Identification of the Six Ligands to Manganese(II) in Transition-State-Analogue Complexes of Creatine Kinase: Oxygen-17 Superhyperfine Coupling from Selectively Labeled Ligands†

George H. Reed* and Thomas S. Leyh

ABSTRACT: The complete coordination scheme for Mn(II) in transition-state-analogue complexes with creatine kinase has been determined by electron paramagnetic resonance (EPR) spectroscopy. Perturbations in the EPR spectra for Mn(II) due to superhyperfine coupling to ^{17}O of selectively labeled ligands have been used to identify oxygen ligands in the first coordination sphere of the metal ion. The results show that in the complex of enzyme·MnADP·formate·creatine, Mn(II) is bound to oxygen ligands from both the α- and β-phosphate groups of ADP, to an oxygen from the carboxylate group of formate, and to three water molecules. In the complex with thiocyanate replacing formate as the stabilizing anion, previous infrared experiments [Reed, G. H., Barlow, C. H., & Burns, R. A., Jr. (1978) *J. Biol. Chem. 253*, 4153–4158] indicated that the nitrogen from thiocyanate was bound to the Mn(II). The magnitudes of the ^{17}O superhyperfine coupling constants from the O^- ligands of the ADP phosphate groups and from the formate carboxylate are approximately equal and are larger than that for the water ligands. The symmetry of the zero-field-splitting tensor for Mn(II) indicates that the oxygens from the α- and β-phosphate groups of ADP and the ligand donor atom from the anion occupy mutually cis positions in the octahedral coordination geometry. Water proton relaxation time measurements show that the three water molecules which are bound to Mn(II) are not in free exchange with the bulk solvent. Hence, an enclosed structure at the active site is indicated. The results suggest that for creatine kinase the activating metal ion is bound to all three phosphate groups in the transition state of the reaction.

There has been a continuing interest in the structures of metal ion–nucleotide complexes and in the specificity of various enzymes for particular structures of the complexes in binding and catalysis (Martin & Miriam, 1979; Eckstein, 1979). There have been several recent advances in methodology for probing the structure of metal–nucleotide complexes at the active sites of enzymes. Among these are methods based on (1) the exchange-inert Cr(III) and Co(III)$(NH_3)_4$ complexes of the nucleotides (Cleland & Mildvan, 1979; Danenberg & Cleland, 1975; Cornelius et al., 1977; Dunaway-Mariano et al., 1979), (2) soft and hard metal ion influences on the stereoselectivity of enzyme-catalyzed reactions of stereoisomers of nucleoside phosphorothioates (Jaffe & Cohn, 1979; Pillai et al., 1980), (3) ^{31}P NMR[1] studies of enzyme-bound nucleotides (Cohn & Nageswara Rao, 1979), and (4) X-ray diffraction studies of crystalline enzyme–metal–nucleotide complexes (Evans & Hudson, 1979; Banks et al., 1979). Each of these methods has contributed new insight into the structure of metal ion–nucleotide complexes at the active sites of the enzymes. One ambiguity which has arisen in comparison of the results for exchange-inert metal complexes and for substitution-labile metal complexes is the question of possible migration of the latter class of metal ions from one site to another along the phosphate chain prior to or following the phosphoryl transfer step (Dunaway-Mariano et al., 1979; Dunaway-Mariano & Cleland, 1980). Structural investigations of transition-state-analogue complexes hold promise for providing information which relates to the activated state of the complex (Wolfenden, 1972). In the present paper, ^{17}O superhyperfine coupling in EPR spectra for transition-state-analogue complexes of creatine kinase has been used to determine the composition of the first coordination sphere of the metal ion.

Superhyperfine coupling between the unpaired electron spins of a transition metal ion and magneticaly active nuclei of ligand atoms can provide an unambiguous indication of direct bonding (Goodman & Raynor, 1970). The scalar component of the superhyperfine interaction requires a through-bond transfer of spin density from orbitals of the metal ion to an s orbital of the ligand atom, and for nonconjugated ligands the magnitude of such spin transfer is severely attenuated by intervening bonds (Goodman & Raynor, 1970). Superhyperfine coupling from ^{17}O ($I = {}^5/_2$) has been used in EPR studies of, for example, Cu^{2+}–enzymes (Deinum & Vänngard, 1975), heme proteins (Gupta et al., 1979), and Co(II)-substituted heme proteins (Gupta et al., 1975) to identify ligands for the paramagnetic metal ion and to characterize the metal ligand bonding. Very recently ^{17}O superhyperfine coupling has been reported for Mo(V) enzyme complexes (Bray, 1979; Cramer et al., 1979). There is also a recent report of ^{17}O superhyperfine coupling to Co(II) in complexes with carboxypeptidase A (Makinen & Kuo, 1980).

With few exceptions (Tsay & Helmholz, 1969; Tinkham, 1956; Windsor et al., 1963), splittings due to ligand superhyperfine interactions in EPR spectra for complexes of Mn(II) are much smaller in magnitude than the intrinsic line widths of the electron spin transitions, and ligand superhyperfine effects have not been observed in enzymatic complexes of Mn(II). However, in some cases, complexes of Mn(II) with enzymes in solution yield high-quality EPR spectra in which the line widths of individual transitions are of the order of a

† From the Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104. *Received May 12, 1980.* This work was supported by U.S. Public Health Service Grant AM 17517.

[1] Abbreviations used: NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid; TLC, thin-layer chromatography; high-performance LC, high-performance liquid chromatography; PEI, poly(ethylenimine); TEA, triethylamine; Ap_5A, diadenosine pentaphosphate; P-enolpyruvate, phosphoenolpyruvate.

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few gauss (Reed & McLaughlin, 1973; Buttlaire et al., 1975; Michaels et al., 1975; Villafranca et al., 1976). Such line widths are of the proper magnitude such that ligand superhyperfine effects from a nucleus such as ^{17}O could become a measurable perturbation in the EPR signals. Among the enzymatic complexes of Mn(II) which exhibit such high-quality EPR spectra are the transition-state-analogue complexes of creatine kinase (Reed & Cohn, 1972; Reed & McLaughlin, 1973). Milner-White & Watts (1971) first reported that planar anions such as nitrate stabilized the dead-end complex of creatine kinase which contained enzyme, metal ion, ADP, and creatine. Moreover, these authors proposed that the action of these anions related to their ability to mimic the migrating phosphoryl group, leading to a complex with a structure which resembles that of the transition state of the phosphoryl transfer reaction. Previous spectroscopic studies have indicated that the anions SCN^-, NO_3^-, and N_3^- are directly bound to the metal ion in these complexes (Reed et al., 1978). The present investigation was initiated to identify other directly bound ligands. The results suggest that for creatine kinase coordination of metal ion to all three phosphate groups is involved in the transition state of the reaction.

