Stereochemical Control over Mn(II)–Thio versus Mn(II)–Oxy Coordination in Adenosine 5′-*O*-(1-Thiodiphosphate) Complexes at the Active Site of Creatine Kinase†

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ABSTRACT: The stereochemical configurations of the Mn(II) complexes with the resolved epimers of adenosine 5′-*O*-(1-thiodiphosphate) (ADPαS), bound at the active site of creatine kinase, have been determined in order to assess the relative strengths of enzymic stereoselectivity versus Lewis acid/base preferences in metal–ligand binding. Electron paramagnetic resonance (EPR) data have been obtained for Mn(II) in anion-stabilized, dead-end (transition-state analogue) complexes, in ternary enzyme–Mn^{II}ADPαS complexes, and in the central complexes of the equilibrium mixture. The modes of coordination of Mn(II) at P_α in the nitrate-stabilized, dead-end complexes with each epimer of ADPαS were ascertained by EPR measurements with (R_p)-[α-^{17}O]ADPαS and (S_p)-[α-^{17}O]ADPαS. The EPR spectrum for the complex with (R_p)-[α-^{17}O]ADPαS showed inhomogeneous broadening due to unresolved superhyperfine coupling from coordinated ^{17}O at P_α. By contrast, the EPR spectrum for Mn(II) in complex with (S_p)-[α-^{17}O]ADPαS is indistinguishable from that obtained for a matched sample with unlabeled (S_p)-ADPαS. A reduction in the magnitude of the ^{55}Mn hyperfine coupling constant in the spectrum for the complex containing (S_p)-ADPαS is indicative of Mn(II)–thio coordination at P_α. Thus, in nitrate-stabilized, dead-end complexes with either epimer of ADPαS, the Δ-screw-sense configuration of the α,β-chelate ring of Mn^{II}ADPαS is conserved—even though a less favorable Mn(II)–thio coordination occurs in the complex with (S_p)-ADPαS. EPR data for dead-end complexes with other anions in place of nitrate (i.e., nitrite, azide, formate, and thiocyanate) also show that a rigid selectivity for the Δ-screw-sense isomer of Mn^{II}ADPαS prevails over the hard Lewis base coordination preference of Mn(II). The strict selectivity of the enzyme for the Δ-screw-sense configuration of the α,β-chelate ring in the Mn(II)–adenosine α-phosphorothioate complexes is also expressed in the active central complexes of the enzyme-bound, equilibrium mixture of substrates. By contrast, in the ternary complex enzyme–$Mn^{II}(S_p)$-ADPαS, "nonproductive" binding of the Λ-screw-sense configuration predominates. The results indicate that a strict discrimination for a unique configuration of the metal–nucleotide substrate is expressed upon binding of all of the substrates to form the active complex (or an analogue thereof). This enzymic stereoselectivity provides sufficient binding energy to overcome an intrinsic preference for the hard Lewis acid Mn(II) to coordinate to the hard Lewis base oxygen.

The prochiral centers at the α- and β-phosphates in nucleoside triphosphates and at the α-phosphate in nucleoside diphosphates lead to stereoisomeric polymorphs (screw-sense isomers) in the resulting metal–nucleotide complexes. Enzymes typically exhibit selectivities for the screw-sense configurations of their metal–nucleotide substrates, and these selectivities have been investigated by various chemical, kinetic, and spectroscopic methodologies (Cleland & Mildvan, 1979; Jaffe & Cohn, 1979; Dunaway-Mariano & Cleland, 1980; Leyh et al., 1982). Isomers of nucleoside phosphorothioates (with stereospecific substitution of sulfur for one of the diastereotopic oxygens at a prochiral center) have proven to be useful probes for such stereochemical investigations (Cohn, 1982; Eckstein, 1985). These thio analogues are accepted as substrates for many enzymes, and the influences of the stereo- and regiospecifically placed sulfur substituent on binding and reactivity have been reported for numerous enzymes (Eckstein, 1985; Cohn, 1982). The presence of the sulfur substituent in the polyphosphate moiety creates perturbations that include alterations in charge distribution (Frey & Sammons, 1985), steric effects, altered H-bonding interactions (Eckstein, 1985), and a bias in the preferred stereochemical configurations of the respective complexes formed with divalent metal ions (Cohn, 1982). The latter effect can be influenced by the Lewis acid properties of the companion metal ion. Thus, Jaffe and Cohn (1978, 1979) exploited differences in the tendencies of different species of metal ion for coordination to sulfur versus oxygen to reverse the epimer selectivity of yeast hexokinase

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for ATPβS.[1] A reversal in the epimer selectivity of an enzyme for ATPβS (or for ATPαS) with hard versus soft metal ions provides an indication of the position of coordination of the metal ion (α or β, or α and β) as well as information on the preferred stereochemical configuration of the metal–nucleotide complex.

The methodology introduced by Jaffe and Cohn (1979) has since been applied to many other enzymes (Cohn, 1982; Eckstein, 1985). Results from steady-state kinetic assays have not always yielded to a straightforward interpretation, because the degrees of epimer selectivities observed with different enzymes have been variable (Jaffe et al., 1982; Eckstein, 1985). In some cases, kinetic data indicate that stereochemical requirements may actually override the substantial preference of the hard Lewis acid Mg(II) for binding to the hard Lewis base oxygen (Connolly et al., 1980; Armstrong et al., 1979). Pecoraro et al. (1984) have noted that this kinetic methodology works, in part, because of inhibition due to appreciable nonproductive binding to the enzyme of the wrong screw-sense isomer of the less active epimer. Hence, these authors pointed out the importance of interpretations based on metal ion reversals in the kinetic parameter, V_{max}/K_m, as opposed to reversals in V_{max} or in initial velocities.

In contrast to the substantial volume of data available regarding the kinetic behavior of the nucleoside phosphorothioates, confirmation of the structures of the respective enzymic complexes through independent means has been lacking. Complexes of creatine kinase with nucleoside phosphorothioates provide attractive candidates for structural investigations. The enzyme accepts the adenosine phosphorothioates as substrates and exhibits an intriguing pattern of metal ion dependent stereoselectivity—especially with respect to the epimers of the α-phosphorothioates (Burgers & Eckstein, 1980). With Mg(II) as the activator, creatine kinase processes either epimer of ATPαS with little difference in K_m and with only an 8-fold reduction in V_{max} for the S_p isomer. This modest selectivity for (R_p)-ATPαS [Δ screw sense for the α,β-chelate ring with Mg(II)–oxy coordination at P_α] was reversed (in initial velocity values) with Mn(II), Co(II), and Cd(II) as activators. Mn(II) is a hard Lewis acid (Pearson, 1968), and the relaxation in stereoselectivity observed with this ion presents an ambiguity with respect to two contradictory interpretations. The enzyme might possess a low selectivity for the α-screw-sense configuration of the α,β-chelate ring of the metal–ATP substrate such that either Δ- or Λ-screw-sense isomers bind and undergo reaction. Alternatively, the stereoselective pressure of the enzyme may be sufficient to overcome the preference of a hard metal ion, such as Mn(II) or Mg(II), to coordinate with an oxygen, rather than with a sulfur donor ligand.

