Stereochemistry of Metal Ion Coordination to the Terminal Thiophosphoryl Group of Adenosine 5′-*O*-(3-Thiotriphosphate) at the Active Site of Pyruvate Kinase†

Jenny L. Buchbinder,‡ Janina Baraniak,§ Perry A. Frey, and George H. Reed*

Institute for Enzyme Research, Graduate School, and Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin—Madison, Madison, Wisconsin 53705

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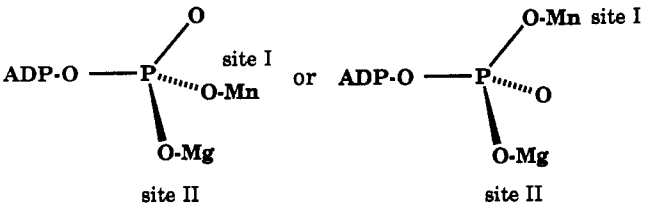
ABSTRACT: Epimers of [γ-^{17}O]adenosine 5′-*O*-(3-thiotriphosphate) ([γ-^{17}O]ATPγS) have been used to determine the stereochemistry of Mn^{2+} coordination to the terminal thiophosphoryl group in complexes of pyruvate kinase, oxalate, ATPγS, and Mg^{2+}, Zn^{2+}, Co^{2+}, or Cd^{2+}. The complex of pyruvate kinase with oxalate and ATP binds 2 equiv of divalent cation per active site. The terminal phosphoryl group of ATP in this enzymic complex becomes a chiral center as a result of coordination to both divalent metal ions. Electron paramagnetic resonance (EPR) data for complexes of pyruvate kinase with R_p- or S_p-[γ-^{17}O]-ATPγS, [^{17}O]oxalate, and mixtures of Mn^{2+} with Mg^{2+}, Zn^{2+}, or Co^{2+} show that Mn^{2+} binds selectively at the site defined by coordination to oxalate and the *pro-R* oxygen of the thiophosphoryl group of ATPγS. In mixtures containing Mn^{2+} and Cd^{2+} with Tl^+ as the monovalent cation, two hybrid complexes form, enzyme–oxalate–Mn^{II}–ATPγS–Cd^{II} and enzyme–oxalate–Cd^{II}–ATPγS–Mn^{II}, as in the analogous complexes with ATP and K^+ or Tl^+ (Buchbinder, J. L., & Reed, G. H. (1990) *Biochemistry 29*, 1799-1806). In the enzyme–oxalate–Mn^{II}–ATPγS–Cd^{II} species, Mn^{2+} binds exclusively to the *pro-R* oxygen of the thiophosphoryl group. In the enzyme–oxalate–Cd^{II}–ATPγS–Mn^{II} species, Mn^{2+} binds to the *pro-R* oxygen (60%) and to the *pro-S* oxygen (40%). The reluctance of the sulfur substituent to coordinate to Zn^{2+}, Co^{2+}, and Cd^{2+} at the nucleotide site suggests that all three pendant oxygens from the γ-phosphate group of ATP interact with positively charged centers.

Studies over the last several years have exploited the chirality of nucleoside phosphorothioate analogues to reveal the stereoselectivities of enzymes for the configurations of their metal–nucleotide substrates (Knowles, 1980; Cohn, 1982; Eckstein, 1985; Frey, 1989). Isotopically chiral forms of the γ-thio analogues have also been used to determine the stereochemical consequences of phosphoryl-transfer events in catalytic cycles of enzymes (Eckstein, 1985; Frey, 1989). The γ-phosphorus of nucleoside triphosphates becomes a chiral center in complexes with enzymes as a consequence of specific interactions of the peripheral oxygens with three different binding determinants. These binding determinants must also be intimately involved in stabilizing the trigonal planar form of the $-PO_3$ group in the transition state of phosphoryl group transfer.

Complexes of ATP with pyruvate kinase are attractive candidates for detailed studies of the binding interactions involving the terminal phosphoryl group of the nucleoside triphosphate. The enzyme needs 2 equiv of divalent metal ion (Gupta et al., 1976; Gupta & Mildvan, 1977; Baek & Nowak, 1982) and a monovalent cation (Boyer et al., 1942) per active site for activity, and all three of these inorganic ions are positioned within the active site. In complexes with ATP and oxalate, an analogue of enolpyruvate, the γ-phosphoryl group of ATP is a bridging ligand for the two divalent cations (Lodato & Reed, 1987; Buchbinder & Reed, 1990). Coordination of the terminal phosphoryl group of ATP to two divalent metal ions at the active site of pyruvate kinase creates a chiral center from a PO_3 moiety (Chart 1).

Chart 1



The chirality of the enzyme-bound–PO_3 moiety provides an opportunity to use stereochemical methods to probe the environment of the third or "unliganded" oxygen of this group. Substitution of epimers of [γ-^{17}O]ATPγS[1] for ATP in these enzymic complexes allows one to use EPR spectroscopy to determine the stereochemical configuration of this chiral center in complexes in which Mn^{2+} occupies one of the sites for binding of divalent cations. Moreover, the spectroscopic readout of the orientation of the chiral thiophosphoryl group permits one to assess the influences of different species of companion metal ion, which have different Lewis acid character, on the position of the thio substituent. The orientation of the chiral thiophosphoryl moiety, in turn, reveals the strength of binding interactions experienced by the thio substituent at the active site. The present paper reports the results of this stereochemical investigation.

