Use of Site-Directed Mutagenesis To Identify Residues Specific for Each Reaction Catalyzed by Chorismate Mutase−Prephenate Dehydrogenase from *Escherichia coli*†

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ABSTRACT: Site-directed mutagenesis was performed on the bifunctional enzyme chorismate mutase−prephenate dehydrogenase in order to identify groups important for each of the two reactions. We selected two residues for mutagenesis, Lys37 and His131, identified previously by differential peptide mapping to be essential for activity [Christendat, D., and Turnbull, J. (1996) *Biochemistry 35*, 4468−4479]. Kinetic studies reveal that K37Q exhibits no mutase activity while retaining wild-type dehydrogenase activity, verifying that Lys37 plays a key role in the mutase. By contrast His131 is not critical for the dehydrogenase; H131A is a reasonably efficient catalyst exhibiting 10% dehydrogenase and 30% mutase activity compared to the wild-type enzyme. Chemical modification of H131A by diethyl pyrocarbonate further inactivated the dehydrogenase, suggesting that a different histidine is now accessible to modification. To identify this group, the protein's remaining eight histidines were changed to alanine or asparagine. A single substitution, H197N, decreased the dehydrogenase activity by 5 orders of magnitude while full mutase activity was retained. In H197N, the Michaelis constants for prephenate and NAD^+ and the mutant's elution profile from Sepharose−AMP were similar to those of wild-type enzyme, indicating that catalysis rather than substrate binding is altered. Log *V* for the dehydrogenase reaction catalyzed by H197N is pH-independent and is in contrast to wild-type enzyme, which shows a decrease in activity at low pH and p*K* of about 6.5. We conclude that His197 is an essential catalytic residue in the dehydrogenase reaction.

Chorismate mutase−prephenate dehydrogenase is a bifunctional enzyme that catalyzes two sequential reactions in the tyrosine biosynthetic pathway in *Escherichia coli* and other enteric bacteria (*1*, *2*). These reactions (Scheme 1) involve the rearrangement of chorismate **1** to prephenate **3** and, in the presence of NAD^+ ,[1] the oxidative decarboxylation of prephenate to (4-hydroxyphenyl)pyruvate **4**. Through the action of an aromatic aminotransferase, the product **4** undergoes conversion to tyrosine, which acts as an end-product inhibitor of both activities (*3*−*5*).

The enzyme from *E. coli* is homodimeric with a molecular weight of 84 000 (*6*−*8*). Alignment of the primary sequence of chorismate mutase−prephenate 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 encodes the mutase activity (*7*, *9*). Crystal structures for the chorismate mutase domain of the *E. coli* mutase−dehydratase ("minimutase") (*10*) and of the monofunctional mutases from *Saccharomyces cerevisiae* (*11*) and *Bacillus subtilis* (*12*) show that the active sites of these mutases are electrostatically similar even though there is no resemblance in overall secondary or tertiary structure. The structure of the minimutase complexed with *endo*-oxabicyclic diacid **5** (Figure 1), a compound that mimics the chairlike transition state **2** (*13*, *14*), has provided valuable insights as to the amino acid residues that may participate in the pericyclic rearrangement of chorismate to prephenate (*10*, *15*). Moreover, the active site residues in the minimutase are well conserved in the mutase portion of *E. coli* mutase−

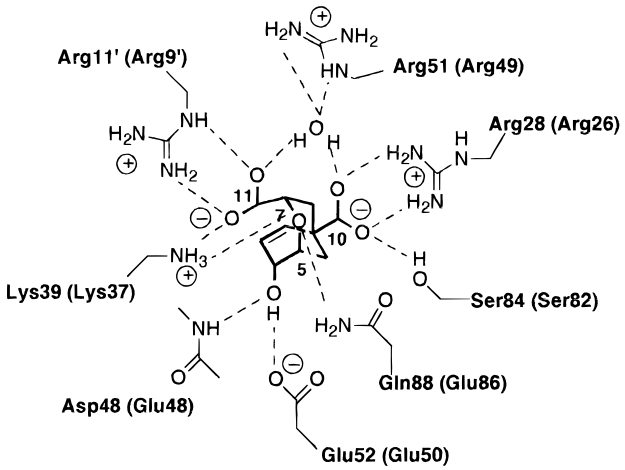

FIGURE 1: Active site of the independently expressed chorismate mutase from *E. coli* mutase−dehydratase bound to the transition-state analogue **5**; adapted from Lee et al. (*10*). Analagous residues for the mutase portion of chorismate mutase−prephenate dehydrogenase are shown in parentheses.

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[1] Abbreviations: CD, circular dichroism; DEPC, diethyl pyrocarbonate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; *endo*-oxabicyclic diacid, (3-*endo*-8-*exo*)-8-hydroxy-2-oxabicyclo[3.3.1]-non-6-ene-3,5-dicarboxylic acid; IPTG, isopropyl β-D-thiogalactoside; MES, 2-(*N*-morpholino)ethanesulfonic acid; NAD^+, nicotinamide adenine dinucleotide; SDS−PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

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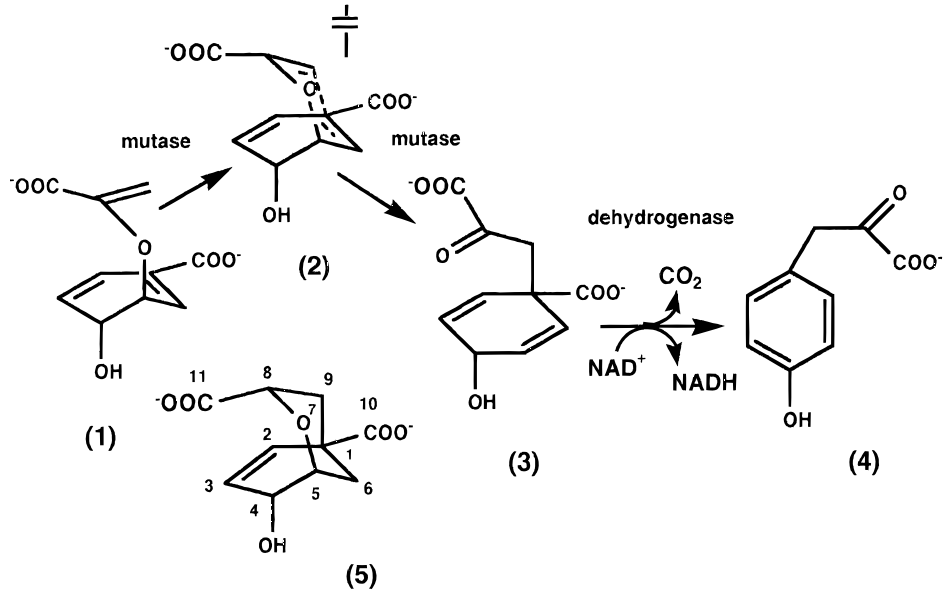
Scheme 1: Reactions Catalyzed by Chorismate Mutase−Prephenate Dehydrogenase



dehydrogenase (*9*, *10*). The importance of many of these active-site groups, which include Lys39, Gln88, Glu52, and arginine residues at positions 11 and 28 in the minimutase and homologous positions in the other monofunctional mutases, has been confirmed recently by site-directed mutagenesis (*16*−*21*). To date, there have been no mutagenesis studies reported for the bifunctional mutase−dehydrogenase.

