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Mechanistic Studies on Chorismate Mutase-Prephenate Dehydrogenase from *E.coli*

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Abstract

Mechanistic Studies on Chorismate Mutase-Prephenate Dehydrogenase from *E.coli*

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Chorismate mutase-prephenate dehydrogenase is a bifunctional enzyme that catalyzes two sequential reactions in the biosynthesis of tyrosine in *E.coli* and other enteric bacteria. Chorismate mutase catalyzes the pericyclic rearrangement of chorismate to prephenate which is subsequently converted to (4-hydroxyphenyl)pyruvate through an oxidative decarboxylation reaction catalyzed by prephenate dehydrogenase. Through chemical modification and site-directed mutagenesis we have identified residues that are critical in both the mutase and dehydrogenase mechanisms and have provided evidence that these two reactions occur at separate active sites on the enzyme. We have identified Lys37 as a residue that is important in the mutase reaction by differential peptide mapping. This result was confirmed by site-directed mutagenesis. The crystal structures of other chorismate mutases indicate that Lys37 may provide important hydrogen bonds in the transition state of the mutase reaction.

We chemically modified mutase-dehydrogenase to show that a histidine is important for dehydrogenase activity. The pH rate profile for the dehydrogenase reaction indicates that a protonated residue is important for catalysis. By comparing the pH profile for wild-type and mutant mutase-dehydrogenase we have identified His197 as this catalytic group. Sequence alignments with other prephenate dehydrogenase enzymes show that this histidine is highly conserved. We propose that His197 may be the catalytic base involved in the hydride transfer from prephenate to NAD^+.

We have identified three positively charged residues, Lys178, Arg286 and Arg294, that are conserved amongst prephenate dehydrogenases from different organisms. We conducted site-directed mutagenesis on these residues and compared the wild-type

enzyme and selected mutants with respect to their stability, pH rate profiles and ability to bind prephenate and a series of inhibitory substrate analogues. Our results indicate that Arg294 plays an important role in prephenate binding by interacting electrostatically with the ring carboxylate of the substrate. Our studies also show that a group with a pK of 8.8 interacts with the pyruvyl side chain carboxylate of prephenate. Further characterization of mutant proteins is being conducted to determine if this binding group is either Lys178 or Arg286.

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To my Loving Mom, Dad, Brother and Three Sisters

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List of Abbreviations

AMP	adenosine monophosphate
Arg (R)	arginine
C	carbon
°C	Celsius
CD	circular dichroism
cm	centimeter
CMPD	chorismate mutase prephenate dehydrogenase
CMPDtase	chorismate mutase prephenate dehydratase
DAHP	3-deoxy-D-arabino-heptulosonate 7-phosphate
DEPC	diethylpyrocarbonate
DHQ	5-dehydroquinate
DTT	dithiothreitol
E4P	erythrose-4-phosphate
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EPSP	5-enoylpyruvoylshikimate-3-phosphate
FTIR	fourier transform infrared spectroscopy
g	grams
Gln (N)	glutamine
His (H)	histidine
HPLC	high pressure liquid chromatography

IPTG	isopropyl β-D-thiogalactoside
hr	hour
Lys (K)	lysine
μ	micro
M	molar
mL	milliliter
MES	2-(N-morpholino)ethanesulfonic acid
min	minute
mm	millimeter
MS	mass spectrometry
N	amino
NAD^+	nicotinamide adenine dinucleotide
nm	nanometer
NMR	nuclear magnetic resonance
O	oxygen
OH	hydroxyl
PAGE	polyacrylamide gel electrophoresis
PEP	phosphoenolpyruvate
Phe (F)	phenylalanine
rpm	revolutions per minute
s	second
SDS	sodium dodecyl sulfate
TFA	trifluoroacetic acid
TNBS	trinitrobenzene sulfonate

Trp (W)	tryptophan
Tyr (Y)	tyrosine

CHAPTER 1

GENERAL INTRODUCTION

Biosynthesis of aromatic amino acids

The biosynthetic pathways for aromatic amino acids (Figures 1.1 and 1.2) are well studied in *E.coli* and other micro-organisms (1, 2, 3, 4). The enzymatic steps leading to the synthesis of chorismate are common for all three aromatic amino acids (tyrosine, phenylalanine and tryptophan) and some aromatic compounds (folate, ubiquinone, menaquinone and enterochelin) (5). Carbon flow through the shikimate pathway is highly regulated by feedback inhibition of the first enzyme, DAHP synthase (3-deoxy-D-arabino-heptulosonate 7-phosphate), through the action of the three aromatic amino acids.

Shikimate Pathway

The first committed step in the shikimate pathway (Figure 1.1) starts with an aldol condensation of phosphoenolpyruvate (PEP) and erythro-4-phosphate (E4P) to give a 7-carbon compound DAHP (6). This step is catalyzed by the highly regulated DAHP synthases, a collection of three isozymes, DAHP synthase (Phe), (Tyr), and (Trp) (7, 8), classified according to the amino acid which serves as the feed-back inhibitor. For example, DAHP synthase (Phe) is more than 95% inhibited by phenylalanine, however, neither tyrosine nor tryptophan affect the activity of this isozyme (1). Interestingly, while the Phe and Tyr isoforms are more than 95% inhibited by their individual end-products, tryptophan inhibition of the Trp isozyme does not exceed 40% (1). The inability of tryptophan to completely inhibit DAHP synthase (Trp) ensures sufficient flow through the shikimate pathway to produce other aromatic compounds such as folate and enterochelin (9).

Figure 1.1 The shikimate pathway

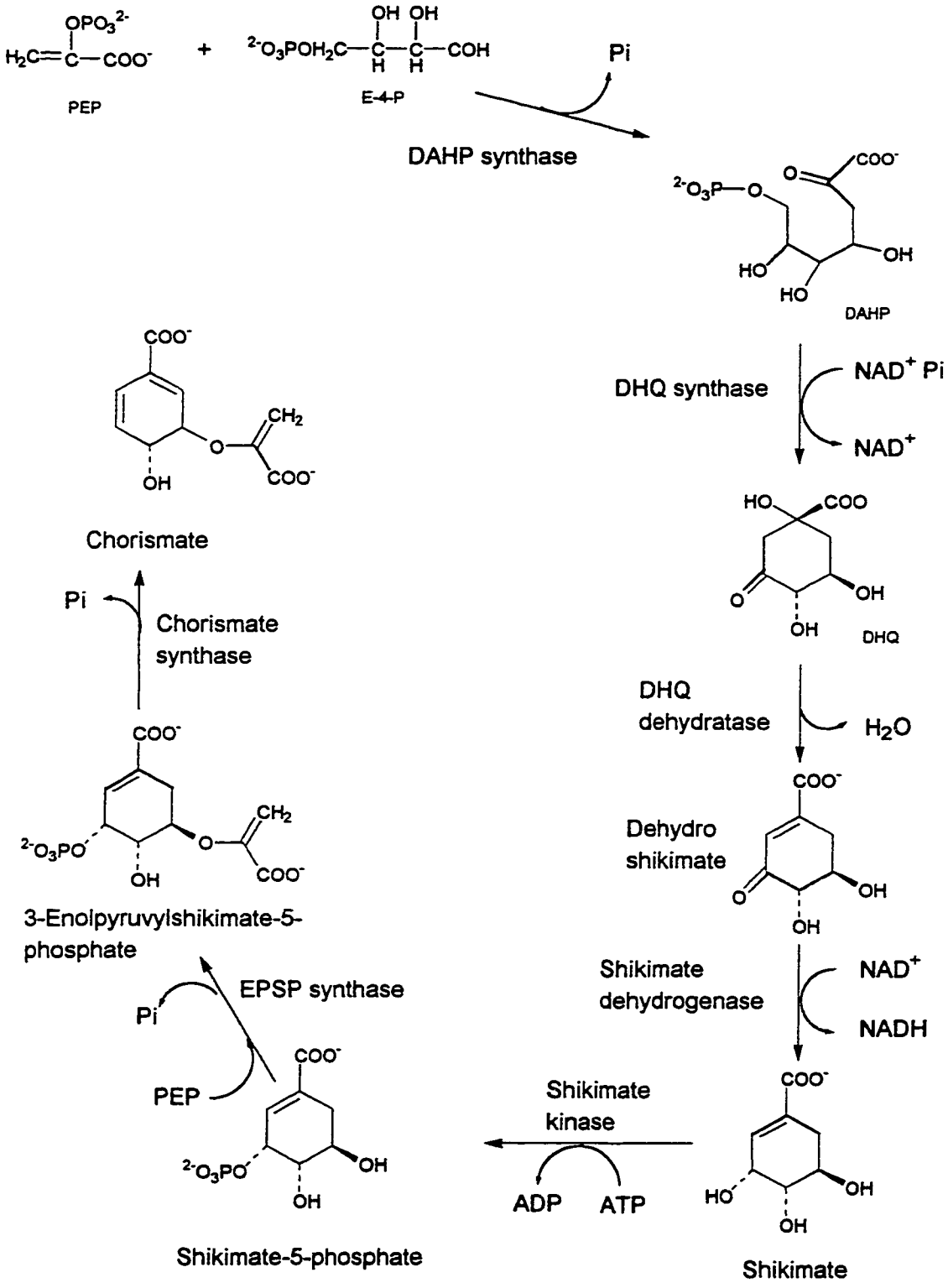
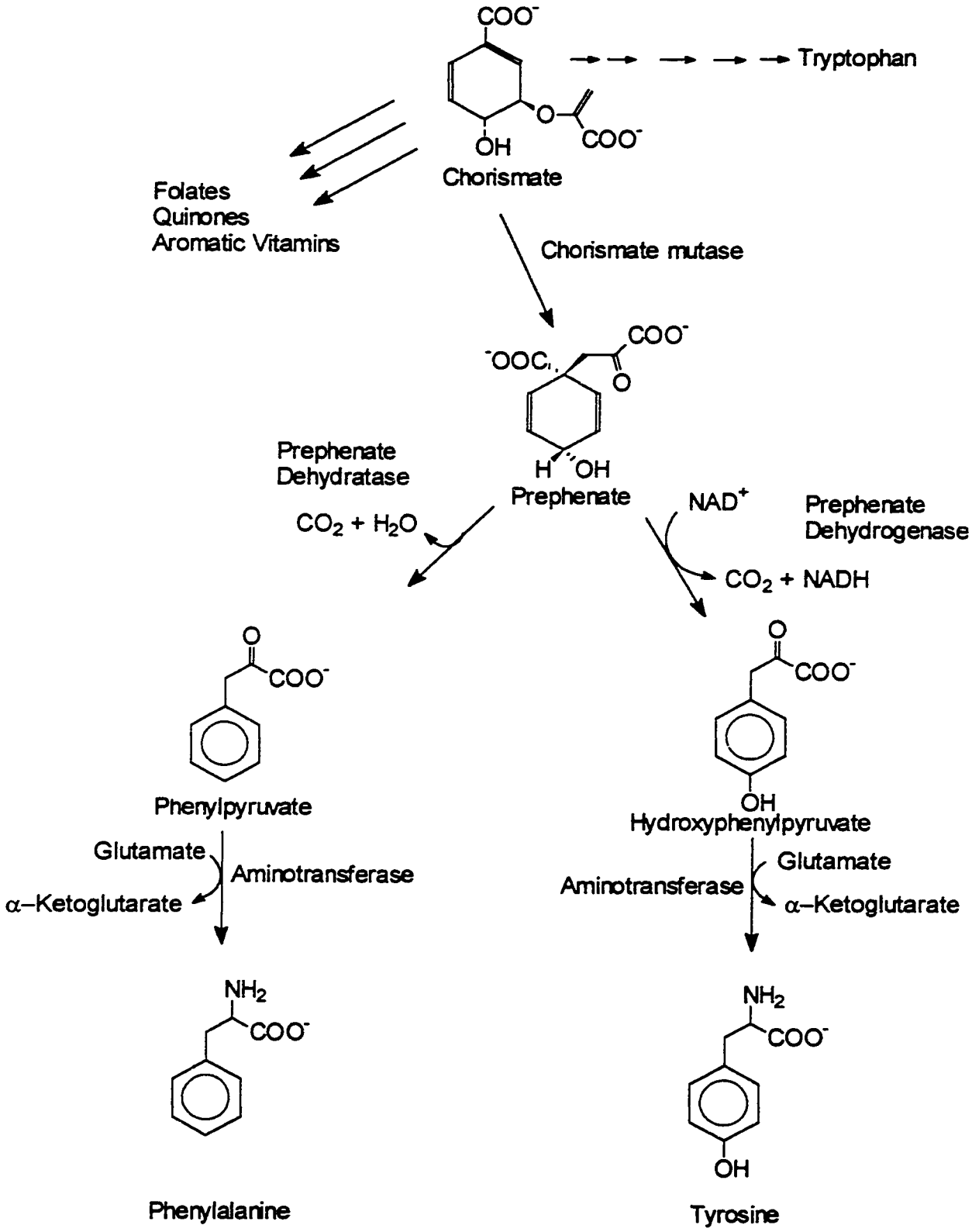


Figure 1.2 The branch pathway



Therefore, these isozymes allow the cell to moderate its overall rate of synthesis in response to changes in the level of a particular amino acid.

The next three reactions in the pathway are unregulated. DAHP is cyclized to 5-dehydroquinate (DHQ) by DHQ synthase (10). DHQ synthase is constitutively expressed and was first identified as an important catalyst in the biosynthesis of aromatic amino acids in 1953 (10). DHQ synthase exists as a monomer of 57 kDa and requires both NAD^+ and Co^{+2} for catalysis (11).

DHQ dehydratase catalyzes the dehydration of DHQ to 3-dehydroshikimate and introduces the first double bond into the ring destined to become chorismate. This enzyme was first isolated and purified from *A.aerogenes* and *E.coli* by Mitsuhashi and Davis (12), and was shown to be a dimer of 60 kDa.

The reduction of the carbonyl of 3-dehydroshikimate to shikimate is catalyzed by a monomeric 32 kDa protein, shikimate dehydrogenase. This protein is constitutively expressed in *E.coli* (13).

The phosphorylation of shikimate to shikimate 3-phosphate is catalyzed by shikimate kinase isozymes, SKI and SKII (14) and is regulated by Tyr and Trp.

The next step in the pathway involves the enzyme enolpyruvoylshikimate-3-phosphate (EPSP) synthase, which catalyzes the transfer of PEP to shikimate 3-phosphate to give 5-enolpyruvoylshikimate-3-phosphate (EPSP) (15).

The last step in the shikimate pathway involves the conversion of EPSP to chorismate by chorismate synthase. This enzyme, first isolated from *E.coli*, is sensitive to oxygen and requires a reducing environment for activity (16).

Branched Pathway

Chorismate serves as the branch point for the synthesis of the three aromatic amino acids as well as other aromatic compounds (Figure 1.2). Chorismate mutase catalyzes the conversion of chorismate to prephenate and plays a central role in the biosynthesis of two aromatic amino acids, phenylalanine and tyrosine, in plants and micro-organisms (17). The chorismate mutase reaction is unique because it is the only known enzyme-catalyzed Claisen rearrangement in nature. Chorismate mutases exist as both monofunctional and bifunctional forms. Bifunctional refers to the fact that chorismate mutase is associated with another enzyme of the pathway and that the two activities reside on a single polypeptide. Both bifunctional and monofunctional chorismate mutases have been isolated from micro-organisms, however, only monofunctional forms have been isolated from plants (18, 19).

Biosynthesis of Tyrosine

We are interested in the *E.coli* bifunctional enzyme, chorismate mutase-prephenate dehydrogenase also known as the T-protein, which is situated in the tyrosine biosynthetic pathway (Figure 1.2). Chorismate mutase catalyzes the rearrangement of chorismate to prephenate, while the prephenate dehydrogenase activity is responsible for the oxidative decarboxylation of prephenate to (4-hydroxyphenyl)pyruvate in the presence of NAD^+ (Figure 1.2). An aromatic aminotransferase then catalyzes the transfer of an amino group from glutamate to the α-keto acid producing tyrosine. The mutase-dehydrogenase enzyme is highly regulated by the end-product of its pathway, tyrosine. However, prephenate dehydrogenase activity is inhibited to a greater extent than the chorismate mutase reaction (20). Moreover, NAD^+ enhances tyrosine inhibition of both mutase and dehydrogenase activities by increasing the enzyme's affinity for the modulator (21, 22).

Biosynthesis of Phenylalanine

The first two reactions in the biosynthesis of phenylalanine from chorismate in *E.coli* also involve a bifunctional enzyme, chorismate mutase-prephenate dehydratase, also known as the P-protein (Figure 1.2). The first activity converts chorismate to prephenate, while prephenate dehydratase catalyzes the dehydration and decarboxylation of prephenate to yield phenylpyruvate. This product undergoes a transamination to form phenylalanine (Figure 1.2). The end-product phenylalanine allosterically inhibits both mutase and dehydratase activities, but the dehydratase activity is affected significantly more (23).

Arogenate Pathway

This is an ancestral pathway, which is thought to coexist with the branch pathway (24). As shown in Figure 1.3, the first reaction of the pathway involves the production of L-arogenate via transfer of an amino group from glutamate or aspartate to prephenate (24). The enzyme arogenate dehydrogenase catalyzes the oxidative decarboxylation of L-arogenate to form tyrosine, in the presence of either NAD^+ or $NADP^+$. Arogenate dehydrogenase has been isolated from a variety of sources (24) and was shown to be inhibited by tyrosine. Another enzyme, arogenate dehydratase, catalyzes the decarboxylation and dehydration of L-arogenate to form phenylalanine. This enzyme isolated from *Euglena gracilis* (25) and *P.diminuta* (26) has been shown to be regulated by phenylalanine.

Mechanisms of Chorismate Rearrangement

The rearrangement of chorismate to prephenate, as catalyzed by chorismate mutase, can occur in the absence of the enzyme, however the rate of the reaction is accelerated by over a million-fold in the presence of chorismate mutase (27, 28).

