Toward Identification of Acid/Base Catalysts in the Active Site of Enolase: Comparison of the Properties of K345A, E168Q, and E211Q Variants†

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ABSTRACT: High-resolution crystallographic data show that Glu 168 and Glu 211 lie on opposite surfaces of the active site from Lys 345. Two different proposals for general base catalysis have emerged from these structural studies. In one scheme, the carboxylate side chains of Glu 168 and Glu 211 are proposed to ionize a trapped water molecule and the OH^- serves as the base [Lebioda, L., & Stec, B. (1991) *Biochemistry 30,* 2817−2822]. In the other proposal, the ϵ-amino group of Lys 345 functions in general base catalysis [Wedekind, J. E., Poyner, R. R., Reed, G. H., & Rayment, I. (1994) *Biochemistry 33,* 9333−9342]. Genes encoding site specific mutations of these active site residues of yeast enolase, K345A, E168Q, and E211Q, have been prepared. The respective protein products of the wild type and mutant genes were expressed in *Escherichia coli* and isolated in homogeneous form. All three mutant proteins possess severely depressed activities in the overall reaction—< 1 part in 10^5 of wild type activity. Properties of the three mutant proteins in partial reactions were examined to define more clearly the roles of these residues in the catalytic cycle. The K345A variant fails to catalyze the exchange of the C-2 proton of 2-phospho-D-glycerate with deuterium in D_2O, whereas both the E211Q and E168Q mutant proteins are functional in this partial reaction. For E211Q and E168Q enolases, exchange is essentially complete prior to appearance of product, and this observation provides further support for an intermediate in the normal reaction. K345A enolase is inactive in the ionization of tartronate semialdehyde phosphate (TSP), whereas both E168Q and E211Q proteins alter the tautomeric state or catalyze ionization of bound TSP. Wild type enolase catalyzes hydrolysis of *(Z)*-3-chloro-2-phosphoenolpyruvate by addition of OH^- and elimination of Cl^- at C-3. This reaction mimics the addition of OH^- to C-3 of phosphoenolpyruvate in the reverse reaction with the normal product. All three mutant proteins are depressed in their abilities to carry out this reaction. In single-turnover assays, the activities vary in the order K345A > E168Q ≫ E211Q. These results suggest that Lys 345 functions as the base in the ionization of 2-PGA and that Glu 211 participates in the second step of the reaction.

Enolase catalyzes the reversible dehydration of 2-PGA[1] to form P-enolpyruvate in glycolysis. The reaction is nearly isoenergetic having an equilibrium constant of ∼4 off the enzyme and ∼1 on the enzyme (Burbaum & Knowles, 1989). Despite the thermodynamic balance between substrate and product, the kinetic barrier of the reaction is substantial. The reaction is believed to proceed in a stepwise manner through a carbanion (enolate) intermediate generated by abstraction of the C-2 proton of 2-PGA (Dinovo & Boyer, 1971; Anderson et al., 1994). The acidity of the proton at C-2 is weak (pK_a ∼28−32), and the enzymatic mechanisms which facilitate rapid ionization of this weak carbon acid and stabilization of the resulting intermediate are of considerable current interest (Gerlt & Gassman, 1992, 1993). Catalysis of this apparent stepwise elimination requires a base to remove the proton from C-2 of the substrate 2-PGA, a means of stabilizing the intermediate, and an acid or other electrophile to activate the C-3 hydroxyl as a leaving group. The two divalent metal ions required by enolase (Faller et al., 1977) are believed to stabilize the intermediate through coordination to an *aci* carboxylate form of the intermediate (Poyner & Reed, 1992; Wedekind et al., 1994). However, the identities of residues functioning in general acid/base catalysis are not yet established unambiguously. Rose and co-workers (Cohn et al., 1970) showed that the enolase-catalyzed elimination reaction is specifically *anti*—a finding which implies the existence of distinct catalytic groups on opposite sides of the substrate.

High-resolution crystallographic analysis shows that the active site of enolase is replete with charged and polar amino acid side chains (Lebioda & Stec, 1991) such that several candidates for acid/base catalysts are present. Thus, Lebioda and Stec (1991) focused attention on the carboxylate side chains from Glu 168 and Glu 211 and an intervening water molecule in a "charge shuttle mechanism" for general base

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[1] Abbreviations: 2-PGA, 2-phospho-D-glycerate; P-enolpyruvate, phosphoenolpyruvate; *(Z)*- 3-Cl-P-enolpyruvate, *(Z)*- 3-chloro-phosphoenolpyruvate; TSP, tartronate semialdehyde phosphate; PhAH, phosphonoacetohydroxamate; PEG, polyethylene glycol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; P-enolpyruvate phosphomutase, phosphoenolpyruvate phosphomutase; NOE, nuclear Overhauser effect.

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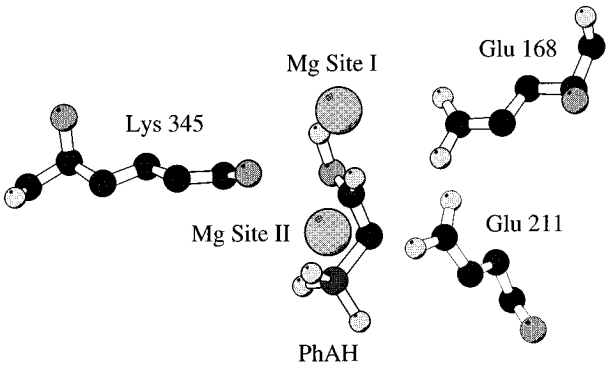


FIGURE 1: Positions of Lys 345, Glu 211, and Glu 168 in relation to Mg^{2+} and PhAH in the active site of enolase. The coordinates are from Wedekind et al. (1994). The figure was generated using the program MOLSCRIPT (Kraulis, 1991).

catalysis. This proposal was based on the apparent locations of the carboxylate and hydroxymethyl moieties of 2-PGA soaked into crystals at pH 6 in 3 M $(NH_4)_2SO_4$ with only the high-affinity metal site occupied. This proposed scheme was compatible with the low fractionation factor attributed to the general base catalyst as inferred from a ^2H isotope effect on the inhibition constant of TSP (Weiss et al., 1987; Cleland, 1992). The importance of Glu 168 in overall catalysis was also demonstrated by $\sim 10^4$ lower activity of the E168Q variant of enolase reported by Brewer et al. (1993). Lebioda and Stec (1991), however, noted that the distinction between the hydroxymethyl and carboxylate moieties of the substrate in their electron density map was ambiguous.

