Magnetic Resonance and Kinetic Studies of Pyruvate, Phosphate Dikinase. Interaction of Oxalate with the Phosphorylated Form of the Enzyme†

George Michaels, Yoram Milner,‡ and George H. Reed*

ABSTRACT: Pyruvate, orthophosphate dikinase (EC 2.7.9.1) carries out its catalytic function in three successive partial reactions, the final step being the reaction of pyruvate with a stable phosphoenzyme intermediate to give phosphoenolpyruvate and free enzyme (Evans, H. J., and Wood, H. G. (1968), *Proc. Natl. Acad. Sci. U.S.A. 61*, 1448). Interactions of oxalate, a structural analog of enolpyruvate, with the phosphorylated form of the enzyme have been investigated by kinetic inhibition measurements and by magnetic resonance studies of manganous ion complexes with the enzyme. Oxalate inhibits the reaction catalyzed by pyruvate, phosphate dikinase, and the inhibition is linearly competitive with respect to pyruvate. The inhibitor constant for oxalate of 25 μM is fourfold lower than the Michaelis constant for pyruvate. The enhancement in the longitudinal relaxation rate of water protons (PRR) which occurs upon binding of Mn(II) to the enzyme has been used to monitor binding of oxalate to Mn(II)-enzyme complexes. PRR titrations indicate that the dissociation constant of oxalate from the Mn(II) complex of the free form of the enzyme is an order of magnitude weaker than the kinetically determined K_i. On the other hand, titrations of solutions which contain the phosphorylated form of the enzyme reveal a much stronger binding of oxalate. Moreover, the strength of oxalate binding to the phosphorylated enzyme is a function both of the species and of the concentration of monovalent cations in the solution. In the presence of Tl^+, which has the most favorable activator constant for the final partial reaction, the dissociation constant for oxalate from its complex with the phosphorylated enzyme is less than 1 μM. Electron paramagnetic resonance (EPR) spectra for the enzyme-bound Mn(II) are sensitive to structural perturbations which occur upon binding of substrates or of oxalate to the enzyme. The EPR spectrum for the Mn(II)-phosphoenzyme-oxalate species is distinguished from spectra for other complexes of the enzyme by unusually narrow line widths and consequent resolution of fine structure from electronic quadrupole splitting. The narrow lines in the EPR spectrum are indicative of a rigid, pseudocrystalline environment for the bound Mn(II). The magnitude and frequency dependence of the PRR for the Mn(II)-phosphoenzyme-oxalate complex indicate that if any water molecules are bound to the Mn(II), their exchange with the bulk water is severely retarded. The kinetic and magnetic resonance studies support the hypothesis that oxalate mimics the reactive intermediate, enolpyruvate, in a complex with the phosphorylated enzyme which may resemble the structure of the transition state of the final partial reaction.

The enzyme pyruvate, orthophosphate dikinase (EC 2.7.9.1) catalyzes the reversible phosphorylation of pyruvate and inorganic phosphate yielding P-enolpyruvate[1] and inorganic pyrophosphate at the expense of a single molecule of ATP.

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

The reaction mechanism proposed for the enzymes from *Propionibacterium shermanii* and *Bacteroides symbiosus* involves three independent partial reactions (Evans and Wood, 1968; Milner and Wood, 1972a):

$$\text{ATP} + \text{enzyme} \rightleftharpoons \text{AMP} + \text{enzyme-PP} \quad (1a)$$

$$P_i + \text{enzyme-PP} \rightleftharpoons PP_i + \text{enzyme-P} \quad (1b)$$

$$\text{pyruvate} + \text{enzyme-P} \rightleftharpoons \text{P-enolpyruvate} + \text{enzyme} \quad (1c)$$

The validity of this mechanism is supported by three experimental observations. First, the existence of exchange reactions 1a, 1b, and 1c in the presence of enzyme and the appropriate substrates has been demonstrated (Evans and Wood, 1968; Milner and Wood, 1972a). Second, both the phosphorylated and pyrophosphorylated forms of the enzyme have been isolated from appropriate incubation mixtures by gel chromatography, and their kinetic competence demonstrated (Milner and Wood, 1972a). Finally, a steady-state kinetic analysis of the reaction is in accord with a tri (uni-uni) ping-pong mechanism (cf. Cleland, 1963), which requires the existence of three separate forms of the enzyme (Milner and Wood, 1972b). The analysis of the kinetic data also indicates that the mechanism is not the "classical" ping-pong type, but rather each substrate pair (ATP, AMP; P_i, PP_i; pyruvate, P-enolpyruvate) has a dis-

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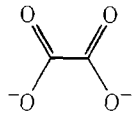
[1] Abbreviations used are: P-enolpyruvate, phosphoenolpyruvate; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; PRR, proton relaxation rate; ϵ^*, the observed PRR enhancement for water; ϵ_a, ϵ_b, and ϵ_t are, respectively, the characteristic PRR enhancements for the Mn(II)-substrate, Mn(II)-enzyme, the Mn(II)-enzyme-substrate complexes. E, Ep, and Epp represent the free, monophosphorylated, and pyrophosphorylated forms of the enzyme, respectively.

tinct binding site on the enzyme (Milner and Wood, 1972b).

