Identifying Groups Involved in the Binding of Prephenate to Prephenate Dehydrogenase from *Escherichia coli*†

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ABSTRACT: Site-directed mutagenesis was used to investigate the importance of Lys178, Arg286, and Arg294 in the binding of prephenate to the bifunctional enzyme chorismate mutase−prephenate dehydrogenase. From comparison of the kinetic parameters of wild-type enzyme and selected mutants, we conclude that only Arg294 interacts specifically with prephenate. The R294Q substitution reduces the enzyme's affinity for prephenate without affecting V/E_t of the dehydrogenase reaction or the kinetic parameters of the mutase reaction. Arg294 likely interacts with the ring carboxylate at C-1 of prephenate since the dissociation constants for a series of inhibitors missing the ring carboxyl group were similar for wild-type and R294Q enzymes. The pH dependencies of log ($V/K_{prephenate}E_t$) and of pK_i for hydroxyphenyllactate show that the wild-type dehydrogenase possesses a group with a pK of 8.8 that must be protonated for binding prephenate to the enzyme. None of the three conserved residues is this group since its titration is observed in the $V/K_{prephenate}E_t$ profiles for the mutants K178Q, R286A, and R294Q. This group is also seen in the pH−rate profiles of the binding of two substrate analogues, hydroxyphenyllactate and deoxoprephenate. Their only common structural feature at C-1 is the side chain carboxylate, indicating that the protonated residue (pK 8.8) must interact with prephenate's side chain carboxylate. Gdn-HCl-induced denaturation was conducted on wild-type and selected mutant proteins. Unfolding of the wild-type enzyme proceeds through a partially unfolded dimer which dissociates into unfolded monomers. The order of stability is wild-type = R294Q > K178Q > R286A > K178R. The least unstable mutants have reduced mutase and dehydrogenase activities.

Chorismate mutase−prephenate dehydrogenase (EC 5.4.995/1.3.1.12) is a bifunctional enzyme that catalyzes the conversion of chorismate to prephenate and the oxidative decarboxylation of prephenate to (4-hydroxyphenyl)pyruvate in the presence of NAD^+.[1] The two reactions are essential for the biosynthesis of tyrosine in enteric bacteria and other microorganisms, yeast, and plants (*1*−*5*). The enzyme in *Escherichia coli* is a homodimer with a molecular mass of 42 kDa per monomer (*6*−*8*). Studies on the *E. coli* enzyme indicate that tyrosine, the end-product of this pathway, modulates both mutase and dehydrogenase activities (*2, 7, 9, 10*). Alignment of the primary sequence of mutase−dehydrogenase with that of chorismate mutase−prephenate dehydratase (a bifunctional enzyme involved in the conversion of chorismate to phenylalanine) indicates that the N-terminal third of each subunit may encode the mutase activity, while the remaining two-thirds is responsible for the dehydrogenase activity (*11, 12*). In addition, studies show that distinct enzymic groups participate in the transition states of the mutase and dehydrogenase reactions (*13*−*15*) and that the sites at which the two reactions occur are likely separate (*14*−*17*).

The kinetic mechanism of prephenate dehydrogenase conforms to a rapid equilibrium, random mechanism with catalysis as the rate-limiting step (*18*), while hydride transfer and decarboxylation occur in a concerted manner (*19*). Studies on the pH dependency of the kinetic parameters V/E_t and V/KE_t for the dehydrogenase reaction have indicated that ionizable groups are involved in the chemical mechanism (*10, 19*). A deprotonated group with a pK value of about 6.5 is required for catalysis. The findings from temperature and solvent perturbation studies (*19*) and from chemical modification of the bifunctional enzyme with DEPC (*14*) indicate that a histidine may be the catalytic residue involved in the dehydrogenase reaction. Furthermore, our recent site-directed mutagenesis studies involving an alanine and asparagine scan of all histidine residues in the protein have implicated His197 as this catalytic group (*15*). These findings support the proposal in which a histidine, His197, might assist in the dehydrogenase reaction by polarizing the 4-hydroxyl group of prephenate, facilitating hydride transfer to NAD^+ and concomitant decarboxylation (*15, 19*). It has also been proposed that a protonated group with a pK value of about 8.8 participates in the binding of prephenate to the enzyme−NAD^+ complex (*10, 19*). However, this has yet to be confirmed by examining the pH dependence of the binding of inhibitory substrate analogues. Moreover, specific residues involved in prephenate binding have not been identified.

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[1] Abbreviations: CD, circular dichroism; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Gdn-HCl, guanidine hydrochloride; MES, 2-(*N*-morpholino)ethanesulfonic acid; NAD^+, nicotinamide adenine dinucleotide; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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Until recently, amino acid sequences from only a few prephenate dehydrogenases were available, making it difficult to predict conserved residues that may play an essential role in the catalytic mechanism of the *E. coli* enzyme. There is no crystal structure available for any prephenate dehydrogenase. However, amino acid sequences from a variety of organisms have now become available. The alignments of several bacterial enzymes (Figure 1) indicate that His197, an essential catalytic residue, and the positively charged residues Lys178, Arg286, and Arg294 of *E. coli* mutase−dehydrogenase are conserved among the prephenate dehydrogenases compared. Since a protonated enzymic group is thought to participate in prephenate binding, we hypothesized that one or more of these conserved cationic residues in the dehydrogenase interacts with the ring carboxylate or side chain pyruvyl group at C-1 of the substrate (Figure 2).

In the present study, we have conducted site-directed mutagenesis on Lys178, Arg286, and Arg294 (the only conserved arginine and lysine residues in the dehydrogenase portion of the enzyme), and have compared the kinetic properties of selected mutant proteins to those of the wild-type enzyme. Furthermore, we have examined the pH dependencies of the reactions catalyzed by wild-type and several mutant dehydrogenases, and have compared the ability of wild-type and R294Q mutant to bind a series of prephenate analogues modified at C-1. Our findings revealed that the ring carboxylate and both the carbonyl and carboxyl groups on the pyruvyl side chain of prephenate are important for the interaction of prephenate with the enzyme. However, Arg294 is the only conserved residue of the three under study that is essential for prephenate binding. We also provide the first study examining the Gdn-HCl-induced unfolding of wild-type and selected mutant proteins.

EXPERIMENTAL PROCEDURES

Materials. Chorismate was isolated from *Klebsiella pneumonia* 62-1 as described by Rieger and Turnbull (*28*) with a slight modification. The tryptophan concentration was increased to 60 mg/L in the accumulation media, resulting in 3-fold improvement in the yield of chorismate from this organism. Sodium prephenate was prepared as previously described (*29*). NAD^+ was obtained in free acid form from Boehringer Mannheim. (4-Hydroxyphenyl)propionate (HPpropionate), (4-hydroxyphenyl)lactate (HPlactate), (4-hydroxyphenyl)pyruvate (HPpyruvate), and 4-hydroxybenzoate (Hbenzoate) were purchased from Sigma-Aldrich. Oligonucleotides were obtained from BioCorp Inc. Restriction enzymes, T4 DNA ligase, and T4 DNA polymerase were purchased from Pharmacia Biotech or MBI Fermentas. All other chemicals were of the highest purity available commercially. Wild-type chorismate mutase−prephenate dehydrogenase was isolated from *E. coli* strain XL2-Blue carrying the plasmid as described by Christendat et al. (*15*). The specific activities of the purified wild-type mutase and dehydrogenase were 37 and 40 units/mg of protein, respectively. A unit of enzyme is defined as the amount of enzyme required to produce 1 μmol of product/min at 30 °C. These values are in agreement with those previously reported for this expression system (*15*).

