^{\circ})^{-1}], \quad (1)$$

where $(T_{1p})^{-1}$ and $(T_{1p}^{o})^{-1}$ are the paramagnetic contributions to the relaxation rate in the presence and absence, respectively, of molecules that bind CrADP. The paramagnetic contribution to longitudinal relaxation rate is calculated by subtracting the observed relaxation rate in the absence of the paramagnetic components from that observed in the presence of all components. A general equation relating the observed enhancement to characteristic enhancements of all of the complexes present in solution can be obtained as:

$$\epsilon_{obs}^{Cr} = \frac{[CrADP]_f}{[CrADP]_T}\epsilon_a^{Cr} + \frac{[E\cdot CrADP]}{[CrADP]_T}\epsilon_b^{Cr} + \frac{[E\cdot CrADP\cdot G6P]}{[CrADP]_T}\epsilon_t^{Cr}. \quad (2)$$

In Eq. 2, subscripts f and T refer to free and total CrADP; G6P refers to glucose-6-P; ϵ_a^{Cr} would be 1 in the absence of glucose-6-P and was found to be 1.18 in the presence of glucose-6-P (10 mM); ϵ_b^{Cr} is the enhancement of the binary enzyme·CrADP complex; ϵ_t^{Cr} is the enhancement of the ternary enzyme·CrADP·glucose-6-P complex. Only the first and third terms of Eq. 2 need to be considered in the titration of hexokinase in the presence of saturating levels (10 mM) of glucose-6-P. Under these conditions rearrangement of Eq. 2 yields:

$$\epsilon_{obs}^{Cr} = (\epsilon_t^{Cr} - \epsilon_a^{Cr})\frac{[E\cdot CrADP\cdot G6P]}{[CrADP]_T} + \epsilon_a^{Cr}. \quad (3)$$

A dissociation constant for CrADP from the hexokinase·CrADP·glucose-6-P complex was obtained by fitting the observed enhancements to calculated concentrations at several levels of the enzyme according to Eq. 3.

Electron-Nuclear Dipolar Correlation Times and Distances

The total effect of an enzymic complex containing CrADP on the longitudinal and transverse relaxation rates is usually a sum of the paramagnetic $(T_{1p})^{-1}$ and the diamagnetic $(T_{1d})^{-1}$ contributions. When the longitudinal relaxation is not exchange-limited (20) and the hyperfine contact contribution to $1/T_{1p}$ is negligible (i.e., either the contact hyperfine coupling is small or the contact term has dispersed away), then the distance-dependence of the paramagnetic component of the longitudinal relaxation rate $(1/T_{1p})$ that forms the basis of distance calculations, is given by the well known Solomon-Bloembergen equation (21–23):

$$\frac{1}{(fqT_{1p})_b} = \frac{2S(S+1)\gamma_I^2 g^2\beta^2}{15r^6}\left\{\frac{3\tau_{c1}}{1+\omega_I^2\tau_{c1}^2} + \frac{7\tau_{c2}}{1+\omega_S^2\tau_{c2}^2}\right\}, \quad (4)$$

where f is the ratio of concentrations of the paramagnet and the relaxing ligand, q is the relative stoichiometry of the bound ligand and bound paramagnet (i.e., the coordination number), S is the electron spin, γ_I is the nuclear gyromagnetic ratio, g is the electronic g-factor, β is the Bohr magneton, r is the metal-nucleus distance, and ω_I and ω_S are the nuclear and electron precession frequencies. $(T_{1p})_b^{-1}$ is the paramagnetic effect arising from the complex of interest. It should be pointed out that if several paramagnetic complexes are present in a given solution, the effect of all complexes other than the one of interest must be subtracted and the data normalized appropriately before Eq. 4 can be applied. τ_{ci} in Eq. 4 are the correlation times defined as:

