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Structures of Manganese(II) Complexes with ATP, ADP, and Phosphocreatine in the Reactive Central Complexes with Creatine Kinase: Electron Paramagnetic Resonance Studies with Oxygen-17-Labeled Ligands†

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ABSTRACT: Coordination of Mn(II) to the phosphate groups of the substrates and products in the central complexes of the creatine kinase reaction mixture has been investigated by electron paramagnetic resonance (EPR) spectroscopy with regiospecifically ^{17}O-labeled substrates. The EPR pattern for the equilibrium mixture is a superposition of spectra for the two central complexes, and this pattern differs from those observed for the ternary enzyme–Mn(II)–nucleotide complexes and from that for the dead-end complex enzyme–Mn(II)ADP–creatine. In order to identify those signals that are associated with each of the central complexes of the equilibrium mixture, spectra were obtained for a complex of enzyme, Mn(II)ATP, and a nonreactive analogue of creatine, 1-(carboxymethyl)-2-iminoimidazolidin-4-one, which is a newly synthesized competitive inhibitor. This inhibitor permits an unobstructed view of the EPR spectrum for Mn(II)ATP in the closed conformation of the active site. The EPR spectrum for this nonreactive complex with Mn(II)ATP matches one subset of signals in the spectrum for the equilibrium mixture, i.e., those due to the enzyme–Mn(II)-ATP–creatine complex. Chemical quenching of the samples followed by chromatographic assays for both ATP and ADP indicates that the enzyme–Mn(II)ADP–phosphocreatine and the enzyme–Mn(II)ATP–creatine complexes are present in a ratio of approximately 0.7 to 1. A similar value for the equilibrium constant for enzyme-bound substrates is obtained directly from the EPR spectrum for the equilibrium mixture. Spectra for samples of the equilibrium mixture set up initially with creatine and either [α-^{17}O]ATP, [β-^{17}O]ATP, or [γ-^{17}O]ATP show equivalent inhomogeneous broadening of signals from both central complexes irrespective of the position of the ^{17}O label. Similar measurements with the nonreactive complex of 1-(carboxymethyl)-2-iminoimidazolidin-4-one and the ^{17}O-labeled forms of ATP show that Mn(II) is coordinated to all three phosphate groups of ATP in this complex. These results show that an α,β,γ-tridentate complex of Mn(II)ATP is the substrate in the forward direction and Mn(II) remains coordinated to the α- and β-phosphates of ADP and to phosphocreatine in the product complex. A definitive model for the involvement of the divalent metal ion in the entire reaction sequence emerges from these results.

The mode of coordination of metal ions to nucleotides in free solution and in complexes with enzymes has been a topic of considerable interest because metal ion–nucleotide complexes are the reactive species in virtually all enzyme-catalyzed reactions that involve nucleotides. Nucleotides possess several functional groups that are potential ligands for metal ions, and knowledge of the isomer of the metal–nucleotide complex that is favored by a given enzyme is necessary to understand the role of the metal ion in the catalytic cycle.

Creatine kinase (EC 2.7.3.2) catalyzes the reversible reaction

$$MgATP^{2-} + \text{creatine} \rightleftharpoons MgADP^{-} + \text{phosphocreatine}^{2-} + H^{+}$$

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This enzyme is especially well suited for detailed studies of metal ion activation of enzymic phospho transfer reactions because only a single equivalent of divalent cation is required for activity, several species of cation will activate the reaction, and the metal ion apparently does not interact directly with functional groups from the protein (Dunaway-Mariano & Cleland, 1980; Reed & Leyh, 1980).

Although high-resolution structures for the enzyme and its complexes are not yet available, several key structural features of the active site have been revealed by various chemical, spectroscopic, and kinetic methods (Kenyon & Reed, 1983). In particular, activity studies with isomers of adenosine phosphorothioate substrates (Burgers & Eckstein, 1980) and with isomers of the exchange-inert Cr(III)ATP complexes (Dunaway-Mariano & Cleland, 1980), as well as EPR studies of the Mn(II) complexes with ^{17}O-labeled ligands (Reed & Leyh, 1980; Leyh et al., 1982), have provided information on the position of metal ion coordination to the nucleotide substrates and on the preferred stereochemical configuration of the metal–nucleotide complex. The major ambiguities that remain relate to the questions of when metal ion coordination to the α-phosphate group of the nucleotides occurs and whether or not the metal ion migrates among phosphate groups during the catalytic cycle.

Results of kinetic studies with isomers of adenosine phosphorothioate substrates with hard vs. soft metal ions as activators suggested that the α- and β-phosphates of the nucleotide coordinate to the metal ion at a rate-limiting step in the catalytic cycle (Burgers & Eckstein, 1980). On the other hand, a β,γ-bidentate isomer of Cr(III)ATP gave between 1 and 2% of a single turnover even though α,β-bidentate Cr(III)ADP was a much better inhibitor than β-monodentate Cr(III)ADP (Dunaway-Mariano & Cleland, 1980). EPR studies with Mn(II) species of the anion-stabilized, dead-end complexes of creatine kinase showed that Mn(II) was coordinated to the α- and β-phosphate groups of ADP and to the anion that is a surrogate for the transferable phospho group in these complexes (Reed & Leyh, 1980). Although the latter studies involved inhibitory complexes, the transition-state analogue characteristics of these complexes (Milner-White & Watts, 1972) provided support for a tridentate coordination scheme between the metal ion and phosphate groups of the substrates in the activated state.

In the present study Mn(II) EPR methods have been extended to the equilibrium mixture of substrate and product complexes in order to determine the coordination schemes between metal ion and substrates in the two reactive central complexes. A new competitive inhibitor of creatine that elicits a substrate-like response at the active site of the enzyme has also been characterized.

Experimental Procedures

Creatine kinase was isolated from rabbit skeletal muscle according to method B of Kuby et al. (1954a). Gel filtration over a column (2.5 × 180 cm) of Sephacryl S-200 (Pharmacia) with 50 mM Hepes[1]/KOH, pH 8.0, as the equilibration and running buffer was substituted for the crystallization step. Specific activities of the preparations were in excess of 50 IU/mg in the coupled assay (Tanzer & Gilvarg, 1959) at 21 °C. Adenosinetriphosphatase (ATPase) activities of the preparations were less than 10^{-4} of the kinase activity, and there was less than 10% loss of ATP after incubation for 15 min at 0 °C in solutions with the same concentrations of enzyme and $Mn(OAc)_2$ that were used in the EPR experiments. The EPR measurements required less than 12 min for completion, and there were no changes in spectra recorded immediately after mixing and after incubation for up to 45 min.[2] Solutions of the enzyme were concentrated with a collodion bag apparatus. High concentrations of enzyme were used to promote saturation of the central complexes and to provide high signal-to-noise ratios in the EPR spectra.