In the present investigation, the resolved epimers of ADPαS and the α-^{17}O-labeled forms of these analogues have been used together with Mn(II) in EPR spectroscopic investigations of metal coordination at P_α of the adenosine α-phosphorothioates in complexes with creatine kinase. Experiments with ternary enzyme–Mn^{II}ADPαS complexes, with transition-state analogue complexes, and with the central complexes of the enzyme-bound, equilibrium mixture of substrates reveal the likelihood of a sequential expression of a rigid stereoselectivity in binding events leading to catalysis.

Experimental Procedures

Materials. Creatine kinase was isolated from rabbit skeletal muscle by the method of Kuby et al. (1954), with the following chromatographic procedures being substituted for the original crystallization step. The enzyme was subjected to chromatography over a column (5 × 12 cm) of Blue Sepharose CL-6B (Pharmacia) and concentrated to approximately 40 mg/mL (Hershenson et al., 1986). Prior to EPR measurements the enzyme was chromatographed on a column (2.5 × 95 cm) of Sephacryl S-200 (Pharmacia) with an equilibration and elution buffer of 50 mM Hepes/KOH, pH 8.0, and was subsequently concentrated with a collodion bag apparatus. Enzyme used in the infrared measurements was treated as described, except for substitution of 10 mM Tris-HCl, pH 8.0, with 50 mM creatine, for the Hepes buffer. Preparations of the enzyme had specific activities in excess of 40 IU/mg in the coupled assay with pyruvate kinase and lactate dehydrogenase at 25 °C. Phosphocreatine was obtained from Sigma as the disodium salt. Water enriched in ^{17}O (50 atom %) was obtained from Monsanto.

The two epimers of [α-^{17}O]ADPαS were prepared from the respective β-cyanoethyl derivatives by using methodology developed for synthesis of the epimers of ADP with stereospecific incorporation of isotopes of oxygen at P_α (Sammons & Frey, 1982; Leyh et al. 1982). The R_p and S_p epimers of β-cyanoethyl-[α-^{17}O]ADPαS were separated by reversed-phase HPLC, and the β-cyanoethyl blocking group was removed by treatment with base (Sammons & Frey, 1982). (S_p)-ADPαS was prepared enzymatically from AMPS and ATP in the reaction catalyzed by rabbit muscle adenylate kinase (Sheu & Frey, 1977). (R_p)-ADPαS was separated from a racemic mixture of ADPαS by selective conversion of the (S_p)-ADPαS into (S_p)-ATPαS in the reaction catalyzed by rabbit muscle pyruvate kinase, under conditions where the concentration of phosphoenolpyruvate was limited to a slight excess over the concentration of (S_p)-ADPαS present in the mixture (Jaffe, 1979). Labeled and unlabeled nucleotides were purified by anion-exchange chromatography on DEAE-Sephadex (A-25) with a gradient of triethylammonium bicarbonate (20–600 mM). Chemical purity of the nucleotides was assessed by HPLC using a Whatman Partisil 10-SAX analytical column eluted isocratically with 0.4 M $NH_4H_2PO_4$. Stereochemical purity of the labeled and unlabeled nucleoside phosphorothioates was assessed by HPLC using a Pharmacia PepRPC (HR 5/5) reversed-phase analytical column eluted isocratically with 60 mM KH_2PO_4/KOH, pH 6.0. Isotopic enrichment of the α-^{17}O-labeled phosphorothioates was quantitated by mass spectrometry following degradation to AMPS and conversion to triethyl phosphate (Iyengar et al., 1984).

Nitrate enriched in ^{17}O was prepared by exchange of nitrous acid in $H_2{}^{17}O$ (Van Etten & Risley, 1981), followed by neutralization with KOH and subsequent oxidation of the ^{17}O-enriched nitrite with a slight excess of bromine. A control sample of unlabeled nitrate was prepared, in parallel, in an analogous fashion.

Spectroscopic Measurements. EPR spectra were obtained at 35 GHz (Q-band) with a Varian E-109Q spectrometer. The spectrometer was interfaced with an IBM PC/AT for data acquisition. EPR spectral simulations were carried out as described previously (Reed & Markham, 1984; Moore & Reed, 1985). The dissociation constants for complexes of Mn^{II}ADPαS from the ternary complexes with the enzyme

[1] Abbreviations: ATPβS, adenosine 5′-*O*-(2-thiotriphosphate); ATPαS, adenosine 5′-*O*-(1-thiotriphosphate); ADPαS, adenosine 5′-*O*-(1-thiodiphosphate); AMPS, adenosine 5′-*O*-thiomonophosphate; HPLC, high-performance liquid chromatography; EPR, electron paramagnetic resonance; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; IR, infrared; NMR, nuclear magnetic resonance; $A(^{55}Mn)$, ^{55}Mn hyperfine coupling constant.

were determined from titrations in which the enhancement in the longitudinal relaxation rate of water protons served as a measure of binding of $Mn^{II}ADP\alpha S$ to the enzyme (Reed et al., 1970). The longitudinal relaxation times (T_1's) of water protons were measured at 24 MHz with a pulsed NMR spectrometer, as described previously (Reed & Leyh, 1980). The titration curves were analyzed with a nonlinear least-squares method (Reed et al., 1970). The dissociation constants for the binary, $Mn^{II}ADP\alpha S$, complexes were determined by EPR at 9 GHz (X-band) by the method of Cohn and Townsend (1954). Infrared spectra were obtained with IBM IR/44 and with Nicolet 5PC interferometers. Samples were contained in Barnes demountable cells with CaF_2 windows and 30-μm spacers.

EPR Methodology with Mn(II). EPR spectra for Mn(II) bound in macromolecular complexes exhibit powder pattern line shapes for this $S = {}^5/_2$ spin system. The dominant signals in the spectrum are due to the central fine structure transition ($M_s = {}^1/_2 \leftrightarrow M_s = -{}^1/_2$) (Reed & Markham, 1984). Additional splitting of the component signals of the ^{55}Mn ($I = 5/2$) hyperfine sextet for this transition reflects the symmetry and magnitude of the zero-field splitting interaction. The zero-field splitting interaction (represented in the spin Hamiltonian as a tensor) is an indicator of the symmetry of the electronic environment of the ion, which is influenced by the composition of ligands and their geometric arrangement in the coordination sphere. The magnitude of the hyperfine interaction between the unpaired electron spin and the nuclear spin of ^{55}Mn is useful in the present investigation, because this parameter is an indicator of the unpaired spin density centered on the metal ion and responds to the degree of covalency in the metal–ligand bond (Reed & Markham, 1984). For example, the ^{55}Mn hyyperfine coupling constant in model complexes is reduced (relative to that for oxygen or nitrogen ligands) by the presence of sulfur ligands (Van Wieringen, 1955; Lifshitz & Francis, 1982). In addition, the effects of sulfur ligands on the magnitude of $A(^{55}Mn)$ appear to be additive (Lifshitz & Francis, 1982). The superhyperfine coupling interaction between the unpaired electron spin of Mn(II) and the nuclear spins of ligand nuclei (e.g., ^{17}O) provides an unequivocal means for identification of the ligand donor groups. The magnitude of superhyperfine coupling between the unpaired electron spin of Mn(II) and the nuclear spin of ^{17}O ($I = {}^5/_2$) is smaller than the intrinsic widths of the EPR signals. The superhyperfine coupling for directly coordinated ^{17}O ligands can, however, be detected as an inhomogeneous broadening of the EPR signals whenever the intrinsic widths of the signals are of the same order as the width of the superhyperfine manifold (Reed & Leyh, 1980).