$$\tau_{ci}^{-1} = \tau_R^{-1} + \tau_{Si}^{-1} + \tau_M^{-1}. \quad (5)$$

τ_R is the correlation time for rotation of the paramagnetic complex and τ_{S1} and τ_{S2} are the longitudinal and transverse electron spin relaxation times, respectively, and τ_M is the residence time for the nucleus under consideration in the paramagnetic complex. Over the range of magnetic fields used in this study, $\omega_S^2 \tau_{c2}^2 >> 1$ and therefore the contribution of the second term in Eq. 4 is negligibly small. Further, over the range of magnetic fields used and for the enzymic complexes considered in this study, τ_{ci} are dominated by τ_{Si}. The electron-spin relaxation times τ_{Si}, and hence τ_{ci} in Eq. 5, may also be magnetic field-dependent. The magnetic field-dependence of τ_{S1} is given by the Bloembergen-Morgan equation (24):

$$\frac{1}{\tau_{S1}} = B\left(\frac{\tau_v}{1 + \omega_S^2 \tau_v^2} + \frac{4\tau_v}{1 + 4\omega_S^2 \tau_v^2}\right), \tag{6}$$

where B is a constant that is a function of the symmetry of the ligand field, and τ_v is the correlation time for the random distortions of the ligand field around the paramagnetic metal ion.

RESULTS

Test of β-Monodentate and α,β-Bidentate CrADP as Substrates for Hexokinase

The reverse reaction of hexokinase involves the magnesium-activated transfer of a phosphoryl group from glucose-6-P to ADP resulting in the formation of glucose and ATP. The ability of chromium complexes of ADP to undergo phosphorylation was tested by following the formation of free glucose by using glucose oxidase and horseradish peroxidase in the coupled assay system shown in the following scheme:

$$\text{Glucose-6-P} + \text{ADP} \overset{\text{hexokinase}}{\rightleftharpoons} \text{Glucose} + \text{ATP}$$

$$\text{Glucose} + \text{H}_2\text{O} + \text{O}_2 \overset{\text{glucose oxidase}}{\rightleftharpoons} \text{Gluconate} + \text{H}_2\text{O}_2$$

$$\text{H}_2\text{O}_2 + \text{o-dianisidine} \overset{\text{peroxidase}}{\rightleftharpoons} 2\text{H}_2\text{O} + \text{brown-colored product.}$$

Formation of brown-colored product (i.e., the oxidation of o-dianisidine in the assay mixture) was followed spectrophotometrically at 436 nm to give a rate for formation of free glucose. α,β-Bidentate CrADP was found to be inactive in promoting phosphoryl transfer from glucose-6-P, because the rate of glucose formation using this complex as phosphoryl acceptor was ≤ 0.05% of the rate measured with MgADP.

β-Monodentate CrADP slowly converts to α,β-bidentate CrADP when the pH of a solution containing monodentate CrADP is raised above pH 3. A half-time for this decomposition was estimated to be ~ 5 min at pH 6.5. Assays for glucose formation with β-monodentate CrADP were limited, therefore, to somewhat incompletely characterized and changing mixtures of mono- and bidentate chromium complexes. It was clear, however, that no glucose formation (≤ 0.03% of the rate found with MgADP) was occurring when β-monodentate CrADP (unbuffered pH 2.8) was added to the assay mixture buffered at pH 6.5. A substantial

concentration of β-monodentate CrADP ($\geq$ 76%) would be expected to be present at pH 6.5 after fast mixing of the solution and would persist over the first 2 min during which the rate was monitored. Neither β-monodentate nor α,β-bidentate CrADP are thus found to be active in the reverse reaction of hexokinase.