Experimental Procedure

Creatine kinase was isolated from rabbit skeletal muscle by method B of Kuby et al. (1954). The specific activities of the preparations were of the order of 50 IU in the coupled assay with pyruvate kinase and lactate dehydrogenase at 21 °C (Tanzer & Gilvarg, 1959). Creatine was purchased from Pfanstiehl Laboratories. Unlabeled nucleotides were from Sigma Chemical Co. Hepes was obtained from Calbiochem. A sample of normalized $H_2{}^{17}O$ (40.64% enrichment) was purchased from Yeda Research and Development Co. Ap_5A was from Boehringer. Enzymes which were used in assays or in the synthesis of labeled nucleotides were obtained from commercial sources or were isolated according to published procedures.

The chemical purity of labeled nucleotides was assayed by TLC with PEI plates (Brinkmann) which were developed with 1.2 M LiCl. The purity was also assayed by high-performance LC with a Whatman Partisil-10 SAX ion-exchange column with an equilibration and running buffer of 0.5 M ammonium phosphate, pH 4.3.

EPR Measurements. EPR spectra at X-band (9.1 GHz) were recorded with a Varian E-3 spectrometer and at K-band (35 GHz) with a Varian 4503 spectrometer. The sample temperature was maintained to ± 1 °C with a standard Varian temperature controller. Difference spectra were obtained with a Varian 1024 computer with a calibrated attenuator in the input circuit or by digitizing the experimental spectra for subsequent subtraction with a computer.

NMR Measurements. ^{31}P NMR spectra were obtained at 24.3 MHz with a Varian NV-14 spectrometer which was modified for pulsed Fourier transform spectroscopy. The longitudinal relaxation times (T_1) of water protons were measured with a custom-built, variable frequency, pulsed spectrometer by using the $180°-\tau-90°$ pulse sequence.

Preparation of β-^{17}O-Enriched ADP. The four steps in the synthesis of ADP enriched in the nonbridging oxygens with ^{17}O are shown in reactions 1–4. Reactions 3 and 4 provide

$$PCl_5 + 4H_2{}^{17}O \rightarrow H_3P^{17}O_4 + 5HCl \quad (1)$$

$$\text{isocyanate} + P^{17}O_4 \rightarrow \text{carbamyl-}P^{17}O_4 \quad (2)$$

$$\text{carbamyl-}P^{17}O_4 + \underset{\text{(trace)}}{\text{ADP}} \xrightarrow[Mg^{2+}]{\text{carbamate kinase}} ATP(\gamma\text{-}^{17}O_3) \quad (3)$$

$$\underset{\text{(XS)}}{\text{AMP}} + ATP(\gamma\text{-}^{17}O_3) \xrightarrow[Mg^{2+}]{\text{adenylate kinase}} ADP(\beta\text{-}^{17}O_3) + ADP \quad (4)$$

a cyclical pathway for utilization and production of ADP with the yield of $ADP(\beta\text{-}^{17}O_3)$ controlled by the amount of labeled carbamyl phosphate. The major product of the pathway is $ADP(\beta\text{-}^{17}O_3)$. The above reactions have been sketched for isotopically pure $H_2{}^{17}O$. Since water enriched to 40.6% in ^{17}O was used in reaction 1, the product ADP contains maximally a 40.6% enrichment in the three nonbridging oxygen atoms of the β-phosphate group. Reaction 1 was carried out in a drybox according to the method of Hackney et al. (1980), except that the order of addition of PCl_5 and H_2O was reversed. Reaction 2 was carried out according to the procedure described by Cohn & Hu (1980), as were the enzyme-catalyzed steps of reactions 3 and 4. The reaction mixture from the last step was acid quenched with concentrated HCl and then immediately brought back to pH 9 with concentrated KOH. The supernatant was loaded onto a column (3 × 80 cm) of Sephadex A-25 preequilibrated with 10 mM TEA/HCO_3^- buffer, pH 7.8. The column was eluted with 3 L of a linear gradient, 10–800 mM, of TEA/HCO_3^-. Fractions containing labeled ADP were pooled, and the buffer was removed by evaporation to dryness with a rotary evaporator. The residue was twice resuspended in methanol, evaporated to dryness, and finally taken up in 0.5 mL of water. The solution of $ADP(\beta\text{-}^{17}O)$ was applied to a 1.9 × 5 cm column of acid-washed Chelex 100 to remove magnesium ions. The chemical purity of the compound was checked by TLC on PEI–cellulose plates and by high-performance LC. The isotopic enrichment of the sample was estimated from the ^{31}P NMR spectrum by the method of Tsai (1979), using the relative areas of the narrow components of the signals for the α- and β-phosphate groups. This assay gave a value of 31 ± 5% ^{17}O enrichment in the nonbridging oxygens of ADP.

Preparation of α-^{17}O-Enriched ADP. Synthesis of ADP enriched with ^{17}O in the nonbridging oxygens of the α position and in the α,β bridge oxygen is outlined in reactions 5–10.

$$PCl_5 + H_2{}^{17}O \rightarrow P^{17}OCl_3 + 2\,HCl \quad (5)$$

$$P^{17}OCl_3 + \text{adenosine} \xrightarrow{\text{triethyl phosphate}} A\text{–}{}^{16}O\text{–}P^{17}OCl_2 + HCl \quad (6)$$

$$A\text{–}{}^{16}O\text{–}P^{17}OCl_2 + 2H_2{}^{17}O \xrightarrow{\text{triethyl phosphate}} A\text{–}{}^{16}O\text{–}P^{17}O_3 + 2HCl + 2H^+ \quad (7)$$

$$A\text{–}{}^{16}O\text{–}P^{17}O_3 + \text{ATP (trace)} \xrightarrow[Mg^{2+}]{\text{adenylate kinase}} ADP(\alpha\text{-}^{17}O_3) + ADP \quad (8)$$

$$ADP(\alpha\text{-}^{17}O_3) + \text{P-enolpyruvate} \xrightarrow[Mg^{2+},\,K^+]{\text{pyruvate kinase}} ATP(\alpha\text{-}^{17}O_3) + \text{pyruvate} \quad (9)$$

$$ATP(\alpha\text{-}^{17}O_3) + \text{glucose} \xrightarrow[Mg^{2+}]{\text{hexokinase}} ADP(\alpha\text{-}^{17}O_3) + \text{glucose-P} \quad (10)$$

Again the reactions have been outlined for isotopically pure $H_2{}^{17}O$ for simplicity. Reactions 8 and 9 provide a cyclical pathway for utilization and production of ATP, with the yield of ATP controlled by the amount of labeled AMP.

Reaction 5 was carried out according to the procedure of Abbott et al. (1979). Reaction 6 was carried out in dry triethyl phosphate at −20 °C for 8 h. A slight excess over 2 equiv of labeled water was then added to give the labeled AMP. A

fraction of the reaction mix containing 200 μmol of labeled AMP was then loaded onto a 2.6 × 45 cm DE-52 column which had been equilibrated with 10 mM TEA/HCO_3^- buffer, pH 7.8. The column was eluted with 3 L of a linear gradient, 10–500 mM, of TEA/HCO_3^-. Fractions containing labeled AMP were pooled. The fractions were concentrated to dryness, resuspended in methanol and brought to dryness twice, and then resuspended in H_2O and brought to dryness. Finally, the residue was resuspended in 10 mL of 50 mM Hepes/KOH, pH 8.00. To this solution was added 250 μmol of tricyclohexylammonium P-enolpyruvate, 30 μL of 2 M $MgCl_2$ (6 mM $MgCl_2$), 150 μL of 3 M KCl (0.1 M KCl), 25 units of pyruvate kinase, 25 units of adenylate kinase, and a 0.25% trace of ATP. The reaction was allowed to run at room temperature overnight. The progress of the reaction was followed by TLC on PEI plates.

