Coordination Scheme and Stereochemical Configuration of Manganese(II) Adenosine 5′-Diphosphate at the Active Site of 3-Phosphoglycerate Kinase†

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ABSTRACT: The structure of the $Mn^{II}ADP$ complex at the active site of 3-phosphoglycerate kinase from yeast has been investigated by electron paramagnetic resonance (EPR) spectroscopy. Inhomogeneous broadening in the EPR signals for Mn(II) resulting from unresolved superhyperfine coupling to ^{17}O regiospecifically incorporated into ADP shows that Mn(II) is coordinated to the α- and β-phosphate groups of ADP at the active site of the enzyme. The EPR pattern for the enzyme–$Mn^{II}ADP$ complex is characteristic of a predominantly axially symmetric zero-field splitting tensor. The symmetry and magnitude of the zero-field splitting interaction suggest that there is an additional negatively charged oxygen ligand in the coordination sphere of Mn(II). EPR measurements for solutions of the enzyme–$Mn^{II}ADP$ complex in ^{17}O-enriched water indicate that there are also two or three water molecules in the coordination sphere of the metal ion. EPR data for complexes with the two epimers of [α-^{17}O]ADP have been used to determine the stereochemical configuration of the $Mn^{II}ADP$ complex at the active site. EPR spectra for Mn(II) in the enzymic complex with (R_P)-[α-^{17}O]ADP show an inhomogeneous broadening due to superhyperfine coupling with ^{17}O whereas spectra for (S_P)-[α-^{17}O]ADP complexes are indistinguishable from those for matched samples with unlabeled ADP. These results show that 3-phosphoglycerate kinase selectivity binds the Λ configuration of the α,β chelate of $Mn^{II}ADP$. Addition of 3-phosphoglycerate to form the dead-end complex (enzyme–$Mn^{II}ADP$–3-phosphoglycerate) does not alter the EPR spectrum, but addition of vanadate to this complex causes marked changes in the spectral parameters. Experiments with the ^{17}O-labeled forms of ADP show, however, that ADP remains bound as an α,β-bidentate ligand to Mn(II) in the dead-end complex with vanadate.

The enzyme 3-phosphoglycerate kinase (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) catalyzes the transfer of a phospho group from 1,3-diphosphoglycerate to ADP, in the first ATP-producing reaction of glycolysis. Enzymes isolated from a wide variety of sources are all monomers of molecular weight approximately 45 000 (Scopes, 1973). Detailed structural information has been obtained from X-ray diffraction data for single crystals of the enzyme from horse muscle (Banks et al., 1979) and from yeast (Watson et al., 1982). The polypeptide chain folds into two domains, and the catalytic site involves residues from each region (Watson et al., 1982; Banks et al., 1979). The two domains apparently come together to enclose the substrates during catalysis in a "hinge-bending" mechanism. Hydrodynamic data (Roustan et al., 1980) support the existence of two distinct conformational states of the protein that are interconverted by appropriate combinations of substrates or sulfate ions. Substrate-induced changes in the radius of gyration of the protein have also been detected by low-angle X-ray scattering experiments (Pickover et al., 1979).

Crystals of the enzyme will take up metal–nucleotide complexes when the crystals are soaked with the appropriate solution. X-ray diffraction results from such crystals of the enzyme from horse muscle (Banks et al., 1979) with Mn(II) or Mg(II) complexes of ADP show that Mn(II) is bound to the α- and β-phosphates of ADP, as well as to an enzyme carboxylate group (Asp-374). In the corresponding complex with ATP, the metal ion is positioned near the γ-phosphate group and is not within bonding distances to the α- and β-phosphate groups. Similar structural features are reported from studies of the crystalline enzyme from yeast, including coordination of the metal ion to the carboxylate group from Asp-372 (Watson et al., 1982).

Interest in the role of metal ions in the activation of enzyme-catalyzed phospho-transfer reactions has stimulated a number of investigations of metal ion–nucleotide interactions at the active site of 3-phosphoglycerate kinase. Chemical methods used to probe metal ion coordination include inhibition studies with exchange-inert complexes of $Cr^{III}ATP$ (Dunaway-Mariano & Cleland, 1980), as well as the metal ion dependent stereospecificity of the enzyme for phosphorothioate analogues of ADP and ATP (Jaffe et al., 1982). Magnetic resonance methods have also been used to investigate enzyme–substrate complexes of 3-phosphoglycerate kinase (Tanswell et al., 1976; Chapman et al., 1977; Nageswara Rao et al., 1978).

