Coordination of Manganous Ion at the Active Site of Pyruvate,Phosphate Dikinase: The Complex of Oxalate with the Phosphorylated Enzyme[†]

James L. Kofron,[‡] David E. Ash,[§] and George H. Reed*[,‡]

Institute for Enzyme Research, Graduate School, and Department of Biochemistry, College of Agricultural and Life Sciences, The University of Wisconsin, Madison, Wisconsin 53705, and Department of Biochemistry and Biophysics, The University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Received October 26, 1987; Revised Manuscript Received February 3, 1988

ABSTRACT: Electron paramagnetic resonance spectroscopy has been used to investigate the structure of the complex of manganous ion with the phosphorylated form of pyruvate,phosphate dikinase (E_p) and the inhibitor oxalate. Oxalate, an analogue of the enolate of pyruvate, is competitive with respect to pyruvate in binding to the phosphorylated form of the enzyme [Michaels, G., Milner, Y., & Reed, G. H. (1975) *Biochemistry 14*, 3213–3219]. Superhyperfine coupling between the unpaired electrons of Mn(II) and ligands specifically labeled with ^{17}O has been used to identify oxygen ligands to Mn(II) in the complex with oxalate and the phosphorylated form of the enzyme. Oxalate binds at the active site as a bidentate chelate with Mn(II). An oxygen from the 3′-*N*-phosphohistidyl residue of the protein is in the coordination sphere of Mn(II), and at least two water molecules are also bound to Mn(II) in the complex. Oxalate also binds directly to Mn(II) in a complex with nonphosphorylated enzyme. The structure for the E_p–Mn(II)–oxalate complex implies that simultaneous coordination of a phospho group and of the attacking nucleophile to the divalent cation is likely an important factor in catalysis of this phospho-transfer reaction.

Pyruvate,phosphate dikinase (EC 2.7.9.1, pyruvate,orthophosphate dikinase) catalyzes reversible phosphorylation of pyruvate and inorganic phosphate (P_i)[1] with a single equivalent of ATP:

$$\text{ATP} + \text{pyruvate} + P_i \rightleftarrows \text{AMP} + \text{P-enolpyruvate} + PP_i \quad (1a)$$

The enzyme is found in plants with the C_4 pathway and in some species of bacteria. Results from steady-state kinetic studies and from studies of exchange reactions[2] with the enzyme from bacteria indicate that the reaction proceeds with a ping-pong mechanism involving two covalent enzymic intermediates, E_{pp} and E_p (Evans & Wood, 1968; Milner & Wood, 1976; Milner et al., 1978; Phillips & Wood, 1986):

$$E + \text{ATP} \rightleftarrows E_{p_\beta p_\gamma} + \text{AMP} \quad (1b)$$

$$E_{p_\beta p_\gamma} + P_i \rightleftarrows E_{p_\beta} + PP_i \quad (1c)$$

$$E_{p_\beta} + \text{pyruvate} \rightleftarrows \text{P-enolpyruvate} + E \quad (1d)$$

The phospho group of E_p is in an acid-labile phosphoramidate linkage with a 3′ nitrogen of a histidyl residue (Spronk et al., 1976; Milner et al., 1978), and a peptide containing a 3′-*N*-phosphohistidyl residue has been isolated and sequenced (Goss et al., 1980). The stereochemical courses of the transfer events have been elucidated, and the results are consistent with the covalent intermediates in the mechanism described above (Cook & Knowles, 1985). More recently, treatment of the labile pyrophosphorylated intermediate with diazomethane has allowed isolation of a pyrophosphohistidine from hydrolysates (Phillips & Wood, 1986).

The enzyme requires divalent cations as cofactors in each of the three partial reactions. The third partial reaction (1d) also exhibits a specific requirement for activation by monovalent cations (Michaels & Milner, 1974). Ammonium ion and thallous ion provide the highest levels of activity among the monovalent cations examined (Michaels, 1975). Oxalate, a structural analogue of the enolate of pyruvate, is a competitive inhibitor with respect to pyruvate in the third partial reaction (Michaels et al., 1975). Oxalate binds with high affinity to the phosphorylated form of the enzyme, and the properties of the E_p–oxalate complex suggest that oxalate mimics the reactive enolate of pyruvate (Michaels et al., 1975).

The coordination scheme of the divalent metal ion in the complex with E_p–oxalate is of considerable interest because the structure of this species may hold important clues regarding the function of the metal ion both in the phospho-transfer reaction and in activation of pyruvate. The narrow EPR signals for Mn(II) bound in the complex with E_p–oxalate (Michaels et al., 1975) provide an opportunity to determine the identity of oxygen ligands to the divalent cation through observation of superhyperfine coupling interactions between the unpaired electron spin of Mn(II) and the nuclear spin of ^{17}O selectively incorporated into potential ligand groups (Reed & Leyh, 1980; Reed & Markham, 1984). The present paper reports the results of such EPR measurements.

EXPERIMENTAL PROCEDURES

Materials. Pyruvate,orthophosphate dikinase was isolated from *Clostridium symbiosum* (formerly *Bacteroides sym-*

[†] This work was supported by Grant GM35752 from the National Institutes of Health.

[‡] Present address: Institute for Enzyme Research, University of Wisconsin—Madison, 1710 University Ave., Madison, WI 53705.

[§] Present address: Department of Biochemistry, Temple University School of Medicine, Philadelphia, PA 19140.

[1] Abbreviations: P-enolpyruvate, phosphoenolpyruvate; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; E_p, the phosphorylated form of pyruvate,phosphate dikinase; E_{pp}, the pyrophosphorylated form of pyruvate,phosphate dikinase; D and E, respectively, the axial and rhombic zero-field splitting parameters in the spin Hamiltonian for Mn(II); trien, triethylenetetramine; P_i, inorganic phosphate; Tris, tris(hydroxymethyl)aminomethane.