Interaction of CrADP with Hexokinase

Binding of α,β-bidentate CrADP to the hexokinase·glucose-6-P complex enhances the paramagnetic effect of CrADP on the nuclear relaxation rate $1/T_1$ of water protons. This enhancement, in a titration of CrADP with varying concentration of hexokinase, in the presence of a saturating concentration of glucose-6-P, is shown in Fig. 1. The experimental decrease in the observed enhancement with decreasing concentration of hexokinase at a fixed concentration of CrADP can be fit to a curve generated from Eq. 3 assuming an ϵ_i^{Cr} (the enhancement of the ternary hexokinase·CrADP·glucose-6-P complex) value of 2.7 ($\pm$ 0.3) and a dissociation constant of 2.0 ($\pm$ 0.4) mM. The K_D value obtained from the water relaxation titration is in good agreement with the kinetic inhibition constant ($\sim$ 2 mM) previously reported by Danenberg and Cleland for bidentate CrADP as a competitive inhibitor versus MgATP (6).

The addition of glucose-6-P to a solution containing hexokinase and CrADP enhances the paramagnetic effect of CrADP on the longitudinal relaxation rate of water protons twofold, suggesting the formation of a ternary hexokinase·CrADP·glucose-6-P complex. This finding is consistent with the earlier observations of an enhancement of water relaxation of the (enzyme + MnADP) system in the presence of glucose (25). The existence of a ternary enzyme·CrADP·glucose-6-P complex is confirmed later in this paper by the observation of a paramagnetic effect of CrADP on the longitudinal relaxation rates of ^{31}P and protons of enzyme-bound glucose-6-P.

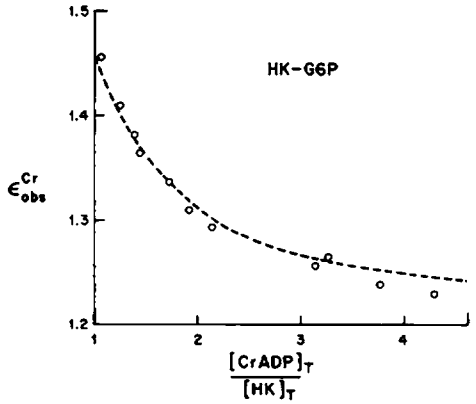

FIGURE 1 Titration measuring the observed enhancement, ϵ_{obs}^{Cr}, of the paramagnetic effect of CrADP on the relaxation rate of water protons as a function of $[CrADP]_T/[HK]_T$ in the enzyme·glucose-6-P (+CrADP) system. The solution contained 0.5 mM CrADP, 10 mM glucose-6-P, 0.1 M KCl, and 0.05 M K^+ Pipes buffer (pH 6.6). The dotted line is a theoretical curve generated from Eq. 3 using K_D = 2 mM and ϵ_i^{Cr} = 2.7. T = 22. HK, hexokinase.

Determination of the Correlation Time (τ_c) for Electron-Proton Dipolar Interaction

Calculation of distances between the paramagnetic Cr^{3+} center and magnetic nuclei of the glucose-6-P ligand requires that several assumptions be justified and necessitates determination of the correlation time, τ_c, for the paramagnetic dipolar interaction. We found it most convenient to obtain τ_c for the enzyme·CrADP·glucose-6-P complex by studying the magnetic field dependence of the water proton relaxation rates in this complex (Table I). The determination of τ_c from this magnetic field dependence using Eqs. 4–6 assumes fast chemical exchange, no outer-sphere contributions, and no hyperfine contact contributions. These assumptions are considered more fully later in this paper with reference to the paramagnetic effects of Cr^{3+} on substrate glucose-6-P. However, it should be noted at this point that water proton exchange is rapid (18, 24, 26) compared to the longitudinal relaxation rate of water protons in the inner sphere of Cr^{3+} in other complexes. For free CrADP, the absence of exchange-limitation is indicated by our observation of a ratio of 35 ± 5 between the paramagnetic contributions to protons and deuterons of HDO at pH 6.5. In the presence of exchange-limited paramagnetic proton relaxation, this ratio is expected to be much lower than γ_H^2: γ_D^2 which is equal to 42.6. The presence of a fivefold magnetic field-dependence in the paramagnetic contribution of enzyme-bound CrADP on the water proton relaxation rate (Table I) suggests, but does not establish, the absence of exchange-limitation on water relaxation in the hexokinase·CrADP·glucose-6-P complex.

