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Magnetic Resonance Studies on Manganese–Nucleotide Complexes of Phosphoglycerate Kinase†

Bogden E. Chapman,‡ William J. O'Sullivan,*,§ Robert K. Scopes, and George H. Reed

ABSTRACT: Measurements of the relaxation rate of water protons (PRR) have been used to study the interaction of yeast phosphoglycerate kinase with the manganous complexes of a number of nucleotides. The results indicate that phosphoglycerate kinase belongs to the same class of enzymes as creatine kinase, adenylate kinase, formyltetrahydrofolate synthetase, and arginine kinase, with maximal binding of metal ion to the enzyme in the presence of the nucleotide substrate. However, an analysis of titration curves for a number of nucleoside diphosphates (ADP, IDP, GDP) showed that there is a substantial synergism in binding of the metal ion and nucleotide to the enzyme in the ternary complex. The metal–substrate binds to the enzyme approximately two orders of magnitude more tightly than the free nucleotide. Other evidence for an atypical binding scheme for Mn(II)–nucleoside diphosphates was obtained by electron paramagnetic resonance (EPR) studies; the EPR spectrum for the bound Mn(II) in the enzyme–MnADP complex differed substantially from those obtained for other kinases. An identical EPR spectrum is observed with the MnADP complex with the rabbit muscle enzyme as with the yeast enzyme. In contrast, the dissociation constant for the enzyme–MnATP complex is approximately fourfold lower than that for enzyme–ATP, and there are no substantial changes in the electron paramagnetic resonance spectrum of $MnATP^{2-}$ when the complex is bound to phosphoglycerate kinase. A small but significant change in the PRR of water is observed on addition of 3-phosphoglycerate (but not 2-phosphoglycerate) to the MnADP-enzyme complex. However, addition of 3-phosphoglycerate to enzyme–MnADP did not influence the EPR spectrum of the enzyme-bound Mn(II).

Phosphoglycerate kinase (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) catalyzes the reaction, $MATP^{2-}$ + 3-phosphoglycerate ⇌ $MADP^{-}$ + 1,3-diphosphoglycerate, where the divalent metal ion may be Mg^{2+}, Mn^{2+}, Ca^{2+}, or Co^{2+}. The enzyme has been isolated from a number of sources (see Scopes, 1971, for a review), though it is the enzyme from yeast that has been subjected to the most detailed investigation. For example, much work has been done on the kinetics of the yeast enzyme (Larsson-Razniekiewicz, 1964, 1967, 1970; Krietsch and Bücher, 1970). Recently, extensive x-ray crystallographic studies have been carried out (Wendell et al., 1972; Bryant et al., 1974) and high resolution NMR[1] studies of the enzyme–substrate complexes have been reported (Tanswell et al., 1976).

In this paper, we present the results of magnetic resonance studies on complexes formed between the enzyme and various Mn–nucleotide complexes. Results from PRR measurements of water indicate an unusual synergism in the binding of Mn–nucleoside diphosphates but not Mn–nucleoside triphosphates to the enzyme. These differences are reflected in qualitative differences in the respective EPR spectra. Evidence from both techniques for the formation of an abortive quaternary complex, enzyme–MnADP–3-phosphoglycerate, is also presented. A preliminary account of some of this work has appeared elsewhere (Chapman et al., 1974).

† From the Department of Medicine, University of Sydney, Sydney, N.S.W. 2006, Australia (B.E.C. and W.J.O'S), Department of Biochemistry, LaTrobe University, Bundoora, Victoria 3083, Australia (R.K.S.), and the Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19174 (G.H.R.). *Received October 14, 1976.* This work was supported by the Australian Research Grants Commission (B.E.C.), the National Health and Medical Research Council of Australia (W.J.O'S), in part, by grants from the United States Public Health Service (GM 12446, AM 17517), the National Science Foundation (GB 32168X), and by a Research Career Development Award to G.H.R. (1-K04-AM 70134) from the National Institute of Arthritis, Metabolic and Digestive Diseases.

‡ Present address: Department of Physical Chemistry, University of Sydney, Sydney, N.S.W. 2006, Australia.

§ Present address: School of Biochemistry, The University of New South Wales, Kensington, N.S.W. 2033, Australia.