Sample Preparation. Samples for which EPR line shapes were examined for ^{17}O-induced inhomogeneous broadening were prepared from a common stock solution that contained all of the components except for the ADPαS. Quantitative additions of concentration-matched solutions of labeled or unlabeled forms of the appropriate epimer of the nucleoside phosphorothioate to aliquots of the stock solution provided an analytical precision of ±2% with respect to the amplitudes of the EPR signals for Mn(II). Spectra for some complexes were obtained from samples in the frozen state, and the reproducibility of the amplitudes of EPR signals for the frozen samples was of the order of ±5%.

Results and Discussion

EPR Data for the Nitrate- and Nitrite-Stabilized, Dead-End Complexes. The dead-end complex, enzyme–$Mn^{II}ADP$–creatine, is stabilized by a specific class of anions

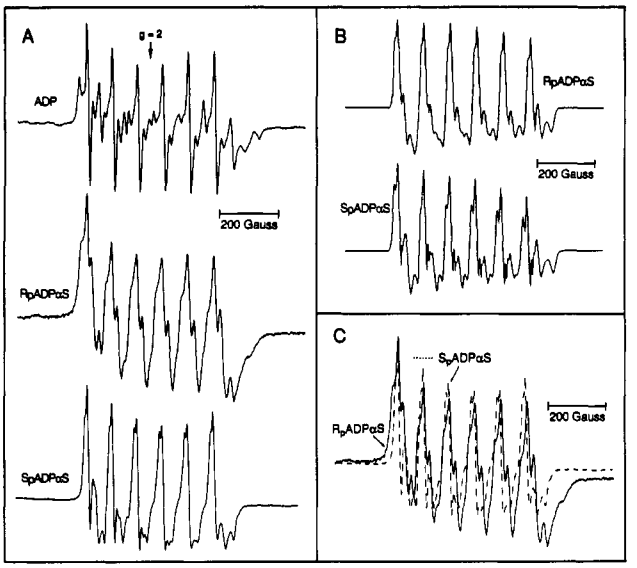

FIGURE 1: Comparison of the experimental (A and C) and simulated (B) EPR spectra (35 GHz) for the complex creatine kinase–$Mn^{II}ADP(\alpha S)$–nitrate–creatine with ADP, (R_p)-ADPαS, or (S_p)-ADPαS. Spectra represent the central fine structure transition ($M_s = {}^1/_2 \leftrightarrow M_s = -{}^1/_2$). Experimental spectra (A and C) were recorded with samples in the liquid phase (−5 °C). Solutions contained 5.4 mM enzyme active sites, 2.1 mM $Mn(CH_3COO)_2$, 6.2 mM nucleotide, 62 mM KNO_3, saturated creatine, and 50 mM Hepes/KOH, pH 8.0. The simulated spectrum for the complex with (R_p)-ADPαS (B, top) was modeled by using the following spin-Hamiltonian parameters: D = 325 G, E = 65 G, line width = 4 G, and $A(^{55}Mn)$ = 90 G. The simulation also included a Gaussian distribution of nine values for D with a half-width of 25 G. The simulated spectrum for the complex with (S_p)-ADPαS (B, bottom) was modeled by using the following parameters: D = 375 G, E = 70 G, line width = 4 G, and $A(^{55}Mn)$ = 88 G. The simulation also included a Gaussian distribution of nine values for D with a half-width of 22 G.

that possess planar or linear geometries (Milner-White & Watts, 1971; Kenyon & Reed, 1983). The stabilizing anions occupy the position of the migrating phospho group (Reed et al., 1978; Reed & Leyh, 1980), to give a structure analogous to the transition state (Milner-White & Watts, 1971). Among these complexes those with nitrate and nitrite have the highest thermodynamic stabilities (Milner-White & Watts, 1971; Reed & McLaughlin, 1973).

EPR spectra obtained for Mn(II) in the nitrate-stabilized, dead-end complexes with ADP and with the two epimers of ADPαS are compared in Figure 1A. Differences in the EPR patterns for the three complexes indicate that the zero-field splitting tensor for Mn(II) senses both the presence of the sulfur substituent at P_α and the stereochemistry of this substitution. Quantitative differences in the zero-field splitting tensor for Mn(II) in the complexes with the epimers of ADPαS are elaborated in simulations of the respective EPR patterns (Figure 1B). The other notable difference in EPR spectral properties for Mn(II) in the complexes with the epimers of ADPαS is a decrease (1.9 G) in the magnitude of the ^{55}Mn hyperfine coupling constant in the spectrum for the complex with (S_p)-ADPαS. The decrease in $A(^{55}Mn)$ leads to an approximate 10-G contraction in the overall width of the ^{55}Mn hyperfine sextet, illustrated in Figure 1C.

EPR spectra for complexes with the α-^{17}O-labeled forms of (R_p)-ADPαS and (S_p)-ADPαS (Figure 2) were obtained in order to determine the mode of coordination of Mn(II) at P_α in the complexes. The EPR data reveal an ^{17}O-induced inhomogeneous broadening of the signals for Mn(II) in the complex with (R_p)-[α-^{17}O]ADPαS, whereas the spectrum for the complex with (S_p)-[α-^{17}O]ADPαS is superimposable with that obtained for a sample with unlabeled (S_p)-ADPαS. The

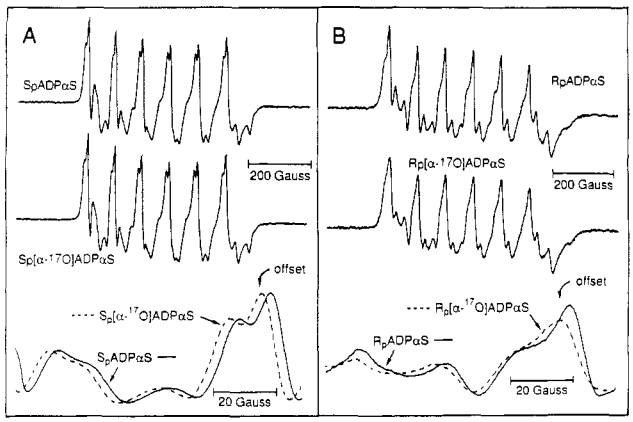

FIGURE 2: Comparison of EPR spectra (35 GHz) for the complex creatine kinase–Mn^{II}ADP(αS)–nitrate–creatine with (S_p)-ADPαS (S_p)-[α-^{17}O]ADPαS, (R_p)-ADPαS, or (R_p)-[α-^{17}O]ADPαS. Upper spectra represent the central fine structure transition ($M_s = {}^1/_2 \leftrightarrow M_s = {}^1/_2$). Lower spectra respresent a 10-fold expansion of the second lowest field hyperfine line in the central fine structure transition. Solutions were supercooled in the liquid phase (−12 °C) and contained 6.1 mM enzyme active sites, 1.6 mM $Mn(CH_3COO)_2$, 6.6 mM nucleotide, 26 mM KNO_3, saturated creatine, and 50 mM Hepes/KOH, pH 8.0. Isotopic enrichment of the α-^{17}O-labeled phosphorothioates was 50 ± 5%.

