An Active-Site Lysine in Avian Liver Phosphoenolpyruvate Carboxykinase†

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Received April 25, 1991

ABSTRACT: The participation of lysine in the catalysis by avian liver phosphoenolpyruvate carboxykinase was studied by chemical modification and by a characterization of the modified enzyme. The rate of inactivation by 2,4-pentanedione is pseudo-first-order and linearly dependent on reagent concentration with a second-order rate constant of 0.36 ± 0.025 M^{-1} min^{-1}. Inactivation by pyridoxal 5′-phosphate of the reversible reaction catalyzed by phosphoenolpyruvate carboxykinase follows bimolecular kinetics with a second-order rate constant of 7700 ± 860 M^{-1} min^{-1}. A second-order rate constant of inactivation for the irreversible reaction catalyzed by the enzyme is 1434 ± 110 M^{-1} min^{-1}. Treatment of the enzyme with pyridoxal 5′-phosphate gives incorporation of 1 mol of pyridoxal 5′-phosphate per mole of enzyme or one lysine residue modified concomitant with 100% loss in activity. A stoichiometry of 1:1 is observed when either the reversible or the irreversible reactions catalyzed by the enzyme are monitored. A study of k_{obs} vs pH suggests this active-site lysine has a pK_a of 8.1 and a pH-independent rate constant of inactivation of 47 700 M^{-1} min^{-1}. The phosphate-containing substrates IDP, ITP, and phosphoenolpyruvate offer almost complete protection against inactivation by pyridoxal 5′-phosphate. Modified, inactive enzyme exhibits little change in Mn^{2+} binding as shown by EPR. Proton relaxation rate measurements suggest that pyridoxal 5′-phosphate modification alters binding of the phosphate-containing substrates. ^{31}P NMR relaxation rate measurements show altered binding of the substrates in the ternary enzyme-Mn^{2+}-substrate complex. Circular dichroism studies show little change in secondary structure of pyridoxal 5′-phosphate modified phosphoenolpyruvate carboxykinase. These results indicate that avian liver phosphoenolpyruvate carboxykinase has one reactive lysine at the active site and it is involved in the binding and activation of the phosphate-containing substrates.

P-Enolpyruvate carboxykinase [GTP:oxaloacetate carboxylyase (transphosphorylating) (EC 4.1.1.32)] catalyzes the reversible decarboxylation of oxalacetate with a concomitant transfer of the terminal phosphate of GTP (or ITP) to yield the products phosphoenolpyruvate (P-enolpyruvate),[1] GDP (IDP), and CO_2:

$$\text{oxalacetate} + \text{GTP(ITP)} \rightleftharpoons \text{P-enolpyruvate} + CO_2 + \text{GDP(IDP)}$$

The primary role of this enzyme in higher organisms is the catalysis of the formation of P-enolpyruvate from oxalacetate as the first committed step in gluconeogenesis. The enzyme from avian liver is a monomeric protein with a molecular weight of 70 000 (Hebda & Nowak, 1982a; Weldon et al., 1990).

P-Enolpyruvate carboxykinase also catalyzes a second, experimentally irreversible decarboxylation of oxalacetate to yield pyruvate (Noce & Utter, 1975):

$$\text{oxalacetate} \xrightarrow{\text{NDP}} \text{pyruvate} + CO_2$$

This reaction requires a nucleotide diphosphate as a cofactor. The physiological significance, if any, of this reaction is presently unknown.

P-Enolpyruvate carboxykinases are reported to be present in a wide variety of animals, bacteria, and plants (Utter & Kolenbrander, 1972). The intracellular distribution of this enzyme in animals is species-dependent. In some species, or tissues within a species, the enzyme is located within the mitochondria: in other species, it is located in the cytosol, and in others, it is located in both the mitochondria and the cytosol (Utter & Kolenbrander, 1972). The mitochondrial and cytosolic forms of P-enolpyruvate carboxykinase have been reported to be different proteins (Ballard & Hanson, 1969; Utter & Chuang, 1978).

Studies of P-enolpyruvate carboxykinase have primarily focused on its regulation, kinetic mechanism, and stereochemistry. In contrast, relatively little work has been performed on the characterization of amino acid residues at the enzyme active site. The majority of P-enolpyruvate carboxykinases studied contain a cysteine residue essential for activity. Among these are the enzymes from rat liver cytosol (Carlson et al., 1978; Jadus et al., 1981; Lewis et al., 1989), pig liver mitochondria (Chang & Lane, 1966), and sheep kidney mitochondria (Barns & Keech, 1968, 1972). Makinen and Nowak (1989) have located a reactive cysteine in the enzyme from chicken liver, but this group appears to be too remote from the binding site to be participating in the catalytic mechanism. Malebrân and Cardemil (1987) reported two functional arginyl residues per enzyme subunit in yeast P-enolpyruvate carboxykinase. Cheng and Nowak (1989a) recently reported essential arginine residues at the active site of the avian liver enzyme. An active-site histidine has also been located by Cheng and Nowak (1989b). Recently, Cardemil and co-workers (Saavedra et al., 1988) have sug-

† This research was supported by a research grant from the National Institutes of Health (DK17049).

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[1] Abbreviations: P-enolpyruvate, phosphoenolpyruvate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PLP, pyridoxal 5′-phosphate; PRR, water proton longitudinal relaxation rate; DTT, dithiothreitol; IDP, inosine 5′-diphosphate; ITP, inosine 5′-triphosphate; CD, circular dichroism.

0006-2960/91/0430-8851$02.50/0

gested an important lysine residue in yeast P-enolpyruvate carboxykinase from affinity labeling of the enzyme.

Lysine ϵ-amino groups are found to participate in catalysis by several enzymes that utilize phosphorylated substrates. Included among these enzymes are several of the kinases, such as muscle pyruvate kinase (Johnson & Deal, 1970; Hollenberg et al., 1971), sheep heart phosphofructokinase (Setlow & Mansour, 1972), rabbit muscle creatine kinase (James & Cohn, 1974), pig liver phosphomevalonate kinase (Bazaes et al., 1980), and spinach leaf phosphoribulokinase (Miziorko et al., 1990). Hanson and co-workers (Cook et al., 1986) have suggested the presence of lysyl residues in both the nucleotide and P-enolpyruvate binding regions of P-enolpyruvate carboxykinase. This prediction is based on comparisons of the amino acid sequences of cytosolic P-enolpyruvate carboxykinases (Cook et al., 1986) with those of other proteins that bind guanine nucleotides and P-enolpyruvate (McCormick et al., 1985; Jurmak, 1985; Stalker et al., 1985; Schultz et al., 1984).

Pyridoxal 5′-phosphate is a chemical modifying reagent highly specific for amino groups located on enzymes (Lundblad & Noyes, 1984; Eyzaguirre, 1987). It is useful for the modification of lysine residues because of solubility, selectivity of reaction, spectral properties of the modified residue, reversibility of reaction, and the establishment of stoichiometry by reduction of the Schiff base initially formed between pyridoxal 5′-phosphate and lysine with tritiated borohydride (Lundblad & Noyes, 1984).

Identification of catalytically important amino acid residues at the active site of this enzyme further aids in understanding the mechanism and structure of the active site of P-enolpyruvate carboxykinase. In the present study, pyridoxal 5′-phosphate was selected as the reagent of choice for modifying lysine residues at the active site of P-enolpyruvate carboxykinase. Avian liver P-enolpyruvate carboxykinase is inactivated by pyridoxal 5′-phosphate by selective modification of an essential lysine residue at the active site of the enzyme. A physical characterization of the lysine-modified enzyme was performed.

Experimental Procedures

Materials

Chicken liver P-enolpyruvate carboxykinase was purified as previously described (Lee & Nowak, 1984). The concentration of P-enolpyruvate carboxykinase was determined with an extinction coefficient $\epsilon_{280} = 16.5 \pm 0.1$ mg/mL (Hebda & Nowak, 1982a) and a molecular weight of 70000 (Weldon et al., 1990). The enzyme used for these studies had a specific activity of 4.0–7.0 μmol of oxalacetate formed min^{-1} mL^{-1} mg^{-1} (units/mg) at 22 ± 1 °C. Malate dehydrogenase (pig heart) was purchased from Boehringer Mannheim Corp. The reagents IDP, ITP, P-enolpyruvate, NADH, *cis*-oxalacetate, pyridoxal 5′-phosphate, HEPES, and PIPES were purchased from Sigma. 2,4-Pentanedione was from Aldrich. NaB^3H_4 (600 mCi/mmol) was purchased from New England Nuclear, Du Pont Co. Chelex-100 and P-6DG resins were from Bio-Rad. All other reagents were of the highest purity commercially available. All solutions were made by using distilled water that was passed through a mixed-bed deionizing column.