Pyruvate, phosphate dikinase is similar to pyruvate kinase (Boyer, 1962; Kayne, 1973) in its requirement for both divalent cation and monovalent cation activators (Reeves et al., 1968). For the dikinase, the divalent cation (Mg(II), Mn(II), or Co(II)) is required for all three partial reactions, whereas the monovalent cation (NH_4^+ or Tl^+) requirement is specific for reaction 1c (Michaels and Milner, 1974). Since Mn(II) satisfies the divalent cation requirement for the enzyme, we have used the paramagnetic properties of this cation as a probe to monitor substrate and inhibitor binding and structural changes at the enzyme's active site.

Several enzymes which use pyruvate as a substrate are inhibited by the dianion of oxalic acid (Novoa et al., 1959; Schmitt et al., 1966; Mildvan et al., 1966; Northrop and Wood, 1969; Reed and Morgan, 1974). In several cases the inhibitor constant for oxalate is substantially lower than the Michaelis constant for pyruvate. Similarities in the structures of oxalate (I) and the enolate of pyruvate (II) may



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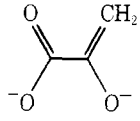

II

provide an explanation for the potent interaction of oxalate with such enzymes. Thus, if enolpyruvate is involved as an intermediate in the catalytic reaction, then oxalate may bind by virtue of its resemblance to the presumed reactive intermediate.

We have examined the interaction of oxalate with pyruvate, phosphate dikinase to test for an involvement of enolpyruvate in the transition state of reaction 1c. In the present paper the interactions of oxalate with the various forms of pyruvate, phosphate dikinase are examined by kinetic inhibition patterns and by NMR and EPR measurements on the Mn(II)–enzyme complexes.

Experimental Procedure

Materials. Pyruvate, phosphate dikinase was prepared from *B. symbiosus* by the method of Milner et al. (1975). The specific activity of the preparation was approximately 12 IU in the direction of pyruvate formation. The enzyme was homogeneous on polyacrylamide disc gel electrophoresis and sedimentation equilibrium ultracentrifugation gave a linear plot of log c vs. r^2. The enzyme was stored at 4° as a precipitate in 80% ammonium sulfate. Before use, aliquots of the enzyme were dialyzed exhaustively against 20 m*M* Tris-acetate buffer (pH 7.2) and were concentrated with an Amicon A-25 apparatus.

Substrates were of the highest purity commercially available. Unless otherwise noted, all substrates were used as their tetramethylammonium, sodium, or tricyclohexylammonium salts.

Kinetic Assays. In the direction of pyruvate formation the reaction was followed using the dinitrophenylhydrazone assay (Milner et al., 1975). In the direction of P-enolpyruvate formation an assay modified from the inorganic pyrophosphatase assay of Cartier and Thuiller (1971) was used where ^{32}P incorporation into PP_i from $^{32}P_i$ was used to follow the reaction (Michaels and Wood, manuscript in preparation).

Magnetic Resonance Measurements. EPR spectra were recorded at approximately 9.1 and 35 GHz using Varian E-3 and 4502 spectrometers, respectively. The aqueous samples were contained in high-purity quartz tubing. The longitudinal relaxation times, T_1's, of water protons were measured at frequencies of 8.13, 15.0, 24.3, and 40.0 MHz using a pulsed NMR spectrometer as described previously (Reuben and Cohn, 1970). Both the EPR and NMR spectrometers were equipped for sample temperature control.

Analysis of Frequency Dependence of PRR. The strategies involved in using the magnitude of the PRR of water and its dependence on the nuclear resonance frequency (magnetic field strength) to evaluate the residual hydration number for Mn(II) bound in a macromolecular complex have been outlined in detail previously (cf. Peacocke et al., 1969; Reuben and Cohn, 1970; Reed et al., 1971; Buttlaire et al., 1975). Briefly, if the influence of Mn(II) on the longitudinal relaxation rate of water protons is a function of the nuclear resonance frequency (magnetic field strength), this frequency dependence can be used to estimate the correlation time for modulation of the dipolar coupling between the electron and nuclear spins. Knowledge of the correlation time then allows a calculation of the hydration number of the Mn(II) from the magnitude of the paramagnetic ion's contribution to relaxation rate of the bulk solvent. The validity of such a calculation hinges on whether or not fast exchange conditions (Swift and Connick, 1962) are satisfied and also on an estimate of the Mn(II) to water proton distance in the complex of interest. The relationship which is important in the frequency range from 8 to 40 MHz is (Peacocke et al., 1969):

$$T_{1P} = (1/3B\tau_c)(1 + \omega_I^2\tau_c^2) \quad (2)$$

In eq 2 B contains constants characteristic of the electron and nuclear spins, the mole fraction of water molecules bound to the ion and the inverse sixth power of the distance between the water protons and Mn(II), ω_I is the nuclear Larmor frequency, and τ_c is the correlation time. Fast exchange of water molecules between the first coordination sphere of Mn(II) and the bulk solution is also implied. If the correlation time itself does not vary with frequency (magnetic field strength), eq 2 predicts a linear relationship between T_{1P} and the square of the nuclear resonance frequency (Peacocke et al., 1969). The slope to intercept ratio of a plot of T_{1P} vs. ω_I^2 gives τ_c^2. For water protons at 2.86 Å from Mn(II) (Reuben and Cohn, 1970), the constant B has a numerical value of 9.48×10^{12} sec^{-2} M^{-1} per water molecule.