The present paper reports results from EPR[1] investigations of the $Mn^{II}ADP$ complexes with 3-phosphoglycerate kinase. Inhomogeneous broadening of the EPR signals due to unresolved superhyperfine coupling to ^{17}O from regiospecifically labeled forms of ADP has been used to determine the coordination scheme of Mn(II) to the nucleotide phosphate groups in the enzyme–$Mn^{II}ADP$ ternary complex. In addition, ^{17}O effects from the stereospecifically labeled nucleotide substrates, (R_P)-[α-^{17}O]ADP and (S_P)-[α-^{17}O]ADP were used to deter-

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[1] Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; ADPαS, adenosine 5′-*O*-(1-thiodiphosphate); ATPβS, adenosine 5′-*O*-(2-thiotriphosphate); EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; *D*, axial zero-field splitting parameter; *E*, rhombic zero-field splitting parameter.

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mine the stereochemical configuration of $Mn^{II}ADP$ at the active site of the enzyme. Binding of vanadate to the enzyme–$Mn^{II}ADP$–3-phosphoglycerate complex has also been investigated by EPR and NMR methods.

Experimental Procedures

Materials. 3-Phosphoglycerate kinase was isolated from bakers' yeast according to the affinity elution method (Fifis & Scopes, 1978). The enzyme was further purified by crystallization according to the method of Scopes (1971). Crystals from these preparations were dissolved in and dialyzed against 50 mM Hepes/KOH, pH 7.5, and the solution was concentrated with a collodion bag apparatus. Specific activities of the preparations were approximately 650 IU/mg in the coupled assay with glyceraldehyde-3-phosphate dehydrogenase (Bucher, 1955) at 21 °C. EPR spectra for $Mn^{II}ADP$ complexes with enzyme prepared in this way were stable for at least 1 h, and no complications[2] from adenylate kinase activity were detected, even at very high concentrations of protein. The possibility that the enzyme, as isolated, contained tightly bound 3-phosphoglycerate was also investigated. Some preparations were dialyzed against a solution of 0.5 M sodium sulfate as described by Conroy et al. (1981), followed by dialysis against 50 mM Hepes/KOH, pH 7.5. EPR spectra for samples of enzyme subjected to these extra steps were identical with those for samples that did not undergo treatment with sodium sulfate. Moreover, the specific influences of vanadate on the dead-end complex, enzyme–$Mn^{II}ADP$–3-phosphoglycerate, were not present upon addition of vanadate to solutions of the enzyme–$Mn^{II}ADP$ complex without exogenous 3-phosphoglycerate (see Results).

3-Phosphoglyceric acid (free acid and tricyclohexylammonium salt) and unlabeled nucleotides were from Sigma Chemical Co. Glyceraldehyde-3-phosphate dehydrogenase was obtained from Boehringer-Mannheim. Unlabeled nucleotides were purified by chromatography on DEAE-Sephadex (A-25) and Chelex 100 as described elsewhere (Reed & Leyh, 1980). [α,α-$^{17}O_2,\alpha\beta$-^{17}O]ADP, [β,β,β-$^{17}O_3$]ADP, (R_P)-[α-^{17}O]ADP, and (S_P)-[α-^{17}O]ADP were prepared and purified as described previously (Reed & Leyh, 1980; Leyh et al., 1982). The chirally labeled samples were generously provided by Dr. Perry A. Frey, University of Wisconsin. The chemical purity of the nucleotides was assessed by high-performance liquid chromatography with a Whatman Partisil-10 SAX ion-exchange column eluted with 0.5 M ammonium phosphate, pH 4.3. Regio- and stereospecific ^{17}O enrichment of the labeled nucleotides was determined by ^{31}P NMR (Tsai, 1979).

Magnetic Resonance Measurements. EPR spectra were recorded at 35 GHz with a hybrid Varian spectrometer (Leyh et al., 1982). Concentration-matched samples for EPR measurements were prepared from a common stock solution. At the concentration of Mn(II) used, spectral amplitudes for separate samples gave a precision of ±2%. Longitudinal relaxation times, T_1's, of water protons were measured at 24 MHz by the 180°–τ–90° pulse sequence with a custom spectrometer as described previously (Reed & Leyh, 1980). The enhancement is defined as the ratio of the paramagnetic contribution to water proton relaxation rates in the presence of Mn(II) and complexing agents (enzyme and nucleotide) to those for $MnCl_2$ in buffer (Mildvan & Cohn, 1970). ^{31}P NMR spectra were obtained at 145 MHz with a Bruker WH-360 spectrometer.