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* Address correspondence to this author at Institute for Enzyme Research, University of Wisconsin, 1710 University Ave., Madison, WI 53705.

‡ Present address: Department of Biochemistry and Biophysics, University of California, San Francisco, California.

§ Present address: Polish Academy of Sciences Centre of Molecular and Macromolecular Studies, 90-363 ul Sienkicwwixcza 112, Lódz, Poland.

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[1] Abbreviations: ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); ADPαS, adenosine 5′-*O*-(1-thiodiphosphate); ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); ADPαS, adenosine 5′-*O*-(1-thiodiphosphate); AMPS, adenosine 5′-*O*-thiophosphate; GppNp, β,γ-imidoguanosine 5′-triphosphate; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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EXPERIMENTAL PROCEDURES

Materials. Pyruvate kinase was isolated from rabbit skeletal muscle by the procedure of Tietz and Ochoa (1958) using additional gel filtration steps as described previously (Ash et al., 1984). Preparations of the enzyme had specific activities of ~300 IU mg^{-1} at pH 7.0 and 22 °C in the coupled assay with lactate dehydrogenase. Water enriched to 52 atom % in ^{17}O was from the Mound Facility of Monsanto. Organic solvents (Aldrich) were distilled and dried over molecular sieves and CaH_2 before use.

Synthesis of R_p- and S_p-[γ-^{17}O]ATPγS. Synthesis of R_p- and S_p-[γ-^{17}O]ATPγS was adapted from the scheme described by Richard and Frey (1982) for synthesis of R_p- and S_p-[β-^{18}O]ADPβS and R_p-[γ-^{18}O]ATPγS. AMPS$^{17}O_2$ was prepared by reaction of adenosine with $SPCl_3$ followed by reaction with $H_2{}^{17}O$. The AMPS$^{17}O_2$ was coupled to diphenyl phosphorochloridate activated phosphate to give an epimeric mixture of [α-^{17}O]ADPαS. The R_p and S_p epimers of [α-^{17}O]-ADPαS were separated by HPLC on a preparative C18 reversed-phase column as described by Sammons and Frey (1982) for separating the epimers of β-cyanoethyl-ADPαS. The resolved epimers of [α-^{17}O]ADPαS were coupled to diphenyl phosphorochloridate activated 2',3'-*O*-(methoxymethylidine)-AMP. The unblocked ribosyl ring was cleaved with periodate. Excess periodate was reduced with ethylene glycol, and the resulting iodate was reduced with thiophosphate. The methoxymethylidine blocking group was removed from the other ribosyl ring by treatment with dilute acid. Alkaline elimination of the periodate-cleaved nucleoside gave the product, R_p- or S_p-[γ-^{17}O]ATPγS. The epimers of [γ-^{17}O]-ATPγS were initially purified by chromatography over DEAE-Sephadex A25 (Leyh et al., 1985). The products were further purified by a paired-ion reversed-phase chromatography procedure adapted from that described by Juengling and Kammermeier (1980). The mobile phase was 28% (v/v) acetonitrile, 9 mM tetra-*n*-butylammonium dihydrogen phosphate, and 48 mM KH_2PO_4, pH 5.9. The stationary phase was a preparative HPLC column: Phenomenex Bondclone-10 C18. Phosphate was removed by chromatography on DEAE-Sephadex A25 using a 3-L linear gradient (0.1 to 0.8 M) of triethylammonium bicarbonate. The R_p- and S_p-[γ-^{17}O]ATPγS and a sample of unlabeled compound were converted to tetramethylammonium salts by chromatography on SP-Sephadex. The ^{17}O enrichment in [γ-^{17}O]ATPγS was estimated from ^{31}P NMR (Tsai, 1979).

EPR Measurements. EPR spectra were obtained at 35 GHz with a Varian E109Q spectrometer. The spectrometer was interfaced with an IBM AT microcomputer for data acquisition. Spectra were recorded for samples in the liquid phase at ~ 0 °C. Samples were contained in quartz capillary tubing as described previously (Buchbinder & Reed, 1990). Oxygen ligands were identified from the presence of ^{17}O-induced inhomogeneous broadening in the EPR signals of Mn^{2+} (Reed & Leyh, 1980). Samples for EPR measurements were taken from a common stock solution that contained all of the components except the ligand under investigation. Equal volumes of labeled or unlabeled ligand were then added to the samples, usually prepared in triplicate. Spectra of matched samples were recorded using the same sample tube and identical spectrometer settings, including iris coupling of cavity and waveguide. Analytical precision in sampling and measurement resulted in ≤3% error in the amplitudes of signals in separate matched samples.

RESULTS AND DISCUSSION

Stereochemistry of ATPγS in Hybrid Complexes with Mn^{2+} and Mg^{2+}. Previous experiments have shown that, in solutions of enzyme, oxalate, ATP, Mn^{2+}, and Mg^{2+}, Mn^{2+} binds selectively at a site (site I) where it accepts ligands from oxalate, Pγ of ATP, the protein, and a water molecule. In this metal hybrid complex, Mg^{2+} binds selectively to the ATP (site II) (Lodato & Reed, 1987). As demonstrated earlier (Lodato, 1986), addition of Mg^{II}ATPγS to solutions of enzyme, oxalate, and Mn^{2+} produces a characteristic EPR spectrum (see Figure 1). EPR signals from samples prepared with [^{17}O]oxalate show an inhomogeneous broadening (Figure 1B) that results from unresolved superhyperfine coupling between ^{17}O and Mn^{2+}. Difference spectra confirm that oxalate is a bidentate ligand for Mn^{2+} in this enzymic complex (data not shown). These spectroscopic data establish that, as in the analogous complex with Mg^{II}ATP, Mn^{2+} binds selectively at site I in a complex of the form enzyme–oxalate–Mn^{II}–ATPγS–Mg^{II}.