Generation, Expression, and Purification of Mutant Mutase−Dehydrogenases. The mutagenesis of *TyrA*, the gene encoding mutase−dehydrogenase in the plasmid pSE380 (Invitrogen), was based on the elimination of a unique site in the plasmid (*30*) as outlined in Christendat et al. (*15*). Mutants were initially screened for elimination of the unique restriction site. In most cases (see below), plasmids carrying the mutation at the unique site were then sequenced to confirm the presence of the desired mutation. Sequencing was performed using the Sequenase version 2 DNA sequencing kit (U. S. Biochemicals) by the method of Sanger et al. (*31*) or using an Applied Biosystems Model 373A DNA sequencer at the Sheldon Biotechnology Research Facility, McGill University. DNA was prepared for sequencing using a Promega Wizard-prep kit.

The following synthetic oligonucleotides were used for mutagenesis: U.S.E., 5′- CAT GTA CAG AGC GCG AGA AGT AC-3′; K178R, 5′-GCA TCA GTG CGC AAT GGG CCA TTA C-3′; K178N, 5′-GCA TCA GTG AAC AAT GGG CCA TTA C-3′; K178Q, 5′-GCA TCA GTG *CAG* AAT GGG CCA TTA C-3′; R286Q, 5′-CG ATT TAC CAA CTT GAG CTG G-3′; R286K, 5′-CG ATT TAC AAA CTT GAG CTG G-3′; R286(K/T/N/Q/P/H/D/E/A), 5′-CG ATT TAC (C/A/G) (A/C) (A/T) CTT GAG CTG C-3′; R294K/Q, 5′-GCG ATG GTC GGG (C/A)AA CTG TTT GCT C-3′. The base changes introduced are underlined. The U.S.E. oligonucleotide eliminates a unique *Xho*I site in the multiple cloning region of pSE380. All mutations at positions 178 and 294 were verified by sequencing. Several colonies believed to house mutations at position 286 were further screened by determining mutase and dehydrogenase activities on crude cell extracts. Activities were detected in several extracts, but only two active mutants (R286A and R286H) could be identified by sequencing. The DNA sequences from several colonies yielding inactive enzyme could not be verified. Attempts were made to generate R286K and R286Q from primers specific for each of the two amino acids. However, cell extracts derived from colonies housing the proposed mutations were inactive, and the DNA of these plasmids could not be sequenced.

Mutant enzymes that were stable and active, and whose sequences could be verified (Table 1), were purified as outlined previously (*15*). These proteins were over 90% pure as judged by Coomassie Blue stained SDS−PAGE (*32*). The expression of the mutant proteins in crude cell extracts was monitored and compared to that of the wild-type enzyme by running nondenaturing gels and staining with Coomassie Blue.

Determination of Enzyme Activity. Mutase and dehydrogenase activities were measured at 30 °C in the presence of the three-component buffer system of 0.1 M MES, 0.051 M *N*-ethylmorpholine, 0.051 M diethanolamine, 1 mM EDTA, and 1 mM DTT. Over the pH range of 5.8−9.5, for which the initial velocities were measured, the ionic strength of this buffer remains essentially constant (*33*). The total reaction volume was 1.0 mL. The conversion of chorismate to prephenate was monitored at 274 nm while the oxidative decarboxylation of prephenate in the presence of NAD^+ was followed at 340 nm (*8*). Both reactions were monitored by using a double-beam spectrophotometer (GBC Model 918) fitted with a Pelletier-Effect temperature-controlled cuvette holder. The pH of the assay mixture was determined at 30 °C before and after the reaction. Rate constants were calculated using a subunit molecular weight of 42 000.

Maximum velocity (V/E_t) is expressed in units of s^{-1} and the apparent second-order rate constant (V/KE_t) in units of M^{-1} s^{-1}. Protein concentration was estimated using the ESL protein determination kit (Boehringer Mannheim) with bovine serum albumin as a standard.

Analysis of Kinetic Data. Kinetic data were fitted to the appropriate rate equation using GraFit (Erathicus Software) or the programs of Cleland (*34*). Initial velocity data were obtained by varying the concentrations of chorismate, prephenate, or NAD^+ (A) and were fitted to eq 1 to yield values for the maximal velocity (V/E_t), the Michaelis constant (K), and the apparent second-order rate constant for the interaction of enzyme with substrate (V/KE_t).

$$v = \frac{VA}{K + A} \tag{1}$$

$$v = \frac{VA}{K(1 + I/K_{is}) + A} \tag{2}$$

To determine the inhibition constant at different pH values, the concentration of inhibitor was varied at a fixed concentration of substrate and the data were fitted to eq 3 to obtain a value for an apparent inhibition constant [$K_{i(app)}$]. The true inhibition constant (K_i) was then determined by using the relationship given in eq 4, where A represents the fixed substrate concentration and K denotes the Michaelis constant for substrate.

$$v = \frac{V}{1 + (A/K_{i(app)})} \tag{3}$$

$$K_i = \frac{K_{i(app)}}{1 + (A/K)} \tag{4}$$

The variation of the values for V/E_t, V/KE_t, or $1/K_i$ as a function of pH was fitted to the log form of eq 5 or eq 6:

$$y = \frac{C}{1 + (H/K_A) + (K_B/H)} \tag{5}$$

$$y = \frac{C}{1 + (H/K_A)} \tag{6}$$

where y represents the value of V/E_t, V/KE_t, or $1/K_i$ at a particular pH value, C represents the pH-independent value of the parameters, K_A and K_B are acid dissociation constants, and H is the hydrogen ion concentration.

Equilibrium Denaturation by Gdn-HCl. (A) Circular Dichroism. Mutase−dehydrogenase was dialyzed for 12 h in 50 mM potassium phosphate, pH 7.4. A stock solution of 8 M Gdn-HCl was prepared in the same buffer and the pH adjusted to 7.4 with NaOH. This stock solution was diluted with phosphate buffer to prepare samples of different concentrations of Gdn-HCl containing 0.1 mg/mL (2.2 μM monomer) mutase−dehydrogenase. The protein samples were then incubated at room temperature for 24 h. Denaturation of mutase−dehydrogenase was monitored by circular dichroism (CD) using a Jasco J-710 spectropolarimeter. Spectra were recorded from 300 to 200 nm in a 0.1 cm quartz cuvette at room temperature. The change in the α-helical signal of the protein at 222 nm (1 nm bandwidth) was recorded as a function of denaturant concentration. Spectra represented the average of four wavelength scans performed at a speed of 50 nm/min in 0.2 nm steps and with a signal averaging time of 0.25 s. Measurements were then corrected for background signal from the buffer and subsequently adjusted to correct for dilution of the protein. To verify that the denaturation was a reversible process, mutase−dehydrogenase (2.2 μM monomer final concentration) was incubated in 6 M Gdn-HCl for 24 h at room temperature. Aliquots of the sample were then dialyzed against 0, 1, 2, 3, and 4 M Gdn-HCl in 50 mM phosphate buffer, pH 7.4, for 24 h at 4 °C. The samples were equilibrated at room temperature, and the degree of renaturation was monitored under conditions as described above for unfolding.

(B) Fluorescence. Gdn-HCl-induced denaturation of wild-type mutase−dehydrogenase was followed by fluorescence using an SLM Aminco Bowman Series 2 spectrofluorimeter. Protein samples were prepared at different concentrations of denaturant as described for the CD experiments except that the final concentration of protein was 0.02 mg/mL (0.40 μM monomer). Fluorescence was measured by excitation at 275 nm and emission from 290 to 390 nm. Measurements were performed at room temperature in a 1 cm quartz cuvette.

(C) Gel Filtration. The denaturation of dimeric wild-type mutase−dehydrogenase was investigated by Gdn-HCl gradient size-exclusion column chromatography (*35, 36*). Experiments were performed at room temperature using a Superose 12 (10/30) FPLC column from Pharmacia (1 × 30 cm, volume ~25 mL). A linear gradient of Gdn-HCl in 50 mM potassium phosphate buffer, 0.1 M KCl (pH 7.4) was formed by mixing 75 mL of buffer containing 6 M GdnHCl with 75 mL of buffer containing 0 M Gdn-HCl (flow rate of 0.4 mL/min). This gradient yielded incremental decreases in Gdn-HCl concentration of about 0.5 M every 30 min. Successive 200 μL samples of mutase−dehydrogenase (22 μM monomer, previously dialyzed against phosphate buffer without denaturant) were injected about every 40 min, and the elution profile was monitored by recording the absorbance at 280 nm.