In a separate study, Wedekind et al. (1994) determined the structure of the bis Mg^{2+} complex of enolase and the intermediate analog, PhAH, from crystals grown at pH 8 from PEG. This structure confirmed the presence of a μ-bridging ligand between the two metal ions, the carbonyl oxygen of PhAH, that had been detected by EPR measurements on Mn^{2+} complexes (Poyner & Reed, 1992). Moreover, the structure of the PhAH complex indicated that the C-2 proton of 2-PGA would be exposed to Lys 345 if the carboxylate moiety of the substrate occupied a position analogous to that of the carbonyl oxygen of PhAH in the active site.

The present experiments with site specific mutant forms of enolase were initiated to gain further insight into the possible roles of Glu 168, Glu 211, and Lys 345 in enolase catalysis. These three residues are highly conserved in all known enolase sequences, and the catalytic activities of the separate mutant proteins are severely depressed ($> 10^5$) with respect to wild type activity. The stepwise nature of the reaction, however, allows assay of partial reactions catalyzed by the mutant proteins in a manner analogous to that employed by Landro et al. (1991) in studies of mutant forms of mandelate racemase. The mutant forms of enolase exhibit distinctive behavior in partial reactions that effectively assay the first and second steps in the catalytic cycle. The present paper reports the properties of these mutant proteins.

EXPERIMENTAL PROCEDURES

Selection of Mutations. The positions of Lys 345, Glu 168, and Glu 211 with respect to the inhibitor, PhAH, are illustrated in Figure 1 (Wedekind et al., 1994). Lys 345 was mutated to Ala in order to allow attempts at chemical rescue experiments with exogenous amines (Toney & Kirsch, 1989). The conservative mutations, E168Q and E211Q, were made to assess the roles of the carboxyl(ate) side chains of these residues.

Materials for DNA Manipulation. Restriction endonucleases, Deep Vent DNA polymerase, and T4 DNA ligase were purchased from New England Biolabs. Lysozyme and DNAse I were purchased from Boehringer Mannheim. The pGEM-7Zf plasmid and the Wizard DNA Mini prep kit were obtained from Promega. The expression vector pET-22b and *Escherichia coli* strain BL21(DE3) were obtained from Novagen. Tryptone and yeast extract were obtained from Acumedia. Oligonucleotides were purchased from Integrated DNA Technologies, Inc.

Construction of the Enolase Expression Vector. The plasmid peno46 (Holland et al., 1981), which contains a genomic clone of the yeast enolase gene, was a generous gift of Dr. Michael Holland, University of California—Davis. The coding region of the enolase gene was amplified using PCR with the following primers: (1) 5′-AAA TCT AGA CAT ATG GCT GTC TCT AAA GTT TAC GCT AGA-3′; (2) 5′-AAA GGA TCC TTA TAA TTT GTC ACC GTG GTG GAA GTT-3′.

Primer 1 contains a *Xba*I restriction site (double underlined) and a *Nde*I site (underlined) 5' to the start codon (ATG). Primer 2 has a *Bam*HI site (underlined). The PCR was carried out for 30 cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 2 min. The amplified product and the pGEM-7Zf plasmid were digested with the restriction endonucleases *Xba*I and *Bam*HI and purified electrophoretically in a low-melting-point agarose gel. The fragments were cut out of the gel and ligated directly in the agarose overnight. The product of the ligation reaction was melted and used to transform competent DH5α cells which were plated on LB−ampicillin plates. Colonies were picked and grown in liquid culture. The plasmids were isolated from the resulting cultures and screened for the insertion of the enolase gene by digestion with *Xba*I and *Bam*HI and subsequent agarose gel electrophoretic analysis. The enolase gene was excised from the pGEM-Enolase construct with *Nde*I and *Bam*HI and ligated into similarly digested pET-22b plasmid. The resulting construct, designated pET−ENOL, was sequenced with the Sequenase v.2 Kit (Amersham) to verify absence of spurious mutations in the coding region of enolase and used to transform competent BL21(DE3) cells for expression.

Site-Directed Mutagenesis. The following primers were synthesized for introducing the site specific mutations: K345A, 5′-CCG ATT TGG TTA ACC GCC AAC AAC AAA GC-3′; E168Q, 5′-AGC AAT CAT AAA CTG CTG CAG AGC CAA AGC-3′; E211Q, 5′-AC ACC ACC TTG GTC ACC AAC GTT ACC GG-3′.

The doubly underlined regions indicate the mutagenic codons, while the underlined regions indicate the silently introduced restriction site for selection of mutants. The primers complement the coding strand of the yeast enolase gene. Mutagenesis was carried out by the method of Michael (1994) which employs Taq ligase, a thermostable polymerase, primers 1 and 2, and a mutagenic primer in a PCR reaction that yields a full length gene product with the mutagenic primer incorporated by the ligase. Thirty cycles of 94 °C for 1 min, 58 °C for 1 min, and 65 °C for 4 min were carried out to synthesize the mutant DNA products. The products

were cloned into the pGEM-7Zf vector in the same way as the wild type clone. Mutant clones were selected by digesting with the following restriction enzymes: *Hpa*I for K345A, *Pst*I for E168Q, and *Psp*1406I for E211Q. The entire coding regions of the mutant constructs were sequenced to verify the absence of unwanted mutations. The mutant genes were cloned into the pET-22b vector and expressed and purified in the same manner as the wild type enolase.

Expression and Purification of Recombinant Enolase. Wild type enolase was isolated from bakers' yeast as described previously (Wedekind et al., 1994). Concentrations of enzyme given in units of molar refer to monomer (i.e., active site). Recombinant forms of enolase were obtained from large scale fermentations which were carried out in 2 L Erlenmeyer flasks. Each 1 L of culture medium contained 10 g of tryptone, 5 g of yeast extract, 16 g of lactose, 0.4 g of ampicillin, and M9 salts. Cultures were inoculated with 10 mL/L of a small culture grown on the same medium minus lactose to an OD_{600nm} of ~0.5. The flasks were then shaken at 37 °C for 12−14 h until the OD_{600nm} was 6−7. Cells were then harvested by centrifugation and stored frozen at −20 °C.