A K_D value of 2 mM for the dissociation of CrADP from hexokinase·CrADP·glucose-6-P was used to determine normalized relaxation rates $(1/fT_{1p})_b$ for water protons at 24.3, 100, and 360 MHz, and these values are listed in Table I. A computer analysis of the experimentally measured $(1/fT_{1p})_b$ values at the various frequencies via Eqs. 4–6, assuming no exchange limitation ($\tau_M \ll T_{1M}$) or a partial exchange limitation ($\tau_M = T_{1M}$) on the observed paramagnetic effects on $1/T_1$, yielded best fit τ_c values in the range 7 (± 3) × 10^{-10} s. The possibility $\tau_M > T_{1M}$ is ruled out by the observed magnitudes and frequency dependence of the paramagnetic effects on $1/T_1$.

TABLE I

MAGNETIC FIELD-DEPENDENCE OF NORMALIZED LONGITUDINAL RELAXATION RATE ($1/fT_{1p}$) OF WATER PROTONS DUE TO PARAMAGNETIC EFFECT OF CrADP*

Complex	$(fT_{1p})_b^{-1}$ s^{-1}			$\tau_c \times 10^{10}$ s
	24.3 MHz	100 MHz	360 MHz	
CrADP (+ glucose-6-P)	3.0×10^5	3.0×10^5	2.7×10^5	1.8 ± 0.4‡
E · CrADP · glucose-6-P	7.4×10^5	6.8×10^5	1.5×10^5	7 ± 3§

*All solutions contained 0.1 M KCl and 0.1 M Pipes buffer at pH 6.7. Estimated experimental error in the relative values of $(fT_{1p})_b^{-1}$ is ≤5%, and that in the absolute values is ≤10%.

‡Magnetic field-independent.

§Magnetic field-dependent. The reported range of τ_c values (7 ± 3) × 10^{-10} s is applicable to a magnetic field of 23.5 kG, corresponding to nuclear resonance frequencies of 100 MHz for ^1H and 40.5 MHz for ^{31}P. Calculations of τ_c values were made assuming no exchange limitation ($\tau_M \ll T_{1M}$) or a partial exchange limitation ($\tau_M \sim T_{1M}$) on the observed paramagnetic effects. The possibility $\tau_M > T_{1M}$ is ruled out by the observed magnitudes and frequency dependence of the paramagnetic effects on $1/T_1$.

Free α,β-bidentate CrADP exhibits a first derivative electron paramagnetic resonance spectrum consisting of a dispersion absorbance at $g = 2.0$ with a peak-to-peak width of ~ 300 G. This sets a lower limit of 2×10^{-10} s on the electron spin relaxation time of free CrADP. Estimation of the rotational correlation time for free CrADP from Stokes law gives a value of 1.6×10^{-10} s at 23°. We calculate a value for τ_c of 1.8×10^{-10} s for free CrADP from the magnetic field-dependence of the water proton relaxation rates (Table I), which is in agreement with Stokes law calculations.

Effect of CrADP on Relaxation Rates of the Phosphorus Atom and the Protons of Glucose-6-P

The 100 MHz ^{1}H NMR spectrum of glucose-6-P at pH 6.7 (Fig. 2) shows it to be a mixture of α- and β- anomers of the cyclic pyranose. Integration of the area under the doublets assignable to the α-anomeric proton (5.6 ppm) and the β-anomeric proton (4.4 ppm) indicates that 38% of glucose-6-P is present as the α-isomer, in good agreement with the values reported by Schray and Benkovic (27). The half-life for the conversion of the α- to the β-anomer has earlier been reported to be 5 s (27). We confirmed that the interconversion of anomers is slow on the NMR time scale by irradiating the anomeric proton of one isomer and observing only small cross saturation effects ($\leq 10\%$) on the other anomeric proton in the NMR spectrum. Unlike protons, the 40.5 MHz ^{31}P NMR spectrum of glucose-6-P shows only a single peak for the phosphorus atom linked to the 6-hydroxymethyl group of glucose.