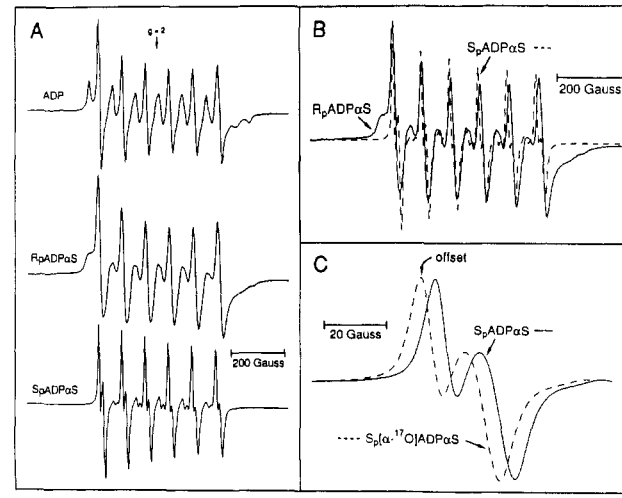

FIGURE 3: Comparison of EPR spectra (35 GHz) for the complex creatine kinase–Mn^{II}ADP(αS)–nitrite–creatine with ADP, (R_p)-ADPαS, or (S_p)-ADPαS (A and B), and with (S_p)-ADPαS or (S_p)-[α-^{17}O]ADPαS (C). Spectra represent the central fine structure transition ($M_s = {}^1/_2 \leftrightarrow M_s = {}^{-1}/_2$) (A and B) and a 10-fold expansion of the lowest field hyperfine line in the fine structure transition for the complex with (S_p)-ADPαS or (S_p)-[α-^{17}O]ADPαS (C). Solutions were in the liquid phase (−4 °C) and contained (A and B) 4.9 mM enzyme active sites, 2.1 mM $Mn(CH_3COO)_2$, 6.2 mM nucleotide, 64 mM $NaNO_2$, saturated creatine, and 50 mM Hepes/KOH, pH 8.0; or (C) 4.2 mM enzyme active sites, 1.65 mM $Mn(CH_3COO)_2$, 4.1 mM nucleotide, 79 mM $NaNO_2$, saturated creatine, and 50 mM Hepes/KOH, pH 8.0. Isotopic enrichment of the (S_p)-[α-^{17}O]ADPαS was 50 ± 5%.

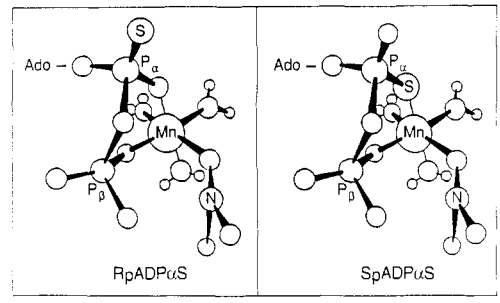

FIGURE 4: Schematic representation of the structures of the Mn(II)–nucleotide chelates bound at the active site of creatine kinase in the nitrate-stabilized, dead-end complexes with either (R_p)-ADPαS or (S_p)-ADPαS. In both structures, the α,β-chelate ring has the Δ-screw-sense configuration.

presence of nitrate in the coordination sphere of Mn(II) in all three complexes was confirmed in separate EPR measurements with ^{17}O-enriched nitrate (data not shown). The data of Figure 2 show that Mn(II) coordinates to the oxygen at P_α of (R_p)-ADPαS to give the Δ configuration of the α,β-chelate ring. The EPR data also show that Mn(II) does not coordinate to the oxygen at P_α of (S_p)-ADPαS. The absence of Mn(II)–oxy coordination at P_α of (S_p)-ADPαS implies that the metal ion coordinates to the sulfur substituent at P_α to give the preferred Δ configuration of the α,β-chelate ring. This inference is supported by the observation of a decrease in the magnitude of the ^{55}Mn hyperfine coupling constant (noted above) in the EPR spectrum for the complex with (S_p)-ADPαS. Thus, the reduction in the magnitude of $A(^{55}Mn)$, observed in spectra for samples with (S_p)-ADPαS, is an indicator of Mn(II)–thio coordination in this complex.

EPR spectra obtained for the respective dead-end complexes with nitrite in place of nitrate are shown in Figure 3A. In the series with nitrite, the spectrum for the complex with (S_p)-ADPαS stands out, because the line shape switches from one characteristic of rhombic symmetry [for complexes with ADP and with (R_p)-ADPαS] to one characteristic of axial symmetry. Again, Mn(II)–thio coordination at P_α in the complex with (S_p)-ADPαS is indicated by the decrease in the magnitude of the ^{55}Mn hyperfine coupling constant [$\Delta A(^{55}Mn)$ = −2.0 G] (Figure 3B). Further, spectra for samples with (S_p)-ADPαS and with (S_p)-[α-^{17}O]ADPαS are indistinguishable (Figure 3C)—confirming the absence of Mn(II)–oxy coordination at P_α.

EPR data for complexes of ADPαS with nitrate and with nitrite as the stabilizing anions show that the Δ configuration of the α,β-chelate ring of Mn^{II}ADPαS is preserved in complexes with either epimer. In the case of (S_p)-ADPαS, this stereospecific binding comes at the expense of a less favorable Mn(II)–thio coordination. These structural conclusions are summarized for the nitrate-stabilized, dead-end complexes by the schematic drawings shown in Figure 4.

EPR Data for Dead-End Complexes with Other Anions. Spectra for the dead-end complexes with ADPαS stabilized with other anions in place of nitrate were examined to ascertain whether or not the stereoselectivity would prevail in complexes with lower thermodynamic stabilities and with differing geometries for the stabilizing anion. The magnitudes of the ^{55}Mn hyperfine coupling constants obtained from spectra for dead-end complexes with (S_p)-ADPαS and azide [$\Delta A(^{55}Mn)$ = −1.9 G], bicarbonate [$\Delta A(^{55}Mn)$ = −2.0 G], and formate [$\Delta A(^{55}Mn)$ = −1.8 G] are reduced, due to the presence of Mn(II)–thio coordination. Thus, the selectivity of the enzyme for the Δ configuration of the α,β-chelate ring predominates over the preferred Mn(II)–oxy coordination in each of these complexes with (S_p)-ADPαS.