Enolase was purified by adaptation of previous procedures (Wedekind et al., 1994). *E. coli* cells were lysed by shaking at 37 °C for 3 h in 100 mL per 10 g of cells of 20 mM Tris/HCl, 2 mM EDTA pH 8.0 containing 0.5 mg/mL lysozyme, 0.1 mg/mL PMSF, and 1 μg/mL DNAse. Cell debris was removed by centrifugation. Solid ammonium sulfate was added to the supernatant to 40% saturation, and the resulting precipitate was removed by centrifugation. The supernatant was brought to 80% saturation with ammonium sulfate, and the precipitate which contained enolase was collected by centrifugation. The resulting pellet was resuspended, desalted, and treated with DEAE cellulose as described previously (Wedekind et al., 1994). A second anion exchange treatment similar to the first was performed on the mutant enzymes to ensure complete removal of *E. coli* enolase because *E. coli* enolase binds tightly to anion exchange resins under these conditions (Spring & Wold, 1971a). The combined filtrate from DEAE cellulose was applied to a 2.5 × 25 cm column of DEAE Sephadex A-50 equilibrated with 20 mM Tris/HCl pH 8.0. The column was washed with the same buffer, and the combined eluates, which contained enolase, were pooled. The eluate was further purified by cation exchange on CM Sephadex, concentrated, and stored according to the procedure of Wedekind et al. (1994). A typical 10 L growth under the conditions described above yielded ~100 g of cell paste from which ~450 mg of purified enolase or variant could be obtained.

Enolase Activity Assays. Mutant forms of enolase were assayed for activity in the conversion of 2-PGA to P-enolpyruvate using a coupled assay with pyruvate kinase and lactate dehydrogenase (mixture from Boehringer Mannheim, desalted before use). Reaction was initiated by adding ~1 mg total enzyme to a cuvette containing 1 mL of Tris/HCl pH 7.8, $I = 0.05$, 2 mM $Mg(OAc)_2$, 45 μM NADH, 0.22 mM ADP, 1.2 units each of pyruvate kinase and lactate dehydrogenase, and between 0.04 and 4 mM 2-PGA. The decrease in absorbance at 340 nm was monitored for 1 h using a Hewlett-Packard diode array spectrophotometer. Initial rates were determined by fitting the linear portion of the progress curve. Kinetic constants were determined by a nonlinear least-squares fit to the data (Cleland, 1979). Recombinant wild type enolase and enolase isolated from bakers' yeast were indistinguishable kinetically.

Chemical Rescue Experiments. Ammonia and *n*-propylamine were tested for an ability to stimulate the catalytic activity of K345A enolase. Coupled assays were performed in 50 mM Ches/KOH buffer pH 9.0, with or without 0.9 M amine (adjusted to pH 9.0 with HCl). Other conditions were the same as described above for enzyme assays.

EPR Spectroscopy. Enolase and variants were exchanged into 50 mM Hepes/KOH pH 7.5 by gel filtration and freed of metal ions using a column of Chelex 100 as described by Poyner and Reed (1992). EPR spectra were recorded at 35 GHz with a Varian E-109Q spectrometer as described previously (Poyner & Reed, 1992). Spectra were subjected to resolution enhancement using the Fourier transform method described by Latwesen et al. (1992).

TSP Binding Assays. Enolase mutants were tested for the ability to bind and ionize TSP by UV difference spectroscopy (Spring & Wold, 1971b). TSP was synthesized enzymatically using the pyruvate kinase catalyzed, ATP dependent phosphorylation of 3-hydroxypyruvic acid (Ash et al., 1984; Weiss et al., 1989). Assays were carried out in a two-chambered cuvette, and spectra were recorded using a Hewlett-Packard diode array spectrophotometer. Each side of the cuvette contained 0.9 mL of 50 mM Hepes/KOH pH 7.7, 0.1 M KCl, and 2 mM $MgCl_2$. The enolase side of the cuvette contained 60 μM enolase, and the TSP side of the cuvette contained 0.1 mM ATP and 0.12 mg/mL pyruvate kinase. TSP formation was initiated by adding 10 μL of 10 mM 3-hydroxypyruvate. The pyruvate kinase reaction was allowed to proceed for 10 min. A blank spectrum was recorded, the two sides of the cuvette were mixed, and the resulting spectrum was recorded at regular intervals for the next 30 min.

2-PGA ^{1}H Exchange Measurements. Enolase mutants were tested for the capacity to catalyze the exchange of the C-2 proton of 2-PGA by ^{1}H NMR spectroscopy in D_2O solutions. Samples contained 23 mM 2-PGA, 2 mM $MgSO_4$, and 2 mM DSS in imidazole/DCl buffer $I = 0.1$, pD 7.8. A reference spectrum was recorded, and the reaction was initiated by addition of an aliquot of concentrated mutant enolase to give a final concentration of 55 μM. Spectra were then recorded at suitable intervals until the signal of the C-2 proton of 2-PGA had disappeared or (in the case of K345A) until significant signals for P-enolpyruvate were detected. All spectra were obtained as the average of 32 scans on a Bruker WP 200 SY spectrometer. The amount of [2-^{1}H]2-PGA remaining in the sample was calculated on the basis of the relative peak heights of the signals of [2-^{1}H]2-PGA and [2-^{2}H]2-PGA.

Synthesis of (Z)-3-Cl-P-enolpyruvate. Synthesis of 3-Cl-P-enolpyruvate using the Perkow reaction between 3,3-dichloropyruvic acid and trimethyl phosphite was reported to yield predominantly the *Z* isomer (Liu et al., 1990). However, ^{1}H to ^{31}P NMR NOE measurements (at 24.3 MHz) on the two isomers indicate that the dominant product of the Perkow reaction is actually the *E* isomer. An alternative synthesis was therefore developed to produce a higher yield of *(Z)*-3-Cl-P-enolpyruvate . This new synthesis relies on the ability of P-enolpyruvate phosphomutase to convert *(R,S)*-3-Cl-3-phosphonopyruvic acid into a mixture of equal

amounts of *(E)*- and *(Z)*- 3-Cl-P-enolpyruvate. P-enolpyruvate phosphomutase (Bowman et al., 1990) was a generous gift from Dr. Debra Dunaway-Mariano, University of Maryland.