Stock solutions of phosphocreatine were made up from the disodium salt (Boehringer Mannheim) immediately before use. Unlabeled nucleotides (Sigma) were purified chromatographically on DEAE-Sephadex and Chelex 100 as described previously (Reed & Leyh, 1980). Water with 52% ^{17}O was obtained from Monsanto. ATP's with ^{17}O enrichment in the α-phosphate or β-phosphate were prepared from α-^{17}O-enriched ADP or [β-^{17}O]ADP by pyruvate kinase catalyzed phosphorylation with phosphoenolpyruvate. The ^{17}O-enriched ADP species were prepared as described previously (Reed & Leyh, 1980). ATP with ^{17}O enrichment in the γ-phosphate group was prepared from ADP by carbamate kinase catalyzed phosphorylation with ^{17}O-enriched carbamyl phosphate (Cohn & Hu, 1980). The ^{17}O-enriched species of ATP were purified by DEAE-Sephadex and Chelex 100 chromatography (Reed & Leyh, 1980). The chemical purity of the nucleotides was assayed by high-performance LC with a Whatman Partisil-10 SAX column eluted with 0.5 M ammonium phosphate at pH 4.3. The ^{17}O enrichment in the three samples of ATP was determined by ^{31}P NMR at 145 MHz according to the method of Tsai (1979).

Synthesis of Guanidine-N,N-diacetic Acid. Cyanamide (5.2 g) (Aldrich Chemical Co.) (freed from stabilizer prior to use by extraction with ether) was added to 2.5 mL of water in a 25-mL flask. Iminodiacetic acid (13.4 g) (Aldrich Chemical Co.) was added slowly with mixing, and 13 mL of aqueous ammonia (58%) was added dropwise. A white crystalline precipitate formed upon incubation of the solution for several days at room temperature. The precipitate was filtered, redissolved in a minimum amount of water, and recrystallized by addition of ethanol. The yield was 70%. The solid decomposed at 270 °C. ^1H NMR spectra in D_2O showed a single resonance at δ 3.91. Addition of starting material (iminodiacetic acid) to the sample tube gave a second signal at δ 3.66. ^{13}C NMR spectra showed three resonances (dioxane as standard): δ 53.81 (α-carbon), 158.19 (guanidino carbon), and 175.21 (carboxylate carbon). A microanalysis on the monoammonium salt gave the following results: Anal. Calcd for $C_5H_{12}N_4O_4$: C, 31.25; H, 6.29; N, 29.15. Found: C, 31.28; H, 6.51; N, 28.99.

Synthesis of 1-(Carboxymethyl)-2-iminoimidazolidin-4-one. This compound was prepared by heating of guanidine-*N,N*-diacetic acid in water. A sample of guanidinediacetic acid was dissolved in a minimum amount of water, and the solution was heated with a heat gun for 10 min. Crystals of 1-(carboxymethyl)-2-iminoimidazolidin-4-one formed upon cooling the solution. The compound was recrystallized from water. ^1H NMR spectra of the compound in D_2O showed single resonances with equal intensities at δ 4.31 and 4.11. Upon prolonged incubation in D_2O the intensity of the signal at δ 4.31 (ring methylene protons) diminished due to exchange with the

[1] Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; high-performance LC, high-performance liquid chromatography; *D*, the axial zero-field splitting parameter; *E*, the rhombic zero-field splitting parameter; EPR, electron paramagnetic resonance.

[2] The ATPase activity is inhibited when creatine is bound together with ATP.

solvent deuterons. This exchange behavior is consistent with that of the related compound, creatinine (Kenyon & Rowley, 1971). The proton-decoupled ^{13}C NMR spectrum of the compound in a mixture of H_2O and D_2O exhibited signals at δ 54.40 (ring methylene) and at δ 48.19 (exocyclic methylene). The signal for the ring methylene carbon had a lower nuclear Overhauser enhancement due to partial exchange of protons. In proton-coupled spectra the ring methylene carbon had a J_{C-H} of 150 Hz, typical of a creatinine-like methylene carbon, whereas the exocyclic methylene carbon had a J_{C-H} of 141 Hz, characteristic of a creatine-like methylene carbon (Dietrich et al., 1980a). Microanalysis gave the following results: Anal. Calcd for $C_5H_7N_3O_3$: C, 38.22; H, 4.49; N, 26.74. Found: C, 38.29; H, 4.47; N, 26.49.

Finally, liquid secondary-ion mass spectra in glycerol/methanol showed a positive ion at m/e 158. This corresponds to the expected molecular weight of the molecular ion (M) plus a proton. A strong peak at m/e 250 that corresponds to M + glycerol + 1 was also observed.

EPR Measurements. EPR spectra were obtained at 35 GHz with a hybrid Varian spectrometer as described previously (Leyh et al., 1982). Samples that were used for comparison of EPR line shapes for labeled and unlabeled species of nucleotide were made up from a common stock solution that contained enzyme, $Mn(OAc)_2$, and creatine. Equal volumes of concentration-matched solutions of the nucleotides were added to matched aliquots from the stock solution, and the spectra were recorded immediately after mixing of the sample. The overall precision in sampling and spectral measurement was such that the standard deviation in the amplitudes for EPR signals from separate matched samples was less than 2%.

Instrumental Methods. ^1H NMR spectra were obtained at 240 MHz with a hybrid spectrometer system at the University of California, San Francisco. ^{13}C NMR spectra were obtained with a Varian FT-80 or Varian XL-100 spectrometer. ^{31}P NMR spectra were obtained at 145 MHz with a Bruker spectrometer. Liquid secondary-ion mass spectra were obtained with a Kratos MS-50 mass spectrometer.[3]

Reaction Quenching. Equilibrium constants for the creatine kinase reaction with a catalytic amount of enzyme and with an excess of enzyme over nucleotide substrates (i.e., under conditions analogous to those employed in the EPR measurements) were determined by quenching enzyme activity, followed by high-performance LC assays for ATP and ADP. Solutions of the equilibrium mixture were injected from a Hamilton 800 type microliter syringe pipet into a 10-fold excess volume of a solution of 10% trichloroacetic acid. Denatured protein was packed by centrifugation, and aliquots from the supernatant were assayed for ATP and ADP by high-performance LC with the Whatman Partisil 10-SAX column. All solutions and the syringe were kept at the appropriate temperature until after enzyme activity had been quenched. Control experiments without enzyme indicated that there was less than 1% hydrolysis of ATP upon exposure to the trichloroacetic acid solution during the interval required for the assay procedure.

RESULTS

EPR Spectra for the Equilibrium Mixture. In applications of EPR to solutions that contain Mn(II) complexes in equilibrium, differences in resonant frequencies[4] for Mn(II) in different coordination environments normally result in a concentration-weighted superposition of spectra for each paramagnetic complex. In order to obtain straightforward spectral information from the reactive, central complexes, it is necessary to find conditions under which the fraction of Mn(II) bound in these complexes predominates over the fraction in other complexes. With high concentrations of enzyme (i.e., >4 mM in active sites), the limiting factor in achieving virtual saturation of Mn(II) into the central complexes is the relatively high K_M for creatine [K_M = 1 mM at 0 °C (McLaughlin et al., 1972)]. Although levels of creatine well above the K_M can be employed to saturate this binding step, elevated levels of creatine also promote formation of the dead-end complex enzyme–Mn(II)ADP–creatine (Morrison & James, 1965).