Results

Inhibition Studies. Oxalate strongly inhibits the reaction catalyzed by pyruvate, phosphate dikinase. Double reciprocal plots shown in Figure 1 indicate that oxalate is competitive with respect to pyruvate. A secondary plot of the slopes of the lines in Figure 1 vs. oxalate concentration is linear and gives an inhibition constant for oxalate of 25 μM. The activator constant for Tl^+ of 0.5 m*M* is an order of magnitude lower than that for NH_4^+ (K_A = 5 m*M*) (Michaels and Milner, unpublished results). Accordingly, oxalate inhibition in the presence of Tl^+ (not shown) indicates that the inhibitor constant for oxalate is at least tenfold lower than in the presence of NH_4^+.

Binding Studies. Binding of Mn(II) to the free form of the enzyme was evaluated by measuring the amplitude of the EPR signals for the equilibrium amount of unbound Mn(II) in solution as described previously (cf. Cohn and

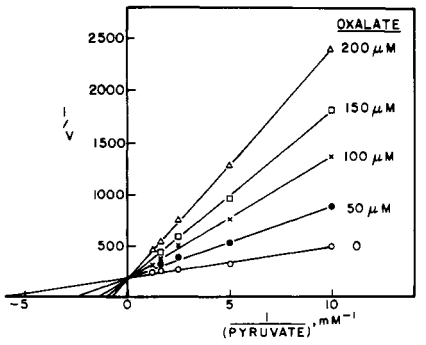

FIGURE 1: Double reciprocal plots of the influence of oxalate on the initial velocity of the pyruvate, phosphate dikinase reaction. Velocities are in units of micromoles of product formed per minute. Reaction assayed in the direction of P-enolpyruvate formation with pyruvate as the variable substrate. Assay contained in a volume of 0.25 ml: Tris-acetate (pH 7.5), 25 μmol; bovine serum albumin, 0.4 mg; $MgCl_2$, 2.5 μmol; $NH_4^+Cl^-$, 10 μmol; $^{32}P_i$, 2 μmol; ATP, 0.5 μmol; enzyme, 7.6 μg; pyruvate and oxalate as indicated.

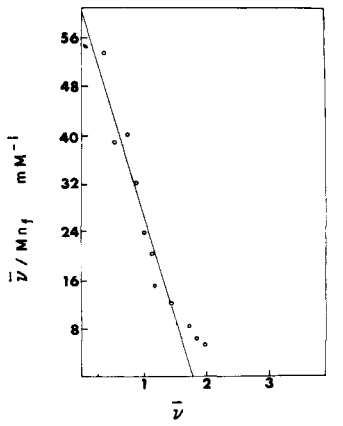

FIGURE 2: Scatchard plot of the binding of Mn(II) to pyruvate, phosphate dikinase. In addition to enzyme and $MnCl_2$ the solutions contained 20 m*M* Tris-acetate (pH 7.2) and 50 m*M* $NH_4^+Cl^-$. EPR measurements of free Mn(II) were made at 20°. $\bar{\nu}$ is moles of Mn(II) bound divided by moles of enzyme and Mn_f is the concentration of free Mn(II).

Townsend, 1954; Reuben and Cohn, 1970). Results of a titration of pyruvate, phosphate dikinase in 50 m*M* $NH_4^+Cl^-$ medium with $MnCl_2$ are shown in the form of a Scatchard plot in Figure 2. The Scatchard plot indicates that approximately 2 mol of Mn(II) is bound per 160,000 g of protein (G. Michaels, Y. Milner, and H. G. Wood, manuscript in preparation). The dissociation constant[2] of the enzyme–Mn(II) complex determined from the intercept on the ordinate and the above stoichiometry is approximately 30 μ*M*.

The binary, Mn(II)–enzyme complex enhances[3] the PRR of water substantially. An enhancement factor, ϵ_b, of 16 ± 0.5 (24.3 MHz, 21°) is obtained from EPR and PRR measurements on the same solutions (Reuben and Cohn, 1970; Mildvan and Cohn, 1963).

Additions of oxalate to solutions of enzyme, $MnCl_2$, and thallium acetate reduce ϵ^* as shown in Figure 3, curve A. The slight decrease in the observed enhancement with increasing concentrations of oxalate can be attributed either to the formation of a ternary complex, Mn(II)–E–oxalate, with $\epsilon_t < \epsilon_b$, or to formation of the binary Mn(II)–oxalate complex, $\epsilon_a = 1.2$ (Reed and Morgan, 1974) at the expense of the binary Mn(II)–enzyme complex, or to both factors. A lower limit for the dissociation constant of oxalate from the Mn(II)–E–oxalate complex of about 200 μ*M* can be estimated from the concentration of oxalate which produces one-half the total change in ϵ^*.

In contrast, addition of oxalate to solutions in which

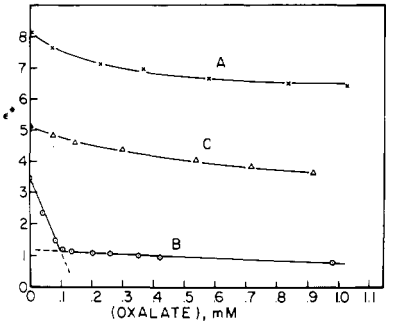

FIGURE 3: PRR titrations of pyruvate, phosphate dikinase with oxalate at 24.3 MHz and 22°: (A) solutions contained Tris-acetate (pH 7.2), 20 m*M*; enzyme, 17 mg/ml; thallium acetate, 10 m*M*; $MnCl_2$, 100 μ*M*; (B) titration as in A except 1 m*M* P-enolpyruvate also present; (C) titration as in B except thallium acetate omitted. Solid curves are sketched through experimental points.