(R,S)- 3-Cl-3-phosphonopyruvic acid was synthesized in two steps from 3-bromopyruvic acid as follows: 1 g of 3-bromopyruvic acid was dissolved in 10 mL of dry dioxane in a dry round bottom flask to which 2.5 mL of tris-(trimethylsilyl) phosphite was then added. The flask was sealed, and the reaction continued overnight at 25 °C. The contents of the flask were then diluted to 100 mL with water, and the pH was adjusted to 8 with Tris base. The product was then applied to a 1.5 × 25 cm column of Bio Rad AG-MP-1 anion exchange resin, 200−400 mesh, HCO_3^- form. The column was eluted at 2 mL/min with a 2 L linear gradient from 0.25 to 0.38 M triethylammonium bicarbonate, pH 7.5. Fractions containing 3-phosphonopyruvate were identified by absorbance at 254 nm and by enzymatic assay with P-enolpyruvate mutase. These fractions were pooled and dried by rotary evaporation. The dried product was then redissolved in methanol and redried twice to remove traces of triethylammonium bicarbonate. The triethylammonium salt of 3-phosphonopyruvate was converted to the free acid by passage over a small column of Dowex 50W X8, H^+ form. The acid was stored as a frozen aqueous solution at −20 °C until use. 3-Phosphonopyruvic acid was chlorinated using *N*-chlorosuccinimide. *N*-Chlorosuccinimide (0.24 g) was added to 5 mL of a 0.26 M solution of 3-phosphonopyruvic acid. This mixture was shaken in a sealed tube until all of the *N*-chlorosuccinimide had dissolved (∼40 min). The reaction mixture was then diluted to 50 mL with water and adjusted to pH 7.5 with Tris base, and 2 M $MgSO_4$ was added to give a final concentration of 2 mM. One hundred units of P-enolpyruvate mutase was then added, and the mixture was left to react overnight. The P-enolpyruvate mutase reaction mixture was diluted to 0.25 L with water and applied to the regenerated AG-MP-1 column and eluted at 2 mL/min with a 2 L linear gradient from 0 to 0.5 M triethylammonium bicarbonate pH 7.5. Two major peaks of material absorbing at 254 nm of roughly equal area emerged corresponding to the *E* and *Z* isomers of 3-Cl-P-enolpyruvate. The peak fractions were pooled, dried, and freed of triethylammonium bicarbonate as described above. The compounds were then dissolved in water, and the solution was adjusted to pH 7.5 with Tris base prior to storage at −20 °C. *(Z)*-3-Cl-P-enolpyruvate was further purified using a Pharmacia Mono-Q HR 10/10 column to remove trace contaminants. The neutralized solution of the *Z* isomer was diluted to 10 mL with water and applied to the Mono-Q column. The column was eluted at 2 mL/min with a 1 L linear gradient from 0 to 0.25 M triethylammonium bicarbonate. Column fractions were tested for purity by HPLC (see below) prior to pooling. Fractions which were >90% pure were pooled, dried, freed of triethylammonium bicarbonate, and neutralized as before. The neutralized *(Z)*-3-Cl-P-enolpyruvate was then converted to the K^+ salt by chromatography over a 1 × 15 cm column of SP Sephadex S-25, K^+ form. Following this treatment, the pH was adjusted to 7.5 with HCl, and the compound was concentrated by rotary evaporation. The ^1H NMR spectrum of the compound consisted of a doublet at ∼6.8 ppm (J_{H-P} ∼ 4 Hz), in agreement with the values reported for the minor product of the Perkow synthesis (Liu et al., 1990).

Enzymatic Assays of Pyruvate Derivatives. The concentrations of stock solutions of 3-Cl-P-enolpyruvate, 3-phosphonopyruvate, and 3-Cl-3-phosphonopyruvate were determined by enzymatic end point assays. In a typical assay an aliquot of the solution to be quantitated was added to a cuvette containing 0.2 mM NADH, 1 mM ADP, 0.1 M KCl, 10 mM $MgCl_2$, and 50 mM Hepes/KOH pH 7.0. The absorbance of this mixture at 340 nm was noted, and the decrease was recorded following addition of separate aliquots of 3−30 units of each of the following enzymes: lactate dehydrogenase, pyruvate kinase, and P-enolpyruvate mutase. (The higher amounts of enzyme were used with chlorinated compounds.) Addition of the enzymes separately allows measurement of up to three components of the sample (e.g., lactate dehydrogenase measures pyruvate and chloropyruvate, pyruvate kinase measures P-enolpyruvate and 3-Cl-P-enolpyruvate, and P-enolpyruvate mutase measures 3-phosphonopyruvate and 3-Cl-3-phosphonopyruvate).

Assay of Enolase-Catalyzed Hydrolysis of (Z)-3-Cl-P-enolpyruvate. The rate of consumption of *(Z)*-3-Cl-P-enolpyruvate by wild type enolase and mutant forms was measured using a discontinuous HPLC assay under single-turnover conditions. Samples contained 0.44 mM enzyme, 0.41 mM *(Z)*-3-Cl-P-enolpyruvate , 0.41 mM benzamide (internal standard), 17 mM Hepes/KOH pH 7.7, and 1.7 mM $Mg(OAc)_2$. Aliquots of 10 μL were removed at various time points and quenched by addition to 40 μL of 5 mM K^+ EDTA pH 8. Protein was removed by filtration using a miniature centrifugal device (Millipore Ultrafree-MC). The filtrates were analyzed on a 45 × 250 mm Rainin Microsorb-MV C18 reverse phase column eluted isocratically at 1 mL/min with 15% acetonitrile, 9 mM tetra-*n*-butylammonium dihydrogen phosphate, and 48 mM KH_2PO_4. Detection was at 226 nm using a LKB Uvicord-S detector, and the signal was integrated using a Hewlett Packard integrator. The amount of *(Z)*-3-Cl-P-enolpyruvate remaining in the sample was calculated on the basis of the ratio of the area of the *(Z)*-3-Cl-P-enolpyruvate peak to the area of the benzamide peak.