[1] Abbreviations used: NMR, nuclear magnetic resonance; PRR, proton relaxation rate; EPR, electron paramagnetic resonance; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; T_{1p}, the paramagnetic contribution to the longitudinal proton relaxation time of water; T_{1M}, the longitudinal proton relaxation time of water in the first coordination sphere of Mn(II); ϵ_a, ϵ_b, and ϵ_t are the characteristic PRR enhancement factors for the Mn(II)–substrate, Mn(II)–enzyme, and Mn(II)–substrate–enzyme complexes, respectively; 3-PGA, 3-phosphoglycerate; ω, the nuclear Larmor precessional frequency; τ_c, correlation time for a Mn(II) complex; τ_r, the rotational correlation time; τ_e, the electron spin–lattice relaxation time; τ_M, the residence time of a water molecule in the first coordination sphere of Mn(II); XDP, xanthosine diphosphate; Ap(CH_2)p, α,β-methylene analogue of ADP; App(CH_2)p, β,γ-methylene analogue of ATP.

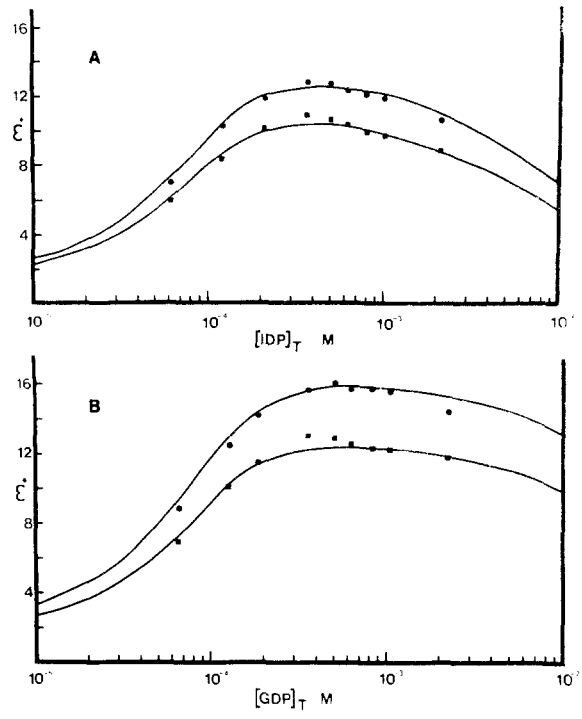

FIGURE 1: (A) PRR titration data for phosphoglycerate kinase and $MnCl_2$ (0.1 mM) with IDP in 50 mM Hepes-KOH, pH 8.0. Upper curve: phosphoglycerate kinase = 0.272 mM. Lower curve: phosphoglycerate kinase = 0.181 mM. Temperature = 25 °C. Solid curves drawn with K_D = 1 mM, ϵ_b = 4.3, K_1 = 0.3 mM, ϵ_a = 1.7, K_2 = 0.14 mM, K_S = 2.8 mM, ϵ_t = 21. Values of K_2, K_S, and ϵ_t taken from minimum percentage of SD (4.2) in regression analysis. (B) PRR titration data for phosphoglycerate kinase and $MnCl_2$ (0.1 mM) with GDP. Upper curve: phosphoglycerate kinase = 0.278 mM. Lower curve: phosphoglycerate kinase = 0.158 mM. Solid curves drawn with K_2 = 0.10 mM, K_S = 10.0 mM, ϵ_t = 23. Other constants and conditions as in A. Values of K_2, K_S, and ϵ_t taken from minimum percentage of SD (5.0) in regression analysis.

Materials and Methods

Enzyme. Crystalline phosphoglycerate kinase was obtained from yeast as described previously (Scopes, 1971). The enzyme had a specific activity in the forward direction of 700–900 μmol of product formed per min per mg of enzyme. The rabbit muscle enzyme was prepared by the method of Scopes (1969) and had a specific activity of approximately 300 μmol of product min^{-1} (mg of protein)$^{-1}$.

Reagents. ADP, d-ADP, and ATP were obtained from Sigma; GDP, xanthosine diphosphate (XDP), UDP, and CDP were from Calbiochem; IDP was from P-L Biochemicals; α,β-methylene-ADP ($Ap(CH_2)p$) and β,γ-methylene-ATP ($App(CH_2)p$) were from Miles Laboratories. 3-Phosphoglycerate (3-PGA) and 2-phosphoglycerate (2-PGA) were obtained as the sodium salts from Sigma and Calbiochem, respectively. Hepes was obtained from Sigma.