Nuclear relaxation rates of the ^{31}P and the α- and β-anomeric protons of glucose-6-P in the presence of CrADP and hexokinase were determined and are summarized in Tables II and III. The paramagnetic effects of CrADP on the nuclear relaxation rates of glucose-6-P were calculated from the respective differences between the relaxation rates measured with and without CrADP. The partial saturation (83–91%) of hexokinase sites with glucose-6-P was taken into account in calculating the effect of the enzymic complex containing CrADP on the longitudinal and transverse relaxation rates of the glucose-6-P nuclei. Under our conditions, $\sim$ 25% of CrADP was complexed to the enzyme. The paramagnetic effect of free

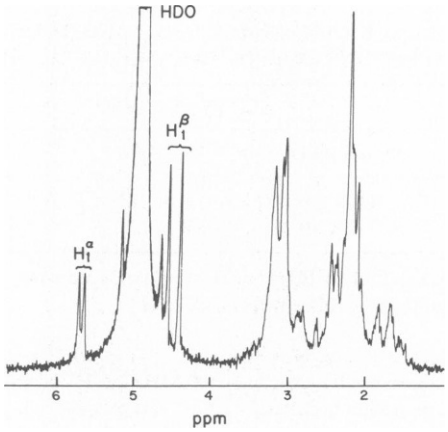

FIGURE 2 100-MHz proton NMR spectrum of glucose-6-P at pH 6.7 (μ = 0.1 M).

TABLE II

EFFECT OF CrADP ON THE LONGITUDINAL $(T_1)^{-1}$ AND TRANSVERSE $(T_2)^{-1}$ RELAXATION RATES OF THE PHOSPHORUS NUCLEUS OF GLUCOSE-6-PHOSPHATE AT 40.5 MHz*

Enzyme sites	[glucose-6-P]	[CrADP]	$(T_1)^{-1}$	$[CrADP]_b$	$(fT_{1p})^{-1}$	$(fT_{1p})_b^{-1}$	$(T_2)^{-1}$	$(fT_{2p})^{-1}$	$(fT_{2p})_b^{-1}$
mM	*mM*	*mM*	s^{-1}	*mM*	s^{-1}	s^{-1}	s^{-1}	s^{-1}	s^{-1}
	10.4		0.14				0.2		
	10.2	0.50	3.4		66				
	9.9	0.97	5.8		58		8.0	80	
0.68	10.4		0.14				1.9		
0.68	10.4	0.11	1.7	0.026		510‡			
0.67	10.3	0.21	3.6	0.050		620‡	8.4		1,300‡
0.66	10.2	0.47	5.5	0.104		360‡			

*All solutions contained 0.1 M KCl and 0.1 M Pipes buffer at pH = 6.7 (uncorrected pH meter reading) in 80% H_2O + 20% D_2O.

‡A 17% correction for partial saturation (83%) of the enzyme sites with glucose-6-P was necessary.

CrADP was subtracted from the total observed effect by using appropriate controls (Tables II and III).

Distances can be calculated from the $(1/fT_{1p})_b$ values by using Eq. 4 if hyperfine contact and outer-sphere contributions can be neglected and chemical exchange-limitation can be ruled out. The hyperfine contact contribution to $1/T_{1p}$ must be negligible, because $\omega_S^2\tau_{c2}^2 >> 1$, and therefore the contact contribution vanishes. Furthermore, the outer-sphere effects must also be negligibly small because displacement experiments, in which 10 mM glucose is used to compete for the active site on hexokinase with 10 mM glucose-6-P, show a 50 ± 10% reduction in the observed paramagnetic effect of enzyme-bound CrADP on the ^{31}P nucleus of glucose-6-P. Displacement of glucose-6-P from the enzyme should eliminate only the