RESULTS AND DISCUSSION

Activity Assays. All three of the mutant forms of enolase showed ∼10^5-fold reduction in k_{cat} and small changes in K_m compared to wild type enolase (Table 1). Differences in K_m among the enzymes (assayed in parallel with the same stock solutions) and between the mutants and wild type *E. coli* enzyme[2] indicate that the measured activity is not due to trace contamination by the latter enzyme. The severely depressed activities for all of the mutant proteins indicate that all three of the residues mutated are essential for catalysis. Activities in the overall reaction, however, provide little insight into the individual roles of each residue. Hence, the capacities of the enzymes to catalyze the $^1H/^2H$ exchange at C-2 of 2-PGA, the ionization of TSP, and the hydrolysis of *(Z)*-3-Cl-P-enolpyruvate were examined to assay the first and second steps in the catalytic cycle.

2-PGA $^1H/^2H$ Exchange Reaction. One measure of the viability of the catalytic base of enolase is the capacity of the enzyme to ionize the C-2 proton of 2-PGA to generate

[2] The K_m of *E. coli* enolase is reported to be indistinguishable from that of yeast enolase (Spring & Wold, 1971a).

Table 1: Kinetic Constants of Yeast Enolase and Variants

	2-PGA dehydration		$^1H/^2H$ exchange of 2-PGA	hydration of *(Z)*-3-Cl-P-enolpyruvate
	K_m (mM)	k_{cat} (s^{-1})	k_{ex}/k_{cat}[a]	k_{cat} (s^{-1})
wild type	0.30 ± 0.06	78 ± 4	0.23 ± 0.006[b]	>0.05
K345A	0.68 ± 0.1	$(6.3 \pm 0.4) \times 10^{-4}$	<0.5	$(8.8 \pm 0.7) \times 10^{-4}$
E211Q	0.12 ± 0.02	$(6.6 \pm 0.3) \times 10^{-4}$	$(2.0 \pm 0.1) \times 10^{3}$	$(8.8 \pm 0.7) \times 10^{-6}$
E168Q	0.11 ± 0.04	$(4.0 \pm 0.4) \times 10^{-4}$	$(2.5 \pm 0.2) \times 10^{3}$	$(2.3 \pm 0.3) \times 10^{-4}$

[a] In this quotient, k_{cat} is for the dehydration of 2-PGA. For wild type and K345A enolase, $k_{ex}/k_{cat} < 1$ indicates a substantial forward commitment (Anderson et al., 1994). [b] Value from Anderson et al. (1994).

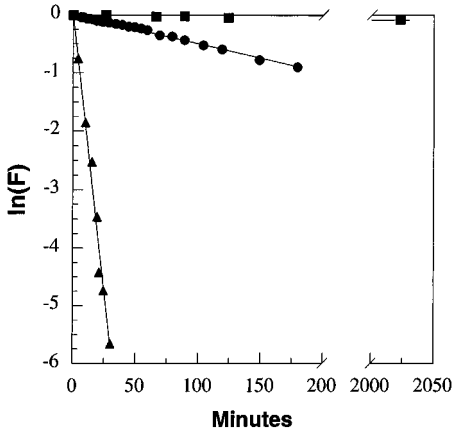


FIGURE 2: Time courses of the exchange of the C-2 proton of 2-PGA with D_2O catalyzed by mutant forms of enolase. *F* is the fraction of [2-^{1}H]2-PGA remaining: (▲) E211Q; (●) E168Q; (■) K435A. Samples contained 55 μM enolase, 23 mM 2-PGA, 2 mM $MgSO_4$, and 2 mM DSS in imidazole/DCl buffer $I = 0.1$, pD 7.8; $T = 20$ °C.

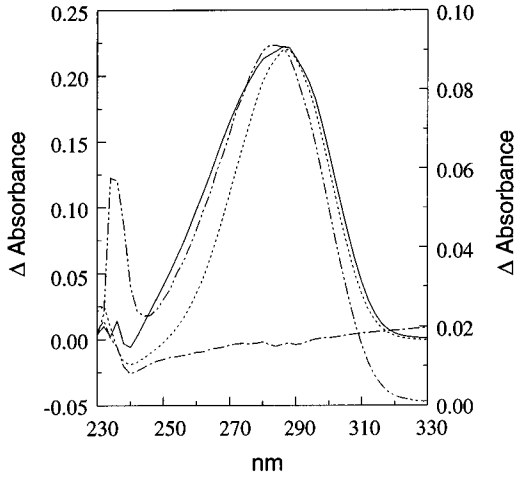


FIGURE 3: Left ordinate applies to UV difference spectra of complexes of enolase variants with TSP: (—) wild type; (···) E211Q; (—·—) K435A. Samples contained 30 μM enolase or variant, ~25 μM TSP, 50 mM Hepes/KOH, pH 7.7, 0.1 M KCl, and 2 mM $MgCl_2$, plus 60 μg/mL pyruvate kinase and 0.1 mM ATP/ADP from the enzymatic synthesis of TSP. Right ordinate applies to the UV difference spectrum of wild type enolase and *(Z)*-3-Cl-P-enolpyruvate after 5 h of incubation(—··—). The reaction was carried out in a two-chambered cuvette as described for the TSP difference spectra. The solution composition was 18 μM *(Z)*-3-Cl-P-enolpyruvate, 14 μM enolase, 2 mM $MgSO_4$, and 5 mM Hepes/KOH pH 7.7.

the intermediate. This partial reaction can be assayed as an exchange of the C-2 proton with solvent D_2O (Stubbe & Abeles, 1980). The time courses of exchange reactions catalyzed by the three mutant forms of enolase are shown in Figure 2, and corresponding first-order rate constants for the exchange are given in Table 1. As illustrated by the data of Figure 2, K345A enolase exhibits virtually no activity in this exchange reaction. Exchange of the C-2 proton of 2-PGA in the assay with K345A enolase was only detectable after a measurable amount of P-enolpyruvate had formed, making it difficult to distinguish exchange from loss of the proton due to the reversal of the overall reaction. The other two mutant proteins, E211Q and E168Q, showed nearly complete exchange before significant amounts of P-enolpyruvate appeared. Furthermore, the capacity of the latter two mutants to carry out exchange before appreciable product formation supports the prevailing view (Anderson et al., 1984; Anderson et al., 1994) that the reaction is indeed stepwise.

