Identification of Active Site Residues of Chorismate Mutase−Prephenate Dehydrogenase from *Escherichia coli*†

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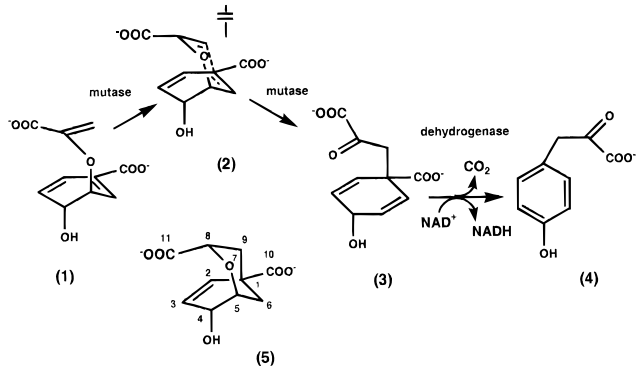
ABSTRACT: Chemical modification studies of the bifunctional enzyme chorismate mutase−prephenate dehydrogenase and mass spectral analysis of peptide fragments containing modified residues are presented. The reaction with diethyl pyrocarbonate (DEPC) results in the modification of several enzymic groups, including a single histidine group essential for dehydrogenase activity and a single lysine residue essential for mutase activity. This conclusion is based on the following evidence. (1) Hydroxylamine rapidly restores dehydrogenase activity to the DEPC-inactivated enzyme without restoring mutase activity. (2) Mutase activity is also lost upon treatment of the enzyme with trinitrobenzene sulfonate. (3) The reactivity of the dehydrogenase to DEPC increases with pH, suggesting the participation of a group with a pK_a of 7.0 in the dehydrogenase reaction. (4) Two peptides identified by differential peptide mapping had mass values matching those calculated for peptides comprising residues 127−135 (containing His131) and residues 36−48 (containing Lys37). In support of the idea that the residues being modified are within the active sites, we show that the substrates prephenate and nicotinamide adenine dinucleotide (NAD^+) offer protection against inactivation of dehydrogenase activity while inactivation of mutase activity can be prevented by prephenate and a transition state analogue (3-*endo*-8-*exo*)-8-hydroxy-2-oxabicyclo[3.3.1]-non-6-ene-3,5-dicarboxylic acid (*endo*-oxabicyclic diacid). Lys37 is conserved among several chorismate mutases and may participate in catalysis by interacting with an ether oxygen between C-5 and C-8 of chorismate in the transition state. His131 may be assisting in a hydride transfer from prephenate to NAD^+ in the dehydrogenase reaction.

Chorismate mutase−prephenate dehydrogenase (EC 5.4.99.5 and EC 1.3.1.12; 4-hydroxyphenylpyruvate synthase) is a bifunctional enzyme that catalyzes two sequential reactions in the biosynthesis of L-tyrosine in *Escherichia coli* and other enteric bacteria (Cotton & Gibson, 1965; Koch et al., 1971). Chorismate mutase catalyzes the Claisen rearrangement of chorismate (1) to prephenate (3), which is believed to proceed via a chairlike transition state (2) (Andrews et al., 1973; Sogo et al., 1984). Prephenate dehydrogenase is responsible for the oxidative decarboxylation of prephenate to (4-hydroxyphenyl)pyruvate (4) in the presence of NAD^+ [1] (Scheme 1). The enzyme from *E. coli* is homodimeric with a molecular weight of 78 000 to 88 000 (SampathKumar & Morrison, 1982a; Hudson et al., 1984; Turnbull et al., 1990). Both activities are associated with each subunit and are allosterically inhibited by the end product of the pathway, L-tyrosine (Christopherson, 1985; Hudson et al., 1983; Turnbull et al., 1991b).

The mutase activity of chorismate mutase−prephenate dehydrogenase is very similar to that of chorismate mutase−prephenate dehydratase (a bifunctional enzyme involved in the conversion of chorismate to phenylalanine), and the N-terminal portions of their amino acid sequences are highly homologous (Hudson & Davidson, 1984; Hudson et al., 1984). In contrast, two other well-studied chorismate mutases in *Saccharomyces cerevisiae* (Schmidheini et al., 1990) and *Bacillus subtilis* (Gray et al., 1990a,b) bear little sequence homology to each other or to the *E. coli* enzymes. Crystal structures are now available for these two mutases and for the chorismate mutase domain of the mutase−dehydratase ("mini-mutase") (Chook et al., 1993; Xue et al., 1994; Lee et al., 1995a), and these confirm that there is no resemblance in secondary or tertiary structure. However, the active sites of the mutase from *B. subtilis* and *E. coli* are electrostatically similar (Lee et al., 1995a,b).

Mechanistic studies on the mutase from *B. subtilis* indicate that this enzyme catalyzes the rearrangement by a simple

Scheme 1: Reactions Catalyzed by Chorismate Mutase−Prephenate Dehydrogenase



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[1] Abbreviations: CD, circular dichroism; DEPC, diethyl pyrocarbonate; *endo*-oxabicyclic diacid, (3-*endo*-8-*exo*)-8-hydroxy-2-oxabicyclo-[3.3.1]non-6-ene-3,5-dicarboxylic acid; MES, 2-(*N*-morpholino)-ethanesulfonic acid; mu, mass unit; NAD^+, nicotinamide adenine dinucleotide; TFA, trifluoroacetic acid; TNBS, trinitrobenzene sulfonate; HPLC, high-performance liquid chromatography.

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pericyclic process similar to the uncatalyzed reaction, where the chemistry is an inherent property of chorismate (Gray & Knowles, 1994; Gray et al., 1990b; Rajogopalan et al., 1993). By contrast, the results from pH profiles (Turnbull et al., 1991a) and isotope effects (Addadi et al., 1983; Guilford et al., 1987) together suggest that the bifunctional *E. coli* mutase−dehydrogenase has enzyme groups which act as acid or base catalysts. The structure of the mini-mutase complexed with the *endo*-oxabicyclic diacid (5), which mimics the bicyclic structure of the transition state, has provided valuable insight as to the amino acid residues that may participate in the rearrangement (Lee et al., 1995a). These groups include Lys39, Gln88, Asp48, Glu52, Ser84, and arginine residues at positions 11, 28, and 51.

The kinetic mechanism of the prephenate dehydrogenase reaction conforms to a rapid equilibrium random mechanism with catalysis as the rate-limiting step (SampathKumar & Morrison, 1982b). Studies of the pH dependence of the kinetic parameters *V* and *V*/*K* for the dehydrogenase reaction have determined that ionizable groups are involved in substrate binding and catalysis (Hermes et al., 1984; Turnbull et al., 1991a). These studies have shown that a deprotonated group is required for catalysis while a protonated group is required for substrate binding. The results of temperature and solvent perturbation studies by Hermes et al. (1984) implied that the catalytic group is a cationic acid, possibly a histidine residue. These authors hypothesized that the histidine could accept a hydrogen bond from the 4-hydroxyl group of prephenate to facilitate hydride transfer and the concomitant decarboxylation to yield (4-hydroxyphenyl)-pyruvate (Hermes et al., 1984).

Since the product of the first reaction is a substrate for the second reaction, many studies have centered on the spatial relationship between the sites at which the two reactions occur. Evidence in favor of a single combining site comes from several studies on the mutase−dehydrogenase from *E. coli* and *A. aerogenes*. Subjecting the enzyme to heat, urea, limited proteolysis (Heyde, 1979), and cysteine-modifying agents (Turnbull & Morrison, 1990; Hudson et al., 1984) has led to a coordinate loss of both activities. Moreover, inactivation of both activities by alkylation can be prevented by prephenate, NAD^+, and tyrosine plus NAD^+ (Turnbull & Morrison, 1990; Hudson et al., 1984). Kinetic data obtained by Heyde and Morrison (1978) and the results of investigations of the inhibition of mutase and dehydrogenase activities of the enzyme with compounds that are clearly analogues of either chorismate or prephenate (Christopherson et al., 1983) tended to indicate a common binding site or two overlapping sites. There is evidence that some of the prephenate formed from chorismate is converted directly to (4-hydroxyphenyl)pyruvate (Heyde, 1979). However, these results are also consistent with two catalytic sites in close proximity. The results differ from those obtained for the mutase−dehydratase, for which there are clearly two distinct active sites (Duggleby et al., 1978; Stewart et al., 1990).