Methods

P-Enolpyruvate Carboxykinase Assay. The carboxylation of P-enolpyruvate to form oxalacetate, catalyzed by P-enolpyruvate carboxykinase, was assayed by the method of Hebda and Nowak (1982a). In this continuous assay, P-enolpyruvate carboxykinase is coupled to malate dehydrogenase, and the disappearance of NADH is continuously monitored with time at 340 nm on a Gilford Model 240 or 250 spectrophotometer thermostated at 22 ± 1 °C.

Initial velocity measurements of the irreversible reaction catalyzed by P-enolpyruvate carboxykinase, in which pyruvate is formed from oxalacetate, were accomplished with a continuous assay. This assay couples the P-enolpyruvate carboxykinase catalyzed formation of pyruvate to lactate dehydrogenase, and the decrease in absorbance at 340 nm due to oxidation of NADH was monitored. A base-line activity was first measured due to the nonenzymatic decarboxylation of OAA to form pyruvate. The reaction was then initiated by the addition of P-enolpyruvate carboxykinase, and the slope was converted to concentration units by using the extinction coefficient of NADH (Noce & Utter, 1975).

Lysine Modification. P-Enolpyruvate carboxykinase (0.39 mg/mL; 5.4 μM) was incubated with varying concentrations of 2,4-pentanedione or of pyridoxal 5′-phosphate at 22 ± 1 °C. These modifiers were freshly prepared in 50 mM HEPES, pH 7.4. In order to minimize photodecomposition, stock solutions and modification reactions involving pyridoxal 5′-phosphate were protected from light by covering them with aluminum foil. In the inhibition experiments, controls were run with buffer in place of modifier. At various periods of time, 0.02-mL aliquots of enzyme were withdrawn and added directly into standard enzyme assays. The activity of modified enzyme was measured and expressed as percent remaining activity. The controls showed no activity loss.

2,4-Pentanedione modification of P-enolpyruvate carboxykinase followed pseudo-first-order kinetics, and the activity of the modified enzyme was expressed as the log of the percent remaining activity. First-order rate constants were calculated from the slope of the plots by a linear least-squares fit of the data. Inactivation by pyridoxal 5′-phosphate followed a typical bimolecular process according to the equation:

$$k_2(A - B)t = \ln \{[B(A - P)]/[A(B - P)]\} \qquad (1)$$

where k_2 is the second-order rate constant; A and B are initial concentrations of PLP and P-enolpyruvate carboxykinase, respectively. P is the concentration of inactivated enzyme and equals (B)(fractional activity loss) at time t. A plot of ln $(A - P)/(B - P)$ vs t yields a straight line with a slope of $k_2(A - B)$ from which k_2 was calculated.

Substrate Protection. P-Enolpyruvate carboxykinase (0.39 mg/mL) was incubated in the presence of the substrates P-enolpyruvate, IDP, $KHCO_3$, $MnCl_2$, oxalacetate, ITP, or combinations of these substrates as specifically described in the figure legends. The protection against inhibition was measured in the presence of 20 μM PLP at 22 ± 1 °C. Periodically, 0.02-mL aliqouts were withdrawn and added into the standard assay mixture. The remaining activity of this lysine-modified enzyme was compared to the apoenzyme control.

Synthesis of [^3H]Pyridoxal 5′-Phosphate. [^{3}H]Pyridoxal phosphate was prepared by using the methods described by Johansson et al. (1974). PLP was reduced with NaB^3H_4 (specific activity, 600 mCi/mmol), reoxidized with manganese(IV) oxide, and purified on a Bio-Rad AG1X-8 (acetate) column using an acetic acid gradient (0–5 M). After ~300 mL of eluant volume, the fractions containing [^{3}H]pyridoxal 5′-phosphate eluted from the column (50 mL-total volume; 584 μCi). The λ_{max} and extinction coefficient of pyridoxol 5′-phosphate and pyridoxal 5′-phosphate are pH-dependent and can be used to distinguish between the two compounds (Peterson & Sober, 1954). At neutral pH, PLP has a λ_{max} of 388 nm and $\epsilon = 4900$ M^{-1} cm^{-1}, while pyridoxol 5′-phos-

phate has a λ_{max} of 325 nm and ϵ = 7400 M^{-1} cm^{-1}. The specific activity of [^{3}H]pyridoxal 5'-phosphate (2.83 Ci/mol) was determined in 0.1 N HCl: λ_{max} = 295 nm and ϵ = 6700 M^{-1} cm^{-1}.

Pyridoxal 5'-Phosphate Incorporation. P-Enolpyruvate carboxykinase (2.37 mg/mL, 32 μM), in 50 mM HEPES, pH 7.4, was inactivated by 50 μM PLP. Samples of the enzyme modification mixture were removed at various time intervals and assayed for residual enzyme activity. At the same time, 0.423-mL aliquots were withdrawn and reduced with 100 mM $NaBH_4$ for 60 min. The PLP-labeled enzyme samples were then precipitated with 5% trichloroacetic acid and stored on ice for 30 min. The enzyme precipitate was collected by centrifugation and washed 3 times with cold 5% trichloroacetic acid and once with cold acetone to remove unbound PLP. The samples were allowed to air-dry, and then the PLP incorporation was determined by quantitation of the inorganic phosphate covalently bound utilizing the molybdenum blue method for determining nucleotide phosphate (Hurst, 1964). A standard curve of A_{700} vs [PLP] was prepared and used to quantitate the amount of PLP incorporated into the P-enolpyruvate carboxykinase samples. Control experiments were carried out identically but in the absence of PLP and showed no phosphate incorporation.

The stoichiometry of PLP incorporation was also determined by utilizing [^{3}H]PLP. P-Enolpyruvate carboxykinase (2.2 mg/mL) was modified with 300 μM [^{3}H]PLP (specific actvity 2.83 Ci/mol), in the presence of 5 mM $NaCNBH_3$, in an analogous fashion as described above. Residual enzyme activity was correlated with [^{3}H]PLP incorporation.

Amino Acid Analysis. P-Enolpyruvate carboxykinase (1.58 mg) was modified with PLP to 5% residual activity, reduced, washed, and collected as described for PLP incorporation. The PLP-labeled protein was dissolved in 1 mL of 6 N constant-boiling HCl (Pierce), purged with N_2, and hydrolyzed for 39 h at 110 °C. A 1.58-mg enzyme sample, in the absence of PLP, was treated identically and served as a control. Amino acid analysis was performed to determine possible loss of amino acid residues.

pH-Dependent Inactivation Studies. Pyridoxal 5'-phosphate was used to modify P-enolpyruvate carboxykinase at pH values ranging from 6 to 9. PIPES buffer was used for the pH range 6–6.8, and HEPES was used for the pH range 7–9. Enzyme (0.39 mg/mL, 5.4 μM) was incubated in 100 mM buffer at the appropriate pH for 10 min before enzyme modification was initiated with 7.5 μM PLP. The concentrations of enzyme and modifier provide bimolecular reaction conditions. Samples (0.02 mL) were taken at various times and assayed for activity. Values for $k_{2,obs}$ were determined at each pH value from the time-dependent decrease in percent residual activity. The effect of pH on inactivation was analyzed according to the following equation (Lindley, 1962; Schmidt & Westheimer, 1971):

$$1/k_{2,obs} = [H^+]/kK + 1/k \quad (2)$$

where $k_{2,obs}$ is the observed second-order rate constant at a given pH, related to the total concentration of lysine in all ionic forms, k is the true second-order rate constant, related to the reactive forms of lysine, and K is the ionization constant of the lysine group. From eq 2, a plot of $1/k_{2,obs}$ vs [H^+] is linear with an intercept of $1/k$ on the $1/k_{2,obs}$ axis, and the ratio of the intercept to the slope is equal to K. The points were determined experimentally, and the curves were generated from the equation.

Electron Paramagnetic Resonance Spectroscopy Studies. The binding of Mn^{2+} to P-enolpyruvate carboxykinase was determined by using EPR and PRR techniques (Townsend & Cohn, 1954; Mildvan & Cohn, 1963). P-Enolpyruvate carboxykinase was modified by PLP to 15–25% residual activity and reduced with $NaBH_4$ as described above. The lysine-modified enzyme was desalted through a P-6DG column having a 1-cm layer of Chelex-100 on top and was concentrated with a collodion bag, vacuum filtration apparatus. A control was treated under the same conditions but in the absence of PLP. The binding of Mn^{2+} was performed with a Varian E-9 EPR spectrometer. Binding was measured as previously described (Hebda & Nowak, 1982a). Binding data were treated by the method of Scatchard (1949).