The reaction mixture was then acid quenched with concentrated HCl and then immediately brought back to pH 9 with concentrated KOH. Ap_5A was then added (10 μM) to further protect the mix from residual adenylate kinase activity. The lack of appreciable activity for pyruvate kinase and adenylate kinase in the mix was verified by standard assay procedures. The reaction mix was then made 30 mM in (+)-α-D-glucose, which was followed by addition of 2 μL of 2 M $MgCl_2$ and 25 units of hexokinase. The reaction was allowed to run at room temperature overnight. The progress of the reaction was followed by TLC. The solution was then loaded onto a DE-52 column which had been equilibrated with 10 mM TEA/HCO_3^- buffer, pH 7.8. The ADP α-$^{17}O_3$ was eluted with 3 L of a linear gradient of the TEA/HCO_3^- buffer, 10–500 mM. The fractions containing ADP(α-^{17}O) were checked by PEI–TLC and were pooled. The pooled fractions were concentrated to dryness in a rotary evaporator and then resuspended in methanol and brought to dryness. Finally the residue was resuspended in ~150 μL of water. The chemical purity of the ADP(α-^{17}O) was checked by TLC and high-performance LC. The isotopic enrichment of 30 ± 6% was estimated from the ^{31}P NMR spectrum by the method of Tsai (1979).

Preparation of ^{17}O-Enriched Formate. Concentrated formic acid (Fisher) (90.7%) was diluted 20:1 with 40% $H_2{}^{17}O$, and the solution was kept at room temperature for 24 h. Solid Tris base was then added to neutralize the acid. The ^{17}O enrichment was assayed by mass spectrometry, and this assay procedure indicated an enrichment of 22 ± 6% in the carboxylate oxygens of formate.

Preparation of Samples for EPR Measurements. For comparison of EPR spectra for samples with labeled and unlabeled ligands, a stock solution was prepared which contained all of the components except for the ligand in question. Identical volumes of solutions containing either labeled or unlabeled ligand were added to matched aliquots from the stock solution. The composition of the samples was such that all components were present in sufficient quantities to ensure a stoichiometric binding of the limiting component of the complex, which was Mn(II). The reproducibility of this procedure with respect to the amplitudes of EPR signals for bound Mn(II) was of the order of the noise level of the instrument (1–3%). Reproducibility between samples which originated from different stock solutions with the same concentration of Mn(II) was also within the noise level of the instrument.

For experiments in which the ^{17}O label in water was investigated, a stock solution containing the full complement of components and buffer was prepared in normal water.

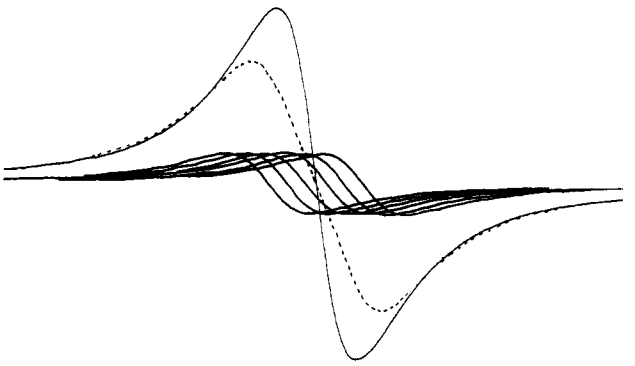

FIGURE 1: Simulation of unresolved superhyperfine splitting from a nucleus with $I = {}^5/_2$ in a first derivative EPR signal. The solid curve of greatest amplitude represents the unperturbed signal. The smaller solid curves represent the individual components of the nuclear spin manifold which contribute to the inhomogeneously broadened signal (dashed curve). For the simulation, a superhyperfine coupling constant equal to 20% of the unperturbed line width was used.

Matched aliquots from the stock solution were lyophilized, and the samples were then taken up in equal volumes of labeled or unlabeled water. The reproducibility of this procedure with respect to the amplitudes of the EPR signals for Mn(II) was also of the order of the noise level of the instrument.

Strategy for Analysis of EPR Spectra. The ^{17}O NMR results of Zetter et al. (1978) provide an estimate of ~6 MHz for the superhyperfine coupling constant, A/h, between $H_2{}^{17}O$ ligands and Mn(II) in its complexes with ADP and ATP in free solution. The ^{17}O nucleus has a spin of ${}^5/_2$ so that ^{17}O superhyperfine structure from a single $H_2{}^{17}O$ ligand would split each EPR transition into six approximately equally spaced components with an ~2-G separation for an overall pattern width of ~10 G. The line widths of individual transitions in the X-band EPR spectra for the formate and thiocyanate transition-state-analogue complexes of creatine kinase are of the order of 10–15 G (Reed & McLaughlin, 1973). Thus the superhyperfine structure from an ^{17}O ligand would not be resolved in the EPR spectrum; however, the unresolved superhyperfine splitting contributes an inhomogeneous broadening to line widths of the signals, as illustrated in Figure 1. For unresolved hyperfine splitting, the approximate[2] line width is given by eq 11, where ΔH is the observed line width and

$$\Delta H \simeq (\Delta H_i^2 + \Delta H_{HF}^2)^{1/2} \qquad (11)$$

ΔH_i and ΔH_{HF} are respectively the intrinsic line width and width of the superhyperfine pattern (Norris et al., 1971). The amplitudes of EPR signals in the normal first derivative detection are proportional to the inverse square of the line width (Poole, 1967); hence the amplitudes of the EPR signals are sensitive indicators of changes in the line width brought about by superhyperfine coupling to ^{17}O ligands. It should also be noted that since the ^{17}O enrichment in the samples is maximally 40%, the signals which are observed are a sum of those for labeled and unlabeled ligands.

Superhyperfine coupling contains contributions from through-bond (scalar) and through-space (dipolar) interactions of the electron and nuclear spins. The value of A/h given above relates to the scalar contribution (Zetter et al., 1978).

[2] Equation 1 is valid for Gaussian line shapes or for line shapes for which the second moment is proportional to the square of the line width (Norris et al., 1971). Computer simulations of the spectral line shapes for the complexes in question indicate that the experimental curves are represented reasonably well with a Gaussian line shape (G. D. Markham and G. H. Reed, unpublished results).

The dipolar contribution to the hyperfine coupling can be estimated[3] from eq 12, where the terms have their usual

$$\text{total splitting} = \frac{2I\gamma_I}{r^3}(3\cos^2\theta - 1) \qquad (12)$$

meaning (Goodman & Raynor, 1970). For a Mn to oxygen bond distance of 2.23 Å (Richards et al., 1964), the maximum pattern width from dipolar coupling is ~3.4 G, which is appreciably smaller than the ~10-G width due to a scalar interaction of the magnitude reported by Zetter et al. (1978). In any case, for an ^{17}O nucleus to exert a measurable influence in the EPR spectrum through either dipolar or scalar coupling, the ^{17}O nucleus must be in the first coordination sphere of the Mn(II).