[2] More recent experiments with the enzyme from *C. symbiosum* indicate ATP/AMP exchange (reaction 1b) requires the presence of P_i (Wang et al., 1988).

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biosis) grown on tryptone/yeast extract/glucose media. Purification of the enzyme was by a method similar to that described by South and Reeves (1975). The active fractions from the hydroxylapatite chromatography were pooled and were further purified by liquid chromatography on a Pharmacia Mono Q column. The elution buffer was 20 mM imidazole/HCl and 20 mM NH_4OH, pH 6.85. The HR 10/10 Mono Q column was eluted with a 40-mL linear gradient, 0–0.25 M NaCl, to remove the major impurity, and the enzyme was subsequently eluted with a second linear gradient, 0.25–0.3 M NaCl (80 mL). The enzyme had specific activities of 12–24 IU/mg when assayed in the reverse direction in a coupled assay with lactic acid dehydrogenase. The enzyme was stored as an ammonium sulfate suspension at 4 °C. Before use, solutions of the enzyme were dialyzed exhaustively versus 20 mM Hepes/NH_4OH, pH 6.8, 70 mM NH_4Cl, and 0.2 mM 2-mercaptoethanol (buffer A). Some samples were prepared by dialysis versus 30 mM Hepes/$(CH_3)_4NOH$, pH 6.8, and 0.5 mM 2-mercaptoethanol. Following dialysis, the solutions were brought to 8 mM in thallium acetate (buffer B). Solutions of the enzyme were concentrated with an Amicon Minicon A-25 concentrator or with a collodion bag apparatus.

Water enriched in $H_2{}^{17}O$ was obtained from Yeda Research and Development Co. and Monsanto Research Corp. Oxalic acid enriched in ^{17}O was prepared by dissolving 1.3 mg of the $(COOH)_2 \cdot 2H_2O$ in 20 μL of 59.3 atom % $H_2{}^{17}O$. A control sample was prepared in normal water. The samples were sealed in ampules and incubated in the dark at room temperature for 2–4 weeks. The samples were neutralized with solid Tris base and diluted to a concentration of 0.1 M. The ^{17}O enrichment in the oxalate was assayed by mass spectrometry. Samples of [β,β-$^{17}O_2$,β-γ-^{17}O]ATP were prepared and assayed as described by Leyh et al. (1985). P-enolpyruvate with ^{17}O enrichment in the phospho and carboxyl groups was prepared by the acidic exchange method of O'Neal et al. (1983) in 56 atom % $H_2{}^{17}O$. The exchange took place in 1 N HCl over 5.5 min at 98 °C. A control sample of P-enolpyruvate was treated in an identical fashion in normal water. The ^{17}O enrichment in the phospho group of P-enolpyruvate was assayed by ^{31}P NMR at 162 MHz. A solution of PP_i contained in a coaxial insert served as an intensity reference. The intensity of this solution of PP_i was calibrated with a standard solution of unlabeled P-enolpyruvate. The ^{17}O enrichment in the phospho group was 46 ± 5%.

EPR Measurements. EPR spectra were obtained at 35 GHz with a Varian E-109-Q spectrometer equipped with a standard accessory for control of sample temperature. The spectrometer was interfaced with an IBM AT microcomputer. Spectral simulations were performed according to procedures outlined previously (Reed & Markham, 1984; Moore & Reed, 1985).

Samples that were used to compare EPR spectra for labeled and unlabeled oxalate were prepared from a common stock solution that contained enzyme, $MnCl_2$, and P-enolpyruvate in buffer B. This solution produces the equilibrium mixture of reaction 1d. Identical volumes of solutions of concentration-matched samples of labeled or unlabeled oxalate were added to matched aliquots from the stock solution. The high affinity[3] of oxalate for the E_p–Mn(II)–oxalate complex (Michaels et al., 1975) effectively pulls reaction 1d in the direction of formation of E_p. The high affinity of Mn(II) for the E_p–Mn(II)–oxalate complex ensures a virtually stoichiometric binding of Mn(II) to the enzymic complex in the presence of a slight excess of E_p and oxalate. At the level of Mn(II) used in these experiments, the precision in sampling and EPR measurements was >97% as determined from reproducibility in the amplitudes of EPR signals for separate samples.

Labeling of the phosphohistidyl residue of the enzyme with ^{17}O was accomplished (via reaction 1d) by addition of ^{17}O-enriched P-enolpyruvate to a stock solution of enzyme, $MnCl_2$, and oxalate to form E_p–Mn(II)–oxalate with ^{17}O enrichment in the enzymic phosphoramidate group. A matched sample was obtained by addition of unlabeled P-enolpyruvate (treated in the same manner as the labeled P-enolpyruvate) to an aliquot from the same stock solution.

For determination of water ligands, aliquots from a stock solution containing enzyme, $MnCl_2$, P-enolpyruvate, and oxalate in buffer A were lyophilized and redissolved either in normal water or in water enriched in $H_2{}^{17}O$. For this protein, reproducibility in subsequent dissolution of the lyophilized samples was facilitated by allowing the samples to freeze "slowly" in a freezer at −20 °C prior to lyophilization. Reliable reproducibility in the amplitudes of EPR signals for samples that were lyophilized was, however, limited to protein concentrations of ~60 mg/mL. This operational limit on protein concentration limited the signal-to-noise ratios in these experiments. Samples were prepared in ^2H_2O by successive cycles of dilution of protein in buffer B prepared in ^2H_2O and reconcentration.