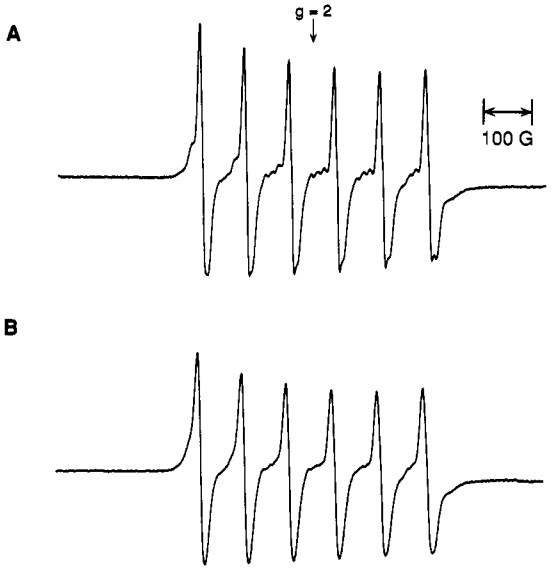


FIGURE 1: Effect of ^{17}O in oxalate on EPR spectra (Q-band) of samples containing Mn^{2+}, Mg^{2+}, oxalate, and ATPγS. Samples contained 50 mM Hepes/KOH, pH 7.5, 150 mM KCl, 2.7 mM enzyme active sites, 1.8 mM $MnCl_2$, 6 mM $MgCl_2$, 5 mM ATPγS, and 9 mM unlabeled or ^{17}O-enriched oxalate. Spectra were obtained in the liquid phase at 0 °C. (A) Spectrum of sample with unlabeled oxalate. (B) Spectrum of sample with ^{17}O-enriched oxalate.

The stereochemical configuration of the terminal thiophosphoryl group of ATPγS in the enzymic complex with oxalate, Mg^{2+}, and Mn^{2+} is revealed in EPR measurements with the resolved epimers of [γ-^{17}O]ATPγS (see Figure 2). EPR signals from the sample with R_p-[γ-^{17}O]ATPγS (Figure 2A, dashed curve) are broadened relative to those from the sample of unlabeled ATPγS (Figure 2A, solid curve). In contrast, EPR signals from the sample with S_p-[γ-^{17}O]ATPγS (Figure 2B, dashed curve) are indistinguishable from those originating from the sample of unlabeled ATPγS (Figure 2B, solid curve). These EPR data establish that Mn^{2+} (at site I) binds stereospecifically to ^{17}O in the *pro-R* position of ATPγS. Oxalyl phosphate (Kofron & Reed, 1990) and ATPγS (Orr et al., 1978) are substrates of pyruvate kinase, and the combination of enzyme, oxalate, and ATPγS is likely to be active in reversible thiophosphoryl transfer. The observed stereospecificity of the ^{17}O-induced inhomogeneous broadening confirms an absence of epimerization of the chiral center as a result of any reversible thiophosphoryl group transfer.

The stereoselective binding of Mn^{2+} to the *pro-R* oxygen at Pγ of ATPγS confirms expectations that the thio substituent would adopt only one of the three possible positions normally held by an oxy substituent at Pγ of ATP. The available options for binding of the thio substituent comprise two hard Lewis acid centers, Mn^{2+} and Mg^{2+}, and a third, as yet uncharac-

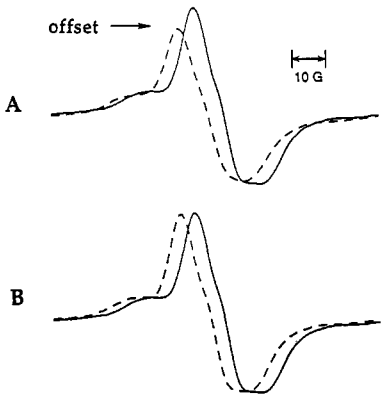


FIGURE 2: Effect of ^{17}O in ATPγS on EPR signals (lowest field $^{55}Mn^{2+}$ hyperfine transition) of samples containing Mn^{2+}, Mg^{2+}, oxalate, and ATPγS. Spectra were obtained in the liquid phase at 0 °C. Samples contained 50 mM Hepes/KOH, pH 7.5, 150 mM KCl, 3 mM enzyme active sites, 2 mM $MnCl_2$, 6 mM $MgCl_2$, 5 mM ATPγS, and 9 mM oxalate. (A) Unlabeled ATPγS (solid line) and R_p-[γ-^{17}O]ATPγS (dashed line offset 5 G on the abscissa). (B) Unlabeled ATPγS (solid line) and S_p-[γ-^{17}O]ATPγS (dashed line offset 5 G on the abscissa).