(D) Data Analysis. Data for the unfolding of mutase−dehydrogenase monitored by CD were analyzed by assuming a two-state transition from native (N) to unfolded protein (U) shown in eq 7:

$$N_2 \rightleftarrows 2U \tag{7}$$

The equilibrium constant for the unfolding reaction at any given denaturant concentration is given by eq 8:

$$K_{eq} = \frac{[U]^2}{[N_2]} = 2P_t\frac{F_u^{\,2}}{1 - F_u} \tag{8}$$

where K_{eq} is the equilibrium constant between the unfolded and native state, P_t is the total concentration of polypeptide, and F_u is the fraction of mutase−dehydrogenase in the unfolded state (*37*). F_u was calculated at each denaturant concentration as described by Pace et al. (*38*) using nonlinear regression analysis with Sigma Plot (*39*). K_{eq} is related to the Gibbs free energy of denaturation (ΔG_d) by eq 9:

$$\Delta G_d = -RT \ln K_{eq} \tag{9}$$

where R is the universal gas constant (1.987 cal deg^{-1} mol^{-1})

FIGURE 1: Amino acid sequence alignment of *E. coli* chorismate mutase−prephenate dehydrogenase with other known bacterial bifunctional mutase−dehydrogenases (B) and monofunctional prephenate dehydrogenases (M). The *E. coli* dehydrogenase domain starts at about residue 100. Invariant and nearly invariant residues are noted by stars and colons, respectively. Lys178, Arg286, and Arg294 are denoted by periods. Sequences are from (a) *Escherichia coli* (B)(*11*), (b) *Erwinia herbicola* (B) (*20*), (c) *Haemophilus influenza* (B)(*21*), (d) *Aquifex aeolicus* (M)(*22*), (e) *Helicobacter pylori* (M)(*23*), (f) *Bacillus subtilis* (M)(*24*), (g) *Lactococcus lactis* (M)(*25*), (h) *Synechocystis sp.* (M)-(*26*), and (i) *Zymomonas mobiliz* (M) (*27*).

and T is the ambient temperature in degrees kelvin (295 K). ΔG_d and K_{eq} depend on the concentration of denaturant according to eq 10:

$$\Delta G_d = \Delta G_{d,aq} - m[\text{denaturant}] \qquad (10)$$

where $\Delta G_{d,aq}$ is the value of ΔG_d at zero concentration of denaturant in aqueous solution and m is the dependence of ΔG_d on denaturant concentration. Linear extrapolation of ΔG_d to 0 M denaturant yields $\Delta G_{d,aq}$. The value of m is derived from the slope of the line. Combining eqs 8, 9, and 10 allows the expression of F_u as a function of m, the concentration of denaturant (x), and $\Delta G_{d,aq}$ shown in eq 11 (36):

$$F_u = \{-e^{[-mx-\Delta G_{u,aq}]/RT} + (e^{[-2mx-2\Delta G_{u,aq}]/RT} + 8P_t e^{[-mx-\Delta G_{u,aq}]/RT})^{1/2}\}/4P_t \qquad (11)$$

Nonlinear least-squares fitting of eq 11 to the data employed Sigma Plot to determine the values of the parameters, $\Delta G_{d,aq}$ and m, shown in Table 4, and the curves in Figure 4.

RESULTS

Expression and Kinetic Parameters of Wild-Type and Mutant Mutase−Dehydrogenases. On the basis of the amino acid sequence alignments from nine bacterial prephenate dehydrogenases (Figure 1), we targeted the conserved cationic residues Lys178, Arg286, and Arg294 for site-directed mutagenesis to determine if they played a role in prephenate binding. We designed several amino acid replacements at each of the three positions. These substitutions were, for the most part, conservative. The effect of charge was examined by replacing the cationic group with a polar or neutral side chain, while steric factors were addressed by substituting the residue for another positively charged group of a different size. Only those five substitutions shown in Table 1 yielded mutant proteins that were detectable by Coomassie Blue staining of nondenaturing gels (data not shown) and were active. However, these included at least one mutant at each of the three positions. The mutations of greatest interest are those which alter the kinetic parameters for the dehydrogenase reaction without affecting those for the mutase reaction.

K178Q mutant protein was well expressed, and the kinetic parameters of the reactions were not remarkably different from those of the wild-type enzyme. The maximum velocity of the dehydrogenase reaction was reduced by a factor of about 2. In addition, the binding of NAD^+ was reduced by a factor of 4, as expected since this residue is located within the nucleotide binding domain of the protein (*11*). The asparagine mutant was also well expressed but suprisingly exhibited no detectable mutase or dehydrogenase activity in crude cell extracts. The K178R mutant was more poorly

Table 1: Summary of Kinetic Data for Wild-Type and Mutant Mutase−Dehydrogenases Measured at pH 7.2 and 30 °C

	mutase activity[b]			dehydrogenase activity[c]					
	chorismate			prephenate			NAD^+		
protein[a]	K_M (μM)	V/E_t (s^{-1})	V/KE_t (M^{-1} s^{-1})	K_M (μM)	V/E_t (s^{-1})	V/KE_t (M^{-1} s^{-1})	K_M (μM)	V/E_t (s^{-1})	V/KE_t (M^{-1} s^{-1})
WT	45 ± 7	27 ± 1	6.0×10^5	44 ± 8.5	27 ± 1	6.2×10^5	103 ± 11	27 ± 1	2.6×10^5
K178Q	24 ± 1.5	29 ± 0.4	1.2×10^6	66 ± 13	8.3 ± 0.5	1.2×10^5	400 ± 12	13 ± 1.4	3.2×10^4
K178R	366 ± 80	0.93 ± 0.09	2.5×10^3	1660 ± 470	4.8 ± 0.44	2.8×10^3	1630 ± 230	5.3 ± 0.3	3.3×10^3
R286A	184 ± 17	15 ± 0.5	8.1×10^4	65 ± 7	10 ± 0.3	1.5×10^5	239 ± 32	11 ± 0.5	4.6×10^4
R286H	25 ± 3	13 ± 0.3	5.2×10^5	85 ± 15	11 ± 0.6	1.3×10^5	178 ± 13	12 ± 0.3	6.7×10^4
R294Q	68 ± 7	26 ± 1	3.8×10^5	5700 ± 550	25 ± 1	4.3×10^3	380 ± 50	25 ± 1	6.7×10^4

[a] Data shown only for those mutants that were significantly expressed. [b] Chorismate concentrations were varied from 0.025 to 0.80 mM for assaying wild-type (WT), K178Q, R286H, and R294Q enzymes and from 0.05 to 1.2 mM for K178R and R286A. [c] Prephenateconcentrations were varied from 0.025 to 1.0 mM for WT, K178Q, R286H, and R286A and from 0.025 to 8.0 mM for K178R. NAD^+ concentrations were varied from 0.05 to 2.0 mM for WT, K178Q, R286H, and R286A and from 0.20 to 6.0 mM for K178R. For R294Q, linear initial velocity data were obtained by varying prephenate from 0.4 to 16 mM and NAD^+ from 0.05 to 1.0 mM; then the data were fitted to the following equation: $v = VAB/(K_{ia}K_b + K_aB + K_bA + AB)$.

expressed as judged by SDS−PAGE and less effective than wild-type protein at catalyzing both mutase and dehydrogenase reactions. Values of V/KE_t obtained with either chorismate, prephenate, or NAD^+ as the variable substrate were at least 2 orders of magnitude less than the wild-type protein (Table 1). The Michaelis constants for all three substrates were elevated over 10-fold by the mutation, but that for prephenate in the dehydrogenase reaction was altered the most, at 40 times the value for wild-type enzyme. In contrast, V/E_t for the mutase reaction was reduced by a factor of 30, compared to a factor of 6 for that of the dehydrogenase.