Recently, there has been more direct evidence in favor of two distinct active sites for the *E. coli* mutase−dehydrogenase. The two activities show different pH rate profiles (Turnbull et al., 1991a) and are inhibited to different degrees by tyrosine (Turnbull et al., 1991b), malonic acid derivatives, and other substrate analogues (Turnbull & Morrison., 1990). The putative transition state analogue *endo*-oxabicyclic diacid is a very selective inhibitor of the mutase (Turnbull & Morrison, 1990). In addition, sequence homology with other bifunctional chorismate mutases suggests that the mutase activity is located on the N-terminal third of the protein (Hudson & Davidson, 1984). This has been confirmed by Maruya et al. (1987), who constructed separate plasmids encoding the hypothetical mutase and dehydrogenase domains and noted that under certain growth conditions the plasmids were able to complement the defect in a host strain which had an inactivated mutase−dehydrogenase.

In the work presented here, our goal was to identify amino acids important for catalysis of each reaction. We have used a combination of chemical modification and mass spectrometry of peptides from proteolytic digests to identify two residues, one of which is important for each activity. Protection studies support the hypothesis that these groups are at distinct active sites.

EXPERIMENTAL PROCEDURES

Materials. Chorismate was isolated from *Klebsiella pneumonia* strain 62-1 as described by Gibson (1968) with modifications that significantly improved the yield of chorismate from this organism (Rieger & Turnbull, 1996). Prephenate was prepared as previously described (Dudzinski & Morrison, 1976). NAD^+ was obtained in free acid form from Boehringer Mannheim. DEPC was obtained from ICN Biochemicals, while TNBS as a 2% solution in water was from Aldrich. HPLC-grade TFA and aceotnitrile were from Baker. Hydroxylamine was obtained from Fisher, while trypsin and *Staphylococcus aureus (S. aureus)* V8 protease were both from Sigma. (3-*endo*-8-*exo*)-8-hydroxy-2-oxabicyclo[3.3.1]non-6-ene-3,5-dicarboxylic acid bis(dicyclohexylammonium) salt (*endo*-oxobicyclic diacid) was a generous gift from Professor Paul Bartlett of the University of California, Berkeley. All other chemicals were obtained commercially and were of the highest quality available.

Source of Chorismate Mutase−Prephenate Dehydrogenase. Chorismate mutase−prephenate dehydrogenase was obtained from an overproducing strain of *E. coli* (JFM30) that carries a multicopy plasmid (Bhosale et al., 1982). Cells were suspended in a buffered solution and then disrupted by repeated freeze−thawing followed by treatment with lysozyme (Cull & Millard, 1990). The enzyme was purified to homogeneity by a procedure that involves affinity chromatography on Matrex Blue A and Sepharose AMP (Turnbull et al., 1990). Subsequent chromatography on DEAE-Sepharose removed all traces of contaminating chorismate mutase−prephenate dehydratase. The enzyme preparation was over 95% pure as judged by silver staining of a denaturing polyacrylamide gel.

Determination of Enzyme Activity and Protein Concentration. Kinetic investigations on the mutase−dehydrogenase were performed at 30 °C in the presence of the three-component buffer system (Ellis & Morrison, 1982) of 0.10 M MES, 0.051 M *N*-ethylmorpholine, 0.051 M diethanolamine (pH 7.2), 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM dithiothreitol (DTT). The total volume of reaction was 1.0 mL. Prephenate dehydrogenase activity was measured by monitoring at 340 nm the formation of NADH from NAD^+ in the presence of prephenate, while chorismate mutase activity was determined following the disappearance of chorismate at 274 nm (Turnbull et al., 1990). Saturating amounts of the substrates prephenate (0.5

mM), NAD^+ (2 mM), and chorismate (0.5 mM) were used. A double beam spectrophotometer (GBC model 918) fitted with thermostatically controlled cuvette holders was used. Protein concentration was estimated using the Bio-Rad protein assay kit with bovine serum albumin as a standard and by recording the absorbance at 205 nm (Scopes, 1994). The results by the two methods were in good agreement.

Inactivation Kinetics of Mutase−Dehydrogenase with Diethyl Pyrocarbonate. A stock solution of DEPC was prepared before each experiment by dilution into ice-cold absolute ethanol. The concentration of DEPC was determined spectrophotometrically by reaction with 10 mM imidazole (pH 7.2) and monitoring the increase in absorbance at 240 nm due to the formation of *N*-carbethoxyimidazole (ϵ = 3400 M^{-1} cm^{-1}) (Miles, 1977; Lundbland, 1995). Inactivation experiments were performed by incubation of mutase−dehydrogenase (22 μM monomer concentration) with DEPC in 50 mM potassium phosphate buffer (pH 7.2) at 25 °C in the absence or presence of ligands (prephenate, NAD^+, or *endo*-oxabicyclic diacid) (0.1 mL total volume). Aliquots of 10 μL were withdrawn from the incubation mixture at various time intervals and placed in 1.0 mL of buffer containing 50 mM *N*-ethylmorpholine (pH 7.2), which rapidly quenched the reaction. The residual activities of mutase and dehydrogenase were determined as described above. The final concentration of ethanol was always kept lower than 5% (v/v) of that of the total incubation mixture and was shown to have no effect on enzyme activity.

The pH dependence of prephenate dehydrogenase inactivation by 0.3 mM DEPC was determined in an incubation buffer of 50 mM potassium phosphate over the pH range of 5.2−8.6 at 25 °C. The first-order rate constant for the decomposition of DEPC (k') was calculated by measuring the time-dependent decomposition of DEPC in a separate control experiment. In this buffer, the value of k' was estimated to be 0.001 28 s^{-1} at pH 7.2 (half-time, 9 min) and increased only by 7% at pH 8.6. All data values in this study were corrected for this decomposition using the value of k' at pH 7.2.

Restoration of Mutase−Dehydrogenase Activities Using Hydroxylamine. Mutase−dehydrogenase (22 μM monomer concentration) was incubated with 0.3 mM DEPC in 50 mM potassium phosphate (pH 7.2) at 25 °C for 1 min, at which time less than 10% of the original mutase activity and about 20% of the dehydrogenase activity remained. The reaction was rapidly quenched with 100 mM imidazole buffer (pH 7.0) to a final concentration of 2 mM. Hydroxylamine adjusted with NaOH to pH 7.0 was added to the quenched sample to give a final concentration of 0.3 M. Aliquots from the resulting mixture were assayed at regular time intervals to follow the restoration of mutase and dehydrogenase activities. In a control reaction using unmodified enzyme, hydroxylamine did not affect the activity of the unmodified enzyme.

UV-Difference Spectroscopy and Stoichiometry of Histidine Modification. The extent of N-carbethoxylation of histidine residues was determined by monitoring the time-dependent increase in the absorbance at 240 nm in a reaction volume of 50 mM potassium phosphate buffer (pH 7.2) at 25 °C. Absorbance readings were recorded over a wavelength range of 200−310 nm for a sample containing buffer and appropriate amounts of mutase−dehydrogenase and DEPC. These readings were computer-corrected using a control containing the same components but without DEPC, scanned over the same wavelength range. The number of modified histidine residues as a function of time was approximated from the extinction coefficient for *N*-carbethoxyhistidine, 3400 M^{-1} cm^{-1}. The stoichiometry of histidine modification was correlated with enzyme activity by monitoring in a parallel experiment the time-dependent loss of dehydrogenase activity.

Isolation of Active Site Peptides by Differential Peptide Mapping. The general strategy employed for the isolation and identification of peptides presumably at the mutase and dehydrogenase sites is outlined here. Mutase−dehydrogenase was reacted with an appropriate modifying reagent in the absence or presence of ligands which protect against reagent-mediated enzyme inactivation. In the dehydrogenase reaction, DEPC was the modifying reagent and prephenate was the protecting ligand. By contrast, trinitrobenzene sulfonate (TNBS) was used to modify the mutase and DEPC served to shield the reactive enzymic residue from TNBS modification. Following proteolytic digestion of the enzyme preparation, peptides containing chromophoric adducts were isolated with HPLC by following the absorbance of the appropriate derivatives, carbethoxyhistidine at 240 nm (Lundblad, 1995) and TNB-lysine at 350 nm (Nakayama et al., 1992). The correct peptide in each case was identified using the chromatogram of digests of the protected enzymes as a reference. The masses of the isolated peptides were determined by mass spectrometry and then compared with the known molecular weights of the fragments in the peptide map to deduce the exact site of the derivitization.

Carbethoxyhistidyl Peptide. Mutase−dehydrogenase (22 μM monomer concentration) in 50 mM potassium phosphate buffer (pH 7.2) was incubated for 1 min at 25 °C with 0.3 mM DEPC in the absence or presence of 2 mM prephenate which was also dissolved in phosphate buffer. The reaction was then rapidly quenched by the addition of imidazole to a final concentration of 2 mM. To this reaction mixture was added a solution of trypsin, in the same phosphate buffer, at a substrate to protease ratio of 100:1 (w/w). Digestion proceeded at 37 °C for 3−4 h and was terminated by freezing the sample. This slowed the rate of degradation of the *N*-carbethoxyhistidine derivative in addition to the proteolytic action of trypsin until the samples could be analyzed. All digests were analyzed by reverse-phase HPLC on a Vydac C_{18} column (25 cm, 4.6 mm i.d., 5 μm particle size, 300 Å pore size). Chromatograms were developed at room temperature at a flow rate of 1 mL/min with a linear gradient of 5 to 70% acetonitrile in 0.05% potassium phosphate buffer at pH 6.4. Eluting peptides were monitored spectrophotometrically at 240 nm to detect those that contained N-carbethoxylated histidine residues. The peptide of interest was collected, and buffer was removed by elution through a Waters C_{18} Sep Pak column with 70% acetonitrile in 0.1% TFA. The sample was then snap frozen in liquid nitrogen, lyophilized on a speed vac, and then stored at −86 °C until mass spectral analysis could be conducted.