In order to find conditions that would permit a clear spectral observation of the central complexes, spectra were obtained for each of the ternary complexes, enzyme–Mn(II)–ATP and enzyme–Mn(II)–ADP, for the dead-end complex, and for the complete reaction mixture with various levels of creatine or of phosphocreatine added initially. EPR spectra obtained from such experiments are shown in Figure 1. The data of Figure 1 show that when all of the components required for the reaction are present, a sharp spectral pattern appears that is distinct from those given by either ternary complex or from that of the dead-end complex.

In samples either with an initial ratio of creatine to ATP less than 1 or with an initial ratio of phosphocreatine to ADP less than 1, the spectra show the expected superposition of patterns for the respective ternary complex and those due to the central complexes.[5] At stoichiometric or greater ratios either of creatine or of phosphocreatine to the respective nucleotide substrate, the spectrum characteristic of the central complexes dominates until at very high ratios of creatine to ATP signals characteristic of the dead-end complex begin to appear (see Figure 1A).

All of the narrow features in the spectra are from the $M_s = -{}^1/_2$ to $M_s = {}^1/_2$ fine-structure transition. The resonant fields for the six ^{55}Mn hyperfine components of this transition are influenced by second-order and higher order terms from the zero-field splitting interaction. The powder pattern that is observed for each ^{55}Mn hyperfine component maps the angular dependence of the resonant fields, and the pattern reflects the symmetry and strength of the zero-field splitting interaction (Markham et al., 1979; Reed & Markham, 1984). The zero-field splitting parameters D and E characterize the electronic influence of the ligands on the unpaired spin of the central metal ion.

Analysis of the spectrum for the equilibrium mixture is slightly more complicated than normal because it consists of a superposition of spectra for the two central complexes. As a first step in deconvolution of this composite spectrum, an attempt was made to simulate the spectrum for one of the

[3] Mass spectra were obtained at the Bioorganic, Biomedical Mass Spectrometry Resource (A. L. Burlingame, Director), supported by NIH Grant RR-01614.

[4] The effective time scale for exchange averaging in EPR spectra is governed by the difference in resonant frequencies for the exchanging species. A difference in magnetic field position of 1 G corresponds to a frequency difference of 1.76×10^7 rad/s.

[5] At this substoichiometric ratio of substrates a difference exists in the amount of central complex expressed between the ATP plus creatine direction and the ADP plus phosphocreatine direction. This asymmetry is likely due to the fact that [Mn(II)] is limiting in these samples and the central complexes compete more effectively for Mn(II) whenever the alternative is the enzyme–Mn(II)–ADP ternary complex as opposed to the enzyme–Mn(II)–ATP complex. With stoichiometric or greater ratios of either creatine or phosphocreatine to the respective nucleotide substrate, the spectral patterns are independent of the direction of approach.

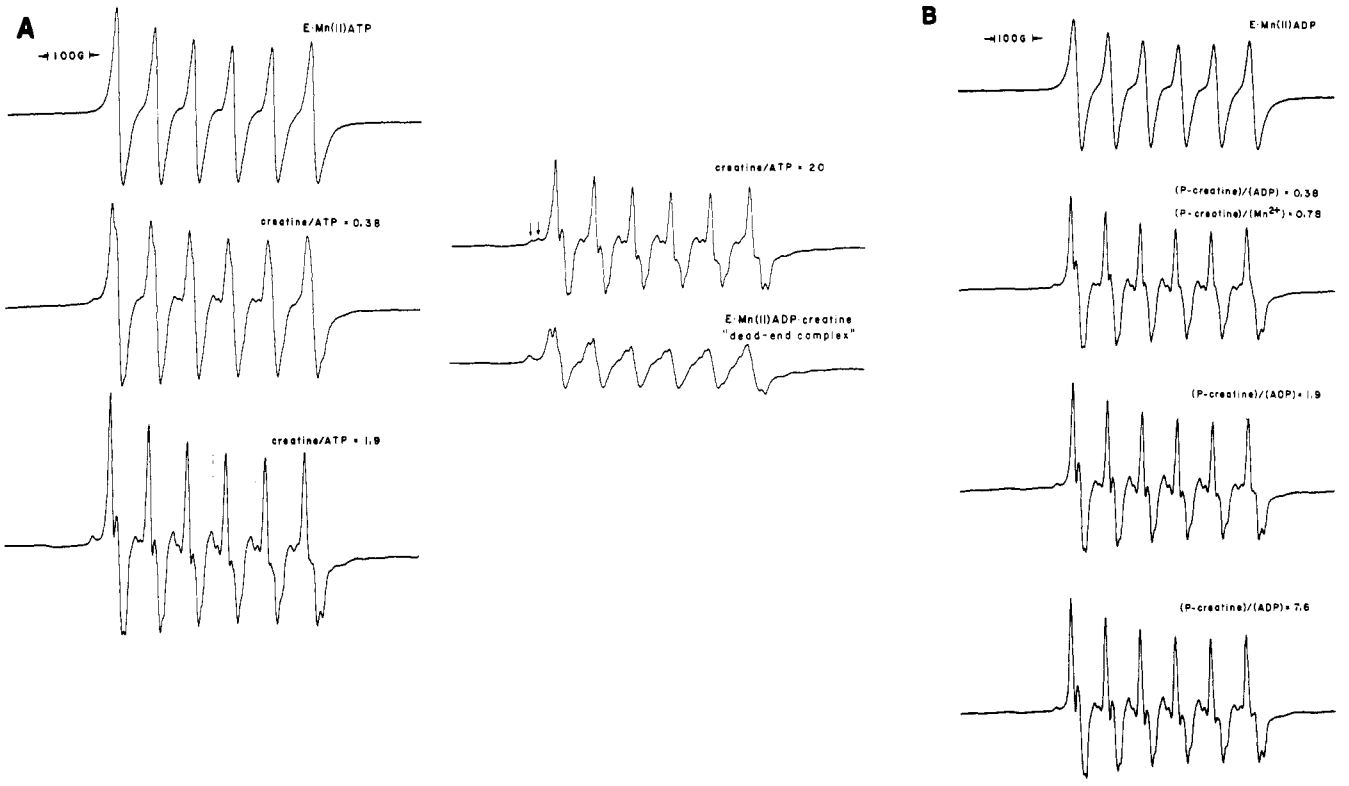


FIGURE 1: Comparison of 35-GHz EPR spectra for solutions of the creatine kinase reaction mixture from the ATP–creatine direction (A) and from the ADP–phosphocreatine direction (B). All the solutions contained 50 mM Hepes/KOH, pH 8.0, 1.6 mM $Mn(OAc)_2$, and 5.8 mM enzyme sites. Spectra were recorded in solution at −4 °C. (A) The first four samples were set up initially with 3.3 mM ATP and creatine as indicated. The sample of the dead-end complex contained 4.1 mM ADP and was saturated with creatine. Vertical arrows in the left spectrum on the right-hand side point to resolved low-field features—one due to the central complexes and one due to the dead-end complex. All spectra were recorded with the same spectrometer settings. (B) The samples were set up initially with 3.3 mM ADP and phosphocreatine (P-creatine) as indicated. All spectra were recorded with the same spectrometer settings.

contributing species (Markham et al., 1979). The more conspicuous signals in the spectrum are reproduced fairly accurately in simulations with a single set of spin Hamiltonian parameters (see Figure 2). Several key features in the experimental spectrum are not reproduced in this simulation. For example, the small signal to the low-field side of the first large signal (see arrow in Figure 2) is not associated with the pattern[6] represented by the simulation. This low-field feature does not belong to the dead-end complex although the spectrum for the dead-end complex does have a similar signal at an even lower field value. However, the two signals are resolved in samples that contain appreciable amounts of the dead-end complex (see Figure 1A). There are also two weak signals at the high-field end of the spectrum that are not associated with the pattern represented in the simulation. These spectral features that are peripheral to the major pattern (as well as some signals that occur between the six major peaks) are part of a second spectrum that has a larger zero-field splitting than that used in the simulation. The more intense signals that belong to this second spectrum are obscured because they overlap with those for the companion complex whose spectral line shape is well represented by the simulation in Figure 2. This overlap is also partly responsible for discrepancies in resolution between the experimental and simulated spectra.