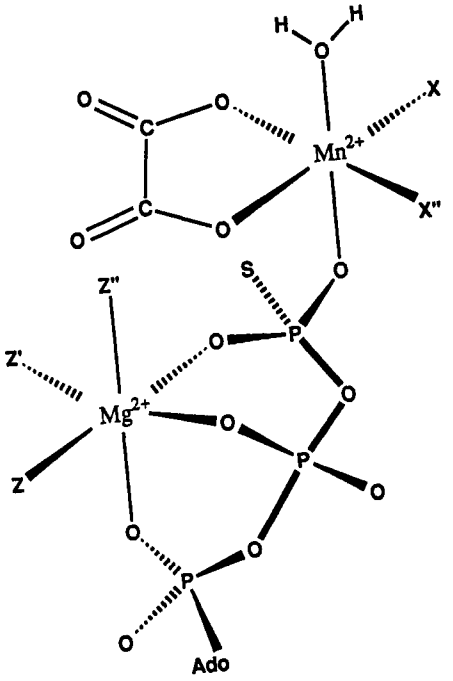
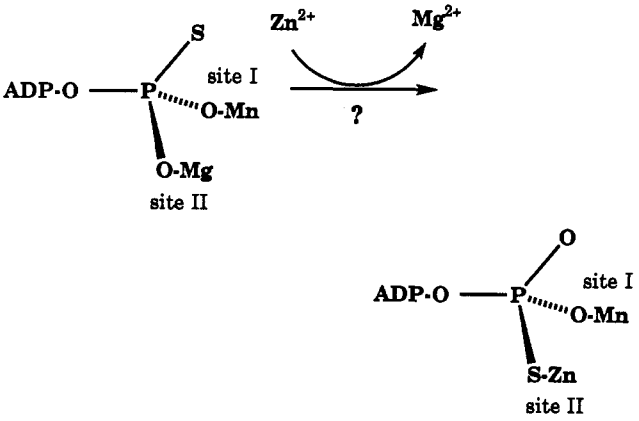


FIGURE 3: Schematic drawing showing the active site of pyruvate kinase with oxalate and ATPγS bound in the presence of Mn^{2+} and Mg^{2+}. Experiments with [^{17}O]oxalate and with the R_p and S_p epimers of [γ-^{17}O]ATPγS show that Mn^{2+} binds at site I in this complex, where it coordinates to the *pro-R* oxygen of ATPγS. The α,β,γ-tridentate structure of the MgATP is derived from EPR results on enzyme–oxalate–Cd^{II}–ATP–Mn^{II} (Buchbinder & Reed, 1990), and the β-Δ, α-exo configuration of the Mg^{II}ATP that is shown is consistent with the findings of Lu et al. (1993) with α,β,γ-tridentate $Rh(H_2O)_3$-ATP. The identities of ligands labeled X, X′, Z, Z′, and Z″ are not presently known, although recent results of Lu et al. (1993) make it virtually certain that the Z ligands are water molecules. Ligands labeled X and X′ are contriubted by the protein.

terized, binding determinant. Of the two divalent cations, Mn^{2+} would have the greater propensity to accommodate a thio ligand (Smithers et al., 1989), and the data of Figure 2 clearly show that Mn^{2+} coordinates to the *pro-R* oxygen. It is probable, therefore, that Mg^{2+} (at site II) coordinates to the *pro-S* oxygen in preference to the thio substituent. The stereochemical configuration of the thiophosphoryl coordination is given in Figure 3.

Stereochemistry of ATPγS in Hybrid Complexes of Mn^{2+} with Zn^{2+} and Co^{2+}. The results presented above indicate

Scheme 1



that the thio substituent at Pγ occupies the as yet uncharacterized position for the terminal phosphoryl group of ATP. The third oxy substituent at Pγ of ATP could interact with one or more hydrogen bond donors or a positively charged entity, e.g., the side chain of an arginine, the protonated ε-amino group of a lysine residue, or the monovalent cation. In comparison to oxygen, sulfur has weak hydrogen-bonding tendencies. If this binding site comprises exclusively hydrogen bond donors, then interactions with the thio substituent of ATPγS would be weaker than interactions with the root oxy substituent of ATP. Substitution of a softer Lewis acid for Mg^{2+} at site II in the complex might then provide a more favorable binding interaction for the thio substituent, thereby effecting a 120° counterclockwise rotation of the γ-thiophosphoryl moiety according to Scheme 1. Such a rotation would bring the *pro-S* oxygen into the coordination sphere of the metal ion at site I.

Complexes with the softer Lewis acids, Zn^{2+} and Co^{2+}, in place of Mg^{2+} were investigated in order to determine whether or not rotation of the γ-thiophosphoryl group could be induced by the presence of either of these softer metal ions at site II. EPR spectra from samples of the hybrid mixtures Mn^{2+}/Zn^{2+} and Mn^{2+}/Co^{2+} are shown[2] in Figure 4. EPR signals obtained from the samples with [^{17}O]oxalate (data not shown) exhibit ^{17}O-induced inhomogeneous broadening which confirms that Mn^{2+} remains bound to oxalate at site I in the hybrid complexes with either Zn^{2+} or Co^{2+}. The selectivities of sites I and II for these mixtures of Mn^{2+} with either Zn^{2+} or Co^{2+} are thus equivalent to those found for the respective complexes with ATP (Buchbinder & Reed, 1990). Spectra from the samples with R_p and with S_p-[γ-^{17}O]ATPγS (see Figure 4A,B parts b and c) show that ^{17}O in the R_p epimer produces inhomogeneous broadening, whereas signals for the complex with the S_p epimer are superimposed on those of the sample of unlabeled ATPγS. Therefore, Mn^{2+} (at site I) remains bound to the *pro-R* oxygen at Pγ in the hybrid complexes with either Zn^{2+} or Co^{2+} bound at site II. Hence, the thio substituent does not alter its position upon substitution of Zn^{2+} or of Co^{2+} for Mg^{2+} at site II, and the hypothetical rotation of the thiophosphoryl group in Scheme 1 does not take place in a detectable[3] fraction of the sample. If Mg^{2+} at site II binds to the *pro-S* oxygen, then Zn^{2+} and Co^{2+} also bind to the *pro-S* oxygen. The failure of either Co^{2+} or Zn^{2+}