Mutants carrying conservative replacements at position 286 (R286K and/or R286Q) were identified preliminarily through screening colonies by restriction analysis (*30*), but their sequences could not be verified, even after multiple trials in different labs. This phenomenon has been noted previously with other site-directed mutants constructed for this enzyme (*15*). In vitro mutase and dehydrogenase assays conducted on crude cell extract derived from these selected colonies indicated that putative mutant proteins were inactive. These replacements may have generated unstable or improperly folded proteins. The replacement of arginine with alanine or histidine at position 286, however, was verified by DNA sequencing, and these mutations yielded proteins whose expression was similar to wild-type mutase−dehydrogenase (data not shown). Both mutants exhibited significant but small changes in the kinetic parameters of the reactions compared to the wild-type enzyme (Table 1). Values of V/E_t for the dehydrogenase and mutase reactions catalyzed by both mutant proteins were reduced by a factor of 2, while the binding of NAD^+ and chorismate to R286A was decreased by 2- and 4-fold, respectively. We concluded that an arginine at this position may be important for full mutase and dehydrogenase activities but was not an essential residue in the binding of prephenate.

The R294K mutant protein was very poorly expressed and was inactive in crude cell extracts (data not shown). In contrast, R294Q protein was obtained in high yield and exhibited kinetic parameters for the mutase reaction similar to wild-type enzyme (Table 1). The dehydrogenase was also active, but only in the presence of high concentrations of prephenate. Analysis of the data obtained by varying both prephenate and NAD^+ yielded the values shown in Table 1. Relative to that of the wild-type dehydrogenase, the Michaelis constant for prephenate for R294Q was increased about 120-fold, while that for NAD^+ was essentially unaffected.

pH Dependence of the Dehydrogenase Reaction. To establish if the ionization state of any of the three conserved cationic residues might affect enzyme activity within the experimentally accessible pH range (pH 5.8−9.5), the pH dependency of the dehydrogenase reaction catalyzed by selected mutant proteins was determined. As a comparison, pH−rate profiles were obtained for the wild-type enzyme, and the pK values of groups involved in substrate binding versus catalysis were established. The pH dependency of the values of V/E_t and $V/K_{prephenate}E_t$ for wild-type dehydrogenase was studied from pH 5.8 to 9.5 by varying the concentration of prephenate at a fixed concentration of NAD^+ that was 15 times greater than its Michaelis constant at every pH. The V/E_t profile approximated a half-bell with slopes of +1 and zero (Figure 3A), indicating that a group must be unprotonated to achieve maximum activity. Fitting the data to eq 6 yielded a pK of 6.63 ± 0.05 for this group (Table 2). In contrast, the $V/K_{prephenate}E_t$ profile was bell-shaped, showing the ionization of two groups associated with prephenate and/or the enzyme−NAD^+ complex (Figure 3A). Fitting the data to eq 5 yielded pK values of 6.31 ± 0.06 and 8.72 ± 0.18 on the acidic and basic limbs, respectively (Table 2). As the ionization of the group of about pK 8.7 is not seen in the V/E_t profile, this implies that the protonated residue must be involved in binding prephenate to the enzyme−NAD^+ complex. The results are in agreement with those previously reported (*10*, *15*).

To determine if the pK values observed in the V/KE_t profile are either true or apparent values, and to establish which ionizable groups in the profiles play a role in substrate binding, we examined the effect of pH on the binding of HPlactate to the wild-type enzyme. HPlactate structurally resembles the product of the dehydrogenase reaction, HP-pyruvate (Figure 2), but is stable over a wide pH range. HPlactate functions as a linear competitive inhibitor with respect to prephenate in the dehydrogenase reaction (data not shown) and gave rise to a bell-shaped K_i profile with limiting slopes of +1 and −1 (Figure 3B). A fit of the data to eq 5 yielded pK values of 6.11 ± 0.06 and 8.86 ± 0.07 on the acidic and basic limbs, respectively (Table 2). These values were comparable to those determined from the $V/K_{prephenate}E_t$ profile and indicated that over this pH range

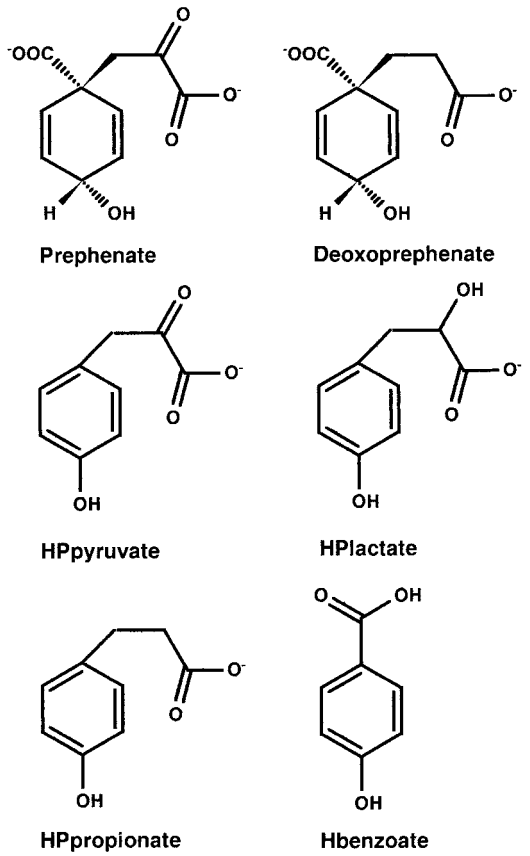

FIGURE 2: Structures of substrates and inhibitory substrate analogues of the prephenate dehydrogenase reaction. Substrates include prephenate and deoxoprephenate, while inhibitory substrate analogues include (4-hydroxyphenyl)pyruvate (HPpyruvate), (4-hydroxyphenyl)lactate (HPlactate), (4-hydroxyphenyl)propionate (HPpropionate), and (4-hydroxy)benzoate (Hbenzoate).

at least two ionizable groups were involved in prephenate binding.

The pH dependencies of $V/K_{prephenate}E_t$ for the dehydrogenase reaction catalyzed by the mutant enzymes R294Q, R286A, and K178Q were determined in order to ascertain if the ionizable binding group with pK of about 8.8 is one of the conserved cationic residues. Particular attention was focused on Arg294, that we have shown plays a role exclusively in the binding of prephenate. Since high concentrations of prephenate were required to monitor saturation kinetics for R294Q, initial velocity data for this mutant were obtained by varying prephenate at three concentrations, one-fifth its Michaelis constant and lower, while keeping NAD^+ saturating. The V/KE_t value at each pH was derived from the slope of the lines. The mutant protein gave rise to a bell-shaped profile (Figure 3A) with pK values similar to those obtained for the wild-type enzyme (Table 2). For the other mutants, reaction rates at only four pH values were used to establish the pH dependence of V/KE_t. pH values of 7.4, 8.4, 8.8, and 9.5 were selected for R286A while values of 7.4, 8.0, 8.4, and 8.8 were chosen for K178Q. (The latter enzyme was unstable at pH 9.5.) The profiles for these mutants (data not shown) were identical in shape to those observed for the wild-type enzyme and R294Q (Figure 3A). Both profiles show a decrease in the value of V/KE_t at high pH, decreasing 0.6 log unit from pH 7.4 to 9.5 (R286A) and 0.5 log unit from pH 7.4 to 8.8 (K178Q).