Trinitrobenzyllysyl Peptide. Mutase−dehydrogenase (22 μM monomer concentration) in 50 mM potassium phosphate buffer (pH 7.2) was incubated with 0.3 mM DEPC for 1 min, and then the reaction was rapidly quenched by the addition of imidazole (final concentration of 2 mM). The modified enzyme was separated from carbethoxyimidazole by passage of the mixture through a 5 mL Sephadex G-25

column equilibrated in the same phosphate buffer. Next, DEPC-modified and unmodified mutase−dehydrogenase were incubated at 25 °C with 0.4 mM TNBS in 50 mM potassium phosphate buffer (pH 7.2) for 10 min. Further reaction was terminated by passage of each of the mixtures through a 5 mL Sephadex G-25 column. To each of the isolated protein solutions was added *S. aureus* V8 protease at a final ratio of substrate to protease of 100:1 (w/w). Digestion was carried out overnight at 37 °C, and the samples were then frozen until they were further analyzed by HPLC. Chromatograms were obtained at room temperature at a flow rate of 1 mL/min with a linear gradient of 5 to 70% acetonitrile in aqueous 0.1% TFA (pH 2.0). The elution of TNB-lysyl peptides was monitored spectrophotometrically at 350 nm. The peptide of interest was collected manually and then lyophilized in a speed vac. The peptide was reconstituted in 50 mM potassium phosphate buffer (pH 7.2). It was then redigested with V8 protease overnight at 37 °C. Following a second chromatography step by reverse-phase HPLC, peptides were collected and then further purified through a Waters Sep-Pak column. The sample was snap frozen, lyophilized, and stored at −86 °C until mass spectral analysis was performed.

Mass Spectrometry of Isolated Active Site Peptides. Isolated peptides were analyzed using a matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometer (Finnegan Lasermat). Approximately 2.5 pmol of each peptide, previously desalted, was mixed with a peptide standard (substance P) and a matrix material (α-cyano-4-hydroxycinnamic acid) at a ratio of 1:1:2 in 70% acetonitrile/30% water/0.1% TFA. The solution was spotted onto a stainless steel plate and then allowed to air dry before being loaded into the mass spectrometer. Spectra were obtained by summing the data from multiple pulses of a laser (355 nm emission wavelength).

Additional Spectroscopic Measurements. Circular dichroism (CD) measurements were recorded from 190 to 360 nm using a JASCO J-710 spectropolarimeter. Fluorescence measurements using excitation and emission wavelenths of 280 and 224 nm, respectively, were recorded on a Shimadzu RF5000U spectrofluorimeter.

Data Analysis. The kinetic data were fitted to the appropriate equations using the computer programs of Cleland (1979) or GraFit (Version 3.0, Leatherbarrow).

Steady-state kinetic data were analyzed using the equations outlined in Turnbull and Morrison (1990) and Turnbull et al. (1991a) to obtain values for the Michaelis constants (K_m) for prephenate and NAD^+ and for the dissociation constants for the interaction of enzyme with prephenate in the dehydrogenase reaction (K_{ia}) and in the mutase reaction (K_i).

Data for the time-dependent inactivation of enzyme activity by DEPC, corrected for the decomposition of DEPC, were fitted to eq 1 to obtain values for k_1I_o. A/A_o is the

$$\ln(A/A_o) = -(k_1/k')I_o(1 - e^{-k't}) \quad (1)$$

fraction of activity remaining at time t, I_o is the intial concentration of DEPC, k_1 is the second-order rate constant for the reaction of the enzyme with reagent, and k' is the first-order rate constant for the hydrolysis of the reagent (Gomi & Fujioka, 1983). Since the concentrations of DEPC were normally well in excess of enzyme concentration, pseudo-first-order conditions are assumed so that k_1I_o represents k_{obs}, the observed pseudo-first-order rate constant of enzyme inactivation.

Data for the effect of ligands on the pseudo-first-order rate constant of inactivation by DEPC were fitted to eq 2 to obtain values for the dissociation constant for the enzyme−ligand complex (K_s) and the rate constant for the combination of free enzyme with DEPC (k_1). I and L represent the

$$k_{obs} = IK_sk_1/(K_s + L) \quad (2)$$

concentrations of DEPC and ligand, respectively (Mildvan & Leigh, 1964).

The variation with pH of the values for the pseudo-first-order rate constant of inactivation was fitted to eq 3 to obtain a value for the pH-independent value of k_{obs}, $(k_{obs})_{max}$, and for the acid dissociation constant (K_a). H is the hydrogen ion concentration.

$$k_{obs} = (k_{obs})_{max}/(1 + H/K_a) \quad (3)$$

Data yielding linear plots were fitted by linear regression analysis.

RESULTS

Kinetics of DEPC Inactivation of Mutase-Dehydrogenase

Prephenate Dehydrogenase. Incubation of mutase−dehydrogenase with DEPC at 25 °C in 50 mM potassium phosphate buffer (pH 7.2) results in a time-dependent loss of prephenate dehydrogenase activity. Figure 1A shows a plot of the natural log of the remaining activity against effective incubation time $[(1 - e^{-k't})/k']$, corrected for the decompostion of DEPC, for various concentrations of DEPC. The data follow first-order kinetics and are fitted to eq 1. The pseudo-first-order rate constants (k_{obs}), as deduced from the slope of inactivation kinetics in Figure 1A, vary linearly with DEPC concentration (Figure 1B). The data also intersect the origin and, taken together with the linear dependence, show that this inactivation is a simple irreversible bimolecular process that is not dependent on the reversible formation of an active enzyme−DEPC complex prior to inactivation (Church et al., 1985). The second-order rate constant for the inactivation of dehydrogenase by DEPC calculated from the slope of the line in Figure 1A is 60 ± 5 M^{-1} s^{-1}. The double natural log plot of k_{obs} versus DEPC concentration is also linear (Figure 1B inset). The slope of this line yields a reaction order (stoichiometry) of 1.05 ± 0.11 and implies that the loss of dehydrogenase activity is due to the modification of a single group per catalytic unit (Levy et al., 1963). The apparent pK_a of this reacting group was estimated by following the pH dependence of the pseudo-first-order rate constant for DEPC inactivation of the dehydrogenase in the phosphate buffer. Over the pH range of 5.2−8.6, the ionization of a single group was observed (Figure 2). A pK_a value of 7.02 ± 0.14 and a maximum value of k_{obs} of 0.0218 ± 0.0002 s^{-1} were determined by fitting the data to eq 3.

Chorismate Mutase. Incubation of mutase−dehydrogenase (22 μM) with DEPC (0−0.4 mM) also results in a rapid loss of mutase activity which approximates pseudo-first-order kinetics (data not shown). Replots of the data are represented in Figure 1B and show that, as with the dehydrogenase, the inactivation follows a simple bimolecular process. The