Proton Relaxation Rate Measurements. The formations of the binary enzyme·Mn^{2+} complex and ternary enzyme·Mn^{2+}·substrate complexes were investigated by PRR techniques. A detailed theory and application of this technique have been described elsewhere (Mildvan & Cohn, 1970; Nowak, 1981). The longitudinal relaxation rate ($1/T_1$) of water protons was measured with a Seimco pulsed NMR spectrometer operating at 24.3 MHz using the Carr–Purcell (Carr & Purcell, 1954) 180°–τ–90° pulse sequence. The dissociation constants and the enhancements of the ternary complexes, ϵ_T, were determined by a computer fit to the PRR titration data which minimized the percent standard deviation of ϵ_T and ϵ_{obs} by systematically varying the values of K_3 and K_S. The constants for the other complexes considered were the same as those previously used to fit such data (Cheng & Nowak, 1989). The Fortran computer program, which was based on the program of Reed et al. (1970), was adapted for a VAX 750 computer by Joe Weber, Department of Physics, University of Notre Dame.

^{31}P NMR Relaxation Rate Studies. ^{31}P relaxation rate measurements were carried out at 121.5 MHz on a Nicolet NTC 300 spectrometer interfaced with a 239 A pulse system and 1180 E computer. The 10-mm sample tubes contained 2.0–2.5-mL sample volume, and Teflon plugs were used as vortex suppressors. The spectrometer was field-frequency-locked on internal ^{2}H present as 50% ^2H_2O in the solvent. The spectra were proton-decoupled to increase the signal to noise ratio. All experiments were run at 22 ± 1 °C. The $1/T_1$ values were measured by the inversion recovery method. The $1/T_2$ values were estimated from the line widths of the resonance of the normal Fourier-transformed spectra from the relationship:

$$1/T_2 = \pi(\nu_{1/2} - B)$$

where $\nu_{1/2}$ is the spectral line width at half-height, in hertz, and B is the artificial line broadening obtained when the spectra were processed. Values for B were usually 0.5 Hz.

In the Mn^{2+} titration experiments, microliter quantities of $MnCl_2$ solutions were titrated into buffered solutions of the specific sample, and $1/T_1$ and $1/T_2$ values for each resonance were measured at each increment of $MnCl_2$ added. In each experiment performed, the concentrations of ligand and enzyme were chosen such that virtually 100% of the Mn^{2+} added is in the enzyme·Mn^{2+}·substrate complex. These results were based on calculations made in this study and from previous work by Nowak and co-workers (Hebda & Nowak, 1982b; Lee & Nowak, 1984; Duffy & Nowak, 1985). The nucleotide-containing samples, prepared in 50% D_2O, contained 50 mM HEPES, pH 7.4, 100 mM KCl, 5–7 mM nucleotide, and 105–160 μM PLP-labeled P-enolpyruvate carboxykinase in a total volume of 2.5 mL. The P-enolpyruvate-containing sample was prepared in 50% D_2O and contained 50 mM HEPES, pH 7.4, 100 mM KCl, 20 mM P-enolpyruvate, and 102 μM PLP-labeled enzyme. $MnCl_2$ concentrations were

varied from 0 to 7 μM for the nucleotide measurements and from 0 to 10 μM for the P-enolpyruvate measurements. PLP-Labeled P-enolpyruvate carboxykinase, modified to 14% residual activity, was used for these experiments.

The Luz–Meiboom and Swift–Connick equations (Luz & Meiboom, 1964; Swift & Connick, 1962) (eq 3 and 4) relate

$$\frac{1}{pT_{1p}} = \frac{1}{T_{1M} + \tau_m} \quad (3)$$

$$\frac{1}{pT_{2p}} = \frac{1}{T_{2M} + \tau_m} \quad (4)$$

the normalized measured relaxation rates $[1/(pT_p)]$ to the relaxation times of the nuclei of the ligands in the metal complex, T_{1M} and T_{2M}, and the mean residence, τ_m, of the ligand in the metal complex. The longitudinal relaxation rates of the nuclei of a ligand in the enzyme·Mn^{2+}·ligand complex $(1/T_{1M})$ may be used to calculate interatomic distances between the paramagnetic ion and the magnetic nuclei by using the dipolar correlation time, τ_c (Solomon & Bloembergen, 1956). Detailed descriptions of the analysis of nuclear relaxation rate data have been previously described [i.e., see Nowak (1981)]. The longitudinal relaxation rate of the nucleus is described by the simplified form of the Solomon–Bloembergen equation (Solomon, 1955; Bloembergen, 1957) as

$$1/T_{1M} = (C/r^6)f(\tau_c) \quad (5)$$

and the transverse relaxation rate as

$$1/T_{2M} = (C/2r^6)f'(\tau_c) + SF \quad (6)$$

The term C is a collection of constants, r is the electron–nuclear distance, $f(\tau_c)$ and $f'(\tau_c)$ are correlation time functions, and the term SF is a scalar function. When the longitudinal relaxation is in fast exchange ($\tau_m \ll T_{1M}$), the value of r can be calculated by eq 5. For Mn^{2+}–^{31}P interactions, eq 5 can be simplified to

$$r\ (Å) = 601[T_{1M}f(\tau_c)]^{1/6} \quad (7)$$

Fast exchange conditions have been previously shown to exist for the ternary complexes of unmodified P-enolpyruvate carboxykinase by Nowak and co-workers (Lee & Nowak, 1984; Duffy & Nowak, 1985).

Circular Dichroism Studies. The circular dichroism (CD) spectra were measured on an AVIV Model 60DS spectropolarimeter. P-Enolpyruvate carboxykinase was modified to 30% residual activity, reduced with $NaBH_4$, and desalted on a P-6DG column equilibrated with 5 mM phosphate buffer, pH 7.4. Native and PLP-modified P-enolpyruvate carboxykinase (0.098 mg/mL) were prepared in 5 mM phosphate buffer, pH 7.4. Prior to analysis, the sample was purged with N_2, and the measurements were conducted in a N_2 atmosphere. The analysis was carried out in the far-UV region (185–250 nm) employing a 0.1-cm cell. In all cases, a buffer base line was determined under the exact conditions of the experiments with enzyme, and subtracted from this latter spectrum, by employing the software package provided. The resulting values are expressed as differences in the molar extinction coefficient ($\Delta\epsilon$) of the left-handed (ϵ_L) and right-handed (ϵ_R) components of circularly polarized light, utilizing a mean residue weight of 109.1. The CD spectra were analyzed by using a Fortran computer program described by Castellino et al. (1986). This method is based upon a best fit of the experimental data to 5 vector-expressed orthogonal-basis CD spectra and their vector-expressed secondary structures that have been generated from 15 proteins and 1 helical polypeptide of known X-ray structure (Hennessey & Johnson, 1981).

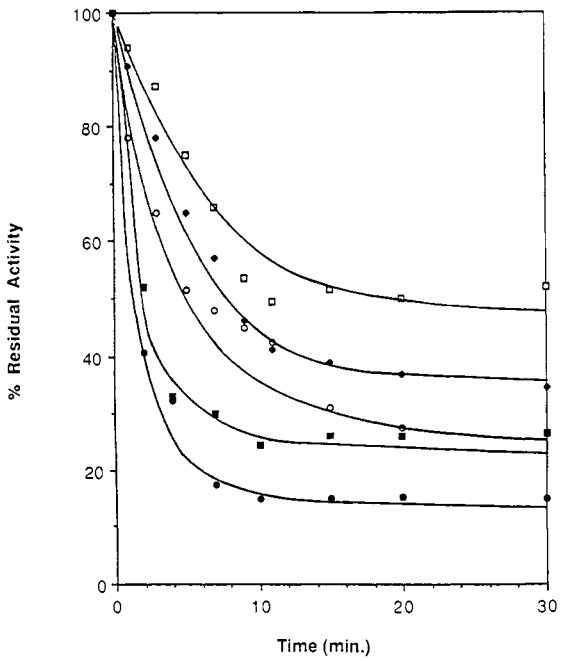

FIGURE 1: Inactivation of P-enolpyruvate carboxykinase (5.4 μM) by various concentrations of PLP. The reversible reaction catalyzed by P-enolpyruvate carboxykinase is assayed. Percent residual activity verus time is plotted for (□) 5, (♦) 10, (○) 20, (■) 50, and (●) 100 μM PLP. Biomolecular rate constants were calculated by linear least-squares fit of the initial slopes as described under Experimental Procedures.