TABLE III

EFFECT OF CrADP ON THE LONGITUDINAL (T_1^{-1}) RELAXATION RATES OF ANOMERIC PROTON OF α D-GLUCOSE-6-P AND β D-GLUCOSE-6-P AT 100 MHz*

Enzyme sites	[glucose-6-P]		[CrADP]	T_1^{-1}		$[CrADP]_b$	$(fT_{1p})^{-1}$		$(fT_{1p})_b^{-1}$	
	α	β		α	β		α	β	α	β
mM	*mM*		*mM*	s^{-1}		*mM*	s^{-1}		s^{-1}	
	7.6	12.4		0.7	1.1					
	7.5	12.3	0.49	2.1	2.0		56	36		
	7.5	12.2	0.98	2.5	2.8		37	34		
0.81	7.1	12.5		1.1	2.7					
0.81	7.0	12.5	0.46	4.7	4.9	0.12			480‡	290‡
0.80	7.0	12.4	0.92	6.4	6.0	0.21			400‡	220‡

*All solutions contained 0.1 M KCl and 0.1 M Pipes buffer at pH 6.4 (uncorrected pH meter reading) in D_2O.

‡The ratio of total [Glucose-6-P] to bound $[CrADP]_b$ was used for calculating $(fT_{1p})_b^{-1}$ and a 10% correction was made for the partial saturation (90%) of enzyme sites with glucose-6-P. It has been implicitly assumed that the anomeric distribution of the enzyme-bound glucose-6-P is similar to that of free glucose-6-P. This assumption is justified by the similarity of the K_M values of α- and β-anomers of glucose in the forward reaction of hexokinase (29).

TABLE IV

DISTANCES BETWEEN Cr^{3+} OF HEXOKINASE-BOUND CrADP AND THE PHOSPHORUS NUCLEUS AND ANOMERIC PROTONS OF D-GLUCOSE-6-P

Nucleus	$(fT_{1p})_b^{-1}$	τ_c	$f(\tau_c)$	Distance from Cr^{3+}
	s^{-1}	$\times 10^{10} s$	$\times 10^{9} s$	$\AA$
^{31}P	490 ± 130	7 ± 3	2.0 ± 0.8	6.6 ± 0.7
H_1^{α}	440 ± 80	7 ± 3	1.7 ± 0.6	8.9 ± 0.8
H_1^{β}	250 ± 75	7 ± 3	1.7 ± 0.6	9.7 ± 1.1

inner-sphere contributions to $1/T_{1p}$. The extent of the observed displacement is consistent with the dissociation constants of glucose-6-P (6) and glucose from the dimeric enzyme (28) under our conditions, which would predict a displacement of 54%. This clearly supports the absence of significant outer-sphere contributions from enzyme-bound CrADP to the observed paramagnetic effects.

The largest value of the transverse relaxation rate of 8.4 s^{-1} (Table II) when normalized by the appropriate ratio ([glucose-6-P]:[enzyme sites] for T_{2d}^{-1} and [glucose-6-P]:$[CrADP]_b$ for T_{2p}^{-1}) sets a lower limit of ~ 1310 s^{-1} on the rate of dissociation of glucose-6-P from the enzyme-site. Because the normalized total longitudinal relaxation rates of ^{31}P and ^{1}H ($1/fT_{1\,\text{total}}$) are 2.7–5.2-fold smaller than the normalized transverse relaxation rate ($1/fT_{2\,\text{total}}$) of ^{31}P measured in the present studies (Tables II and III), the rate of chemical exchange between free and enzyme-bound glucose-6-P must be faster in comparison to the longitudinal relaxation rates in the enzyme-bound state. These considerations permit us to calculate the distances between the enzyme-bound glucose-6-P and the Cr^{3+} atom of enzyme-bound CrADP via Eq. 4. The results are summarized in Table IV and Fig. 3.