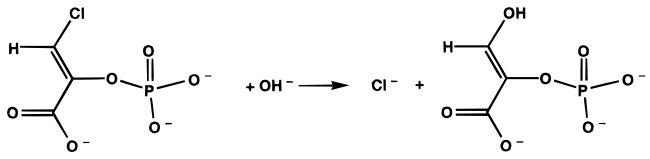
TSP Ionization. A second way to measure the competency of the catalytic base of enolase is through the ability of the respective proteins to ionize bound TSP. Binding of TSP to wild type enolase results in a characteristic increase in the UV absorbance at ~288 nm. This increase in absorbance has been attributed to the ionization (or tautomerization) of TSP (Spring & Wold, 1971b). Thus, mutant forms of enolase which retain the catalytic base should produce this increase in absorbance while forms which lack the catalytic base should not. Brewer et al. (1993) have demonstrated the appearance of this characteristic absorbance change upon binding of TSP to E168Q enolase. A similar spectrum (Figure 3) is generated when TSP binds to E211Q enolase, and the difference spectrum is fully developed within the mixing time, ~15 s. Sangadala et al. (1995) report[3] a similar difference spectrum upon binding of TSP to E211Q enolase. In contrast to the positive difference spectra elaborated upon binding of TSP to E211Q and E168Q enolase, a slight decrease in absorbance occurs upon mixing of TSP with K345A enolase (Figure 3).

Hydrolysis of (Z)-3-Cl-P-enolpyruvate. Stubbe and Kenyon (1972) reported an enolase-catalyzed degradation of *(Z)*-3-F-P-enolpyruvate. The product of the reaction was a potent, reversible inhibitor of the enzyme. Although the identity of the product of the reaction of *(Z)*-3-F-P-enolpyruvate with enolase was not reported (Stubbe & Kenyon, 1972), the predicted product of the reaction of enolase with 3-halo-P-enolpyruvate is 3-halo-2-phosphoglycerate, which should eliminate HX to give the enol of TSP. The reaction is analogous to that of the reverse reaction of enolase wherein OH^- adds to C-3 of P-enolpyruvate. These earlier experiments suggested that enolase might also catalyze hydrolysis of *(Z)*-3-Cl-P-enolpyruvate (Scheme 1).

When wild type enolase is mixed with an equivalent amount of *(Z)*-3-Cl-P-enolpyruvate, an initial increase in UV absorbance at 250 nm occurs that is likely due to the enol

[3] A shoulder at 282 nm develops over a longer time period following binding of TSP to E211Q enolase. Sangadala et al. (1995) attribute this slower spectral change to the ionization of TSP.

Scheme 1



of TSP. Upon standing, this spectrum changes to match closely that seen upon binding of TSP to the enzyme (Figure 3). HPLC assays show that the single-turnover reaction with wild type enolase is complete in less than 30 s. Multiple-turnover measurements were not attempted since potent product inhibition by TSP is expected to occur.

Mutant forms of enolase which lack the ability to activate a water molecule for attack at C-3 should show reduced activity in hydrolysis of *(Z)*-3-Cl-P-enolpyruvate. The course of degradation of *(Z)*-3-Cl-P-enolpyruvate catalyzed by mutant forms of enolase which can activate water but lack the catalytic base could also differ from the reaction catalyzed by wild type enzyme. Attack of an activated water at C-3 could still occur in the absence of the base. Such a reaction could result in direct displacement of Cl^-, or solvent could donate a proton to the intermediate to complete the reaction. Progress curves for degradation of *(Z)*-3-Cl-P-enolpyruvate by mutant forms of enolase are shown in Figure 4, and the corresponding rate constants are included in Table 1.

In order to determine whether or not the decomposition of *(Z)*-3-Cl-P-enolpyruvate led to covalent modification of the enzyme, a sample containing K345A enolase and *(Z)*-3-Cl-P-enolpyruvate was incubated for a period long enough to ensure complete degradation of the substrate. The sample was then quenched with EDTA (to allow dissociation of Mg^{2+} and products). Subsequently the sample was subjected to three cycles of dilution with buffer (50 mM Hepes/KOH pH 7.5) and concentration via ultrafiltration. Enzyme recycled in this fashion retained activity in the dehydration of 2-PGA, indicating that *(Z)*-3-Cl-P-enolpyruvate does not covalently inactivate the enzyme.

As indicated by the data of Figure 4 and Table 1, none of the three mutant forms of enolase catalyze hydrolysis of *(Z)*-3-Cl-P-enolpyruvate as effectively as the wild type enzyme. However, K345A and E168Q proteins both catalyzed breakdown of *(Z)*-3-Cl-P-enolpyruvate at appreciable rates in single-turnover assays. In contrast, hydrolysis of *(Z)*-3-Cl-P-enolpyruvate catalyzed by E211Q enolase was much slower than that by either of the other variants. The unique behavior of E211Q in this reaction implicates the carboxylate of Glu 211 in activation water in the reverse reaction and the conjugate acid in activation of the C-3-OH of 2-PGA in the forward reaction.