Non-Enzymatic Rearrangement of Chorismate

The uncatalyzed reaction of chorismate is an intramolecular mechanism that proceeds via a transition state of chair-like geometry (29) (Figure 1.4). Chorismate exists in two distinct conformers in aqueous solution, the diequatorial and the diaxial conformer. Proton NMR studies have indicated that 10 to 20% of chorismate, with its hydroxyl and enolpyruvyl group in the diaxial conformer, exists in equilibrium with the more stable

Figure 1.3 The arogenate pathway

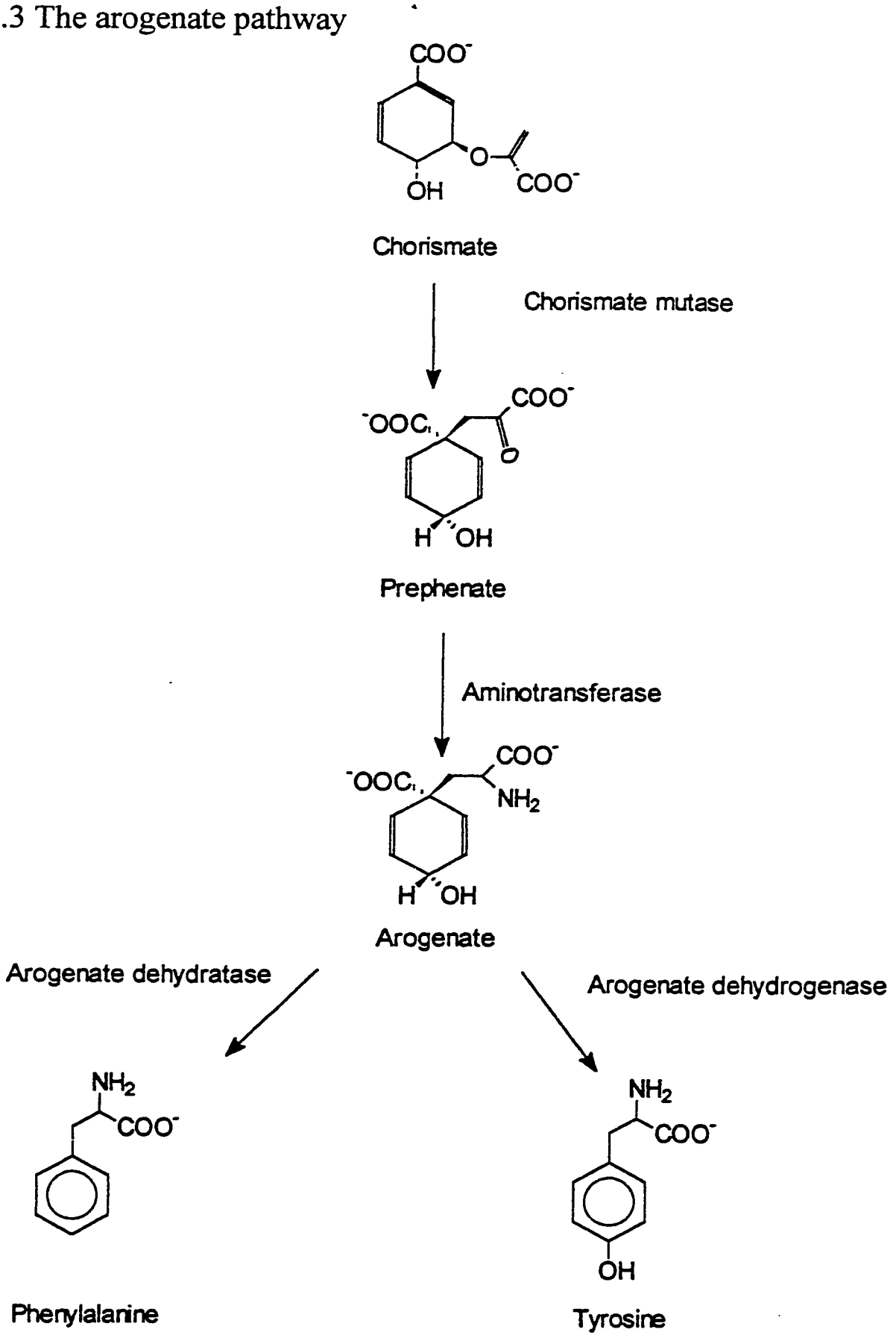
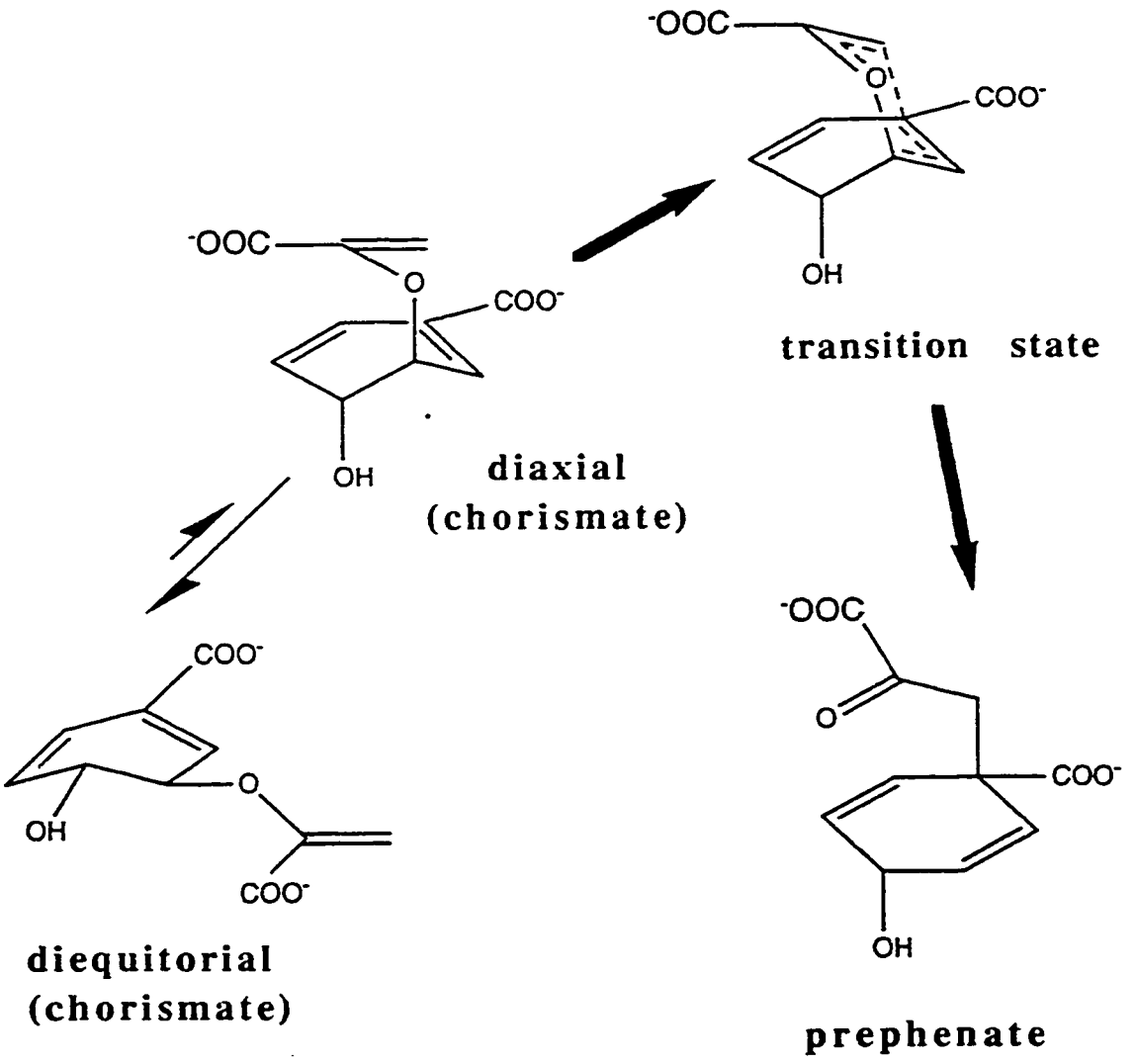


Figure 1.4 Uncatalyzed rearrangement of chorismate



diequitorial form (30). The non-enzymatic reaction occurs via the diaxial conformer of chorismate. In aqueous solution, the enolpyruvyl group of the diaxial conformer of chorismate is stabilized by hydrogen bonding to water molecules thus facilitating bond breakage between C-5 and the oxygen of chorismate. Secondary tritium isotope studies have implicated an asymmetric transition state in which this C_5–O bond breakage precedes bond formation at C-1 (31).

Enzymatic Rearrangement of Chorismate

Similar to the uncatalyzed rearrangement, chorismate mutase also selectively discriminates against the diequatorial conformer of chorismate (30). The structural features required for catalysis have been determined by the synthesis and characterization of numerous chorismate analogues (32, 33). Neither the 5,6-olefinic nor the 4-hydroxyl are necessary, but mutases require the allyl vinyl ether and the two carboxylate groups for binding chorismate to the active site (32). Two catalytic mechanisms (dissociative and non-dissociative pathways) have been proposed through which the enzymatic rearrangement may occur (31, 34, 35).

The Dissociative Pathway

This mechanism involves protonation of the ether bond on the enolpyruvyl side chain of chorismate by a general acid catalyst followed by a nucleophilic attack at C-5 by an enzymic group resulting in cleavage of the C-5 ether bond. This enolate intermediate is stabilized by the electrostatic environment in the active site. The next step is an S_N2 attack at C-1 of the cyclohexadiene by the resulting enolate pyruvate moiety to complete

the rearrangement process. D_2O solvent isotope studies on the mutase from *E.coli* mutase-dehydrogenase showed a significant effect on k_{cat} of about 2.2, indicating that the reaction is sensitive to the solvent proton isotope and thus supporting the hypothesis for general acid catalysis by the enzyme (35). The pH dependency of the rearrangement reaction catalyzed by the mutase component of mutase-dehydrogenase yielded a wave in the *V* profile indicating that deprotonation of a residue with a p*K* of 7.1 influenced the acceleration, but was not absolutely required for the rearrangement process over the pH range studied. However, the $(V/K)_{chorismate}$ profile showed three ionizable groups that are involved in chorismate binding and catalysis; one group that must be unprotonated and two groups that must be protonated. It was suggested that chorismate binds to only the correctly protonated form of the enzyme (36). Hence, a catalytic group seen in the *V/K* profile may not be seen in the *V* profile. This catalytic group, possibly a lysine, would be important for the protonation of the ether oxygen thereby facilitating cleavage of the C_5-O bond of chorismate.

Non-Dissociative Pathway

This mechanism, proposed for the monofunctional chorismate mutase from *B.subtilis,* is one of a simple unimolecular reaction in which the diaxial conformer of chorismate is selectively stabilized by the enzyme (34). Chorismate mutase lowers the entropy of activation of the reaction by restricting the conformational freedom of chorismate and by reducing the enthalpy of activation of the reaction through hydrogen-bond interactions in the enzyme-chorismate complex (37). Fourier transform infra-red spectroscopy (FTIR) studies with the monofunctional chorismate mutase from *B.subtilis* have indicated that

the carbonyl stretching for enzyme-bound prephenate is similar to that of prephenate in water (34). This observation indicates that the enzyme-assisted rearrangement may proceed by a similar transition state to that of the uncatalyzed reaction. Hence, this mechanism alone brings about the enhanced rate acceleration in the enzymic reaction. The *V* and *V/K* pH rate profiles for the rearrangement catalyzed by the mutase from *B.subtilis* are pH-independent between pH 5-9 (38). This observation suggests that enzymic acid/base are not involved in the catalyzed reaction.

Three-Dimensional Structures of Chorismate Mutases

The crystal structures of monofunctional chorismate mutase from *S.cerevisiae, B.subtilis* and from the engineered "minimutase" containing the first 109 amino acids from the *E.coli* mutase-dehydratase are now available (39, 40, 41, 42). The structures for *B.subtilis* (Figure 1.5) and *E.coli* (Figure 1.6) enzymes have been solved with an analogue resembling the transition state of the mutase reaction complexed at the active site of these enzymes (40, 41, 42). The transition state analogue referred to as *endo*-oxabicyclic diacid was synthesized by Bartlett and Johnson (43) and was shown to be a potent and selective inhibitor of chorismate mutase; it binds to the enzyme about 250-fold more tightly than chorismate (20, 44). The structure of the *E.coli* "minimutase" and the *B.subtilis* chorismate mutase complexed with *endo*-oxabicyclic diacid has provided valuable insights as to the active site residues that may participate in the Claisen rearrangement of chorismate (40, 41, 42). Although alignment of the primary sequences of these three chorismate mutases revealed little sequence similarity, their crystal

Figure 1.5 The structure of *B.subtilis* chorismate mutase complexed with *endo*-oxabicyclic diacid

This figure was adapted from Lee *et al.* (42).

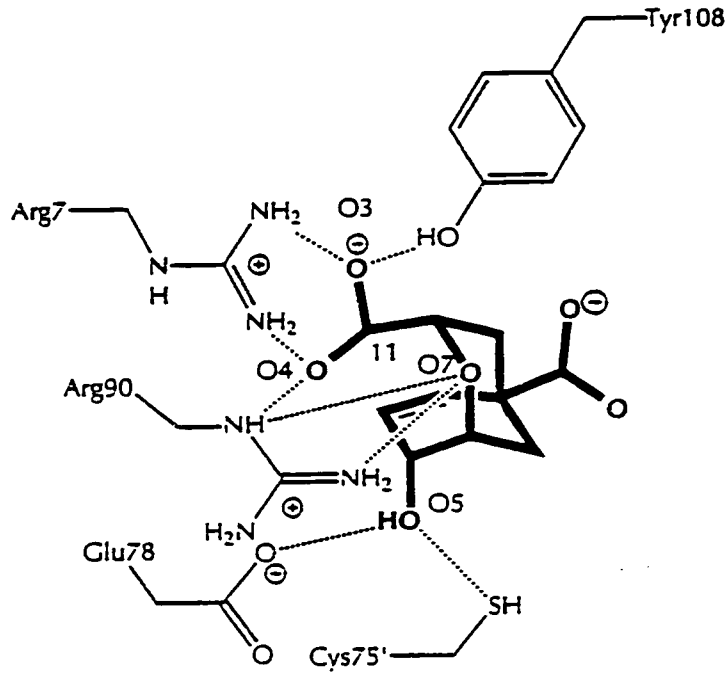
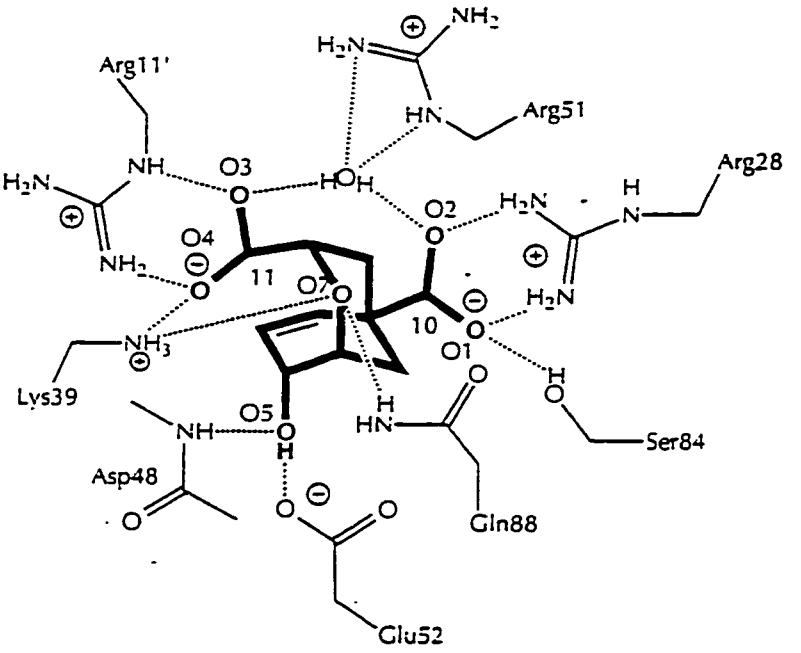


Figure 1.6 The structure of *E.coli* "minimutase" complexed with the *endo*-oxabicyclic diacid

This figure was adapted from Lee *et al.* (42).



structures showed that most of their active site residues are conserved. While the crystal structure of chorismate mutase from *S.cerevisiae* is available, the active site has not been experimentally identified. Xue and Lipscomb (45) have simulated an active site complexed with the transition state analogue by comparing the sequence and structure of *S.cerevisiae* chorismate mutase to that of the *E.coli* "minimutase". Interestingly, four of the conserved active site residues are located in the crevice of the molecule (45). When the active sites of the *E.coli* "minimutase" and the *B.subtilis* chorismate mutase are compared, a common motif emerges showing an extensive network of positively charged residues that interact with the enolpyruvyl group of chorismate. It is proposed that these positively charged residues are strategically placed to orient and lock chorismate in the requisite conformer for the rearrangement.

The overall three-dimensional structure of chorismate mutase from *B.subtilis* was shown to be different from that of *S.cerevisiae* and the *E.coli* "minimutase" (39, 41, 42). The *B.subtilis* enzyme is monofunctional and exists as a trimer with shared active sites at the subunit interfaces. The peptide backbone of *B.subtilis* chorismate mutase adopts a five-stranded mixed β-sheet together with a single α-helix and two 3_{10}-helices. Interestingly, the active site is open and accessible to solvent which may explain why its transition state is similar to the uncatalyzed reaction (41).

In contrast, the *E.coli* "minimutase" and the monofunctional chorismate mutase from *S.cerevisiae* are predominantly helical (39, 42). The *E.coli* "minimutase" is a dimer and each monomer folds into three helices connected by two loops. The structure of

chorismate mutase complexed with the *endo*-oxabicyclic diacid shows that residues from each monomer contribute to a complete active site which is located in a four-helix bundle buried at the subunit interface (42). This enclosed active site may explain why the proposed *E.coli* chorismate catalyzed rearrangement is different from that of the *B.subtilis* enzyme. Chorismate mutase from *S.cerevisiae* is a homodimer of 60 kDa. In contrast to the *E.coli* "minimutase", each monomer folds into twelve helices. The active site has been elucidated by computer modelling and was also shown to be located at the interface of a four-helix bundle (39).

Endo-oxabicyclic diacid was also used to engineer murine antibodies that are capable of catalyzing the rearrangement of chorismate to prephenate (46, 47, 48, 49). Interestingly, of the three antibodies generated, 11F1-2E11, 1F7 and AZ-28, the most efficient catalyst is the AZ-28 antibody, which showed a 10^5-fold increase over the uncatalyzed reaction (49). The three-dimensional crystal structure of the complex AZ-28 with *endo*-oxabicyclic diacid revealed an extensive network of binding interactions in the active site (49). It is thought that these electrostatic interactions provide AZ-28 with its catalytic properties for the rearrangement of chorismate.

The structures of chorismate mutase complexed with *endo*-oxabicyclic diacid for both the *E.coli* "minimutase" and the *B.subtilis* enzyme have been used to target active site residues for mutagenesis (50, 51). Results from studies on these active site mutants suggest a simple Claisen rearrangement mechanism for both the *E.coli* "minimutase" and the *B.subtilis* chorismate mutase. This mechanism occurs when protonated active site

residues exert conformational control in the enzyme-chorismate complex and hydrogen bonding with the O_7 of chorismate increases its ground state enthalpy to facilitate the rearrangement process. Interestingly, studies on the activation entropy from different chorismate mutases revealed two ranges of entropy values for the enzymic reaction (37). A large unfavourable decrease in entropy of activation, similar to that of the uncatalyzed reaction, was observed for *B.subtilis* chorismate mutase and the catalytic antibody IF7, which supports the non-dissociative mechanism. However, changes in activation entropy values close to zero were observed for other chorismate mutases including the *E.coli* "minimutase" and the catalytic antibody 11F1-2E11, which may indicate the existence of other catalytic mechanisms (ie. general acid catalysis).

Prephenate Dehydrogenase

The oxidative decarboxylation of prephenate to (4-hydroxyphenyl)pyruvate in the presence of NAD^+ is catalyzed by prephenate dehydrogenase (Figure 1.2). This reaction is essentially irreversible being driven by the aromaticity of the ensuing product. While the uncatalyzed reaction is not observed, prephenate can be rapidly decarboxylated under acidic conditions to give phenylpyruvate (52). The acid-assisted decarboxylation follows a stepwise mechanism (52). Protonation of the hydroxyl group on prephenate results in the formation of a resonance stabilized carbonium ion followed by decarboxylation to produce phenylpyruvate. In contrast, the enzyme-catalyzed reaction proceeds with hydride transfer and decarboxylation occurring via a concerted mechanism (52). This mechanism was derived from isotope effect studies conducted with prephenate and prephenate analogues by Hermes *et al.*, (52). Using the substrate analogue

deoxoprephenate, deuterated at C-4, Hermes (52) observed an isotope effect for the hydride transfer to NAD^+. In addition, a carbon isotope effect was observed for cleavage of the C-C bond between the cyclohexadiene ring and the ring carboxylate using the natural abundance of ^{13}C in the substrate. Interestingly, the ^{13}C isotope effect was larger in the presence of deuterium versus hydrogen at C-4 suggesting that both the ^{13}C and the deuterium isotope effects are in the same transition state; that is, deuterium has made the ^{13}C-sensitive step more rate-limiting by slowing it down (52).

It has been suggested that the mechanism of the dehydrogenase reaction involves a catalytic residue, most likely a histidine, that assists in the hydride transfer to NAD^+ and the concomitant decarboxylation (52). This conclusion is based on studies of the pH dependence of the kinetic parameters *V* and *V/K* for the reaction of prephenate with the enzyme-NAD^+ complex. These studies show that ionizable groups are involved in substrate binding and catalysis (36). The variation of *V* with pH yields a half-bell profile and indicates that an unprotonated group is essential for catalysis. In contrast, the *V/K* profile is bell-shaped indicating two ionizable groups are required for enzyme activity: an unprotonated group involved in catalysis and a protonated group likely involved in the binding of prephenate. The p*K* value of this catalytic group is consistent with a histidine residue.

Studies by Hermes *et al.* (52) and Turnbull *et al.* (57) have elucidated some of the structural requirements for substrates of prephenate dehydrogenase. These studies

indicated that the absence of the carbonyl group of the pyruvyl moiety of prephenate had no effect on catalysis, but saturation of one of the double bonds caused a 15-fold decrease in the maximum velocity of the reaction. Moreover, the position of the double bond in the ring structure was also critical. A 1000-fold drop in the maximum velocity occurred when the ring was completely saturated. A change of the carbonyl group to an amino group, to produce arogenate, caused a 10-fold reduction in binding of the substrate and in maximum activity.

The kinetic mechanism of the dehydrogenase reaction catalyzed by mutase-dehydrogenase has been investigated in *E.coli* (53) and *A.aerogenes* (54). Initial velocity patterns, product and dead-end inhibition patterns, along with isotope partition experiments using ^{14}C-NAD$^+$ established that the binding of substrates and release of products occurs via a rapid equilibrium, random mechanism with catalysis as the rate-limiting step of the reaction. This is in contrast to many other dehydrogenases which follow an ordered reaction with NAD$^+$ binding first (52).