phosphorylated enzyme is present produces an abrupt change in ϵ^*. Phosphorylated enzyme is produced upon addition of P-enolpyruvate to solutions of enzyme, Mn(II), and an activating monovalent cation (Michaels and Milner, 1974). Figure 3, curve B, shows the results of a titration similar to that depicted in curve A with the exception of an addition of P-enolpyruvate prior to titration with oxalate. The presence of P-enolpyruvate lowers the initial enhancement and this effect can be attributed to formation of complexes, e.g. Mn(II)–Ep–pyruvate, Mn(II)–E–P–enolpyruvate, and Mn(II)–Ep, which have lower enhancements than the simple, binary complex of Mn(II) with the free enzyme (G. Michaels, Y. Milner, and G. H. Reed, unpublished observations). Subsequent titration of the solution with oxalate shows a decrease in ϵ^* which is virtually linear with the concentration of oxalate added, up to the breakpoint at 0.1 m*M* oxalate. The breakpoint in the titration curve coincides with the analytical concentration of Mn(II) which is the limiting component of the solution. The titration curve indicates the formation of a complex with a dissociation constant of less than 1 μ*M*. Figure 3, curve C, shows the results of a titration similar to that given in curve B with the exception that no activating monovalent cation is present in the solution. Elimination of an appropriate monovalent cofactor precludes formation of phosphorylated enzyme, and in the

[2] The enzyme is stable for a period of 2–4 days at concentrations of several milligrams per milliliter when stored at 4° in the Tris-acetate buffer. A 10 to 15% decrease in the specific activity of the enzyme is observed over a period of several days in this medium. This slight loss in specific activity parallels a decrease in the enzyme's affinity for Mn(II) as measured by EPR. Moreover, Mn(II) binding experiments suggest that only the "active" form of the enzyme has a high affinity for Mn(II), i.e. a constant value for the dissociation constant of the Mn(II)–enzyme complex was obtained by correcting the protein concentration for the small fraction of inactive enzyme.

[3] The enhancement is defined as:

$$\epsilon^* = \frac{1/T_1^* - 1/T_{10}^*}{1/T_1 - 1/T_{10}} = \frac{1/T_{1P}^*}{1/T_{1P}}$$

where the subscript 0 denotes a measurement in the absence of Mn(II) and the asterisk denotes a measurement in the presence of a complexing agent, e.g. an enzyme or substrate or both (Mildvan and Cohn, 1970).

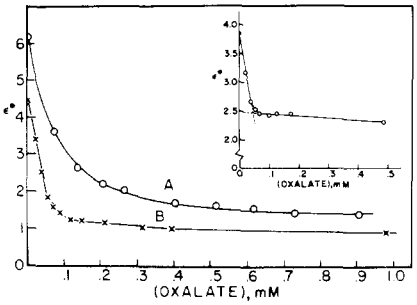

FIGURE 4: PRR titration of pyruvate, phosphate dikinase with oxalate at 24.3 MHz and 22°. Inset: Titration with an excess of Mn(II) over enzyme sites. Solutions contained Tris-acetate (pH 7.2), 20 m*M*; enzyme, 10.6 mg/ml; $MnCl_2$, 200 μM; thallium acetate, 10 m*M*; P-enolpyruvate, 1 m*M*. Titrations with oxalate at 24.3 MHz at two different concentrations of $NH_4^+Cl^-$: (A) solutions contained Tris-acetate (pH 7.2), 20 m*M*; enzyme, 17.1 mg/ml; P-enolpyruvate, 1 m*M*; $MnCl_2$, 100 μM; $NH_4^+Cl^-$, 5 m*M*; (B) titration as in A except enzyme, 15.9 mg/ml, and $NH_4^+Cl^-$, 50 m*M*.

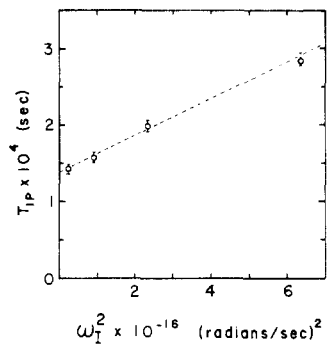

FIGURE 5: T_{1P} for water protons in a solution of Mn(II)-Ep-oxalate vs. the square of the nuclear resonance frequency. The solution contained 20 m*M* Tris-acetate (pH 7.2); 17 mg/ml of enzyme; 100 μM $MnCl_2$; 5 m*M* thallium acetate; 4 m*M* oxalate ($(CH_3)_4N^+$ salt); initially, 2 m*M* P-enolpyruvate (tricyclohexylammonium salt). Measurements were made at 21°. The error bars were calculated from the uncertainties in the T_1 measurements at each frequency.

absence of this form of the enzyme the potent binding of oxalate is not apparent.