The kinetic mechanism of the prephenate dehydrogenase reaction conforms to a rapid equilibrium, random mechanism with catalysis as the rate-limiting step (*22*), while hydride transfer and decarboxylation occur in a concerted manner (*23*). Studies of the pH dependence of the kinetic parameters *V* and *V/K* for the dehydrogenase reaction have indicated that an unprotonated group is required for catalysis while a protonated residue is required for binding prephenate to the enzyme−NAD^+ complex (*23*, *24*). The results from temperature and solvent perturbation studies (*23*) indicate that this catalytic group may be a histidine. These mechanstic studies have led to the hypothesis that the histidine could accept a hydrogen bond from the 4-hydroxyl group of prephenate to facilitate hydride transfer to NAD^+ (*23*, *24*). Our recent chemical modification studies with diethyl pyrocarbonate (DEPC) provided further evidence that a histidine residue is essential for dehydrogenase activity (*25*).

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. While there is some evidence derived from kinetics (*26*, *27*) and protein chemistry studies (*7*, *28*) in favor of a single combining site, more recent studies support the idea of two separate active sites. The two activities show different pH−rate profiles (*24*) and are inhibited to different degrees by tyrosine (*5*) and selected substate analogues (*28*, *29*). Moreover, the putative transition-state analogue, *endo*-oxabicyclic diacid **5**, is a very selective inhibitor of mutase activity (*28*, *30*). In our previous paper (*25*), we used a combination of chemical modification and mass spectrometry of peptides from proteolytic digests of the bifunctional enzyme to identify two residues, one of which is important for each activity. These studies indicated that Lys37 and His131 may play a role in the mutase and dehydrogenase reactions, respectively. Moreover, protection studies support the hypothesis that these groups are at or near the active sites and that the sites are distinct.

In this study, we have constructed mutant proteins of Lys37 and His131 as well as eight other histidine residues within the dehydrogenase portion of the protein. Steady-state kinetic analysis of the mutant proteins confirms that Lys37 plays a key catalytic role in the mutase reaction, while it is His197 rather than His131 that plays a role as an essential hydrogen-bond acceptor in the dehydrogenase reaction. Our results are in keeping with the observation that Lys37 and His197 are highly conserved in mutases and dehydrogenases whose primary sequences have recently been reported (*9*, *31*−*35*).

EXPERIMENTAL PROCEDURES

Materials. Chorismate was isolated from *Klebsiella pneumonia* 62-1 as described by Rieger and Turnbull (*36*), while prephenate was prepared as outlined by Dudzinski and Morrison (*37*). NAD^+ was obtained in free acid form from Boehringer Mannheim. DEPC was obtained from ICN Biochemicals. HPLC-grade TFA and acetonitrile were purchased from Baker. Trypsin was obtained from Sigma while T4 DNA ligase, T4 DNA polymerase, and restriction enzymes were purchased from Pharmacia Biotech or MBI Fermentas. DNA sequencing was performed with the Sequenase version 2 DNA sequencing kit from U.S. Biochemical Corp. by the dideoxy chain-termination method (*38*). The *TyrA* gene, which codes for mutase−dehydrogenase, was cloned from the plasmid pKB45 (*39*) into an inducible expression vector pSE380 (Invitrogen) by the polymerase chain reaction (PCR). The resulting plasmid, pVIV1, produces mutase−dehydrogenase that is free from contaminating mutase−dehydratase activity and with a yield that is about 10-fold higher per gram wet weight of cells than reported earlier (*8*).

Site-Directed Mutagenesis. The mutagenesis of *TyrA* in pSE380 was based on the elimination of a unique site in the

Table 1: Oligonucleotides Used for Site-Directed Mutagenesis

Mutants	Oligonucleotide Sequences[a]
K37A	5'-GGC GAG GTG **GCG** AGC CGT TTG G-3'
K37Q	5'-GGC GAG GTG **CAG** AGC CGT TTG G-3'
H131A	5'-CTG GAG CAA **GCG** GAC TGG GAT CG-3'
H153A	5'-GTG CCA ATC **GCG** GTT ACT GAG C-3'
H153N	5'-GTG CCA ATC **AAC** GTT ACT GAG C-3'
H189A	5'-G CTG GTG GCG **GCG** GAT GGT CCG G-3'
H189N	5'-G CTG GTG GCG **AAC** GAT GGT CCG G-3'
H197A	5'-G CTG GGG CTA **GCG** CCG ATG TTC G-3'
H197N	5'-G CTG GGG CTA **AAC** CCG ATG TTC G-3'
H238A	5'-GCT CGG CTG **GCG** CGT ATT AGC G-3'
H238N	5'-GCT CGG CTG **AAC** CGT ATT AGC G-3'
H245A	5'-GCC GTC GAG **GCG** GAT CAG AAT ATG G-3'
H245N	5'-GCC GTC GAG **AAC** GAT CAG AAT ATG G-3'
H257A	5'-GCA CTG CGC **GCG** TTT GCT ACT TTT GC-3'
H265A	5'-GCT TAC GGG CTG **GCG** CTG GCA GAA G-3'
H347A	5'-GC AAG GTG GAG **GCG** TGG TTC GGC-3'
H347N	5'-GC AAG GTG GAG **AAC** TGG TTC GGC-3'
U.S.E.[b]	5'-CAT GTA CAG AGC **G**CG AGA AGT AC- 3'

[a] The mutated nucleotides are underlined. [b] U.S.E. eliminates a unique *Xho*I site in the multiple cloning region of pSE380.

plasmid (*40*). The procedure employs two mutagenic oligonucleotide primers. One primer contains the desired mutation and the second contains a mutation for the unique nonessential restriction site, *Xho*I, that is downstream of *TyrA* in pVIV1 (see Table 1). Mutants were initially screened for elimination of the unique restriction site. Plasmids that had the mutation at the unique site were then sequenced to confirm the presence of the desired mutation. The correct nucleotide sequence was verified for all mutants listed in Table 1 except for H153A, H197A, and H245A. Regrettably, the plasmid DNA from constructs for these mutations could not be sequenced, even after several attempts. We hypothesize that these mutations were very detrimental to the cell resulting in modified, unsequenceable DNA. No further work was done with these constructs.