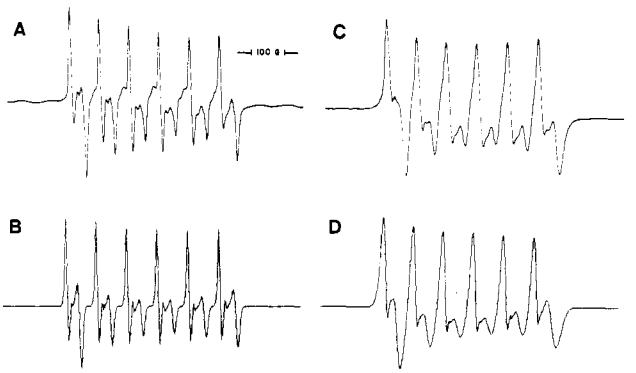

FIGURE 1: EPR spectra at 35 GHz of the creatine kinase–$Mn^{II}ADP$–formate–creatine (A and B) and the 3-phosphoglycerate kinase–$Mn^{II}ADP$ (C and D) complexes. (A) The solution contained 1.8 mM $Mn(OAc)_2$, 73 mM sodium formate, 3.9 mM ADP, saturating creatine, 4 mM enzyme active sites, and 50 mM Hepes/KOH, pH 8.0. [The broad signals on the high- and low-field side of the spectrum are from the $+^3/_2 \leftrightarrow +^1/_2$ fine structure transition.] (B) Simulation of the spectrum in (A) with D = 320 G, E/D = 0.05, a line width of 3 G, and a ^{55}Mn hyperfine coupling constant of 90 G. For this spectrum, a Gaussian distribution of D with a half-width of 15 G and nine values of D were used. (C) The solution contained 1.7 mM $MnCl_2$, 3.9 mM enzyme, 6 mM ADP, and 50 mM Hepes/KOH, pH 7.5. (D) Simulation of the spectrum in (C) with D = 355 G, E/D = 0.1, a line width of 7 G, and a ^{55}Mn hyperfine coupling constant of 90 G. For this spectrum, a distribution of D with a half-width of 40 G and four values of D were used. Spectra in (A) and (C) were recorded in the liquid phase at −5 °C.

Analysis of EPR Spectra. EPR spectra at 9.1 GHz for Mn(II)–nucleotide complexes with 3-phosphoglycerate kinase have been reported by Chapman et al. (1977). These low-frequency spectra do not have sufficient resolution to permit observation of perturbations due to unresolved superhyperfine coupling to ^{17}O or to allow an accurate estimate of the zero-field splitting parameters. There is a marked improvement in spectral resolution in spectra obtained at 35 GHz (see Figure 1). The dominant signals in the spectrum are from the $M_s = -^1/_2 \leftrightarrow {}^1/_2$ fine structure transition of Mn(II). This transition is split into a sextet pattern through hyperfine coupling to the ^{55}Mn nuclear spin ($I = {}^5/_2$). Each of the ^{55}Mn hyperfine components shows additional structure that is due to second-order effects from the zero-field splitting interaction [see Reed & Markham (1984)]. The zero-field splitting parameters were determined by simulation of the spectra as described by Reed & Markham (1984) with inclusion of the orientation and ^{55}Mn nuclear-spin dependence of the transition probabilities (Allen, 1965; Eidels-Dubovi & Beltran-Lopez, 1978).

The extent of perturbations in EPR signals due to unresolved superhyperfine coupling depends on the width of the superhyperfine splitting pattern relative to the natural line width of the signals (Norris et al., 1971). Even though the overall line widths of the EPR signals for the complexes with 3-phosphoglycerate kinase are relatively broad, the degree of resolution of the second-order fine structure in the signals provides a sensitive measure of ^{17}O-induced inhomogeneous broadening.

Results

3-Phosphoglycerate Kinase–$Mn^{II}ADP$ Ternary Complex. EPR spectra for the $Mn^{II}ADP$ complex of 3-phosphoglycerate kinase and the complex of creatine kinase with $Mn^{II}ADP$, creatine, and formate are compared in Figure 1. The major difference in the spectral patterns for the two complexes is the line width. Both spectra exhibit a variation in the apparent

[2] The enzyme–$Mn^{II}ADP$ and enzyme–$Mn^{II}ATP$ complexes give different EPR spectra. Trace contamination of the enzyme by adenylate kinase would be revealed in a time-dependent conversion of the EPR spectrum for enzyme–$Mn^{II}ADP$ to the spectrum characteristic of enzyme–$Mn^{II}ATP$.