The inset in Figure 4 shows the results of a titration similar to that given in Figure 3, curve B, except that in this titration the concentration of $MnCl_2$ exceeds the concentration of enzyme sites. The concentration of oxalate at the breakpoint in the titration curve occurs at the concentration of the limiting component of the complex—in this case, the concentration of enzyme sites which have been phosphorylated and thereby bind oxalate with a high affinity. The break point in the titration occurs at 45 μM oxalate, which corresponds to about 0.7 mol of oxalate bound per 160,000 g of enzyme. Three separate determinations of the amount of oxalate bound all gave similar values (i.e. in each case slightly less than 1 mol of oxalate bound per 160,000 g of protein). Figure 4 (curves A and B) shows the results of titrations similar to that shown in curve B of Figure 3 except for substitution of $NH_4^+Cl^-$, at two concentrations, for thallium acetate in the solution.[4]

The results given in Figure 4 indicate that the apparent dissociation constant for oxalate from the complex with phosphorylated enzyme decreases with increasing (NH_4^+). Furthermore, even with 50 m*M* $NH_4^+Cl^-$ present, oxalate binding is not as strong as in the presence of 10 m*M* thallium acetate (cf. Figures 3B and 4B). A detailed analysis of the titration curves in Figure 4 is not yet feasible since the equilibrium constant for partial reaction (1c) is not available and dissociation constants of the substrates pyruvate and P-enolpyruvate from the free and phosphorylated forms of the enzyme have not yet been evaluated. However, the "half-point" of the titration curve with 50 m*M* $NH_4^+Cl^-$ present occurs at about 30 μM oxalate, and this value represents an upper limit for the dissociation constant of oxalate from the Mn(II)-Ep-oxalate complex. This approximate value for the dissociation constant compares favorably with the K_i of 25 μM obtained from the kinetic inhibition data.

Frequency Dependence of PRR for Mn(II)-Ep-Oxalate. The low PRR enhancement factor for the Mn(II)-Ep-oxalate complex suggests that the correlation time for modulation of the electron spin-nuclear spin interaction is in a region which is not conducive for nuclear spin relaxation or that first coordination sphere water molecules (if any are present on Mn(II)) are not free to exchange with the bulk solvent. One can differentiate between these two possibilities if the correlation time can be determined from the dependence of the PRR on the nuclear resonance frequency (cf. Experimental Procedure). T_{1P} for water protons in solutions of the Mn(II)-Ep-oxalate complex is given as a function[5] of ω_I^2 in Figure 5. The slope to intercept ratio gives a correlation time of 5.4×10^{-9} sec. A correlation time of $\sim 7 \times 10^{-9}$ sec is estimated from the sum of contributions from the rotational correlation time of the protein ($\sim 6 \times 10^{-8}$ sec obtained from Stokes' law) and the electron spin relaxation time ($\sim 8.2 \times 10^{-9}$ sec obtained from the 8-G line width in the EPR spectrum for the complex). This suggests an additional contribution to the correlation time from the lifetime of water molecules in the vicinity of the paramagnetic center.

For a correlation time of 5.4×10^{-9} sec and a single first coordination sphere water in rapid exchange with bulk solvent, eq 2 predicts a value of 11.6 sec^{-1} for $1/T_{1P}$ (at 15 MHz in a 1×10^{-4} *M* solution of the Mn(II) complex). The experimental value of 0.63 sec^{-1} for these conditions falls far below the value predicted for even a single rapidly exchanging water molecule. The large discrepancy between the predicted and experimental values for $1/T_{1P}$ indicates that if any water molecules remain in the first coordination sphere of Mn(II) in the Mn(II)-Ep-oxalate complex, then they are not free to exchange with the bulk solvent. That an exchange-limited contribution from first coordination sphere water molecules does not dominate the observed value for $1/T_{1P}$ is evidenced by an approximately 20% increase in $1/T_{1P}$ with a decrease in sample temperature of 20°. An exchange-limited condition would exhibit the opposite temperature dependence (Swift and Connick, 1962).

EPR Spectra of Enzyme-Bound Mn(II). Interactions of

[4] The decrease in ϵ^* in Figure 4, curve B, in the absence of oxalate is due to a competition between NH_4^+ and Mn(II) for weak binding sites on the protein. In the absence of NH_4^+ or Tl^+ Scatchard plots show that Mn(II) interacts at two classes of sites and that the weaker class of sites is eliminated by sufficient concentrations of an activating monovalent cation (e.g. 50 m*M* NH_4^+, cf. Figure 2). The release of Mn^{2+} from the weaker class of sites by NH_4^+ reduces ϵ^*.

[5] The linear dependence of T_{1P} on ω_I^2 indicates that τ_c is not frequency (magnetic field) dependent over this frequency (magnetic field) region. The frequency independence of τ_c is compatible with the observation that the line widths of EPR signals for the Mn(II)-Ep-oxalate complex do not vary appreciably at magnetic field strengths of 3.2 kG (X-band) and 12.5 kG (K-band).