[2] EPR signals of the hybrid complex with Mn^{2+} and Co^{2+} are broader than the signals in Mn^{2+} hybrids with other metals, e.g., Mg^{2+} or Zn^{2+}. The observation of broader signals in the hybrid complex of Mn^{2+}/Co^{2+} is analogous to that described earlier (Buchbinder & Reed, 1990) for Mn^{2+}/Co^{2+} hybrid complexes with ATP and oxalate. The increment in line width in the latter case could be modeled as a homogeneous relaxation effect (Buchbinder & Reed, 1990).

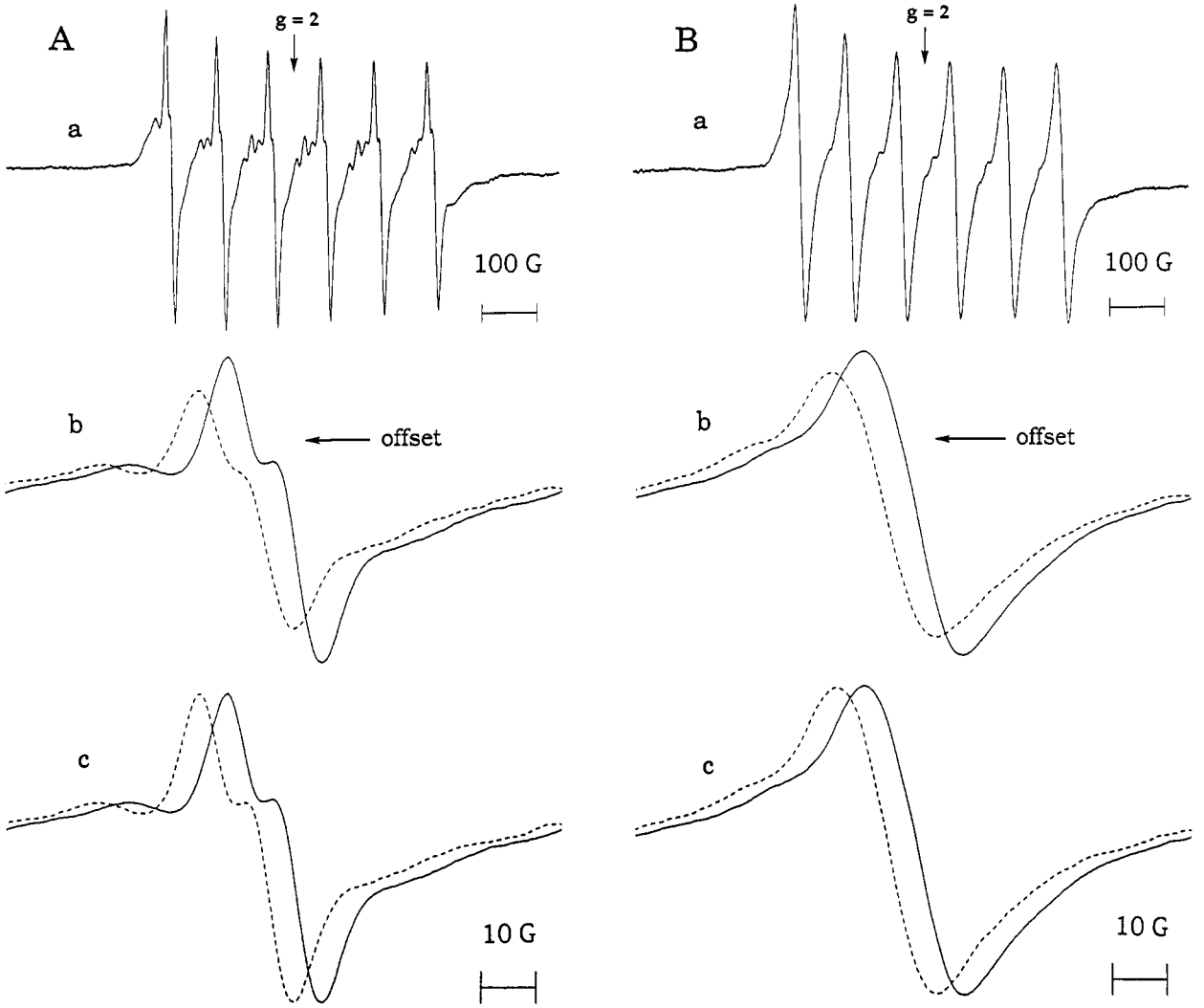


FIGURE 4: Effect of ^{17}O in S_p- or R_p-[γ-^{17}O]ATPγS on EPR spectra of samples containing oxalate and ATPγS and a combination of either Mn^{2+}/Zn^{2+} (A) or Mn^{2+}/Co^{2+}(B). Spectra were obtained in the liquid phase at 0 °C. Samples contained 50 mM Hepes/KOH, pH 7.5, 150 mM KCl, 3.3 mM enzyme active sites, 2 mM $MnCl_2$, 5 mM ATPγS (or ^{17}O-labeled ATPγS), 9 mM oxalate, and either 6 mM $Zn(OAc)_2$ (A) or 6 mM $CoCl_2$ (B). In panels A and B, part a is the full spectrum of the unlabeled sample: parts b and c are expansions of the lowest field $^{55}Mn^{2+}$ hyperfine components in the spectra of the samples with R_p-[γ-^{17}O]ATPγS in b (dashed curves) or with S_p-[γ-^{17}O]ATPγS in c (dashed curves). In parts b and c, the dashed lines are offset 5 G on the abscissa and the solid lines are signals from concentration-matched samples with unlabeled ATPγS.

to attract the thio substituent as a ligand implies either that the sulfur experiences favorable interactions at its adopted site or that steric crowding associated with the bulkier sulfur atom and the longer PS bond precludes rotation of the thiophosphoryl group.

Stereochemistries of ATPγS in Hybrid Complexes of Mn^{2+} with Cd^{2+}. Previous experiments on hybrid mixtures of Cd^{2+} and Mn^{2+} with ATP and oxalate revealed partially resolved EPR spectra from a pair of hybrid complexes, enzyme–oxalate–Mn^{II}–ATP–Cd^{II} and enzyme–oxalate–Cd^{II}–ATP–Mn^{II} (Buchbinder & Reed, 1990). EPR spectra (data not shown) obtained from samples of enzyme, Mn^{2+}, oxalate, Cd^{2+}, and ATPγS in a medium of 0.15 M