The field positions of the peripheral signals could, in principle, be used to simulate the parts of the spectrum that are obscured by overlap with signals from the partner complex. Confidence in deconvolution of the spectra would be enhanced, however, if one could view the experimental spectrum for one or both of the complexes in isolation. Isolation of one of the

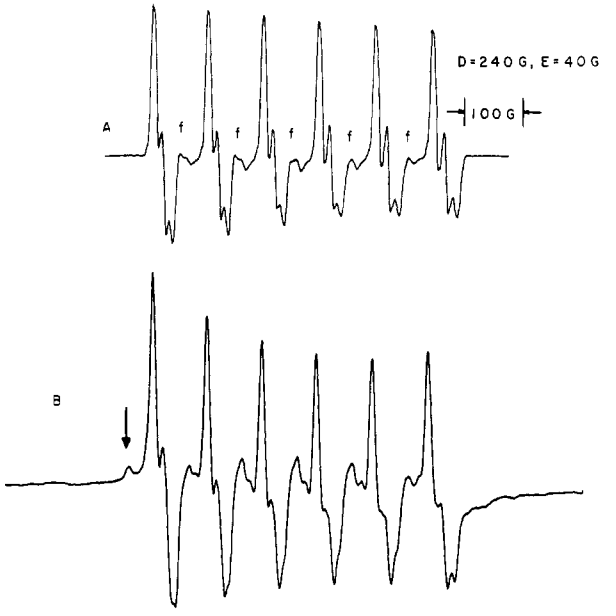


FIGURE 2: Comparison of simulated (A) and experimental (B) EPR spectra for the equilibrium mixture of creatine kinase. (A) The powder pattern was simulated with D = 240 G, E = 40 G, a line width of 5 G, and a ^{55}Mn hyperfine coupling constant of 92 G. A total of 2500 crystal orientations were summed. The small signals labeled f represent forbidden hyperfine transitions. (B) The sample contained 50 mM Hepes/KOH, pH 8.0, 5.8 mM enzyme sites, and 1.6 mM $Mn(OAc)_2$. The sample was set up initially with 3.3 mM ATP and 6.3 mM creatine. The spectrum was recorded in the solution phase at −4 °C. The arrow marks the low-field signal that is not present in the simulated pattern.

[6] The line width of this signal and its position are inconsistent with those of a component from one of the outer fine-structure transitions.