EPR spectra for the transition-state-analogue complexes of Mn(II) with creatine kinase in solution exhibit powder pattern line shapes (Reed & Cohn, 1972). The central fine structure transition ($M_s = +^1/_2 \leftrightarrow M_s = -^1/_2$) dominates the central region of the spectrum. The signals for each of the six ^{55}Mn hyperfine components of this transition are split by second-order terms in the zero-field splitting interaction, and at X-band frequencies forbidden hyperfine transitions which occur between the major ^{55}Mn hyperfine components have appreciable intensities (Bleaney & Rubins, 1961). Of particular significance for the present investigation is the fact that spectra for the individual transition-state-analogue complexes can be fitted with a single set of spin Hamiltonian parameters (Markham et al., 1979). If the complex consisted of an equilibrium mixture of species each with a different set of ligands to Mn(II), then the EPR spectra for each species would give rise to a distinct EPR spectrum. In such a case the observed spectrum would be a sum over the spectra for individual complexes. Thus, the EPR spectra for the complexes in question reflect the electronic symmetry for a single coordination scheme. Moreover, the interpretations are not subject to ambiguities such as exchange averaging.

Although the positions of individual transitions in the spectra can be described with a "solid-state" analysis, the line widths of the signals are still sensitive to molecular motion (Meirovitch & Poupko, 1978). Low temperatures and high concentrations of protein lead to decreased line widths, and both lower temperatures and higher concentrations of protein slow the rotational motion of the protein.

Results

EPR Studies with $H_2{}^{17}O$. X-band EPR spectra for matched samples of the thiocyanate-stabilized dead-end complex of creatine kinase with normal water and with 40% $H_2{}^{17}O$-enriched water as a solvent are shown in Figure 2. K-band (35 GHz) spectra for a pair of samples of the formate complex in $H_2{}^{16}O$ and in $H_2{}^{17}O$ are shown in Figure 3. The ^{17}O-induced perturbations in the amplitudes and widths of the EPR signals for the $H_2{}^{17}O$-enriched samples indicate the presence of water ligands in the first coordination sphere of Mn(II) in this complex.

Since the ^{17}O enrichment in the solvent is 40%, spectra for the enriched samples represent a sum of spectra for complexes with different combinations of $H_2{}^{16}O$ and $H_2{}^{17}O$ in the first coordination sphere.[4] For example, if there were one coordination site for water in the complex, 60% of the spectrum would be due to the normal $H_2{}^{16}O$ complex. For two or three water ligands in the complex, the spectral contribution from species without an $H_2{}^{17}O$ ligand falls to 36% and 22%, respectively. Because the splitting patterns for any number of $H_2{}^{17}O$ ligands lead to local maxima and minima at the same positions as those in the corresponding spectrum for the $H_2{}^{16}O$ sample, subtraction of too large a fraction of the spectrum for

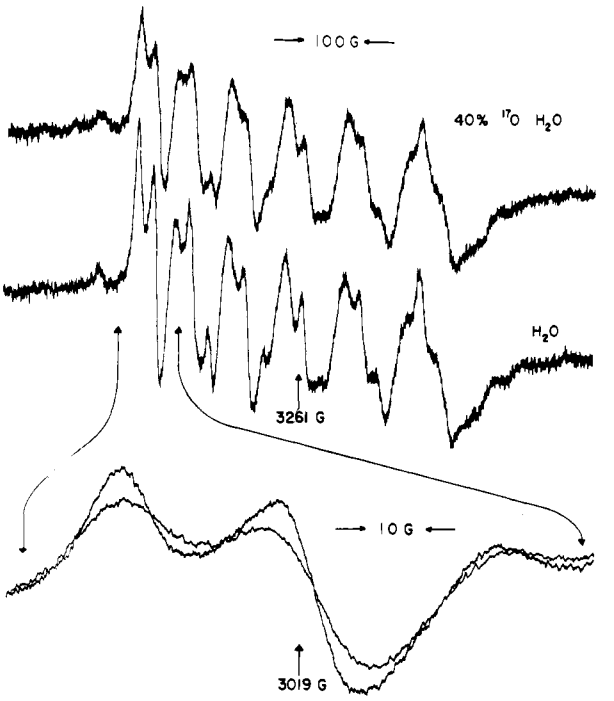

FIGURE 2: X-band EPR spectra for concentration matched samples of the creatine kinase·MnADP·thiocyanate·creatine complex in normal water and in water enriched to 40% in ^{17}O. The lower curves are expansions of the region of the spectrum indicated by the arrows. Spectra were recorded at 1 °C with a modulation amplitude of 2 G. The composition of the solutions was creatine kinase 180 mg/mL, $Mn(OAc)_2$ 2.0 mM, ADP 4.0 mM, creatine saturated solution (~100 mM), KSCN 25 mM, Hepes/KOH 50 mM; pH 8.0.

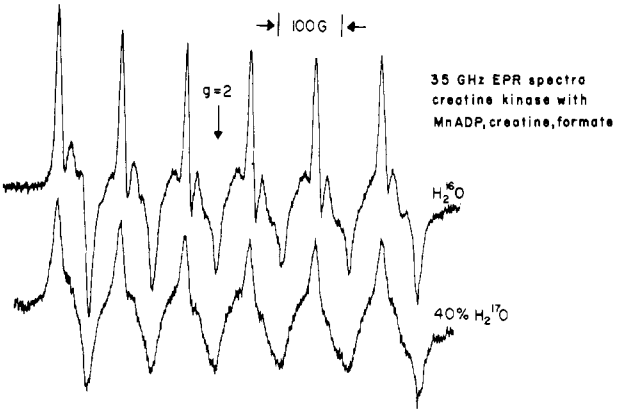

FIGURE 3: K-band EPR spectra for concentration matched samples of the creatine kinase·MnADP·formate·creatine complex in normal water and in water enriched to 40% in ^{17}O. Spectra were recorded at 1 °C with a modulation amplitude of 1 G. The composition of the solutions was creatine kinase 190 mg/mL, $Mn(OAc)_2$ 1.0 mM, ADP 1.8 mM, creatine saturated solution, formate 40 mM, Hepes/KOH 50 mM; pH 8.0.

[3] The dipolar contribution may be slightly greater than the estimate provided by eq 2 because in the presence of a scalar coupling some spin density is transferred to the oxygen orbitals. The nucleus is therefore closer to a small fraction of the unpaired spin (Goodman & Raynor, 1970).

[4] The stock sample of $H_2{}^{17}O$-enriched water also contained an appreciable enrichment in $H_2{}^{18}O$. However, since the ^{18}O nucleus is nonmagnetic like ^{16}O, the ^{18}O-containing species are treated as part of the ^{16}O fraction.