E.coli Chorismate Mutase-Prephenate Dehydrogenase

Chorismate mutase-prephenate dehydrogenase from *E.coli*, is a homodimer with molecular weight of 84 kDa (55, 56, 57). The enzyme encodes both mutase and dehydrogenase activities on each subunit and has been shown to be allosterically inhibited by the end-product of the pathway, tyrosine (56, 57, 58). Since the product of the first reaction, prephenate, is a substrate for the second reaction, there has been much interest in determining the spatial geometry of the active site(s) in which the two

reactions occur. There is evidence supporting a common binding site for prephenate and chorismate, as well as evidence for two distinct non-interacting yet closely situated active sites. Studies supporting a single site include those of Koch *et al.*, (59), Heyde (60) and Hudson *et al.*, (56). These authors showed that a variety of inactivating agents such as urea, chemical modifying reagents, extreme pH, and thermal denaturation led to parallel loss of both mutase and dehydrogenase activities. Kinetic studies showed that the two reactions are catalyzed with similar turnover numbers (53). Moreover, Heyde (60) showed that prephenate acting as a substrate in the dehydrogenase reaction or as an inhibitor of the mutase reaction bound with similar affinities. The same author also showed that there was very little channelling of prephenate as an intermediate in the overall reaction. Early attempts to produce monofunctional enzymes through random mutagenesis resulted in an active mutase, however loss of dehydrogenase activity was due to the disruption of the nucleotide-binding site (61).

The genetic approach has been used more recently to support the idea of two separate sites. Maruya *et al.* (62) used molecular genetic techniques to subclone the proposed *E.coli* mutase and dehydrogenase domains separately and demonstrated the independence of these two domains under specific growth conditions. However, the proteins were never isolated and characterized. Recently Xia *et al.*, (63) produced a mutant mutase-dehydrogenase from *E.herbicola*, missing the first 37 N-terminal amino acids, that retained only prephenate dehydrogenase activity. Turnbull and Morrison (20) provided kinetic evidence that demonstrated the existence of distinct mutase and dehydrogenase active sites. They showed that a transition state analogue of the mutase reaction, the

endo-oxabicyclic diacid, selectively inhibited the mutase activity without affecting prephenate binding to the dehydrogenase site.

The crystal structures of chorismate mutase from *E.coli* minimutase and *B.subtilis* revealed a highly charged network of active site residues. Determining the roles of these positively charged residues will help to elucidate the mechanism through which chorismate mutase accelerates the rearrangement of chorismate to prephenate. Before the initiation of these studies, the identity of the residues involved in the oxidative decarboxylation reaction catalyzed by prephenate dehydrogenase were unknown and little was known about the structural requirements for substrate binding.

Scope of this thesis

In this project, we have conducted mechanistic studies on the *E.coli* bifunctional enzyme, chorismate mutase-prephenate dehydrogenase. The project has been divided into five sections:

1) The overexpression, purification and characterization of recombinant mutase-dehydrogenase;

2) Identification through chemical modification, differential peptide mapping and site-directed mutagenesis of an essential active site residue for the mutase reaction;

3) Verification that a histidine residue is important for dehydrogenase activity by chemical modification and site-directed mutagenesis. The role of this histidine in the dehydrogenase reaction was then deduced by kinetic studies;

4) Identification of an active site residue that electrostatically interacts with the ring carboxylate of prephenate that is lost during the decarboxylation process;

5) Determination of the spatial geometry between the mutase and dehydrogenase active sites.

The *TyrA* gene, encoding mutase-dehydrogenase, was subcloned from its operon into a commercially available expression vector, pSE380 (Invitrogen). The resulting vector, pVIV1 produced mutase-dehydrogenase with a final yield of about 150 mg of pure protein per L of culture. The enzyme was purified using a modified procedure of Turnbull *et al.*, (36) which increased the yield of purified protein by about 35-fold. This recombinant enzyme was shown to have similar kinetic and physical properties to those of the native enzyme.

We investigated the role of an essential ionizable residue for the mutase reaction. Chemical modification of mutase-dehydrogenase with diethylpyrocarbonate (DEPC) resulted in the parallel loss of both mutase and dehydrogenase activities. However, the kinetics of inactivation of the mutase were consistent with the modified group being a lysine. Chorismate mutase was also inactivated by the lysine-specific reagent, trinitrobenzene sulfonate (TNBS). By a combination of HPLC and mass spectrometry, we identified Lys37 as the group that was initially modified by DEPC and was essential for mutase activity. Site-directed mutagenesis confirmed our finding that this lysine residue was indeed essential for mutase activity. From the recent crystal structures for the monofunctional mutases from *E.coli* "minimutase" and *B.subtilis,* which show this conserved cationic group, we conclude that Lys37 forms important interactions with chorismate in the transition state of the reaction.

Previous studies indicated that a catalytic group, perhaps a histidine, was important in the hydride transfer from prephenate to NAD^+. Chemical modification studies with DEPC implicated histidine as a group important for dehydrogenase activity. Sequence alignment and an alanine/asparagine scan of histidine residues by site-directed mutagenesis identified this group as His197. Examination of the pH dependency of the reaction catalyzed by wild-type enzyme and H197N confirmed that His197 was this catalytic group and that it had to be unprotonated to assist in the hydride transfer step.

Prephenate consists of two carboxylates that are ionized at physiological pH. We proposed that positively charged groups on the enzyme electrostatically interacted with these carboxylates. Sequence alignment of many prephenate dehydrogenases revealed three absolutely conserved residues, a lysine and two arginine groups. Site-directed mutagenesis and characterization of wild-type and mutant proteins with prephenate analogues indicated that Arg294 was important for prephenate binding and interacted with the ring carboxylate of prephenate.

The spatial relationship between the sites at which the two reactions occur is of special interest in this bifunctional enzyme. We have provided some convincing evidence supporting two separate active sites for mutase-dehydrogenase rather than one combined site for the binding of prephenate and chorismate. Through chemical modification, we can inactivate both mutase and dehydrogenase activities but can selectively regain only the dehydrogenase activity. We have isolated mutant proteins generated through site-directed mutagenesis that retain only mutase or dehydrogenase activity. Inhibition

studies conducted on a mutant protein show that chorismate cannot combine at the prephenate binding site.

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CHAPTER 2

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

FOOTNOTES

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

[2] In a separate experiment, double digestion by V8 protease of DEPC-modified mutase-dehydrogenase afforded a mass signal at m/z = 2075.4. This corresponds to the value for a peptide of sequence VGEVKSRFGLPIYVERE (amino acids 33 to 50, $[M + H]^+$ = 2075.4)-- a fragment that upon complete digestion would have yielded the peptide comprising amino acids 36 to 48. The spectrum also showed the multiplet containing the TNB-lysyl adduct and putative fragments of this adduct.

[3] Cysteine adducts with DEPC exhibit a maximum absorbance at 230 nm, and their cleavage can be promoted by hydroxylamine. However, this adduct is only formed in carboxylate buffers; it is not known to form in phosphate (1). In addition, the half-life of the DEPC cysteine adduct at pH 6.4 is about 2 hr compared to 55 hr for carbethoxyimidazole (2).

SUMMARY

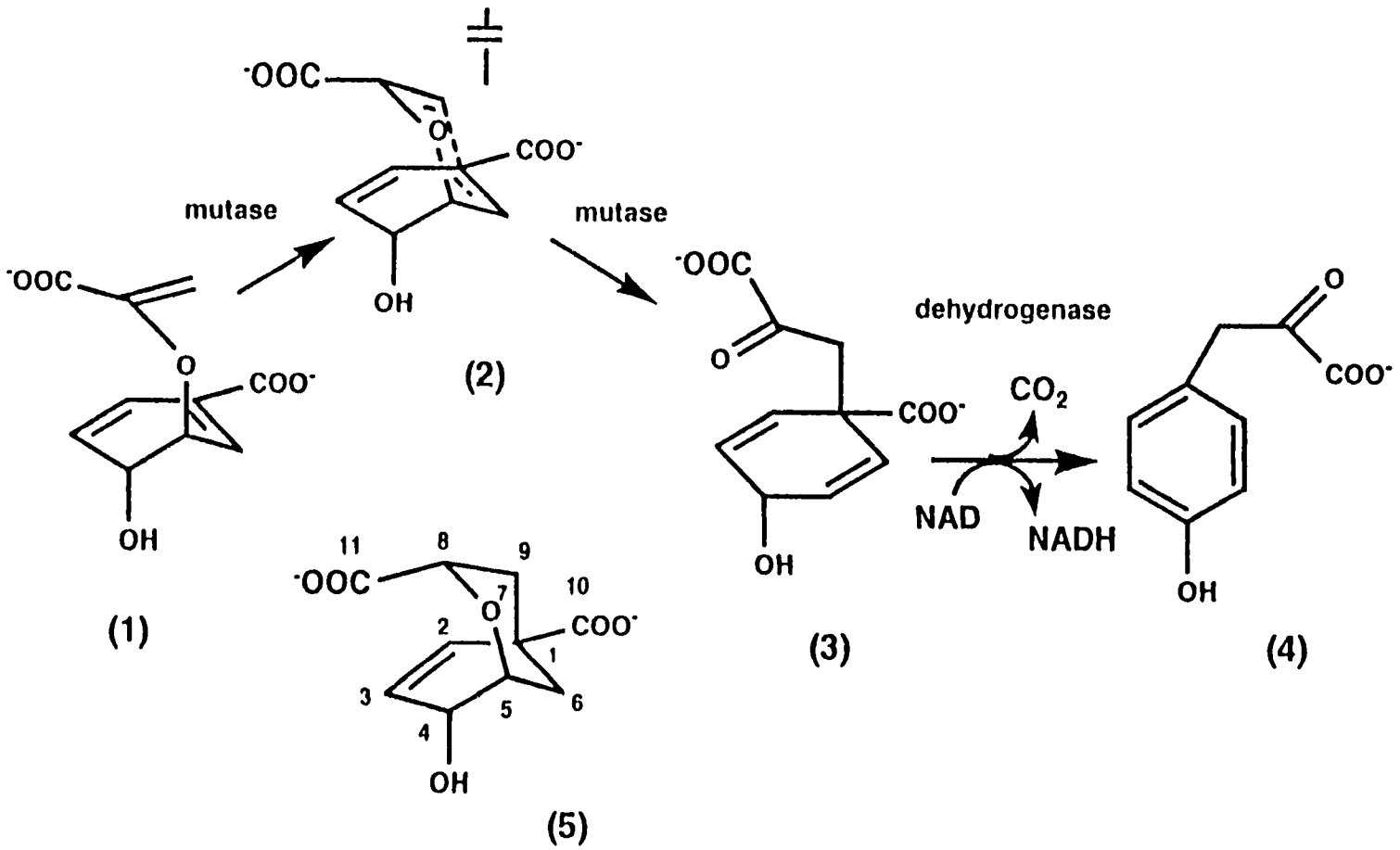
Chemical modification studies of the bifunctional enzyme chorismate mutase-prephenate dehydrogenase and mass spectral analysis of peptide fragments containing modified residues are presented. The reaction with diethylpyrocarbonate (DEPC) results in the modification of several enzymic groups including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

INTRODUCTION

Chorismate mutase-prephenate dehydrogenase (EC 5.4.99.5 and EC 1.3.1.12; 4-hydroxyphenylpyruvate synthase) is a bifunctional enzyme that catalyzes two sequential reactions in the biosynthesis of L-tyrosine in *Escherichia coli* and other enteric bacteria (3, 4). Chorismate mutase catalyzes the Claisen rearrangement of chorismate (**1**) to prephenate (**3**), which is believed to proceed via a chair-like transition state (**2**) (5, 6). Prephenate dehydrogenase is responsible for the oxidative decarboxylation of prephenate to (4-hydroxyphenyl)pyruvate (**4**) in the presence of NAD^+ [1] (Figure 2.1). The enzyme from *E. coli* is homodimeric with a molecular weight of 78,000 to 88,000 (7, 8, 9). Both activities are associated with each subunit and are allosterically inhibited by the end product of the pathway, L-tyrosine (10, 11, 12).

The mutase activity of chorismate mutase-prephenate dehydrogenase is very similar to that of chorismate mutase-prephenate dehydratase (a bifunctional enzyme involved in the conversion of chorismate to phenylalanine), and the N-terminal portions of their amino acid sequences are highly homologous (13, 8). In contrast, two other well studied chorismate mutases in *Saccharomyces cerevisiae* (14) and *Bacillus subtilis* (15, 16) bear little sequence homology to each other or to the *E.coli* enzymes. Crystal structures are now available for these two mutases and for the chorismate mutase domain of the mutase-dehydratase ("minimutase") (17, 18, 19), and these confirm that there is no resemblance in secondary or tertiary structure. However, the active sites of the mutases from *B.subtilis* and *E.coli* are electrostatically similar (19, 20).

Figure 2.1 The reactions catalyzed by chorismate mutase-prephenate dehydrogenase.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OH
(5)
(3)
(4)

Mechanistic studies on the mutase from *B.subtilis* indicate that this enzyme catalyzes the rearrangement by a simple pericyclic process similar to the uncatalyzed reaction, where the chemistry is an inherent property of chorismate (16, 21, 22). By contrast, the results from pH profiles (23) and isotope effects (24, 25) together suggest that the bifunctional *E.coli* mutase-dehydrogenase has enzyme groups which act as acid or base catalysts. The structure of the "minimutase" complexed with the *endo*-oxabicyclic diacid (**5**), which mimics the bicyclic structure of the transition state, has provided valuable insight as to the amino acid residues that may participate in the rearrangement (19). These groups include Lys39, Gln88, Asp48, Glu52, Ser84 and arginine residues at positions 11, 28 and 51.

The kinetic mechanism of the prephenate dehydrogenase reaction conforms to a rapid equilibrium random mechanism with catalysis as the rate-limiting step (26). Studies of the pH dependence of the kinetic parameters *V* and *V/K* for the dehydrogenase reaction have determined that ionizable groups are involved in substrate binding and catalysis (12, 27). These studies have shown that an unprotonated group is required for catalysis while a protonated group is required for substrate binding. The results of temperature and solvent perturbation studies by Hermes *et al.*, (27) implied that the catalytic group is a cationic acid, possibly a histidine residue. These authors hypothesized that the histidine could accept a hydrogen bond from the 4-hydroxyl group of prephenate to facilitate hydride transfer and the concomitant decarboxylation to yield (4-hydroxyphenyl)pyruvate (27).

Since the product of the first reaction is a substrate for the second reaction, many studies have centered on the spatial relationship between the sites at which the two reactions occur. Evidence in favour of a single combining site comes from several studies on the mutase-dehydrogenase from *E.coli* and *A.aerogenes*. Subjecting the enzyme to heat, urea, limited proteolysis (28) and cysteine modifying agents (8, 29) has led to a coordinate loss of both activities. Moreover, inactivation of both activities by alkylation can be prevented by prephenate, NAD^+, and tyrosine plus NAD^+ (8, 29). Kinetic data obtained by Heyde and Morrison (30) and the results of investigations of the inhibition of mutase and dehydrogenase activities of the enzyme with compounds that are clearly analogues of either chorismate or prephenate (31) tended to indicate a common binding site or two overlapping sites. There is evidence that some of the prephenate formed from chorismate is converted directly to (4-hydroxypheny)pyruvate (28). However, these results are also consistent with two catalytic sites in close proximity. The results differ from those obtained for the mutase-dehydratase for which there are clearly two distinct active sites (32, 33).

Recently, there has been more direct evidence in favour of two distinct active sites for the *E.coli* mutase-dehydrogenase. The two activities show different pH rate profiles (23) and are inhibited to different degrees by tyrosine (12), malonic acid derivatives and other substrate analogues (9). The putative transition state analogue *endo*-oxabicyclic diacid is a very selective inhibitor of the mutase (9). In addition, sequence homology with other bifunctional chorismate mutases suggests that the mutase activity is located on the N-terminal third of the protein (13). This has been confirmed by Maruya *et al.* (34), who constructed separate plasmids encoding the hypothetical mutase and dehydrogenase

domains and noted that under certain growth conditions, the plasmids were able to complement the defect in a host strain which had an inactivated mutase-dehydrogenase. In the work presented below, our goal was to identify amino acids important for catalysis of each reaction. We have used a combination of chemical modification and mass spectrometry of peptides from proteolytic digests to identify two residues, one of which is important for each activity. Protection studies support the hypothesis that these groups are at distinct active sites.

EXPERIMENTAL PROCEDURES

Materials

Chorismate was isolated from *Klebsiella pneumonia* strain 62-1 as described by Gibson (35) with modifications that significantly improved the yield of chorismate from this organism (36). Prephenate was prepared as previously described (37). NAD^+ was obtained in free acid form from Boehringer Mannheim. DEPC was obtained from ICN Biochemicals while TNBS as 2 % solution in water was from Aldrich. HPLC-grade TFA and acetonitrile were from Baker. Hydroxylamine was obtained from Fisher while trypsin and *Staphylococcus aureus (S.aureus)* V8 protease were both from Sigma. (3-endo-8-exo)-8-hydroxy-2oxa (3.3.1) non-6-ene-3,5-dicarboxylic acid bis (dicyclohexyl-ammonium) salt, (*endo*-oxabicyclic diacid), was a generous gift from Professor Paul Bartlett of the University of California, Berkeley. All other chemicals were obtained commercially and were of the highest quality available.

Source of Chorismate Mutase-Prephenate Dehydrogenase

Chorismate mutase-prephenate dehydrogenase was obtained from an overproducing strain of *E.coli* (JFM30) that carries a multicopy plasmid (38). Cells were suspended in a buffered solution then disrupted by repeated freeze-thawing followed by treatment with lysozyme (39). The enzyme was purified to homogeneity by a procedure that involves affinity chromatography on Matrex Blue A and Sepharose AMP (9). Subsequent chromatography on DEAE-Sepharose removed all traces of contaminating chorismate mutase-prephenate dehydratase. The enzyme preparation was over 95 % pure as judged by silver staining of a denaturing polyacrylamide gel.

Determination of Enzyme Activity and Protein Concentration

Kinetic investigations on the mutase-dehydrogenase were performed at 30°C in the presence of the three-component buffer system (40) of 0.10 M MES, 0.051 M N-ethylmorpholine, 0.051 M diethanolamine (pH 7.2), 1 mM EDTA and 1 mM DTT. The total volume of reaction was 1.0 mL. Prephenate dehydrogenase activity was measured by monitoring at 340 nm the formation of NADH from NAD^+ in the presence of prephenate, while chorismate mutase activity was determined following the disappearance of chorismate at 274 nm (9). Saturating amounts of the substrates prephenate (0.5 mM), NAD^+ (2 mM) and chorismate (0.5 mM) were used. A double beam spectrophotometer (GBC Model 918) fitted with thermostatically controlled cuvette holders was used. Protein concentration was estimated using the Bio-Rad protein assay kit with bovine serum albumin as a standard and by recording the absorbance at 205 nm (41). The results by the two methods were in good agreement.

Inactivation Kinetics of Mutase-Dehydrogenase with Diethylpyrocarbonate

A stock solution of DEPC was prepared before each experiment by dilution into ice-cold absolute ethanol. The concentration of DEPC was determined spectrophotometrically by reaction with 10 mM imidazole (pH 7.2) and monitoring the increase in absorbance at 240 nm due to the formation of N-carbethoxyimidazole (ε = 3400 M^{-1} cm^{-1}) (42, 43). Inactivation experiments were performed by incubating mutase-dehydrogenase (22 μM monomer concentration) with DEPC in 50 mM potassium phosphate buffer (pH 7.2) at 25°C in the absence or presence of ligands (prephenate, NAD^+ or *endo*-oxabicyclic diacid) (0.1 mL total volume). Aliquots of 10 μl were withdrawn from the incubation mixture at various time intervals and placed in 1.0 mL of buffer containing 50 mM N-

ethylmorpholine (pH 7.2), which rapidly quenched the reaction. The residual activities of mutase and dehydrogenase were determined as described above. The final concentration of ethanol was always kept lower than 5 % (v/v) of the total incubation mixture and was shown to have no effect on enzyme activity.

The pH dependence of prephenate dehydrogenase inactivation by 0.3 mM DEPC was determined in an incubation buffer of 50 mM potassium phosphate over the pH range of 5.2 to 8.6 at 25°C. The first-order rate constant for the decomposition of DEPC (k') was calculated by measuring the time-dependent decomposition of DEPC in a separate control experiment. In this buffer the value of k' was estimated to be 0.00128 s^{-1} at pH 7.2 (half time 9 min) and increased only by 7 % at pH 8.6. All data values in this study were corrected for this decomposition using the value of k' at pH 7.2.