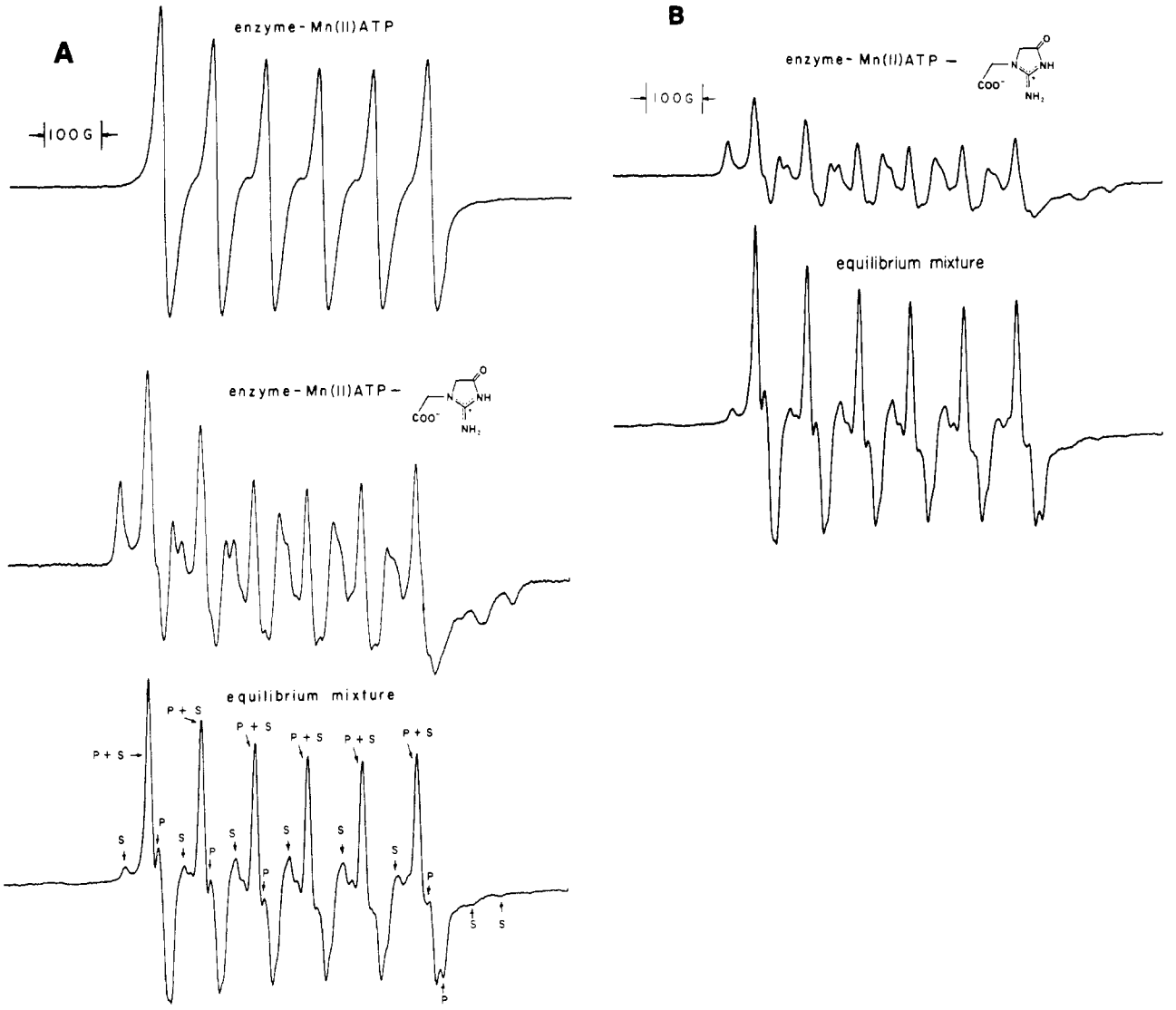


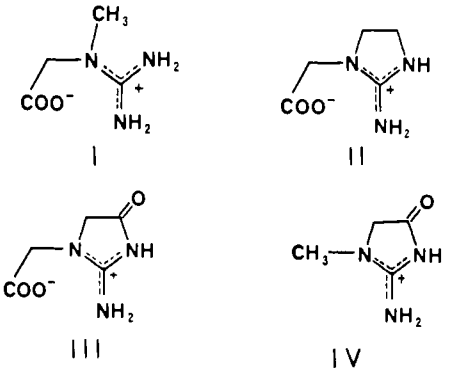
FIGURE 3: Comparison of 35-GHz EPR spectra for creatine kinase complexes with Mn(II)ATP, with Mn(II)ATP and 1-(carboxymethyl)-2-iminoimidazolidin-4-one (III), and with the equilibrium mixture of substrates. All solutions contained 50 mM Hepes/KOH, pH 8.0, 5.7 mM enzyme sites, and 1.6 mM $Mn(OAc)_2$. (A) Solutions for the top two spectra contained 3.5 mM ATP, and the sample for the middle spectrum contained 30 mM 1-(carboxymethyl)-2-iminoimidazolidin-4-one. The solution for the bottom spectrum is described in the legend for Figure 2. Assignments for the signals in the bottom spectrum are the following: s signals predominantly from the substrate, ATP–creatine, central complex; p signals or splittings predominantly from the product, ADP–phosphocreatine, central complex; p + s signals that contain contributions from both central complexes. The middle spectrum was taken at higher spectrometer-gain settings. (B) Comparison of spectra for the equilibrium mixture of substrates and the enzyme–Mn(II)ATP complex with 1-(carboxymethyl)-2-iminoimidazolidin-4-one. Spectra in (B) were taken with the same concentrations of Mn(II) and with the same spectrometer settings.

reactive complexes requires a considerable shift in the position of equilibrium for enzyme-bound reactants. Although there are some shifts in the position of equilibrium for bound reactants with temperature or pH, the EPR spectrum for the mixture loses resolution at higher temperatures or at lower pH values (data not shown). A more promising approach to this problem involves the use of substrate analogues.

EPR Spectra for ATP Complexes. All of the potential phosphate ligands are present in the simple ternary complex enzyme–Mn(II)–ATP. However, the spectrum for the enzyme–Mn(II)–ATP ternary complex does not exhibit resolved fine structure (see Figure 1) undoubtedly because the presence of the second substrate is required to promote enclosure of the active site (Reed & Cohn, 1972). In the open conformation of the active site the specificity of the enzyme for a particular isomer of the metal–ATP complex may not yet be expressed, and the EPR spectrum for the ternary complex is inhomogeneously broadened. The EPR signals for the ternary complex may also be broadened by motional perturbations that are due to the access of solvent to the site (Reed & Cohn, 1972). It is apparent that the site for creatine must be occupied to bring about the changes at the active site that will permit observation of the EPR spectrum for the reactive species of Mn(II)ATP.

The glycocyamidine family of compounds was examined because glycocyamidines [e.g., creatinine (IV)] are not substrates for creatine kinase but might serve as competitive inhibitors[7] (Kuby et al., 1954b; Gercken & Doring, 1974). Given the structure–function relationships among creatine and analogues that interact favorably with creatine kinase, 1-(carboxymethyl)-2-iminoimidazolidin-4-one (III) appeared to

[7] Phosphocreatinine is a competitive inhibitor with respect to phosphocreatine in the creatine kinase reaction (Gercken & Doring, 1974). In phosphocreatinine the phospho group is on the exocyclic nitrogen (Zeile & Meyer, 1938). By contrast, in the kinase reaction, cyclocreatine is phosphorylated on the ring nitrogen (Struve et al., 1977; Phillips et al., 1979).



be an attractive candidate as it bears a close structural relationship to the two most reactive compounds, creatine (I) and cyclocreatine (II); yet chemically it is analogous to the unreactive compound, creatinine (IV). Indeed, 1-(carboxymethyl)-2-iminoimidazolidin-4-one (III) turns out to be a nonreactive[8] competitive inhibitor of creatine (K_i = 2 mM at 30 °C) in the creatine kinase reaction (Nguyen, 1983).

In contrast to the ternary complex, the EPR spectrum for the enzyme–Mn(II)ATP complex with III (see Figure 3) exhibits a well-resolved pattern of fine-structure splitting. Moreover, the spectrum for the complex with ATP and III includes signals whose positions match fairly closely those signals in the spectrum for the equilibrium mixture of substrates that were not explained by the simulation of Figure 2. The close correspondence between spectral features indicates that the electronic symmetry of Mn(II) in the complex with ATP and III is virtually equivalent to that for Mn(II) in one of the central complexes of the equilibrium mixture—namely, that for the enzyme–Mn(II)ATP–creatine complex. The component with the less anisotropic zero-field splitting tensor (i.e., that with the spectrum that matches the simulation in Figure 2) is assigned to the enzyme–Mn(II)ADP–phosphocreatine central complex.

The zero-field splitting parameters for the enzyme–Mn(II)ATP complex with III are large enough such that features from adjacent ^{55}Mn components overlap. The extreme low-field and high-field parts of the spectrum are free from overlap, and features in these two regions show that the powder pattern reflects an intermediate degree of rhombic character. The spectral line shape also indicates that there is a distribution of zero-field splitting parameters (analogous to g strain) (Meirovitch & Poupko, 1978). This distribution contributes an inhomogeneous broadening that is most pronounced in features that are furthest removed from the respective isotropic resonance positions. The overall character and extent of the spectrum are qualitatively reproduced in simulations with 500 G < D < 540 G and E/D = 0.22 ± 0.2 (see Figure 4). The spread in zero-field splitting parameters has been modeled by Gaussian distributions in either D or E. Slightly better results are obtained with variations in the rhombic parameter with a fixed value for D. Residual discrepancies between the experimental and calculated spectra may be due to the inexact model for variations in the underlying parameters, to effects of the crystal field on the relative transition probabilities of ^{55}Mn hyperfine components (Allen, 1965), or to the perturbation approximation for zero-field splitting parameters of this magnitude (Reed & Markham, 1984).

The relative concentrations of the two central complexes may be estimated by a quantitative comparison of the signals that are unique to the enzyme–Mn(II)ATP–creatine complex with the analogous features in a spectrum for the enzyme–Mn(II)ATP complex with III. This comparison is possible because spectra for the enzyme–Mn(II)ATP–creatine complex and for the analogue complex have virtually the same line shape. Results from such experiments are included in Table I.