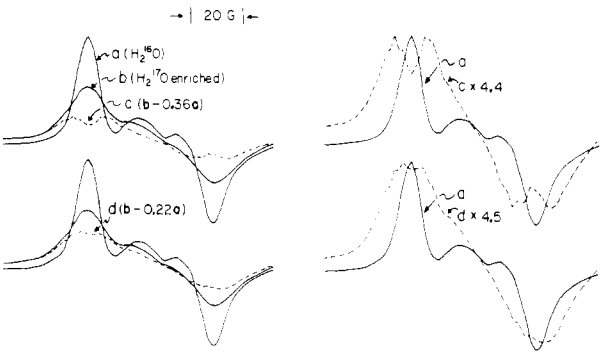

FIGURE 4: Comparison of experimental and difference EPR spectra for the lowest field ^{55}Mn hyperfine component for the formate stabilized complex of creatine kinase. Spectra were recorded at 35 GHz at 1 °C with 1-G modulation amplitude. The solid curves represent the experimental signals for samples in normal water (a) and in 40% ^{17}O water (b). The dashed curves represent difference spectra obtained by subtracting 36% (curve c) and 22% (curve d) of curve a from curve b. The composition of the samples is given in the legend for Figure 3.

the $H_2{}^{16}O$ sample from the spectrum for the enriched sample will be evident from the appearance of "gaps" in the difference spectrum at positions which should correspond to maxima or minima in the line shape. The maximal contribution of the species containing only $H_2{}^{16}O$ ligands can therefore be obtained by a deconvolution procedure in which different fractions of the spectrum for the sample in $H_2{}^{16}O$ are subtracted from the spectrum for the $H_2{}^{17}O$-enriched sample. This procedure is illustrated for one of the ^{55}Mn hyperfine transitions in the K-band spectrum of the formate complex in Figure 4. The deconvolution procedure shows that there must be at least three water molecules bound to Mn(II) in the complex. It should be noted that the difference spectrum (curve d) is itself a sum of spectra for complexes containing one, two, and three $H_2{}^{17}O$ ligands. The EPR results are especially significant because water proton relaxation measurements for solutions of the transition-state-analogue complexes indicate that none of the bound water molecules are in free exchange with the bulk solvent (see below).

Water Proton Relaxation Measurements. The magnitude of the paramagnetic contribution to the longitudinal relaxation time of water protons can be used to determine the number of water ligands to the paramagnetic metal ion which are in rapid exchange with the bulk solvent (Peacocke et al., 1969). Previous studies of the nitrate-stabilized dead-end complex of creatine kinase indicated that there was less than one rapidly exchanging water ligand (Reed et al., 1972). Although the thiocyanate and formate complexes exhibit similar low enhancement of water proton relaxation (Reed & McLaughlin, 1973), the number of rapidly exchanging water ligands had not been evaluated.

In order to calculate the number of water ligands from NMR relaxation data, one must determine the correlation time for modulation of the dipole–dipole interaction between the electron spin and the protons. The correlation time can be evaluated from the frequency dependence of the paramagnetic contribution to the relaxation time (Peacocke et al., 1969). Figure 4 shows the results of measurements of the water proton relaxation times at frequencies between 8 and 50 MHz for the thiocyanate and formate complexes of creatine kinase. Correlation times are obtained from the square root of the slope to intercept ratio of the plots in Figure 5. The results which are summarized in Table I indicate that none of the water molecules which are bound to Mn(II) in the complex are in rapid exchange with the bulk solvent.

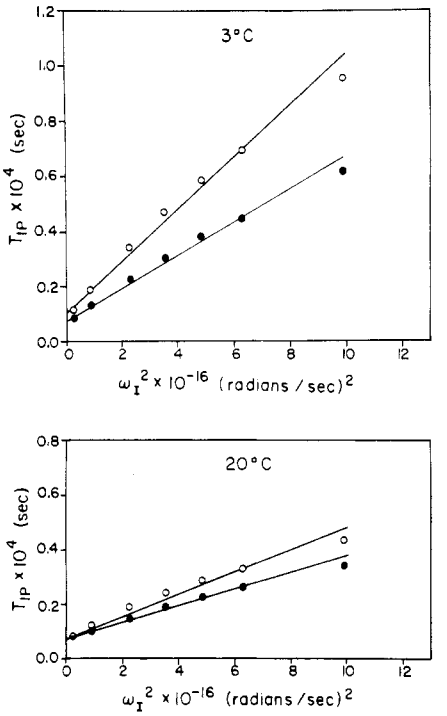

FIGURE 5: Frequency dependence of the proton relaxation rate of water in solutions of the formate (●) and thiocyanate (○) complexes of creatine kinase. The composition of the solutions was creatine kinase 15 mg/mL, $Mn(OAc)_2$ 0.1 mM, ADP 0.2 mM, creatine saturated solution, formate (●) 40 mM, KSCN (○) 25 mM, Hepes/KOH 50 mM; pH 8.0.

Table I: Correlation Times and Number of Exchangeable Water Molecules Bound to Mn(II) in Solutions of Anion-Stabilized Dead-End Complexes of Creatine Kinase[a]

anion	temp (°C)	τ_c (ns)	n[b]
formate	3	9.1 ± 0.2	0.5
formate	20	6.6 ± 0.2	0.7
thiocyanate	3	9.7 ± 0.2	0.3
thiocyanate	20	7.6 ± 0.2	0.5

[a] The correlation times, τ_c, were obtained from the square root of the slope to intercept ratio of the plots in Figure 5. The composition of the solutions is given in the legend for Figure 5. [b] The number of exchangeable water molecules, n, is obtained from the ratio of the observed value for $1/T_{1p}$ to that expected for a single water molecule which is in rapid exchange with the bulk solvent (Reed et al., 1972).

The correlation times are in a region which provides for very efficient relaxation of protons over the range of frequencies studied, and to satisfy fast exchange conditions, the residence time of the water which is bound to Mn(II) must be less than ~0.2 μs. The residence time of water bound to Mn(II) in its complex with ADP in free solution is ~0.01 μs (Zetter et al., 1978). Thus, in the transition-state-analogue complexes of thiocyanate and formate with creatine kinase, the rate of water exchange from Mn(II) is retarded by at least an order of magnitude.

EPR Results for Complexes with β-^{17}O-Enriched ADP. EPR spectra for matched samples of the thiocyanate-stabilized complex of creatine kinase with ADP and with β-^{17}O-enriched ADP are shown in Figure 6. Spectra for the β-^{17}O-enriched ADP exhibit a reproducible decrease in amplitude relative to those for the unlabeled sister samples. Similar results were obtained for the formate complex with β-^{17}O-enriched ADP. This observation provides direct evidence for coordination of Mn(II) to an oxygen of the β-phosphate group of ADP in these complexes.