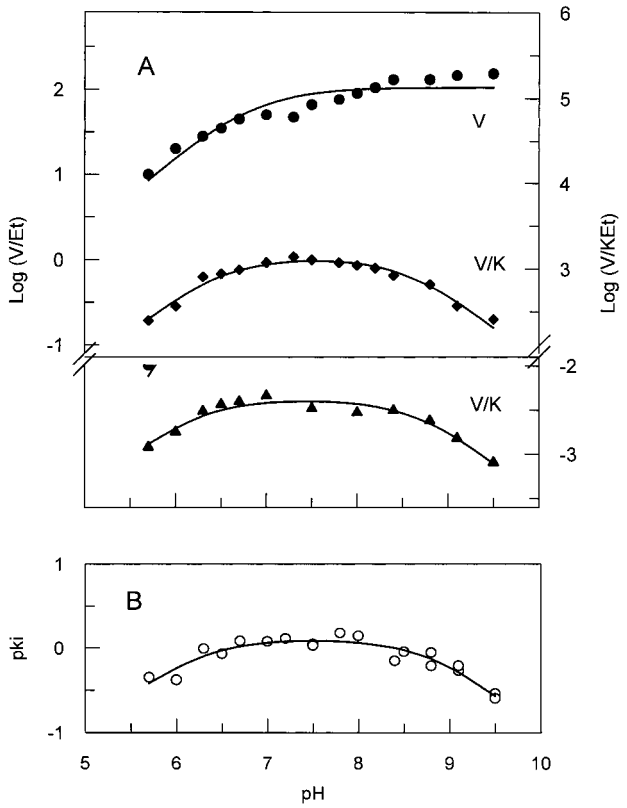

FIGURE 3: Variation with pH of (A) log V/E_t (●) and log ($V/K_{prephenate}E_t$) (◆) for the reaction catalyzed by wild-type prephenate dehydrogenase and of log ($V/K_{prephenate}E_t$) (▲) for the reaction catalyzed by R294Q prephenate dehydrogenase at pH 7.2 and 30 °C. Variation with pH of (B) pK_i (○) for the reaction catalyzed by wild-type prephenate dehydrogenase in the presence of HPlactate. The units for V/E_t , V/KE_t, and K_i are s^{-1}, M^{-1} s^{-1}, and M^{-1}, respectively. The curves for V/KE_t and V/E_t in panel A represent best fits of the data to eq 4 and eq 5, respectively, while the curve for panel B represent best fit of the data to eq 4. The values of the parameters used to draw the curves are given in Table 2.

Steady-State Velocity Patterns in the Presence of Substrate Analogues. Studies were undertaken on the inhibition of the activity associated with wild-type and R294Q prephenate dehydrogenases by analogues of prephenate. This provided information concerning the importance of prephenate's ring carboxylate and side chain carbonyl group in substrate binding. The substrate analogues (shown in Figure 2) possess an aromatic rather than a cyclohexadiene ring characteristic of prephenate, and are missing the ring carboxylate that is lost during the decarboxylation of prephenate. Hbenzoate also lacks the pyruvyl side chain at C-1. HPpropionate is missing the carbonyl group on the side chain of prephenate, while an alcohol replaces the carbonyl group in HPlactate. For the range of concentrations over which prephenate was used, these inhibitors gave rise to linear competitive inhibition with respect to prephenate (data not shown). Analysis of the data yields the true values of the dissociation constants for the interaction of the enzyme−NAD^+ complex with inhibitor (Table 3).

Circular Dichroism Spectroscopy and Equilibrium Denaturation of Wild-Type and Mutant Dehydrogenases. Far-UV CD was employed as a probe of the secondary structure of the wild-type mutase−dehydrogenase and mutant proteins K178R, K178Q, R286A, and R294Q. Spectra obtained between 250 and 200 nm were identical in shape for all of

Table 2: Values for p*K*s and pH-Independent Kinetic Parameters for the Reaction Catalyzed by Wild-Type and R294Q Prephenate Dehydrogenases

protein	conditions	parameters determined	pH-independent value of parameter	pK_A	pK_B
wild-type	prephenate varied	$V/K_{pre}\,E_t$ ($\mu M^{-1}\,s^{-1}$)	1.4 ± 0.1	6.31 ± 0.06	8.72 ± 0.18
		V/E_t (s^{-1})	105 ± 5	6.63 ± 0.05	−
	HPlactate varied	K_i (mM)	0.75 ± 0.05	6.11 ± 0.08	8.86 ± 0.06
R294Q	prephenate varied	$V/K_{pre}\,E_t$ ($\mu M^{-1}\,s^{-1}$)	$4.4 \times 10^{-3} \pm 3.5 \times 10^{-4}$	6.14 ± 0.06	8.82 ± 0.07

Table 3: Kinetic Constants for the Interaction of Substrate Analogues with Wild-Type and R294Q Prephenate Dehydrogenases at pH 7.2 and 30 °C[a]

protein	inhibitor	K_i(E−NAD$^+$ + I) (mM)	K_M for prephenate (mM)
wild-type	HPpyruvate	0.18 ± 0.03	0.053 ± 0.006
	HPpropionate	1.8 ± 0.2	0.070 ± 0.006
	HPlactate	0.35 ± 0.06	0.067 ± 0.008
R294Q	HPpyruvate	0.23 ± 0.08	6.6 ± 0.5
	HPpropionate	1.7 ± 0.2	5.5 ± 0.6
	HPlactate	0.66 ± 0.08	5.3 ± 0.5

[a] Data were obtained by varying prephenate at six different concentrations ranging from 0.025 to 0.80 mM (wild-type) and from 0.40 to 10 mM (R294Q), and varying the inhibitor at six different concentrations for both wild-type and mutant ranging from 0.10 to 1.0 mM (HPpyruvate and HPlactate) and from 0.10 to 10 mM (HPpropionate). NAD$^+$ concentration was held constant at 3 mM. Each experiment allowed the independent determination of K_m for prephenate. The constants were determined by fitting the data to eq 2.

the proteins (data not shown). The curves showed a distinct double minimum at 222 and 208 nm, and matched the spectrum expected for an α-helical protein (*36*, *40*, *41*). Moreover, the relative intensities of the peaks were very similar. These findings implied that the amino acid substitutions did not impart any large global changes in the secondary structure of the protein.

To determine if the mutations caused any alterations in the stability of the protein, wild-type and mutant proteins were analyzed further by monitoring the change in the CD signal at 222 nm as a function of the concentration of the denaturant, Gdn-HCl. Urea was used initially as a denaturant, but it was found that the protein did not fully unfold, even at concentrations of 8 M urea. Hence, a stronger denaturant than urea was required. The fraction of unfolded protein, F_u, was calculated as described under Experimental Procedures. The pattern of unfolding of selected proteins in Gdn-HCl is shown in Figure 4, and values of the concentration of denaturant at the midpoint in the transition from folded to unfolded conformation, $D_{1/2}$, are given in Table 4. The patterns of unfolding of wild-type enzyme and R294Q were virtually superimposable, yielding $D_{1/2}$ values of 2.60 M. In contrast, shallower patterns were observed for the other three mutants (only the tracing for K178R is shown in Figure 4); $D_{1/2}$ values were the lowest for K178R (at 2.35 M) followed by R286A (at 2.40 M) and K178Q (at 2.50 M).

Unfolding of the wild-type enzyme was reversible as determined spectroscopically and enzymatically (data not shown). Protein refolded by dialyzing with Gdn-HCl solutions of 0, 1, 2, 3, and 4 M yielded CD signals at 222 nm similar to those obtained at the same concentration during the unfolding process. In addition, greater than 85% of the enzymatic activities were recovered after refolding by dilution.