Detection of Superhyperfine Coupling. Superhyperfine coupling between the unpaired electron spin of Mn(II) and the nuclear spin of ^{17}O ($I = {}^5/_2$) splits each EPR transition into a sextet pattern. The superhyperfine coupling constant is smaller than the intrinsic line widths of the EPR signals, so that the coupling is unresolved in the spectrum. The coupling does, however, add an inhomogeneous contribution to the widths of the signals, and this increment in line width can be readily observed whenever the intrinsic signal widths are of the same order as the width of the sextet pattern from the superhyperfine coupling (Reed & Leyh, 1980). Previous experiments with two epimers of [α-^{17}O]ADP in complexes with creatine kinase demonstrate that ^{17}O must be in the first coordination sphere to produce a detectable inhomogeneous broadening in the EPR signals for Mn(II) (Leyh et al., 1982). EPR spectra for samples with unlabeled ligands, together with the isotopic enrichment of the labeled samples, can be used in a spectral substraction procedure to determine the number of coordination sites occupied by the ligand (Reed & Leyh, 1980; Lodato & Reed, 1987).

RESULTS

Description of EPR Spectra. The dominant signals in the EPR spectrum for the E_p–Mn(II)–oxalate complex are due to the central fine structure transition ($M_s = {}^1/_2 \leftrightarrow M_s = -{}^1/_2$) for Mn(II) ($S = {}^5/_2$). As shown in Figure 1, the experimentally observed pattern is reproduced reasonably well in simulations with a single set of spin Hamiltonian parameters (D = 240 G, E/D = 0.19). Thus, all of the features in the spectrum arise from Mn(II) in a unique coordination environment. The intrinsically narrow signals in the spectrum, together with the appearance of partially resolved, second-order fine structure, facilitate observation of inhomogeneous broadening from superhyperfine coupling to ^{17}O of directly bound oxygen ligands.

The influence of monovalent cations on the EPR signals for Mn(II) in the complex with E_p–oxalate is shown in Figure 2.

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[3] An upper limit of 2 μM for the dissociation constant of oxalate from the complex with E_p and Mn(II) is obtained from the titration data given by Michaels et al. (1975). By contrast, the K_m for pyruvate in reaction 1d is 80 μM (Michaels, 1975).

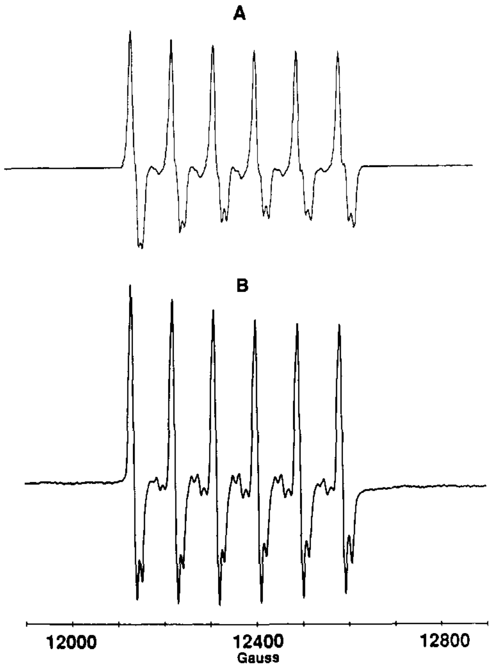

FIGURE 1: Simulated (A) and experimental (B) EPR spectra for the E_p–Mn(II)–oxalate complex. The simulated spectrum was calculated with the following set of spin Hamiltonian parameters: $D = 240$ G, $E = 40$ G, A (^{55}Mn) = 90.4 G, and a Lorentzian line width of 4 G. Forbidden ^{55}Mn hyperfine transitions were included in the calculation. The line shape was modeled with a Gaussian distribution in D with a half-width of 10 G. The simulated spectrum is a sum of seven spectra with amplitudes weighted according to the Gaussian distribution. The experimental spectrum (B) was obtained with a solution containing Hepes/tetramethylammonium hydroxide, pH 6.8, mg/mL enzyme, 440 μM $MnCl_2$, 5.6 mM P-enolpyruvate, 5.6 mM tetramethylammonium oxalate, and 9 mM thallium(I) acetate.

The features that are characteristic of the spectrum for the E_p–Mn(II)–oxalate complex are apparent with $(CH_3)_4N^+$ present.[4] Addition of the strongly activating Tl^+, however, produces a striking improvement in the resolution of these features. There is a further increase in the resolution of spectral features for samples dissolved in ^2H_2O. This latter effect is due to a loss (by $^2H/^1H$ exchange) of unresolved ^{1}H–Mn(II) superhyperfine coupling. The unusually narrow signals observed for the complex in ^2H_2O provided an opportunity to search for features due to a substantial ^{205,203}Tl–Mn(II) superhyperfine coupling. A 25–30 G ^{205,203}Tl–VO^{2+} coupling has been observed in complexes with pyruvate kinase (Lord & Reed, 1987) and with *S*-adenosylmethionine synthetase (Markham & Leyh, 1987). There is, however, no evidence for ^{205,203}Tl–Mn(II) coupling of $>\sim 2$ G in the EPR signals.