EPR and IR Data for the Thiocyanate-Stabilized, Dead-End Complex. EPR spectra obtained for dead-end complexes with thiocyanate as the stabilizing anion are shown in Figure 5A,B. In this series, the spectrum for the sample with (S_p)-ADPαS also shows a pattern that differs substantially from those obtained from samples with ADP and with (R_p)-ADPαS. The spectrum for the complex with (S_p)-ADPαS actually consists of two sets of signals (Figure 5C). One set of signals ("N") has approximately the same line shape (except for a reduced ^{55}Mn hyperfine coupling) as in spectra for the analogous complexes with ADP and with (R_p)-ADPαS.

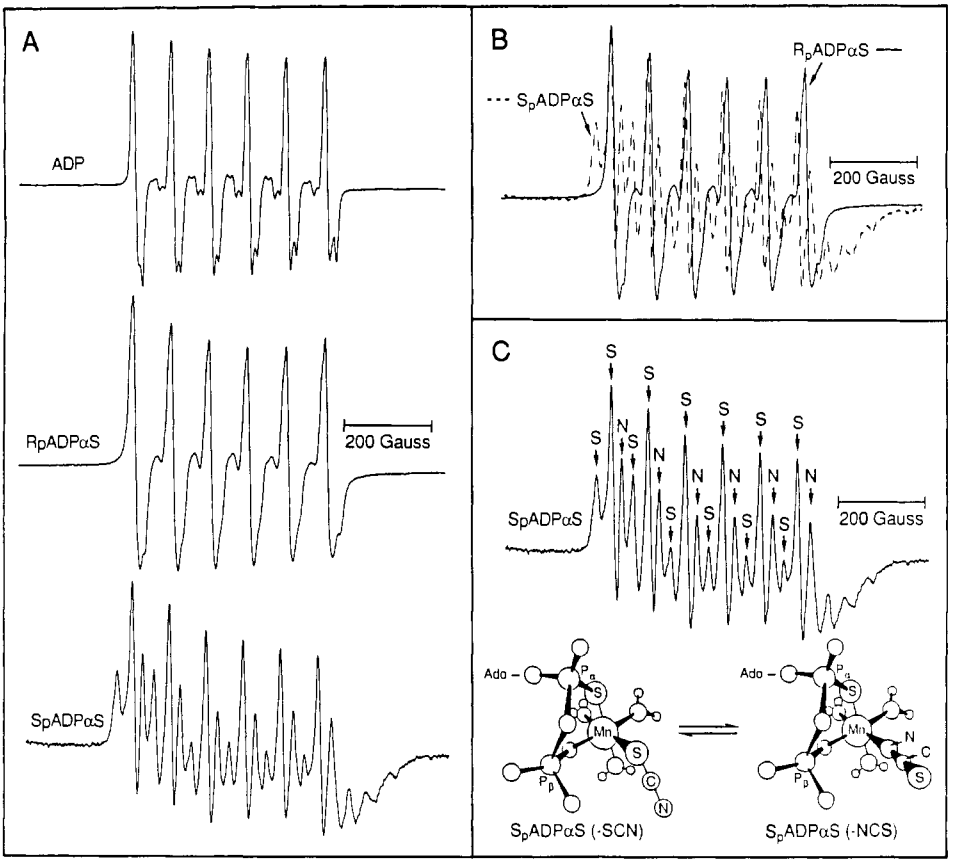

FIGURE 5: Comparison of EPR spectra (35 GHz) for the complex creatine kinase–Mn^{II}ADP(αS)–thiocyanate–creatine with ADP, (R_p)-ADPαS, or (S_p)-ADPαS. Spectra represent the central fine structure transition ($M_s = {}^1/_2 \leftrightarrow M_s = -{}^1/_2$). Solutions were in the liquid phase (−5 °C) and contained 5.4 mM enzyme active sites, 2.1 mM $Mn(CH_3COO)_2$, 6.2 mM nucleotide, 86 mM KSCN, saturated creatine, and 50 mM Hepes/KOH, pH 8.0. Signals in the spectrum for the complex with (S_p)-ADPαS (C) have been tentatively assigned as follows. Transitions arising predominantly from the complex containing Mn(II)–SCN coordination are labeled "S", while those arising predominantly from the complex containing Mn(II)–NCS coordination are labeled "N". The proposed linkage isomerization of the SCN^- ligand in the complex with (S_p)-ADPαS is illustrated by the schematic representation of the structures of the respective Mn^{II}(S_p)-ADPαS–thiocyanate complexes (C, bottom).

The line shape for the other set of signals ("S") corresponds to a much larger zero-field splitting anisotropy. Furthermore, the magnitude of the ^{55}Mn hyperfine coupling constant in this set is reduced [$\Delta A(^{55}Mn) = -3.8$ G] to a greater extent (approximately 2-fold) than in spectra for complexes with (S_p)-ADPαS stabilized by other anions. These observations suggest that the two sets of signals in the spectrum may be from the two species that result from linkage isomerization of the thiocyanate ligand. The signals labeled "S" are tentatively assigned to complexes in which thiocyanate coordinates to Mn(II) through the sulfur whereas the signals marked "N" are assigned to complexes with nitrogen coordination of thiocyanate. The species with the more anisotropic spectrum ("S" signals) is more abundant because this set of derivative signals, from a more anisotropic powder line shape, has higher amplitudes than the more isotropic set ("N" signals).

Thiocyanate is an ambidentate ligand, and the propensity for sulfur versus nitrogen coordination in complexes with this ligand is normally dictated by the Lewis acid character of the metal ion (Burmeister, 1975). Metal ions with hard Lewis acid character can, however, be "softened" through coordination to ligands with soft Lewis base character (Jorgensen, 1964) (i.e., the presence of one soft ligand promotes coordination of additional soft ligands in the phenomenon of "ligand symbiosis"). It is therefore possible that coordination of Mn(II) to the sulfur at P_α of (S_p)-ADPαS promotes an isomerization in the linkage of Mn(II) to thiocyanate (i.e., coordination through sulfur rather than through nitrogen) in a major fraction of the complexes. The proposed linkage isomerization of the thiocyanate ligand in the complex with (S_p)-ADPαS is illustrated by the schematic drawings of the respective structures shown in Figure 5C.

The frequency of the carbon–nitrogen stretching vibration of thiocyanate (ν_3 normal mode) is sensitive to metal coordination. This vibration occurs at 2093 cm^{-1} for thiocyanate bound to Mn(II) in the complex enzyme–Mn^{II}ADP–NCS–creatine (Reed et al., 1978) and in the Mn^{II}NCS complex in free solution (Fronaeus & Larsson, 1962a,b). The reliability of the frequency of this normal mode in responding to linkage isomerism is less certain (Burmeister, 1975). IR spectra for solutions of the complex enzyme–Mn^{II}(S_p)-ADPαS–SCN–creatine, and for the corresponding complex with (R_p)-ADPαS, exhibit a C–N stretching frequency for bound thiocyanate at 2099 ± 2 cm^{-1}. The absorption at 2099 cm^{-1} from the sample with (S_p)-ADPαS may be due to the fraction of the complex with Mn(II)–nitrogen linkage. No other new bands were resolved in the 2000–2200-cm^{-1} region of the spectrum for this sample. The extinction coefficient for the C–N absorption is expected to be substantially smaller for S-linked thiocyanate (Burmeister, 1975), and failure to observe any new IR absorptions from an S-linked species may be due to the lower absorptivity of this linkage isomer. Thus, the IR data are inconclusive with respect to the proposed linkage isomerism.