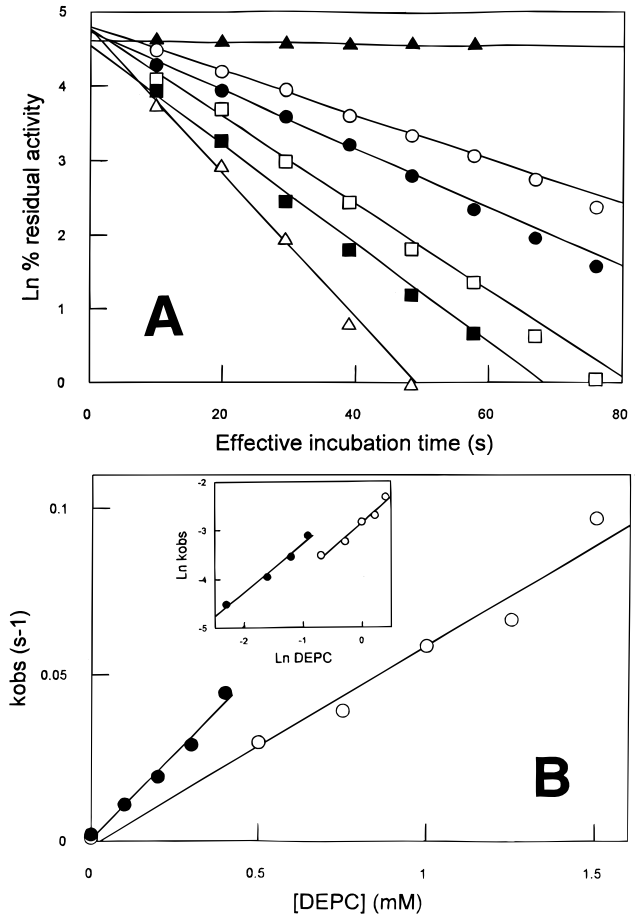


FIGURE 1: Kinetics of inactivation of prephenate dehydrogenase by DEPC. (A) Dependence of residual activity on the effective incubation time. Mutase−dehydrogenase (22 μM) was incubated with increasing concentrations of DEPC in 50 mM potassium phosphate buffer (pH 7.2, 25 °C), and then samples were withdrawn at various time intervals for the determination of residual activity. The concentrations of DEPC used were 0 mM (▲), 0.50 mM (○), 0.75 mM (●), 1.00 mM (□), 1.25 mM (■), and 1.50 mM (△). The data for A were fitted to eq 1 to obtain values for the pseudo-first-order rate constants. (B) Plots of the concentration dependence of the pseudo-first-order rate constants for the inactivation of mutase (●) and dehydrogenase (○).

second-order rate constant for the inactivation of the mutase is 104 ± 8 M^{-1} s^{-1}. The double natural log plot of k_{obs} against DEPC concentration shown in the Figure 1B inset is also linear, yielding a slope of 0.99 ± 0.09. These results are in accord with the idea that the modification of a single group per catalytic unit results in the loss of mutase activity.

Ligand Protection against DEPC-Mediated Inactivation of Mutase−Dehydrogenase

Substrates of the dehydrogenase reaction, prephenate and NAD^+, and an inhibitory substrate analogue of the mutase reaction, *endo*-oxabicyclic diacid, were tested for their abilities to prevent the DEPC-mediated loss of either the mutase or dehydrogenase activities. The prior addition of increasing concentrations of prephenate (0−0.4 mM) protected against the inactivation of both dehydrogenase and mutase by 0.3 mM DEPC. A representative plot of the type of data obtained is shown for the dehydrogenase reaction (Figure 3). When the reciprocals of the slopes of the lines in Figure 3 are varied with respect to prephenate concentration, a straight line is obtained (data not shown). Such a

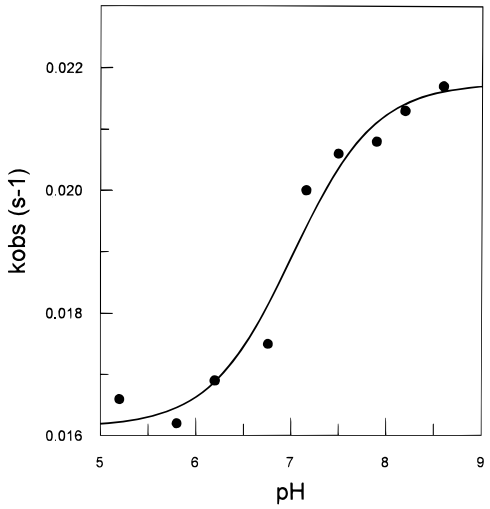


FIGURE 2: pH dependence of the pseudo-first-order rate constant for inactivation of prephenate dehydrogenase by DEPC. Mutase−dehydrogenase (22 μM) was incubated at 25 °C with 0.3 mM DEPC in 50 mM potassium phosphate buffer at various pH values. Aliquots were removed every 30 s over 4 min, and dehydrogenase activity was measured. The curve represents the best fit of the data to eq 3.

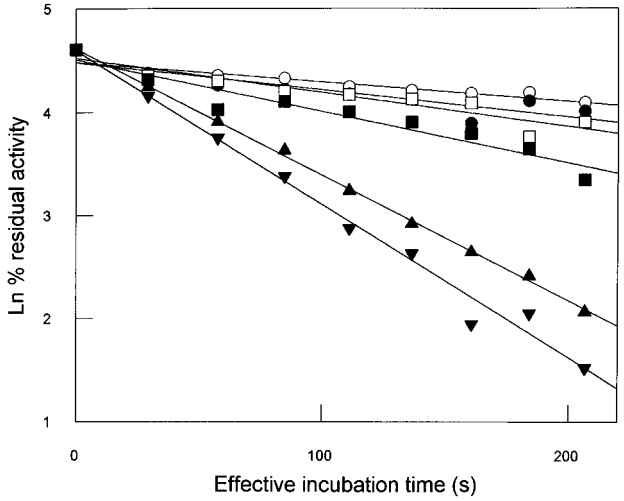


FIGURE 3: Protection by prephenate against inactivation of prephenate dehydrogenase by DEPC. The dependence of residual activity on the effective incubation time in 50 mM potassium phosphate buffer (pH 7.2) at 25 °C is shown for the inactivation of prephenate dehydrogenase (22 μM) by 0.3 mM DEPC in the presence of 0 mM (○), 0.05 mM (●), 0.10 mM (□), 0.20 mM (■), 0.30 mM (▲), and 0.4 mM (▼) of prephenate. The data were fitted to eq 1 to obtain values for k_{obs}.

result implies that prephenate totally protects against DEPC-mediated inactivation; the reagent cannot inactivate an enzyme−ligand complex (Mildvan & Leigh, 1964).

Data for these protection experiments were fitted to eq 2 to obtain values of the dissociation constants (K_s) for the interaction of the ligand with the free enzyme. The values for K_s obtained for the interaction of prephenate acting as a substrate of the dehydrogenase reaction (54 ± 3 μM) and as an inhibitor of the mutase reaction (140 ± 9 μM) in the protection studies are similar to those obtained from the analysis of steady-state kinetic data for the two reactions at pH 7.2. Steady-state kinetic data were analyzed assuming that the dehydrogenase reaction conforms to a rapid equilibrium random mechanism with catalysis being rate-limiting (SampathKumar & Morrison, 1982b). From the analyses, a

value of 63 ± 10 μM was obtained for the dissociation constant (K_{ia}) for the interaction of prephenate with the free enzyme in the dehydrogenase reaction and a value of 95 ± 9 μM for the inhibition constant (K_i) of prephenate in the mutase reaction. Regretably, reliable quantitative information could not be easily derived from protection studies using NAD^+ as this ligand reacted with free DEPC, thereby lowering its effective concentration and that of the modifying reagent in the inactivation experiments. However, 1 mM NAD^+ did afford 75% protection of DEPC-mediated inactivation of dehydrogenase activity, while under identical assay conditions, inactivation of the mutase was not prevented, even up to an NAD^+ concentration of 2 mM.

The inhibitory transition-state analogue *endo*-oxabicyclic diacid completely prevented the inactivation of the mutase but had little effect on the dehydrogenase (data not shown). Only stoichiometric amounts of *endo*-oxabicyclic diacid were required for total protection of the mutase, precluding the determination of a dissociation constant for this enzyme−ligand complex by these studies. A dissociation constant for this ligand from the mutase of 0.11 ± 0.01 μM has been derived from steady-state kinetic data at pH 7.2 (Turnbull & Morrison, 1990). In contrast, *endo*-oxabicyclic diacid protected the dehydrogenase against DEPC-mediated inactivation only weakly. The data (not shown) for the time-dependent inactivation of the dehydrogenase by 0.3 mM DEPC at six concentrations of *endo*-oxabicyclic diacid (0−2 mM) were fit to eq 2 to obtain a K_s value of 212 ± 7 μM for the interaction of *endo*-oxabicyclic diacid with the dehydrogenase.

Characterization of DEPC-Modified Mutase−Dehydrogenase

The difference spectrum of the DEPC-modified and untreated enzyme from 200 to 310 nm shows an absorption maximum at 240 nm (Figure 4A) which is characteristic of the carbethoxylation of histidine residues. The maximum absorbance change of 0.48 occurs after 15 min at a DEPC concentration of 1.5 mM and corresponds to the modification of six of the nine histidine groups in the protein. However, when the extent of modification of histidine residues is correlated with the inactivation of the dehydrogenase (Figure 4B), the data suggest that only one of the histidine groups modified by DEPC is critical for dehydrogenase activity. With time, additional histidine residues are carbethoxylated, but these do not significantly contribute to additional inactivation. Moreover, the time required to modify 0.5 mol of histidine/subunit (25 s) correlated exactly with the half-time of inactivation (data not shown). Partially inactivated enzyme (20% residual activity) had K_m values for prephenate (45 ± 5 μM) and NAD^+ (198 ± 20 μM) that were similar to those of the native enzyme (29 ± 4 and 107 ± 33 μM, respectively), ruling out the possibility that the loss of activity after modification may be due to a large increase in K_m values.