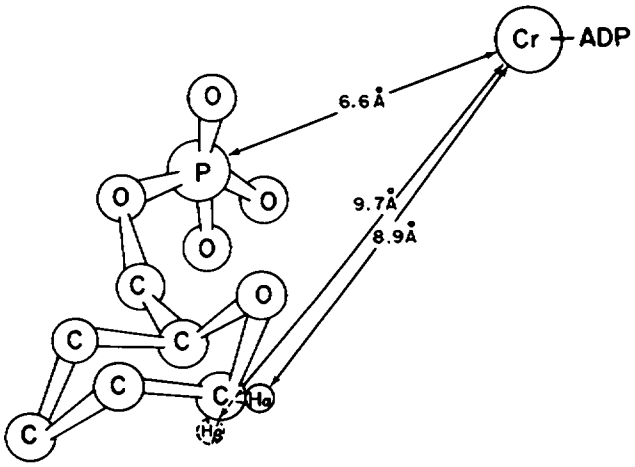

FIGURE 3 Spatial arrangement of glucose-6-P with respect to the Cr^{3+} atom of CrADP in the hexokinase · ADPCr · glucose-6-P complex in accord with the distances of Table IV.

DISCUSSION

Our observation of paramagnetic effects of enzyme-bound CrADP on the anomeric protons of both the α- and the β-anomers of glucose-6-P indicates that both forms bind to hexokinase, consistent with the activity of both anomeric forms of glucose in the forward reaction of the enzyme with comparable K_M and V_{max} values (29).

The distance of 6.6 Å from the phosphorus atom of glucose-6-P to the metal of hexokinase-bound ADP is well beyond an appropriate distance of 3.0 ± 0.1 Å for direct inner-sphere coordination. Assuming CrADP and MgADP interact with the same site on the enzyme, as is indicated by the kinetic studies of Danenberg and Cleland (6), it would appear that considerable metal ion migration (~ 3.6 Å) toward the transferred phosphoryl group of glucose-6-P would be necessary before coordination of this phosphoryl group to the metal could occur. Metal migration could take place in MgADP accompanying breaking of the coordination bond to the α-phosphoryl group of ADP. Kinetic inertness to coordination bond-breaking in Cr^{3+} compounds, however, would prevent such migration for CrADP. Our results show that neither α,β-bidentate nor β-monodentate CrADP can function as a substrate for the hexokinase catalyzed phosphoryl transfer from glucose-6-P. This is in contrast to the ability of β,γ-bidentate CrATP to phosphorylate glucose in the forward reaction of hexokinase, as shown by Danenberg and Cleland (6). If the first step in the reaction mechanism of hexokinase were the coordination of the phosphoryl group of glucose-6-P by the nucleotide-bound metal, the inertness of mono- or bidentate CrADP to substitution would block the reaction by prohibiting ligation of the transferred phosphoryl group.

A two-step mechanism for the hexokinase-catalyzed reaction of glucose-6-P with ADP is suggested from the spatial arrangement of glucose-6-P and CrADP at the active site of hexokinase and the inability of α,β-bidentate and β-monodentate CrADP to accept a phosphoryl group from glucose-6-P while β,γ-CrATP can phosphorylate glucose. This is shown schematically in Fig. 4. Step I can be viewed as ligand substitution in which the phosphoryl group of glucose-6-P enters the inner coordination sphere of Mg^{2+} while the α-phosphoryl group of MgADP dissociates. Metal coordination is followed by Step II in which phosphoryl transfer occurs. Step I cannot take place with either α,β-bidentate or β-monodentate CrADP. Therefore, CrADP is not a substrate for the hexokinase reaction but provides a useful paramagnetic probe for the intersubstrate distance measurements. It should, however, be pointed out that the two-step mechanism postulated in Fig. 4 for the enzyme reaction does not rule out the possibility that it may also be a concerted mechanism.

Even though the phosphoryl group of glucose-6-P is not in the inner-sphere of the nucleotide-bound metal on hexokinase, the Cr^{3+} to glucose-6-P distances in the enzyme·ADPCr·glucose-6-P complex obtained here, and the suggested mechanism of phosphoryl transfer, indicate some spatial overlap, at least of the order of the size of an oxygen atom, between the phosphoryl groups of ATP and glucose-6-P, which would prevent their simultaneous binding to the enzyme.