Magnetic Resonance Measurements. Measurements of the longitudinal relaxation time, T_1, of water protons were made at 30 MHz in 50 mM Hepes-KOH, pH 8.0, at 25 °C using a pulsed nuclear magnetic resonance spectrometer as described previously (O'Sullivan et al., 1972). The relationship between ϵ^*, the observed enhancement of the PRR, and ϵ_a, ϵ_b, and ϵ_t, the characteristic enhancements for the metal-substrate, metal-enzyme, and enzyme-metal-substrate complexes, have been described[2] (Mildvan and Cohn, 1970; Reed et al., 1970; O'Sullivan et al., 1973). Titration data were analyzed as described by Reed et al. (1970). Values obtained from analysis were ϵ_t and the constants, K_2 and K_S, for the dissociation of metal-substrate and substrate from enzyme-metal-substrate and enzyme-substrate complexes, respectively. The values obtained previously for ϵ_a (1.7) and the dissociation constant K_1 of Mn-nucleoside diphosphate (0.03 mM) and of Mn-nucleoside triphosphate (0.01 mM) were used (O'Sullivan and Cohn, 1966).

Electron paramagnetic resonance spectra were recorded at ~9.1 GHz using a Varian E-3 spectrometer with a standard accessory for temperature control. Samples were contained in high purity quartz capillary tubing as described previously (Reed and Cohn, 1973).

TABLE I: Enhancements and Dissociation Constants for the Binding of Manganese Nucleotides to Phosphoglycerate Kinase.[a]

Nucleotide	ϵ_t	K_2 (mM)	K_S (mM)	% SD[b]
ADP	21	0.08	3.2	3.7
IDP	21	0.14	2.8	4.2
GDP	23	0.10	10.0	5.0
dADP[c]	11	0.4	7.4	13
ATP	10	0.25	1.0	7
$App(CH_2)p$	9.5	0.05	0.14	5

[a] ϵ_t refers to the enhancement of the ternary enzyme-metal-substrate (EMS) complex, K_2 is the dissociation constant for the metal nucleotide from the ternary complex, and K_S the dissociation constant for the free nucleotide from the binary enzyme-nucleotide complex. [b] Percent standard deviation from the mean of ϵ_t, calculated for each data point. [c] Values are only approximate because of significant degeneracy in the analysis (see text).

Results

Binary Complexes. Addition of phosphoglycerate kinase to a solution of Mn(II) results in a small enhancement of the PRR of water. Analysis of a titration of a solution of $MnCl_2$ with enzyme gives a best fit with an ϵ_b of 4.3 and a dissociation constant of 1.0 mM assuming a single binding site (Chapman et al., 1974).

Mn-3-Phosphoglycerate Complex. The dissociation constant of Mn-3-PGA, in 50 mM Hepes-KOH, pH 8.0, was estimated[3] by following the decrease in the amplitude of the EPR signal of free Mn(II) on addition of 3-PGA (Cohn and Townsend, 1954). The average value from five experiments was 830 (±90) μM. This value is lower by a factor of three than the value of 2.6 mM reported previously (Larsson-Raznikiewicz, 1972). The discrepancy between the two values may be attributed, in part, to differences in experimental conditions since the latter value was obtained in 0.2 M KCl.

Ternary Complexes. Weak interactions between either free metal (ϵ_b, 4.3, and K_D, 1.0 mM) of free ADP^{3-} (K_S, 3.2 mM) and the enzyme but relatively strong interactions (ϵ_t, 21.5, and K_2, 0.08 mM) between $MnADP^-$ and the enzyme have been observed (Chapman et al., 1974). Very similar titration curves are obtained with $MnIDP^-$ and $MnGDP^-$ (Figure 1). The initial increase in ϵ^* which occurs with increasing concentra-

[2] The equation for the observed enhancement is $\epsilon^* = \Sigma_i([M_i]/[M]_T)\epsilon_i$ where ϵ_i is the enhancement of the ith species containing paramagnetic metal ion at a concentration of $[M_i]$ and $[M]_T$ is the total concentration of paramagnetic metal ion.

[3] When Mn-liquid complexes have a net negative charge, interactions between the complex and $Mn(H_2O)_6^{2+}$ broaden the EPR spectrum for $Mn(H_2O)_6^{2+}$ slightly. This leads to a small determinate error in the $Mn(H_2O)_6^{2+}$ concentration measured by EPR (Hu, A., Cohn, M., and Reed, G. H., unpublished results).