EPR Spectra for Complexes with Labeled Oxalate. Oxalate binds with high affinity to the E_p form of the enzyme, and addition of a slight excess of oxalate to solutions of the equilibrium mixture of reaction 1d leads to a virtually stoichiometric conversion of the enzyme to the E_p–Mn(II)–oxalate species (Michaels et al., 1975). EPR spectra for concentration-matched samples with labeled (54 ± 4 atom % ^{17}O) and unlabeled oxalate are shown in Figure 3 for the E_p–Mn(II)–oxalate complex. Signals obtained for the sample with labeled oxalate exhibit a strong inhomogeneous broadening, and this observation shows that oxalate binds directly to Mn(II) at the active site of the phosphorylated enzyme. The number of coordination sites occupied by oxylate was obtained by the difference spectra method (Reed & Leyh, 1980; Lodato & Reed, 1987). The fractional contribution to the spectrum from complexes that have Mn(II) coordinated exclusively to nonmagnetic isotopes of oxygen from oxalate is related to the enrichment of the labeled oxalate and the number of coordination sites occupied by oxalate. This fraction, F_n, is given by $F_n = [f(^{16}O) + f(^{18}O)]^n$, where $f(^{16}O) + f(^{18}O)$ is the fractional composition of nonmagnetic isotopes of oxygen in the enriched oxalate and n is the number of coordination sites for oxalate. F_n, hence n, is estimated from difference spectra: $L_d = L_e - F_n L_u$. In this expression L_d, L_e, and L_u are, respectively, the line shapes of the difference spectrum, the spectrum for the enriched sample, and the spectrum for the unenriched sample (Lodato & Reed, 1987). Anomalies in L_d of Figure 3B, curve c (at ~12 135 G), for the monodentate model ($n = 1$) show that oxalate is a bidentate ligand for Mn(II) in the E_p–M(II)–oxalate complex.

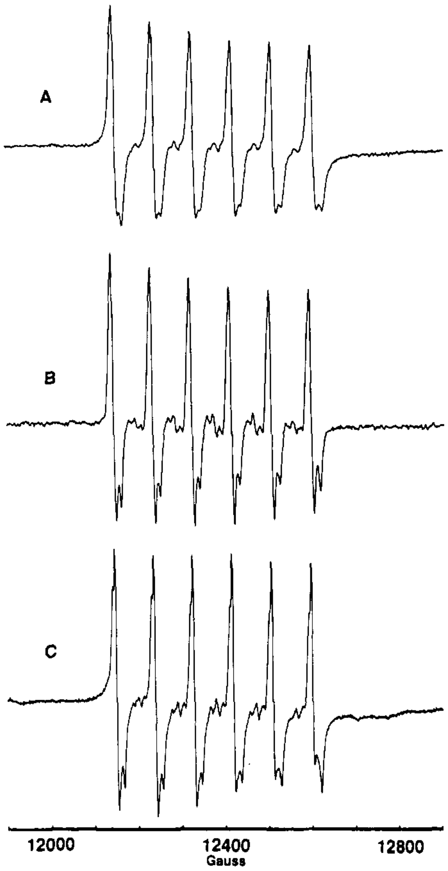

FIGURE 2: EPR spectra (Q-band) for solutions of E_p–Mn(II)–oxalate. (A) Spectrum for a solution containing Hepes/tetramethylammonium hydroxide, pH 6.8, 75 mg/mL) enzyme, 440 μM $MnCl_2$, 5.6 mM P-enolpyruvate, and 5.6 mM tetramethylammonium oxalate. (b) Same concentrations as (A) with the addition of 9 mM thallium(I) acetate. (C) Essentially the same components as (B) after dilution with ^2H_2O and reconcentration. All spectra were obtained in the solution phase at −1 °C.

Oxalate binds to the E form of the enzyme with a much lower affinity than for E_p (Michaels et al., 1975). EPR spectra for Mn(II) in the complex E–Mn(II)–oxalate (i.e., the form of the enzyme that lacks the phosphohistidyl group) with labeled and unlabeled oxalate (see Figure 4) show that Mn(II) also binds directly to oxalate in this analogue of the dead-end complex, E–pyruvate. The intrinsic widths of the EPR signals for the E–Mn(II)–oxalate species are too large to permit a

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[4] It is clear from the EPR spectrum that the tricyclohexylammonium salt of P-enolpyruvate in the $(CH_3)_4N^+$ medium has reacted to form the E_p–oxalate species. $(CH_3)_4N^+$ is not a strong monovalent cation activator (Michaels, 1975). It is not possible to rule out activation of the reaction by a trace contaminating level of another monovalent cation such as ammonium ion.