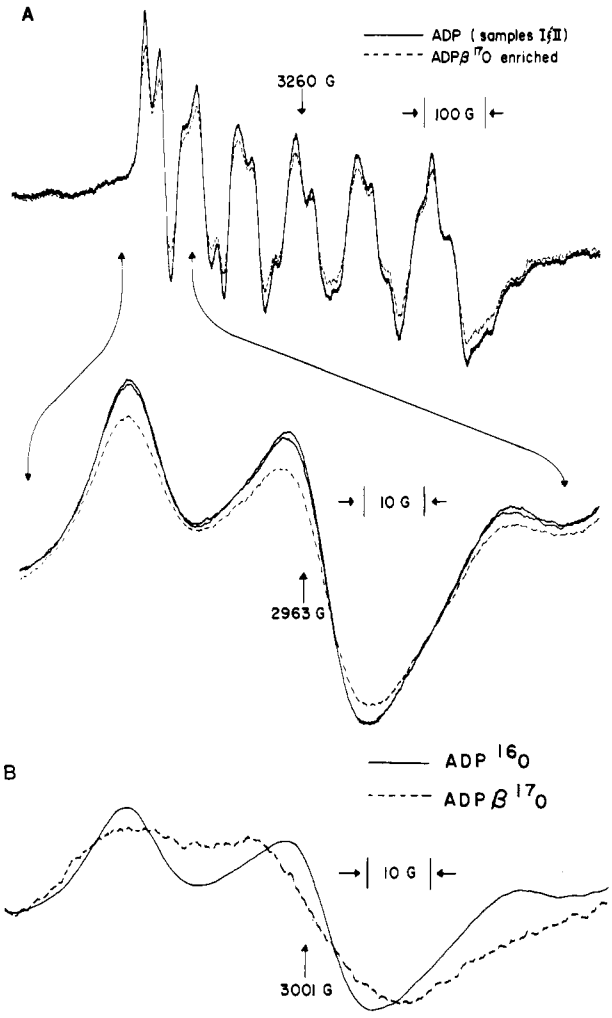

FIGURE 6: (A) X-band EPR spectra for concentration matched samples of the creatine kinase·MnADP·thiocyanate·creatine complex with normal ADP and with ADP enriched to 31 ± 5% with ^{17}O in the β-phosphate. The solid curves represent spectra for two samples with normal ADP, and the dashed curve is for the sister sample with β-^{17}O-enriched ADP. The botton curves are expansions of the region of the spectrum indicated by the arrows. The experimental conditions are identical with those given in the legend for Figure 2. The composition of the solutions was creatine kinase 190 mg/mL, $Mn(OAc)_2$ 0.9 mM, ADP, normal and enriched, 2.3 mM, creatine saturated solution, KSCN 25 mM, Hepes/KOH 50 mM; pH 8.0. (B) Difference spectrum (dashed curve) and spectrum for normal ADP (solid curve). The difference spectrum was obtained by subtracting 30% of the spectrum for the normal ADP sample from the spectrum from the β-^{17}O-enriched sample. The amplitude of the difference spectrum has been scaled to match that of the experimental spectrum.

Quantitatively, the spectral perturbations from ^{17}O enrichment in the β-phosphate group of ADP are smaller than those observed for ^{17}O enrichment in the water because only a single oxygen from the β-phosphate group is a ligand for Mn(II). The relative spectral contribution from species with an ^{17}O ligand is equal to the fractional enrichment of ^{17}O in the nonbridge oxygens of the β-phosphate group. A difference spectrum for the low-field ^{55}Mn hyperfine component of the thiocyanate complex is compared with the normal ^{16}O-ADP spectrum in Figure 6B. This difference spectrum corresponds to the EPR spectrum for the complex with an ^{17}O from the β-phosphate group bound to the Mn(II). The perturbation in the peak-to-peak line width of the signal due to the presence of unresolved ^{17}O superhyperfine coupling is of the order of 9–10 G. Application of eq 1 gives an estimate of ~3.5 G for the ^{17}O superhyperfine coupling constant. A curve-fitting

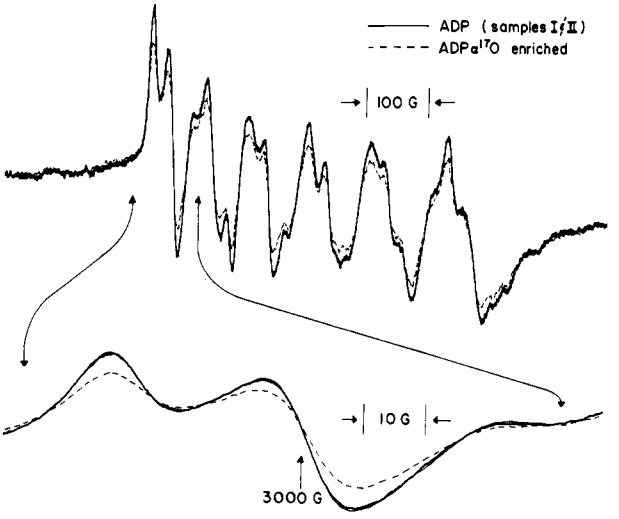

FIGURE 7: X-band EPR spectra for concentration matched samples of the creatine kinase·MnADP·thiocyanate·creatine complex with normal ADP and with ADP enriched to 30 ± 6% in ^{17}O in the α-phosphate (see text). The solid curves represent spectra for two samples with normal ADP, and the dashed curve is for the sister sample with [α-^{17}O]ADP. The bottom curves are an expansion of the region of the spectrum indicated by the arrows. Conditions for recording of the spectra are given in the legend for Figure 2. The composition of the solutions was creatine kinase 190 mg/mL, $Mn(OAc)_2$ 0.9 mM, ADP, normal and enriched, 1.7 mM, creatine saturated solution, KSCN 25 mM Hepes/KOH 50 mM; pH 8.0.

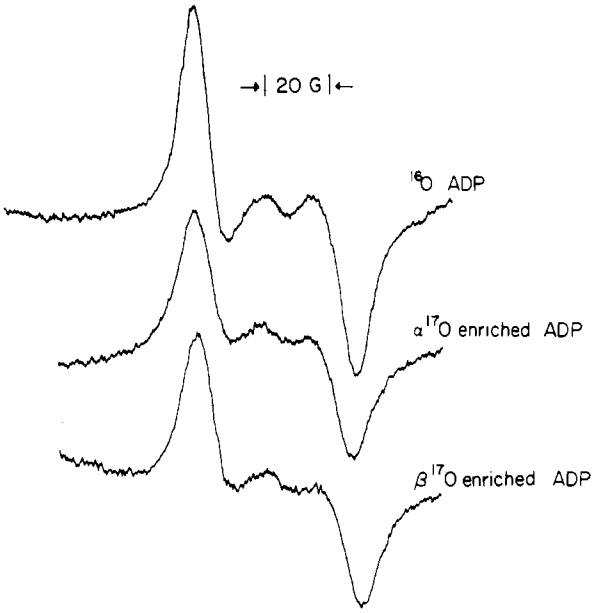

FIGURE 8: Comparison of K-band EPR spectra for the creatine kinase·MnADP·formate·creatine complex with normal ADP, α-^{17}O-enriched ADP, and β-^{17}O-enriched ADP. Spectra were recorded at 1 °C with 1-G modulation amplitude. The composition of the solutions was creatine kinase 190 mg/mL, $Mn(OAc)_2$ 1.0 mM, ADP, normal and enriched samples, 1.7 mM, formate 40 mM, creatine saturated solution, Hepes/KOH 50 mM; pH 8.0. The spectra are for the lowest field ^{55}Mn hyperfine component of the spectrum.

procedure in which the signal for the [^{16}O]ADP sample was split into six overlapping components to match the line shape of the difference spectrum also gave a value of 3–4 G for the ^{17}O superhyperfine coupling constant.