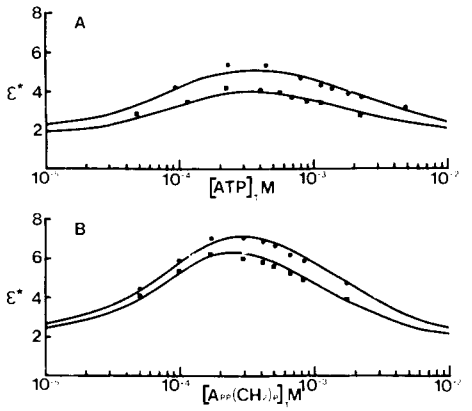

FIGURE 2: (A) PRR titration data for phosphoglycerate kinase and $MnCl_2$ (0.1 mM) with ATP. Upper curve: phosphoglycerate kinase = 0.28 mM. Lower curve: phosphoglycerate kinase = 0.16 mM. Solid curves drawn with K_1 = 0.01 mM, K_2 = 0.25 mM, K_S = 1.0 mM, ϵ_t = 10. Other constants and conditions as for Figure 1. Values of K_2, K_S, and ϵ_t taken from minimum percentage of SD (7.0) in regression analysis. (B) PRR titration data for phosphoglycerate kinase and $MnCl_2$ (0.1 mM) with App(CH_2)p. Upper curve: phosphoglycerate kinase = 0.37 mM. Lower curve: phosphoglycerate kinase = 0.25 mM. Solid curves drawn with K_2 = 0.05 mM, K_S = 0.14 mM, ϵ_t = 9.5, percentage of SD, 4.9. Other conditions as above.

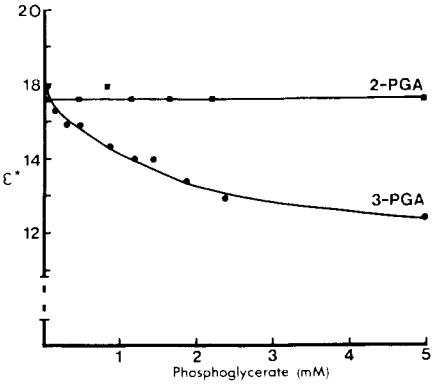

FIGURE 3: Comparison of PRR titration of MnADP-phosphoglycerate kinase with 3-phosphoglycerate and 2-phosphoglycerate. Concentrations were: ADP, 0.5 mM; $MnCl_2$, 0.1 mM; enzyme, 0.49 mM. Temperature = 25 °C. Other conditions as for Figure 1.

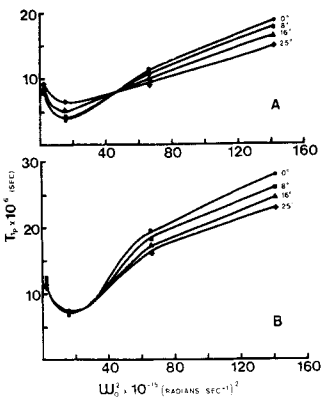

FIGURE 4: (A) Frequency dependence of molar relaxation time of the MnADP-phosphoglycerate kinase complex at 0, 8, 16, and 25 °C. The solution contained: enzyme, 0.39 mM; $MnCl_2$, 0.1 mM; ADP, 0.55 mM; Hepes-KOH, pH 8.0, 50 mM. (B) Frequency dependence of molar relaxation time of the MnADP-phosphoglycerate kinase-3-PGA complex. Data same as for A with the addition of 5 mM 3-phosphoglycerate.

tions of nucleotide is indicative of the formation of an enzyme-Mn-nucleotide complex. Very high concentrations of nucleotide are required to lower ϵ* in the region beyond the maximum, indicating a weak competition of free nucleotide for the metal-nucleotide binding site on the enzyme. Values obtained for the dissociation constants and ternary complex enhancement factors for a number of nucleotides are summarized in Table I. The K_S:K_2 ratios, 20-100, for ADP, IDP, and GDP reflect the much tighter binding of metal-nucleotide to the enzyme than of free nucleotide.

Titration of solutions of Mn(II) and enzyme with dADP gave a curve with smaller observed enhancements than those for ADP, IDP, and GDP. Because of the limited region of the saturation fraction covered in the dADP titrations, there is considerable degeneracy in the computer fits, and the values of ϵ_t, K_2, and K_S were strongly correlated. No binding of the pyrimidine compounds, UDP and CDP, was detected.

The enhancement curves for the titration of phosphoglycerate kinase and Mn(II) with ATP (Figure 2A) were more symmetrical than those for the nucleoside diphosphates and also gave lower maximum observed enhancements. Analysis of the curves gave K_2, 0.25 mM, K_S, 1.0 mM, and ϵ_t, 10.[4] Results similar to those for ATP were obtained for App(CH_2)p (Figure 2B). The values for K_2 and K_S, 0.05 and 0.14 mM, respectively, indicate that the inhibitor binds more tightly to the enzyme than does the substrate, ATP.