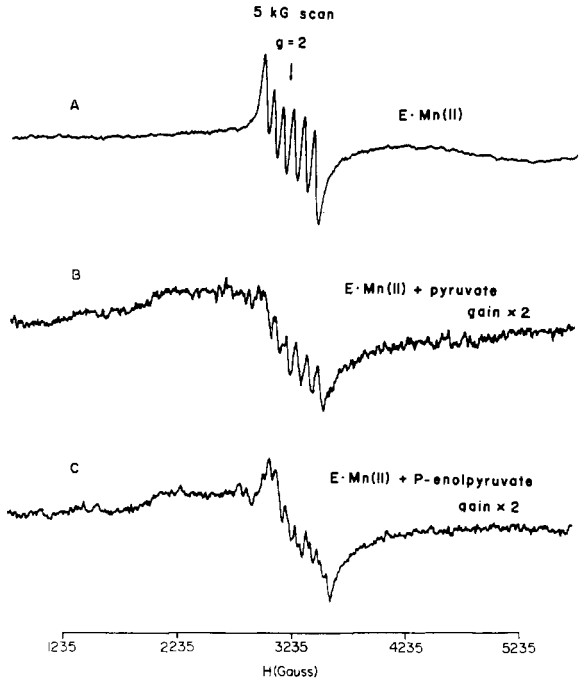

FIGURE 6: X-Band EPR spectra for Mn(II) complexes of pyruvate, phosphate dikinase. All solutions contain 20 m*M* Tris-acetate (pH 7.2): (A) solution contains enzyme, 150 mg/ml; 310 μM $MnCl_2$; (B) solution contains enzyme, 80 mg/ml; 410 μM $MnCl_2$; 1 m*M* pyruvate, $(CH_3)_4N^+$ salt; two scans of a buffer solution were subtracted from the accumulated sample scans; (C) solution contains enzyme, 80 mg/ml; 410 μM $MnCl_2$; 1 m*M* P-enolpyruvate, tricyclohexylammonium salt. Two scans of the sample were collected in a computer of average transients and two scans of a buffer solution were subtracted from the accumulated sample scans. Spectra taken at ~1°. The solutions did not contain activating monovalent cations.

oxalate with pyruvate, phosphate dikinase can also be examined by monitoring the EPR spectrum of Mn(II) bound to the enzyme. The EPR spectrum for a solution of enzyme and $MnCl_2$ is given in Figure 6, curve A. The presence of a large excess of enzyme sites over Mn(II) and the high affinity of the enzyme for Mn(II) ensure that the concentration of $Mn(H_2O)_6^{2+}$ in this solution is suppressed so that it does not contribute to the observed EPR spectrum. The absence of a substantial contribution to the spectrum from free $Mn(H_2O)_6^{2+}$ was verified by increasing the temperature of the sample (Reed and Ray, 1971; Reed and Cohn, 1973). In this case there was virtually no change in the amplitude of the EPR spectrum at the higher temperature whereas trace amounts of free $Mn(H_2O)_6^{2+}$ are distinguished by increases in signal amplitude with increases in temperature.

The EPR spectrum of the enzyme-bound Mn(II) is sensitive to the presence of either P-enolpyruvate or pyruvate at the active site as shown in Figure 6, curves B and C, respectively. In contrast to the relatively isotropic spectrum of E-Mn(II), the ternary complexes which are formed with P-enolpyruvate or with pyruvate give broad, anisotropic EPR signals, and the spectral patterns are spread over several kilogauss in magnetic field. The presence of monovalent cofactors for the reaction of pyruvate and P-enolpyruvate with the enzyme (NH_4^+ or Tl^+) does not influence the EPR line shape for the ternary complexes of these substrates with the enzyme.

The influences of oxalate on EPR spectra for the enzyme-bound Mn(II) are illustrated in Figure 7. Addition of oxalate in the absence of P-enolpyruvate produces only a slight broadening of the EPR signals (curve A). Moreover,

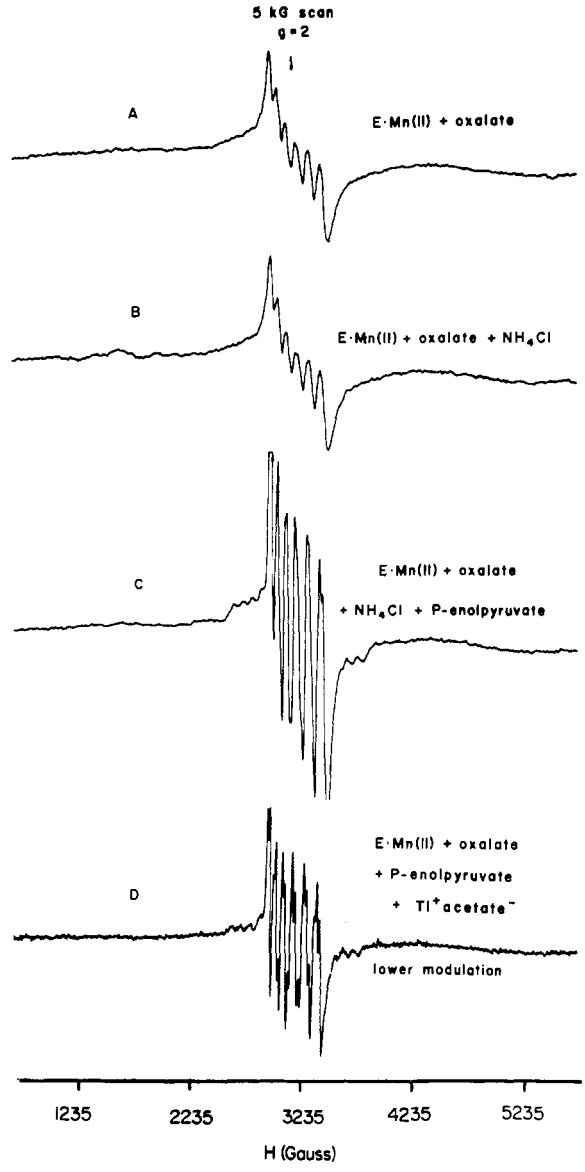

FIGURE 7: X-Band EPR spectra for Mn(II) complexes of pyruvate, phosphate dikinase with oxalate present. All solutions contain 20 m*M* Tris-acetate (pH 7.2): (A) solution contains enzyme, 86 mg/ml; 510 μmol of $MnCl_2$; 1.8 m*M* oxalate, $(CH_3)_4N^+$ salt; (B) same components as in A with 70 m*M* $NH_4^+Cl^-$; (C) same components as in B with 1.8 m*M* P-enolpyruvate present initially; (D) solution contains initially, enzyme, 115 mg/ml; 480 μM $MnCl_2$; 3 m*M* oxalate, $(CH_3)_4N^+$ salt; 4.7 m*M* P-enolpyruvate (tricyclohexylammonium salt) present initially; 24 m*M* thallium acetate. Spectra taken at ~1°.