Treatment of the DEPC-modified enzyme at pH 7.0 with 0.3 M hydroxylamine, a reagent known to cleave carbethoxy adducts from histidine groups, resulted in a time-dependent regeneration of over 80% of the original dehydrogenase activity (Figure 5). Moreover, the K_m values for prephenate (54 ± 13 μM) and NAD^+ (140 ± 48 μM) in the dehydrogenase reaction for the regenerated enzyme (after removal of excess hydroxylamine) were very similar to those values for the native, underivatized enzyme. This futher supports the idea that the inactivation of the dehydrogenase is due to the reversible modification of a histidine group by DEPC (Miles, 1977). By contrast, treatment of the carbethoxylated enzyme with 0.3 M hydroxylamine did not restore mutase activity (Figure 5). This result is consistent with the idea that the mutase is inactivated by the reaction of DEPC with a primary amine such as a lysine residue (Miles, 1977).

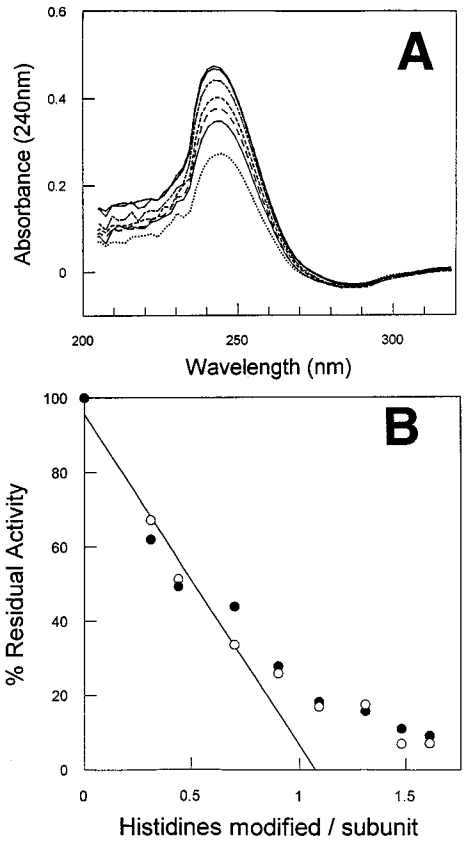


FIGURE 4: Modification of mutase−dehydrogenase by DEPC. (A) Typical difference spectra for the modification of mutase−dehydrogenase by DEPC illustrating the signal, representative of *N*-carbethoxyhistidine, appearing at 240 nm over time. The curves were obtained over increasing time intervals of 0.5, 1, 1.5, 2, 5, 10, and 15 min. (B) Relationship between residual activity and the number of histidine residues modified by DEPC. The number of histidine residues modified per subunit was determined by a difference spectrum obtained by monitoring the time-dependent absorbance at 240 nm of the reaction of mutase−dehydrogenase (22 μM) with 0.3 mM DEPC in 50 mM potassium phosphate buffer (pH 7.2, 25 °C). Percent residual activity was determined in a parallel experiment under identical conditions. Aliquots from the incubation mixture were removed at specific time intervals, and residual dehydrogenase activity was measured as described in Experimental Procedures. The results from two independent experiments (○ and ●) showing the linear extrapolation were based on the first four time points from each experiment.

Effect of TNBS Modification of Mutase−Dehydrogenase

Further chemical modification studies of mutase−dehydrogenase provided additional evidence to indicate a lysine is carbethoxylated in the mutase. When mutase−dehydrogenase was incubated for 20 min with 0.1 mM TNBS, at a ratio of reagent to enzyme of 4:1, approximately 95% of the mutase activity was lost (data not shown). By contrast, only 40% of the dehydrogenase activity was lost (data not shown). TNBS reacts reasonably specifically with lysine

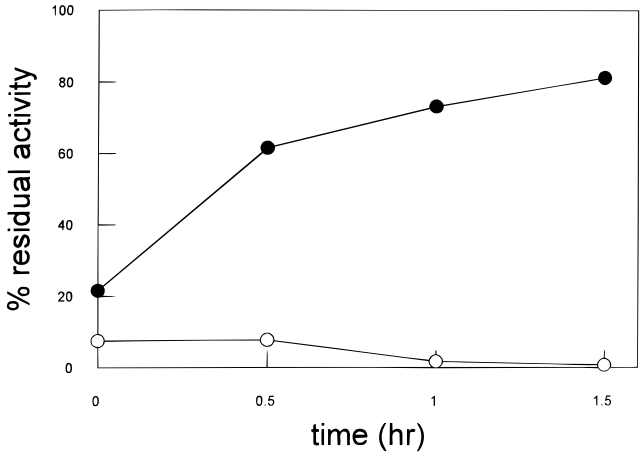


FIGURE 5: The ability of hydroxylamine to restore mutase−dehydrogenase activity. The restorations of mutase (○) and dehydrogenase (●) activity by 0.3 M hydroxylamine at pH 7.0 and 25 °C in 50 mM potassium phosphate buffer were followed separately for a DEPC-inactivated enzyme. A control (not shown) using unmodified enzyme treated with hydroxylamine under the same conditions showed no significant change in either activity.

groups, yielding an adduct that absorbs strongly at 350 nm (Salvucci, 1993; Nakayama et al., 1992). However, this reagent will also more slowly react with cysteine residues, yielding a weakly absorbing adduct (Lundblad, 1995).

Generation, Isolation, and Identification of Active Site Peptides

Our data indicate that an active site histidine residue is involved in the catalytic mechanism of the dehydrogenase reaction while an active site lysine is involved in the mutase reaction. In order to locate each of these two residues in the amino acid sequence of mutase−dehydrogenase, we employed differential peptide mapping with mass spectrometry as outlined in Experimental Procedures.

Prephenate Dehydrogenase-Containing Peptide. Mutase−dehydrogenase samples modified with DEPC in the presence and absence of prephenate were digested with trypsin and fractionated by reverse-phase HPLC in a solvent system maintained at pH 6.4. At this pH, the adduct is sufficiently stable. Peptides were detected at 240 nm to monitor the elution of those fragments containing *N*-carbethoxyhistidine groups. Comparison of the HPLC tracings at 240 nm for the elution of samples derivatized in the presence of prephenate (Figure 6A) or in its absence (Figure 6B) shows that one region in the peptide map eluting at about 14 min is substantially protected by ligand. Prephenate absorbs at 240 nm and elutes at about 4 min (Figure 6A). Other regions within the maps showed much less pronounced changes which were not reproducible between separate experiments. The material eluting at 14 min was collected and then desalted. Although the adduct is not expected to survive the acidic desalting conditions of the second column, this step is a prerequisite to mass spectrometry. The mass spectrum of the isolated peptide along with a peptide standard is shown in Figure 8A. One signal belongs to the standard substance P ($[M + H]^+$, $m/z = 1348.6$) and the other to a peptide at $m/z = 1212.3$. Comparison of this latter mass value with those predicted for the 43 trypsin-generated fragments of mutase−dehydrogenase yielded a single match with sequence ILEQHDWDR ($[M + H]^+ = 1212.6$),

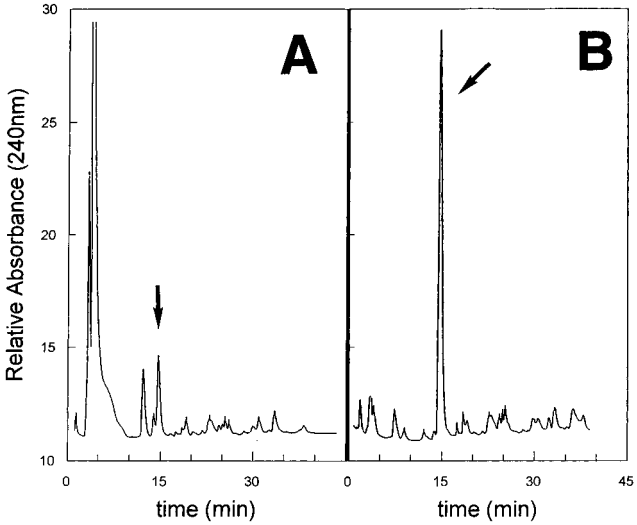


FIGURE 6: Reverse-phase HPLC chromatogram of tryptic digests of mutase−dehydrogenase derivatized by DEPC in the presence (A) and absence (B) of prephenate. Peptides were eluted with a linear solvent system of 5 to 70% acetonitrile in 0.05% potassium phosphate buffer (pH 6.4) as described in Experimental Procedures. Those peptides containing *N*-carbethoxyhistidines were detected at 240 nm. Similar quantities of protein were injected. The altered peak is indicated by an arrow.

corresponding to residues 127−135 containing His131. The next closest mass is 1191.7, comprising a peptide of amino acids 40−49 which lacks a histidine residue. The nearest histidine-containing peptide was 1408.6, comprising amino acids 346−356.