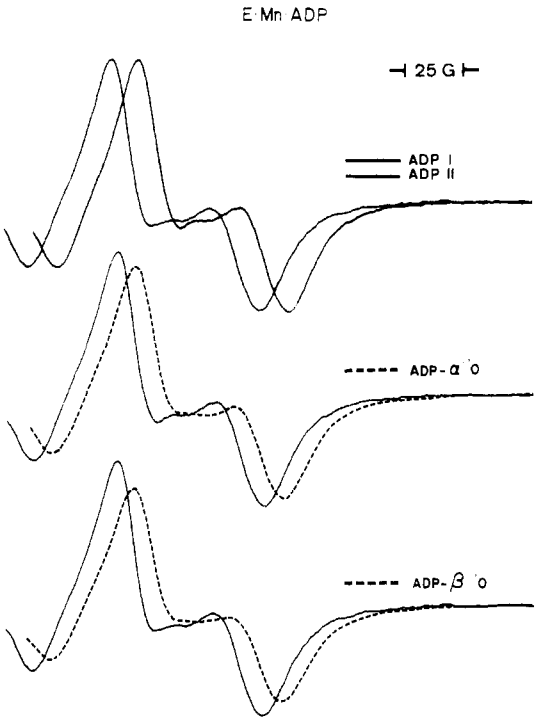

FIGURE 2: EPR spectra at 35 GHz of the 3-phosphoglycerate kinase–Mn^{II}ADP complex for concentration-matched samples containing unlabeled ADP, [α,α-$^{17}O_2$,$\alpha\beta$-^{17}O]ADP, and [β,β,β-$^{17}O_3$]ADP. The top spectra were recorded for two separate samples with unlabeled ADP. The solutions contained 3.9 mM enzyme, 1.7 mM $MnCl_2$, 5.6 mM nucleotide, and 50 mM Hepes/KOH, pH 7.5. Spectra were recorded in the liquid phase at −5 °C. The region of the spectrum shown represents the highest field ^{55}Mn hyperfine transition. Isotopic enrichment of [α,α-$^{17}O_2$,$\alpha\beta$-^{17}O]ADP was 40 ± 4% and of [β,β,β-$^{17}O_3$]ADP was 44 ± 4% in each labeled position.

amplitudes of the ^{55}Mn hyperfine transitions. Allen (1965) pointed out that this variation is due to the influence of the zero-field splitting on the relative transition probabilities. Spectral simulations with expressions that include the corrected transition probabilities provide a reasonably accurate representation of the experimental line shapes (see Figure 1B,D).

The influence of sulfate on EPR spectra for solutions of the enzyme–Mn^{II}ADP complex was examined since sulfate has been reported to effect the activity (Scopes, 1978) and conformational state (Roustan et al., 1980) of the enzyme. However, concentrations of Na_2SO_4 up to 50 mM did not influence the EPR spectra. [The apparent dissociation constant for sulfate at its high-affinity site on the enzyme is between 1 and 4 mM (Roustan et al., 1980; Tanswell et al., 1976).] EPR spectra for the enzymic complexes were also obtained in Tris-HCl and in triethanolamine hydrochloride buffers to eliminate potential complications due to the presence of a sulfonic acid group in the buffer (Hepes). Spectra were identical in all three buffer systems.

Representative EPR spectra obtained for matched samples of the enzyme–Mn^{II}ADP complex[3] with unlabeled ADP and with ADP labeled regiospecifically with ^{17}O at the α-phosphate or at the β-phosphate are shown in Figure 2. Resolution of the second-order splittings in EPR signals for the ^{17}O-labeled samples is reproducibly diminished relative to spectra for

[3] The binding data of Chapman et al. (1974) show that an excess of ADP over Mn(II) promotes formation of the ternary complex, enzyme–Mn^{II}ADP, and that with a sufficient excess of ADP the amount of ternary complex is insensitive to variations in the concentration of free ADP. It is therefore straightforward to select experimental conditions that ensure virtually stoichiometric binding of Mn(II) in the ternary complex.

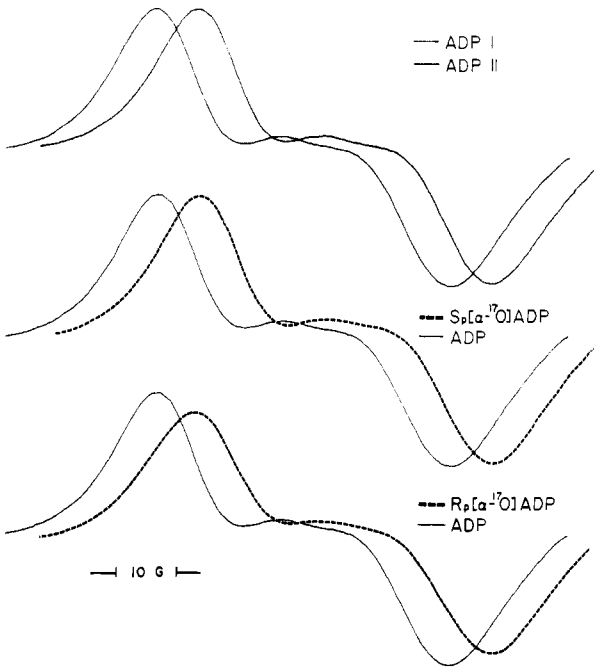

FIGURE 3: EPR spectra at 35 GHz of the 3-phosphoglycerate kinase–Mn^{II}ADP complex for concentration-matched samples containing unlabeled ADP, (R_P)-[α-^{17}O]ADP, and (S_P)-[α-^{17}O]ADP. Solution compositions were as described in Figure 2, with the exception that the nucleotide concentration was 4.9 mM. Spectra were recorded in the liquid phase at −5 °C. The region of each spectrum shown represents the lowest field ^{55}Mn hyperfine transition. Isotopic enrichment of the two epimers of [α-^{17}O]ADP was 45 ± 5%.

unlabeled ADP. These observations show that Mn(II) is coordinated to an oxygen from the α-phosphate and from the β-phosphate of ADP.