Quaternary Complexes. Addition of the substrate, 3-phosphoglycerate, to the ternary Mn-ADP-phosphoglycerate kinase complex caused a decrease in the observed enhancement (Chapman et al., 1974). The decrease in enhancement was attributed to the formation of the abortive complex, enzyme-Mn-ADP-3-PGA. However, the possibility for a slight decrease in ϵ* due to competition between 3-PGA and enzyme-ADP for Mn(II) was considered. Titrations of solutions of enzyme, and MnADP with 2-phosphoglycerate, show that this compound has no significant effect on ϵ* over the same concentration range in which ϵ* is lowered by 3-PGA (Figure 3). The differential effects of 2-phosphoglycerate and 3-phosphoglycerate suggest that 3-PGA influences the observed enhancement by binding to the enzyme-MnADP complex.

Titration of the ternary complex with 3-phosphoglycerate to near-saturating levels decreases the enhancement by approximately 25%.[5] Assuming that the curve demonstrated saturation behavior, a dissociation constant of approximately 0.5 mM for 3-phosphoglycerate from the quaternary complex (Chapman et al., 1974) was estimated, in good agreement with the kinetically determined value of 0.6 mM (Larsson-Razniekiewicz, 1970).

Frequency and Temperature Dependence of PRR. Values of T_{1p} (molar) for the ternary Mn-ADP-phosphoglycerate kinase complex and for the quaternary Mn-ADP-enzyme-3-phosphoglycerate complex are plotted as a function of frequency squared in Figures 4A and 4B, respectively, at four temperatures 0, 8, 16, and 25 °C. A linear relationship between

[4] The possibility that breakdown of ATP, by transfer of a phosphoryl group to the enzyme (Walsh and Spector, 1971), could perturb the experimental results was explored by incorporating [^{14}C]ATP in one of the solutions being measured ($MnCl_2$, 0.1 mM; enzyme, 0.17 mM; ATP from 0.08 mM to a final concentration of 0.2 mM). The breakdown of ATP was less than 5% during the course of the experiment (approximately 20 min).

[5] No significant changes were observed on the addition of nitrate ion to the quaternary complex, in contrast to results obtained with creatine kinase (Reed and Cohn, 1972) and arginine kinase (Buttlaire and Cohn, 1974a).

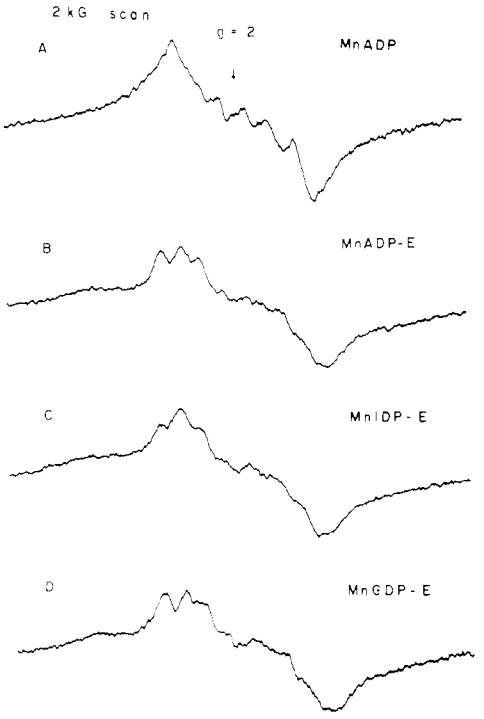

FIGURE 5: EPR spectra for Mn–nucleoside diphosphate complexes. All solutions contained $MnCl_2$, 0.3 mM, in 40 mM Hepes–KOH buffer, pH 8.0. Additional components were: (A) ADP, 2.0 mM; (B) ADP, 0.9 mM; phosphoglycerate kinase, 0.7 mM; (C) IDP, 1.0 mM; phosphoglycerate kinase, 0.7 mM; (D) GDP, 1.0 mM; phosphoglycerate kinase, 0.7 mM. Spectra were recorded at 2 °C.