Restoration of Mutase-Dehydrogenase Activities Using Hydroxylamine.

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

UV-Difference Spectroscopy and Stoichiometry of Histidine Modification

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

Isolation of Active Site Peptides by Differential Peptide Mapping

The general strategy employed for the isolation and identification of peptides presumably at the mutase and dehydrogenase sites is outlined here. Mutase-dehydrogenase was reacted with an appropriate modifying reagent in the absence or presence of ligands which protect against reagent-mediated enzyme inactivation. In the dehydrogenase reaction, DEPC was the modifying reagent and prephenate was the protecting ligand. By contrast, trinitrobenzenesulfonate (TNBS) was used to modify the mutase and DEPC served to shield the reactive enzymic residue from TNBS modification. Following proteolytic digestion of the enzyme preparation, peptides containing chromophoric adducts were isolated with HPLC by following the absorbance of the appropriate derivatives, carbethoxyhistidine at 240 nm (43) and TNB-lysine at 350 nm (44). The

correct peptide in each case was identified using the chromatogram of digests of the protected enzymes as a reference. The masses of the isolated peptides were determined by mass spectrometry, then compared with the known molecular weights of the fragments in the peptide map to deduce the exact site of the derivitization.

Carbethoxyhistidyl Peptide

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

Trinitrobenzyllysyl Peptide

Mutase-dehydrogenase (22 μM monomer concentration) in 50 mM potassium phosphate buffer pH 7.2 was incubated with 0.3 mM DEPC for 1 min. The reaction was rapidly quenched by the addition of imidazole (final concentration of 2 mM). The modified enzyme was separated from carbethoxyimidazole by passing the mixture through a 5 mL Sephadex G-25 column equilibrated in the same phosphate buffer. Next, DEPC-modified and unmodified mutase-dehydrogenase were incubated at 25°C with 0.4 mM TNBS in 50 mM potassium phosphate buffer (pH 7.2) for 10 min. Further reaction was terminated by passing each of the mixtures through a 5 mL Sephadex G-25 column. To each of the isolated protein solutions was added *S.aureus* V8 protease at a final ratio of substrate to protease of 100:1 (w/w). Digestion was carried out overnight at 37°C and the samples were then frozen until further analyzed by HPLC. Chromatograms were obtained at room temperature at a flow rate of 1 mL/min with a linear gradient of 5 to 70 % acetonitrile in aqueous 0.1 % TFA (pH 2.0). The elution of TNB-lysyl peptides was monitored spectrophotometrically at 350 nm. The peptide of interest was collected manually and then lyophilized in a Speed Vac. The peptide was reconstituted in 50 mM potassium phosphate buffer pH 7.2. It was then redigested with V8 protease overnight at 37°C. Following a second chromatography step by reverse phase HPLC, peptides were collected then further purified through a Waters Sep-Pak column. The sample was snap frozen, lyophilized and stored at -86°C until mass spectral analysis was performed.

Mass Spectrometry of Isolated Active Site Peptides

Isolated peptides were analyzed using a Matrix-Assisted Laser Desorption Time-of-Flight (MALDI-TOF) mass spectrometer (Finnegan Lasermat). Approximately 2.5 pmol of

each peptide, previously desalted, was mixed with a peptide standard (substance P) and a matrix material (α-cyano-4 hydroxycinnamic acid) at a ratio of 1:1:2 in 70 % acetonitrile / 30 % water / 0.1 % TFA. The solution was spotted onto a stainless steel plate then allowed to air dry before loading into the mass spectrometer. Spectra were obtained by summing the data from multiple pulses of a laser (355 nm emission wavelength).

Additional Spectroscopic Measurements

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

Data Analysis

The kinetic data were fitted to the appropriate equations using the computer programs of Cleland (45) or GraFit (Version 3.0, Leatherbarrow). Steady-state kinetic data were analyzed using the equations outlined in Turnbull and Morrison (29) and Turnbull *et al.* (23) to obtain values for the Michaelis constants (K_m) for prephenate and NAD^+, and for the dissociation constants for the interaction of enzyme with prephenate in the dehydrogenase reaction (K_{ia}) and in the mutase reaction (K_i).

Data for the time-dependent inactivation of enzyme activity by DEPC, corrected for the decomposition of DEPC, were fitted to equation 1 to obtain values for $k_1 I_0$. A/A_0 is the fraction of activity remaining at time t, I_0 is the initial concentration of DEPC, k_1 is the

second-order rate constant for the reaction of the enzyme with reagent and k' is the first-order rate constant for the hydrolysis of the reagent (46). Since the concentrations of DEPC were normally well in excess of enzyme concentration, pseudo first-order conditions are assumed so that $k_I I_O$ represents k_{obs}, the observed pseudo first-order rate constant of enzyme inactivation.

$$\ln (A/A_O) = -(k_I/k') I_O (1 - e^{-k't}) \qquad (2.1)$$

Data for the effect of ligands on the pseudo-first-order rate constant of inactivation by DEPC were fitted to equation 2 to obtain values for the dissociation constant for the enzyme-ligand complex (K_S) and the rate constant for the combination of free enzyme with DEPC (k_I). I and L represent the concentrations of DEPC and ligand respectively (47).

$$k_{obs} = (I K_S k_I) / (K_S + L) \qquad (2.2)$$

The variation with pH of the values for the pseudo first-order rate constant of inactivation was fitted to equation 2.3 to obtain a value for the pH-independent value of k_{obs}, $(k_{obs})_{max}$, and for the acid dissociation constant (K_a). H is the hydrogen ion concentration.

$$k_{obs} = (k_{obs})_{max} / [1 + (H / K_a)] \qquad (2.3)$$

Data yielding linear plots were fitted by linear regression analysis.

RESULTS

Kinetics of DEPC Inactivation of Mutase-Dehydrogenase

Prephenate Dehydrogenase

Incubation of mutase-dehydrogenase with DEPC at 25 °C in 50 mM potassium phosphate buffer (pH 7.2) results in a time-dependent loss of prephenate dehydrogenase activity. Figure 2.2 shows a plot of ln of the remaining activity against effective incubation time [$(1 - e^{-k't}) / k'$], corrected for the decomposition of DEPC, for various concentrations of DEPC. The data follow first-order kinetics and are fitted to equation 2.1. The pseudo first-order rate constants (k_{obs}), as deduced from the slope of inactivation kinetics in Figure 2.2, vary linearly with DEPC concentration (Figure 2.3). The data also intersect the origin and taken together with the linear dependence, show that this inactivation is a simple irreversible bimolecular process that is not dependent on the reversible formation of an active enzyme-DEPC complex prior to inactivation (48). The second-order rate constant for the inactivation of dehydrogenase by DEPC calculated from the slope of the line in Figure 2.2 is 60 ± 5 $M^{-1}s^{-1}$. The double ln plot of k_{obs} versus DEPC concentration is also linear (Figure 2.3 inset). The slope of this line yields a reaction order (stoichiometry) of 1.05 ± 0.11 and implies that the loss of dehydrogenase activity is due to the modification of a single group per catalytic unit (49). The apparent pK_a of this reacting group was estimated by following the pH dependence of the pseudo first-order rate constant for DEPC inactivation of the dehydrogenase in the phosphate buffer. Over the pH range of 5.2 to 8.6, the ionization of a single group was observed (Figure 2.4). A pK_a value of 7.02 ± 0.14 and a maximum value of k_{obs} of 0.0218 ± 0.0002 s^{-1} was determined by fitting the data to equation 2.3.

Figure 2.2 Inactivation of prephenate dehydrogenase by DEPC

The dependence of residual activity on the effective incubation time. Mutase-dehydrogenase (22 μM) was incubated with increasing concentrations of DEPC in 50 mM potassium phosphate buffer (pH 7.2, 25°C) then samples were withdrawn at various time intervals for the determination of residual activity. The concentrations of DEPC used were 0 mM (▲), 0.50 mM (○), 0.75 mM (●), 1.00 mM (□), 1.25 mM (■), 1.50 mM (△). The data for A were fitted to equation 2.1 to obtain values for the pseudo first-order rate constants.

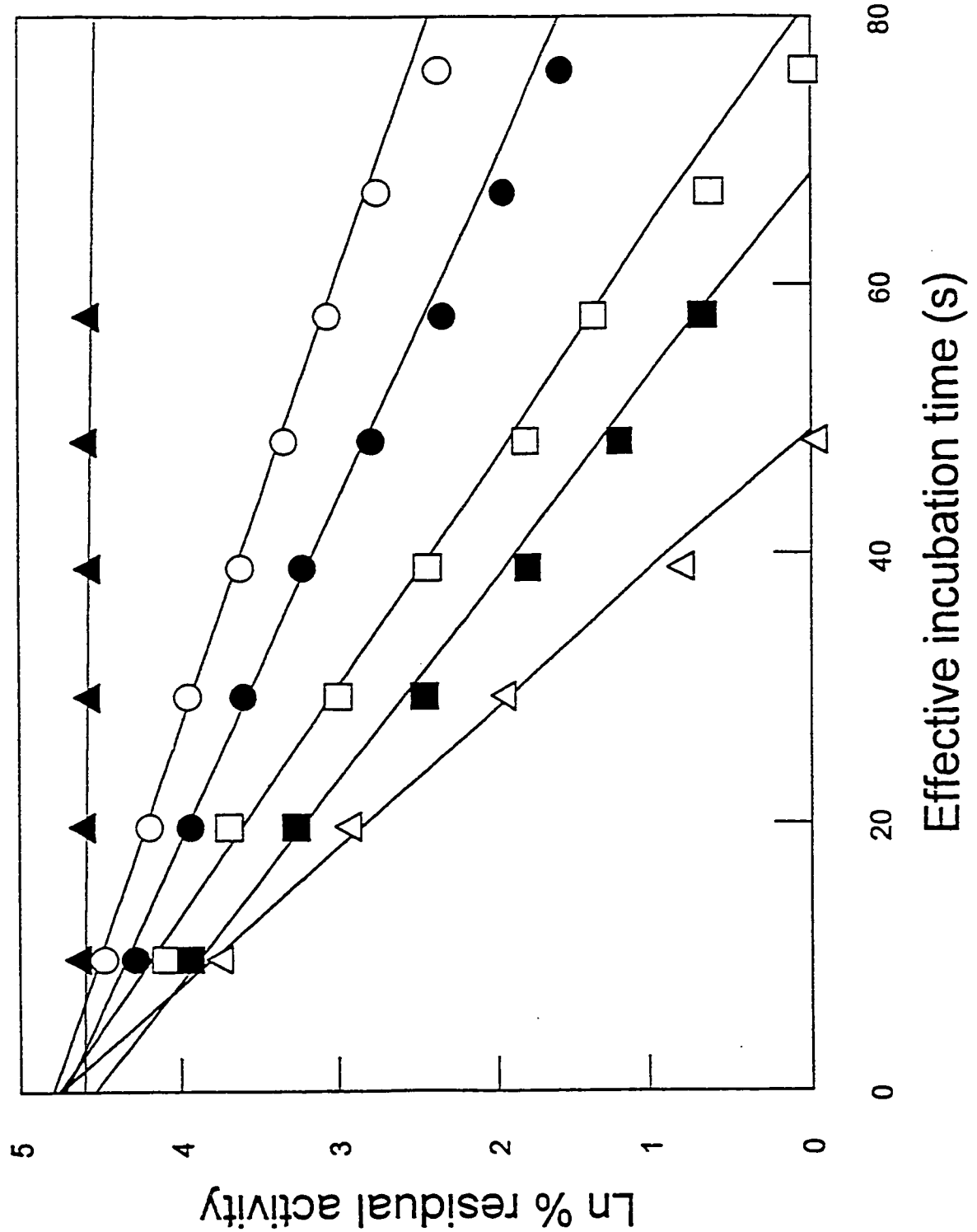

Ln % residual activity
5
4
3
2
1
0
0
20
40
60
80
Effective incubation time (s)

Figure 2.3 Kinetics of inactivation of prephenate dehydrogenase by DEPC

Plots of the concentration dependence of the pseudo first-order rate constants for the inactivation of mutase (●) and dehydrogenase (○).

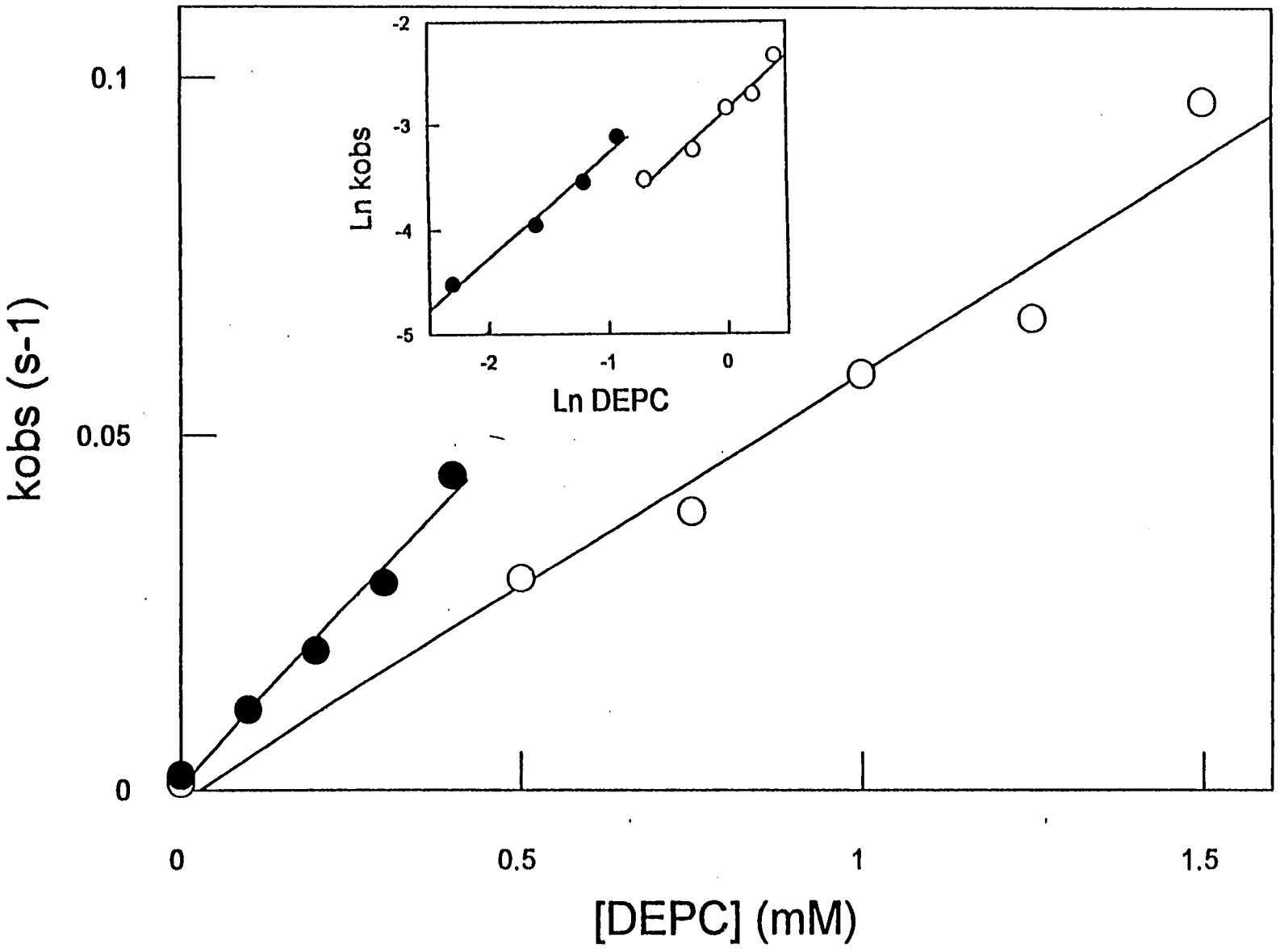
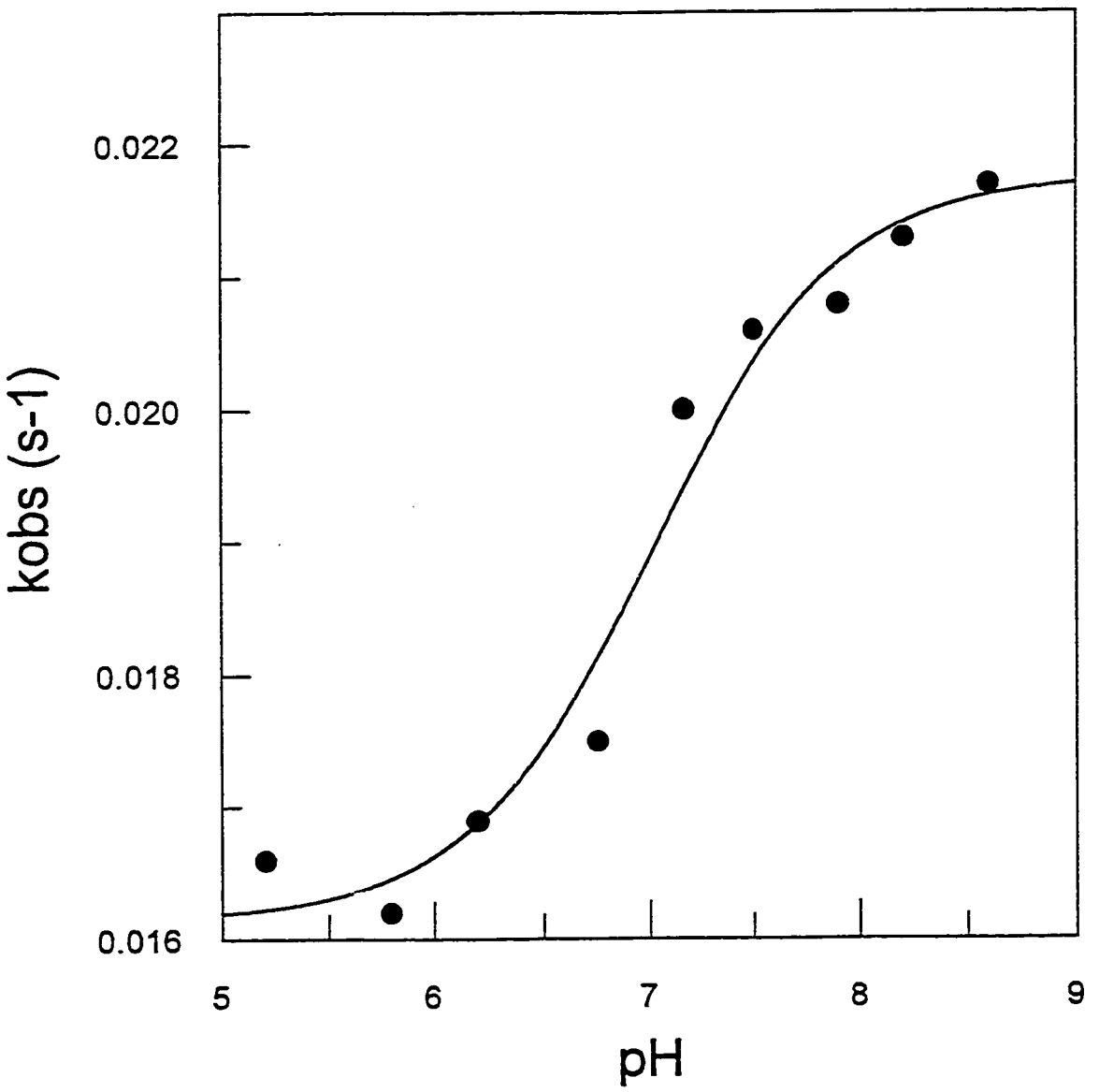

kobs (s-1)
0.1
0.05
0
0
0.5
1
1.5
[DEPC] (mM)
Ln kobs
-2
-3
-4
-5
-2
-1
0
Ln DEPC

Figure 2.4 pH dependence of pseudo-first-order rate constant for inactivation of prephenate dehydrogenase by DEPC

Mutase-dehydrogenase (22 μM) was incubated at 25°C with 0.3 mM DEPC in 50 mM potassium phosphate buffer at various pH values. Aliquots were removed every 30 s over 4 min and dehydrogenase activity measured. The curve represents the best fit of the data to equation 2.3.


0.022
0.020
0.018
0.016
kobs (s-1)
5
6
7
8
9
pH

Chorismate Mutase.