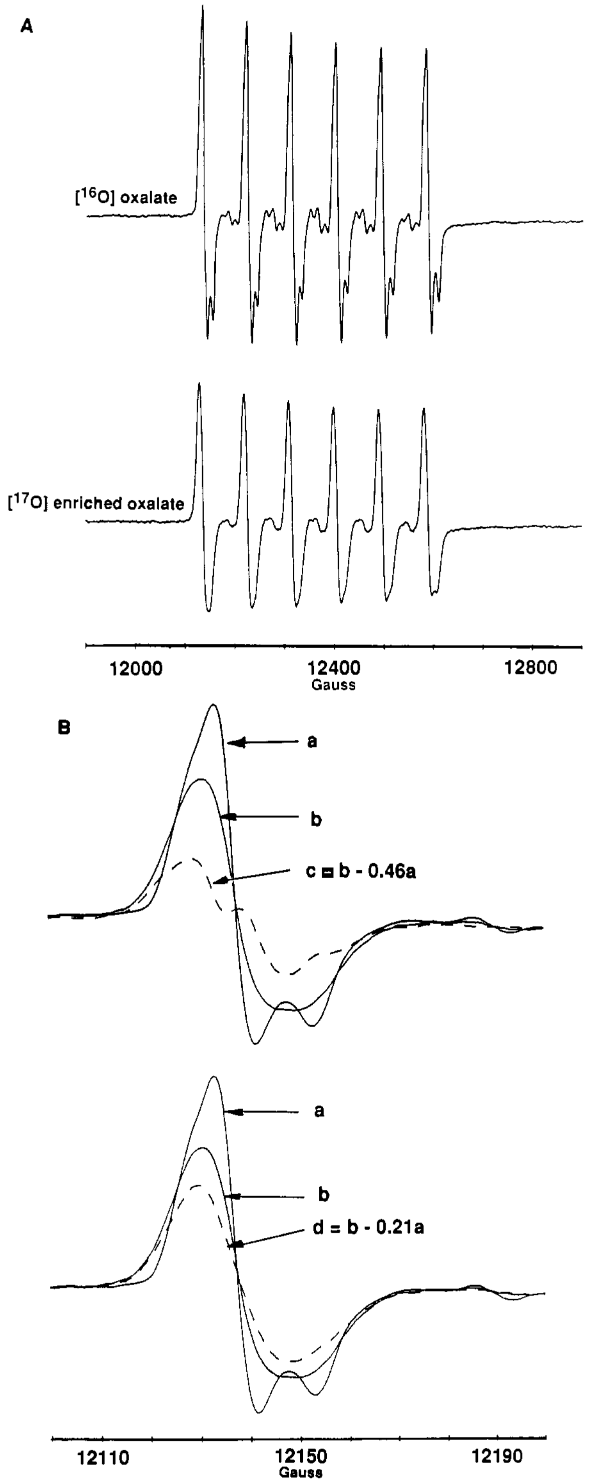

FIGURE 3: EPR spectra (Q-band) for solutions of E_p–Mn(II)–oxalate–Tl^+ with ^{17}O-labeled and unlabeled oxalate. (A) Solutions contained Hepes/tetramethylammonium hydroxide, pH 6.8, 70 mg/mL enzyme, 370 μM $MnCl_2$, 4.6 mM P-enolpyruvate, 7.5 mM thallium(I) acetate, 0.5 mM 2-mercaptoethanol, and 9.1 mM unlabeled or labeled sodium oxalate. Spectra were obtained in the solution phase at −1 °C. (B) Expansions of the low-field ^{55}Mn hyperfine component. Spectra labeled a and b are for the unlabeled and labeled oxalate spectra, respectively. Dashed curves are difference spectra (representing the spectrum for the ^{17}O-liganded species) obtained for a monodentate (c) or a bidentate (d) coordination scheme for oxalate. The difference spectrum for the monodentate model exhibits an anomalous feature at a position that corresponds to the maxima in the spectrum for the unlabeled sample. The appearance of this feature indicates that a smaller fraction of the sample exists with nonmagnetic isotopes of oxygen as ligands (i.e., oxalate is a bidentate ligand). The distribution of the Mn(II) species in the ^{17}O-enriched sample is 21% $^{17}O_0$, 50% $^{17}O_1$, and 29% $^{17}O_2$, where the subscript refers to the number of resident ^{17}O ligands. The dashed curve (d) is a superposition of spectra for the two ^{17}O-liganded species: 50% $^{17}O_1$ and 29% $^{17}O_2$.

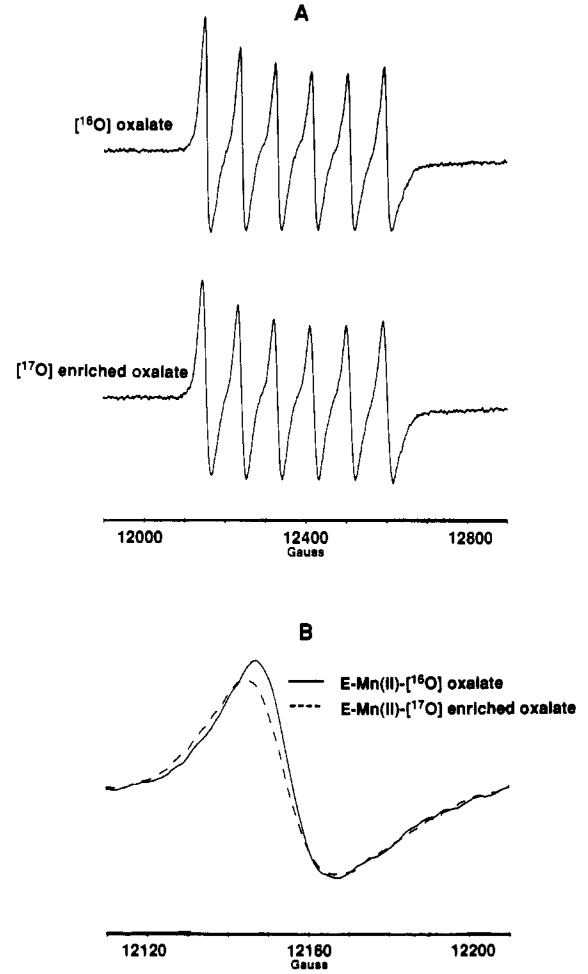

FIGURE 4: EPR spectra (Q-band) for solutions of pyruvate,phosphate dikinase–Mn(II) with ^{17}O-labeled and unlabeled oxalate. Solutions contained Hepes/tetramethylammonium hydroxide, pH 6.8, 80 mg/mL enzyme, 470 μM $MnCl_2$, and 6.3 mM ^{17}O-labeled or unlabeled oxalate. (B) Expansion of the upfield ^{55}Mn hyperfine component for samples with unlabeled and ^{17}O-labeled oxalate. Spectra were obtained for the solution phase at −1 °C.

reliable determination, from difference spectra, of whether oxalate binds as a monodentate or bidentate ligand in this complex. The fact that ^{17}O-induced inhomogeneous broadening is readily apparent in these broad signals, however, makes it highly likely that oxalate functions as a bidentate chelator in the complex [i.e., with chelation there is a higher statistical probability for an ^{17}O to be directly coordinated to Mn(II)].

EPR Spectra for Complexes with a Labeled Phosphatidyl Residue. The active-site histidyl residue of the enzyme was phosphorylated as described under Experimental Procedures with normal and ^{17}O-enriched P-enolpyruvate. Spectra for the E_p–Mn(II)–oxalate complex with the labeled and unlabeled forms of E_p are shown in Figure 5. Inhomogeneous broadening from the ^{17}O-labeled phosphoramidate moiety is clearly evident in the EPR signals. These data show that the phosphohistidyl residue contributes an oxygen ligand for Mn(II) in the complex.