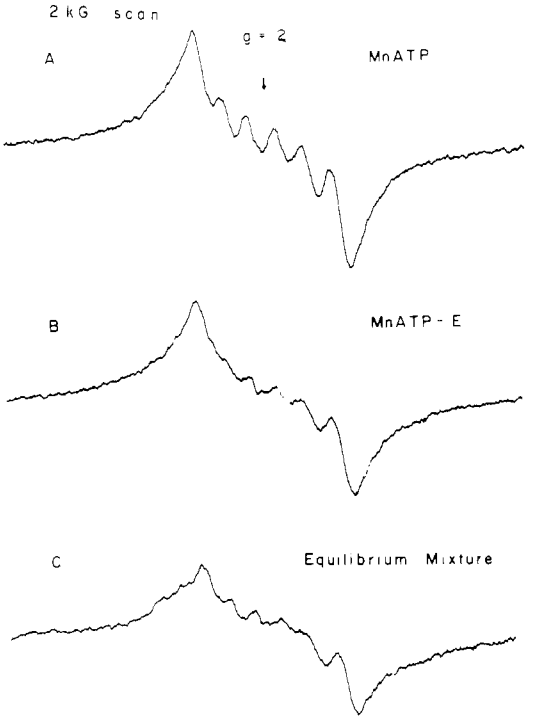

FIGURE 6: EPR spectra for Mn–ATP complexes. Solutions contained $MnCl_2$, 0.3 mM, in 40 mM Hepes–KOH buffer, pH 8.0. Additional components were: (A) ATP, 0.8 mM; (B) ATP, 0.8 mM; phosphoglycerate kinase, 0.7 mM; (C) ATP, 0.8 mM; phosphoglycerate kinase, 0.7 mM; 3-phosphoglycerate, 4 mM (equilibrium mixture). Spectra were recorded at 2 °C.

T_{1p} and ω^2 is expected from the frequency dependent term $\tau_c/(1 + \omega^2\tau_c^2)$ in the Solomon–Bloembergen equation for T_{1M} (Solomon, 1955; Bloembergen, 1957); but as observed for a number of other ternary enzyme systems (Reuben and Cohn, 1970; Reed et al., 1972), the plots pass through a minimum in the low frequency region at all temperatures. This behavior indicates that the correlation time, τ_c, is itself frequency dependent. As $1/\tau_c = 1/\tau_r + 1/\tau_c + 1/\tau_M$ and as the rotational correlation time, τ_r, and the residence time for a water molecule in the first coordination sphere of manganous ion, τ_M, are both independent of frequency, the electron spin relaxation time, τ_e, must make a significant contribution to τ_c.

EPR Spectra of Phosphoglycerate Kinase Complexes. EPR spectra for solutions of MnADP in the presence and absence of enzyme are shown in Figure 5. The spectra show that binding of MnADP to phosphoglycerate kinase leads to an alteration in the characteristic six-line spectrum of MnADP which is manifested in amplitudes of signals and in the introduction of new fine structure. A similar experiment with phosphoglycerate kinase from rabbit muscle gave an essentially identical spectrum. A different situation is found with creatine kinase (Reed and Cohn, 1972), arginine kinase (Buttlaire and Cohn, 1974b), and formyltetrahydrofolate synthetase (Buttlaire et al., 1975), where differences in the EPR line shapes for the binary and ternary complexes are not as striking as for phosphoglycerate kinase. Addition of 3-phosphoglycerate (4 mM) with and without monovalent anions (NO_3^- or SCN^- at approximately 5 mM) did not produce any further detectable alteration in the spectrum. However, denaturation of the protein by heating resulted in reversion to the spectrum for MnADP.

EPR spectra for the enzymic complexes of MnIDP, MnGDP (see Figure 6), and MnXDP were all similar to that for enzyme–MnADP. As indicated from the PRR results, MndADP bound much more weakly to the enzyme and though similar features as with MnADP were observed, a spectrum free from interference of the binary complex was not obtained.

In contrast to the results with the nucleoside diphosphates, binding of MnATP to the enzyme does not result in new structure in the EPR spectrum but rather a general broadening of the entire pattern (Figure 6). Further addition of 3-phosphoglycerate to the solution containing $MnATP^{2-}$ and enzyme, to give the equilibrium mixture, results in a spectrum which appears to correspond to that of a mixture of enzyme–MnADP and enzyme–MnATP (Figure 6C).

Similar measurements for the phosphonate analogue of ADP, $Ap(CH_2)p$, and the β-γ-methylene analogue of ATP, $App(CH_2)p$, give no significant change in the EPR spectrum on binding of the manganous complex to phosphoglycerate kinase. In neither case did the addition of 3-phosphoglycerate or nitrate ion significantly affect the spectrum.