KCl, however, do not exhibit the pattern of signals that are characteristic of a hybrid complex. Rather, increases in the concentration of Cd^{II}ATPγS produce increases in EPR signals that are superimposed on those of free Mn^{II}ATPγS. These observations suggest that, with ATPγS in place of ATP, Cd^{2+} competes more effectively with Mn^{2+} for binding at both sites and that EPR-silent complexes of the form enzyme–oxalate–Cd^{II}–ATPγS–Cd^{II} are the predominant enzymic species. The Mn^{2+} is therefore chased from the active site and combines with free ATPγS in solution.

Buchbinder and Reed (1990) reported influences of the species of monovalent cation on the EPR patterns of the Cd^{2+}/Mn^{2+} mixtures in the analogous ATP–oxalate complexes. Substitution of Tl^+ for K^+ in complexes with ATPγS promotes the formation of hybrid complexes. The two sets of EPR signals (see Figure 5A) that appear in the presence of Tl^+ indicate that, as in the case of complexes of ATP (with either K^+ or Tl^+), a pair of hybrid complexes are present that differ in the positions of Mn^{2+} and Cd^{2+}. Selective broadening of one set of signals (Figure 5B, signals I) by [^{17}O]oxalate identifies these I signals with the complex enzyme–oxalate–Mn^{II}ATPγSCd^{II}. The other set of signals (II) is assigned to the complex enzyme–oxalate–Cd^{II}–ATPγS–Mn^{II}. As in the case of the Cd^{2+}/Mn^{2+} hybrid complexes of ATP (Buchbinder & Reed, 1990), the enzyme–oxalate–Cd^{II}–ATPγS–Mn^{II} is the more abundant complex, as indicated by the fact that the

[3] In principle, a complex with the opposite stereochemistry could be detected if its relative concentration were greater than the experimental uncertainty in signal amplitude (i.e., ≥3%). In practice, the limit of detection would be more of the order of ≥6%. In the case of Mn^{2+}/Zn^{2+} hybrids, the EPR signals are so narrow that a hypothetical, broader component corresponding to a complex with Zn^{2+}–thio coordination might be missed at a somewhat higher percentage.

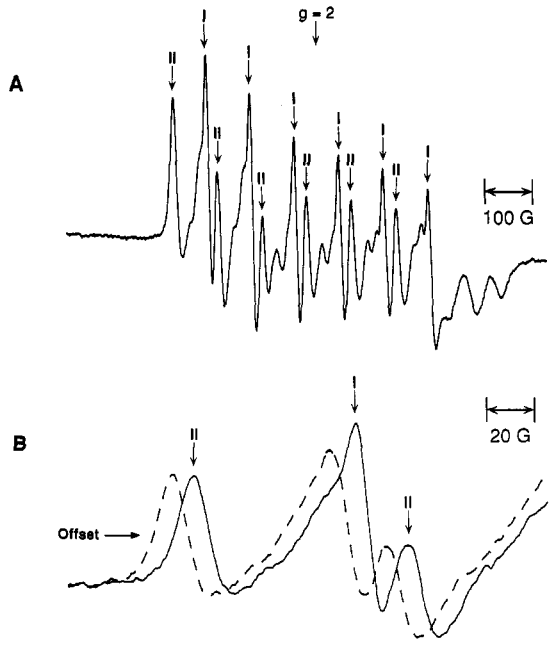
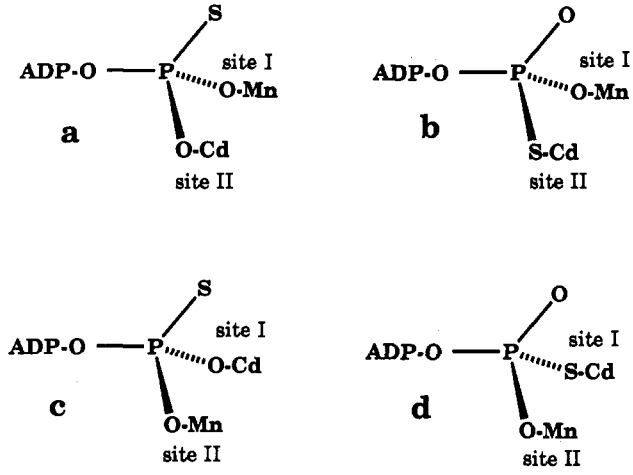