Results

Kinetics of P-Enolpyruvate Carboxykinase Inactivation. The reagent 2,4-pentanedione has been previously shown to react with the ε-amino groups of lysine residues in proteins under neutral pH to form an enamine product (Gilbert & O'Leary, 1975, 1977). Incubation of avian liver P-enolpyruvate carboxykinase with various concentrations of 2,4-pentanedione at pH 7.4 results in a time-dependent loss of enzyme activity. The inactivation process follows pseudo-first-order kinetics with respect to the enzyme concentration. A replot of the observed inactivation rate constant (k_{obs}) vs 2,4-pentanedione concentration is linear, yielding a second-order rate constant of 0.36 ± 0.025 M^{-1} min^{-1} (data not shown). P-Enolpyruvate carboxykinase was treated with 0.1 M 2,4-pentanedione to give 10% residual activity, the sample was desalted, and a UV spectrum was taken (data not shown). An absorbance maximum at 312 nm was observed, characteristic of enamine formation (Gilbert & O'Leary, 1975). This was further confirmed by treatment of the modified enzyme with 0.5 M hydroxylamine, after which the enamine was converted back to an amine as evidenced by the loss of the 312-nm enamine absorbance (Gilbert & O'Leary, 1975). The kinetics of inhibition of UV analysis suggest that specific modification of lysine by 2,4-pentanedione leads to loss of enzymatic activity.

The lysine-specific modifying reagent pyridoxal 5'-phosphate (PLP) was also used as an inactivator of P-enolpyruvate carboxykinase. PLP reversibly inactivates P-enolpyruvate carboxykinase through a typical bimolecular process. This inactivation can be made irreversible by reduction of the PLP-modified enzyme with $NaBH_4$ or $NaCNBH_3$. Figure 1 shows the effect of various PLP concentrations on the rate of inactivation of the enzyme. At longer periods of time, the data are no longer linear, typical of enzyme inactivations by

PLP (Lundblad & Noyes, 1984; Eyzaguirre, 1987). The formation of the Schiff base is an equilibrium process. The linear portion of the inactivation is treated as described under Experimental Procedures. The second-order rate constant of inactivation, obtained from the initial slopes (Figure 1), is calculated as 7700 ± 860 M^{-1} min^{-1}.

Analogous inactivation experiments using PLP as the chemical modifying reagent were carried out for the irreversible reaction catalyzed by P-enolpyruvate carboxykinase in which oxalacetate is irreversibly decarboxylated to form pyruvate. An increase in the concentrations of PLP causes an increased rate of inactivation for the enzyme-catalyzed irreversible reaction. The second-order rate constant of inactivation for the enzyme-catalyzed irreversible reaction, k_2, is 1430 M^{-1} min^{-1}. Additionally in a separate experiment, P-enolpyruvate carboxykinase was inactivated by PLP, and the enzyme activity was assayed for both the reversible (P-enolpyruvate → oxalacetate) and irreversible (oxalacetate → pyruvate) catalytic activities at identical time points. The time course of inactivation (percent residual activity vs time) for both activities was essentially parallel and identical (data not shown). These results suggest that the same lysine residue is participating in both enzyme-catalyzed reactions and that these two separate activities occur at the same catalytic site.

The mechanism of PLP inactivation was tested by adding lysine to a sample of PLP-treated enzyme which retained 24% residual activity. Lysine addition produced a 90% recovery of activity after 45 min. If the PLP-treated enzyme was reduced with $NaBH_4$ before addition of lysine, no reactivation occurred. Control experiments demosntrated that $NaBH_4$ treatment caused no inactivation of the enzyme at concentrations up to 100 mM (data not shown). Pyridoxal was much less effective at inactivating the enzyme and required much greater concentrations to elicit any inactivation. The rate constant of inactivation by pyridoxal is 6.8 M^{-1} min^{-1} (data not shown). PLP demonstrates a much greater reactivity for the enzyme than 2,4-pentanedione or pyridoxal. When treated with $NaBH_4$, PLP exhibits an absorbance maximum at 325 nm characteristic of a reduced Schiff base (data not shown). Because of these favorable properties, it was the reagent of choice for further detailed studies of lysine modification of P-enolpyruvate carboxykinase.

Pyridoxal 5'-Phosphate Incorporation. The specificity of lysine modification was investigated by determining the stoichiometry of PLP incorporated as a function of activity. Several initial attempts were made to label the enzyme with tritium by reducing the Schiff base formed between PLP and lysine with NaB^3H_4. Although this method for labeling PLP-modified enzymes is generally utilized, it was unsuccessful for P-enolpyruvate carboxykinase due to the abnormally high concentrations of $NaBH_4$ required to irreversibly modify the enzyme. The high concentrations of $NaBH_4$ resulted in nonspecific ^3H labeling of the protein, rendering a high tritium background, and prevented accurate determination of the stoichiometry. Therefore, the amount of PLP incorporated into the enzyme was accomplished by quantitating the amount of phosphate irreversibly attached to P-enolpyruvate carboxykinase by utilizing the molybdenum blue method for determining phosphorus (Hurst, 1960). A plot of residual enzymatic activity as a function of PLP incorporation (data not shown) allows extrapolation to 0% enzyme activity, indicating 1.07 ± 0.06 mol of PLP incorporated/mol of enzyme. At longer times of modification, nonspecific PLP incorporation can occur. Control experiments carried out under identical conditions but in the absence of PLP show no phosphate incorporation. An analogous experiment was performed utilizing [^3H]PLP and also showed incorporation of 1.0 mol of PLP incorporated/mol of enzyme at 0% residual enzyme activity (data not shown).

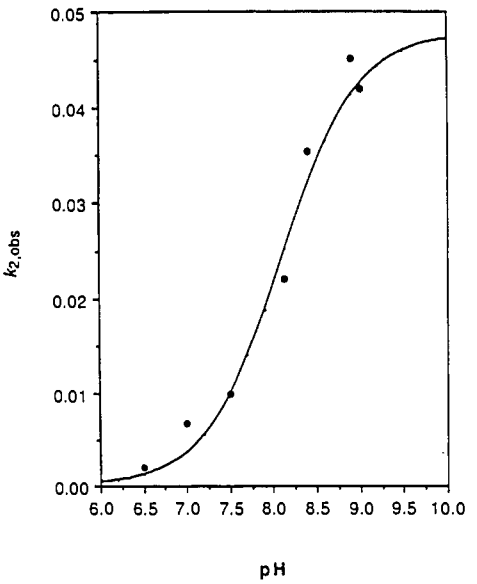

FIGURE 2: Effect of pH on the observed biomolecular rate constant of inactivation of P-enolpyruvate carboxykinase by pyridoxal 5'-phosphate. The pK_a was determined to be 8.1 ± 0.2. The pH titration curve is generated on the basis of a pK_a of 8.1, as described under Experimental Procedures.

To verify the modification of one lysine residue in the complete inactivation of P-enolpyruvate carboxykinase, amino acid analysis was carried out on PLP-labeled enzyme. P-Enolpyruvate carboxykinase was modified to 5% residual activity and reduced with $NaBH_4$, and amino acid analysis was carried out to determine the loss in lysine content. Control experiments were carried out in an identical fashion with native enzyme but without PLP. The amount of each amino acid present in the samples was determined by comparing the integrated area for each amino acid peak to the integrated area of a known quantity of amino acid standard. The data were analyzed by comparing the lysine content to each individual amino acid that is not destroyed by acid hydrolysis and by comparing it to the total amino acid content in the sample (internal controls). Native enzyme contains 20 lysine residues; therefore, modification of 1 lysine in the enzyme would result in a 5% change in lysine content. The above precautions were taken to maximize accuracy. When the lysine content in the PLP-modified enzyme was compared to the lysine content in native enzyme, an average value of 93.9% ± 2.7% was obtained by comparing each individual amino acid to lysine.[2] A value of 95% was obtained when comparing the lysine content to the total amino acid content in the protein (data not shown). This 5% loss in lysine content in the PLP-modified enzyme corresponds to a loss of 1 lysine residue upon inactivation, or the total lysine content decreasing from 20 to 19 residues. The results of experiments that determined phosphate content incorporated per inactivation, and the amino acid analysis results, demonstrate that modification of one lysine residue gives complete inactivation of P-enolpyruvate carboxykinase.

[2] Several of the amino acids, where the ratios of Lys/amino acid are compared for the native and PLP-modified P-enolpyruvate carboxykinase, respectively, are indicated. The percent lysine found in the modified sample is indicated in parentheses: Glu, 0.3360 and 0.3013 (90.0%); Ser, 0.5264 and 0.4990 (94.8%); His, 1.419 and 1.339 (94.4%); Gly, 0.2656 and 0.2519 (94.8%).