EPR spectra for the enzyme–Mn^{II}ADP ternary complex with unlabeled ADP and with the R_P and S_P epimers of [α-^{17}O]ADP are shown in Figure 3. Signals for the sample with (R_P)-[α-^{17}O]ADP show inhomogeneous broadening relative to the signals obtained for concentration-matched samples either with unlabeled ADP or with (S_P)-[α-^{17}O]ADP. Coordination of Mn(II) to the *pro-R* oxygen at the α-phosphate of ADP gives rise to the Λ configuration of the resulting α,β-chelate ring of the Mn^{II}ADP complex at the active site of the enzyme. Amplitudes for spectra of the complex with the R_P epimer were reproducibly diminished by 10% relative to those for samples with ADP. The precision in the measurement of amplitudes for theses samples is 2%. Amplitudes of signals for the S_P epimer were equal to those for unlabeled ADP within the limit of precision, and these observations establish a lower limit of 5:1 for the screw-sense selectivity of the enzyme for the α,β-chelate ring of the metal–ADP complex.

EPR spectra for samples of the enzyme–Mn^{II}ADP complex in normal water and in water enriched to 40% in $H_2{}^{17}O$ are shown in Figure 4. The EPR spectrum for the sample in ^{17}O-enriched water is the sum of spectra for complexes with various combinations of $H_2{}^{16}O$ and $H_2{}^{17}O$ ligands, with statistical weights dependent on the number of water ligands in the complex and on the ^{17}O enrichment of the water. Complexes that contain only $H_2{}^{16}O$ ligands have a line shape that is identical with that for the control sample in unenriched water, and the fractional contribution of this complex may be determined by the deconvolution procedure described previously (Reed & Leyh, 1980; Webb et al., 1982). Such a deconvolution (see Figure 4) shows that there are at least two water molecules in the coordination sphere of Mn(II). The

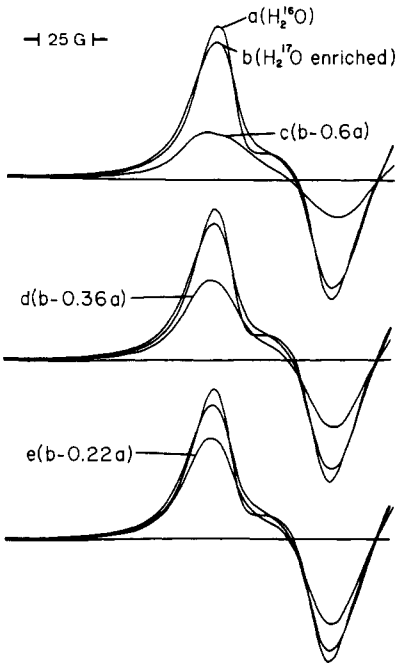

FIGURE 4: Deconvolution of EPR spectra at 35 GHz of 3-phosphoglycerate kinase–Mn^{II}ADP complexes in ^{17}O-enriched water. The lowest field ^{55}Mn hyperfine component is shown. The ^{17}O enrichment in the water was 40%. (a) Spectrum in normal water; (b) spectrum in ^{17}O-enriched water; (c–e) difference spectra.

EPR signals are, however, too broad to permit a definitive distinction between two and three water ligands.

3-Phosphoglycerate Kinase–Mn^{II}ADP–3-Phosphoglycerate Dead-End Complex: Effects of Vanadate. Chapman et al. (1977) reported that addition of 3-phosphoglycerate to solutions of the enzyme–Mn^{II}ADP ternary complex did not alter the 9.1-GHz EPR spectrum for the complex. There is no noticeable change in the spectrum (not shown) for this complex at 35 GHz with an excess (30 mM) of 3-phosphoglycerate present. Moreover, 50 mM Na_2SO_4 did not alter the spectrum, although sulfate does markedly enhance protection of a glutamyl residue from chemical modification with this dead-end combination of substrates (Roustan et al., 1980). The absence of any measurable spectral response upon binding of 3-phosphoglycerate to form the dead-end complex indicates that the missing phospho group may be essential in bringing about "hinge bending" for enclosure of the active site. The hydrodynamic measurements on the dead-end combination of substrates support this concept (Roustan et al., 1980). Experiments with anions, other than sulfate, were initiated in an attempt to find a substitute for the missing phospho group in the dead-end complex.