Purification and Expression of Wild-Type and Mutant Mutase−Dehydrogenase. Mutase−dehydrogenase was purified by the method of Turnbull et al. (*8*) but with several modifications. Wild-type enzyme was expressed in *E. coli* strain XL2-Blue (Novagen) following transformation by pVIV1. The resulting strain was grown at 37 °C in Luria−Bertani (LB) medium containing 50 μg/mL ampicillin to an $OD_{600\ nm}$ of 0.8 before induction with 0.5 mM IPTG. The cells were harvested 4 h after induction with a yield of 11 g wet weight/L of culture and were kept frozen at −20 °C until required. Cells were suspended in a buffered solution containing lysozyme and disrupted by repeated freeze−thawing cycles (*41*) followed by sonication with 6 × 30 s bursts for every 10 g of cells. The lysate was centrifuged at 14000*g* for 15 min and then subjected to ammonium sulfate fractionation (32−50%) as described elsewhere (*8*). The enzyme mixture was dialyzed for 24 h against two changes of 2 L each of buffer B containing 0.1 M *N*-ethylmorpholine, pH 7.4, 1 mM EDTA, 1 mM DTT, 1 mM sodium citrate, and 10% (v/v) glycerol. The dialysate was centrifuged at 14000*g* for 15 min at 4 °C and the supernatant was applied to a Q-Sepharose Fast Flow column (2.6 × 15 cm) equilibrated with buffer B. After the column was washed with five volumes of buffer B, mutase−dehydrogenase was eluted with a linear gradient of 0−0.25 M KCl in the same buffer. Enzymatically active fractions were pooled, dialyzed, and chromatographed on Sepharose−AMP by the method of Turnbull et al. (*8*) with the following modifications: the pH of the dialysate was maintained at 7.5 rather than adjusted to pH 6 prior to chromatography, and the protein was eluted with a 0−1.5 M KCl gradient in buffer B. The enzyme preparation was over 95% pure as judged by Coomassie Blue-stained SDS−PAGE (*42*). The plasmid pVIV1 housing specific site-directed mutations in *TyrA* was transformed into either strain XL2-Blue or into KB357 (*43*), an *E. coli* strain with the genes disrupted for mutase−dehydrogenase and mutase−dehydratase. Mutant mutase−dehydrogenases were expressed and purified as previously described for the wild-type enzyme except the Sepharose−AMP column was omitted. The purity of the final enzymic preparations was checked by SDS−PAGE with Coomassie Blue staining and was found to be over 90%. Wild-type and mutant proteins were most stable when stored as an ammonium sulfate precipitate at 4 °C and then dialyzed just prior to use in the appropriate buffer. To test whether the expression of mutants (whose sequences were verified) could be detected in the host cells, crude cell extracts made from mutant and wild-type cells were screened first by running nondenaturing gels and staining with Coomassie Blue. Selected mutants were further screened by Western Blot analysis (*44*). For immunoblotting, proteins were separated by SDS−10% PAGE, transferred to a nitrocellulose membrane, and probed with rabbit anti-mutase−dehydrogenase antiserum followed by reaction with goat anti-rabbit antibody linked to horseradish peroxidase. The mutase−dehydrogenase bands were visualized by chemiluminescence with Renaissance according to the instructions supplied by NEM Life Sciences.

Determination of Enzyme Activity. Mutase and dehydrogenase activities were measured spectrophotometrically at 30 °C in a three-component buffer of 0.10 M MES, 0.051 M *N*-ethylmorpholine, 0.051 M diethanolamine (pH 7.2), 1 mM EDTA, and 1 mM DTT as previously described (*25*). Over the pH range for which the initial velocities were measured, the ionic strength of this buffer mixture remained essentially constant (*45*). Mutase activity was monitored by following the disappearance of chorismate at 274 nm, and dehydrogenase activity was measured by monitoring the production of NADH from NAD^+ in the presence of prephenate at 340 nm. For measuring the pH dependence of the kinetic parameters of the dehydrogenase reaction, the pH of the assay mixture was determined at 30 °C before and after the assay. The kinetics of inactivation of mutase−dehydrogenase by DEPC were performed in 50 mM potassium phosphate buffer (pH 7.2) at 25 °C as described by

Table 2: Purification of Chorismate Mutase−Prephenate Dehydrogenase from *E. coli* XL2-Blue Cells[a] Harboring pVIV1

			mutase activity		dehydrogenase activity			
purification step	volume (mL)	protein (mg)	total (units)	sp act. (units/mg)	total (units)	sp act. (units/mg)	purification (*x*-fold)[b]	yield (%)
Cell-free extract	161	2270	11 877	5.2	10 000	4.4	1.0	100
Ammonium sulfate	62	868	11 284	13	9800	11	2.5	95
Q-Sepharose	170	476	9860	20	9500	20	3.9	83
Sepharose-AMP	170	255	9700	38	8772	34	7.3	82

[a] From 23 g of wet cells. [b] Calculated from mutase activity.

Christendat and Turnbull (*25*). Protein concentration was estimated by using the Bio-Rad protein assay kit with bovine serum albumin (BSA) as a standard and by recording the absorbance at 205 nm (*46*). The results by these two methods were in good agreement.

Circular Dichroism Spectroscopy. CD spectroscopy was used to compare the overall secondary structure of wild-type and selected mutant proteins. Measurements were recorded at room temperature from 190 to 250 nm using a Jasco J-710 spectropolarimeter with a 0.05 cm cell and at 0.1 nm wavelength increments. Each spectrum was normalized for protein concentration and the observed ellipticity (θ) was background-corrected against the spectrum obtained for the dialysis buffer.

Data Analysis. The kinetic data were fitted to the following rate equations by using the computer programs of Cleland (*47*) or GraFit (Version 3.0, Leatherbarrow):

$$v = \frac{VA}{K + A} \quad (1)$$

$$y = \frac{C}{1 + (H/K_A) + (K_B/H)} \quad (2)$$

$$y = \frac{C}{1 + (H/K_A)} \quad (3)$$

The initial velocity data obtained by varying the concentration of chorismate, prephenate, or NAD^+ (A) were fitted to eq 1 to yield values for the maximum velocity (V), the Michaelis constant (K), and the apparent first-order rate constant for the interaction of enzyme and substrate (V/K). For the determination of V/K values, the concentration of the fixed substrate was saturating at the pH of the assay. The variation of the values of V and V/K as a function of pH was fitted to the log form of eqs 2 and 3, where y represents the value of V or V/K at a particular pH value, C is the pH-independent value of y, H is the hydrogen ion concentration, and K_A and K_B are acid dissociation constants for groups on the enzyme or substrate.

RESULTS

Expression and Purification of Wild-Type Mutase−Dehydrogenase. In previous studies (*8, 25*) mutase−dehydrogenase was obtained from JFM30, a strain carrying the multicopy plasmid pKB45 coding for mutase−dehydrogenase, mutase−dehydratase, and 3-deoxy-D-arabinoheptulosonate 7-phosphate synthase. In the present study we subcloned the *TyrA* gene coding for mutase−dehydrogenase and placed it under a T7 promoter. This new expression system increased by over 10-fold the amount of mutase−dehydrogenase activity per gram of cell paste. Our new purification scheme (Table 2) removes a Matrex Blue A affinity column previously incorporated to separate mutase−dehydrogenase from mutase−dehydratase. Previously, less than 40% of the activity loaded onto this column was recovered (*8*). By removing this column, we achieve a final yield of protein greater than 80%. Overall, our improvements to the expression and purification procedure resulted in about a 35-fold increase in the yield of mutase−dehydrogenase per gram of cells. The kinetic parameters and molecular weight of the enzyme are similar to those reported elsewhere (*8*).