Attempts at Chemical Rescue of K345A Enolase. There is precedent for chemical rescue, by exogenous amines, of function in enzymes or other proteins in which a critical lysine has been mutated (Toney & Kirsch, 1989; Zhukovsky et al., 1991; Sekimoto et al., 1993; Harpel & Hartman, 1994). The K345A mutation was chosen so that chemical rescue could be attempted. This type of chemical rescue of K345A enolase by exogenous amines could not be demonstrated. When either ammonia or propylamine was added to the assay mixture the rate of reaction decreased slightly. The slight inhibition might be due to the high concentrations of the ammonium forms of the amines which may interfere with

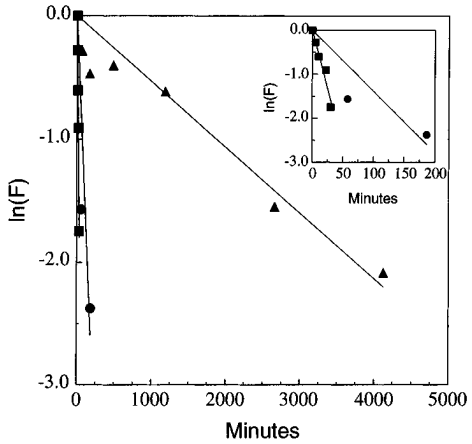


FIGURE 4: Time courses of the breakdown of *(Z)*-3-Cl-P-enolpyruvate catalyzed by mutant forms of enolase. *F* is the fraction of *(Z)*-3-Cl-P-enolpyruvate remaining: (▲) E211Q; (●) E168Q; (■) K345A. Samples contained 0.44 mM enzyme, 0.41 mM *(Z)*-3-Cl-P-enolpyruvate, 0.41 mM benzamide (internal standard), 17 mM Hepes/KOH pH 7.7, and 1.7 mM $Mg(OAc)_2$.

normal divalent cation binding. In light of the other experiments with K345A enolase, however, the negative findings in the rescue assays are inconclusive.

Folding. Problems with proper folding of the mutant enzymes are not expected because the side chains of the residues mutated are not part of the core of the protein and are not directly involved in domain or subunit interfaces. Glu 168, the first residue of β-strand 2 of the $\alpha\beta$-barrel, is the only one of the residues mutated that is part of regular secondary structure. Glu 211 and Lys 345 are located on loops between secondary structural elements. Hence, it seems unlikely that the global structures of the mutant proteins are significantly perturbed, and several lines of evidence support the belief that the mutant proteins are correctly folded.

The mutant enzymes behave similarly to the wild type enzyme in the purification procedure—an observation which would not be expected of improperly folded proteins. Furthermore, E168Q and E211Q proteins bind TSP as evidenced by UV difference spectroscopy. Each mutant catalyzes either the exchange of the C-2 proton of 2-PGA or the displacement of Cl^- from *(Z)*-3-Cl-P-enolpyruvate. Brewer and co-workers (Brewer et al., 1993; Sangadala et al., 1995) report other physical data supporting the contention that E168Q and E211Q enolases are properly folded. Finally, EPR spectra of Mn^{2+} complexes with the mutant proteins show that all three of the mutants retain the ability to bind metal ions. Spectra of the Mn^{2+}/Zn^{2+} hybrid metal complexes of wild type enolase with the equilibrium mixture and K345A enolase with P-enolpyruvate or the equilibrium mixture are shown in Figure 5. In these Mn^{2+}/Zn^{2+} hybrid complexes, Mn^{2+} binds predominately at the lower affinity site (site II) (Poyner, 1993). Sharp signals for the Mn^{2+} complex of K345A enolase indicate that site II, which comprises two ligands from the substrate and two ligands from Ser 39 in the active site loop (Wedekind et al., 1994), is competent in the mutant protein. The depressed activity of K345A enolase also permits observation of the approach to equilibrium by EPR spectroscopy. Thus, the EPR spectrum changes over a period of several hours as the reaction comes to equilibrium. For K345A enolase, the internal equilibrium constant may differ from unity—thus

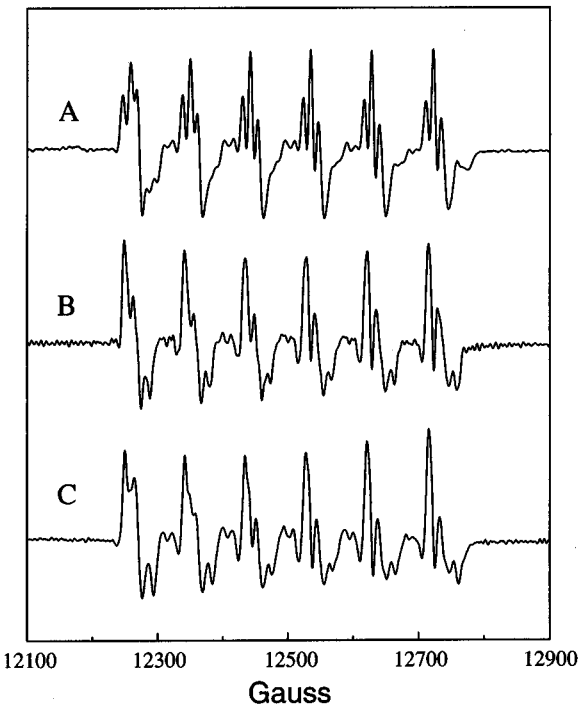
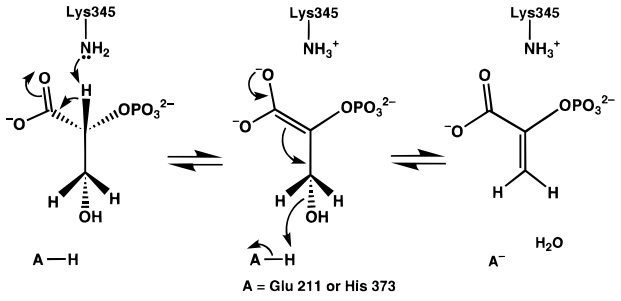


FIGURE 5: Comparison of EPR spectra obtained from hybrid complexes of Zn^{2+}, Mn^{2+}, and substrates with either wild type or K345A enolase. (A) Wild type enolase complexed with an equilibrium mixture of P-enolpyruvate and 2-PGA: 6.4 mM enolase, 6.4 mM $Zn(OAc)_2$, 1.7 mM $MnCl_2$, 10 mM P-enolpyruvate (initial value), and 50 mM Hepes/KOH pH 7.5. (B) K345A enolase complexed with P-enolpyruvate: 5.4 mM K345A enolase, 5.1 mM $Zn(OAc)_2$, 1.0 mM $MnCl_2$, 12 mM P-enolpyruvate (initial value), and 50 mM Hepes/KOH pH 7.5; spectrum recorded within 10 min of addition of P-enolypruvate. (C) Spectrum of B after 48 h of incubation at 22 °C to allow equilibrium to be reached. The change in the spectrum from B to C reflects the slow conversion of P-enolpyruvate to an equilibrium mixture of P-enolpyruvate and 2-PGA. Spectra were acquired from liquid samples at ~2 °C.