Vanadate is an inhibitor of several enzymes that catalyze phospho-transfer reactions (Simons, 1979). The inhibitory action of vanadate is attributed to the ability of pentavalent vanadium to adopt a stable trigonal-bipyramidal geometry that is analogous to the transition state in phospho-transfer reactions (Macara, 1980). Climent et al. (1981) have shown that vanadate inhibits the reaction catalyzed by 3-phosphoglycerate kinase. In the absence of 3-phosphoglycerate, 5 mM vanadate does not influence the EPR pattern for the enzyme–Mn^{II}ADP complex. However, with 3-phosphoglycerate present vanadate changes the EPR spectrum for the bound Mn(II) such that the zero-field splitting tensor is decidedly rhombic (Figure 5A). Binding of vanadate to the dead-end mixture of substrates also influences the magnitude of the enhancement in the water proton relaxation rate for the enzyme-bound Mn(II) (Figure 5B). The change in relaxation enhancement may be used in

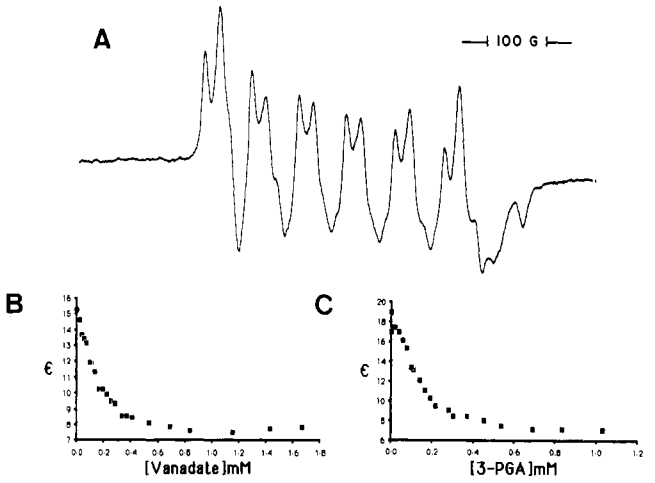

FIGURE 5: (A) EPR spectrum at 35 GHz of the 3-phosphoglycerate kinase–Mn^{II}ADP–3-phosphoglycerate–vanadate complex. The solution contained 2 mM enzyme, 0.9 mM $MnCl_2$, 2.8 mM ADP, 10 mM 3-phosphoglycerate, 5 mM vanadate, and 50 mM Hepes/KOH, pH 7.5. The spectrum was recorded in the liquid phase at −3.5 °C. (B) Titration of the 3-phosphoglycerate–Mn^{II}ADP–3-phosphoglycerate complex with vanadate. Enhancement of the water proton relaxation rate was followed as a function of vanadate concentration. The solution contained 0.44 mM enzyme, 0.1 mM $MnCl_2$, 0.4 mM ADP, 0.5 mM 3-phosphoglycerate, and 50 mM Hepes/KOH, pH 8.2. (C) Titration of the 3-phosphoglycerate kinase–Mn^{II}ADP–vanadate complex with 3-phosphoglycerate. Enhancement of the water proton relaxation rate was followed as a function of 3-phosphoglycerate concentration. The solution contained 0.44 mM enzyme, 0.1 mM $MnCl_2$, 0.4 mM ADP, 1.0 mM vanadate, and 50 mM Hepes/KOH, pH 8.2. Titrations were carried out at 22 °C.

titrations to determine the affinities both of vanadate[4] for the enzyme–Mn^{II}ADP–3-phosphoglycerate complex and of 3-phosphoglycerate for the enzyme–Mn^{II}ADP–vanadate complex (Figure 5B,C). Dissociation constants for vanadate and for 3-phosphoglycerate, estimated from the midpoints of the titration curves, are both 100 ± 10 μM.

EPR spectra for the dead-end complex with vanadate present exhibit inhomogeneous broadening both from α- and from β-^{17}O-labeled forms of ADP (data not shown). These observations demonstrate that Mn(II) remains bound to both phosphate groups of ADP in this complex. Although it is likely that changes in the EPR spectra for Mn(II) upon binding of vanadate are due to direct coordination between Mn(II) and an oxygen of vanadate, it is not possible to test this hypothesis by EPR experiments with ^{17}O-labeled vanadate because vanadate oxygens equilibrate rapidly with the oxygens of water (Murmann, 1976).