Addition of phosphate ion (HPO_4^{2-}), arsenate, or sulfate to a solution containing $MnADP^-$, enzyme, and 3-phosphoglycerate does elicit changes in the EPR spectrum, with loss of the distinctive low-field features and reversion to the usual spectrum seen for Mn–nucleotide complexes. It is likely that the spectral effects are due to free $MnADP^-$, which is released from the enzyme by the presence of both 3-PGA and the anions, though there was the possibility of the formation of a higher order complex, with MnADP, 3-PGA, and HPO_4^{2-} simultaneously bound to the enzyme.

Discussion

The marked synergism in binding of Mn(II) and nucleoside diphosphates to phosphoglycerate kinase and the unique EPR

pattern for the enzyme-metal-nucleoside diphosphate complexes suggest that the structure of this ternary complex differs from those for metal-nucleotide complexes of creatine kinase (Reed and Cohn, 1972), arginine kinase (Buttlaire and Cohn, 1974b), adenylate kinase (Price et al., 1973), and formyltetrahydrofolate synthetase (Buttlaire et al., 1975). Blake and Evans (1974) have suggested a structure for the ternary complex of the horse muscle enzyme in which the metal ion is liganded to the phosphate groups of ADP and to a donor group on the protein. Such a structure appears to be consistent with x-ray data for the yeast enzyme as well (Bryant et al., 1974). While the presence of a ligand from the protein in the coordination sphere of Mn(II) could well account for the synergism in binding of Mn(II) and nucleoside diphosphates to the enzyme and for the unusual EPR spectrum for the ternary complex, it is significant that the protein NMR studies of Tanswell et al. (1976) did not reveal a Mn(II)-protein interaction for the yeast enzyme. However, the line-broadening measurements for the protein resonances were done with a substoichiometric concentration of metal ion, and it is possible that only those protons sufficiently remote from the metal ion would fall into the fast exchange region and be visible in the NMR difference spectrum.

The essentially identical EPR spectra for the purine nucleoside diphosphate complexes with Mn(II) and the yeast enzyme and for the complexes with the rabbit muscle enzyme would indicate effectively identical structures around the manganous ion in these ternary complexes. In this respect, the EPR pattern for these complexes serves as a fingerprint for the active-site structure. Moreover, the inhibitor, $Ap(CH_2)p$, shows a different EPR pattern in its ternary complex with Mn(II) and enzyme.

Though the changes in PRR enhancement on addition of 3-PGA to solutions of the ternary enzyme-Mn-ADP complex were small and no change in the EPR spectrum was observed, the use of 2-phosphoglycerate as a control, however (Figure 3), does provide evidence for the formation of a quaternary MS_1-E-S_2 complex with 3-phosphoglycerate. It is interesting to note that there is evidence from kinetic experiments with red cell phosphoglycerate kinase that 3-phosphoglycerate binds more weakly to the enzyme in the presence of $MgADP^-$ (and vice versa) (Lee and O'Sullivan, 1975). If this situation pertains to the enzyme from other sources, it could explain the difficulty in observing higher order complexes in x-ray diffraction studies (Blake and Evans, 1974). Tanswell et al. (1976) obtained evidence from NMR data for an abortive quaternary complex. However, the NMR data indicated that binding of 3-PGA had very little influence on the nucleotide binding site, a result consistent with the small influence of 3-PGA binding on the PRR enhancement and EPR spectrum for the enzyme-MnADP complex.

The PRR studies at various temperatures and frequencies provide supporting evidence for the formation of a quaternary complex. As observed for similar complexes with other kinases, the electron spin relaxation time makes a significant contribution to the overall correlation time. There are considerable differences in data for the ternary and quaternary mixtures of substrates again inferring the existence of a viable quaternary complex. Unfortunately, the field dependence of the electron spin relaxation rate precluded a more quantitative treatment of the PRR data in terms of the number of exchangeable water molecules in the first coordination sphere of the manganous ion.

The apparent discrepancy with regard to quaternary complexes which have not been detected in the x-ray diffraction studies (Blake and Evans, 1974; Wendell et al., 1972) can largely be attributed to differences in the experimental conditions. In particular, the presence of approximately 2 M sulfate ion in the solutions used for the x-ray work may weaken binding of 3-PGA.