The phosphohistidyl residue can also be labeled from the forward direction starting with [β,β-$^{17}O_2$,β-γ-^{17}O]ATP and P_i. EPR spectra for the E_p–Mn(II)–oxalate complex prepared by this method (data not shown) consistently exhibited a significantly smaller extent of inhomogeneous broadening (for comparable ^{17}O enrichment in the starting compounds) than those prepared from ^{17}O-enriched P-enolpyruvate. The apparent washout of ^{17}O observed in labeling from the β-phos-

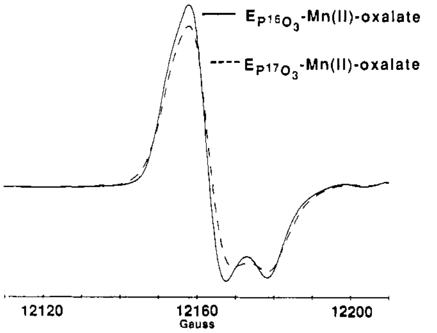

FIGURE 5: EPR spectra (Q-band) for solutions of E_p–Mn(II)–oxalate with ^{17}O-labeled and unlabeled forms of E_p. Solutions contained Hepes/NH_4OH, pH 6.8, 60 mM NH_4Cl, 0.4 mM 2-mercaptoethanol, 115 mg/mL enzyme, 550 μM $MnCl_2$, 3.7 mM sodium oxalate, and 1 mM of either unlabeled or ^{17}O-labeled phosphoenolpyruvate. The low-field ^{55}Mn hyperfine component is shown for each sample. Spectra were obtained at 0 °C.

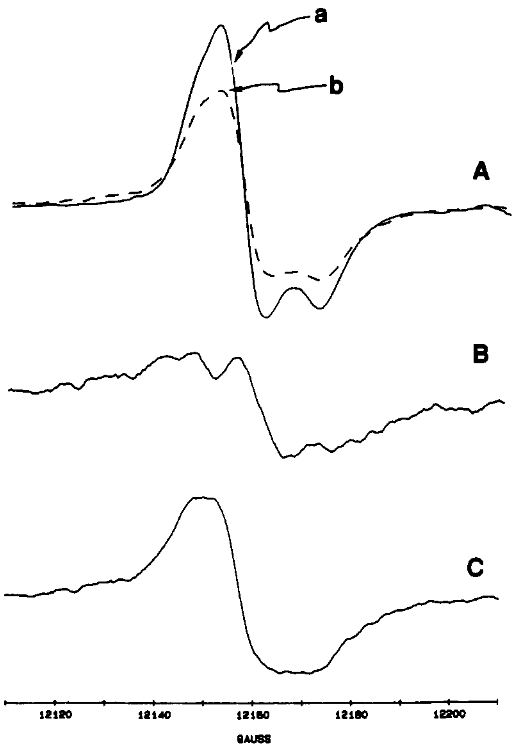

FIGURE 6: EPR spectra (Q-band) of solutions of E_+–Mn(II)–oxalate obtained in water and in water enriched (41 atom %) in ^{17}O. Solutions contained Hepes/NH_4OH, pH 6.8 75 mM NH_4Cl, 0.5 mM 2-mercaptoethanol, 68 mg/mL enzyme, 360 μM $MnCl_2$, and 4.3 mM sodium oxalate. Spectra were obtained at −1 °C in the solution phase. The low-field ^{55}Mn hyperfine component is shown in (A) for both the sample in normal water (a) and the sample in ^{17}O-enriched water (b). The difference spectra for coordination of water to one site [(b) − 0.59(a)] and two sites [(b) − 0.35(a)] are shown in (B) and (C), respectively. The apppearance of anomalous features in the one-site model (B) indicates that a smaller fraction of the sample exists with nonmagnetic isotopes of oxygen as ligands. The difference spectrum for two bound waters (C) has no anomalies, indicating that at least two waters are coordinated to Mn(II) in this complex.

phate of ATP may be due to an adenylate kinase like activity that has been observed with some preparations of the enzyme (Wang et al., 1988).

EPR Spectra for Samples in $H_2{}^{17}O$. Samples of the E_p–Mn(II)–oxalate complex were prepared in normal water and in water enriched to 41 atom % in ^{17}O (see Experimental Procedures). EPR spectra for these samples are compared in Figure 6. Difference spectra (see also Figure 6) indicate that there are at least two water ligands to Mn(II) in the complex.

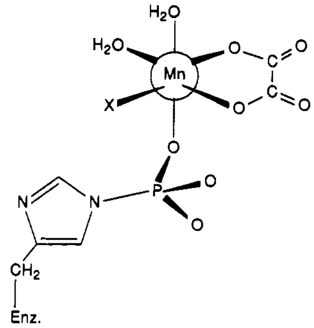

FIGURE 7: Schematic representation of the coordination sphere of the E_p–Mn(II)–oxalate complex. The five oxygen ligand donors have been identified by ^{17}O labeling. The relative positions of the ligands are arbitrary.

The presence of water ligands to the enzyme-bound Mn(II) is also indicated by a measurable reduction in the line widths of signals for the complex E_p–Mn(II)–oxalate obtained in ^2H_2O (see Figure 2). The smaller line widths of signals for samples dissolved in ^2H_2O show that unresolved superhyperfine coupling between the unpaired electron spin of Mn(II) and nearby exchangeable protons makes a measurable contribution to the widths of the signals for the samples in normal water. Protons from the coordinated water ligands are undoubtedly the source of this inhomogeneous broadening.