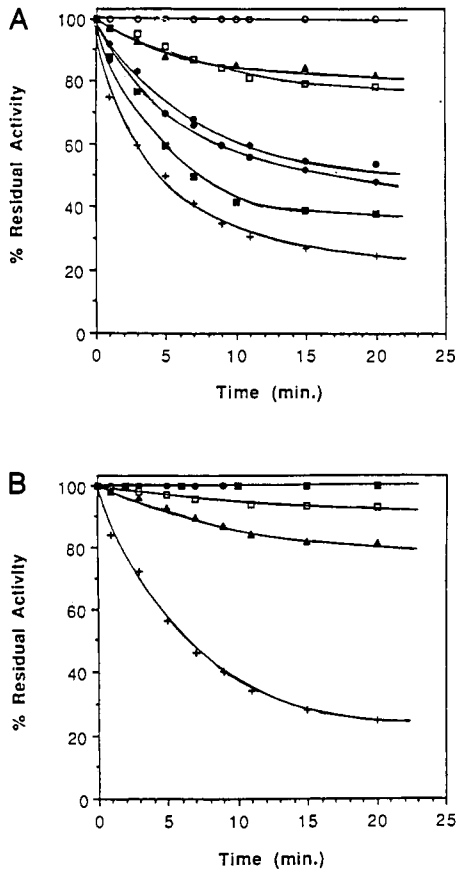

FIGURE 3: Substrate protection against inactivation of P-enolpyruvate carboxykinase by pyridoxal 5'-phosphate. The inactivation was carried out with 20 μM PLP and 5.4 μM P-enolpyruvate carboxykinase. The percent residual activity was measured as a function of time as described under Experimental Procedures. In (A), the substrates employed are as follows: no substrate present (+); 100 mM $KHCO_3$ (■); 1.0 mM oxalacetate (♦); 10 mM $MnCl_2$ (●); 1.25 mM P-enolpyruvate (□); 1.0 mM ITP (▲); 1.0 mM IDP (○). The substrates used in (B) are the following: no substrate present (+); 10 mM $MnCl_2$ + 1.25 mM P-enolpyruvate (▲); 10 mM $MnCl_2$ + 1.0 mM ITP (□); 10 mM $MnCl_2$ + 1.0 mM IDP (●); 10 mM $MnCl_2$ + 1.25 mM P-enolpyruvate + 1.0 mM IDP (■).

pH-Dependent Inactivation Studies. The effect of pH on the inactivation reaction was studied in order to establish the pK_a of the reactive group. The rate of inactivation is dependent on the degree of dissociation of the amino group since the more reactive form of lysine is the unprotonated species. Figure 2 shows the effect of pH on the observed bimolecular rate constant of P-enolpyruvate carboxykinase inactivation by PLP. A plot of $1/k_{2,obs}$ vs $[H^+]$ yields a pK_a of 8.1 ± 0.15 (see Experimental Procedures). The pH-independent second-order rate constant, k (related to the reactive form of lysine), was calculated to be 47700 M^{-1} min^{-1} from these experiments. Figure 2 shows the experimental points and the calculated titration curve generated based on a pK_a of 8.1. These data suggest that the reactive lysine residue in P-enolpyruvate carboxykinase, modified by PLP, has a pK_a of 8.1.

Substrate Protection from PLP Inactivation. Substrate protection from inactivation of the enzyme by PLP was performed to determine the location of the essential lysine residue. The results of these experiments are shown in Figure 3. The percent residual activity is plotted against time in the presence of the various substrates (or combinations of substrates) for P-enolpyruvate carboxykinase. Figure 3A shows that CO_2 offers the least protection and oxalacetate and Mn^{2+} offer slightly better protection. The best protection is seen with the phosphate-containing substrates P-enolpyruvate, IDP, and ITP.

Table I: Kinetic Constants for Native and PLP-Modified P-Enolpyruvate Carboxykinase[a]

enzyme	substrate	V_{max} (units/mg)	K_M (mM)	k_{cat} (s^{-1})
native	$KHCO_3$	2.04 ± 0.07	12.0 ± 0.4	2.45
modified	$KHCO_3$	0.37 ± 0.01	9.5 ± 0.3	0.44
native	PEP[b]	2.65 ± 0.03	0.065 ± 0.007	3.18
modified	PEP	0.67 ± 0.01	0.071 ± 0.007	0.80
native	IDP	2.75 ± 0.03	0.078 ± 0.001	3.30
modified	IDP	0.58 ± 0.07	0.095 ± 0.010	0.70

[a] P-Enolpyruvate carboxykinase was irreversibly modified with PLP to 19% residual activity as described under Experimental Procedures. Kinetic studies were performed immediately after the modified enzyme (modified) was desalted through a P-6DG column. [b] P-Enolpyruvate.

Table II: Mn^{2+} Binding to Native and Modified P-Enolpyruvate Carboxykinase[a]

complex[b]	% remaining activity	n	K_D (μM)
nE	100.0	1.06 ± 0.06	23.3 ± 1.6
mE	25.0	1.20 ± 0.13	46.0 ■ 5.0
nE–PEP[c]	100.0	1.01 ± 0.22	9.2 ± 2.0
mE–PEP	15.0	1.04 ± 0.20	7.4 ± 1.6
nE–IDP[d]	100.0	1.00 ± 0.20	10.6 ± 1.4
mE–IDP	15.0	0.87 ± 0.18	7.8 ± 1.3

[a] Enzyme was modified with PLP to 15–25% residual activity, and samples were prepared as described under Experimental Procedures. Mn^{2+} was added to the complexes listed, and the concentrations of free Mn^{2+} were determined by EPR spectroscopy. The data were treated by Scatchard plots. [b] nE and mE refer to native and modified P-enolpyruvate carboxykinase, respectively. PEP represents phosphoenolpyruvate. [c] Binding constant and number of binding sites taken from Hebda and Nowak (1982b). [d] Values taken from Lee and Nowak (1984).

P-Enolpyruvate and ITP give similar protection against inactivation with the enzyme retaining 80–85% activity. When P-enolpyruvate and Mn^{2+} are present, the protection is similar. When ITP and Mn^{2+} are present, the protection increases to 95% residual activity (Figure 3B). IDP and IDP·Mn^{2+} offer complete protection against inactivation. The combination of P-enolpyruvate, IDP, and Mn^{2+} also gives complete protection against inactivation (Figure 3B). These substrate protection studies are consistent with an active-site lysine residue at the binding site of the phosphate-containing substrates.

Kinetic Constants for Native and Modified P-Enolpyruvate Carboxykinase. P-Enolpyruvate carboxykinase was modified with PLP to 19% residual activity, followed by reduction with $NaBH_4$ and desalting to remove free reagents. The kinetic constants for native and PLP-modified enzyme were determined, and the results are presented in Table I. No significant changes in K_M values for the substrates are observed; however, in each case, the V_{max} and k_{cat} values are decreased to approximately 20% the value of native enzyme. This 80% loss in V_{max} and k_{cat} correlates with the loss in residual activity measured during PLP modification. These results suggest that modification of one lysine residue in the active site of the enzyme causes complete loss of activity.

Mn^{2+} Binding to Native and PLP-Modified P-Enolpyruvate Carboxykinase. EPR and PRR techniques were employed to examine the effect of lysine modification on the binding of Mn^{2+} to P-enolpyruvate carboxykinase. The EPR data were treated by Scatchard plots, and the results are summarized in Table II. Native enzyme has a dissociation constant of 23.3 ± 1.6 μM and 1.06 ± 0.06 metal binding sites. PLP-labeled enzyme has a dissociation constant of 46.0 ± 5 μM and 1.20 ± 0.13 binding sites. PRR measurements of the native and modified enzyme yielded binary enhancement values, ϵ_b, of 12.4 ± 0.8 and 12.5 ± 1.7, respectively (data not shown). The results of Mn^{2+} binding to the binary enzyme·P-enolpyruvate and enzyme·IDP complexes are also listed in

Table III: ^{31}P Relaxation Rates and ^{31}P–Mn^{2+} Distances Calculated for the Ternary Enzyme·Mn^{2+}·Phosphoenolpyruvate, Enzyme·Mn^{2+}·IDP, and Enzyme·Mn^{2+}·GTP Complexes

complex[a]	$1/pT_{1p}$ (s^{-1})	$1/pT_{2p}$ (s^{-1})	r (Å)[b]	r (Å)[c]
nE·Mn·PEP[d]	(0.29 ± 0.05) × 10^3	(2.2 ± 0.3) × 10^4	8.48 ± 0.06	7.92 ± 0.06
mE·Mn·PEP	(0.20 ± 0.01) × 10^3	(2.8 ± 0.1) × 10^4	8.99 ± 0.07	8.46 ± 0.06
nE·Mn·αIDP[e]	(1.65 ± 0.13) × 10^3		6.18 ± 0.09	5.42 ± 0.07
mE·Mn·αIDP	(1.60 ± 0.15) × 10^3	(1.1 ± 0.10) × 10^5	6.13 ± 0.10	5.41 ± 0.09
nE·Mn·βIDP[e]	(2.02 ± 0.27) × 10^3		5.97 ± 0.09	5.25 ± 0.07
mE·Mn·βIDP	(4.60 ± 0.46) × 10^3	(1.3 ± 0.10) × 10^5	5.13 ± 0.10	4.54 ± 0.08
nE·Mn·αGTP[e]	(1.41 ± 0.13) × 10^3	(3.0 ● 0.1) × 10^4	6.34 ± 0.09	5.57 ● 0.08
mE·Mn·αGTP	(3.65 ± 0.15) × 10^3	(2.8 ± 0.2) × 10^4	5.34 ± 0.10	4.71 ± 0.09
nE·Mn·βGTP[e]	(1.76 ± 0.21) × 10^3	(3.4 ± 0.4) × 10^4	6.11 ± 0.09	5.37 ± 0.08
mE·Mn·βGTP	(4.95 ± 0.22) × 10^3	(2.2 ± 0.1) × 10^4	5.08 ± 0.10	4.48 ± 0.08
nE·Mn·γGTP[e]	(1.77 ± 0.09) × 10^3	(3.0 ± 0.5) × 10^4	6.11 ± 0.08	5.37 ± 0.07
mE·Mn·γGTP	(3.95 ± 0.21) × 10^3	(3.5 ± 0.1) × 10^4	5.27 ± 0.08	4.65 ± 0.07