Chorismate Mutase-Containing Peptide. Carbethoxylated lysine adducts do not absorb light above 200 nm (Lundblad, 1995). In order to identify the residue that reacts with DEPC and which is important for mutase activity, mutase−dehydrogenase was modified with TNBS in the presence and absence of DEPC. The modified proteins were then digested with *S. aureus* V8 protease prior to fractionation by reverse-phase HPLC. V8 protease, which cleaves specifically at the C-terminal side of glutamate residues, was used instead of trypsin, which is specific for lysine and arginine groups (Allen, 1989). HPLC tracings at 350 nm for the elution of samples modified in the presence (Figure 7A) or absence (Figure 7B) of DEPC revealed one peptide eluting at about 49 min whose modification was protected substantially in the presence of protecting ligand DEPC. Mass spectrometry indicated that this fragment had a high molecular mass presumably resulting from incomplete proteolytic digestion (data not shown). Consequently, this isolated peptide was redigested overnight. C_{18} reverse-phase chromatography of this redigested fragment resolved one major peak eluting at 36 min and two smaller peaks eluting at 25 and 49 min (Figure 7C). Each peak was collected and subjected to mass spectrometry. The mass of the fragment eluting at 49 min was characteristic of the incompletely digested fragment previously isolated. The peak at 25 min contained either no peptide or an insufficient sample for mass analysis (<2 pmol). The mass spectrum of the predominant peak eluting from the HPLC at 36 min (Figure 7C) is shown in Figure 8B. The signal at $m/z = 1348.6$ belongs to standard substance P ($[M + H]^+$), while all other peaks in the spectrum belong to the isolated peptide. The masses of these peaks were compared with those values predicted for the 29 possible V8 protease-generated fragments of mutase−dehy-

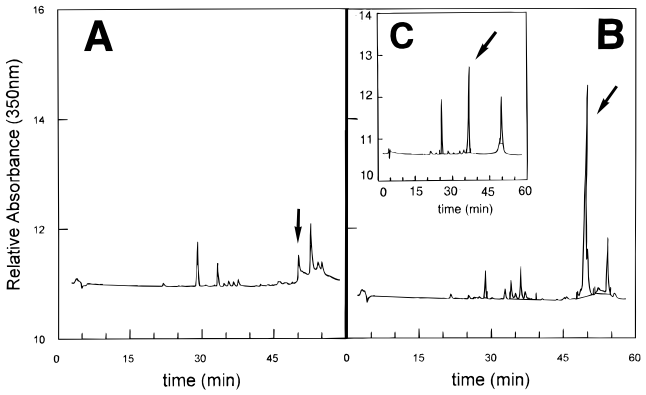


FIGURE 7: Reverse-phase HPLC chromatograms of V8 protease digests of mutase−dehydrogenase derivatized in the presence (A) and absence (B and C) of DEPC. Peptides were separated using a solvent system of 5 to 70% acetonitrile in 0.1% TFA (pH 2.0). For panels A and B, similar quantities of protein were injected. The TNB-lysine-containing peptides were detected at 350 nm, and an arrow indicates a peak whose area is altered by the protecting ligand. The peptide eluting at 49 min in panel B was redigested with V8 protease and then rechromatographed to yield the tracing shown in panel C. The altered peak is indicated by an arrow.

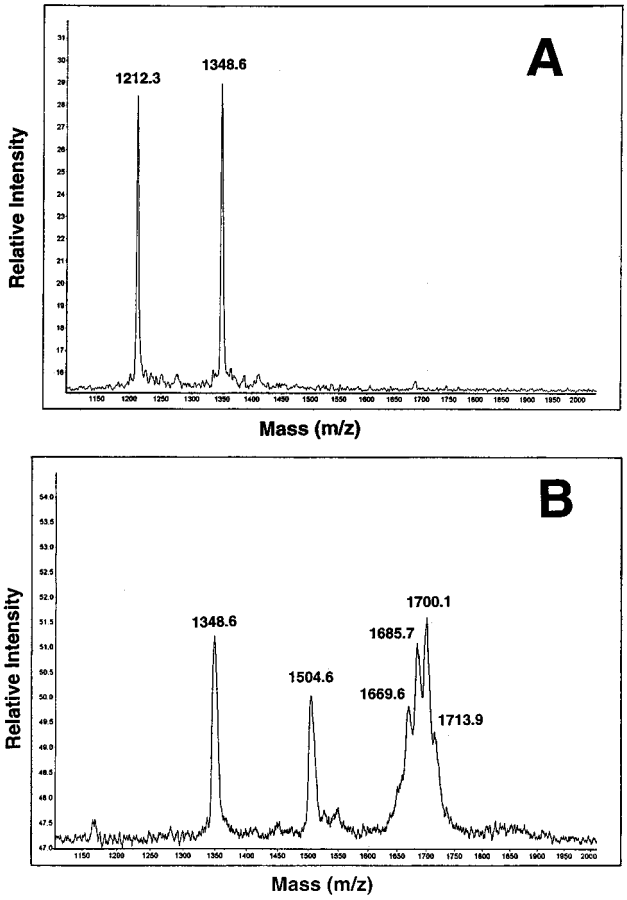


FIGURE 8: Mass spectrometry of an isolated proteolytic peptide from mutase−dehydrogenase modified with DEPC (A) and TNBS (B). In panel A, the tryptic peptide eluting at 14 min in Figure 7A yields a signal at $m/z = 1212.3$. In panel B, the peptide eluting at 36 min in Figure 7C yields signals at $m/z = 1504.6$, 1669.6, 1685.7, 1700.1, and 1713.9. A peptide standard is shown with a $m/z = 1348.6$ in both figures.

drogenase. The peak at $m/z = 1504.6$ was within 1.2 mu of the mass of an underivatized peptide corresponding to amino acids 36−48 ($[M + H]^+ = 1505.8$, VKSRFGLPIYVPE), including a single lysine at position 37. The next closest mass value was 1503.7 and belonged to a lysine-free peptide comprising amino acids 276−288 (QLLALSSPIYRLE). This peptide did not contain residues that would react with TNBS, yielding adducts that absorb significantly at 350 nm (Lundblad, 1995). The next nearest mass of a lysine-containing peptide was 1596.9 (amino acids 15− 28). The other peaks in the mass spectrum were part of a broad, rather poorly resolved multiplet. There is more error associated with the assignment of mass values to these signals. Nevertheless, we noted that the signal at $m/z = 1713.9$ was within 2.9 mu of that predicted for the peptide ($[M + H]^+$) whose sequence is shown above but carrying a trinitrobenzyl adduct on Lys37. It is likely that the other peaks in this multiplet arise from fragmentation of the TNB-lysyl peptide since the absorbance maximum of the derivatized peptide, at 350 nm, is close to the emission wavelength (355 nm) of the mass spectrometer's laser light.[2]

Circular Dichroism and Fluorescence Measurements

We attempted to use circular dichroism and fluorescence to probe for any structural changes in the enzyme upon modification (data not shown). CD measurements in the far-UV region (190−245 nm) indicated that there was no change in secondary structure between the carbethoxylated and unmodified enzyme. Measurements in the near-UV region (245−360 nm) did show a larger negative CD signal at 300 nm in the modified enzyme. The near-UV region of the spectrum is sensitive to environments of UV-absorbing amino acid side chains (Kahn, 1979). Hence, this observation could be due to an intrinsic signal of the adduct. Fluorescence emission did not reveal any differences between the two proteins, but any small changes might be masked by the large intrinsic fluorescence of the mutase−dehydrogenase.

DISCUSSION

In the present investigation, we have identified two residues that are important for the activity of chorismate mutase−prephenate dehydrogenase and provide further support for the idea that the mutase and dehydrogenase reactions are catalyzed at two distinct sites. The basic strategy that we adopted, which relies on chemical modification of the enzyme in the presence and absence of protecting ligands followed by analysis of peptide fragments, has been used successfully by others to identify active site residues (Salvucci, 1993; Chang & Tam, 1993; Deka et al., 1992; Ko et al., 1991).