DISCUSSION

Oxalate is a strong inhibitor of several enzymes for which enolpyruvate is believed to be the reactive form of the substrate pyruvate (Mildvan et al., 1966; Northrop & Wood, 1969; Reed & Morgan, 1974). Since oxalate and pyruvate compete for the same site on the enzyme (Michaels et al., 1975), it is not unreasonable to suspect that the metal ion supplies analogous points for attachment of both compounds. Very recent experiments with a preparation of the mixed anhydride of oxalic and phosphoric acids, oxalyl phosphate, reinforce the notion that oxalate mimics the enolate of pyruvate. Addition of the labile acyl phosphate to a solution of the free enzyme and Mn(II) produces the E_p–Mn(II)–oxalate species (i.e., the phosphorylated form of oxalate is a substrate for reaction 1d) (J. L. Kofron and G. H. Reed, unpublished observations). Hence, the structural features of the E_p–Mn(II)–oxalate complex very likely mimic those for the analogous complex involving the enolate of pyruvate.

The zero-field splitting parameters derived from the EPR pattern for the E_p–Mn(II)–oxalate complex indicate that Mn(II) is bound in normal octahedral coordination geometry with only a slight deviation from cubic electronic symmetry. The narrow widths of the EPR signals show that there is a high degree of fidelity in the structure throughout the entire population of the sample. The present experiments with ^{17}O-labeled ligands have identified five oxygen ligands for Mn(II) in this complex. A plausible structure for the complex is shown schematically in Figure 7. The structure given in Figure 7 shares several features with that of the complex pyruvate kinase–Mn(II)–oxalate–Mg^{II}ATP (Lodato & Reed, 1987). In the complex with pyruvate kinase, Mn(II) binds in a bidentate chelate with oxalate and also coordinates to the γ-phosphate of the Mg^{II}ATP. In the complex with pyruvate,-phosphate dikinase, the γ-phosphate ligand of Mg^{II}ATP is replaced by the phosphatidyl group. In complexes with both enzymes, oxalate is believed to mimic a form of pyruvate that is activated to attack a phospho group to produce a P-enolpyruvate product. The common features of the complexes with the two enzymes suggest that the divalent metal ion plays similar roles in formation of the enolate and in the phosphotransfer steps in the overall reactions of each enzyme.

Although the ^{17}O superhyperfine coupling identifies five of the six ligands to Mn(II) in the complex with E_p and oxalate, the geometrical arrangement of the ligands about Mn(II) shown in Figure 7 is not defined rigorously by the spectroscopic data. The magnitude of the zero-field splitting anisotropy for Mn(II) in the complex does, however, render an end-on configuration of the oxalate ligand improbable, because the distortion in the four-membered chelate ring would likely give rise to a larger zero-field splitting anisotropy. Modeling also suggests that the phosphohistidyl ligand should be bound cis with respect to both of the carboxylate groups of oxalate to require only minimal rearrangement in the transition state for phosphotransfer. The position of the monovalent cation has not been determined. The monovalent cation influences the dissociation constants for Mn(II) and for oxalate (Michaels et al., 1975) as well as the widths of EPR signals for Mn(II) in the complex. A carboxylate group from oxalate may possibly function as a bridging ligand between monovalent and divalent cations. The sixth ligand for Mn(II) is as yet unidentified.

Water proton relaxation measurements in solutions of the E_p–Mn(II)–oxalate complex had shown that if there were any water molecules coordinated to the Mn(II), then the rate of exchange of these ligands was slow on the effective NMR timescale [i.e., the residence time of the water ligands is longer than the longitudinal relaxation time of water protons when bound to Mn(II) (Michaels et al., 1975)]. The two water ligands revealed by the present EPR data are therefore in slow exchange as measured by NMR. Slow exchange of the water ligands suggests that Mn(II) is likely bound in a cleft that is inaccessible to the bulk solvent. The coordinated waters presumably make favorable H-bonding contacts with groups on the protein, and such contacts would contribute to the high stability of the complex and to the high fidelity of the structure. Slow exchange of water ligands is also observed in anion-stabilized complexes of creatine kinase (Reed & Leyh, 1980).

Bidentate coordination of pyruvate and of the corresponding enolate to the metal ion does offer advantages in activation of pyruvate. Chelation of Mn(II) by a carboxylate oxygen and the oxygen at C_2 of pyruvate would stabilize the reactive enolate in the forward direction of reaction 1d. Analogous roles for metal ions in catalysis of the enolization of α-keto acids have been proposed [see Miller and Leussing (1985), Schellenberger and Hubner (1965), and Kluger (1978)]. The reactivity of nucleophiles coordinated to metal ions has been firmly established (Buckingham et al., 1969, 1970), and coordination of Mn(II) to the enolate oxygen of enolpyruvate would not inactivate this nucleophile. Viewed from the other direction, the coordination of Mn(II) to the C–O–P bridging oxygen in the complex with P-enolpyruvate would augment the leaving-group ability of the enolate in the formation of E_p from P-enolpyruvate.

Simultaneous coordination of the divalent metal ion to the phosphohistidyl group and to the prospective attacking nucleophile involves a four-membered ring in the transition state. Phospho transfer from the phosphoramidate to enolpyruvate would also produce a four-membered chelate ring in the immediate product complex with P-enolpyruvate. Cornelius (1980) has presented evidence from studies of the hydrolysis of a bidentate complex, $Co^{III}(NH_3)_4PPP_i$, catalyzed by $Co^{III}(NH_3)_4(H_2O)_2$ that simultaneous coordination of a phospho group and an attacking nucleophile (H_2O) to Co(III) is an important factor in the rate of water attack. The transition state in this nonenzymic hydrolysis would involve a four-membered chelate ring. Farrell et al. (1969) proposed that bidentate coordination of methyl phosphate bound to $Co(trien)^{3+}$ was significant in enhancing the rate of hydrolysis of this ligand. Cyclodiphosphates (with four-membered rings) are involved as intermediates in reactions of nucleoside phosphorothioates with cyanogen bromide (Sammons et al., 1982; Iyengar et al., 1984).