[a] nE refers to native enzyme, and mE refers to lysine-modified enzyme. PEP refers to phosphoenolpyruvate. αIDP and βIDP refer to the α- and β-phosphorus of IDP, respectively. αGTP, βGTP, and γGTP represent the α-, β-, and γ-phosphorus of GTP, respectively. [b] Values calculated by assuming no frequency dependence of τ_c. $\tau_c = 2.2 \times 10^{-9}$ s and $f(\tau_c) = 2.4 \times 10^{-9}$ s for the ternary enzyme·Mn·P-enolpyruvate complex (Duffy & Nowak, 1985). For the ternary enzyme·Mn·IDP complex, $\tau_c = 1.5 \times 10^{-9}$ s, and $f(\tau_c) = 1.9 \times 10^{-9}$ s (Lee & Nowak, 1984). [c] Values calculated by assuming maximal frequency dependence of τ_c. $\tau_c = 0.7 \times 10^{-9}$ s and $f(\tau_c) = 1.6 \times 10^{-9}$ s for the ternary enzyme·Mn·P-enolpyruvate complex (Duffy & Nowak, 1985). For the ternary enzyme·Mn·IDP complex, $\tau_c = 5.5 \times 10^{-9}$ s, and $f(\tau_c) = 0.9 \times 10^{-9}$ s (Lee & Nowak, 1984). [d] Data taken from Duffy and Nowak (1985). [e] Data taken from Lee and Nowak (1984).

Table II. The presence of substrate elicits enhanced Mn^{2+} binding in the lysine-modified enzyme. Similar dissociation constants and number of binding sites are observed for native and modified enzyme. These results demonstrate that modified, inactive enzyme exhibits little change in Mn^{2+} binding. The complex formation of the modified enzyme with P-enolpyruvate and IDP still occurs and also affects Mn^{2+} binding to the enzyme.

Proton Relaxation Rate Measurements of Enzyme·Mn^{2+}·Substrate Complexes. The interactions of the substrates with the native and modified binary P-enolpyruvate carboxykinase·Mn^{2+} complexes were investigated by PRR techniques. The binding character of the modified enzyme, which maintains 15–20% residual activity, is shown in Figure 4. Titrations of native enzyme are also shown for comparison. The substrates P-enolpyruvate and IDP elicit no change in enhancement in the titrations with modified enzyme (Figure 4). The binding of CO_2 and ITP to modified enzyme·Mn^{2+} appears unaffected (data not shown). The binding constants for ITP and CO_2 in the ternary enzyme·Mn^{2+}·substrate complex (K_3), the binding constants for the enzyme·substrate complex (K_s), and the enhancements of the respective ternary enzyme·Mn^{2+}·ligand complexes (ϵ_T) are unchanged from native enzyme (Hebda & Nowak, 1982b). The results suggest that lysine-modified P-enolpyruvate carboxykinase excludes or alters binding of the phosphate-containing substrates P-enolpyruvate and IDP.

Mn^{2+}–^{31}P Nuclei Interactions in the Ternary Enzyme·Mn^{2+}·Ligand Complexes for Lysine-Modified Enzyme. The effects of enzyme-bound Mn^{2+} on the ^{31}P relaxation rates of the substates P-enolpyruvate and IDP were measured at 121.5 MHz. The ^{31}P spectra were ^1H decoupled. Plots of ln ($M_\infty - M_t$) vs τ were linear. Under experimental conditions (concentration of enzyme and ligand), all the Mn^{2+} added is bound in the enzyme·Mn^{2+}·substrate complex. Both P-enolpyruvate and IDP are saturating at >0.5 mM as demonstrated by their synergistic effects in facilitating Mn^{2+} binding to the enzyme. The distribution of Mn^{2+} was calculated by using the binding constants reported herein and by previously determined values (Hebda & Nowak, 1982b; Lee & Nowak, 1984). The calculations were performed with the program used to fit the PRR binding data (Lee & Nowak, 1984). These calculations take into account all possible equilibria present in solution including the increase in Mn^{2+} binding by the enzyme in the presence of substrate. The normalized

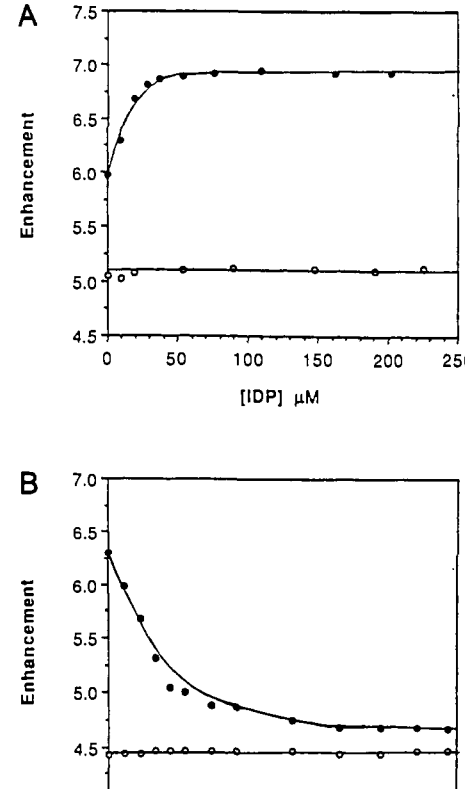

FIGURE 4: Proton relaxation rate (PRR) titration curves of the ternary enzyme·Mn^{2+}·substrate complexes. P-Enolpyruvate carboxykinase was modified with PLP to 15–20% residual activity, and the titrations were carried out as described under Experimental Procedures. The observed enhancement is plotted as a function of substrate concentration. In each case, titrations with native (●) and modified (○) enzyme were carried out and are shown for comparison. (A) Titration with IDP and (B) titration with P-enolpyruvate.

relaxation rates are presented in Table III. In order to use the relaxation rate data to calculate the Mn^{2+}–nuclear distance, r, from eq 7, the ligands must be in fast exchange ($1/pT_{1p} = 1/T_{1M}$) and the correlation time must be known. The ternary complexes for native enzyme have previously been shown to be in fast exchange (Lee & Nowak, 1984; Duffy & Nowak, 1985). The relaxation rates for complexes with

modified enzyme are consistent with fast exchange where $1/pT_{2p} \gg 1/pT_{1p}$. It has previously been demonstrated (Nowak, 1981) that when $1/pT_{2p}$ values are substantially larger than $1/pT_{1p}$ values, $1/pT_{1p}$ is in the rapid exchange domain and is a measure of relaxation ($1/T_{1M}$). Caution must be taken, however, in the quantitative use of $1/T_2$ values (Ray et al., 1988). When the values determined for the correlation times for other P-enolpyruvate carboxykinase·Mn^{2+}·ligand complexes by Nowak and co-workers (Lee & Nowak, 1984; Duffy & Nowak, 1985) are used, the correlation functions $f(\tau_c)$ could be calculated (Nowak, 1981).[3] The values for $f(\tau_c)$ and the values measured for $1/pT_{1p}$ were used to calculate the Mn^{2+}–nuclear distances, r (Table III). The correlation time functions were assumed to be either frequency-independent or frequency-dependent, and calculations were performed on the basis of each assumption. These calculations were performed with the simplified Solomon–Bloembergen equation (eq 7). Table III compares the ^{31}P relaxation rates and calculated distances for the ternary complexes with both native and lysine-modified enzyme. P-Enolpyruvate binds to modified enzyme, but the phosphate group is 0.5 Å further from the Mn^{2+} when compared to native enzyme. The α- and β-phosphorus atoms of IDP are displaced by 0.16 and 0.84 Å, respectively, closer to the bound Mn^{2+}. The α-, β-, and γ-phosphorus atoms of GTP are moved closer to the Mn^{2+} by 1.0, 1.0, and 0.8 Å, respectively. These data demonstrate altered binding of the phosphorylated substrates in the ternary complexes with lysine-modified enzyme.