Incubation of mutase-dehydrogenase (22 μM) with DEPC (0 to 0.4 mM) also results in a rapid loss of mutase activity which approximates pseudo first-order kinetics (data not shown). Replots of the data are represented in Figure 2.3 and show that, as with the dehydrogenase, the inactivation follows a simple bimolecular process. The second-order rate constant for the inactivation of the mutase is 104 ± 8 $M^{-1}s^{-1}$. The double ln plot of k_{obs} against DEPC concentration shown in Figure 2.3 inset is also linear yielding a slope of 0.99 ± 0.09. These results are in accord with the idea that the modification of a single group per catalytic unit results in the loss of mutase activity.

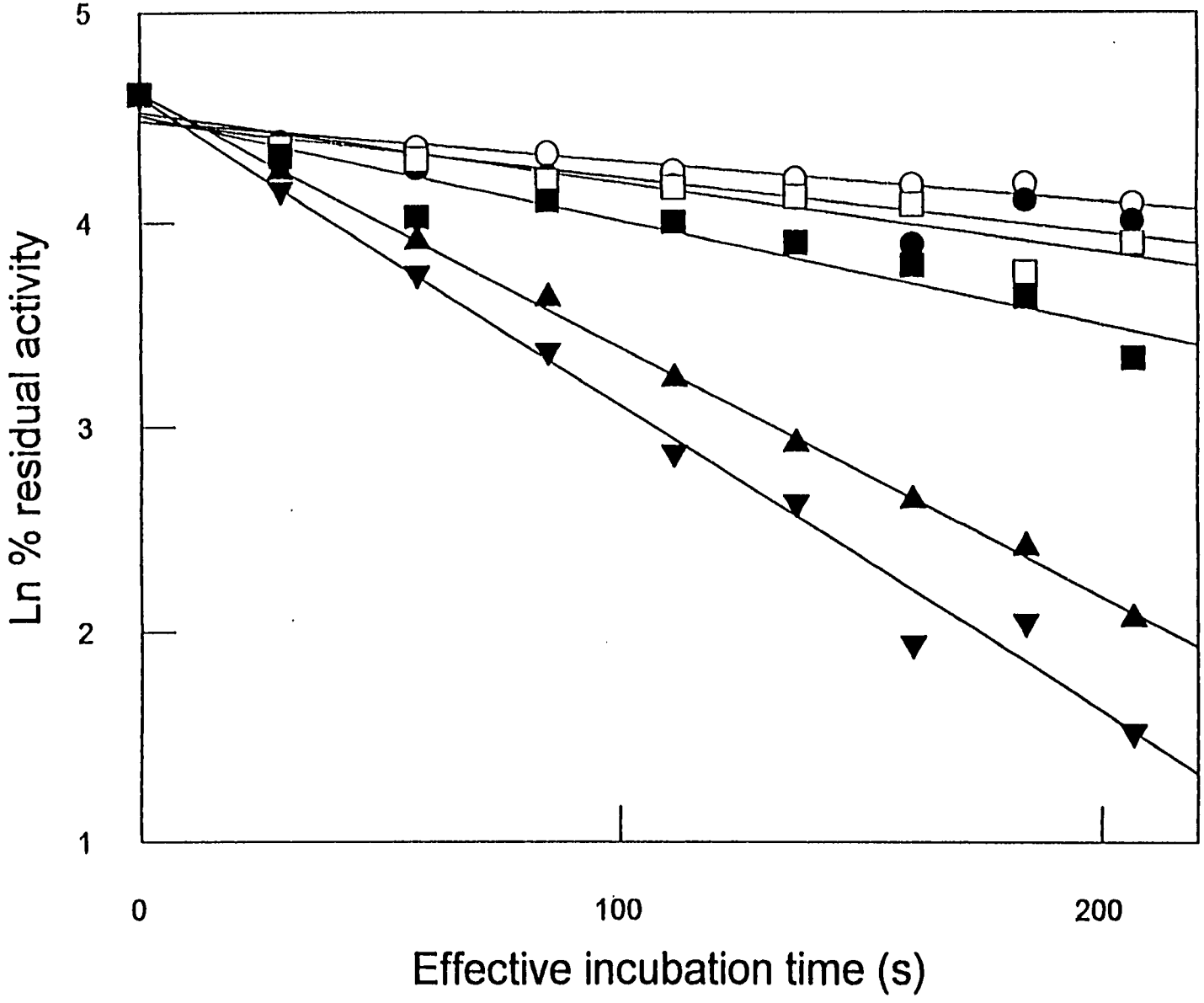
Ligand Protection Against DEPC-Mediated Inactivation of Mutase-Dehydrogenase.

Substrates of the dehydrogenase reaction, prephenate and NAD^+, and an inhibitory substrate analog of the mutase reaction, *endo*-oxabicyclic diacid, were tested for their abilities to prevent the DEPC-mediated loss of either the mutase or dehydrogenase activities. The prior addition of increasing concentrations of prephenate (0 to 0.4 mM) protected against the inactivation of both dehydrogenase and mutase by 0.3 mM DEPC. A representative plot of the type of data obtained is shown for the dehydrogenase reaction (Figure 2.5). When the reciprocals of the slopes of the lines in Figure 2.5 are varied with respect to prephenate concentration, a straight line is obtained (data not shown). Such a result implies that prephenate totally protects against DEPC-mediated inactivation; the reagent cannot inactivate an enzyme-ligand complex (47).

Data for these protection experiments were fitted to equation 2.2 to obtain values of the dissociation constants (K_S) for the interaction of the ligand with the free enzyme.

Figure 2.5 Protection by prephenate against inactivation of prephenate dehydrogenase by DEPC

The dependence of residual activity on the effective incubation time in 50 mM potassium phosphate buffer (pH 7.2) at 25°C are shown for the inactivation of prephenate dehydrogenase (22 μM) by 0.3 mM DEPC in the presence of 0mM (○), 0.05 mM (●), 0.10 mM (□), 0.20 mM (■), 0.30 mM (▲), 0.40 mM (▼) of prephenate. The data were fitted to equation 1.1 to obtain values for k_{obs} .


Ln % residual activity
5
4
3
2
1
0
100
200
Effective incubation time (s)

The values for K_S obtained for the interaction of prephenate acting as a substrate of the dehydrogenase reaction (54 ± 3 μM) and as an inhibitor of the mutase reaction (140 ± 9 μM) in the protection studies are similar to those obtained from the analysis of steady-state kinetic data for the two reactions at pH 7.2. Steady-state kinetic data were analyzed assuming that the dehydrogenase reaction conforms to a rapid equilibrium random mechanism with catalysis rate-limiting (26). From the analyses, a value of 63 ± 10 μM was obtained for the dissociation constant (K_{ia}) for the interaction of prephenate with the free enzyme in the dehydrogenase reaction, and a value of 95 ± 9 μM for the inhibition constant (K_i) of prephenate in the mutase reaction. Regrettably, reliable quantitative information could not be easily derived from protection studies using NAD^+ as this ligand reacted with free DEPC thereby lowering its effective concentration and that of the modifying reagent in the inactivation experiments. However, 1 mM NAD^+ did afford 75 % protection of DEPC-mediated inactivation of dehydrogenase activity while under identical assay conditions, inactivation of the mutase was not prevented, even up to an NAD+ concentration of 2 mM.

The inhibitory transition state analogue *endo*-oxabicyclic diacid completely prevented the inactivation of the mutase but had little effect on the dehydrogenase (data not shown). Only stoichiometric amounts of *endo*-oxabicyclic diacid were required for total protection of the mutase, precluding the determination of a dissociation constant for this enzyme-ligand complex by these studies. A dissociation constant for this ligand from the mutase of 0.11 ± 0.01 μM has been derived from steady state kinetic data at pH 7.2 (29). In contrast, *endo*-oxabicyclic diacid protected the dehydrogenase against DEPC-mediated

inactivation only weakly. The data (not shown) for the time-dependent inactivation of the dehydrogenase by 0.3 mM DEPC at six concentrations of *endo*-oxabicyclic (0 to 2 mM) were fit to equation 2 to obtain a K_S value of 212 $\pm$ 7 μM for the interaction of *endo*-oxabicyclic diacid with the dehydrogenase.

Characterization of DEPC-Modified Mutase-Dehydrogenase

The difference spectrum of the DEPC-modified and untreated enzyme from 200 nm to 310 nm shows an absorption maximum at 240 nm (Figure 2.6) which is characteristic of the carbethoxylation of histidine residues. The maximum absorbance change of 0.48 occurs after 15 min at a DEPC concentration of 1.5 mM and corresponds to the modification of six of the nine histidine groups in the protein. However, when the extent of modification of histidine residues is correlated with the inactivation of the dehydrogenase (Figure 2.7), the data suggest that only one of the histidine groups modified by DEPC is critical for dehydrogenase activity. With time, additional histidine residues are carbethoxylated, but these do not significantly contribute to additional inactivation. Moreover, the time required to modify 0.5 mol histidine/subunit (25 s) correlated exactly with the half-time of inactivation (data not shown). Partially inactivated enzyme (20 % residual activity) had K_m values for prephenate (45 $\pm$ 5 μM) and NAD^+ (198 $\pm$ 20 μM) that were similar to those of the native enzyme (29 $\pm$ 4 mM and 107 $\pm$ 33 μM, respectively), ruling out the possibility that the loss of activity after modification may be due to a large increases in K_m values.

Treatment of the DEPC-modified enzyme at pH 7.0 with 0.3 M hydroxylamine, a reagent known to cleave carbethoxy adducts from histidine groups, resulted in a time-dependent

Figure 2.6 Modification of mutase-dehydrogenase by DEPC.

Typical difference spectra for the modification of mutase-dehydrogenase by DEPC illustrating the signal, representative of N-carbethoxyhistidine, appearing at 240 nm over time. The curves were obtained over increasing time intervals of (0.5, 1, 1.5, 2, 5, 10, 15 min).

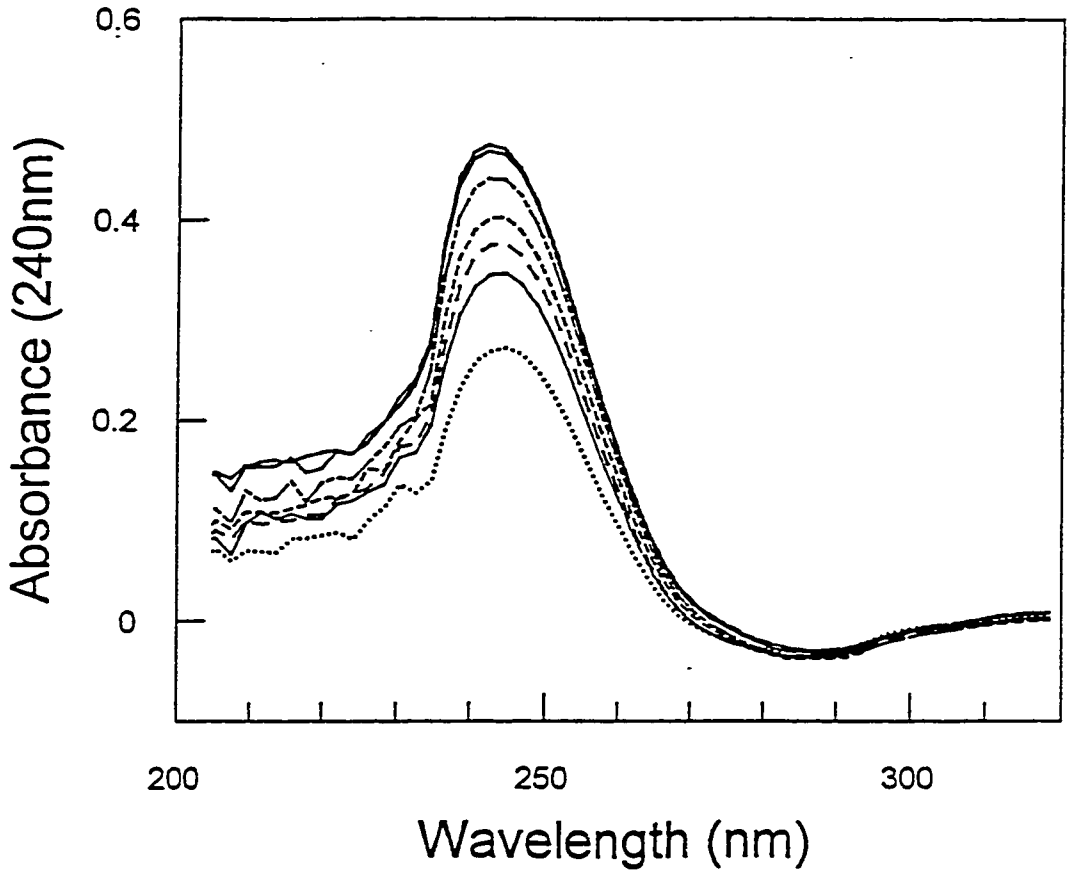
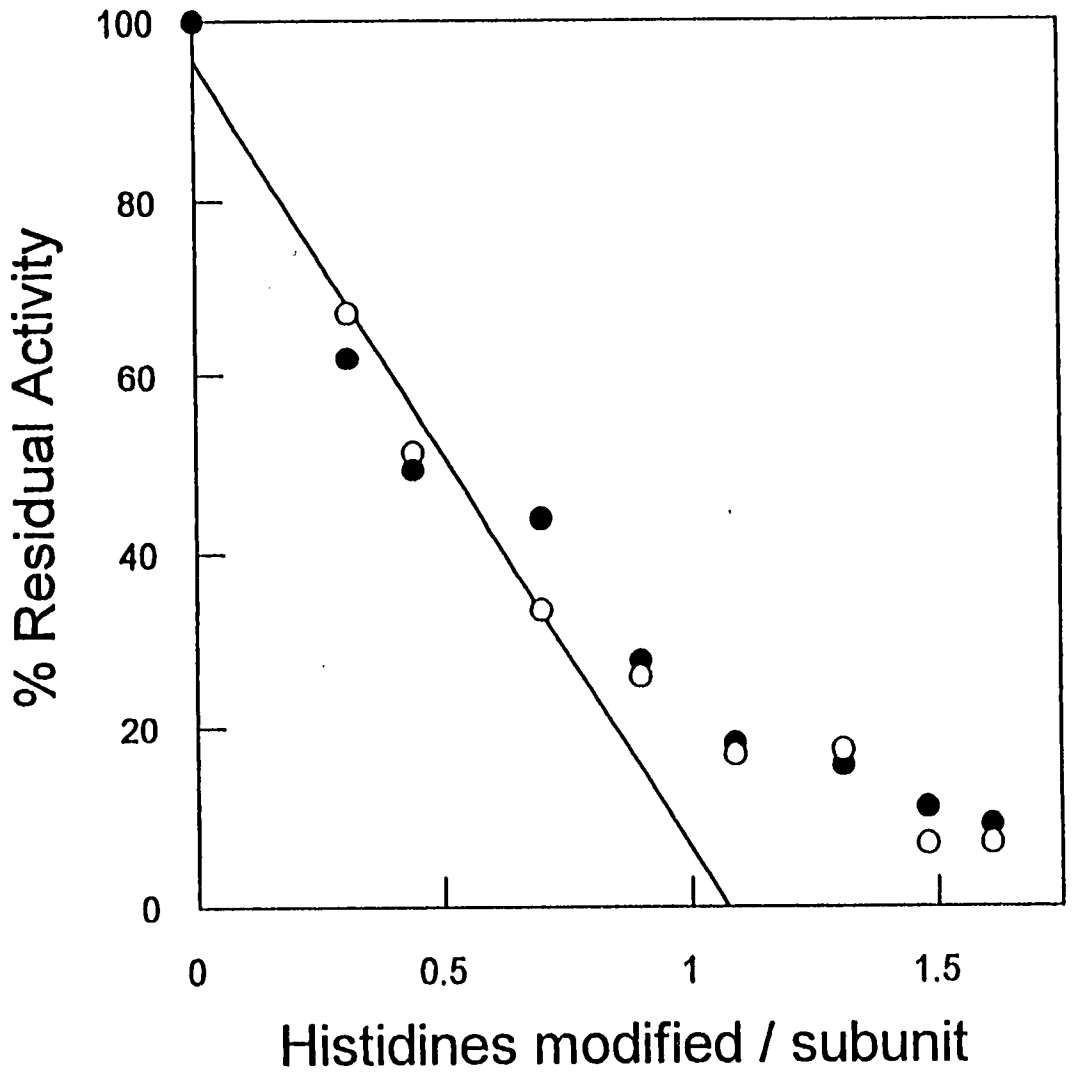


Figure 2.7 Relationship between residual activity and the number of histidine residues modified by DEPC.

The number of histidine residues modified/subunit was determined by a difference spectrum obtained by monitoring the time-dependent absorbance at 240 nm of the reaction of mutase-dehydrogenase (22 μM) with 0.3 mM DEPC in 50 mM potassium phosphate buffer (pH 7.2, 25°C.) Percent residual activity was determined in a parallel experiment under identical conditions. Aliquots from the incubation were removed at specific time intervals and residual dehydrogenase activity was measured as described under "Experimental Procedures". The results from two independent experiments (○ and ●) showing the linear extrapolation was based on the first four time points from each experiment.


100
80
60
40
20
0
% Residual Activity
0
0.5
1
1.5
Histidines modified / subunit

regeneration of over 80 % of the original dehydrogenase activity (Figure 2.8). Moreover, the K_m values for prephenate (54 ± 13 μM) and NAD^+ (140 ± 48 μM) in the dehydrogenase reaction for the regenerated enzyme (after removing excess hydroxylamine) were very similar to those values for the native, underivatized enzyme. This further supports the idea that the inactivation of the dehydrogenase is due to the reversible modification of a histidine group by DEPC (42). In contrast, treatment of the carbethoxylated enzyme with 0.3 M hydroxylamine did not restore mutase activity (Figure 2.8). This result is consistent with the idea that the mutase is inactivated by the reaction of DEPC with a primary amine such as a lysine residue (42).

Effect of TNBS Modification of Mutase-Dehydrogenase

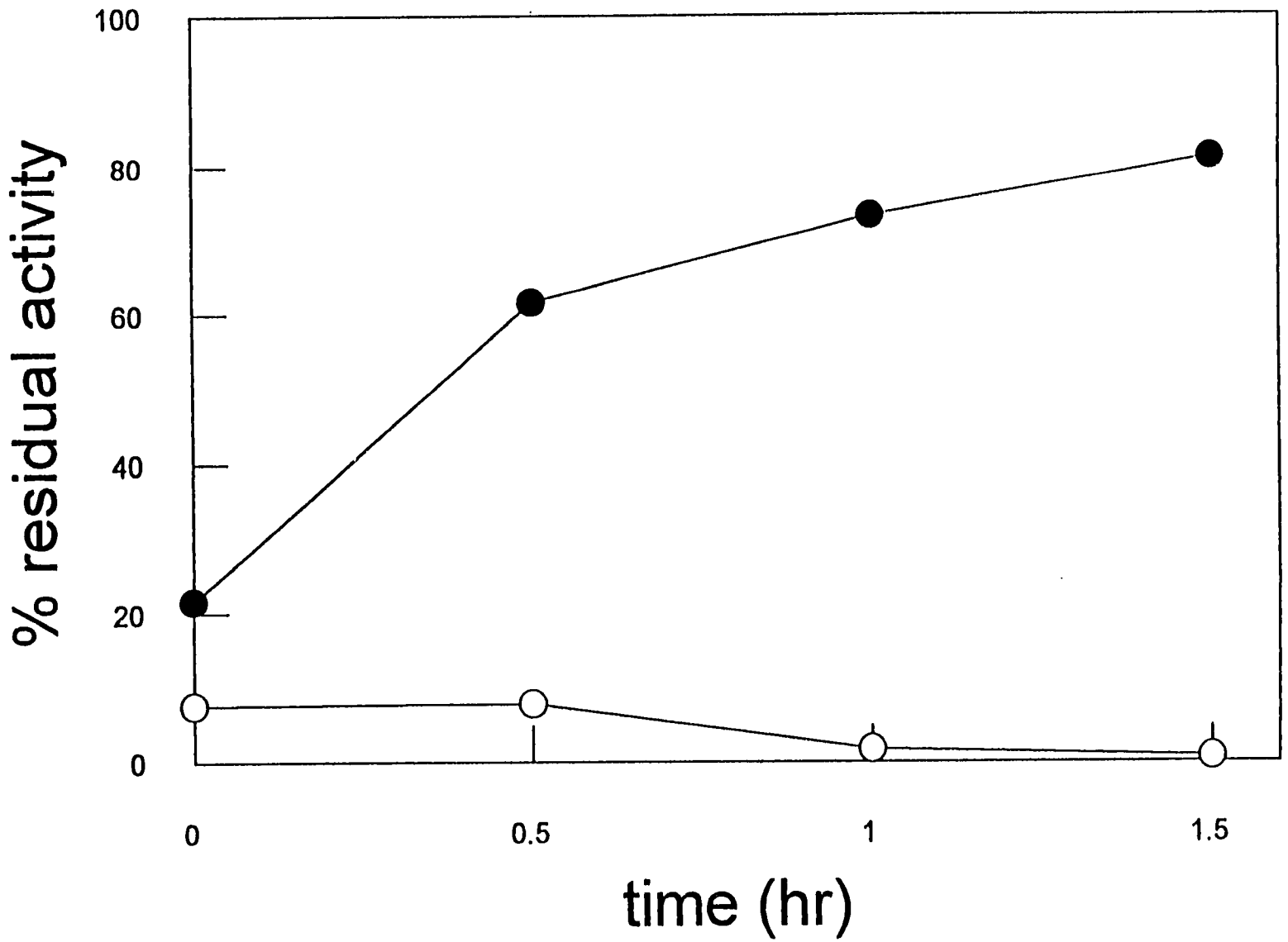
Further chemical modification studies of mutase-dehydrogenase provided additional evidence to indicate a lysine is carbethoxylated in the mutase. When mutase-dehydrogenase was incubated for 20 min with 0.1 mM TNBS, at a ratio of reagent to enzyme of 4:1, approximately 95 % of the mutase activity was lost (data not shown). By contrast only 40 % of the dehydrogenase activity was lost (data not shown). TNBS reacts reasonably specifically with lysine groups yielding an adduct that absorbs strongly at 350 nm (44). However, this reagent will also more slowly react with cysteine residues yielding a weakly absorbing adduct (43).