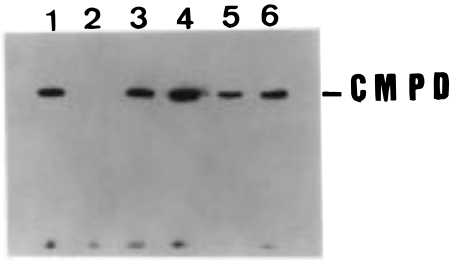


FIGURE 2: Expression of recombinant mutase−dehydrogenase in crude *E. coli* lysates. The levels of expression of wild-type and mutant enzymes are compared by use of an immunoblot probed with anti-rabbit mutase−dehydrogenase antiserum. Cultures of strain KB357 containing either wild-type or mutant plasmids were grown under identical conditions. Following cell lysis (see Experimental procedures), 0.5 μg of crude protein was loaded into separate wells on an SDS−10% polyacrylamide gel. The proteins are represented as follows: lane 1, H189N; lane 2, H189A; lane 3, H197N; lane 4, K37Q; lane 5, K37A; lane 6, wild-type enzyme. The proteins migrate at a molecular weight of 42 000 as predicted for the monomeric form of chorismate mutase−prephenate dehydrogenase (CMPD).

Properties of Mutant Mutase−Dehydrogenase Proteins: (A) K37A/Q. Our previous study using chemical modification and differential peptide mapping identified Lys37 as a residue important for chorismate mutase activity. Replacement of Lys37 with alanine yields a protein that is more poorly expressed than wild-type enzyme as judged by SDS−PAGE (not shown) and immunoblots (Figure 2). Moreover, a crude extract of this mutant did not exhibit any detectable mutase or dehydrogenase activity. We hypothesize that structural changes of the molecule may result in its inactivity and instability. By contrast, the K37Q mutant protein was well expressed (Figure 2) and provided a far-UV CD spectrum similar to that of wild-type enzyme (data not shown). The purified protein showed kinetic parameters for the dehydrogenase reaction similar to wild-type but did not exhibit any detectable mutase activity even upon the addition of 1.5 mg of mutant protein and 1.5 mM chorismate in the assay mixture (Table 3).

It has been previously demonstrated that prephenate is a competitive inhibitor with respect to chorismate in the mutase

Table 3: Summary of Kinetic Data for Wild-Type and Mutant Mutase−Dehydrogenase Measured at pH 7.2 and 30 °C

	mutase activity, chorismate			dehydrogenase activity: prephenate			dehydrogenase activity: NAD^+		
protein[a]	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
WT	45 ± 7	27 ± 0.7	6.0×10^5	44 ± 8	27 ± 1	6.2×10^5	103 ± 11	27 ± 0.7	2.6×10^5
K37Q	ND[b]	ND	*c*	54 ± 9	23 ± 1	4.3×10^5	141 ± 16	22 ± 0.7	1.6×10^5
H131A	53 ± 10	7.2 ± 0.4	1.4×10^5	56 ± 12	3.1 ± 0.2	0.6×10^5	280 ± 37	2.6 ± 0.1	9.3×10^3
H153N	51 ± 10	10 ± 0.5	2.0×10^5	46 ± 5	19 ± 1	4.2×10^5	171 ± 28	19 ± 1.0	1.1×10^5
H189N		$<2.5 \times 10^{-3}$			$<2.5 \times 10^{-3}$			$<2.5 \times 10^{-3}$	
H197N	68 ± 10	16 ± 0.5	2.3×10^5	55 ± 8	3.0×10^{-3}	5.5×10^{-5}	128 ± 19	3.5×10^{-3}	2.7
H239N	126 ± 12	2.3 ± 0.1	0.18×10^5	34 ± 4	5.6 ± 0.4	1.6×10^5	186 ± 18	5.6 ± 0.4	0.3×10^5
H245N	225 ± 1	4.8 ± 0.1	0.21×10^5	100 ± 7	12 ± 0.2	1.2×10^5	185 ± 22	15 ± 0.4	0.8×10^5
H257A	98 ± 2	8.0 ± 0.5	0.81×10^5	99 ± 8	20 ± 0.7	2.0×10^5	142 ± 15	15 ± 0.5	1.0×10^5
H265A	99 ± 25	15 ± 1	1.5×10^5	77 ± 15	8.0 ± 0.4	1.0×10^5	191 ± 28	7.2 ± 0.3	0.4×10^5
H347N	41 ± 8	6.0 ± 0.2	1.5×10^5	28 ± 1	9.8 ± 0.3	3.5×10^5	73 ± 10	4.5 ± 0.2	0.6×10^5

[a] Data shown only for those mutants that were significantly expressed. [b] No detectable activity. [c] Not determined.

reaction (*27*, *48*). Furthermore, the value of the dissociation constant for the interaction of prephenate with the free enzyme in the dehydrogenase reaction (0.17 mM) is similar to the value for prephenate acting as an inhibitor of the mutase reaction (0.21 mM) (*27*). These values represent true dissociation constants since it has been previously determined that binding is rapid relative to the rate-determining chemistry (*22*, *27*). However, despite the structural similarities of the two compounds, chorismate did not inhibit the dehydrogenase reaction catalyzed by K37Q, even up to a concentration of 0.5 mM chorismate in the presence of 25 μM prephenate and 3 mM NAD^+. In fact, the reaction velocity was enhanced by about 10% by the addition of chorismate (data not shown).

(B) H131A. Our previous chemical modification studies implicated His131 as a catalytic residue in the dehydrogenase reaction (*25*). However, the H131A substitution did not eliminate dehydrogenase activity. As shown in Table 3, this mutant retained about 10% dehydrogenase activity and 30% mutase activity compared to the wild-type enzyme. The affinity for substrates, prephenate, NAD^+, and chorismate was only slightly increased over that found for the wild-type enzyme. Moreover, the pH dependence of log *V* for this mutant paralleled that found for wild-type dehydrogenase (data not shown). Together the data imply that His131 is not an essential residue whose protonation state is critical for catalysis or substrate binding.

Chemical modification with DEPC was performed on H131A to determine if another histidine residue rather than His131 could fulfill this catalytic role. Incubation of 0.11 mM active sites of H131A with 0.2 mM DEPC results in a time-dependent loss of prephenate dehydrogenase activity. Moreover, prior addition of 1 mM prephenate completely protects against inactivation (Figure 3). At higher concentrations of DEPC necessary to monitor pseudo-first-order kinetics, the enzyme was inactivated almost instantaneously. This behavior precluded conducting more rigorous inactivation studies as outlined previously (*25*). In any event, the results from our second order kinetics imply that the group(s) modified by DEPC in the H131A mutant protein may also include a highly reactive histidine in the dehydrogenase active site.