Circular Dichroism Analysis and Secondary Structure Calculations of Native and Modified P-Enolpyruvate Carboxykinase. Circular dichroism spectra, taken at 185–250 nm, were used to investigate possible conformational changes between native and PLP-modified P-enolpyruvate carboxykinase. The CD data were fit as described under Experimental Procedures to estimate protein secondary structure. There is no significant change in α-helix and random structure upon inactivation of the enzyme with PLP. These results suggest that the inactivation of P-enolpyruvate carboxykinase by PLP is not caused by a significant change in enzyme structure.

Discussion

Cationic amino acids such as lysine and arginine are frequently found at the active site of enzymes that act on anionic substrates and cofactors. The substrates for P-enolpyruvate carboxykinase are anionic and contain phosphate groups. Hanson and co-workers (Cook et al., 1986) have suggested the presence of lysine residues in the phosphoryl binding regions of P-enolpyruvate carboxykinases, on the basis of comparisons of the amino acid sequences of cytosolic P-enolpyruvate carboxykinase with those of other proteins that bind guanine nucleotides. Arginine is apparently not located at the phosphoryl binding sites of P-enolpyruvate carboxykinase (Cheng & Nowak, 1989a). Thus, lysine is a good candidate for an active-site amino acid residue involved in P-enolpyruvate carboxykinase catalysis. Inactivation of the enzyme by 2,4-pentanedione follows pseudo-first-order kinetics and is linearly dependent on the reagent concentration, giving a second-order rate constant of 0.36 M^{-1} min^{-1}. PLP reversibly inactivates P-enolpyruvate carboxykinase by a bimolecular process. A second-order rate constant of 7700 M^{-1} min^{-1} is obtained when the reversible reaction catalyzed by P-enolpyruvate carboxykinase is assayed. A bimolecular rate constant of inactivation of 1430 M^{-1} min^{-1} is measured when the enzyme is assayed for the irreversible reaction. Although both reactions are inhibited in a parallel fashion, the reason for nonidentical rate constants for inactivation is unclear. Perhaps the different assay conditions affect the equilibrium constant for the Schiff base complex. The inactivation reaction is sufficiently rapid that pseudo-first-order conditions cannot normally be achieved.

The stoichiometry of PLP incorporation and amino acid analysis demonstrate that one lysine residue is critical for enzyme catalysis. This essential lysine has a pK_a value of 8.1 as measured by pH effects on the inactivation rate constant of the enzyme. This pK_a value for the reactive lysine is low compared to that estimated for the ϵ-amino group of lysine in a polypeptide chain [9.5–10.6 (Segal, 1975)]. The pK_a value for the reactive lysine residue agrees with the expected pK_a for a lysine residue in a low-polarity microenvironment (Fersht, 1985). According to the conclusions of Cook et al. (1986), all of the putative binding regions identified by consensus in cytosolic P-enolpyruvate carboxylkinase from chicken occur in hydrophobic regions. This agrees with the previous findings of Silverstein et al. (1980), who had provided evidence of a hydrophobic environment for the active site of mitochondrial hog liver P-enolpyruvate carboxykinase. A hydrophobic environment at the nucleotide site was also indicated in yeast (Encinas et al., 1990) and in rat liver cytosol (Lewis et al., 1989) P-enolpyruvate carboxykinase. Several cases of depressed pK_a's have been observed in enzyme active-site microenvironments resulting in highly reactive ϵ-amino groups. Examples of such reactive lysines in enzymes are oxaloacetate decarboxylase [pK_a = 5.9 (Schmidt & Westheimer, 1971], glutamate dehydrogenase [pK_a = 7.7 (Veronese et al., 1972)], and phosphomevalonate kinase [pK_a = 8.15 (Bazaes et al., 1980)]. The pK_a reported herein is also in good agreement with the pK_a = 8.2 for the putative active-site lysine in this enzyme determined by affinity labeling (Guidinger, 1990) and a pK_a = 8.1 determined for a predicted active-site lysine determined from affinity labeling of yeast P-enolpyruvate carboxykinase (Saavedra et al., 1988). Kinetic measurements were carried out to determine if the 19% residual activity results from 81% of the enzyme being completely inactivated or if all the enzyme is modified and retains partial (19%) activity. No significant changes in the K_M values for the substrates are observed. The V_{max} and k_{cat} values are decreased to values that correlate with the loss in enzyme activity upon PLP modification. This and other data presented indicate that modification of a single lysine residue causes complete loss of activity.

Studies of Mn^{2+} binding to the enzyme show no appreciable change in dissociation constant or number of binding sites for the divalent metal. The presence of substrates enhances Mn^{2+} binding to lysine-modified enzyme as is also observed with native enzyme. The values obtained from these experiments are the same as results obtained for native enzyme (Hebda & Nowak, 1982b; Lee & Nowak, 1984). These studies indicate that modified, inactive enzyme shows no significant changes in Mn^{2+} binding. The substrates can still bind to the active site and elicit a conformational change about the Mn^{2+} environment. The reactive lysine is not directly involved in the interaction of Mn^{2+} at the enzyme active site nor does its modification preclude substrate binding or substrate-induced active-site changes.

[3] A frequency dependence of PRR values for native and PLP-modified binary enzyme–Mn and ternary enzyme–Mn–substrate complexes, where substrate is IDP or P-enolpyruvate, was measured. The value of τ_c estimated for native enzyme complexes was 2.7 ± 0.2 ns, and the value estimated for the PLP-modified enzyme complexes was 2.0 ± 0.1 ns. These values are not significantly different and give no significant differences in the calculation of the dipolar distances that are calculated.

The increased enhancement and the thermodynamic constants obtained for the HCO_3^- titration to native enzyme–Mn and PLP–enzyme–Mn binary complexes suggest similar binding to both enzymes. The results with ITP also imply similar binding in both native and lysine-modified enzyme. The substrates P-enolpyruvate and IDP demonstrated no change in enhancement in the titrations with modified enzyme·Mn^{2+}. These results suggest that lysine-modified P-enolpyruvate carboxykinase alters binding of these substrates. The absence of an observable perturbation in the observed enhancement does not necessarily indicate the lack of formation of the respective ternary complexes. These substrates may bind to the lysine-modified enzyme·Mn^{2+} complex, causing no perturbation of the environment of the metal ion. Alternatively, these substrates may bind near or at the Mn^{2+} and cause fortuitous, compensating changes in q, τ_m, and T_{1M} (Dwek, 1973). The latter explanation is more plausible than total exclusion of substrate binding in light of the ligand-induced change in Mn^{2+} binding to the enzyme·ligand complex and from the high-resolution ^{31}P NMR results presented in Table III. Lee and Nowak (1984) observed a lack of change in observed enhancement upon IDP addition to the native enzyme·Mn^{2+} complex. However, the formation of an enzyme·Mn^{2+}·IDP complex was detected by direct binding studies using an equilibrium gel separation technique. The Mn^{2+}–^{31}P distances in the ternary enzyme·Mn^{2+}·nucleotide complexes were determined, and the results showed that the phosphorus atoms of the nucleotide were in an outer sphere complex to the enzyme-bound Mn^{2+} (Lee & Nowak, 1984). The lack of change in PRR enhancement was attributed to the ligand interacting in an outer sphere complex with the enzyme-bound Mn^{2+} and does not affect the immediate environment of the bound Mn^{2+}. It appears that the altered binding of these substrate ligands in an outer sphere complex with the lysine-modified enzyme–Mn^{2+} causes a lack of observed enhancement with the substrates P-enolpyruvate and IDP.

A compilation of the relaxation rate data and calculated Mn^{2+}–nuclear distances can be seen in Table III. The results show that P-enolpyruvate, IDP, and ITP bind to lysine-modified enzyme in an altered fashion. In all cases, the altered positioning of substrates still results in outer sphere complexes as suggested from the Mn^{2+}–^{31}P distance and PRR data. PLP-Labeled P-enolpyruvate carboxykinase appears to cause P-enolpyruvate to bind further away from Mn^{2+} than in the native enzyme. The nucleotides are repositioned closer to Mn^{2+} in modified enzyme. This altered binding is more drastic in GTP than IDP binding possibly due to the longer polyphosphate chain in GTP. With IDP, the β-phosphate (end of the phosphate chain) is repositioned closer to Mn^{2+} while the α-phosphate is relatively unchanged. The longer polyphosphate chain of GTP could possibly have less room for specific movement of the chain in the active site. A specific interaction by a lysine on the enzyme with the β-phosphate (middle of the chain) could push the whole chain closer to the Mn^{2+}. An interaction of the lysine residue with the γ-phosphate of the nucleotide triphosphate might cause behavior similar to the results with the nucleotide diphosphate, i.e., only altered positioning of the terminal phosphate (γ-P). This altered positioning of substrates (due to blocking of the important lysine residue) appears to distort the substrates from optimal alignment which is proposed to be required for enzymatic catalysis (Lee & Nowak, 1984; Duffy & Nowak, 1985; Lee et al., 1985; Guidinger & Nowak, 1990).