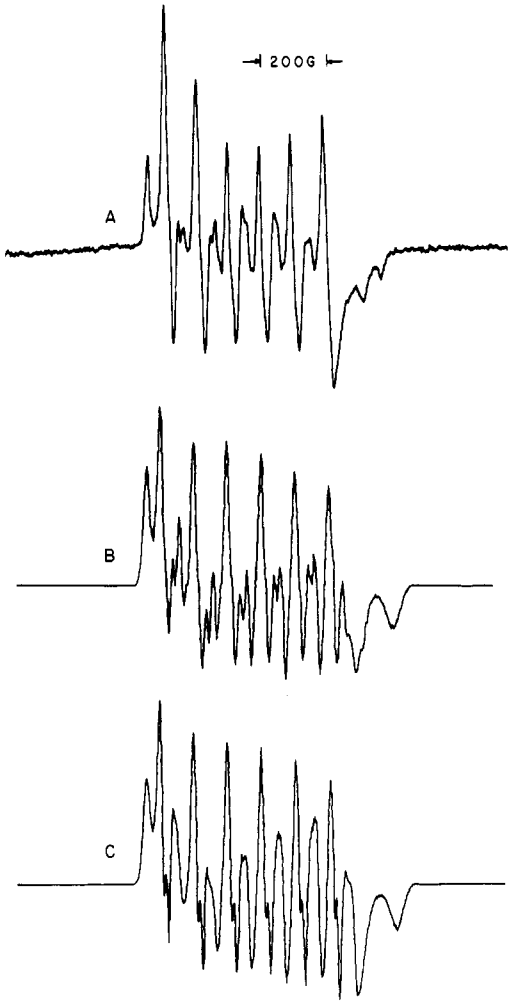


FIGURE 4: Comparison of experimental and simulated EPR spectra for the complex enzyme–Mn(II)ATP–1-(carboxymethyl)-2-iminoimidazolidin-4-one. (A) Experimentally observed spectrum. The solution contained 50 mM Hepes/KOH, pH 8.0, 1.5 mM $Mn(OAc)_2$, 6.6 mM ATP, 5.6 mM enzyme sites, and 30 mM 1-(carboxymethyl)-2-iminoimidazolidin-4-one. (B) Simulated spectrum. The following parameters were used in the simulation: D = 515 G, a line width of 5 G, and a ^{55}Mn hyperfine coupling constant of 92 G. A Gaussian distribution of 16 values for E was used with a center value of 122 G and a half-width of 32 G. A total of 1800 crystal orientations were sampled for each value of E. (C) Simulated spectrum. The following parameters were used in the simulation: E = 116 G, a line width of 6 G, and a ^{55}Mn hyperfine coupling constant of 92 G. A Gaussian distribution of nine values for D was used with a center value of 526 G and a half-width of 33 G. A total of 2500 crystal orientations were sampled for each value of D.

EPR Spectra for Complexes with ^{17}O-Labeled Nucleotides. In order to determine the coordination scheme between Mn(II) and the phosphate groups of the substrates in the two central complexes, solutions of the equilibrium mixture were made up from the ATP plus creatine direction with four separate forms of ATP: (1) unlabeled ATP, (2) [α,β-^{17}O,α-^{17}O]ATP, (3) [β,γ-^{17}O,β-^{17}O]ATP, and (4) [γ-^{17}O]ATP. The ^{17}O enrichment in each of the three forms of labeled ATP was 40 ± 4%. EPR spectra obtained for these samples are shown in

[8] 1-(Carboxymethyl)-2-iminoimidazolidin-4-one shows no activity under normal assay conditions, and there is no activity in samples incubated with a stoichiometric amount of enzyme at 0 °C for up to 30 min.

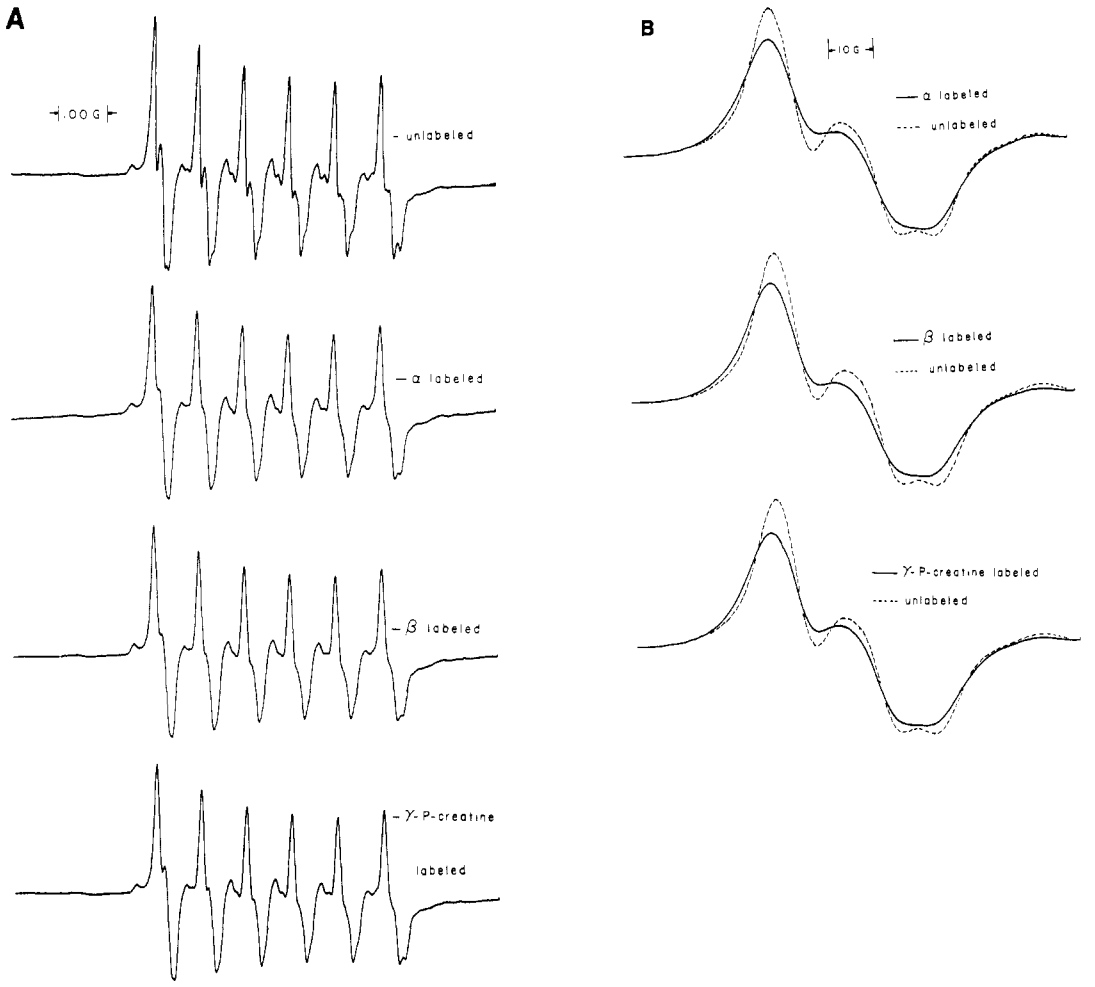


FIGURE 5: EPR spectra for the equilibrium mixture of substrates set up with ATP and with the three regiospecifically labeled forms of [^{17}O]ATP. The solutions were made up with 50 mM Hepes/KOH, pH 8.0, 1.5 mM $Mn(OAc)_2$, 5.6 mM enzyme sites, initial concentrations of ATP or of the labeled forms of ATP of 3.3 mM, and initial concentration of creatine of 6.3 mM. (A) The entire central fine-structure transition for Mn(II) in the complexes. (B) Comparison of the lowest field ^{55}Mn hyperfine component for unlabeled (dashed curves) and labeled (solid curves) samples. The spectra were recorded at −4 °C in the solution phase with identical spectrometer settings. ^{17}O enrichment in the three labeled forms of ATP was 40 ± 4%.