FIGURE 5: Effect of ^{17}O in oxalate on EPR spectra of samples containing enzyme, oxalate, ATPγS, and a combination of Mn^{2+} and Cd^{2+} in the presence of Tl^{+}. Samples contained 50 mM Hepes/$(CH_3)_4NOH$, pH 7.5, 20 mM $(CH_3)_4N(OAc)$, 2.7 mM enzyme active sites, 2 mM $Mn(OAc)_2$, 4 mM $Cd(OAc)_2$, 5.5 mM ATPγS, 24 mM Tl(OAc), and 9 mM unlabeled or ^{17}O-enriched oxalate. Spectra were obtained at −2 °C. (A) Spectrum of sample with unlabeled oxalate. (B) Expansions of the lowest field $^{55}Mn^{2+}$ hyperfine components in the spectra of the samples with unlabeled oxalate (solid line) and labeled oxalate (dashed line, offset 10 G on the abscissa).

Chart 2



spectrum of this complex has greater anisotropy;[4] yet the signals are comparable in amplitude to those of the other complex.

The soft Lewis acid Cd^{2+} could bind the thio ligand when Cd^{2+} is in either site I or site II. With the possibilities for thio or oxy coordination of Cd^{2+} at each of the two divalent cation sites in the hybrid complexes, four isomeric species (**a–d**, Chart 2) are possible.

EPR measurements with the chiral ^{17}O-labeled forms of ATPγS permit an assignment of the isomeric composition of complexes in the sample. Spectra obtained from samples with R_p and S_p-[γ-^{17}O]ATPγS and with a matched sample of unlabeled ATPγS are shown in Figure 6. In Figure 6A signals for the complex with R_p-[γ-^{17}O]ATPγS exhibit inhomogeneous broadening for Mn^{2+} at site I (signals marked I) and at site II (signals marked II). In contrast, the signals for the complex with S_p-[γ-^{17}O]ATPγS (Figure 6B) show inhomogeneous broadening only for Mn^{2+} at site II. A direct comparison of signals for complexes of S_p (solid curve, Figure 6C) and R_p (dashed curve, Figure 6C) emphasizes that signals for Mn^{2+} at site I are not broadened in the complex with the S_p epimer. These data show that Mn^{2+} at site I binds exclusively to the *pro-R* oxygen in the enzyme–oxalate–Mn^{II}–ATPγS–Cd^{II} species (a in Chart 2). The amplitudes of signals from Mn^{2+} at site I in the sample with S_p-[γ-^{17}O]ATPγS match those of the unlabeled sample. The EPR signals of Mn^{2+} at site II (the enzyme–oxalate–Cd^{II}–ATPγS–Mn^{II} species), however, are influenced by ^{17}O in both R_p- and S_p-[γ-^{17}O]ATPγS. This observation shows that isomers c and d are both present in the sample. The fraction of each isomer is estimated from the amplitudes of the signals of Mn^{2+} at site II. Isomer d, where Mn^{2+} binds to the *pro-R* oxygen, represents 62 ± 5% of the site II Mn^{2+}, whereas isomer c represents 38 ± 5% of the Mn^{2+} in this site. Furthermore, the observation of Mn^{2+}–^{17}O interaction in the enzyme–oxalate–Cd^{II}–[γ-^{17}O]ATPγS–Mn^{II} species with either the R_p or *the* S_p isomer shows that the thio substituent is not a ligand for the hard Lewis acid at site II, and this observation reinforces the earlier assertion that Mg^{2+} at site II does not bind to the thio ligand.

The spectral data of Figure 6 show that, of the four possible isomers of the hybrid complex with Cd^{2+} and Mn^{2+}, isomers