The distance of 6.6 ± 0.7 Å from the metal in CrADP to the phosphorus of glucose-6-P is consistent with molecular contact between the phosphorus of glucose-6-P and the terminal oxygen of ADP. This may be deduced as follows. Model building and NMR studies reveal a distance of 4.9 ± 0.4 Å from a metal, such as Mg^{2+}, coordinated to a polyphosphate chain, to

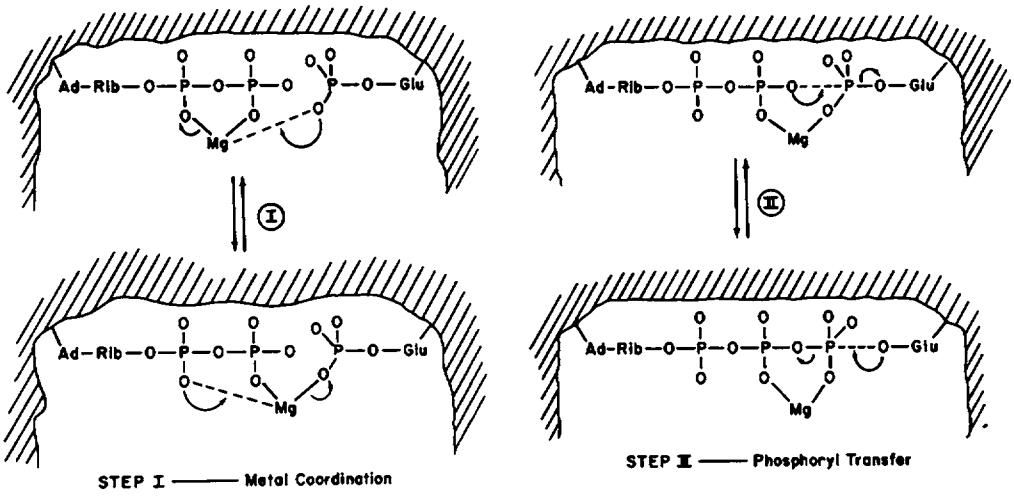

FIGURE 4 Mechanism of hexokinase action consistent with the spatial arrangement of substrates shown in Fig. 3, with the ability of β, γ-bidentate CrATP to act as a substrate and with the inability of CrADP to accept a phosphoryl group from glucose-6-P. Metal-migration occurs via change in the metal ion-chelation of the polyphosphate chain and metal-coordination of the transferable phosphoryl group precedes phosphoryl transfer in the catalytic mechanism.

the first covalently bound phosphoryl phosphorus that is not directly coordinated to the metal (30). This distance would increase by 1.7 Å to 6.6 ± 0.6 Å when the covalent P — O bond length (1.6 Å) is increased to that of a simple van der Waals contact interaction (3.3 Å). The metal to phosphorus distance of 6.6 ± 0.6 Å thus expected for van der Waals contact of a metal pyrophosphate complex with an adjacent phosphoryl group overlaps with the present experimental value of 6.6 ± 0.7 Å. Molecular contact of the transferable phosphoryl group with the phosphoryl acceptor atom would suggest an associative mechanism for hexokinase.

Finally, it should be stated that the mechanistic conclusions in this paper depend on the assumption that CrADP binds to hexokinase in the same manner as MgADP. Further, the Solomon-Bloembergen-Morgan (SBM) equations used in this paper neglect electron delocalization and ligand-field effects (31). The latter are expected to be negligible at the high magnetic fields used, while the internal consistency of our proton and phosphorus distances would argue against the former being a significant source of error in our studies. However, it should be pointed out that in a few water relaxation studies with Mn^{2+}-protein complexes, the point-dipole approximation of the SBM theory appears to underestimate the strength of the paramagnetic dipolar interaction with solvent protons (31, 32).

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