EPR Data for the Ternary Complexes. Binding data (see Experimental Procedures) show that the Mn(II) complexes with (R_p)-ADPαS and with (S_p)-ADPαS have affinities for the enzyme (K_d's ~0.03 mM) that are similar to that for the corresponding complex with ADP (K_d ~0.06 mM). Neither

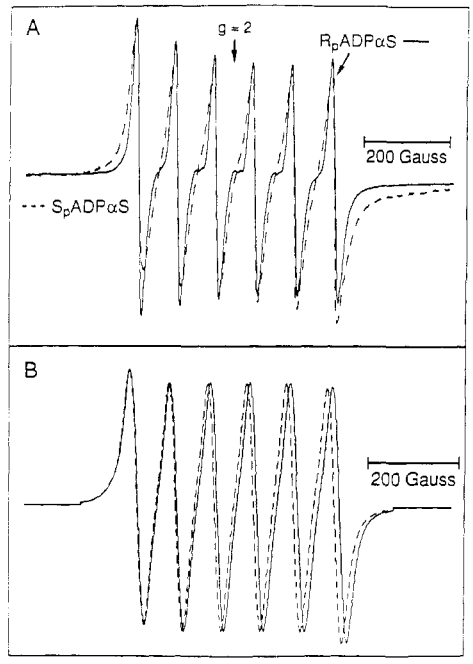

FIGURE 6: Comparison of the experimental (A) and simulated (B) EPR spectra (35 GHz) for the ternary complex creatine kinase–Mn^{II}ADPαS with (R_p)-ADPαS or (S_p)-ADPαS. Spectra represent the central fine structure transition ($M_s = {}^1/_2 \leftrightarrow M_s = -{}^1/_2$). Experimental spectra (A) were recorded on samples in the liquid phase (−3 °C). Solutions contained 6.4 mM enzyme active sites, 2.0 mM $Mn(CH_3COO)_2$, 6.2 mM nucleotide, and 50 mM Hepes/KOH, pH 8.0. The simulated line shapes (B) were modeled by using an isotropically averaged zero-field splitting with a line width = 25 G and $A(^{55}Mn)$ = 90 G (—) or 88 G (---).

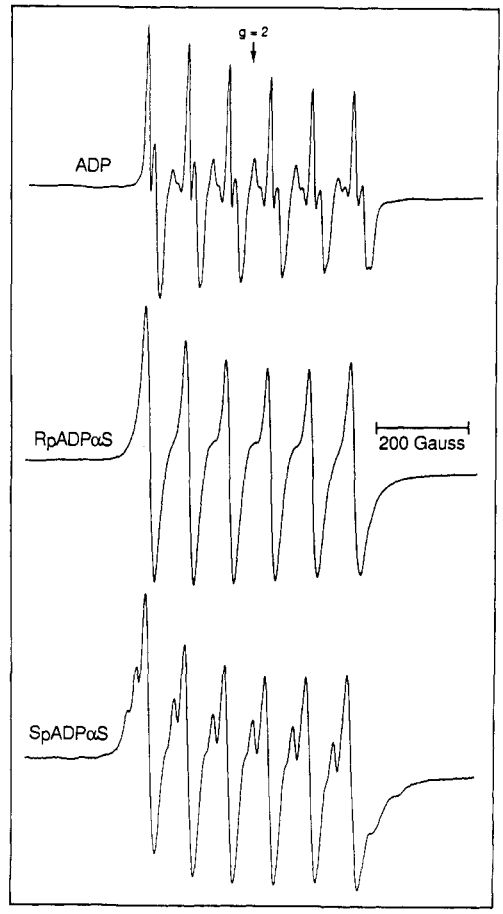

FIGURE 7: Comparison of EPR spectra (35 GHz) for the equilibrium mixture of bound substrates, generated from the complex enzyme–Mn^{II}ADP(αS)–phosphocreatine with ADP, (R_p)-ADPαS, or (S_p)-ADPαS. Spectra represent the central fine structure transition ($M_s = {}^1/_2 \leftrightarrow M_s = {}^1/_2$). Solutions were in the frozen state (−22 °C) and contained 5.5 mM enzyme active sites, 2.1 mM $Mn(CH_3COO)_2$, 4.7 mM nucleotide, 23 mM phosphocreatine (initial concentration), and 50 mM Hepes/KOH, pH 8.0.

the presence of the thio substituent nor its stereochemical position interferes with binding of the Mn^{II}ADPαS in the simple ternary complexes with the enzyme.

EPR signals for Mn(II) in the simple ternary complexes with enzyme and ADPαS are too broad for ready observation of inhomogeneous broadening from superhyperfine coupling to ^{17}O. The influence of sulfur ligands on the ^{55}Mn hyperfine coupling constant can, however, be detected in such spectra. EPR spectra for solutions of the enzyme–Mn^{II}ADPαS complexes are compared in Figure 6A. A superposition of spectra simulated with the expected difference (2 G) in $A(^{55}Mn)$, if the complex with the S_p epimer possessed Mn(II)–thio coordination, is shown in Figure 6B. The simulations indicate that Mn(II)–thio coordination would appear as a measurable contraction in the hyperfine sextet if such coordination were present. Although the spectrum for the complex with (S_p)-ADPαS has somewhat broader signals than those for the complex with (R_p)-ADPαS, the span of the ^{55}Mn hyperfine sextet is the same in spectra for both epimers of ADPαS. The EPR data indicate that the Λ isomer of Mn^{II}(S_p)-ADPαS is the predominant species present in the simple ternary complex. This species is an example of a "nonproductive" complex with the wrong screw-sense configuration of the metal–ADPαS chelate ring. The stereoselective binding interactions at this stage (i.e., the absence of the cosubstrate) of the catalytic cycle are insufficient to promote Mn(II)–thio coordination at P_α of (S_p)-ADPαS.

EPR Data for the Equilibrium Mixture of Substrates. Experiments on the equilibrium mixture of bound substrates and products were initiated in order to determine whether the stereoselectivity of the enzyme for the Δ-screw-sense configuration of the α,β-chelate ring would appear as a predominant interaction in the central (i.e., ground-state) complexes of the equilibrium mixture. EPR spectra for the equilibrium mixture created by addition of phosphocreatine to solutions of the ternary complexes with ADP and with the epimers of ADPαS are compared in Figure 7. The spectra are superpositions of signals for the two central complexes, enzyme–Mn^{II}ATP-(αS)–creatine and enzyme–Mn^{II}ADP(αS)–phosphocreatine (Leyh et al., 1985). The ^{55}Mn hyperfine sextet is contracted in the spectrum for samples made up from (S_p)-ADPαS [$\Delta A(^{55}Mn)$ = −1.6 G]. Moreover, there is no observable ^{17}O-induced inhomogeneous broadening in the signals for samples made up with (S_p)-[α-^{17}O]ADPαS. These observations indicate that the correct Δ-screw-sense configuration of the α,β-chelate ring [with Mn(II)–thio coordination] is present as a dominant species in the central complexes with S_p epimers of the adenosine α-phosphorothioates.