Scheme 2



explaining the differences between spectra for the two forms of the enzyme.

Conclusions. Results of the $^1H/^2H$ exchange experiments with 2-PGA clearly implicate Lys 345 as the catalytic base (Scheme 2). Mutations of Glu 211 and Glu 168 to Gln do not disable exchange of the C-2 proton of 2-PGA, whereas the Lys to Ala mutation at 345 virtually eliminates this exchange. Results of the experiments with TSP also support the contention that Lys 345 is the catalytic base in the first step of the forward reaction. The ability of E211Q and E168Q to generate the increase in the absorbance of TSP solutions at 288 nm, which is characteristic of ionization of the inhibitor, also shows that these enzymes contain a base capable of abstracting the C-2 proton of TSP. On the other hand, the inability of K345A to produce this absorbance change suggests that K345A enolase lacks the base.

The capacity of mutant forms of enolase to catalyze breakdown of *(Z)*-3-Cl-P-enolpyruvate should depend primarily on the ability of the enzyme (conjugate base of an active site acid) to activate a water molecule for attack. The subsequent course of the reaction of *(Z)*-3-Cl-P-enolpyruvate most likely involves protonation of the resulting intermediate by the catalytic base, and this part of the reaction would be altered in the reaction catalyzed by K345A enolase. This change in the protonation step may explain the slower rate, relative to wild type enolase, of the reaction catalyzed by K345A enolase. Mutation of Glu 211 to Gln has, by far, the largest effect on the rate of breakdown of *(Z)*-3-Cl-P-enolpyruvate. Because the E211Q enzyme retains the catalytic base, Lys 345, it should be able to protonate the intermediate formed by attack of water at C-3 of *(Z)*-3-Cl-P-enolpyruvate. Thus, the slow rate of breakdown of *(Z)*-3-Cl-P-enolpyruvate catalyzed by E211Q enolase is most likely due to a deficit in activation of the substrate water molecule for attack at C-3. A water molecule within hydrogen-bonding distance of the γ-carboxyl group of Glu 211 is prominent in several of the available crystal structures of enolase (Lebioda & Stec, 1991; Wedekind et al., 1994). For example, in the structure of the enolase complex with PhAH (Wedekind et al., 1994), a water molecule is within 3 Å of the carboxylates of Glu 211 and Glu 168 and 2.9 Å from the ϵ-nitrogen of His 373. Two explanations remain for the low activity of E211Q enolase in the breakdown of *(Z)*-3-Cl-P-enolpyruvate. One possibility is that Glu 211 is the primary acid/base catalyst which removes the proton from the water in the reverse reaction. The other possibility is that His 373 is the primary acid/base catalyst and that the E211Q mutation perturbs the charge or structure in this region such that the acid/base catalysis is disabled. Both explanations represent departures from previous suggestions that the metal ion at site I coordinates and activates the attacking water molecule in the reverse reaction and that coordination to the metal ion enhances the leaving ability of the hydroxyl in the forward direction (Nowak et al., 1973; Lebioda & Stec, 1991; Poyner & Reed, 1992; Wedekind et al., 1994).

Results of these partial reactions clearly implicate Lys 345 and Glu 211 in the first and second steps of the enolase reaction, respectively. The proposed roles of these residues account for the dramatic losses in overall activity resulting from the corresponding mutations. The catalytic role of Glu 168, however, remains somewhat enigmatic. E168Q enolase functions with intermediate proficiency in the $^1H/^2H$ exchange reaction of 2-PGA, and it is almost as effective as the K345A protein in catalysis of the breakdown of *(Z)*-3-Cl-P-enolpyruvate. E168Q enolase is, however, compromised to virtually the same extent in the overall reaction as the K345A and E211Q proteins. At this point, one can surmise that Glu 168 contributes critically to both steps of the reaction.

The previously proposed roles of Glu 211 and Glu 168 in the charge shuttle mechanism of general base catalysis (Lebioda & Stec, 1991; Cleland, 1992; Brewer et al., 1993) are incompatible with the facile exchange of the C-2 proton of 2-PGA catalyzed by E211Q enolase. In contrast, the properties of K345A enolase in the reactions studied here clearly implicate Lys 345 as the catalytic base. These results support the contentions of Wedekind et al. (1994) that the hydroxamate moiety of PhAH mimics the carboxylate group

of the substrate and that the productive orientation of the hydroxymethyl and carboxylate groups of 2-PGA is opposite to that selected for mechanistic discussions by Lebioda & Stec (1991). The present results and conclusions are also fully compatible with the stereochemical requirements of the *anti* β-elimination reaction (Cohn et al., 1970) in that residues apparently functioning as acid/base catalysts in the first and second steps of the reaction lie on opposite surfaces of the active site (Figure 1).

While this paper was under review, a third proposal for the catalytic base, His 159 (yeast enolase numbering system), for lobster enolase appeared (Duquerroy et al., 1995). This proposal was based on crystallographic data obtained by soaking the inhibitor, 2-phosphoglycolate, into crystals of the lobster enzyme in 2.5−2.7 M $(NH_4)_2SO_4$. Only one of the essential divalent metal ions was present in the active site. The structure of the bis Mg^{2+} complex of enolase and PhAH at 2.1 Å resolution (Wedekind et al., 1994) shows that the side chain of His 159 interacts with an oxygen from the phosphonate moiety of the inhibitor. A structure of the bis Mg^{2+} complex of yeast enolase with the equilibrium mixture of substrate and product at 1.8 Å resolution (T. M. Larsen, J. E. Wedekind, I. Rayment, and G. H. Reed, manuscript in preparation) shows that the side chain of His 159 interacts with a peripheral oxygen of the phosphate group of the substrate/product and that this side chain is also on the wrong side of the substrate to function as the base.

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