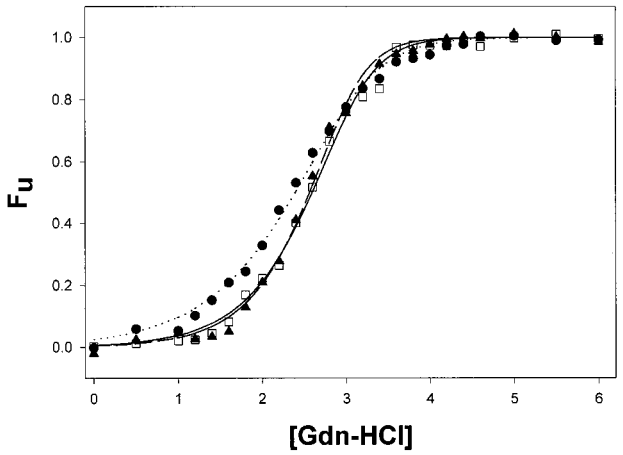

FIGURE 4: Guanidine hydrochloride denaturation of *E. coli* chorismate mutase−prephenate dehydrogenase monitored by circular dichroism at 222 nm. The fraction unfolded (F_u) is plotted as a function of Gdn-HCl concentration for wild-type (□), R294Q (▲), and K178R (●) mutase−dehydrogenase enzymes. Curves are derived from nonlinear least-squares fitting of the data to eq 11 as described under Experimental Procedures.

Table 4: Thermodynamic Parameters for the Unfolding of Wild-Type and Mutant Mutase−Dehydrogenases[a]

protein	$\Delta G_{d,aq}$ (kcal mol^{-1})	m (kcal mol^{-1} M^{-1})	$D_{1/2}$ (M)
wild-type	13.18 ± 0.23	2.14 ± 0.09	2.60
K178Q	11.82 ± 0.16	1.60 ± 0.06	2.50
K178R	10.49 ± 0.09	1.31 ± 0.04	2.35
R286A	11.31 ± 0.13	1.47 ± 0.05	2.40
R294Q	13.65 ± 0.24	2.34 ± 0.09	2.60

[a] Values of $\Delta G_{d,aq}$ and m were calculated by nonlinear regression analysis of unfolding data using eq 11 (see Experimental Procedures). Values of $D_{1/2}$ were estimated from the concentration of denaturant at half-maximal ellipticity.

To estimate the conformational stability of wild-type enzyme and these selected mutants, the free energy of unfolding was determined by assuming the following two-state model:

$$N_2 \rightleftarrows 2U$$

where N_2 is the native dimer and U is the denatured monomer. The data points of F_u versus Gdn-HCl concentration followed closely but not identically with the theoretical curves (see examples in Figure 4), indicating that a simple two-state model of unfolding may not be entirely accurate for the dimeric mutase−dehydrogenase. Nonetheless, analyzing the data for all proteins in the same manner allowed us to establish some comparative values between wild-type and mutant enzymes. F_u was fitted to eq 11 for wild-type and mutant enzyme data sets to yield the curves shown in Figure 4 and the values for the following parameters (Table 4):

$\Delta G_{u,aq}$, which represents the difference in free energy between the native and the denatured state of the protein in the absence of denaturant; *m*, which reflects the slope of the unfolding curve in the transition region. The thermodynamic parameters, listed in Table 4, indicated that K178R is the least stable of all the proteins examined.

We attempted to use tryptophan fluorescence as a probe to detect tertiary structural changes upon unfolding the wild-type dimeric protein. Unfortunately, the protein emission spectra recorded at different concentrations of Gdn-HCl (data not shown) were very complex, reflecting the likelihood that the environments of several of the four tryptophan and nine tyrosine residues were changing during the unfolding process.

Since mutase−dehydrogenase exists in solution as a noncovalently linked dimer (*8*), the possibility arises that the denaturation from fully folded dimer to fully unfolded monomer can proceed through one or more stable intermediates, such as a compact folded monomer and/or a more loosely folded dimer. We have used Gdn-HCl gradient size exclusion chromatography to investigate the oligomeric state of the wild-type enzyme during the unfolding process. Native protein was applied to the gel filtration column at specific time intervals during the gradient run from 6 to 0 M denaturant, and the appearance of peaks corresponding to the fully unfolded monomer, the native dimer, and any intermediate form(s) was monitored. On a gel filtration column, it is expected that a fully unfolded monomer should elute earlier than the compact folded dimer. However, a compact structured monomeric intermediate should elute later than the dimeric protein (*36*). When wild-type mutase−dehydrogenase was analyzed by this techniques (see Experimental Procedures), there was no evidence of a species eluting following the native dimer at any Gdn-HCl concentration. Instead, a peak (presumably corresponding to a partially unfolded dimer) appeared between that of the intact dimer and the fully unfolded monomer. Moreover, the unfolded monomeric form appears only at a Gdn-HCl concentration greater than about 3.5 M. These results imply that the quaternary structure of the mutase−dehydrogenase is very stable and that the dimeric protein unfolds significantly before subunit separation. Moreover, these results, taken together with CD data for the unfolding of the protein's secondary structure, suggest that a two-state model is a reasonable approximation of the unfolding (see Discussion).

DISCUSSION

The purpose of this investigation was to identify groups of *E. coli* prephenate dehydrogenase or the substrate prephenate that are essential for binding prephenate at the enzyme active site. Toward this goal, we conducted site-directed mutagenesis on Lys178, Arg286, and Arg294, the only arginine and lysine residues that are conserved among nine mono- and bifunctional prephenates (Figure 1), and that might interact with the negatively charged carboxyl groups of prephenate. We compared wild-type enzyme and selected mutant proteins with respect to their stability, pH−rate profiles, and ability to bind prephenate and a series of inhibitory substrate analogues carrying substitutions at C-1. Our conclusions are summarized in Figure 5: (i) Arg294 interacts electrostatically with the ring carboxylate of prephenate. (ii) A protonated residue in the dehydrogenase (p*K* of

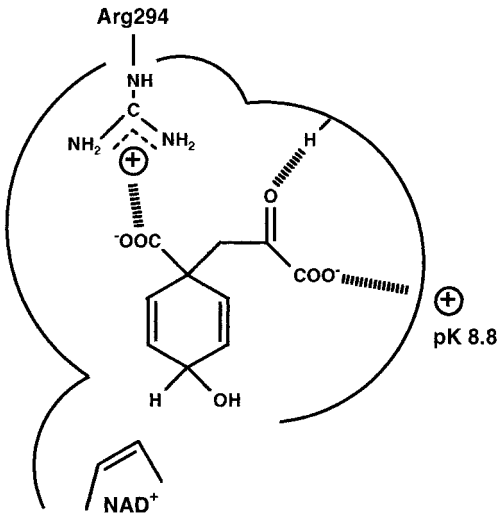

FIGURE 5: Possible interactions of prephenate with prephenate dehydrogenase.

8.8) interacts with the carboxyl group on the pyruvyl side chain of prephenate. However, this residue is not one of the three conserved residues under study. (iii) The carbonyl group on the pyruvyl side chain of prephenate contributes to substrate binding by hydrogen bonding to an enzymic group, but it is unlikely that this enzymic group is Arg286 or Lys178. We also show that replacing lysine for an arginine at position 178 dramatically affects the kinetic parameters of both the mutase and the dehydrogenase reactions, as well as the stability of the enzyme, through steric effects.

Role of Arg294 in Prephenate Binding. To address the importance of R294 in prephenate binding, two mutant proteins R294K and R294Q, were examined. Although the positive charge was conserved in the lysine substitution, the resulting mutant (R294K) was inactive and poorly expressed in a crude cell extract. This result was not too surprising given our findings that K178R was also poorly expressed. Moreover, we have shown previously that *E. coli* mutase−dehydrogenase can be destabilized by other "conservative" point mutations (*15*). The R294Q mutant, by contrast, yielded a highly expressed and active mutase−dehydrogenase. Furthermore, the binding of prephenate was dramatically reduced (by a factor of about 120) while interactions of the mutant with NAD^+ in the dehydrogenase reaction and chorismate in the mutase reaction were unchanged. In addition, the maximum velocities of both the dehydrogenase and mutase reactions as catalyzed by R294Q were unaltered relative to the wild-type enzyme. This implies that Arg294 plays a role specifically in the binding of prephenate as opposed to the binding of other substrates or in catalysis. To verify this hypothesis and to rule out the probability that the mutation grossly distorts the prephenate binding site, we monitored the unfolding of R294Q in Gdn-HCl. As expected, the pattern of unfolding (Figure 4) and the thermodynamic parameters of unfolding for R294Q (Table 4) were nearly identical to the wild-type enzyme.