Conclusions. Experiments with the anion-stabilized dead-end complexes and with central complexes of the equilibrium mixture indicate that the selectivity of creatine kinase for the Δ-screw-sense configuration of the metal–nucleotide complex overrides the preference of the hard metal ion, Mn(II), for oxy versus thio coordination. The predominance of the Δ configuration of the α,β-chelate ring [with Mn(II)–thio coordination] in these complexes with the S_p epimers of the adenosine α-phosphorothioates substantiates the notion that the Δ configuration (α,β) of the metal–nucleotide substrate is the reactive species. The catalytic activity observed with Mn(II) and (S_p)-ATPαS (Burgers & Eckstein, 1980) is therefore attributable to the capacity of the enzyme to force the less

favorable Mn(II)–thio coordination at P_α.

In the absence of the appropriate cosubstrate (i.e., creatine or phosphocreatine), the stereoselectivity of the enzyme for the Δ configuration is weaker such that the "nonproductive" Λ configuration of the $Mn^{II}(S_p)$-ADPαS chelate binds at the active site. The ability of the active site to accommodate the wrong screw-sense isomer in the ternary complex is understandable because the enzyme binds ADP (in the absence of divalent metal ions) with an affinity only 2-fold lower than that of the Mn^{II}ADP complex (Reed et al., 1970). In initial binding steps, the enzyme may capture a metal–nucleotide complex with an incorrect stereochemical configuration. Complexes of nucleotides with Mn(II) [and with Mg(II)] undergo isomerization rapidly, and favorable binding interactions provided by stereospecific contacts in the active site subsequently stabilize the desired isomeric form of the metal–nucleotide complex prior to catalysis. For creatine kinase, stereoselective binding interactions, which are sufficient to promote a Λ to Δ isomerization of the complex with (S_p)-ADPαS, gain control upon binding of the cosubstrate.

Results from chemical modification experiments (Milner-White & Watts, 1971) and from spectroscopic measurements (Reed & Cohn, 1972; Reed & Leyh, 1980) indicate that the active site adopts a closed conformation upon formation of the anion-stabilized, dead-end complexes. Travers et al. (1979) identified protein isomerizations following addition of creatine to the enzyme–Mg^{II}ATP complex. Spectral evidence for an isomerization of the enzyme upon formation of the nitrate-stabilized, dead-end complex has also been presented (Travers & Barman, 1980). The enhanced binding energy available in the closed conformation of the bisubstrate complexes forces the correct configuration of the metal–nucleotide chelate.

It is not yet possible to ascertain whether or not the appreciable activity observed for the Mg(II) complex with (S_p)-ATPαS in the creatine kinase reaction (Burgers & Eckstein, 1980) reflects Mg(II)–thio coordination at P_α. The capacity of the enzyme to force this coordination on another hard metal ion, Mn(II), together with the strict stereochemical requirements of the α,β,γ-tridentate form of the metal–ATP substrate (Leyh et al., 1985; Jarori et al., 1985), implies that Mg(II)–thio coordination may well be responsible for the activity observed with $Mg^{II}(S_p)$-ATPαS (Burgers & Eckstein, 1980).

The structures that have been elucidated for the Mn^{II}ADPαS complexes with creatine kinase indicate that the low extent of kinetic discrimination between the S_p and R_p epimers of ATPαS with Mn(II) as the activator (Burgers & Eckstein, 1980) is actually a consequence of a very high enzymic selectivity for the Δ configuration of the α,β-chelate ring of the metal–nucleotide substrate. As suggested by Pecoraro et al. (1984), a high degree of kinetic discrimination between epimers of nucleoside phosphorothioates may reflect ambivalence of an enzyme with respect to binding of a nonproductive configuration of the metal–nucleotide substrate. Thus, the high kinetic discrimination between epimers of nucleoside phosphorothioates exhibited by enzmes such as hexokinase (Jaffe & Cohn, 1979) and 3-phosphoglycerate kinase (Jaffe et al., 1982) may indicate that these enzymes lack the capacity to force the correct configuration of the metal–nucleoside phosphorothioate substrate when challenged by the hard or soft ligand coordination tendencies of a particular activating metal ion. By contrast, enzymes such as creatine kinase (Burgers & Eckstein, 1980) and arginine kinase (Cohn et al., 1982), which exhibit less dramatic kinetic discrimination for epimers of nucleoside phosphorothioates, may actually possess a much higher degree of stereospecificity in binding of their metal–nucleotide substrates.

Acknowledgments

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Registry No. (R_p)-ADPαS, 118399-03-4; (S_p)-ADPαS, 118399-04-5; Mn-ADP, 69828-68-8; Mn, 7439-96-5; nitrate, 14797-55-8; nitrite, 14797-65-0; azide, 14343-69-2; formate, 64-18-6; thiocyanate, 302-04-5; creatine kinase, 9001-15-4.

References

Armstrong, V. W., Yee, D., & Eckstein, F. (1979) *Biochemistry 18*, 4120–4123.
Burgers, P. M. J., & Eckstein, F. (1980) *J. Biol. Chem. 255*, 8229–8233.
Burmeister, J. L. (1975) in *The Chemistry and Biochemistry of Thiocyanic Acid and its Derivatives* (Newman, A. A., Ed.) pp 68–138, Academic Press, London.
Cleland, W. W., & Mildvan, A. S. (1979) *Adv. Inorg. Biochem. 1*, 163–191.
Cohn, M. (1982) *Acc. Chem. Res. 15*, 326–332.
Cohn, M., & Townsend, J. (1954) *Nature 173*, 1090–1091.
Cohn, M., Shih, N., & Nick, J. (1982) *J. Biol. Chem. 257*, 7646–7649.
Connolly, B. A., von der Haar, F., & Eckstein, F. (1980) *J. Biol. Chem. 255*, 11301–11307.
Dunaway-Mariano, D., & Cleland, W. W. (1980) *Biochemistry 19*, 1506–1515.
Eckstein, F. (1985) *Annu. Rev. Biochem. 54*, 367–402.
Frey, P. A., & Sammons, R. D. (1985) *Science 228*, 541–545.
Fronaeus, S., & Larsson R. (1962a) *Acta Chem. Scand. 16*, 1433–1446.
Fronaeus, S., & Larsson, R. (1962b) *Acta Chem. Scand. 16*, 1447–1454.
Hershenson, S., Helmers, N., Desmueles, P., & Stroud, R. (1986) *J. Biol. Chem. 261*, 3732–3736.
Iyengar, R., Ho, H.-T., Sammons, R. D., & Frey, P. A. (1984) *J. Am. Chem. Soc. 106*, 6038–6049.
Kenyon, G. L., & Reed, G. H. (1983) *Adv. Enzymol. Rel. Areas Mol. Biol. 54*, 367–426.
Kuby, S. A., Noda, L., & Lardy, H. A. (1954) *J. Biol. Chem. 209*, 203–210.
Jaffe, E. K. (1979) Ph.D. Dissertation, University of Pennsylvania.
Jaffe, E. K., & Cohn, M. (1978) *J. Biol. Chem. 253*, 4823–4825.
Jaffe, E. K., & Cohn, M. (1979) *J. Biol. Chem. 254*, 10839–10845.
Jaffe, E. K., Nick, J., & Cohn, M. (1982) *J. Biol. Chem. 257*, 7650–7656.
Jarori, G. K., Ray, B. D., & Nageswara Rao, B. D. (1985) *Biochemistry 24*, 3487–3494.
Jorgensen, C. K. (1964) *Inorg. Chem. 3*, 1201–1202.
Leyh, T. S., Sammons, R. D., Frey, P. A., & Reed, G. H. (1982) *J. Biol. Chem. 257*, 15047–15053.
Leyh, T. S., Goodhart, P. J., Nguyen, A. C., Kenyon, G. L., & Reed, G. H. (1985) *Biochemistry 24*, 308–316.
Lifshitz, E., & Francis, A. H. (1982) *J. Phys. Chem. 86*, 4714–4718.
Milner-White, E. J., & Watts, D. C. (1971) *Biochem. J. 122*, 727–740.