The high affinity of oxalate for the E_p form of pyruvate,-phosphate dikinase coupled with the unusually sharp EPR signals for the E_p–Mn(II)–oxalate species suggested that this complex might mimic the transition state for the final partial reaction of the enzyme (Michaels et al., 1975). The very recent observations regarding substrate activity for oxalyl phosphate (J. L. Kofron and G. H. Reed, unpublished results), however, imply that the oxalate complex with E_p actually mimics an intermediate state, E_p–Mn(II)–pyruvate (enolate).

ACKNOWLEDGMENTS

We thank Drs. Harland G. Wood and Neil H. Goss for providing samples of enzyme for preliminary studies. We thank Drs. Harland G. Wood and Nelson F. B. Phillips for providing a slant culture of *C. symbiosum*. We also thank Drs. Debra Dunaway-Mariano and Daniel H. Rich for helpful discussions and Drs. George D. Markham and Geoffrey W. Smithers for data acquisition software. The ^{31}P NMR spectra were obtained at the National Magnetic Resonance Facility at Madison (NIH Grant RR02301).

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Mechanism of Aromatic Ring Cleavage of a β-Biphenylyl Ether Dimer Catalyzed by Lignin Peroxidase of *Phanerochaete chrysosporium*[†]

Keiji Miki,[‡] Ryuichiro Kondo,[§] V. Renganathan, Mary B. Mayfield, and Michael H. Gold[*]

Department of Chemical and Biological Sciences, Oregon Graduate Center, Beaverton, Oregon 97006-1999

Received December 14, 1987; Revised Manuscript Received February 18, 1988

ABSTRACT: Homogeneous lignin peroxidase (LiP) oxidized 1-(4-methoxyphenyl)-2-(2,6-dimethoxy-4-phenylphenoxy)-1,3-propanediol (I) to yield 1-(4-methoxyphenyl)-1,2,3-propanetriol (II), α-(4-methoxyphenyl)-2-oxo-1,3-dioxolane-4-methanol (III), 5-(4-methoxyphenyl)-2-oxo-1,3-dioxolane-4-methanol (IV), 4-oxo-3-phenyl-2-butenoic acid methyl ester (V), 5-hydroxy-4-phenyl-2(5*H*)-furanone (VI), 4-oxo-3-phenyl-2-pentenedioic acid dimethyl ester (VII), and 5-carbomethoxy-5-hydroxy-4-phenyl-2(5*H*)-furanone (VIII). Products II–VIII are all derived from the opening of the B aromatic ring of the substrate. Oxidation of I in $H_2{}^{18}O$ resulted in 87% and 65% incorporation of ^{18}O into the carbonyl oxygen of III and IV, respectively. Oxidation of I under $^{18}O_2$ resulted in the incorporation of two atoms of oxygen into the aldehyde (V); one of these atoms of incorporated oxygen was exchangeable with H_2O. Oxidation of I under $^{18}O_2$ also resulted in three atoms of oxygen incorporated into the ketone (VII); one of these atoms of incorporated oxygen was exchangeable with H_2O. Oxidation of I under either $^{18}O_2$ or $H_2{}^{18}O$ resulted in the incorporation of three atoms of oxygen into the lactol VI; two atoms of incorporated oxygen originate from O_2 and one from H_2O. Finally, oxidation of I under either $^{18}O_2$ or $H_2{}^{18}O$ indicated that four atoms of oxygen were incorporated into the lactol VIII; three atoms of incorporated oxygen originated from O_2 and one from H_2O. These results are explained by a mechanism involving the one-electron oxidation of the B ring of the substrate to an aryl cation radical by the H_2O_2 oxidized enzyme. This aryl cation radical intermediate undergoes nucleophilic attack by H_2O or the γ or α side-chain hydroxyls followed by coupling with O_2 to yield several possible cyclic peroxide intermediates. These intermediates undergo cleavage reactions to yield various final products. In these oxidative pathways, only the formation of the aryl cation radical is enzyme-catalyzed. Subsequent reactions appear to be nonenzymatic, accounting for the variety of ring-opened products formed.

The lignin-degrading basidiomycete *Phanerochaete chrysosporium* secretes at least two extracellular heme peroxidases during its secondary metabolic phase of growth. These enzymes, manganese peroxidase (MnP)[1] (Glenn & Gold, 1985; Paszczynski et al., 1986; Glenn et al., 1986) and lignin peroxidase (LiP) (Gold et al., 1984; Tien & Kirk, 1984; Renganathan et al., 1985), have been purified to homogeneity. The main isozymic form of lignin peroxidase is a glycoprotein with M_r 41 000, and all forms of the enzyme have a single iron protoporphyrin IX prosthetic group (Renganathan et al., 1985). Electronic absorption spectroscopy (Gold et al., 1984), EPR spectroscopy (Andersson et al., 1985), and resonance Raman spectroscopy (Andersson et al., 1985, 1987; Kuila et al., 1985) indicate that the heme iron in native lignin per-

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[†] This work was supported by Grants DE-FG06-86ER 13550 from the U.S. Department of Energy and DMB 8311441 from the National Science Foundation.

[*] To whom correspondence should be addressed.

[‡] Present address: National Research Institute for Pollution and Resources, Yatabe, Tsukuba, Ibaraki 305, Japan.

[§] Present address: Department of Forest Products, Faculty of Agriculture, Kyushu University, Hakozaki, Higashi-ku, Fukuoka 812, Japan.

[1] Abbreviations: BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; DMF, dimethylformamide; FT-NMR, Fourier transform nuclear magnetic resonance spectrometer; GCMS, gas chromatography–mass spectrometry; HRP, horseradish peroxidase; LiP, lignin peroxidase; MnP, manganese peroxidase; MS, mass spectrum; TMS, trimethylsilyl.

0006-2960/88/0427-4787$01.50/0