Generation, Isolation and Identification of Active Site Peptides

Our data indicate that an active site histidine residue is involved in the catalytic mechanism of the dehydrogenase reaction while an active site lysine is involved in the mutase reaction. In order to locate each of these two residues in the amino acid sequence

Figure 2.8 The ability of hydroxylamine to restore mutase-dehydrogenase activity.

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


100
80
60
40
20
0
% residual activity
0
0.5
1
1.5
time (hr)

of mutase-dehydrogenase, we employed differential peptide mapping with mass spectrometry as outlined in "Experimental Procedures".

Prephenate Dehydrogenase-Containing Peptide

Mutase-dehydrogenase samples modified with DEPC in the presence and absence of prephenate were digested with trypsin and fractionated by reverse-phase HPLC in a solvent system maintained at pH 6.4. At this pH, the adduct is sufficiently stable. Peptides were detected at 240 nm to monitor the elution of those fragments containing N-carbethoxyhistidine groups. Comparison of the HPLC tracings at 240 nm for the elution of samples derivatized in the presence of prephenate (Figure 2.9A) or in its absence (Figure 2.9B) shows that one region in the peptide map eluting at about 14 min is substantially protected by ligand. Prephenate absorbs at 240 nm and elutes at about 4 min (Figure 2.9A). Other regions within the maps showed much less pronounced changes which were not reproducible between separate experiments. The material eluting at 14 min was collected then desalted. Although the adduct is not expected to survive the acidic desalting conditions of the second column, this step is a prerequisite to mass spectrometry. The mass spectrum of the isolated peptide along with a peptide standard is shown in Figure 2.10. One signal belongs to the standard substance P $[M + H]^+$, m/z = 1348.6) and the other to a peptide at m/z = 1212.3. Comparing this latter mass value with those predicted for the 43 trypsin-generated fragments of mutase-dehydrogenase yielded a single match with sequence ILEQHDWDR ($[M + H]^+$ = 1212.6), corresponding to residues 127 to 135 containing His131. The next closest mass is 1191.7, comprising a peptide of amino acids 40 to 49 which lacks a histidine residue. The nearest histidine-containing peptide was 1408.6, comprising amino acids 346 to 356.

Figure 2.9 Reverse-phase HPLC chromatogram of tryptic digests of mutase-dehydrogenase derivatized by DEPC in the presence (A) and absence (B) of prephenate.

Peptides were eluted with a linear solvent system of 5 to 70 % acetonitrile in 0.05 % potassium phosphate buffer (pH 6.4) as described under "Experimental Procedures". Those peptides containing N-carbethoxyhistidines were detected at 240 nm. Similar quantities of protein were injected. The altered peak is indicated by an arrow.

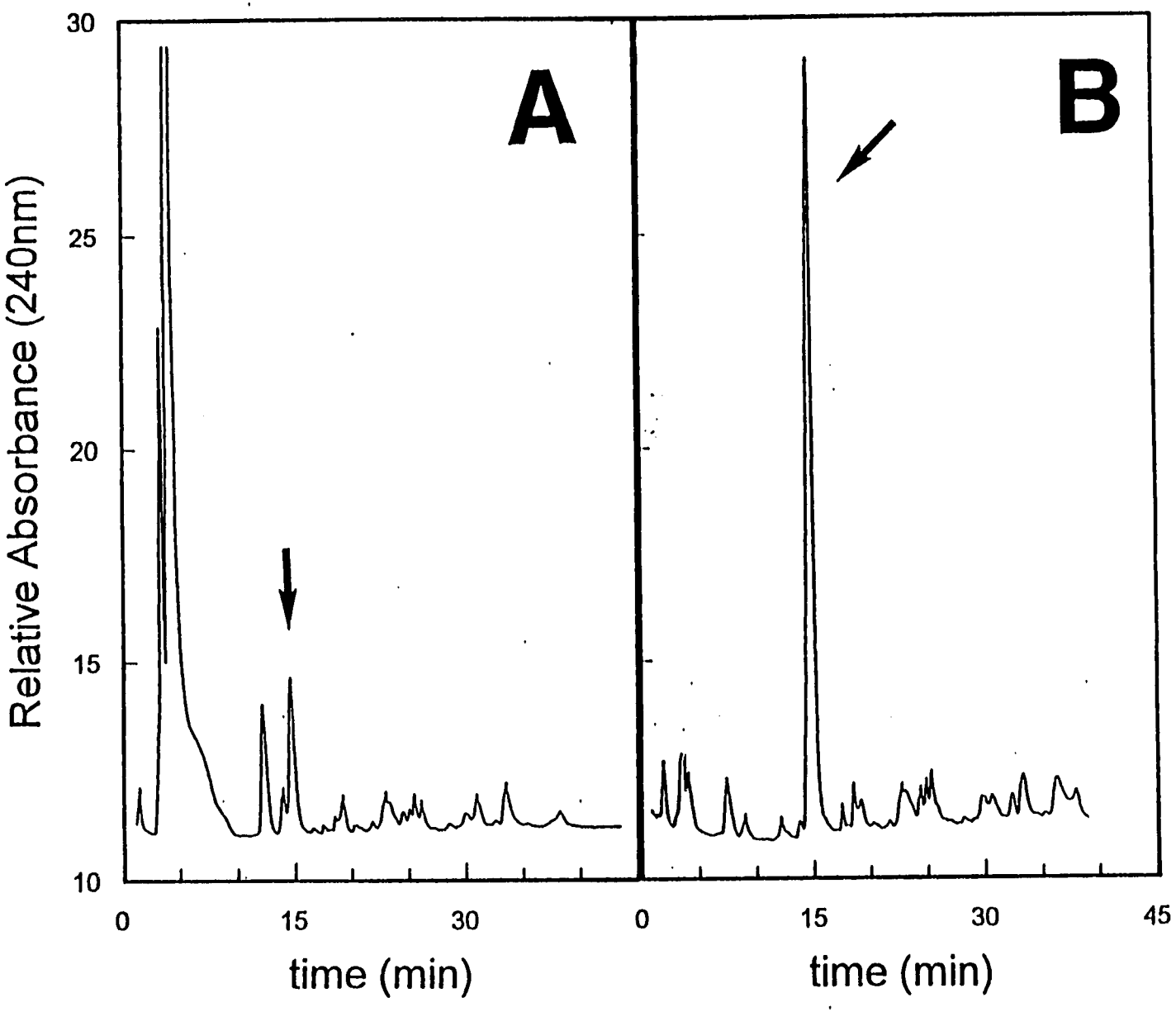
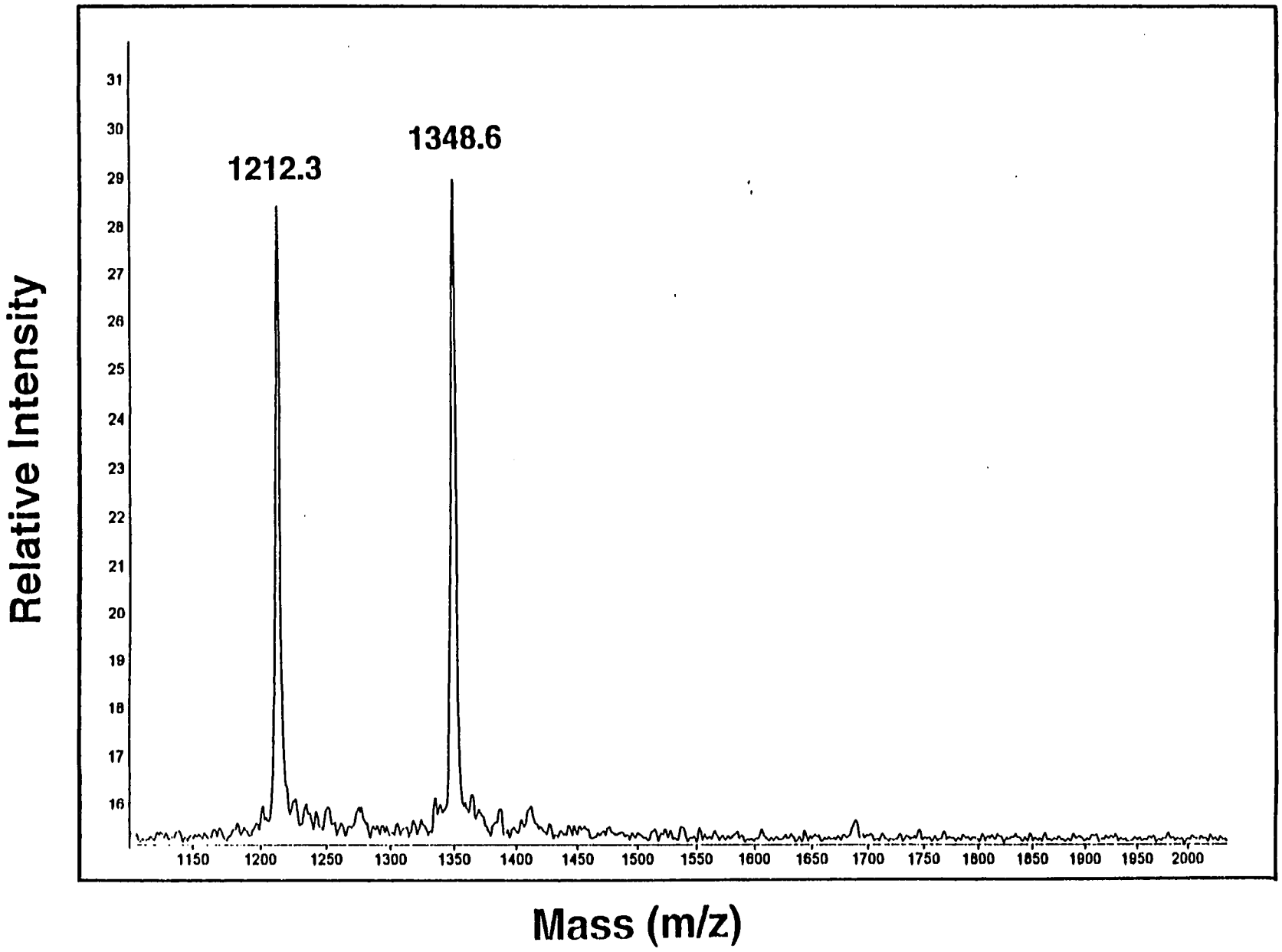

A
B
Relative Absorbance (240nm)
30
25
20
15
10
0
15
30
0
15
30
45
time (min)
time (min)

Figure 2.10 Mass spectrometry of an isolated proteolytic peptide from mutase-dehydrogenase modified with DEPC.

The tryptic peptide eluting at 14 min in Figure 2.9 yields a signal at m/z = 1212.3. A peptide standard is shown with a m/z = 1348.6.


Relative Intensity
31
30
29
28
27
26
25
24
23
22
21
20
19
18
17
16
1212.3
1348.6
1150
1200
1250
1300
1350
1400
1450
1500
1550
1600
1650
1700
1750
1800
1850
1900
1950
2000
Mass (m/z)

Chorismate Mutase-Containing Peptide

Carbethoxylated lysine adducts do not absorb light above 200 nm (43). In order to identify the residue that reacts with DEPC and which is important for mutase activity, mutase-dehydrogenase was modified with TNBS in the presence and absence of DEPC. The modified proteins were then digested with *S.aureus* V8 protease prior to fractionation by reverse-phase HPLC. V8 protease, which cleaves specifically at the C-terminal side of glutamate residues, was used instead of trypsin, which is specific for lysine and arginine groups (50). HPLC tracings at 350 nm for the elution of samples modified in the presence (Figure 2.11A) or absence (Figure 2.11B) of DEPC revealed one peptide eluting at about 49 min whose modification was protected substantially in the presence of protecting ligand DEPC. Mass spectrometry indicated that this fragment had a high molecular mass presumably resulting from incomplete proteolytic digestion (data not shown). Consequently, this isolated peptide was redigested overnight. C_{18} reverse phase chromatography of this redigested fragment resolved one major peak eluting at 36 min and two smaller peaks eluting at 25 min and 49 min (Figure 2.11C). Each peak was collected and subjected to mass spectrometry. The mass of the fragment eluting at 49 min was characteristic of the incompletely digested fragment previously isolated. The peak at 25 min contained either no peptide or insufficient sample for mass analysis (< 2 pmol). The mass spectrum of the predominant peak eluting from the HPLC at 36 min (Figure 2.11C) is shown in Figure 2.12. The signal at m/z = 1348.6 belongs to standard substance P $[M + H]^+$ while all other peaks in the spectrum belong to the isolated peptide. The masses of these peaks were compared with those values predicted for the 29 possible V8 protease-generated fragments of mutase-dehydrogenase. The peak at m/z = 1504.6 was within 1.2 mu of the mass of an underivatized peptide corresponding to

Figure 2.11 Reverse-phase HPLC chromatograms of V8 protease digests of mutase-dehydrogenase derivatized in the presence (A) and absence (B and C) of DEPC.

Peptides were separated using a solvent system of 5 to 70 % acetonitrile in 0.1 % TFA (pH 2.0). For A and B, similar quantities of protein were injected. The TNB-lysine containing peptides were detected at 350 nm and an arrow indicates a peak whose area is altered by the protecting ligand. The peptide eluting at 49 min in Figure 2.11B was redigested with V8 protease then rechromatographed to yield the tracing shown in Figure 2.11C. The altered peak is indicated by an arrow.

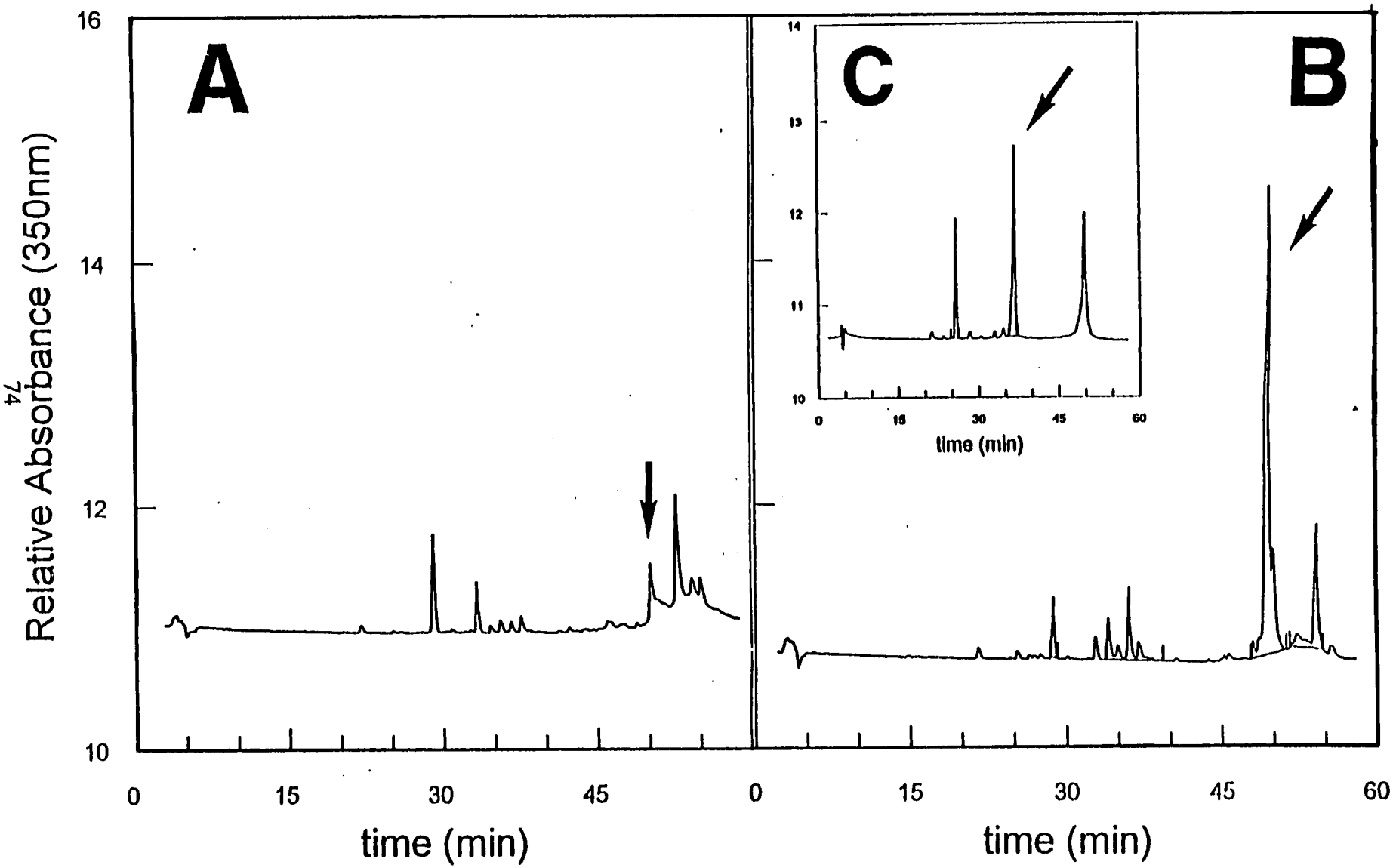
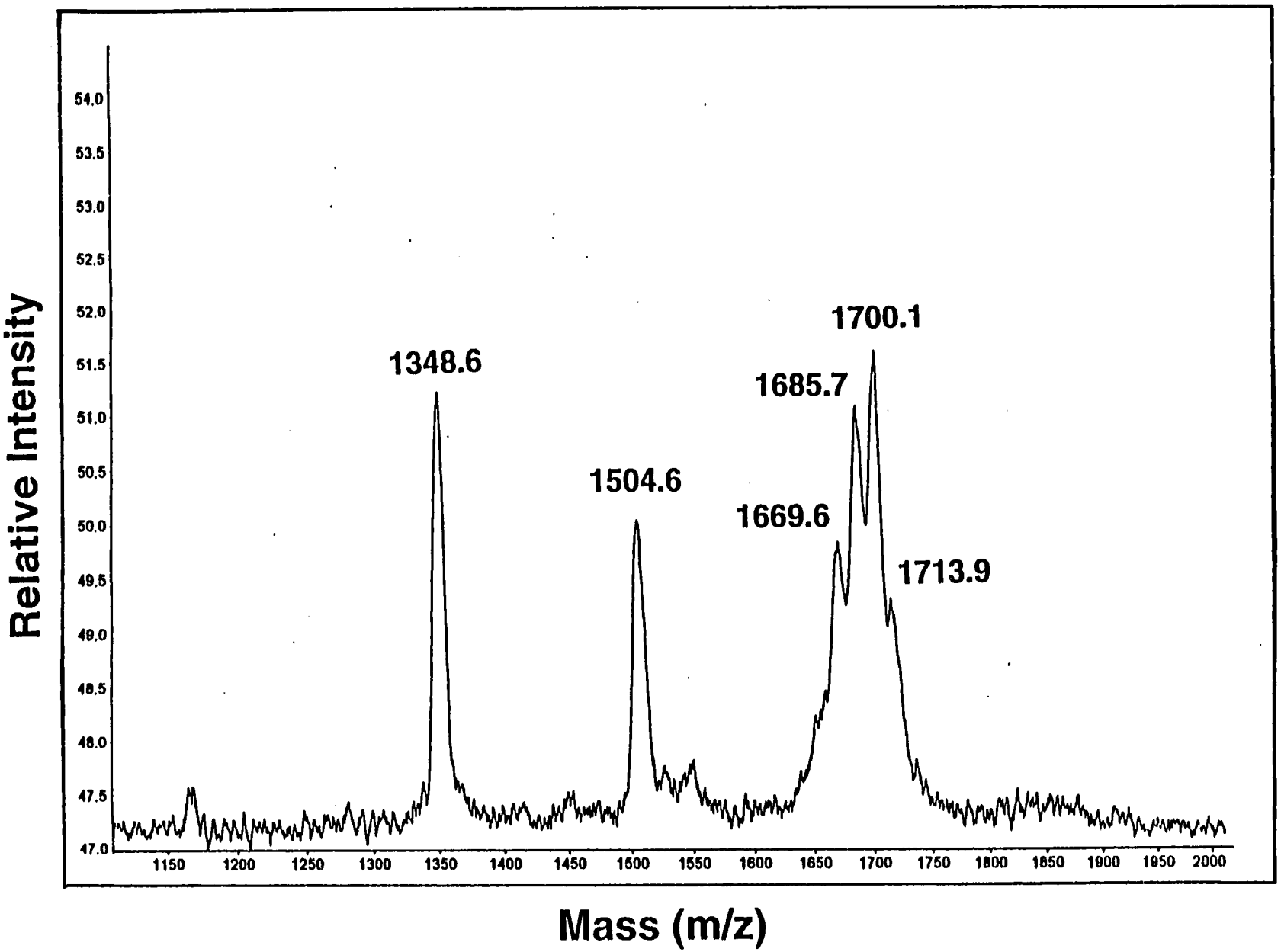

A
B
C
Relative Absorbance (350nm)
time (min)
time (min)
time (min)
16
14
12
10
0
15
30
45
60

Figure 2.12 Mass spectrometry of an isolated proteolytic peptide from mutase-dehydrogenase modified with TNBS.