(C) Alanine/Asparagine Scan. Mutase−dehydrogenase contains only nine histidine residues, all located in the presumed dehydrogenase portion of the polypeptide chain (*9*). Hence, an alanine scan of the eight remaining histidines

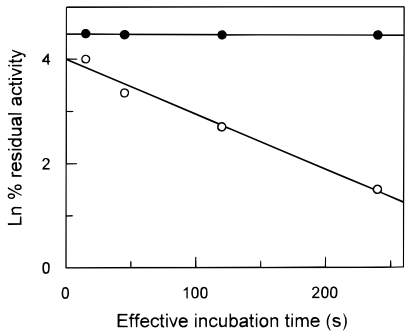


FIGURE 3: Kinetics of inactivation of H131A prephenate dehydrogenase by DEPC in the presence and absence of the protecting ligand, prephenate. Inactivation studies were conducted in 50 mM potassium phosphate buffer (pH 7.2) at 25 °C with H131A mutase−dehydrogenase (110 μM monomer) and 0.2 mM DEPC alone (○) or with 0.2 mM DEPC plus 1 mM prephenate (●).

was attempted in order to identify the key catalytic residue in the dehydrogenase reaction—specifically, a histidine that when replaced would yield a mutant that lacked dehydrogenase activity but still possessed mutase activity. SDS−PAGE of crude cell extracts (data not shown) indicated that two of the alanine mutant proteins (H257A and H265A) were stable and well expressed, whereas the expression of two others (H189A and H238A) could not be detected. The nucleotide sequence for another three (H153A, H197A, and H245A) could not be confirmed and these mutants were not pursued further. However, asparagine substitutions at these latter five positions yielded proteins (H153N, H189N, H197N, H238N, and H245N) that were well expressed. At position 347, only the histidine to asparagine mutation was made and the resulting protein was stable. Immunoblots of selected proteins are shown in Figure 2, while the kinetic properties of the mutant proteins are listed in Table 3. Six of the eight mutant proteins generated from scanning mutagenesis did not exhibit kinetic properties that were remarkably different from wild-type enzyme. The relatively small changes in k_{cat} and in Michaelis constants for substrates may have reflected minor structural changes in the enzymes. In contrast, however, H197N did not exhibit any significant dehydrogenase activity. In fact, the small amount of dehydrogenase activity (recorded in Table 3) could only be detected by assaying with high levels of this mutant protein. However, H197N retained nearly wild-type mutase activity and unaltered Michaelis constants for chorismate, prephenate, and NAD^+. H189N showed less than 0.01% the activity of

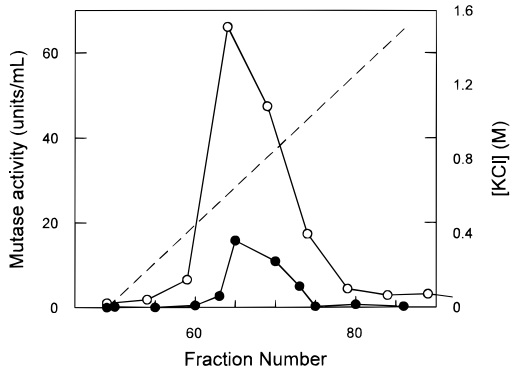


FIGURE 4: Elution patterns of wild-type and H197N mutase−dehydrogenase from Sepharose−AMP. Wild-type (250 mg, ○) and H197A (90 mg, ●) were chromatographed separately on a 2.6 × 10 cm Sepharose−AMP column and eluted with a linear gradient of 0−1.5 M KCl (- - -) (see Experimental Procedures). Fractions of 10 mL were collected and assayed for chorismate mutase activity. Both proteins eluted at 0.55 M KCl.

wild-type mutase and dehydrogenase. The far-UV CD spectra of the stable mutants did not show any significant deviation from that of the wild-type enzyme, indicating that there were no global secondary structural changes occurring with these amino acid replacements (data not shown).

(D) H197N. The selective loss of dehydrogenase activity was not due to an inability of the mutant to bind NAD^+ or prephenate since the Michaelis constants for both substrates were similar to that of the wild-type enzyme (Table 3). For the dehydrogenase reaction, the Michaelis constant is a good estimate of the true dissociation constant of substrate from the binary complex, since binding is at equilibrium (*22*). As added proof that NAD^+ binding was not markedly affected, we chromatographed the mutant on Sepharose−AMP and monitored its elution profile by following mutase activity. Mutase−dehydrogenase is believed to interact with Sepharose−AMP through its nucleotide binding site (*8*). Figure 4 shows that H197N and the wild-type enzyme eluted at a concentration of 0.55 M KCl, implying that both proteins bind to Sepharose−AMP with similar affinities. We attempted to confirm by thermodynamic techniques that the binding of prephenate to the mutant was not significantly different from that of wild-type enzyme. However, the methods employed, which included fluorescence titration, isothermal titration calorimetry, near-UV CD, and capillary electrophoresis, proved unsuccessful in providing a dissociation constant for prephenate bound to either the wild-type or the mutant enzyme.

To determine if the pH dependence of the dehydrogenase reaction was affected by the mutation, we compared the pH−rate profiles for wild-type enzyme and H197N (Figure 5). For the wild-type enzyme, the effect on the dehydrogenase reaction was determined by varying the prephenate concentration at a fixed concentration of NAD^+ that was at least 15 times greater than its Michaelis constant at any given pH. The $(V/K)_{prephenate}$ profile is bell-shaped with slopes of +1, 0, and −1 and illustrates the falloff in the rate of reaction of prephenate with the enzyme−NAD^+ complex at both high and low pH values. The fit of the data to eq 2 yielded pK values of 6.29 ± 0.07 and 8.72 ± 0.10 on the acidic and basic limbs, respectively, and a pH-independent value of the parameter of 1.46 ± 0.16 $\mu M^{-1}\ s^{-1}$. The variation of log V with pH gives rise to a half-bell profile with slopes of +1

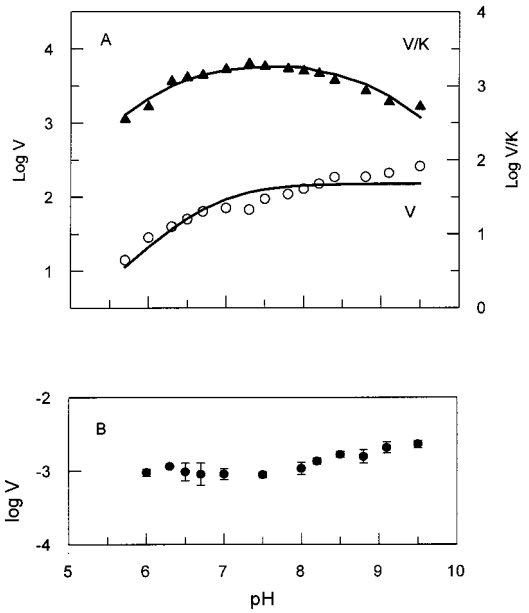


FIGURE 5: Variation with pH of log V and log $(V/K)_{prephenate}$ for the reaction catalyzed by wild-type (A) and H197N prephenate dehydrogenase (B). NAD^+ concentrations were fixed at 3 mM. The points in panel B represent the average from three separate determinations. The units for V and V/K are $second^{-1}$ and $molar^{-1}$ $second^{-1}$, respectively. The curves in panel A represent best fits of the data to eqs 2 and 3, respectively. The values of the parameters used to draw the curves are given in the text.