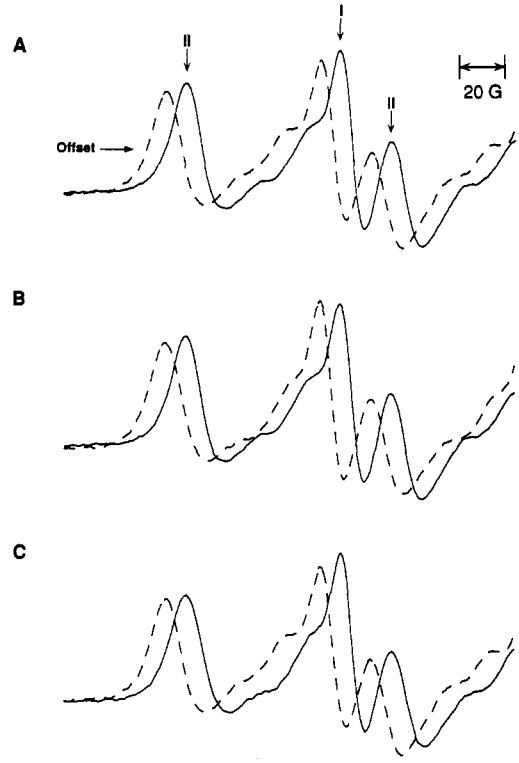


FIGURE 6: Effect of ^{17}O in ATPγS on spectra of samples containing enzyme, oxalate, ATPγS, and a combination of Mn^{2+} and Cd^{2+} in the presence of Tl^{+}. (A) Expansions of the lowest field $^{55}Mn^{2+}$ hyperfine components in the spectra of the samples with unlabeled ATPγS (solid line) and R_p-[γ-^{17}O]ATPγS (dashed line offset 10 G on the abscissa). (B) Expansions of the lowest field $^{55}Mn^{2+}$ hyperfine components in the spectra of the samples with unlabeled ATPγS (solid line) and S_p-[γ-^{17}O]ATPγS (dashed line offset 10 G on the abscissa). (C) Expansions of the lowest field $^{55}Mn^{2+}$ hyperfine components in the spectra of the samples with S_p-[γ-^{17}O]ATPγS (solid line) and R_p-[γ-^{17}O]ATPγS (dashed line offset 10 G on the abscissa).

[4] In comparing EPR spectra of Mn^{2+} species, the magnitude of the zero field splitting anisotropy in the dominant, M_s 1/2 ↔ M_s −1/2, fine-structure transition is greatest in the spectrum in which the signals are displaced furthest from $g = 2$ [see Reed and Markham (1984)].

a, c, and d have appreciable concentrations. The relative abundances of isomers c and d indicate that the soft Lewis acid Cd^{2+} captures the thio substituent as a ligand at site I with an efficiency of ~60%. At site II, however, Cd^{2+} binds virtually exclusively to the *pro-S* oxygen, and isomer b is not detected. The resistance of the thio substituent to pairing with Zn^{2+} or Co^{2+}, or Cd^{2+} at site II, suggests that this substituent is involved in an energetically favorable interaction when not liganded to either of the divalent cations.

The stereochemical configuration at P_γ of ATPγS in its binding interactions with hybrid combinations of divalent cations at the active site of pyruvate kinase provides additional clues to the structure of this site. The P–S bond in phosphorothioates is ~0.5 Å longer than the corresponding P–O bond (Frey & Sammons, 1985). Steric crowding might contribute to the positional preference of the thiophosphoryl group, given the apparent selectivity of the ATP site for the smaller cations. The larger cations Ca^{2+} and Cd^{2+} do, however, populate the ATP site in mixtures with Mn^{2+} (Buchbinder & Reed, 1990), and the ATP site accommodates $Co^{III}(NH_3)_4$-ATP (Dunaway-Mariano & Cleland, 1980). Furthermore, the two large cations Cd^{2+} and Tl^+ bind simultaneously in the active site. Although the steric contributions to the stereochemical configuration cannot be overlooked, it is likely that the characteristics of groups at the active site that interact with the third oxy substituent at Pγ of ATP influence the orientation of the thio substituent of ATPγS.

The third phosphoryl oxygen might bind at a site with multiple hydrogen bond donors (i.e., a site analogous to the oxy anion hole of serine proteases), and the hydrogen bonds might assist in stabilizing the trigonal planar geometry for the $-PO_3$ group in the transition state. As noted above, the preference of the thio substituent for this site over coordination to Zn^{2+}, Co^{2+}, or Cd^{2+} at site II diminishes the likelihood of a site having solely hydrogen-bonding interactions. Rather, interaction of the thio substituent with a positively charged entity would explain the apparent tenacity of the thio moiety for this position in preference to soft divalent cations at site II in the complexes. At present, the two most likely candidates for such a salt link are the guanidium group of an Arg residue and the monovalent cation. Although a protonated ϵ-amino group of a Lys residue could provide such a salt link, Lys-269 appears to be the only conserved Lys in the region of the active site (Muirhead et al., 1986). Jiang et al. (1991) have presented evidence, through site-directed mutagenesis and stereochemical analysis, that Arg-44 in the active site of adenylate kinase interacts with the thio substituent of AMPS. Four conserved Arg residues (R71, R119, R244, and R293) are in the vicinity of the active site of pyruvate kinase (Muirhead et al., 1986). ^{205}Tl NMR relaxation measurements position the monovalent cation 4.5–5 Å from the divalent cation at site I (Reuben & Kayne, 1971). This distance would be compatible with a bridging phosphate ligand between all three inorganic cations, and the monovalent cation remains a viable candidate for the third binding determinant.

The view of the electrostatic environment of the transferable phosphoryl group in the active site of pyruvate kinase that emerges from these studies is strikingly similar to that of the phosphoryl group in the active site of alkaline phosphatase, in which the oxygens of the $-PO_3$ group are bound by two Zn^{2+}'s and the guanidium group of Arg-166 (Kim & Wyckoff, 1991). The constellation of positive charges interacting with the $-PO_3$ group in alkaline phosphatase and pyruvate kinase contrasts with, for example, the surroundings of the γ–PO_3 in the GppNp complex of H-*ras* p21 where Mg^{2+} and the ϵ-amino group of Lys-16 interact with the β- and γ-phosphates and other interactions with γ–PO_3 are H-bonding only (Pai et al., 1990). For pyruvate kinase, however, the clustering of positive charges around the transferable–PO_3 may be a consequence of the high density of negative charge associated with the substrates.

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