Our findings suggest that Arg294 interacts electrostatically with the ring carboxylate of prephenate. The group on the substrate interacting with Arg294 was identified by comparing the Michaelis constant for prephenate in the dehydrogenase reaction and the inhibition constants for the substrate analogues HPpyruvate, HPlactate, and HPpropionate for both

wild-type and R294Q enzymes. The results in Table 3 show that for R294Q, the Michaelis constant of prephenate is markedly increased, while the dissociation constants for the three substrate analogues remain essentially unchanged compared to wild-type dehydrogenase. These inhibitors all possess common structural features; they are aromatic, and lack the ring carboxylate at C-1. The 120-fold increase in the Michaelis constant for prephenate represents a ΔG of about 3 kcal/mol, a value consistent with the loss of an ion pair interaction between enzyme and substrate (*42*).

Hermes et al. (*19*) have shown that the oxidative decarboxylation of prephenate to give HPpyruvate proceeds with decarboxylation and hydride transfer in the same transition state. These authors hypothesized that the two steps are concerted because the ring carboxyl group may lie in a hydrophobic pocket, thus promoting decarboxylation. We speculate that Arg294 resides in this hydrophobic pocket and has its positively charged guanidino group extended to facilitate an electrostatic interaction with the ring carboxylate of prephenate (Figure 5). It is noteworthy that eight residues in the primary sequence surrounding Arg294 are hydrophobic (LAMVGRLFA) (*11*). Moreover, prephenate analogues lacking the ring carboxyl group bind significantly better to R294Q than does the substrate, since neither electrostatic partner (the guanidino group of Arg294 or the carboxyl group of the inhibitor) is present. The hypothesis is also consistent with the results from other mutations at this position. We were unable to isolate an active mutant by replacing Arg294 with a lysine residue. Although also hydrophobic, lysine's side chain is more flexible than that of arginine, and the positive charge of the ϵ-amino group is more localized (*43*). These properties may somehow facilitate an unfavorable interaction between lysine and its environment and destabilize the protein. In contrast, the glutamine substitution did not affect enzyme expression or stability; as an uncharged residue, it might interact more favorably with the other residues in a hydrophobic region.

pH Dependency of the Dehydrogenase Reaction. The pH dependence of the kinetic parameters V/E_t and $V/K_{prephenate}E_t$ for the dehydrogenase reaction as performed in this study and others (*10, 15, 19*) indicates that a protonated group with a p*K* value of about 8.8 is essential for the binding of prephenate to the enzyme−NAD^+ complex. However, this hypothesis has never been verified by examining the binding of competitive inhibitors of prephenate, nor has the identity of this residue been examined. The results from the present investigation confirm this hypothesis but also indicate that this protonated group is not any of these three conserved cationic groups. The $V/K_{prephenate}E_t$ profiles for wild-type and R294Q enzymes are identical (Figure 3), indicating that the residue titrating with a p*K* of 8.8 is still present when this arginine is missing. Similarly, preliminary pH−rate profiles for K178Q and R286A show activity falling off at high pH values, thus eliminating Arg286 and Lys178 as the binding group with p*K* of 8.8. The p*K* values of these conserved enzymic groups are likely greater than 9.5 and outside the experimentally accessible pH range of the dehydrogenase reaction. There are other groups in the dehydrogenase portion of the protein that can be positively charged between pH 6 and 9.5, for example, histidine. However, alanine/asparagine scanning mutagenesis of the nine histidine residues in the enzyme did not identify mutant proteins having Michaelis constants for prephenate that were significantly and selectively elevated (*15*). Hence, these results tend to eliminate a protonated histidine as this binding group. It may be that the role is fulfilled by one of the nine tyrosine groups, or by a nonconserved cationic residue.

To verify that the group with p*K* of 8.8 is involved in the binding of prephenate to the wild-type enzyme, the pH dependence of the K_i for HPlactate, an inhibitory substrate analogue of prephenate, was examined. Since profiles of the pK_i (HPlactate) and $V/K_{prephenate}E_t$ are identical (Figure 3), and as HPlactate is not a substrate, it follows that the ionizable group with a p*K* value of 8.8 observed in the V/KE_t profiles is also a binding group. p*K* values derived from the V/KE_t profile can be displaced upward or downward from their true values if the binding of the substrate to the enzyme is not at equilibrium relative to the catalytic step. However, the pH dependence of pK_i for a competitive inhibitor of the substrate will yield the true p*K* value of ionizing residues in the V/KE_t profile (*44*). The V/KE_t profile for prephenate and the K_i for the binding of HPlactate were identical, yielding a p*K* value on the basic limb of 8.8 (Table 2). Therefore, we can conclude that the p*K* of this binding residue observed in the V/KE_t profile represents a true value.

Both the V/E_t and $V/K_{prephenate}E_t$ profiles for the wild-type dehydrogenase reaction show that the activity falls off at low pH. It has been suggested that the acidic limb reflects a single catalytic group titrating in the binary complex (V/KE_t) with a p*K* of 6.3, and in the ternary complex (V/E_t) with a p*K* of 6.6 (*10, 15, 19*). Surprisingly, the K_i profile for HPlactate presented in this study indicates that an unprotonated group with a p*K* of about 6.1 is also involved in binding the inhibitor, and, by inference, prephenate to the enzyme−NAD^+ complex (Figure 3, Table 2). It may be that the catalytic group is also involved in prephenate binding. However, our previous studies (*15*) do not support this premise, since removal of the putative catalytic base by site-directed mutagenesis (H197N) does not alter prephenate binding. Instead, our data support the idea that two ionizable groups are titrating in the acidic limb of the V/KE_t profile and possess similar p*K* values—one group is involved in binding (p*K* ~6.1) and the other, solely in catalysis (p*K* ~6.3). Regrettably, it was not possible to obtain more data points much below pH 6 to yield a good fit to a model accommodating two titrating groups on the acidic limb because the enzyme is unstable below this pH (*6*).

What group on prephenate interacts with the enzymic residue possessing a p*K* value of about 8.8? The bell-shaped pH dependence for the binding of HPlactate implies that it is not exclusively the ring carboxylate of prephenate since HPlactate does not possess such a group. However, the K_i profile does not allow us to distinguish between the importance of the carbonyl or the carboxylate associated with the pyruvyl side chain of prephenate. HPlactate possesses the side-chain carboxylate group, and the carbonyl group is replaced with an alcohol. Hermes et al. (*19*) showed, however, that the alternate substrate, deoxoprephenate (which lacks the carbonyl on the side chain but retains the ring carboxylate, Figure 2), also yields a bell-shaped V/KE_t profile similar to that for prephenate. Together, these findings implicate the pyruvyl carboxylate as an electrostatic partner with the protonated enzymic residue of p*K* 8.8. These findings will only be confirmed by additional site-directed

mutagenesis studies, by the synthesis of other inhibitory prephenate analogues, and/or by the elucidation of the crystal structure of *E. coli* mutase−dehydrogenase.

Binding of Substrate Analogues. We have examined the dissociation constants for the interaction of the dehydrogenase with inhibitory substrate analogues shown in Figure 2, to draw conclusions concerning the interaction of prephenate with the enzyme. Hbenzoate bound very poorly to the dehydrogenase (K_i > 20 mM), indicating that the pyruvyl side chain on C-1 of prephenate is essential for substrate binding (data not shown). This observation is consistent with other studies (*16, 17*) showing that some short-chain dicarboxylic acids bind with low millimolar affinity to the enzyme, presumably through the carboxylate groups of the inhibitors.