3-Phosphoglycerate Kinase Complexes with ATP. In contrast to the EPR results obtained for the enzyme–Mn^{II}ADP complex, spectra for the enzyme–Mn^{II}ATP complex do not exhibit fine structure splitting (data not shown). The absence of sharp features in the spectrum for enzyme–Mn^{II}ATP precludes straightforward investigations with ^{17}O-labeled forms of ATP.

Discussion

EPR data for the enzyme–Mn^{II}ADP complex with ^{17}O-labeled forms of the nucleotide show that Mn(II) is coordinated to the β-phosphate and to the *pro-R* oxygen at the α-phosphate of ADP to give an α,β-bidentate complex with the Λ configuration. Coordination of Mn(II) to a carboxylate ligand from a residue of the protein is probable[5] because the magnitude

[4] The ionization state and molecular form of vanadate that is responsible for the effects has not been established.

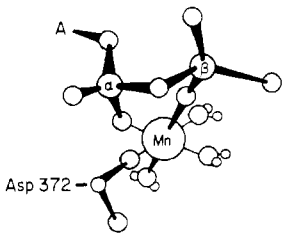

FIGURE 6: Schematic representation of the configuration of the bound $Mn^{II}ADP$ complex at the active site of 3-phosphoglycerate kinase.

and symmetry of the zero-field splitting tensor in the $Mn^{II}ADP$ complex with 3-phosphoglycerate kinase are similar to those for Mn(II) in the formate-stabilized dead-end complex of creatine kinase. In the complex with creatine kinase, a carboxylate oxygen from formate, oxygens from the α- and β-phosphate groups of ADP, and three water molecules are present in the coordination sphere of the metal ion (Reed & Leyh, 1980). Although EPR spectra for the $Mn^{II}ADP$ complex with 3-phosphoglycerate kinase are too broad to permit one to obtain an exact hydration number for Mn(II) in the complex, deconvolution of spectra obtained in solutions enriched in $H_2{}^{17}O$ shows that there are at least two water molecules present as ligands. The predominantly axial zero-field splitting tensor for the complex is compatible with a structure in which a trifacial arrangement of negatively charged oxygen and neutral oxygen ligands produces an effective 3-fold axis of electronic symmetry about the metal ion (Figure 6). The frequency-dependent correlation time for water proton relaxation in the presence of the enzyme–$Mn^{II}ADP$ complex precluded a straightforward evaluation of the number of rapidly exchanging water ligands in previous NMR studies (Chapman et al., 1977).

The shift from a predominantly axial to a rhombic zero-field splitting tensor for Mn(II) in the dead-end complex upon binding of vanadate indicates that there are changes in the coordination sphere of Mn(II). However, ADP remains bound to Mn(II) as an α,β-bidentate chelate in this complex so any ligand exchanges must involve the carboxylate or water ligands that are present in the ternary complex. EPR and NMR observations on the complex with vanadate are consistent with the notion that the enzyme adopts the closed conformation in this inhibitory complex, although the exact mode of binding for this anion has not been established.

Several other investigations have provided information on metal ion–nucleotide coordination at the active site of this enzyme. Kinetic assays with phosphorothioate analogues of ATP and ADP revealed that the enzyme exhibits stereoselectivity for the S_P epimer of ATPαS with Mg(II) as activator as well as a reversal[6] of selectivity (in terms of V_{max}) with the softer metal ions, Co(II) and Zn(II), as activators (Jaffe et al., 1982). The reversal in V_{max} for the epimers with Zn(II) vs. Mg(II) suggested that the metal ion is coordinated to the α-phosphate of the nucleotide at a rate-limiting step in the reaction and that the enzyme prefers the Λ configuration of the α,β-chelate ring. In experiments with ATPβS, a selectivity for the S_P epimer was observed, but the selectivity was not reversed upon switching from hard to soft metal ions (Jaffe et al., 1982). The absence of a metal ion specific reversal or relaxation of stereoselectivity at the β-phosphate group of ATPβS left some ambiguity with regard to the status of metal ion coordination to the β-phosphate of the nucleotide. Thus, Jaffe et al. (1982) pointed out that such a result could be explained by a lack of metal ion coordination to the β-phosphate or by coordination of hard and of soft metal ions to the same substituent, oxygen or sulfur, of (S_P)-ATPβS. Although the EPR data presented here show that the β-phosphate of ADP is coordinated to the metal ion in the ternary complex, there is a possibility that this coordinate interaction does not occur at a rate-limiting step of the reaction.