addition of $NH_4^+Cl^-$ has no effect on the EPR signals (curve B). However, upon addition of P-enolpyruvate to the solution (curve C), phosphorylated enzyme is formed, and the oxalate complex with the phosphorylated enzyme gives an EPR spectrum which differs markedly from those given by the complexes of the enzyme with the substrates, P-enolpyruvate, and pyruvate and from the simple Mn(II)-enzyme species. A virtually identical spectrum is obtained for the Mn(II)-Ep-oxalate complex formed in the presence of Tl^+ as shown in Figure 7, curve D.

Phosphorylated enzyme can also be produced by additions of ATP and P_i to solutions of enzyme and Mn(II) (cf. partial reactions 1a and 1b) in the absence of the monovalent activators (Michaels and Milner, 1974). Figure 8 shows EPR spectra for a solution of enzyme and $MnCl_2$ to which ATP, oxalate, P_i, and $NH_4^+Cl^-$ are successively

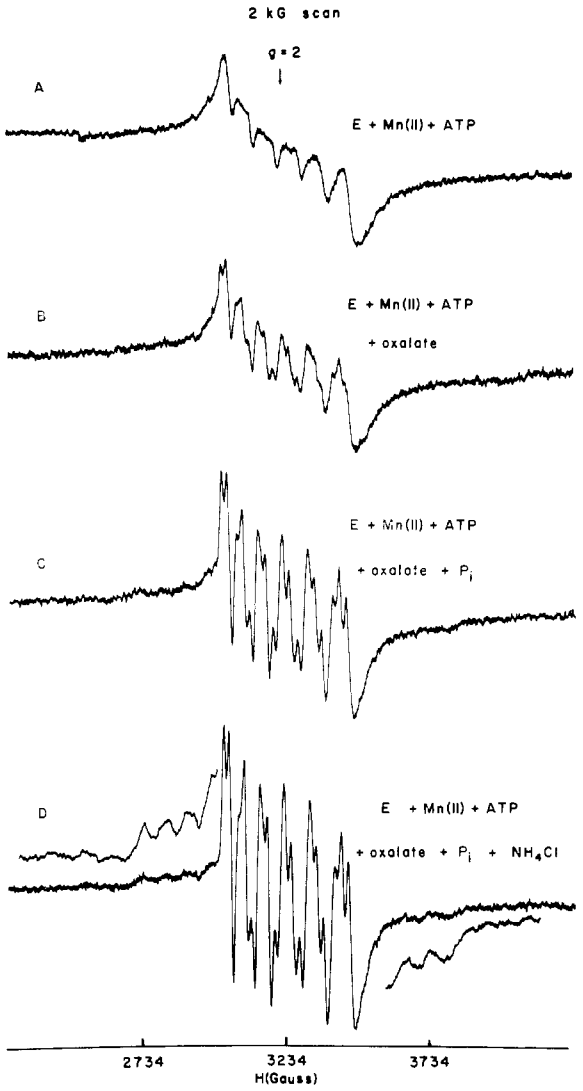

FIGURE 8: X-Band EPR spectra for Mn(II) complexes with pyruvate, phosphate dikinase. All solutions contain 20 m*M* Tris-acetate (pH 7.2): (A) solution contains initially 130 mg/ml of enzyme, 400 μM $MnCl_2$; 1.1 m*M* ATP, sodium salt; (B) same components as in A with 2.7 m*M* oxalate, $(CH_3)_4N^+$ salt; (C) same components as in B with with 5 m*M* P_i, $(CH_3)_4N^+$ salt; (D) same components as in C with 45 m*M* $NH_4^+Cl^-$. Traces above (left) and below (right) D are taken at higher gain and show signals from the $\pm 3/2 \leftrightarrow \pm 1/2$ fine structure sets. Narrow lines in C and D are artificially broadened by the field modulation of 20 G. At lower modulation the narrow lines are of the order of 5 to 8 G peak to peak width. Spectra are obtained at identical spectrometer settings at ~1°.

added. Production of phosphorylated enzyme upon addition of P_i to the solution (following addition of ATP) results in the appearance of a spectral pattern which matches the spectrum obtained in the presence of the monovalent activators (cf. Figure 7C). However, the spectrum for the Mn(II)-Ep-oxalate complex is intensified by the subsequent addition of NH_4^+ to the solution (Figure 8, curve D). Intensification of the EPR spectrum for Mn-Ep-oxalate upon addition of NH_4^+ indicates an increase in the concentration of Mn-Ep-oxalate which is consistent with a lowering of the dissociation constant for oxalate by NH_4^+ (cf. Figure 4, curves A and B).

The spectrum in Figure 8, curve B, shows the presence of a small amount of the Mn(II)-Ep-oxalate complex. A slight contamination of the solution with P_i would account for the appearance of this species. However, in the absence of added P_i the spectrum characteristic of the Mn(II)-Ep-oxalate species grows in intensity with time. After incubation for 18 hr at 4° the spectrum reaches an intensity equivalent to that of the spectrum in Figure 8, curve C. This observation suggests that hydrolysis of Epp to Ep + P_i occurs during this period.