The peptide eluting at 36 min in Figure 2.11C yields signals at m/z = 1504.6, 1669.6, 1685.7, 1700.1 and 1713.9. A peptide standard is shown with a m/z = 1348.6.


Relative Intensity
54.0
53.5
53.0
52.5
52.0
51.5
51.0
50.5
50.0
49.5
49.0
48.5
48.0
47.5
47.0
1348.6
1504.6
1669.6
1685.7
1700.1
1713.9
1150
1200
1250
1300
1350
1400
1450
1500
1550
1600
1650
1700
1750
1800
1850
1900
1950
2000
Mass (m/z)

amino acids 36 to 48 ($[M + H]^+$ = 1505.8, VKSRFGLPIYVPE) including a single lysine at position 37. The next closest mass value was 1503.7 and belonged to a lysine-free peptide comprising amino acids 276 to 288 (QLLALSSPIYRLE). This peptide did not contain residues that would react with TNBS yielding adducts that absorb significantly at 350 nm (43). The next nearest mass of a lysine-containing peptide was 1596.9 (amino acids 15 to 28). The other peaks in the mass spectrum were part of a broad, rather poorly resolved multiplet. There is more error associated with the assignment of mass values to these signals. Nevertheless, we noted that the signal at m/z = 1713.9 was within 2.9 mu of that predicted for the peptide ($[M + H]^+$) whose sequence is shown above but carrying a trinitrobenzyl adduct on Lys37. It is likely that the other peaks in this multiplet arise from fragmentation of the TNB-lysyl peptide since the absorbance maximum of the derivatized peptide, at 350 nm, is close to the emission wavelength (355 nm) of the mass spectrometer's laser light[2].

Circular Dichroism and Fluorescence Measurements

We attempted to use circular dichroism and fluorescence to probe for any structural changes in the enzyme upon modification (data not shown). CD measurements in the far UV (190 to 245 nm) indicated that there was no change in secondary structure between the carbethoxylated and unmodified enzyme. Measurements in the near UV (245 to 360 nm) did show a larger negative CD signal at 300 nm in the modified enzyme. The near UV region of the spectrum is sensitive to environments of UV-absorbing amino acid side-chains (51). Hence, this observation could be due to an intrinsic signal of the adduct. Fluorescence emission did not reveal any differences between the two proteins

but any small changes might be masked by the large intrinsic fluorescence of the mutase-dehydrogenase.

DISCUSSION

In the present investigation, we have identified two residues that are important for the activity of chorismate mutase-prephenate dehydrogenase and provide further support for the idea that the mutase and dehydrogenase reactions are catalyzed at two distinct sites. The basic strategy that we adopted, which relies on chemically modifying the enzyme in the presence and absence of protecting ligands followed by analysis of peptide fragments, has been used successfully by others to identify active site residues (44, 52, 53, and 54).

Identification of His131 by Chemical Modification

Of the nine histidine residues found in mutase-dehydrogenase, six of them are in some way accessible to DEPC. However, at pH 7.2 and 25°C only one histidine residue is particularly reactive and it is the modification of this alone which causes a loss of dehydrogenase activity (Figure 2.7). Moreover, the rate of inactivation was equal to the rate of modification. The second-order rate constant for the inactivation of the dehydrogenase by DEPC, about 60 $M^{-1}s^{-1}$, is at the high end of the range of values usually reported for histidine groups in other proteins at neutral pH and ambient temperature (53, 55, 56, 57, 58). However, the essential histidine residues of some dehydrogenases (59, 60), as well as other enzymes (54, 61, 62) have second-order rate constants for inactivation by DEPC that match or greatly exceed our value. The rate enhancements for some of these histidine residues have been attributed to their involvement in charge-relay systems or proximity to metal.

Further evidence in support of a histidine comes from following the variation with pH of the pseudo first-order rate constant of inactivation (Figure 2.3). A pK_a value of about 7.0 was obtained for the ionizing group, in the range of 5 to 8 normally accepted for imidazole groups in proteins (63). Furthermore, this value is in reasonable agreement with that derived from plots of the variation with pH of log V and V/K for the dehydrogenase reaction which showed that a single group with a pK_a of about 6.7 had to be unprotonated for maximal dehydrogenase activity (27, 23). Hermes *et al.* (27) have reported that dimethyl sulfoxide had no significant effect on the observed p*Ka* value in the V/K profile and that the enthalpy of ionization for this group is low, suggesting that the catalytic group is a cationic acid, most likely a histidine.

While DEPC is commonly used for modifying histidine residues it can also react with lysine, tyrosine and cysteine contributing to inactivation (42, 43). Our data tend to exclude these other possibilities. We rule out the possibility that cysteine and tyrosine residues were carbethoxylated since the absorbance spectrum in Figure 2.6 did not indicate a shoulder at 230 nm, characteristic of an N-acetylcysteine modified with DEPC (64) or a decrease at 278 nm, which would indicate that the phenol side chain of a tyrosine is modified (65). Furthermore, the dehydrogenase activity was almost completely restored upon treating an inactivated enzyme with neutral hydroxylamine for 90 min (Figure 2.8)-- the signature that the cleaving of a carbethoxyhistidine adduct had regenerated an active enzyme. Activity was regained with a half-life of about 30 min at neutral pH, which is in the range normally expected for cleavage of carbethoxyhistidine adducts (42, 43). An adduct with primary amines or cysteine cannot be reversed under these conditions[3], and that with tyrosine is cleaved at a much slower rate (42, 65).

It is noteworthy that mutase-dehydrogenase activity can be lost by modifying one of the three cysteine residues in this protein with cysteine modifying reagents (8, 29). However, unlike the results in our present study, these authors reported both activities are lost in parallel and inactivation of both activities can be prevented by prephenate and NAD^+.

Final confirmation that a single histidine is involved in the DEPC-mediated inactivation of the dehydrogenase comes from the isolation of a peptide from differential peptide mapping containing a histidine at position 131. A mass spectrum of the DEPC-peptide adduct could not be obtained due to its instability a low pH. However, this peptide corresponded to the only significant peak at 240 nm in a chromatogram obtained under conditions where the adduct is stable (Figure 2.9). The protection strategy used to isolate this peptide confirms that this histidine is at or near the prephenate binding site of the dehydrogenase reaction. As expected, this histidine is in the latter two-thirds of the protein sequence, which is believed to contain the dehydrogenase activity (13). Finally, the peptide isolated by differential peptide mapping contains only one residue capable of reacting with DEPC-- His131.

Identification of Lys37 by Chemical Modification

The second-order rate constant for the DEPC-mediated inactivation of the mutase at pH 7.2 and 25°C was almost twice that of the dehydrogenase (Figure 2.3) and was the first indication that modification of distinct groups was responsible for the loss of each activity. It is noteworthy that the value, at about 100 $M^{-1}s^{-1}$, is 50 to 100 times higher than that normally observed for the reaction of an enzymic lysine with DEPC (66, 59).

However, a high reaction rate has also been reported for a lysine in hydroxysteroid dehydrogenase (56) and ribonuclease (67). In the latter case, this reactivity has been attributed to the proximity of an arginine residue which decreased the pK_a of the lysine group. As will be discussed later, our high second-order rate constant is also in accord with the idea that Lys37 has an unusually low pK_a value. For reasons discussed above, the fact that hydroxylamine did not reactivate the DEPC-inactivated mutase is evidence that this group could be a lysine residue. Carbethoxylated lysine adducts do not have a strong UV-VIS signal as do carbethoxylated histidine residues. In order to identify the particular residue responsible for loss of mutase activity, we found it necessary to use another reagent which would modify lysines and also absorb. Our preliminary studies showed that TNBS, a reagent that reacts reasonably specifically with lysine residues (43), almost completely inactivated the mutase. No spectral or kinetic characterization was done specifically to rule out the possibility that modification of cysteine residues, which can also react with TNBS (43), was responsible for the inactivation. However, no cysteine was modified by DEPC, and whatever group is modified by DEPC is probably also modified with TNBS, since the DEPC-modified enzyme did not react with TNBS under these conditions. It is this feature that has allowed us to isolate and identify the Lys37-containing peptide through a scheme of differential peptide mapping similar to that outlined for the dehydrogenase studies. The amino acid sequence alignments that we have performed on the chorismate mutase portion of mutase-dehydrogenase and mutase-dehydratase enzymes from the bacteria *E.coli*, *Erwinia herbicola* and the *Pseudomonads* show that Lys 37 is conserved in all of these mutases. In addition, Gething and Davidson (68) also noted the importance of a lysine residue in the mutase-dehydratase whose modification with TNBS led to a complete loss of mutase activity without significantly

affecting the dehydratase domain, although they did not identify the particular lysine involved.

Interestingly, it was noted that after longer exposure to TNBS, 40% of the dehydrogenase activity was lost. We have not pursued the identification of the dehydrogenase residue modified by TNBS. However, the active site could contain a lysine residue since the profile of pH dependence of *V/K* shows there is at least one group with a pK_a of 8.4 (possibly a lysine) which must be protonated for prephenate to bind to the enzyme-NAD^+ complex (23).

Lys37 and His131 are Associated with Distinct Active Sites.

The results from studies using ligands to protect the mutase-dehydrogenase against DEPC-mediated inactivation suggest that the two reactive groups (His131 and Lys37) are located at distinct active sites. If a ligand protects by binding to its natural binding site on the enzyme, the value of the dissociation constant calculated in the protection experiment should agree with that calculated from steady-state kinetic analysis. The values show that the *endo*-oxabicyclic diacid binds to and preferentially protects the mutase from inactivation on the order of 2000 times more effectively than it does the dehydrogenase. *Endo*-oxabicyclic diacid mimics the proposed transition state for the chorismate mutase reaction (69) and thus was expected to provide protection against modification of residues at the mutase active site. The protection is stoichiometric as expected given its K_i value of about 110 nM at pH 7.2 (29). If the two activities shared a common active site, *endo*-oxabicyclic should have protected both activities equally well. NAD^+ prevented the DEPC-mediated inactivation of the dehydrogenase preferentially over the

mutase. Unfortunately, DEPC reacts with NAD^+, so we cannot reliably obtain a dissociation constant for NAD^+ in this protection experiment. The dissociation constants for the interaction of prephenate with enzyme derived from the protection studies agree well with those obtained from steady-state kinetic analysis performed in this study as well as by Turnbull and Morrison (29). As expected, the results are in accord with the idea that prephenate protects against DEPC-mediated inactivation by binding at either the prephenate subsite of the dehydrogenase or by binding as a product at the active site of the mutase.

The primary sequence locations of Lys37 and His131 support the idea that these residues are in distinct active sites: the N-terminal third of the *E.coli* mutase-dehydrogenase is highly homologous to several chorismate mutase sequences from other bacterial bifunctional enzymes, including the recently crystallized mutase domain of the *E.coli* chorismate mutase-prephenate dehydratase (19). A homologous lysine (Lys39) forms H-bonds to the *endo*-oxabicyclic diacid inhibitor in the crystal structure of the enzyme-inhibitor complex.

Most other prephenate dehydrogenases which have been sequenced are from very closely related bifunctional mutase-dehydrogenases. However, alignments with other dehydrogenases and flavoproteins have shown that His131 is reasonably close (at least in primary structure) to residues 101 to 111, which comprise the adenine binding sites in the Rossman fold, a conserved dinucleotide binding domain found in several FAD- and NAD^+-binding enzymes (13). These findings argue *against* the idea that the

modifications are not in the active site and that enzyme inactivation is elicited through conformational changes in the protein.

The Roles of Lys37 and His131 in the Catalytic Mechanism of Mutase-Dehydrogenase

Lys37

A crystal structure of the highly homologous mutase domain of mutase-dehydratase (minimutase) liganded to *endo*-oxabicyclic diacid compound shows Lys39 (which aligns with Lys37 in mutase-dehydrogenase) interacts with the ligand's ether oxygen-7 as well as the C-11 carboxylate which is believed to occupy the same position as the enol pyruvate side chain in the transition state (see Figure 2.1).

Kinetic studies on the mutase activity of the mutase-dehydrogenase clearly show a pH dependence on *V/K* (23) and a distinct deuterium isotope effect (> 2) on both *V* and *V/K* (25), consistent with a general acid-proton transfer in the rate-limiting step. Of the three ionizable groups reported in the *V/K* pH profile, Turnbull *et al.* (23) have suggested that one group with a pK_a of 7.1 must be protonated for binding chorismate and may also participate in catalysis if the substrate binds to only the correctly protonated form of the enzyme. This group could be Lys 37 in the mutase-dehydrogenase (or Lys39 in the mutase-dehydratase) which might have a depressed pK_a value because of its location in a positively charged active site. Such an idea is consistent with the high reactivity of this lysine towards DEPC: a lysine with a pK_a value of 7 would be 50% unprotonated at the pH of our modification studies and neutral amines are better nucleophiles. Two possibilities for the role of this lysine as an enzymic acid include: (A) protonation of the ether oxygen by an enzymic acid, in conjunction with the attack on the C-1 carbon by an

electron pair on the methylene group of the enolpyruvyl side-chain (23); (B) a dissociative mechanism involving a heterolytic cleavage of the ether bond with general acid catalysis, assisted by the concomitant attack of a nucleophile, leading to a covalently bound intermediate (25). Inherent in both of these mechanisms is the assumption that the enzyme binds the kinetically and thermodynamically less stable diaxial form of chorismate (A) (70).

As an alternate view, Lee *et al.* (19, 20) have proposed an electrostatic basis for catalysis by the *E.coli* mutase. Although the bifunctional mutase-dehydrogenase and mutase-dehydratase are more closely related in primary sequence to each other than either is to the monofunctional *B.subtilis* enzyme (15), the mutase from *B.subtilis* possesses a H-bonding group (Arg 90) involved in a similar interaction (as Lys39) with *endo*-oxabicyclic diacid (17). Moreover, Lee *et al.* (19) noted that in both enzymes these key groups are flanked by an arginine-rich electrostatic wall interacting with the pyruvyl side-chain carboxylate. Kinetic studies on the mutase from *B.subtilis* show that this enzyme catalyzes the rearrangement in the absence of any enzymic acids or bases; it relies mainly on the selective binding of the reactive pseudodiaxial conformer of chorismate, with some additional stabilization of the transition state (21). Reasoning by analogy to the *B.subtilis* mutase, Lee *et al.* (20) have proposed that Lys39 in the minimutase is involved in the conformational trapping of chorismate. That is, Lys39 initially H-bonds to a side chain carboxylate in chorismate when the side-chain is in an extended conformation, then, aided by the arginine groups, brings about the rearrangement of the side chain to one where it is poised in a position like that in the transition state. This model is consistent with the observed acceleration of Claisen rearrangements in polar solvents (71, 72). The pH

studies by Turnbull *et al.* (23) showed that the same groups whose ionizations affect chorismate binding also affect the binding of *endo*-oxabicyclic diacid, and hence can also accommodate this model. Lys39's full catalytic potential is not achieved until its side-chain makes those contacts found in the transition state. Both of the proposed models illustrate the importance of Lys39 in the catalytic mechanism of chorismate mutase from *E.coli* and wait further testing with site-directed mutants of chorismate mutases from both organisms.

His131

Studies by Cleland and colleagues (23, 27) on the pH dependence of the prephenate dehydrogenase reaction have clearly shown that there is a catalytic group involved and this group, with a pK_a value of about 6.7, must be unprotonated for maximum activity of the enzyme. Our study supports this view and extends the findings to show that this group may be His131.

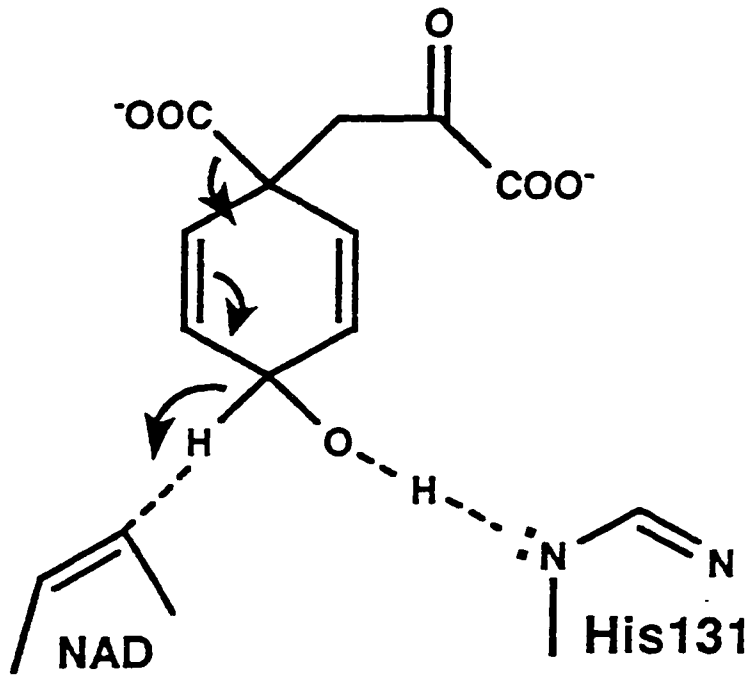
The other $NAD(P)^+$-dependent oxidative decarboxylases which have been studied kinetically operate by a two-step mechanism. The first step is a base catalyzed transfer of a hydride ion from the substrate to NAD^+, generating a β-keto intermediate; once the intermediate is formed it is readily decarboxylated with some assistance from an enzymic acid. Most of these decarboxylases (for example malic enzyme and isocitrate dehydrogenase) also use a metal to stabilize the enolate thus facilitating decarboxylation while only a few (6-phosphogluconate dehydrogenase being the most characterized) are not metal dependent (73). The pH dependency of the reaction catalyzed by 6-phosphogluconate dehydrogenase in *Candida utilis* has led Berdis and Cook (74) to

propose that there is a lysine or a histidine (pK_a of about 7.5) acting as the base. Furthermore, the recently solved crystal structure for the enzyme from sheep liver shows this base is a lysine residue (75).