and 0. Fitting of the data to eq 3 yielded a pK value of 6.70 ± 0.04 and a pH-independent value of the parameter of 80 ± 4 s^{-1}. These values for the wild-type enzyme are in agreement with those previously determined (*23*, *24*) and are in keeping with the idea that the group with pK of about 6.5 titrating in both the V/K and V profiles must be unprotonated for catalysis, while the group with pK of about 8.7 must be protonated and involved in binding prephenate to the enzyme−NAD^+ complex. In contrast, the log V profile for H197N was pH-independent over the same pH range as reported for wild type, implying that His197 may be the group titrating on the acidic limb that is essential for catalysis in the dehydrogenase reaction. The pH-independent value of the parameter was estimated at $3 \times 10^{-3}\ s^{-1}$. Since high levels of mutant protein were required to follow the reaction rate (about 30 μM monomer per assay), the pH dependence of $(V/K)_{prephenate}$ was not examined. Such an experiment would necessitate using equal molar concentrations of substrate and enzyme.

With an unprotonated catalytic group removed from the active site of H197N, we attempted to regenerate a fraction of the lost activity by adding exogenous bases into the assay mixture. Such experiments have been performed with mutant forms of several different enzymes, but most successfully with those carrying histidine to alanine replacements (*49*−*52*). Unfortunately but not suprisingly, the addition of 0.5 M imidazole, free histidine, or hydroxylamine did not increase dehydrogenase activity in H197N, which carries a more bulky asparagine substitution.

DISCUSSION

The present study represents the first site-directed mutagenesis experiments on the bifunctional enzyme chorismate mutase−prephenate dehydrogense from *E. coli*. We have

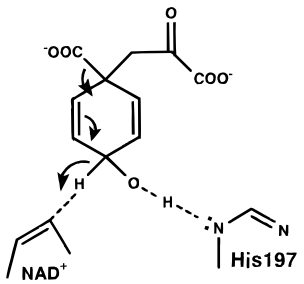
identified two residues that are important for the activity of the enzyme—Lys37, which is essential for the mutase, and His197, which is important for the dehydrogenase.

Chorismate Mutase. Lys37 was previously identified as a residue important for mutase activity by chemical modification of the enzyme by a lysine-specific reagent, trinitrobenzenesulfonate, in the presence and absence of protecting ligands followed by analysis of peptide fragments by mass spectrometry (*25*). The results by site-directed mutagenesis in this report confirm our findings obtained by protein chemistry. Moreover, our findings also agree with other recent mutagenesis experiments on monofunctional chorismate mutases from *B. subtilis* (*16*−*18*) and *S. cerevisiae* (*20*) and on the independently expressed mutase domain (minimutase) associated with the *E. coli* bifunctional enzyme chorismate mutase−prephenate dehydratase (*19, 21*). The lysine homologue in these mutases (Arg90 in *B. subtilis*, Lys39 in *E. coli* minimutase, and Lys168 in yeast), when changed to alanine (yeast, *E. coli* minimutase, *B. subtilis*), asparagine, glutamine, or arginine (*E. coli* minimutase) or lysine (*B. subtilis*) resulted in mutant proteins that were reported to have little or no detectable mutase activity (<0.1% k_{cat}/K_m of wild type). In our study with mutase−dehydrogenase, the K37Q mutant protein also exhibited no detectable mutase activity. Interestingly, however, immunoblots revealed that K37A was more poorly expressed than the wild-type enzyme (Figure 2), whereas the comparable mutant in the other mutases was well expressed. Such an observation may reflect the difference between the bifunctional enzyme, in the case of the mutase−dehydrogenase, versus the monofunctional form of the other mutases examined. From the crystal structure of the highly homologous mutase portion of mutase−dehydratase liganded to the transition-state analogue *endo*-oxabicyclic diacid (*10*), it can be inferred that 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 enolpyruvyl side chain in the transition state (see Figure 1). As concluded from previous findings with monofunctional mutases (*17*−*21*), we propose that Lys37 and other cationic/polar residues in the active site help to orient and lock chorismate in the requisite chain conformation for rearrangement. Lys37 may provide provide additional assistance to catalysis by stabilizing the developing negative charge on the enolpyruvyl side chain initiated by C-5−O-7 bond cleavage (*53*−*55*).

Prephenate Dehydrogenase. The pH dependency of the prephenate dehydrogenase reaction as examined in this study and others (*23, 24*) has clearly shown that there is a catalytic group with a p*K* value of about 6.5 that must be unprotonated for maximum dehydrogenase activity. Our previous studies using chemical modification with DEPC in the absence and presence of prephenate indicated that this residue may be a histidine located at or near the dehydrogenase active site. Of the nine histidines in the mutase−dehydrogenase, one histidine, identified by peptide mapping as His131, was particularly reactive, and we had shown that the modification of this residue alone caused a loss of dehydrogenase activity in the wild-type enzyme (*25*). However, results from the present study using site-directed mutagenesis indicate that the key catalytic histidine is likely not His131. The H131A mutant is an efficient enzyme, possessing wild-type Michaelis constants for its substrates and somewhat reduced but nonetheless significant activity for both mutase and dehydrogenase (Table 3). This result highlights the importance of combining site-directed mutagenesis with chemical modification studies. Moreover, we report that the dehydrogenase associated with H131A can be inactivated by DEPC much more rapidly than the wild-type enzyme. Such an observation implies that another very reactive group, most likely a histidine, has now become accessible to the modifying reagent as a result of the H131A substitution. We speculate that His131 is located at the lip of the dehydrogenase active site exposed for easy carbethoxylation and positioned such that its modification hinders access to the prephenate site. The reaction of His131 with DEPC by a simple bimolecular collision would prevent the substrate prephenate from binding, and likewise, the binding of prephenate could somehow render this residue sterically inaccessible to DEPC. Moreover, either the binding of prephenate or the carbethoxylation of H131 may prevent the modification of a very reactive, catalytically important group located deeper in the active site of the dehydrogenase. Only when His131 is replaced with alanine does this catalytic residue become accessible to DEPC. Verification of this hypothesis awaits a crystal structure.