Identification of His131 by Chemical Modification

Of the nine histidine residues found in mutase−dehydrogenase, six of them are in some way accessible to DEPC. However, at pH 7.2 and 25 °C, only one histidine residue is particularly reactive, and it is the modification of this alone which causes a loss of dehydrogenase activity (Figure 4). Moreover, the rate of inactivation was equal to the rate of modification. The second-order rate constant for the inac-

[2] In a separate experiment, double digestion by V8 protease of DEPC-modified mutase−dehydrogenase afforded a mass signal at $m/z = 2075.6$. This corresponds to the value for a peptide of sequence VGEVKSRFGLPIYVERE (amino acids 33−50, $[M + H]^+ = 2705.4$), a fragment that upon complete digestion would have yielded the peptide comprising amino acids 36−48. The spectrum also showed the multiplet containing the TNB-lysyl adduct and putative fragments of this adduct.

tivation of the dehydrogenase by DEPC, about 60 M^{-1} s^{-1}, is at the high end of the range of values usually reported for histidine groups in other proteins at neutral pH and ambient temperature (Bateman & Hersh, 1987; Pasta et al., 1987; Deka et al., 1992; Perdiguero et al., 1995; Banzon et al., 1995). However, the essential histidine residues of some dehydrogenases (Holbrook & Ingram, 1973; Hennecke & Plapp, 1983), as well as other enzymes (Ferri & Meighen, 1994; Ko et al., 1991; Battaglia et al., 1994), have second-order rate constants for inactivation by DEPC that match or greatly exceed our value. The rate enhancements for some of these histidine residues have been attributed to their involvement in charge-relay systems or proximity to metal.

Further evidence in support of a histidine comes from following the variation with pH of the pseudo-first-order rate constant of inactivation (Figure 2). A pK_a value of about 7.0 was obtained for the ionizing group, in the range of 5−8 normally accepted for imidazole groups in proteins (Fersht, 1985). Furthermore, this value is in reasonable agreement with that derived from plots of the variation with pH of log *V* and *V*/*K* for the dehydrogenase reaction which showed that a single group with a pK_a of about 6.7 had to be deprotonated for maximal dehydrogenase activity (Hermes et al., 1984; Turnbull et al., 1991a). Hermes et al. (1984) have reported that dimethyl sulfoxide had no significant effect on the observed p*K* value in the *V*/*K* profile and that the enthalpy of ionization for this group is low, suggesting that the catalytic group is a cationic acid, most likely a histidine.

While DEPC is commonly used for modification of histidine residues, it can also react with lysine, tyrosine, and cysteine, contributing to inactivation (Miles, 1977; Lundblad, 1995). Our data tend to exclude these other possibilities. We rule out the possibility that cysteine and tyrosine residues were carbethoxylated since the absorbance spectrum in Figure 4A did not indicate a shoulder at 230 nm, characteristic of an *N*-acetylcysteine modified with DEPC (Saluja & McFadden, 1980), or a decrease at 278 nm, which would indicate that the phenol side chain of a tyrosine is modified (Melchior & Fahrney, 1970). Furthermore, the dehydrogenase activity was almost completely restored upon treatment of an inactivated enzyme with neutral hydroxylamine for 90 min (Figure 5), the signature that the cleavage of a carbethoxyhistidine adduct had regenerated an active enzyme. Activity was regained with a half-life of about 30 min at neutral pH, which is in the range normally expected for cleavage of carbethoxyhistidine adducts (Miles, 1977; Lundblad, 1995). An adduct with primary amines or cysteine cannot be reversed under these conditions,[3] and that with tyrosine is cleaved at a much slower rate (Miles, 1977; Melchior & Fahrney, 1970).

It is noteworthy that mutase−dehydrogenase activity can be lost by modification of one of the three cysteine residues in this protein with cysteine-modifying reagents (Hudson et al., 1984; Turnbull & Morrison, 1990). However, unlike the results in our present study, these authors reported that both activites are lost in parallel and inactivation of both activites can be prevented by prephenate and NAD^+.

Final confirmation that a single histidine is involved in the DEPC-mediated inactivation of the dehydrogenase comes from the isolation of a peptide from differential peptide mapping containing a histidine at position 131. A mass spectrum of the DEPC−peptide adduct could not be obtained due to its instability at low pH. However, this peptide corresponded to the only significant peak at 240 nm in a chromatogram obtained under conditions where the adduct is stable (Figure 6). The protection strategy used to isolate this peptide confirms that this histidine is at or near the prephenate binding site of the dehydrogenase reaction. As expected, this histidine is in the latter two-thirds of the protein sequence, which is believed to code for dehydrogenase actvity (Hudson & Davidson, 1984). Finally, the peptide isolated by differential peptide mapping contains only one residue capable of reacting with DEPC His131.

Identification of Lys39 by Chemical Modification

The second-order rate constant for the DEPC-mediated inactivation of the mutase at pH 7.2 and 25 °C was almost twice that of the dehydrogenase (Figure 1B) and was the first indication that modification of distinct groups was responsible for the loss of each activity. It is noteworthy that the value, at about 100 M^{-1} s^{-1}, is 50−100 times higher than that normally observed for the reaction of an enzymic lysine with DEPC (Wells, 1973; Holbrook & Ingram, 1973). However, a high reaction rate has also been reported for a lysine in hydroxysteroid dehydrogenase (Pasta et al., 1987) and ribonuclease (Glazer, 1970). In the latter case, this reactivity has been attributed to the proximity of an arginine residue which decreased the pK_a of the lysine group. As will be discussed later, our high second-order rate constant is also in accord with the idea that Lys37 has an unusually low pK_a value. For reasons discussed above, the fact that hydroxylamine did not reactivate the DEPC-inactivated mutase is evidence that this group could be a lysine residue. Carbethoxylated lysine adducts do not have a strong UV−vis signal as do carbethoxylated histidine residues. In order to identify the particular residue responsible for loss of mutase activity, we found it necessary to use another reagent which would modify lysines and also absorb. Our preliminary studies showed that TNBS, a reagent that reacts reasonably specifically with lysine residues (Lundblad, 1995), almost completely inactivated the mutase. No spectral or kinetic characterization was done specifically to rule out the possibility that modification of cysteine residues, which can also react with TNBS (Lunblad, 1995), was responsible for the inactivation. However, no cysteine was modified by DEPC, and whatever group is modified by DEPC is probably also modified with TNBS, since the DEPC-modified enzyme did not react with TNBS under these conditions. It is this feature that has allowed us to isolate and identify the Lys37-containing peptide through a scheme of differential peptide mapping similar to that outlined for the dehydrogenase studies. The amino acid sequence alignments that we have performed on the chorismate mutase portion of mutase−dehydrogenase and mutase−dehydratase enzymes from the bacteria *E. coli*, *Erwinia herbicola*, and the *Pseudomonads* show that Lys37 is conserved in all of these mutases. In addition, Gething and Davidson (1977) also noted the importance of a lysine residue in the mutase−dehydratase

[3] Cysteine adducts with DEPC exhibit a maximum absorbance at 230 nm, and their cleavage can be promoted by hydroxylamine. However, this adduct is only formed in carboxylate buffers; it is not known to form in phosphate (Garrison & Himes, 1975). In addition, the half-life of the DEPC cysteine adduct at pH 6.4 is about 2 h compared to 55 h for carbethoxyimidazole (Saluja & McFadden, 1980).

whose modification with TNBS led to a complete loss of mutase activity without significantly affecting the dehydratase domain, although they did not identify the particular lysine involved.

Interestingly, it was noted that, after longer exposure to TNBS, 40% of the dehydrogenase activity was lost. We have not pursued the identification of the dehydrogenase residue modified by TNBS. However, the active site could contain a lysine residue since the profile of pH dependence of *V*/*K* shows there is at least one group with a pK_a of 8.4 (possibly a lysine) which must be protonated for prephenate to bind to the enzyme−NAD^+ complex (Turnbull et al., 1991a).

Lys37 and His131 Are Associated with Distinct Active Sites

The results from studies using ligands to protect the mutase−dehydrogenase against DEPC-mediated inactivation suggest that the two reactive groups (His131 and Lys37) are located at distinct active sites. If a ligand protects by binding to its natural binding site on the enzyme, the value of the dissociation constant calculated in the protection experiment should agree with that calculated from steady-state kinetic analysis. The values show that the *endo*-oxabicyclic diacid binds to and preferentially protects the mutase from inactivation on the order of 2000 times more effectively than it does the dehydrogenase. *endo*-Oxabicyclic diacid mimics the proposed transition state for the chorismate mutase reaction (Bartlett et al., 1988) and thus was expected to provide protection against modification of residues at the mutase active site. The protection is stoichiometric as expected given its K_i value of about 110 nM at pH 7.2 (Turnbull & Morrison, 1990). If the two activities shared a common active site, *endo*-oxabicyclic diacid should have protected both activities equally well. NAD^+ prevented the DEPC-mediated inactivation of the dehydrogenase preferentially over the mutase. Unfortunately, DEPC reacts with NAD^+, so we cannot reliably obtain a dissociation constant for NAD^+ in this protection experiment. The dissociation constants for the interaction of prephenate with enzyme derived from the protection studies agree well with those obtained from steady-state kinetic analysis performed in this study as well by Turnbull and Morrison (1990). As expected, the results are in accord with the idea that prephenate protects against DEPC-mediated inactivation either by binding at the prephenate subsite of the dehydrogenase or by binding as a product at the active site of the mutase.