Acknowledgments

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Galactose-1-phosphate Uridylyltransferase: Isolation and Properties of a Uridylyl-Enzyme Intermediate†

Lee-Jun Wong, Kwan-Fu Rex Sheu, Sue-Lein Lee, and Perry A. Frey*

ABSTRACT: Galactose-1-P uridylyltransferase catalyzes the interconversion of UDP-glucose and galactose-1-P with UDP-galactose and glucose-1-P by a double displacement pathway involving a uridylyl-enzyme intermediate. The amount of radioactivity incorporated into the protein by uracil-labeled UDP-glucose is decreased by the presence of UDP-galactose, which competes with UDP-glucose for uridylylating the enzyme. The amount of glucose-1-P released upon reaction of the enzyme with UDP-glucose indicates that the dimeric enzyme contains more than one active site per molecule, 1.7 on the average for the most active preparation obtained. This suggests that there is one uridylylation site per subunit and that the subunits are similar or identical. The uridylyl-enzyme is stable to mild alkaline conditions, 0.10 M NaOH at 60 °C for 1 h, but it is very sensitive to acid, being largely hydrolyzed after 12 h at pH 3.5 and 4 °C. The principal radioactive product resulting from hydrolysis of [*uracil*-2-^{14}C]uridylyl-enzyme under the latter conditions is [^{14}C]UMP. The hydrolytic properties of the uridylyl-enzyme show that the uridylyl moiety is bonded to the protein through a phosphoramidate linkage. Complementary studies on the effects of group selective reagents on the activity of the enzyme suggest that the active site nucleophile to which the uridylyl group is bonded may be a histidine residue. The enzyme is rapidly inactivated by diethyl pyrocarbonate at pH 6 and 0 °C and reactivated by NH_2OH. UDP-glucose at 0.5 mM fully protects the enzyme against diethyl pyrocarbonate while 70 mM galactose-1-P has only a slight protective effect. Uridylyl-enzyme is inactivated by diethyl pyrocarbonate at no more than 2% of the rate for free enzyme. The enzyme is not inactivated by $NaBH_4$ or by $NaBH_4$ in the presence of UDP-glucose. It is not inhibited by 1 mM pyridoxal phosphate or by 0.5 mM 5-nitrosalicylaldehyde at pH 8.5 and it is not inactivated by $NaBH_4$ in the presence of pyridoxal phosphate. The enzyme is inactivated by 5 to 50 μM *p*-hydroxymercuribenzoate at pH 8.5, but substrates exert no detectable protective effect against this reagent. It is concluded that the enzyme contains at least one essential sulfhydryl group which is not located in the active site in such a way as to be shielded by substrates.

Galactose-1-P uridylyltransferase (EC 2.7.7.12) catalyzes the interconversion of UDP-glucose and galactose-1-P with UDP-galactose and glucose-1-P, the second step in the Leloir pathway for converting galactose to glucose-6-P. The first step is the phosphorylation of galactose by ATP catalyzed by galactokinase and the third is the interconversion of UDP-galactose and UDP-glucose catalyzed by UDP-galactose 4-epimerase. Glucose-6-P is produced from glucose-1-P by the action of phosphoglucomutase.

Galactose-1-P uridylyltransferase activity is found in microorganisms, plants, and animals (Kalckar et al., 1953; Maxwell et al., 1955; Kurahashi, 1957; Pazur and Shadaksharaswamy, 1961). A defect in this enzyme is associated with galactosemia (Kalckar, 1960), an inherited disease of humans in which galactose cannot be metabolized to glucose at sufficient rates to prevent the accumulation of galactose and derived metabolities to toxic levels.

The close structural similarities between the uridylyl donors, UDP-glucose and UDP-galactose, and between the acceptors, glucose-1-P and galactose-1-P, as well as the chemical nature of the reaction suggested to us that it would be efficient from the standpoints of both mechanism and structural evolution for the active site to consist essentially of a UDP-hexose binding site capable of binding either uridylyl donor, such that the reaction mechanism could follow a double-displacement pathway involving a uridylyl-enzyme intermediate and ping-pong kinetics. This is a highly restrictive concept for the action of this enzyme, in that it denies the existence of productive ternary complexes and it demands that the uridylyl moiety undergoing transfer be stabilized in some way, probably by covalent bond formation to a nucleophile. This mechanism is outlined in eq 1 and 2, in which –XH symbolizes the functional group and the Michaelis complexes are omitted.

$$\text{UDP-glucose} + \text{E–XH} \rightleftharpoons \text{uridylyl–X–E} + \text{glucose-1-P} \quad (1)$$

$$\text{galactose-1-P} + \text{uridylyl–X–E} \rightleftharpoons \text{E–XH} + \text{UDP-galactose} \quad (2)$$

† From the Department of Chemistry, The Ohio State University, Columbus, Ohio 43210. *Received August 30, 1976.* This work was supported by Grant No. GB-42030 from the National Science Foundation.