Figure 5. The spectra of Figure 5 show that the extent of ^{17}O-induced inhomogeneous broadening in all of the signals in the spectrum is virtually equivalent irrespective of the position of the ^{17}O label in the initial substrate.

The sharpest features in the spectrum for the equilibrium mixture are those associated with the enzyme–Mn(II)ADP–phosphocreatine complex. These sharp features are clearly broadened uniformly by ^{17}O in the α- and β-phosphates of ADP and by ^{17}O in phosphocreatine. The signals in the spectrum due to the enzyme–Mn(II)ATP–creatine complex that do not overlap with those from the companion species have somewhat lower signal-to-noise ratios. In order to be certain of the coordination assignments for the enzyme–Mn(II)-ATP–creatine complex, EPR measurements with the ^{17}O-labeled forms of ATP were repeated with the nonreactive analogue, III, in place of creatine. The uniformity of the ^{17}O-induced inhomogeneous broadening in the EPR spectra for these samples (see Figure 6) shows that ATP functions as an α,β,γ-tridentate ligand for Mn(II) at the active site of the enzyme. The zero-field splitting pattern for this complex is characteristic of an α,β,γ-tridentate complex of Mn(II) and ATP. The presence of this pattern in the spectrum for the equilibrium mixture of substrates together with the data of Figure 5 leaves no doubt that α,β,γ-tridentate Mn(II)ATP is present on the substrate side of the reaction. The data of Figure 5 also show that Mn(II) is coordinated to the α- and β-phosphate groups of ADP and to the phosphoguanidinium moiety of phosphocreatine in the product complex.[9]

Chemical Determination of the Equilibrium Constant. Although the EPR data provide an estimate of the equilibrium constant for enzyme-bound reactants with Mn(II) as the activating cation, chemical quenching methods were also used in order to compare values for Mn(II) with those for Mg(II). Solutions with compositions analogous to those used in the EPR experiments were quenched of enzymic activity, and the resulting solutions were assayed for ADP and ATP (see Experimental Procedures). Results of these experiments are summarized in Table I. The catalytic equilibrium constant

[9] The magnitude of the ^{17}O-induced inhomogeneous broadening is consistent with Mn(II) being coordinated to all phosphate groups all of the time. The reduction in the amplitudes of the signals as a result of the inhomogeneous broadening is quantitatively consistent with the intrinsic line width of the signals in the unlabeled samples, a superhyperfine coupling constant of 4 G, and an isotopic enrichment of 40%. Other scenarios that would give rise to equivalent inhomogeneous broadening would require the existence of several isomers of each central complex with the concentration of each species and coordination of Mn(II) in each combining to give rise to uniform ^{17}O effects from each labeled position. If such a scheme were present, one would expect to find separate spectra for each species. The magnitude of the ^{17}O-induced inhomogeneous broadening would be diminished, because a larger fraction of the signals would be due to complexes in which Mn(II) was not coordinated to the labeled phosphate group.

Table I: Distribution of ATP and ADP in Solutions of the Complete Reaction Mixture for Creatine Kinase either with Excess Concentration of Enzyme Active Sites over Total Nucleotide or with Catalytic Amounts of Enzyme

method	[ADP]/[ATP]	metal ion	temp (°C)
NMR[a]	1.0	Mg(II)	4
acid quench[b]	0.64 ± 0.05	Mn(II)	1
acid quench[b]	0.75 ± 0.08	Mg(II)	1
EPR[c]	0.84 ± 0.2	Mn(II)	0
EPR[c]	0.8 ± 0.3	Mn(II)	−5
acid quench[d] (catalytic amt of enzyme)	0.18 ± 0.04	Mn(II)	1

[a] Values taken from Nageswara Rao & Cohn (1981). Conditions were the following: pH 7.8; 5.1 mM $Mg(OAc)_2$; 4.2 mM enzyme sites; 3.9 mM initial ATP; 3.8 mM initial creatine. [b] Solutions contained the following: 5.6 mM enzyme sites; 4.1 mM $Mn(OAc)_2$ or $Mg(OAc)_2$; 50 mM Hepes/KOH, pH 8.0; 3.3 mM initial ATP; 5.8 mM initial creatine. Error limits are standard deviations from separate determinations. At 4 °C the turnover rate for the enzyme is about 7 s^{-1} (Travers et al., 1979), and with Mg(II) the interconversion of central complexes is 90 s^{-1} (Nageswara Rao & Cohn, 1981). The time constant for the quenching procedure is estimated to be less than 10 ms. [c] The ratio of the two central complexes was determined from EPR spectra as described in the text. Error limits are standard deviations. [d] Quench of solutions allowed to reach equilibrium with a catalytic amount of enzyme. Solutions contained the following: 0.1 mg/mL enzyme; 150 mM Hepes/KOH, pH 8.0; 4.0 mM $Mn(OAc)_2$; 3.2 mM initial ATP; 6.4 mM initial creatine. Solutions were incubated at 1 °C for 30 min.

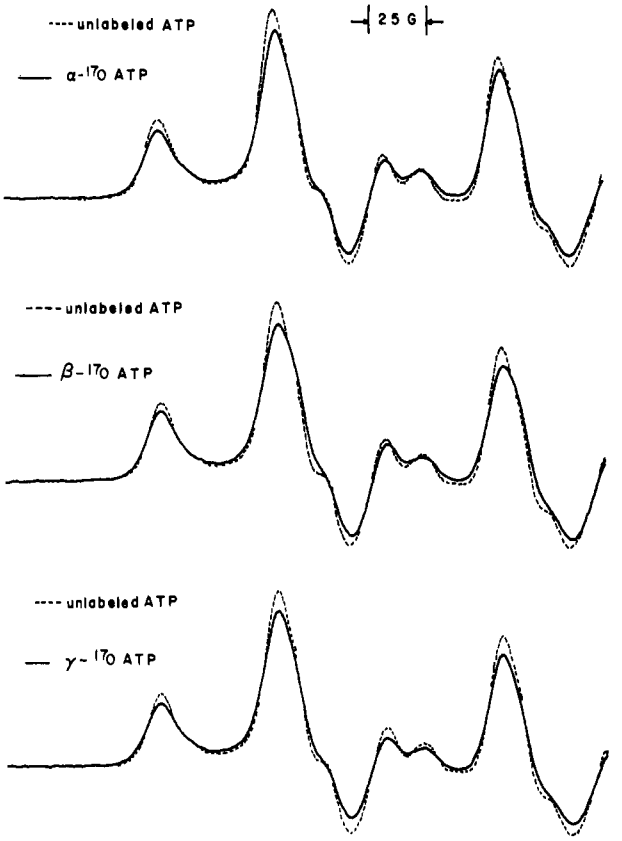


FIGURE 6: EPR spectra for the enzyme–Mn(II)ATP complex with 1-(carboxymethyl)-2-iminoimidazolidin-4-one (III) with ATP and with three regiospecifically labeled forms of [^{17}O]ATP. The samples contained 50 mM Hepes/KOH, pH 8.0, 6.5 mM enzyme sites, 1.8 mM $Mn(OAc)_2$, 30 mM 1-(carboxymethyl)-2-iminoimidazolidin-4-one, and 6.1 mM ATP or labeled ATP. The region of the spectrum shown includes the lowest field ^{55}Mn hyperfine component and part of the second lowest hyperfine component.