EPR Results for Complexes with α-^{17}O-Enriched ADP. EPR spectra for the thiocyanate complex of creatine kinase with ADP and with $\alpha^{17}O$-enriched ADP are compared in Figure 7. Coordination of Mn(II) to an oxygen of the α-phosphate group of ADP is evident from the suppression of

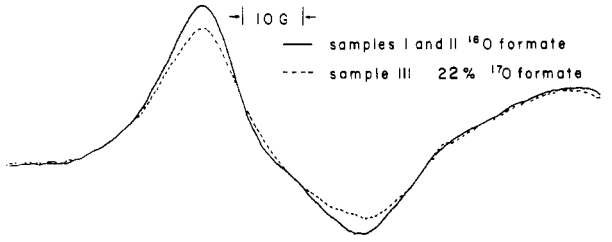

FIGURE 9: X-band EPR spectra for concentration mathced samples of the creatine kinase·MnADP·formate·creatine complex with normal formate and with 22 ± 6% ^{17}O-enriched formate. Experimental conditions are given in the legend for Figure 2. The composition of the solutions was creatine kinase 130 mg/mL, $Mn(OAc)_2$ 1.0 mM, ADP 1.6 mM, creatine saturated solution, formate, normal and enriched, 90 mM, Hepes/KOH 50 mM; pH 8.0. Spectra are for the lowest field ^{55}Mn hyperfine component.

the amplitudes and increases in line width of the ^{17}O-enriched sample. The spectral perturbations from ^{17}O enrichment in the α-phosphate group are quantitatively comparable to those observed with ^{17}O enrichment in the β-phosphate group.[5] For example, K-band EPR spectra for the low-field ^{55}Mn hyperfine component of the formate complex with matched samples of ADP, α-^{17}O-enriched ADP, and β-^{17}O-enriched ADP are compared in Figure 8. The perturbation in the amplitudes of signals for the sample with the α-^{17}O-enriched sample of ADP is slightly larger than that observed for the corresponding sample of β-^{17}O-enriched ADP. Within the uncertainties of the determination of the extent of enrichment of the two forms of enriched ADP, the ^{17}O superhyperfine coupling constants for oxygen on the α and β positions of ADP are approximately equal. Since the superhyperfine coupling constant reflects the character of the Mn(II) to oxygen bond, the results indicate that the coordinate bonds from Mn(II) to the α- and β-phosphate groups have very similar properties.

EPR Results for ^{17}O-Enriched Formate. Previous infrared data indicated that thiocyanate was liganded to Mn(II) in the corresponding complex with creatine kinase (Reed et al., 1978). There was also an indication from NMR shift data that formate was possibly bound to Co(II) in the corresponding complex with creatine kinase (McLaughlin et al., 1976). However, there was some ambiguity in the interpretation of the NMR shift data with Co(II) due to the possibility of pseudocontact contributions to the observed chemical shift changes.

EPR results for concentration-matched samples of formate and ^{17}O-enriched formate are shown in Figure 9. These data show unequivocally that the carboxylate group of formate provides an oxygen ligand for Mn(II) in the complex. Since the extent of ^{17}O enrichment in the formate is less than that of the two forms of labeled ADP, the spectral perturbation observed experimentally is smaller for the enriched formate sample than for the labeled ADP samples. However, within the uncertainties of the measurements of ^{17}O enrichment in the various compounds, the superhyperfine coupling constants for the O^- ligands on either formate or ADP have approximately the same value.

Discussion

EPR data with ^{17}O-enriched ligands have permitted identification of six oxygen ligands to Mn(II) in the complex, creatine kinase·MnADP·formate·creatine. In the corresponding complex with thiocyanate instead of formate, five oxygen ligands are revealed by EPR, and the sixth ligand, the nitrogen of thiocyanate, is known from infrared data (Reed et al., 1978). Although there are a few examples of seven-coordinate complexes with Mn(II) (Richards et al., 1964), EPR spectra for Mn(II) in the complexes with creatine kinase are indicative of an octahedral coordination geometry. Since six ligands have been identified, the composition of the coordination sphere of Mn(II) is established.

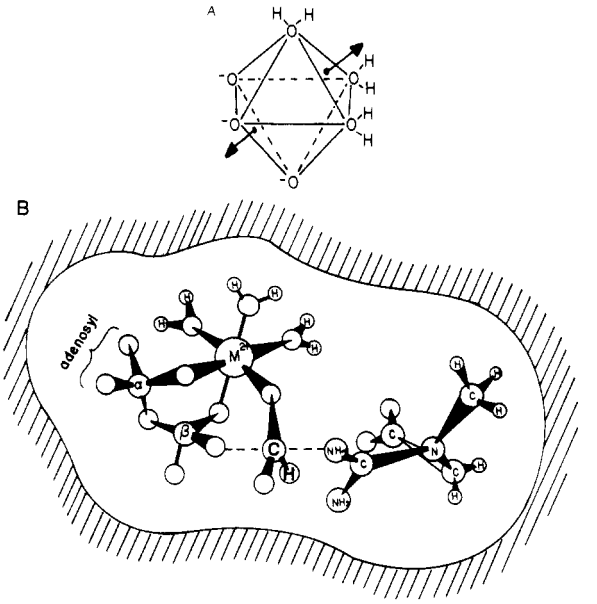

FIGURE 10: (A) Schematic representation of a trigonal distortion of an octahedral complex. (B) Schematic representation of the structure of the creatine kinase·MnADP·formate·creatine complex. The six oxygen ligands to the metal ion have been identified by EPR with ^{17}O labeling. The configuration of the ligands is consistent with the axially symmetric zero-field-splitting tensor for Mn(II) in the complex. The conformation of creatine is taken from the findings of Dietrich et al. (1980) with conformationally restricted analogues of creatine.

There is sufficient information available to determine how the six ligands are placed in the octahedron. The anions substitute for the missing phosphoryl group in the transition-state-analogue complexes, and the present studies show that the metal ion is bound to the two phosphate groups of ADP and to the anion. An examination of molecular models shows that an α,β,γ-tridentate metal ion complex with ATP is most readily achieved with the oxygen donor atoms from the three phosphate groups in a mutually cis configuration. A recent X-ray diffraction study of $Zn^{II}ATP$-2,2′-bipyridyl reveals a mutually cis arrangement of the three phosphate groups (Orioli et al., 1980). In the present complexes the anion which replaces the γ-phosphoryl group of ATP would be cis with respect to the α- and β-phosphate oxygens from ADP. This arrangement of the three O^- ligands explains the axial symmetry of the zero-field-splitting tensor for the formate complex. The ^{17}O superhyperfine coupling constants for the α- and β-phosphate oxygens and for the carboxylate oxygen of formate have approximately the same value (3–4 G), whereas the three neutral oxygen ligands from water have a slightly smaller superhyperfine coupling constant (Zetter et al., 1978). There are effectively two distinct types of Mn to oxygen bonds. With respect to the electronic environment of Mn(II), the arrangement of the two classes of bonds imposes a trigonal distortion as is shown schematically in Figure 10A. The trigonal distortion gives rise to the axially symmetric zero-field-splitting tensor, and the EPR spectrum for Mn(II) in the formate complex shows axial symmetry (Reed &

[5] Even though the nonbridge oxygens in the α-phosphate group of ADP are diastereotopic and Mn(II) may bind stereospecifically with respect to this position, the random labeling pattern at this position makes the probability for Mn(II) binding to an ^{17}O at this position equal to the fractional enrichment of these oxygens.