Dunaway-Mariano & Cleland (1980) found that the mixture of isomers of β,γ-bidentate $Cr^{III}ATP$ inhibited the enzyme competively with respect to $Mg^{II}ATP$ (K_{is} = 63 μM), but no turnover was observed for the Cr(III) complexes in an assay based on the circular dichroism spectra of the complexes. The potent inhibition by bidentate $Cr^{III}ATP$ indicates that a metal–nucleotide complex can bind with high affinity without the aid of coordination to Asp-372. Jaffe et al. (1982) used a sensitive assay with ^{14}C-labeled 3-phosphoglycerate and resolved isomers of β,γ-bidentate $Cr^{III}ATP$ but found no activity for either complex. These exchange-inert complexes of Cr(III) would resist the insertion of a carboxylate ligand from the protein. Moreover, β,γ-bidentate $Cr^{III}ATP$ does not possess α-phosphate coordination, the importance of which was revealed in the studies with ATPαS (Jaffe et al., 1982). Either or both of these coordinate interactions may be essential for catalysis.

The EPR spectrum for the enzyme–$Mn^{II}ATP$ complex differs from that of the corresponding complex of ADP (Chapman et al., 1977), and this observation indicates that there are differences in the coordination sphere of Mn(II) in the two complexes. Signals in the EPR spectrum for the complex with ATP are, however, too broad to permit detailed investigation with ^{17}O-labeled forms of ATP. EPR spectra for Mn(II) complexes with the fully active equilibrium mixture of substrates are, however, suitable for elaboration of the coordination scheme for Mn(II) in the central complex with ATP and 3-phosphoglycerate (J. M. Moore and G. H. Reed, unpublished observations). Results of these investigations will be reported subsequently.

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[5] The zero-field splitting parameters alone do not prove that a carboxylate group is bound to Mn(II) in the complex.

[6] The reversal with Zn(II) and with Co(II) is in V_{max} but not in V_{max}/K_m. Pecoraro et al. (1984) have suggested that the correct kinetic parameter to compare in such studies is V_{max}/k_m. In this respect, the observations of Jaffe et al. (1982) indicate a relaxation of selectivity rather than a reversal.

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Comparison of the Active Sites of Atropinesterase and Some Serine Proteases by Spin-Labeling

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ABSTRACT: The side chain of the serine residue in the active center of atropinesterase (AtrE), α-chymotrypsin (Chymo), and subtilisin A (Sub) was labeled with two paramagnetic reporter groups of different size (label I or II, respectively) by sulfonylation with *N*-[3-(fluorosulfonyl)phenyl]-1-oxy-2,2,5,5-tetramethylpyrroline-3-carboxamide or *N*-[6-(fluorosulfonyl)-2-naphthyl]-1-oxy-2,2,5,5-tetramethylpyrroline-3-carboxamide. ESR spectra of labeled enzymes in 10 mM phosphate buffer, pH 7.4, were measured at temperatures between 133 and 298 K by using a home-built spectrometer operating in the absorption mode at 10-kHz field modulation. The spectra, in particular those at 276–298 K, were analyzed by computer simulation of the overall line shape according to the methods developed by Freed and co-workers, based on eigenfunction expansion. In the case of AtrE for both labels, the best agreement between experimental and simulated solution spectra was obtained with only one mobility component showing anisotropic, axially symmetric reorientation according to the Egelstaff jump–diffusion model. The axis of preferential reorientation was found to lie in the XZ plane at a polar angle of about 30° with the X axis. The corresponding rotational correlation time ($\tau_{\parallel}$) did not show appreciable viscosity/temperature (η/T) dependence but had a constant value of 4.4 and 2.2 ns for labels I and II, respectively. The rotational correlation time associated with rotation around the axes perpendicular to that of preferential reorientation ($\tau_{\perp}$) showed the usual η/T dependence and had a value of 22.0 ns at 276 K for both labels. The above results strongly suggest that in AtrE both nonpolar reporter groups reside in a pocket near the active serine. Contrary to the situation in AtrE, the overall mobility of the –N–O· fragments in Chymo and Sub was found to result from contributions of at least two distinct motional states, strongly and weakly immobilized. In going from label I to label II, the relative contribution of the latter state increases at the expense of that of the former. This is ascribed to an equilibrium between a relatively free state of the aromatic cores and a firmly bound position in the specificity pocket of these proteases. The apparently more rigid embedding of the spin-labels in the enzyme structure of AtrE suggests that the size of the nonpolar binding pocket in the active center region of this esterase allows a deeper penetration of the aromatic portions of the labels than is possible for the specificity pocket of Chymo or Sub.

Stable nitroxide radicals [cf. Rozantsev (1970)] have been widely applied as spin-labels in enzymology (Berliner, 1974, 1978; Morrisett, 1976) and in biomedical research (Chignell, 1979; Piette & Hsia, 1979) because they have proven to be useful reporter groups for probing the structure of specific sites in a biomolecular environment. Their ESR[1] spectra reflect both the effect of interactions between label and biomolecule

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[1] Abbreviations: AtrE, atropinesterase; Chymo, α-chymotrypsin; Sub, subtilisin A; DFP, diisopropyl phosphorofluoridate; ESR, electron spin resonance.

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