with Mg(II) as the activator has been estimated from ^{31}P NMR spectra for solutions of the equilibrium mixture (Nageswara Rao & Cohn, 1981). The results in Table I show good

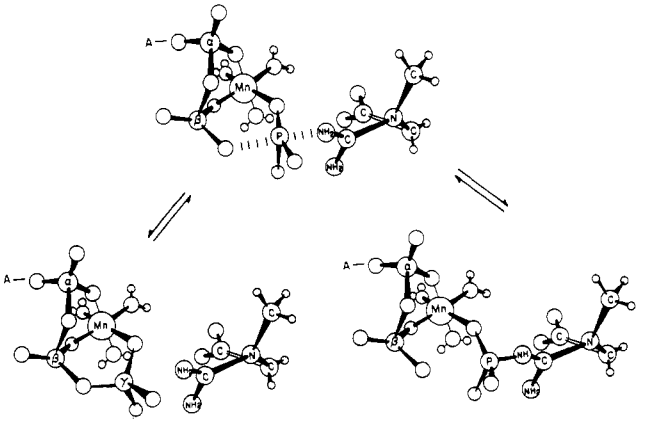


FIGURE 7: Schematic representation of the structures for the two central complexes of creatine kinase and for a plausible transition state. The screw sense of the α,β-chelate ring is taken from the results of Leyh et al. (1982). The screw sense of the β,γ-chelate ring in the ATP complex is based on the findings of Burgers & Eckstein (1980). The conformation of creatine is taken from the findings of Dietrich et al. (1980b). The number and geometry of placement of the three water ligands are taken from the results of Reed & Leyh (1980) for the anion-stabilized dead-end complexes. Results with ^{17}O-enriched water with the equilibrium mixture of substrates (data not shown) indicate that there are at least two (and probably three) water ligands to Mn(II) in the central complexes.

agreement among the values obtained from the chemical quenches and those obtained by ^{31}P NMR with Mg(II) present. Furthermore, the values obtained from the quench experiments with Mn(II) and with Mg(II) show that there is only a slight difference in the equilibrium constant with Mn(II) vs. Mg(II) as the activating cation. This observation provides further assurance that the two metal ions behave virtually isomorphically with this enzyme.

The EPR experiments were necessarily carried out for solutions in which the concentration of Mn(II) was less than that of the other components because the strong, isotropic signal for $Mn(H_2O)_6^{2+}$ would obscure signals for Mn(II) in the enzymic complexes. With a limiting amount of metal ion present, one expects the [ADP]/[ATP] ratio to reflect not only the catalytic equilibrium constant but also the relative affinities of the metal-free complexes of the nucleotides for the enzyme. The EPR signals arise only from complexes that contain Mn(II), and the [ADP]/[ATP] ratio in such complexes is expected to match that obtained for samples with a stoichiometric level of metal ion. The excellent agreement between the value for the equilibrium constant obtained with a stoichiometric level of Mn(II) and that obtained directly from the EPR data confirms this expectation.

Discussion

Results from the present EPR studies provide a clear picture of metal ion–substrate coordination in the two central complexes with creatine kinase. Previous studies of the anion-stabilized dead-end complexes (Reed & Leyh, 1980; Leyh et al., 1982) provided similar information for analogues of the transition state. Structures for the two central complexes and for a plausible transition state are shown schematically in Figure 7. These structures depict an economy of motion, for in order to proceed from either central complex to the transition state the triad of nonbridging oxygens on the transferring phospho group and the central phosphorus atom simply become coplanar. The phospho transfer step occurs with inversion of configuration (Hansen & Knowles, 1981), and such a transformation requires a Walden inversion of the reacting phospho group. The metal ion does not undergo any ligand exchange

reactions during the transformation from substrate to product central complexes, but coordination between Mn(II) and phosphocreatine would be relinquished prior to dissociation of either of the product species.

On the product side of the reaction Mn(II) is bound to the reactive groups of both substrates, ADP and phosphocreatine. This coordination scheme brings together two negatively charged groups. Here the metal ion neutralizes charge and provides an entropic advantage by serving as a template to which the reactive centers attach. On the substrate side, ATP is bound as an α,β,γ-tridentate complex with the metal ion. In this form the polyphosphate chain of ATP is coiled about the metal ion in a compact shape. The contiguous, six-membered chelate rings of the tridentate complex likely possess strain that may be relieved somewhat in the transition state where the scissile bond is stretched. The ring strain that develops in the substrate species may also be a factor in slightly destabilizing the ATP–creatine complex to give more nearly energetically balanced central complexes. It is not yet possible to ascertain whether or not the enzyme-bound Mn(II)ATP is predominantly a tridentate complex prior to binding of creatine. The striking transformation in the EPR spectra upon going from either ternary complex to the equilibrium mixture and upon formation of the inhibitory complex with ATP and III reflects a significant change in the environment of the active site. This creatine-induced structural change is also reflected both in solvent nuclear spin relaxation parameters (Reed & Cohn, 1972) and in chemical reactivity of active site residues (Milner-White & Watts, 1971). Travers et al. (1979) have also identified an isomerization step following binding of creatine to the ATP ternary complex. Results from all of these methods suggest that the active site becomes enclosed when both substrates are bound. The rapid equilibrium–random kinetic scheme for the reaction (Morrison & James, 1965) suggests that binding of either substrate to the enzymic complex of the other substrate can bring about this change in structure. At this stage of the catalytic cycle the intrinsic selectivity of the site for the specific stereoisomer of the tridentate complex of the metal–nucleotide substrate probably materializes.

The metal ion is directly involved in stabilizing the transition-state complex. The negative charge that develops in the phospho group is attracted to the pendent oxygens through their interactions with positive charges (Knowles, 1980; Reed et al., 1978; Kenyon & Reed, 1983). One of these interactions is coordination to the metal ion. The other two oxygens are likely involved with positively charged residues, e.g., the ϵ-ammonium group of lysine (James & Cohn, 1974), the guanidinium group of arginine, or both. These specific charge interactions simultaneously enhance susceptibility of the central phosphorus to nucleophilic attack (Knowles, 1980).

These EPR methods are ideally suited for structural studies of enzymic complexes in dynamic equilibrium. The EPR time scale effectively freezes the chemical interconversion step and provides a spectrum for each reactive complex instead of an exchange-averaged response. Thus, the EPR results provide the structural complement to the dynamic information that is available from ^{31}P NMR studies of the reactive complexes (Nageswara Rao & Cohn, 1981). The spectrum for the enzyme–Mn(II)ATP complex with III is characteristic of the α,β,γ-tridentate species of Mn(II)ATP, and knowledge of this diagnostic pattern should be useful in EPR studies of other enzymes that select this isomer for activity.

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