McLaughlin, 1973). In the thiocyanate complex, a nitrogen ligand is mutually cis to the α- and β-phosphate ligands of ADP, and this asymmetry gives rise to the rhombic zero-field-splitting tensor for Mn(II) in the thiocyanate complex (Markham et al., 1979). The EPR results lead to a self-consistent structure for the coordination sphere of Mn(II) at the active site of creatine kinase, and a schematic representation is shown in Figure 10B.

The three water molecules which are bound to Mn(II) in the complex are not in rapid exchange with the bulk solvent, in contrast to the situation for the ternary complex of enzyme·MnADP (Reed et al., 1972). When the transition state analogue complex is formed, the active site most likely adopts an enclosed structure which retards the free exchange of water ligands. Ruben & Reuben (1976) have predicted that residence times for water bound to metal ions in the "interior" of proteins would be increased due to the more hydrophobic character of the surroundings. An enclosed structure at the active site would increase contacts between the protein and the substrates and thereby contribute to the considerably enhanced thermodynamic stability of the transition-state-analogue complexes relative to those of the ternary and dead-end complexes (Milner-White & Watts, 1971; McLaughlin et al., 1972).

The conclusion from the EPR studies of the transition-state-analogue complexes is that the enzyme stabilizes a structure in which the activating metal ion is bound to both phosphate groups which are present and to an analogue of the missing phosphoryl group. Thus, coordination of the metal ion to all three phosphate groups is implicated in the transition state of the creatine kinase reaction. With respect to the role of the metal ion in the phosphoryl transfer event, perhaps the most significant aspect of the coordination scheme is the attachment of the migrating phosphoryl group directly to the metal ion (Reed et al., 1978; Dunaway-Mariano & Cleland, 1980). However, there is some question about the stage of the reaction in which metal ion coordination to the α-phosphate group of the nucleotide occurs. Metal ion coordination to the α-phosphate at a rate-determining step in the overall reaction is implied by a reversal of the stereoselectively of the enzyme for the two diastereomers of ATPαS when Cd(II) replaces Mg(II) as the activating cation (Burgers & Eckstein, 1980). The α,β,γ-tridentate isomers of Cr(III)ATP also bind to the enzyme with a higher affinity than do the β,γ-bidentate isomers, and the enzyme exhibits stereoselectivity in its inhibition by the two diastereomers of α,β-Cr(III)ADP (Dunaway-Mariano & Cleland, 1980). However, the tridentate isomers of $Cr^{III}ATP$ do not show substrate activity, whereas the Λ isomer of β,γ-bidentate $Cr^{III}ATP$ gives 2% of a single turnover in the creatine kinase reaction (Dunaway-Mariano & Cleland, 1980).

The results for the exchange-inert complexes of the Cr(III) nucleotides have led to the suggestion that Mg(II) migrates away from the α-phosphate group prior to the phosphoryl transfer event and coincident with a conformation change which encloses the active site (Dunaway-Mariano & Cleland, 1980). However, the transition-state-analogue complexes possess the closed conformation of the active site, and in this closed conformation, the enzyme stabilizes the structure with metal ion coordination to the α-phosphate group. If the proposed migration of the metal ion involves a ligand-exchange reaction in which the α-phosphate ligand is replaced by a water molecule (as in the structure of β,γ-bidentate $Cr^{III}ATP$), the process would appear to be an endergonic one and would add to the activation energy.

On the other hand, an examination of molecular models indicates that an α,β,γ-tridentate complex of ATP with a metal ion involves strain. The crystal structure of the $Zn^{II}ATP$–2,2′-bipyridyl complex shows that the bond between Zn(II) and the α-phosphate oxygen is longer than the bonds to the β- and γ-phosphate oxygens (Orioli et al., 1980). In the complexes with the anions and ADP present, there is no such strain because there is no chelate ring connecting the anion to the β-phosphate of ADP. An alternative view of the proposed migration of Mg(II) is a lengthening of the coordinate bond between Mg(II) and the α-phosphate without a bond cleavage. Such a lengthening of the metal ion to α-phosphate linkage would be inhibited in the α,β,γ-tridentate $Cr^{III}ATP$ complex because of the stronger interaction of the Cr(III) with the α-phosphate group.

Another feature of the Cr(III) nucleotide complexes which is related to the above arguments is their inability to undergo a rapid equilibration of chelate ring conformers (Cleland & Mildvan, 1979). The ring-puckering isomers of the Cr(III) complexes are very long-lived and may be separated by column chromatography. This property of the Cr(III) complexes may be especially significant in situations where neither the α,β,γ-tridentate isomers nor the β,γ-bidentate isomers of Cr(III)ATP give a large fraction of a turnover in an enzymatic reaction. Molecular models indicate that it is difficult to cleave the β,γ-bridge bond of ATP in a tridentate complex without involving a puckering motion of the chelate ring formed by the α- and β-phosphate groups and the metal ion. The fixed chelate ring conformation of the tridentate $Cr^{III}ATP$ species could account for the absence of substrate activity for this coordination isomer. On the other hand, the inflexible chelate ring is absent in the β,γ-bidentate $Cr^{III}ATP$; however, this species has weak substrate activity because the enzyme may prefer the α,β,γ-tridentate complex with the activating metal ion. An alternative explanation for the low activity of the β,γ-bidentate species is an unfavorable catalytic equilibrium constant for the Cr(III) complex due to the increased charge on the central metal ion (W. W. Cleland, private communication).

For enzymatic complexes of Mn(II) which give EPR spectra in which the signals have intrinsic line widths of $\leq$15 G, observation of superhyperfine coupling to ^{17}O of ligand groups holds much promise as a method for structure determination. Moreover, stereospecific incorporation of ^{17}O into prochiral groups such as the α- and β-phosphate groups of ATP will extend the scope of such investigations to stereochemical problems as well. However, because the ^{17}O superhyperfine coupling to Mn(II) is relatively weak, the EPR requirements with respect to the intrinsic line width of the signals are restrictive. EPR spectra for Mn(II) complexes with enzymes typically exhibit reduced line widths at higher frequencies (Reed & Ray, 1971; Reed & Cohn, 1972). Higher frequency EPR measurements coupled with higher enrichments of ligands with ^{17}O should extend the range of applicability of the method.

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