Moore, J. M., & Reed, G. H. (1985) *Biochemistry 24*, 5328-5333.
Pearson, R. G. (1968) *J. Chem. Educ. 45*, 581-587.
Pecoraro, V. L., Hermes, J. D., & Cleland, W. W. (1984) *Biochemistry 23*, 5262-5271.
Reed, G. H., & McLaughlin, A. C. (1973) *Ann. N.Y. Acad. Sci. 222*, 118-129.
Reed, G. H., & Leyh, T. S. (1980) *Biochemistry 19*, 5472-5480.
Reed, G. H., & Markham, G. D. (1984) *Biol. Magn. Reson. 6*, 73-142.
Reed, G. H., Cohn, M., & O'Sullivan, W. J. (1970) *J. Biol. Chem. 245*, 6547-6552.
Reed, G. H., Barlow, C. H., & Burns, R. A., Jr. (1978) *J. Biol. Chem. 253*, 4153-4158.
Sammons, R. D., & Frey, P. A. (1982) *J. Biol. Chem. 257*, 1138-1141.
Sheu, K.-F. R., & Frey, P. A. (1977) *J. Biol. Chem. 252*, 4445-4448.
Travers, F., & Barman, T. E. (1980) *Eur. J. Biochem. 110*, 405-412.
Travers, F., Barman, T. E., & Bertrand, R. (1979) *Eur. J. Biochem. 100*, 149-155.
Van Etten, R. L., & Risley, J. M. (1981) *J. Am. Chem. Soc. 103*, 5633-5636.
Van Wieringen, E. (1955) *Discuss. Faraday Soc. 19*, 118-126.

Sulfuryl Transfer Catalyzed by Pyruvate Kinase†

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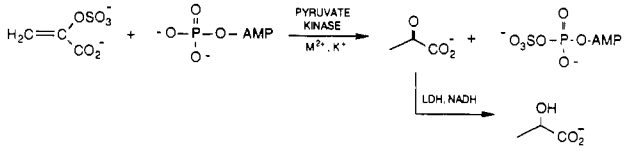
ABSTRACT: Sulfoenolpyruvate, the analogue of phosphoenolpyruvate in which the phosphate ester has been replaced by a sulfate ester, has been synthesized in three chemical steps from ethyl bromopyruvate in 40% overall yield. This compound is a substrate for pyruvate kinase, producing pyruvate and adenosine 5′-sulfatopyrophosphate. The latter compound has been identified by NMR spectroscopy and by comparison with an authentic sample. Sulfuryl transfer from sulfoenolpyruvate is 250–600-fold slower than phosphate transfer from phosphoenolpyruvate under identical conditions. Sulfoenolpyruvate is not a substrate for phosphoenolpyruvate carboxylase. Kinetic studies reveal that it does not bind to the active site; instead, it binds to the site normally occupied by glucose 6-phosphate and activates the enzyme in a manner similar to that shown by glucose 6-phosphate.

Mechanisms of enzyme-catalyzed phosphoryl group transfers continue to be studied by a variety of methods. Substrate analogues in which the phosphate group has been replaced by another anionic group (thiophosphates, phosphonates, etc.) have played an important role in this effort (Eckstein, 1983; Engel, 1977). Sulfate esters are structurally and electronically similar to phosphate esters, and the two families share much chemistry. Although sulfate esters have served as analogues of phosphate esters in a number of connections, we are aware of no reports of sulfuryl transfer catalyzed by enzymes that ordinarily catalyze phosphoryl transfer. We report here the first example of such a reaction.

Because of the central role of phosphoenolpyruvate (PEP)[1] in metabolism (Davies, 1979), this compound has been the target of substantial enzymatic and chemical experimentation. Most of the analogues, such as 3-bromophosphoenolpyruvate, 3-fluorophosphoenolpyruvate (Diaz et al., 1988; Stubbe & Kenyon, 1972; Gonzalez & Andreo, 1988), 1-carboxyallenyl phosphate (Wirsching & O'Leary, 1988), phosphoenol-α-ketobutyrate (Stubbe & Kenyon, 1971; Duffy et al., 1982), and others, involve variations distal to the phosphate group. Work done with phosphoenolthiopyruvate (Sikkema & O'Leary, 1988), thiophosphoenolpyruvate (Orr et al., 1978; Hansen & Knowles, 1982), α-(dihydroxyphosphinylmethyl)-acrylic acid (Stubbe & Kenyon, 1972), and methylphosphoenolpyruvate (Lazarus et al., 1979) represent the fewer cases utilizing variations in the phosphate functionality of PEP. These analogues show little or no activity and low affinity for enzyme active sites. To date, the only catalytically active analogues of PEP in which the phosphate is replaced by another group are the thiophosphates, in which an oxygen of the phosphate has been replaced by sulfur (Hansen & Knowles, 1982; Sikkema & O'Leary, 1988).

Scheme I



This paper describes the synthesis and study of sulfoenolpyruvate (SEP), a sulfate analogue of PEP. We present evidence that pyruvate kinase catalyzes a sulfuryl transfer to ADP to form adenosine 5′-sulfatopyrophosphate (Scheme I).

Materials and Methods

Materials

Sodium cyanoborohydride (Aldrich), DL-lactic acid (Aldrich), sulfur trioxide pyridine complex (Aldrich), sodium pyruvate (Sigma), oxalic acid (Mallinckrodt), tricyclohexylammonium phosphoglycolate (Sigma), disodium NADH (Sigma), and potassium ADP (Sigma) were used as supplied.

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[1] Abbreviations: SEP, sulfoenolpyruvate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PEP, phosphoenolpyruvate; THF, tetrahydrofuran; AP_5A, P^1,P^5-bis(5′-adenosyl) pentaphosphate.

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