Discussion

The use of enzyme-inhibitor complexes to gain insight into the structure of the transition state of the catalytic reaction has been discussed in detail by Wolfenden (1972) and by Lienhard (1973). The arguments set forth by these authors suggest that an enzyme should possess an especially high affinity for compounds whose structure resembles that of the substrate as it is modified in the transition state. Such compounds should be potent competitive inhibitors of the enzymatic reaction and should have inhibition constants which are appreciably lower than the Michaelis constant for the substrate. Furthermore, variables such as solution pH or concentration of activators which influence the ratio of the enzymatic to nonenzymatic rates should produce a parallel influence on the binding affinity of the analog (Wolfenden, 1972). Several examples of inhibitors for specific enzymes which meet one or more of these criteria are documented in the literature (cf. Wolfenden, 1972; Lienhard, 1973).

Kinetic and magnetic resonance experiments with oxalate and pyruvate, phosphate dikinase lend support to the proposed similarities between oxalate and the enolate of pyruvate and to an involvement of enolpyruvate in the transition state of partial reaction 1c. Oxalate is a competitive inhibitor with respect to pyruvate, and the inhibitor constant for oxalate of 25 μM is fourfold lower than the Michaelis constant for pyruvate (Milner et al., 1975). Furthermore, the PRR titrations indicate that oxalate binds most strongly to the phosphorylated form of the enyzme—the enzyme form with which pyruvate reacts (Evans and Wood, 1968). The dissociation constant for oxalate from the complex with phosphorylated enzyme is also sensitive to the species and concentration of monovalent cations present in solution, and the monovalent cations are essential cofactors for reaction of pyruvate with the phosphorylated enzyme (Michaels and Milner, 1974). Finally, the Mn(II)-Ep-oxalate complex exhibits unusually narrow EPR signals and a low PRR enhancement factor, and both of these observations are indicative of a rigid, enclosed structure at the active site.

PRR titrations show that an especially strong interaction between oxalate and the enzyme occurs only under conditions where the phosphoenzyme is present. Furthermore, EPR spectra for the enzyme-boundMn(II) indicate that the Mn(II)-Ep-oxalate species forms by partial reaction 1c or by the combination of partial reactions 1a and 1b. The fact that the characteristic EPR pattern for the Mn(II)-Ep-oxalate species is observed in the absence of monovalent activators (cf. Figure 8C) shows that phosphoenzyme, Mn(II), and oxalate are the only components of the complex which are required to give the characteristic EPR spectrum. However, the data of Figure 3 and of Figure 8 show that the presence of Tl^+ or NH_4^+ (monovalent cofactors for partial reaction 1c) strengthens the interaction of oxalate with the Mn(II)-Ep complex. In this regard, Tl^+ has a tighter activator constant than does NH_4^+ (Michaels and Milner, 1974), and oxalate binds to the Mn(II)-Ep complex with a dissociation constant of less than 1 μM in the presence of 10 m*M* thallium acetate.

The Mn(II)–Ep–oxalate complex is distinguished from other complexes of the enzyme by a low PRR enhancement factor for water and by the strikingly narrow lines in its EPR spectrum. Similar findings have been reported for complexes which mimic the structure and composition of the transition state for creatine kinase (Reed and Cohn, 1972), arginine kinase (Buttlaire and Cohn, 1974), and formyltetrahydrofolate synthetase (Buttlaire et al., 1975). In each of these systems detailed studies of the frequency dependence of the water PRR led to the conclusion that exchange of water molecules from the first coordination sphere of the Mn(II) is severely retarded so that "outer sphere" contributions dominate the observed relaxation effects. This conclusion also accounts for the unusually narrow EPR lines for Mn(II) in these complexes as the Mn(II) coordination sphere is not freely accessible to the buffeting action of bulk solvent. This latter effect is an important electron spin relaxation mechanism for Mn(II) (Bloembergen and Morgan, 1961; Reed et al., 1971). The frequency dependence and magnitude of the PRR for water in solutions of Mn(II)–Ep–oxalate also show that if there are any water molecules bound to the Mn(II), they are not free to exchange with the bulk water. An increase in contact between an enzyme and its substrates in the transition state is compatible with the increase in enzyme–substrate affinity in the transition state. It is likely that enclosure of the active site in the transition state analog complexes reflects the increased contact between the protein and substrates which is predicted by the transition state analog theory (Wolfenden, 1972).

The stoichiometry of oxalate binding to the phosphoenzyme indicates that only 1 mol of oxalate is bound on this dimeric enzyme per 160,000 g of protein. Tracer studies (Spronk and Wood, unpublished observations) indicate that only a single phosphoryl group is covalently linked per mole of enzyme upon incubation of the enzyme with ^{32}P-enolpyruvate. These observations suggest that the dikinase, which appears to be a dimer, may possess the property of "half-of-the-sites reactivity" (cf. Lazdunski, 1972), although further experiments are required to confirm these findings.

It is also noteworthy that Epp formed in partial reaction 1a undergoes a slow hydrolysis to give Ep + P_i. The P_i which is produced in this side reaction is free to react with Epp to give Ep + PP_i so that each hydrolytic step could consume 2 equiv of Epp. The hydrolytic reaction of pyruvate, phosphate dikinase is analogous to the reaction mechanism proposed for the closely related enzyme, P-enolpyruvate synthetase (Cooper and Kornberg, 1967; Berman and Cohn, 1970) where no positive evidence has been found for a stable pyrophosphorylated enzyme.

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