The oxidative decarboxylation of prephenate yields the aromatic product HPpyruvate that binds remarkably well to the dehydrogenase. Its dissociation constant at 180 μM (Table 3) is only 3-fold higher than the Michaelis constant for prephenate, corresponding to a ΔG of about 0.3 kcal/mol. Since HPpyruvate no longer possesses the ring carboxylate that is proposed to interact with the enzyme through an ion pair with Arg294, the increase in free energy associated with the removal of this electrostatic interaction is at least 10-fold less than the predicted range of about 3−10 kcal/mol (*42*). In contrast but as expected, eliminating the interaction between Arg294 and the ring carboxylate of prephenate by an enzymic change (R294Q) yielded a ΔG of about 3 kcal/mol. These findings imply that the dehydrogenase active site is optimized to bind the aromatic ring of the product rather than the cyclohexadiene ring of the substrate. It is worth noting that the end-product of the pathway, tyrosine, is also aromatic and inhibits the dehydrogenase reaction at low micomolar concentrations (*7, 9, 10*) although it remains to be established conclusively if tyrosine binding can occur at the prephenate binding site in addition to an allosteric site.

The presence of an alcohol on the pyruvyl side chain (HPlactate) rather than a carbonyl group (HPpyruvate) does not alter binding interactions. However, removing the side chain carbonyl group of HPpyruvate to yield HPpropionate weakens the interaction by a factor of 10 (ΔG ~1.5 kcal/mol). This is consistent with the loss of a hydrogen bond between the carbonyl or hydroxyl oxygen on the side chain of the inhibitor (acceptor) and an enzymic residue (donor). Entropic factors may also contribute to the reduced binding since HPpropionate's side chain possesses an additional degree of freedom compared to that of HPpyruvate. The results of Hermes et al. (*19*) are also in agreement with our findings. They showed that by removing the carbonyl group from the side chain of prephenate, the binding of the resulting analogue, deoxoprephenate (as a substrate or as an inhibitor), was reduced by 4-fold or 12-fold, respectively. It is unlikely that Lys178 or Arg286 fulfills the enzymic role as an essential hydrogen bond donor since K178Q and R286A mutant proteins bind prephenate with almost the same affinity as the wild-type enzyme. Additional valuable information could have been obtained by examining the interaction of mutant proteins with cyclohexadiene-containing molecules that possess both the ring and the side chain carboxyl groups. However, neither deoxoprephenate nor the inhibitory prephenate analogue, carboxyethyldihydrobenzoate, both of which fulfill these requirements, was available.

Stability of Wild-Type and Mutant Enzymes. In this report, we present the first biophysical study examining the stability of wild-type and mutant forms of the bifunctional *E. coli* enzyme chorismate mutase-prephenate dehydrogenase. It is important to provide some structural assessment of mutant proteins, particularly for those mutants that have markedly different kinetic parameters and/or levels of expression compared to the wild-type protein. Moreover, we show that the least stable mutants are those that exhibit a functional loss of both mutase and dehydrogenase domains. Our point is well illustrated in the case of K178R. Our kinetic studies indicated that this mutation dramatically reduced V/KE_t values for both mutase and dehydrogenase reactions (Table 1). Despite these changes in catalytic efficiency, the far-UV CD spectra of wild-type and K178R proteins indicated that this mutation did not impart any global conformational changes in the protein. However, the CD signal in the far-UV region does not accurately detect tertiary structural changes in a molecule (*36, 37*). Instead, analysis of the unfolding of the backbone of K178R and wild-type enzyme in Gdn-HCl clearly highlighted the differential stability between the two proteins (Table 4). Our results show that K178R unfolds at a Gdn-HCl concentration 10% lower than the wild-type enzyme and is destabilized relative to the wild-type enzyme by about 3 kcal/mol. Moreover, a decrease in the slope of the transition curves, as reflected by a decrease in the value of m from about 2.2 to 1.3 kcal mol^{-1} M^{-1}, indicates that the native mutant enzyme is more loosely structured and tends to unfold over a wider range of denaturant concentrations compared to wild-type enzyme (*45*). Hence, this mutation likely affects the folding and catalytic efficiency of all three "domains", that is, the site of binding for chorismate, for prephenate and for NAD^+. The finding that K178R was poorly expressed is also consistent with these results. In contrast to K178R, the expression, kinetic parameters, and stability of K178Q mutant protein were reasonably similar to the wild-type enzyme. The unexpectedly low value of m (about 1.5 kcal mol^{-1} M^{-1}, Table 4) may reflect the fact that data for F_u for this mutant were more scattered in the transition region compared to the wild-type enzyme (data not shown). In general, the data imply that the positive charge at this position is not critical for the proper folding of the protein or for the chemistry of the reaction. Hence, the destabilization of K178R is a consequence of steric effects elicited by the substitution of arginine, a positively charged but larger amino acid than lysine. The global destabilization caused by the arginine substitution at a position that is clearly in the dehydrogenase domain of the bifunctional enzyme (*11*) is also manifested in the kinetics of the mutase reaction. The efficient acceleration of the pericyclic rearrangement in the mutase reaction is dependent on the precise arrangement of electrostatic residues in the mutase active site (*46*). Subtle structural changes may displace these strategically placed residues, thereby reducing the catalytic efficiency of the enzyme. V/E_t for the mutase reaction catalyzed by K178R was only 3% that of the wild-type enzyme (Table 1). R286A is also quite unstable relative to the wild-type enzyme ($\Delta\Delta G_{u,\,aq}$ ~ 2 kcal/mol, $\Delta D_{1/2}$ ~ 0.2 M), although not as marked as K178R. Structural changes of R286A are also manifested through decreased catalytic efficiencies for both mutase and dehydrogenase reactions (Table 1). However, in this case, the

binding of NAD^+ to the nucleotide binding pocket, rather than the binding of prephenate to its site, is what brings about the reduced V/KE_t values for the dehydrogenase reaction.

The results of gel filtration indicate that unfolding of the wild-type enzyme is not a simple two-state process but is more accurately described by the following scheme:

$$N_2 \rightleftarrows I_2 \rightleftarrows 2U$$

where denaturation of the native dimer (N_2) involves at least one stable intermediate (a partially unfolded dimer, I_2), which then dissociates to unfolded monomers (2U). However, the following two points indicate that the unfolding approximates a simple two-state model closely enough to allow a preliminary estimate of the thermodynamic parameters of unfolding (Table 4). First, gel filtration results confirm that the unfolded monomer is not present in detectable amounts below about 3.5 M Gdn-HCl. At this concentration of denaturant, the enzyme has lost >90% of its secondary structure (Figure 4). Hence, it is likely that the secondary structure of the putative intermediate resembles that of the unfolded monomer. Second, the intermediate is not a predominant species as judged by far-UV CD. Thus, the polypeptide backbone of mutase−dehydrogenase appears to unfold in a single step. The conformational stability of the wild-type bifunctional enzyme, at ∼13 kcal/mol, is within the range documented for other dimeric proteins (∼9−28 kcal/mol) (*37*). A value of ∼40 kcal/mol has recently been reported for the remarkably stable dimeric organophosphorus hydrolase (*47*). The value we obtain, however, is lower than that for the mutase domain of *E. coli* mutase-dehydratase (∼20 kcal/mol) or for the chorismate mutase from the thermophile *Methanococcus jannaschii* (∼26 kcal/mol) (*36*). As our study is only preliminary, a more thorough examination of the unfolding of the bifunctional enzyme is required before quantitative comparisons can be established between the mutases from different sources. Our results indicate qualitatively, however, that interchain interactions make significant contributions to the global stability of the bifunctional mutase−dehydrogenase. Thus, the unfolding of the bifunctional *E. coli* enzyme resembles that of the monofunctional chorismate mutase from *M. jannaschii* (*36*). This dimeric mutase also appears to unfold by a process involving a partially unfolded dimer.

In summary, we have identified that the conserved residue, Arg294, plays a specific role in the binding of prephenate by interacting with the ring carboxyl group of the substrate. An unidentified protonated residue (p*K* 8.8) likely interacts with the side chain carboxylate of prephenate. In addition, we have established conditions that allow more extensive investigations of the conformational stability of *E. coli* mutase−dehydrogenase.

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