The primary sequence locations of Lys37 and His131 support the idea that these residues are in distinct active sites. The N-terminal third of the *E. coli* mutase−dehydrogenase is highly homologous to several chorismate mutase sequences from other bacterial bifunctional enzymes, including the recently crystallized mutase domain of the *E. coli* chorismate mutase−prephenate dehydratase (Lee et al., 1995a). A homologous lysine (Lys39) forms H bonds to the *endo*-oxabicyclic diacid inhibitor in the crystal structure of the enzyme−inhibior complex.

Most other prephenate dehydrogenases which have been sequenced are from very closely related bifunctional mutase−dehydrogenases. However, alignments with other dehydrogenases and flavoproteins have shown that His131 is reasonably close (at least in primary structure) to residues 101−111, which comprise the adenine binding sites in the Rossman fold, a conserved dinucleotide binding domain found in several FAD- and NAD^+-binding enzymes (Hudson & Davidson, 1984). These findings argue *against* the idea that the modifications are not in the active site and that enzyme inactivation is ellicited through conformational changes in the protein.

Roles of Lys37 and His131 in the Catalytic Mechanism of Mutase−Dehydrogenase

Lys37. A crystal structure of the highly homologous mutase domain of mutase−dehydratase (mini-mutase) liganded to the *endo*-oxabicyclic diacid compound shows that Lys39 (which aligns with Lys37 in mutase−dehydrogenase) interacts with the ligand's ether oxygen-7 as well as the C-11 carboxylate which is believed to occupy the same position as the enolpyruvate side chain in the transition state (see Scheme 1).

Kinetic studies on the mutase activity of the mutase−dehydrogenase clearly show a pH dependence on *V*/*K* (Turnbull et al., 1991a) and a distinct deuterium isotope effect (>2) on both *V* and *V*/*K* (Guilford et al., 1987), consistent with a general acid−proton transfer in the rate-limiting step. Of the three ionizable groups reported in the *V*/*K* pH profile, Turnbull et al. (1991a) have suggested that one group with a pK_a of 7.1 must be protonated for binding chorismate and may also participate in catalysis if the substrate binds to only the correctly protonated form of the enzyme. This group could be Lys37 in the mutase−dehydrogenase (or Lys39 in the mutase−dehydratase) which might have a depressed pK_a value because of its location in a positively charged active site. Such an idea is consistent with the high reactivity of this lysine toward DEPC. A lysine with a pK_a value of 7 would be 50% deprotonated at the pH of our modification studies, and neutral amines are better nucleophiles. Two possibilities for the role of this lysine as an enzymic acid include (1) protonation of the ether oxygen by an enzymic acid, in conjunction with the attack on the C-1 carbon by an electon pair on the methylene group of the enolpyruvyl side chain (Turnbull et al., 1991a); and (2) a dissociative mechanism involving a heterolytic cleavage of the ether bond with general acid catalysis, assisted by the concomitant attack of a nucleophile, leading to a covalently bound intermediate (Guilford et al., 1987). Inherent in both of these mechanisms is the assumption that the enzyme binds the kinetically and thermodynamically less stable diaxial form of chorismate (1) (Copley & Knowles, 1987).

As an alternate view, Lee et al. (1995a,b) have proposed an electrostatic basis for catalysis by the *E. coli* mutase. Although the bifunctional mutase−dehydrogenase and mutase−dehydratase are more closely related in primary sequence to each other than either is to the monofunctional *B. subtilis* enzyme (Gray et al., 1990a), the mutase from *B. subtilis* possesses a H-bonding group (Arg90) involved in a similar interaction (as Lys39) with *endo*-oxabicyclic diacid (Chook et al., 1993). Moreover, Lee et al. (1995a) noted that in both enzymes these key groups are flanked by an arginine-rich electrostatic wall interacting with the pyruvyl side chain carboxylate. Kinetic studies on the mutase from *B. subtilis* show that this enzyme catalyzes the rearrangement in the absence of any enzymic acids or bases; it relies mainly on the selective binding of the reactive pseudodiaxial

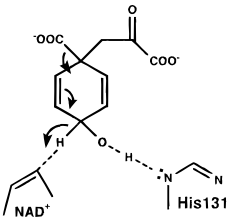
conformer of chorismate, with some additional stabilization of the transition state (Gray & Knowles, 1994). Reasoning by analogy to the *B. subtilis* mutase, Lee et al. (1995b) have proposed that Lys39 in the mini-mutase is involved in the conformational trapping of chorismate. That is, Lys39 initially H bonds to a side chain carboxylate in chorismate when the side chain is in an extended conformation and then, aided by the arginine groups, brings about the rearrangment of the side chain to one where it is poised in a position like that in the transtion state. This model is consistent with the observed acceleration of Claisen rearrangements in polar solvents (Goering & Jackobson, 1958; White et al., 1958). The pH studies by Turnbull et al. (1991a) showed that the same groups whose ionizations affect chorismate binding also affect the binding of *endo*-oxabicyclic diacid, and hence can also accomodate this model. Lys39's full catalytic potential is not achieved until its side chain makes those contacts found in the transition state. Both of the proposed models illustrate the importance of Lys39 in the catalytic mechanism of chorismate mutase from *E. coli* and wait further testing with site-directed mutants of chorismate mutases from both organisms.

His131. Studies by Cleland and collegues (Hermes et al., 1984; Turnbull et al., 1991a) on the pH dependence of the prephenate dehydrogenase reaction have clearly shown that there is a catalytic group involved and this group, with a pK_a value of about 6.7, must be unprotonated for maximum activity of the enzyme. Our study supports this view and extends the findings to show that this group may be His131.

The other NAD(P)$^+$-dependent oxidative decarboxylases which have been studied kinetically operate by a two-step mechanism. The first step is a base-catalyzed transfer of a hydride ion from the substrate to NAD$^+$, generating a β-keto intermediate; once the intermediate is formed, it is readily decarboxylated with some assistance from an enzymic acid. Most of these decarboxylases (for example, malic enzyme and isocitrate dehydrogenase) also use a metal to stabilize the enolate, thus facilitating decarboxylation, while only a few (6-phosphogluconate dehydrogenase being the most characterized) are not metal-dependent (O'Leary, 1992). The pH dependency of the reaction catalyzed by 6-phosphogluconate dehydrogenase in *Candida utilis* has led Berdis and Cook (1993) to propose that there is a lysine or a histidine (pK_a of about 7.5) acting as the base. Furthermore, the recently solved crystal structure for the enzyme from sheep liver shows this base is a lysine residue (Adams et al., 1994).

Isotope effect studies done on the three enzymes mentioned above confirm this stepwise mechanism (Grissom & Cleland, 1983; Hermes et al., 1982; Rendina et al., 1984). By contrast, the oxidative decarboxylation of prephenate by prephenate dehydrogenase follows a concerted mechanism; hydride transfer and decarboxylation occur in the same chemical step without the formation of a vinylogous β-keto intermediate (Hermes et al., 1984). The prephenate dehydrogenase reaction is essentially irreversible because an aromatic product, (4-hydroxyphenyl)pyruvate, is formed. Therefore, a base may not be needed. In support of the idea that it is the incipient aromaticity of the product that lowers the activation energy for decarboxylation, it was found that a partially saturated prephenate analogue, which would not become aromatic after decarboxylation, was reversibly oxidized but could not be decarboxylated (Hermes et al., 1984). This prompted the conclusion from these authors that prephenate dehydrogenase provides only the catalytic machinery necessary to carry out the simple oxidation step. Such being the case, a mechanism was proposed (Scheme 2) in which a histidine (we propose His131) is needed only to polarize the 4-hydroxyl group of prephenate rather than to deprotonate; a vinylogous β-keto acid is never formed. Polarization of the 4-hydroxyl group would lower the activation energy for hydride abstraction by NAD$^+$ and the concomitant decarboxylation (Hermes et al., 1984; Turnbull et al., 1991a). Our present studies support this idea in that His131 is in the presumed dehydrogenase domain of the protein, and it is probably at or near the prephenate binding site, since prephenate does protect against DEPC-mediated inactivation of the dehydrogenase. Our studies do not rule out an alternative possibility that this histidine may play a role in the orientation the cofactor NAD$^+$, since His131 is close in primary structure to the adenine binding site of the dinucleotide fold (residues 101−111) (Hudson & Davidson, 1984) and NAD$^+$ does appear to afford some protection against DEPC-mediated inactivation. In either case, His131 sits in the dehydrogenase site in a good position to assist in the oxidation reaction catalyzed by prephenate dehydrogenase.

Scheme 2: Possible Role of His131 in the Concerted Hydride Transfer and Decarboxylation of Prephenate



The role that His131 and other residues play in the catalytic mechanism is being explored currently with site-directed mutagenesis and will be reported in due course.

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