Our present results from site-directed mutagenesis suggest that His197 is the essential catalytic residue in the dehydrogenase reaction. The H197N substitution eliminates exclusively and essentially completely the dehydrogenase activity (Table 3). Moreover, His197 does not play a role in the binding of prephenate of NAD^+ since the Michaelis constants for these two substrates in the mutant and wild-type enzymes are comparable (Table 3), as is the elution profile of both enzymes from an affinity column showing high specificity for nucleotide-containing proteins (Figure 4). We provide evidence that His197 is likely the residue titrating with a p*K* of about 6.5 in the pH-rate profile for the wild-type enzyme since the pH dependence of log *V* for H197N mutant is missing this acidic limb (Figure 5). How could such a catalytic group participate in the oxidative decarboxylation of prephenate? Other nucleotide-dependent oxidative decarboxylases have been reported to operate via a two-step mechanism where the first step is a base-catalyzed transfer of a hydride ion from the substrate to NAD^+, generating a β-keto intermediate, followed by decarboxylation of the intermediate with the assistance of an enzymic acid (see references in ref *25*). In contrast, the oxidative decarboxylation of prephenate to (4-hydroxyphenyl)pyruvate is a concerted reaction and does not proceed through this intermediate (*23*). However, studies suggest that the dehydrogenase still possesses the necessary catalytic machinery (a enzymic base) to assist in the oxidation step: the enzyme can reversibly oxidize a prephenate analogue unable to undergo decarboxylation (*23*), and the reaction is dependent on a group that must be deprotonated for maximum activity (*23*, *24*; this study). Hence, it has been previously hypothesized (*23*−*25*) that such a residue may assist in the oxidative decarboxylation of prephenate to (4-hydroxyphenyl)pyruvate by polarizing the 4-hydroxyl group of prephenate. Since the ensuing product is aromatic, thus providing much of the driving force behind the reaction, polarization rather than deprotonation of the 4-hydroxyl group to form a vinylogous β-keto acid would be all that is required to lower the

Scheme 2: Possible Role of His197 in the Concerted Hydride Transfer and Decarboxylation of Prephenate



activation energy for hydride abstraction by NAD^+ and the concomitant decarboxylation (Scheme 2).

After these mutagenesis studies were initiated, the primary sequence of other prephenate dehydrogenases became available in the Protein Data Bank. This facilitated effective sequence alignments in order to identify key conserved residues in the prephenate dehydrogenase portion of the *E. coli* bifunctional enzyme. As shown in Figure 6, His197 as well as His245 and His257 are conserved among five prephenate dehydrogenases, both bifunctional and monofunctional forms. While this is in accord with the kinetic results of H197N, by contrast, H245N and H257N are reasonably efficient mutase−dehydrogenases; in fact, the latter two mutations appear to alter mutase more than dehydrogenase activity. The other six histidine residues including His131 are not conserved, and amino acid substitutions at these positions shown in Table 3, except at His189, yield mutant proteins that are reasonably effective catalysts. It is suprising that alanine substitutions at positions 189 and 238 (and presumably 153, 197, and 245) could not be tolerated while asparagine replacements were. This observation suggests that the hydrogen-bonding capabilities of histidine or asparagine at these positions are essential for the expression of stable enzyme. His131 is close in primary sequence 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 (*9*). The fact that the H131A mutant binds NAD^+ about a third as well as the wild-type enzyme (Table 3) implies that His131 may play a minor role in nucleotide binding. H189N's inactivity could stem from tertiary structural changes to the enzyme rather than secondary structural changes that we monitored with far-UV CD. We are investigating this mutant enzyme further as well as examining other residues in the mutase−dehydrogenase (Lys178, Arg286, and Arg294) that are conserved among prephenate dehydrogenases to determine if they play a role in prephenate binding.

Relationship between the Two Activities. The results of this investigation are consistent with the involvement of

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(a)  --MVAELTALRDQIDEVDKALLNLLAKRLELVAEVGEVKSRFGLPIYVPEREASMLASRRAEAEALGVPPDLIEDVLRRVMRESY 83
(b)  --MVAELTALRDQIDSVDKALLDLLAKRLELVAEVGEVKSRYGLPIYVPEREASMLASRRKEAEALGVPPDLIEDVLRRVMRESY 83
(c)  MSFMEALKDLRSEIDSLDRELIQLFAKRLELVSQVGKVKHQHGLPIYAPEREIAMLQARRLEAEKAGISADLIEDVLRRFMRESY 85
(d)  ------------------------------------------------------------MTVFKHIAIIGLG--LIGSSAARAT-KAY 26
(e)  ------------------------------------------------------MNQMKDTILLAGLG--LIGGSIALAIKKNH 28
(f)  ----------------------------------------------------------MKKILIIGLG--LIGSSIALGIKKAH 24
                                                                    *:   ** .      : :
(a)  SSENDKGFKTLCPSLRPVVIVGGGGQMGRLFEKMLTLSGYQVRILEQHDWDRAADIVADAGMVIVSVPIHVTEQVIGKLPP--LP 166
(b)  TSENDKGFKTLCPELRPVVIVGGKGQMGRLFEKMLGLSGYTVKTLDKEDWPQAETLLSDAGMVIISVPIHLTEQVIAQLPP--LP 166
(c)  ANENQFGFKTINSDIHKIVIVGGYGKLGGLFARYLRASGYPISILDREDWAVAESILANADVVIVSVPINLTLETIERLKPY-LT 169
(d)  -----------CPD----VTVSLYD-KSEFVCDRARALNLGDNVTD--D---IQDAVREADLVLLCVPVRAMGIVAAAMAPA-LK 89
(e)  -----------PG---KRIIG-----IDISDEQAVAALKLGVIDDRADS--FISGVKEAATVIIATPVEQTLVMLEELAHSGIE 81
(f)  -----------PE---FEILG-----SDR-EEVENIAQKRGIIDSKVE---LVKGAQEADIIILAVPISVTLELLKQIATFDLK 85
                                              .  :         :*  ::::.*:
(a)  KDCILVDLASVKN---GPLQAMLVAHDGPVLGLHPMFG-----PDSG-SLAKQ---VVVWCDGRKP-EAYQWFLEQIQVWGARLH 238
(b)  EDCILVDLASVKN---RPLQAMLAAHNGPVLGLHPMFG-----PDSG-SLAKQ---VVVWCDGRQP-EAYQWFLEQIQVWGARLH 238
(c)  ENMLLADLTSVKR---EPLAKMLEVHTGAVLGLHPMFG-----ADIA-SMAKQ---VVVRCDGRFP-ERYEWLLEQIQIWGAKIY 241
(d)  KDVIICDTGSVKVSVIKTLQDNLPNHI--IVPSHPLAGTENNGPDAGFAELFQDHPVILTPDAHTPAQAIAYIADYWEEIGGRIN 172
(e)  HELLITDVGSTKQKVVDYADQVLPSRY-QFVGGHPMAGSHKSGVAAAKEFLFENAFYILTPGQKTDKQAVEQLKNLLKGTNAHFV 165
(f)  DGLLITDAGSTKSEIVELANQLFSGTKVKFIGGHPMAGSHKSGVMAADLNLFENAYYVLTEE--S-----QELRELLKGLHAKFI 163
        :: *  *.*          :      .:  Δ*: *        .     :    :: : :  :   .::
(a)  RISAVEHDQNMAFIQALRHFATFAYGLHLA-EENVQLEQLLALSSPIYRLELAMVGRLFAQDPQLYADIIMSSERNLALIKRYY 321
(b)  RISAVEHDQNMAFIQALRHFATFAYGLHLA-EENVNLDQLLALSSPIYRLELAMVGRLFAQDPQLYADIIMSSESNLALIKRYY 321
(c)  QTNATEHDHNMTYIQALRHFSTFANGLHLS-KQPINLANLLALSSPIYRLELAMIGRLFAQDAELYADIIMDKSENLAVIETLK 324
(d)  LMSAEHHDHVLALTSHLPHVIAYQLIGMVSGYEKKSRTPIMRYSAGSFRDAT----RVAASEPRLWQDIMLENAP--ALLPVLD 250
(e)  EMSPEEHDGVTSVISHFPHIVAASLVHQTH-HSENLYPLVKRFAAGGFRDIT----RIASSSPAMWRDILLHNKD--KILDRFD 242
(f)  ILDAKEHDKVTGQVSHFPHILASTLVWQSD-DYAKEHPLVKHLAAGGFRDLT----RIAEADSLMWTSVLLSNPE--ITLERIE 240
       .. .**      . : *. :                :   ::  :*       *:   .. :: .::: .      :
(a)  KRFGEAIELLEQG---DKQAFIDSFRKVEHWFGDYAQR--FQSESRVLLRQANDNRQ------------------------    373
(b)  QRFGEAIALLEQG---DKQAFIASFNRVEQWFGDHAKR--FLVESRSLLRSANDSRP------------------------    373
(c)  QTYDEALTFFENN---DRQGFIDAFHKVRDWFGDYSEQ--FLKESRQLLQQANDLKQG------------------------    377
(d)  HFIADLKKLRTAI---ASQ---D-----EDYLLEHFKE--SQ-KARLALKTDHDIHP------------------------    293
(e)  EWIREIDKIRTYVEQEDAENLFRYFKTAKDYRDGLPLRQKGAIPAFYDLYVDVPDHPGVISEITAILAAERISITNIRIIE    323
(f)  NFKKHLDEIALKITKRDSQAIEHFFEEGKKIRQAMEIH-KGALPNFYDLFISVPDEKGVVLRVLALL--QDFSITNIKINE    318
                        .   :         :              *
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FIGURE 6: Amino acid sequence alignment of *E. coli* chorismate mutase−prephenate dehydrogenase with other known bacterial bifunctional mutase−dehydrogenases (B) and monofunctional prephenate dehydrogenases (M). Invariant and nearly invariant residues are noted by the stars and colons, respectively. His197 is denoted by a triangle. Bold letters highlight the nine histidine residues in the *E. coli* bifunctional enzyme and the three invarant histidines. Sequences are from (a) *E. coli* (B) (*9*), (b) *E. herbicola* (B) (*33*), (c) *Haemophilus influenzae* (B) (*35*), (d) *Zymomonas mobilis* (M) (*34*), (e) *B. subtilis* (M) (*32*), and (f) *Lactobacillus lactis* (M) (*31*).