The values for $1/pT_{2p}$ of the ^{31}P of the nucleotides in the native enzyme have been shown to be ligand exchange rates (Lee & Nowak, 1984). Although the locations of the polyphosphate chains of the nucleotides have been altered at the catalytic site of the enzyme, it appears that the exchange rate for GTP has not changed. The exchange rate for IDP has been estimated to have increased by an order of magnitude, however (Lee and Nowak, unpublished results).

The interactions of nucleotides with P-enolpyruvate carboxykinase have previously been studied by using the stereospecific thiophosphate analogues of GDP and GTP (Lee et al., 1985). The phosphothioate analogues of guanosine (GDPαS, GDPβS, GTPαS, GTPβS, and GTPγS) were utilized to study nucleotide–enzyme interactions. Guanosine 5′-*O*-(2-thiotriphosphate) (GTPβS) (S_P) is a much better substrate (>50 times) than is GTPβS (R_P). The metal ions have little effect on the selectivity. Additionally, the α,β-methylene or β,γ-imido and β,γ-methylene derivatives of the nucleotides all fail to elicit substrate activity or inhibition. These results suggest a specific interaction of the β-phosphate of the nucleotide with the protein. A positively charged amino acid residue of the enzyme (such as lysine) could be involved in the interaction of the β-phosphate to form a "chiral" active site of the enzyme. The β-phosphate appears to interact with the enzyme and with a water molecule bound to the metal ion. It is also conceivable, on the basis of these studies, that the lysine residue is involved in activation of the phosphate of P-enolpyruvate. The possibility that the α- or γ-phosphate of the nucleotide interacts with the lysine residue cannot be unequivocally ruled out. However, on the basis of available information on avian liver P-enolpyruvate carboxykinase, the specific interaction of the β-phosphate of the nucleotide with lysine is a more attractive hypothesis.

An active-site lysine has also been implicated with other GTP- or ITP-utilizing proteins. GTP and phosphoryl binding sites have been predicted from X-ray crystallographic analysis of elongation factor Tu from *Escherichia coli* (McCormick et al., 1985; Jurnak, 1985). Several other proteins that interact with GTP contain the same series of conserved sequences identified for the elongation factor Tu (McCormick et al., 1985), suggesting similar binding sites. Work toward the goal of identifying the location of this lysine in the amino acid sequence of mitochondrial chicken liver P-enolpyruvate carboxykinase is currently in progress in our laboratory.

Enzyme modification by PLP showed little if any change in the CD spectra of modified enzyme when compared to native enzyme. The CD data were used to calculate the secondary structure of the native and modified enzyme. These data suggest that the structure of P-enolpyruvate carboxykinase is not significantly perturbed by PLP modification. The substrates P-enolpyruvate and nucleotide form outer sphere complexes with the enzyme-bound Mn^{2+} (data reported herein; Lee & Nowak, 1984; Duffy & Nowak, 1985). The results obtained from this study are also consistent with data obtained with this enzyme by using the affinity label 2′,3′-dialdehyde derivative of guanosine diphosphate. This analogue has been shown to bind specifically at the nucleotide site of the enzyme and reacts with a lysine residue to cause inhibition (Guidinger, 1990). The identities of that lysine and the lysine labeled by PLP are currently being investigated. Modifying the active-site lysine by PLP causes a repositioning of the substrates and results in enzyme inactivation. Optimal alignment of the substrates, apparently oriented by the bound Mn^{2+} and a lysine residue, is required for enzymatic catalysis.

In summary, there is a single reactive lysine residue at the catalytic site of avian P-enolpyruvate carboxykinase. It appears to play an important role in the proper binding, orientation, and activation of P-enolpyruvate and GDP or of GTP. The lysine is not essential for substrate binding but is critical for the proper binding of substrates. Since the enzyme-bound Mn^{2+} does not serve as a direct template for the binding of the anionic substrates, this role is assumed by active-site amino acids. This lysine is a key residue for such a function.

Registry No. Lys, 56-87-1; PLP, 54-47-7; IDP, 86-04-4; ITP, 132-06-9; Mn, 7439-96-5; phosphoenolpyruvate carboxykinase, 9013-08-5; 2,4-pentanedione, 123-54-6; phosphoenolpyruvic acid, 138-08-9.

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Evidence for Distinct Dehydrogenase and Isomerase Sites within a Single 3β-Hydroxysteroid Dehydrogenase/5-Ene–4-Ene Isomerase Protein

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Received January 28, 1991; Revised Manuscript Received May 9, 1991

ABSTRACT: Complementary DNA encoding human 3β-hydroxysteroid dehydrogenase/5-ene–4-ene isomerase (3β-HSD) has been expressed in transfected GH_4C_1 with use of the cytomegalovirus promoter. The activity of the expressed protein clearly shows that both dehydrogenase and isomerase enzymatic activities are present within a single protein. However, such findings do not indicate whether the two activities reside within one or two closely related catalytic sites. With use of [^{3}H]-5-androstenedione, the intermediate compound in dehydroepiandrosterone (DHEA) transformation into 4-androstenedione by 3β-HSD, the present study shows that 4MA (*N*,*N*-diethyl-4-methyl-3-oxo-4-aza-5α-androstane-17β-carboxamide) and its analogues inhibit DHEA oxidation competitively while they exert a noncompetitive inhibition of the isomerization of 5-androstenedione to 4-androstenedione with an approximately 1000-fold higher K_i value. The present results thus strongly suggest that dehydrogenase and isomerase activities are present at separate sites on the 3β-HSD protein. In addition, using 5α-dihydrotestosterone (DHT) and 5α-androstane-3β,17β-diol as substrates for dehydrogenase activity only, we have found that dehydrogenase activity is reversibly and competitively inhibited by 4MA. Such data suggest that the irreversible step in the transformation of DHEA to 4-androstenedione is due to a separate site possessing isomerase activity that converts the 5-ene-3-keto to a much more stable 4-ene-3-keto configuration.

The activity of 3β-hydroxysteroid dehydrogenase (EC 1.1.1.145)/steroid 5-ene–4-ene isomerase (EC 5.3.3.1), hereafter called 3β-HSD, catalyzes the transformation of 5-ene-3β-hydroxysteroids to the corresponding 4-ene-3-keto configuration (Samuels et al., 1951) and is therefore an essential step in the biosynthesis of all classes of hormonal steroids, namely, progesterone, glucocorticoids, mineralocorticoids, androgens, and estrogens. While different proteins responsible for these two reactions have been isolated separately from bacterial sources (Talalay & Wang, 1955), the two activities appear to reside within a single protein in mammalian tissues as observed for the enzyme purified from bovine ovaries (Cheatum & Warren, 1955) as well as from human placenta (Luu-The et al., 1988, 1989, 1990; Lachance et al., 1990), ovine adrenals (Ford & Engel, 1974), rat adrenals (Ishii-Ohba et al., 1987) and testes (Ishii-Ohba et al., 1986), and bovine adrenals (Eastman & Neville, 1987; Inano et al., 1990).

In addition to being required for the synthesis of all classes of steroids, 3β-HSD activity could well play a role in the preferential transformation of steroid precursors into mineralocorticoids, glucocorticoids, progesterone, or sex steroids. In fact, there is still a debate about the presence of one or more 3β-HSD(s), and clinical data suggest the existence of more than one 3β-HSD in the human (del Carmen Cravioto et al., 1986). The existence of three different substrate-specific 5-ene–4-ene isomerases has been suggested in bovine adrenals (Edwald et al., 1964).

In order to obtain more information about the mechanism of action of 3β-HSD, we have expressed human placental 3β-HSD cDNA (Luu-The et al., 1989) in mammalian cells and we have synthesized [^{3}H]-5-androstenedione, the intermediate product in DHEA transformation into 4-androstenedione. In addition, with the use of 3β-HSD purified from human placenta (Luu-The et al., 1990), the present data show that 4MA inhibits competitively dehydrogenase activity while it is noncompetitive for the isomerase site. On the other hand, trilostane, another potent inhibitor of 3β-HSD, inhibits competitively both dehydrogenase and isomerase activities. The present results thus suggest that the two enzymatic sites are distinct within a single 3β-HSD protein.

Materials and Methods

Materials. NAD^+ and $NADP^+$ were purchased from Sigma Chemical Co. [4,7-^{3}H]Pregnenolone, [1,2-^{3}H]-dehydroepiandrosterone, 5α-[1,2,6,7-^{3}H]dihydrotestosterone, 5α[1,2-^{3}H]androstane-3β,17β-diol, [^{14}C]dehydroepiandrosterone, and [^{14}C]pregnenolone were obtained from New England Nuclear. TLC silica gel plates were from Merck,

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0006-2960/91/0430-8861$02.50/0