distinct active-site residues in the transition states of the two reactions. This study is the first to report the expression of mutants of the bifunctional enzyme that possess solely mutase activity (H197N) or dehydrogenase activity (K37Q). Moreover, while prephenate can act as a product inhibitor of the mutase (*22*, *24*, *27*), we show that chorismate does not act as a competitive inhibitor with respect to prephenate in the dehydrogenase reaction catalyzed by K37Q. Presumably this is because chorismate does not bind to, or interacts only very weakly with, the dehydrogenase site of K37Q. This latter observation could only be examined effectively with a monofunctional dehydrogenase or with a mutant bifunctional enzyme like K37Q, which possesses only dehydrogenase activity and will not convert chorismate to prephenate. Our finding supports the previous studies by Heyde and Morrison (*27*), who showed that, in the presence of NAD^+ and 50 μM prephenate, chorismate enhanced the production of (4-hydroxyphenyl)pyruvate by 50%, presumably by binding to the mutase site and causing a conformational change in the dehydrogenase or channeling some of the prephenate formed from chorismate to the dehydrogenase site. The small enhancement of dehydrogenase activity that we report may reflect the fact that chorismate also binds, albeit poorly, to the mutase portion of K37Q. *Endo*-oxabicyclic diacid, an analogue that mimics the transtion state in the conversion of chorismate to prephenate, does not inhibit the dehydrogenase reaction (*28*). It is not surprising then that chorismate, which resembles prephenate less than the transition-state analogue of the mutase reaction, also does not inhibit the dehydrogenase. This observation is also consistent in terms of regulation of metabolism and provides an example of evolution. Chorismate is stationed at the branch point of aromatic amino acid biosynthesis in the shikimate pathway. If prephenate is in excess, the mutase can be inhibited so that more chorismate can be diverted into other biosynthetic pathways, such as the production of aromatic vitamins and folates rather than the production of tyrosine and phenylalanine. With an abundance of chorismate in the cell, all pathways leading to its utilization should be activated, not inhibited, including the production of (4-hydroxyphenyl)-pyruvate leading to tyrosine as catalyzed by prephenate dehydrogenase.

The close sequence alignment of the mutase portions of mutase−dehydrogenase with mutase−dehydratase tends to suggest that the *E. coli* bifunctional enzymes possess two separately folding units, a mutase and a dehydrogenase (or dehydratase) domain. The premise has been verified for chorismate mutase−prephenate dehydratase by Ganem and collegues, who reported the separate expression and purification of fully active, monofunctional proteins (*10*, *56*, *57*); the mutase encompasses the first 100 amino acids, while the dehydratase is associated with the remainder of the polypeptide. Their studies support previous kinetic findings (*58*) that the mutase and dehydratase sites are distinct and noninteracting. To date, the molecular genetic approach has not been demonstrated as elegantly for the mutase−dehydrogenase. As steps toward this goal however, Maruya et al. (*43*) constructed separate plasmids presumably encoding the mutase and dehydrogenase domains and noted that under certain growth conditions the plasmids were able to complement the defect in a host strain that had an inactivated mutase−dehydrogenase. However, these monofunctional proteins were never expressed and characterized. Moreover, a monofunctional dehydrogenase was generated by deleting 37 amino acids from the N-terminus of mutase−dehydrogenase from *Erwinia herbicola* (*33*). The results are as expected since this region encompasses many key active-site residues of the mutase. Contrary to results obtained for mutase−dehydratase, however, evidence persists that the mutase and dehydrogenase sites, while they may be separate, are not entirely independent. The binding of NAD^+ to its subsite in the dehydrogenase pocket enhances mutase activity (*27*). There is evidence that some of prephenate formed from chorismate is converted directly to (4-hydroxyphenyl)-pyruvate (*26*). Inhibition data obtained with some malonic acid derivatives were consistent with two overlapping sites (*59*). Moreover, we have demonstrated in the present study that substitutions of amino acid clearly in the dehydrogenase portion of the enzyme (H189N, H238N, and H245N) can affect mutase activity. We are currently investigatiging the extent to which the two catalytic sites are structurally and functionally independent. Our results will be presented in due course.

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