Isotope effect studies done on the three enzymes mentioned above confirm this step-wise mechanism (76, 77, 78). By contrast, the oxidative decarboxylation of prephenate by prephenate dehydrogenase follows a concerted mechanism--hydride transfer and decarboxylation occur in the same chemical step without the formation of a vinylogous β-keto intermediate (27). The prephenate dehydrogenase reaction is essentially irreversible because an aromatic product, (4-hydroxyphenyl)pyruvate, is formed. Therefore, a base may not be needed. In support of the idea that it is the incipient aromaticity of the product that lowers the activation energy for decarboxylation, it was found that a partially saturated prephenate analogue, which would not become aromatic after decarboxylation, was reversibly oxidized but could not be decarboxylated (27). This prompted the conclusion from these authors that prephenate dehydrogenase provides only the catalytic machinery necessary to carry out the simple oxidation step. Such being the case, a mechanism was proposed (Figure 2.13) in which a histidine (we propose His131) is needed only to polarize the 4-hydroxyl group of prephenate rather than to deprotonate-- a vinylogous β-keto acid is never formed. Polarization of the 4-hydroxyl group would lower the activation energy for hydride abstraction by NAD^+ and the concomitant decarboxylation (27, 23). Our present studies support this idea in that His131 is in the presumed dehydrogenase domain of the protein and it is probably at or near the prephenate binding site, since prephenate does protect against DEPC-mediated inactivation of the dehydrogenase. Our studies do not rule out an alternative possibility

sible role of His131 in the concerted
n of prephenate.

Figure 2.13 The possible role of His131 in the concerted hydride transfer and decarboxylation of prephenate.



that this histidine may play a role in orienting the cofactor NAD^+, since His131 is close in primary structure to the adenine binding site of the dinucleotide fold (residues 101 to 111) (13) and NAD^+ does appear to afford some protection against DEPC-mediated inactivation. In either case, His131 sits in the dehydrogenase site in a good position to assist in the oxidation reaction catalyzed by prephenate dehydrogenase. The role that His131 and other residues play in the catalytic mechanism is being explored currently with site-directed mutagenesis and will be reported in due course.

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CHAPTER 3

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

FOOTNOTES

[1] 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.

SUMMARY

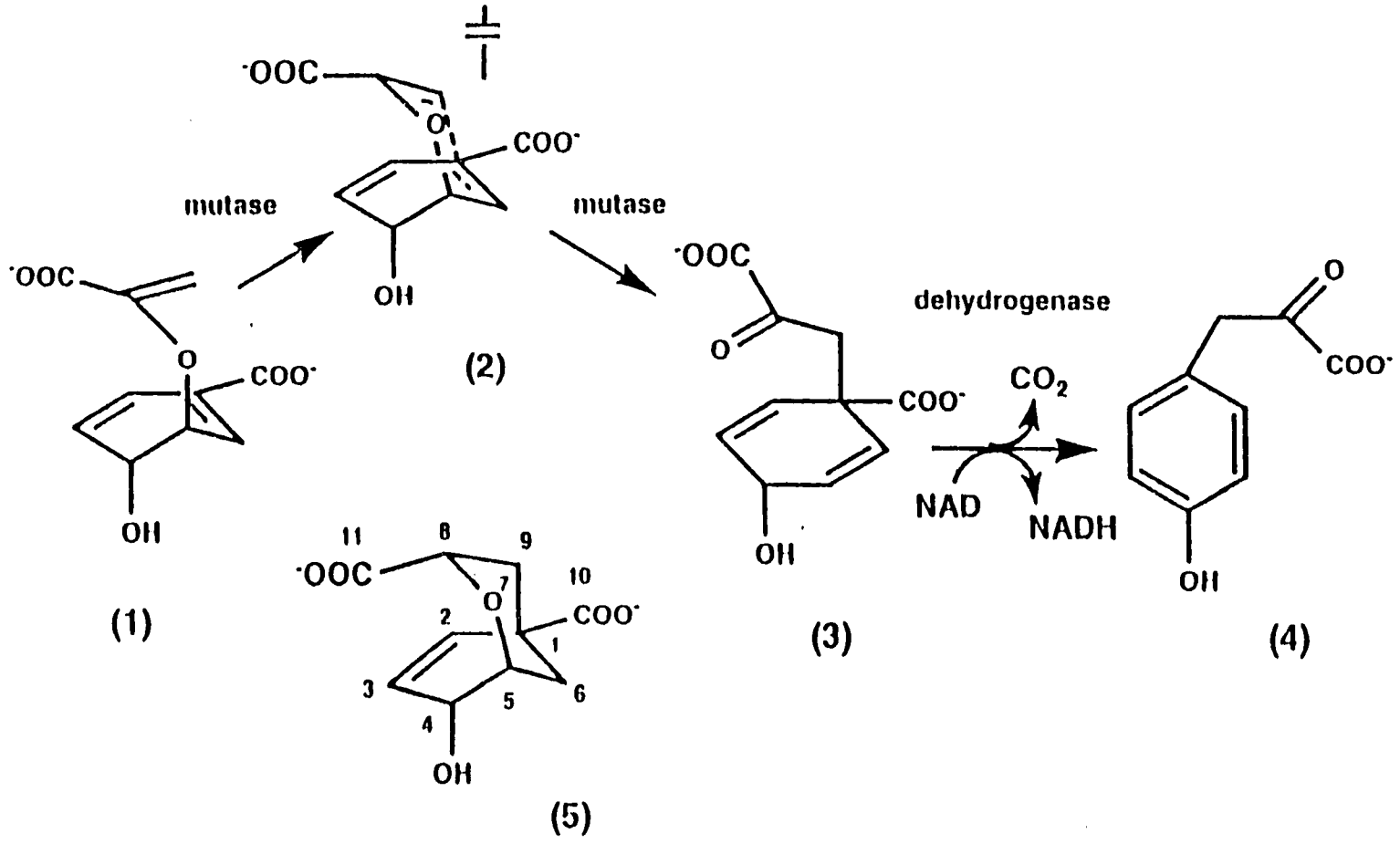
Site-directed mutagenesis was performed on the bifunctional enzyme chorismate mutase-prephenate dehydrogenase in order to identify groups important for each of the two reactions. We selected two residues for mutagenesis, Lys37 and His131, identified previously by differential peptide mapping to be essential 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. In contrast, His131 is not critical for the dehydrogenase; H131A is an efficient catalyst exhibiting 10% dehydrogenase and 30% mutase activity of wild-type enzyme. Chemical modification of H131A by diethylpyrocarbonate (DEPC) 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, inactivated the dehydrogenase by five orders of magnitude while retaining full mutase activity. In H197N, Michaelis constants for prephenate and NAD^+ and its elution profile from Sepharose-AMP were similar to 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 in contrast to the wild-type enzyme which shows a decrease in activity at low pH with a pK of about 6.5. We conclude that His197 assists in the hydride transfer from prephenate to NAD^+.

INTRODUCTION

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 (Figure 3.1) 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, 4, 5).

The enzyme from *E.coli* is homodimeric with a molecular weight of 84,000 (6, 7, 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**, a compound which mimics the chair-like 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 importance 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 mutases have been confirmed recently by site-directed mutagenesis

Figure 3.1 Reactions catalyzed by chorismate mutase-prephenate dehydrogenase


mutase
mutase
dehydrogenase
NAD
NADH
CO_2
(1)
(2)
(3)
(4)
(5)

(16, 17, 18, 19, 20, 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 mechanistic 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 diethylpyrocarbonate (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 substrate 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 confirm that Lys37 plays a key catalytic role in the mutase reaction, while it is His197 rather than His131 that most likely plays a role as the hydrogen bond acceptor in the hydride transfer of 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, 32, 33, 34, 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 USB using 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) using 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 g 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 plasmid (40). The procedure employs two mutagenic oligonucleotide primers. One primer contains the desired mutation and the second contains a mutation for the unique non-essential restriction site, *Xho*I, that is downstream of *TyrA* in pVIV1 (see Table 3.1). 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.

Table 3.1 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.

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_{600nm} 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 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 x 30s bursts for every 10 g of cells. The lysate was centrifuged at 14000g for 15 min 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 14000g for 15 min at 4°C and the supernatant was applied to a Q-Sepharose Fast Flow column (2.6 x 15 cm) equilibrated with buffer B. After washing the column 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 kept at 7.5 rather than adjusted to pH 6 prior to chromatography; 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 Coommassie Blue-stained SDS-PAGE (42). The pVIV1 derivatives housing specific site-directed mutations in *TyrA* were 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 were checked by SDS-PAGE with Coommassie Blue staining and were found to be over 90% pure. Wild-type and mutant proteins were most stable when stored as an ammonium sulfate precipitate at 4°C, then dialyzed just prior to use in the appropriate buffer. To test whether the mutants were expressed in the host cells, crude cell extracts made from each mutant and wild-type cells were screened by immunoblotting (44). The proteins were separated by 10% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with rabbit anti-mutase-dehydrogenase antibody followed by reaction with goat anti-rabbit antibody linked to horseradish peroxidase. The mutase-dehydrogenase bands were visualized by chemiluminescence with Renaissance^{R} 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). 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 Christendat and Turnbull (25). Protein concentration was

estimated using the Bio-Rad protein assay kit with bovine serum albumin (BSA) as a standard and by recording the absorbance at 205 nm (45). 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 mutant proteins. Measurements were recorded at room temperature from 190-250 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 using the computer programs of Cleland (46) or GraFit (Version 3.0, Leatherbarrow).

$$v = \frac{VA}{K + A} \tag{3.1}$$

$$y = \frac{C}{1 + (H/K_A) + (K_B/H)} \tag{3.2}$$

$$y = \frac{C}{1 + (H/K_A)} \tag{3.3}$$

The initial velocity data obtained by varying the concentration of chorismate, prephenate or NAD^+ (*A*) were fitted to equation 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 were fitted to the log form of equations 3.2 and 3.3, 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 g of cell paste. Our new purification scheme (Table 3.2) 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 g of cells. The kinetic parameters and molecular weight of the enzyme are similar to those reported elsewhere (8).

Properties of Mutant Mutase-Dehydrogenase Proteins

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 poorly expressed compared to the wild-type enzyme as judged by SDS-PAGE and immunoblots (Figure 3.2). Moreover, a crude extract of this mutant did not exhibit any detectable mutase or dehydrogenase activity.

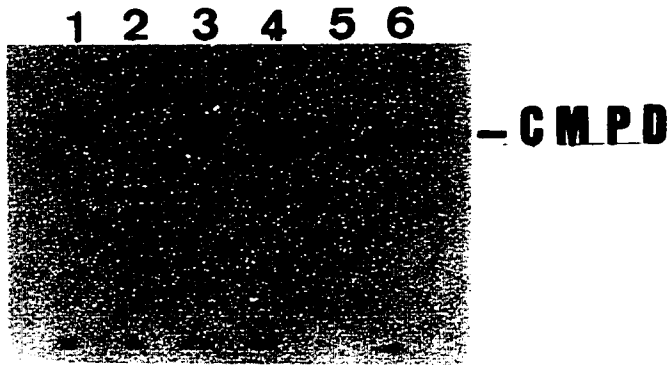
Table 3.2 Purification of Chorismate Mutase - Prephenate Dehydrogenase from *E.coli* XL2-Blue Cells[a] Harboring pVIV1

Purification Step	Volume (mL)	Protein (mg)	Mutase Activity		Dehydrogenase Activity		Purification (x-fold)[b]	Yield (%)
			Total (units)	S.A (units/mg)	Total (units)	S.A (units/mg)		
Cell-free extract	161	2270	11 877	5.2	10 000	4.4	1.0	100
Ammonium sulfate	62	868	11 284	13	9 800	11	2.5	95
Q-Sepharose	170	476	9 860	20	9 500	20	3.9	83
Sepharose-AMP	170	255	9 700	38	8 772	34	7.3	82

[a] 23 g of wet cells [b] calculated from mutase activity

Figure 3.2 Expression of recombinant mutase-dehydrogenase in crude *E. coli* lysates.

The levels of expression of wild-type and mutant enzymes are compared using an immunoblot probed with anti-rabbit mutase-dehydrogenase antiserum. Cultures of strain KB357 containing either wild-type or mutant genes were grown under identical conditions. Following cell lysis (see experimental procedures), 0.5 μg of crude protein was loaded into separate wells on a 10% SDS-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).


1 2 3 4 5 6
CMPD

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 3.2) and provided a far UV-CD spectrum similar to 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 3.3).

It has been previously demonstrated that prephenate is a competitive inhibitor with respect to chorismate in the mutase reaction. Furthermore, the value for the combination of prephenate as a substrate for the dehydrogenase reaction (0.17 mM) is similar to its value as a product inhibitor of the mutase reaction (0.21 mM) (24). In spite of the structural similarities of the two compounds, the reciprocal is not true. In the presence of 25 μM prephenate and 3 mM NAD^+, chorismate did not inhibit the dehydrogenase reaction catalyzed by K37Q, even up to a concentration of 0.5 mM. In fact, the reaction velocity was enhanced by about 10% by the addition of chorismate (data not shown).

H131A

Our previous chemical modification studies have implicated His131 as a catalytic residue in the dehydrogenase reaction (25). However, the H131A substitution did not eliminate dehydrogenase activity. As shown in Table 3.3, 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 were only slightly increased over that found for the wild-type enzyme. Moreover, the pH dependence of log V for this mutant (data not shown) paralleled that found for wild-type dehydrogenase

Table 3.3 Summary of Kinetic Data for Wild-Type and Mutant Mutase-Dehydrogenase

Protein[a]	Mutase Activity			Dehydrogenase Activity					
	Chorismate			Prephenate			NAD^+		
	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

(see Figure 3.4). Together the data imply that His131 is not an essential residue whose protonation state is critical for catalysis or substrate binding.

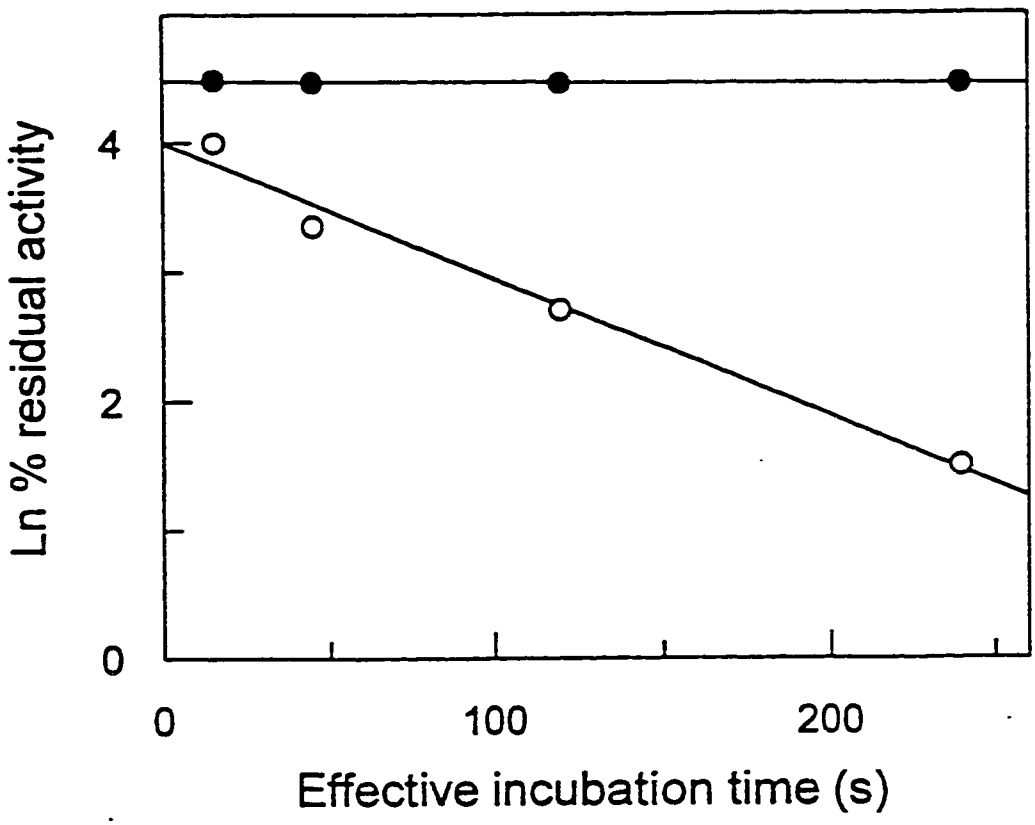
Chemical modification with DEPC was performed on H131A to determine if another histidine residue rather than His131 could fulfill this catalytic role. Incubation of 0.11 mM active sites of H131A with 0.2 mM DEPC results in a time-dependent loss of prephenate dehydrogenase activity. Moreover, prior addition of 1 mM prephenate completely protects against inactivation (Figure 3.3). 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 group(s) modified by DEPC in the mutant may include a particularly reactive histidine previously shielded in the active site of the wild-type enzyme.

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 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 possess mutase activity. Two of the alanine mutant proteins (H257A, H265A) were stable whereas five others (H153A, H189A, H197A, H239A, and H245A) were not expressed and/or were unstable and were changed to asparagine. Of these asparagine mutants five (H153N, H189N, H197N, H239N,

Figure 3.3 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 (O) or with 0.2 mM DEPC plus 1 mM prephenate (•).


Ln % residual activity
4
2
0
0
100
200
Effective incubation time (s)

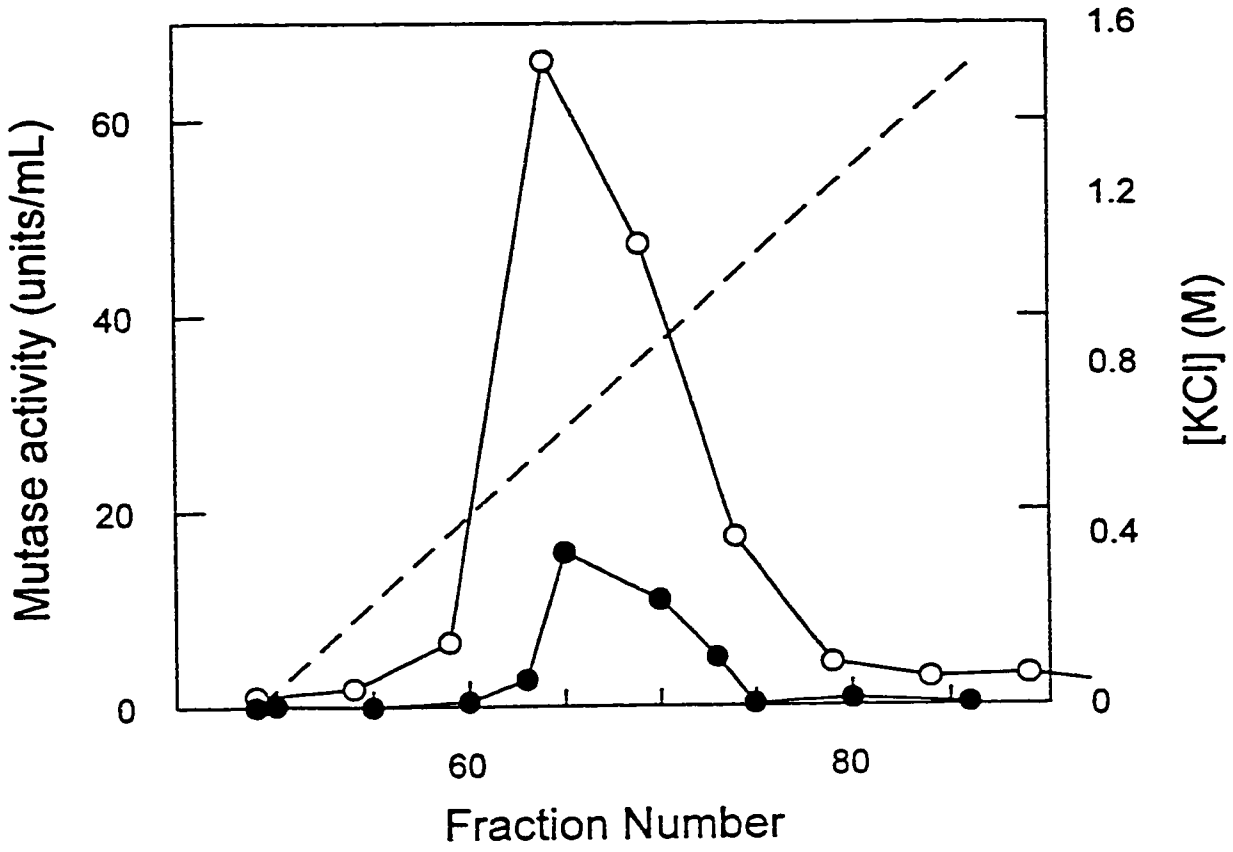
H245N) were well expressed. At position 237, only the histidine to asparagine mutation was made and the resulting protein was stable. Immunoblots of selected proteins are shown in Figure 3.2 while the kinetic properties of the mutant proteins are listed in Table 3.3. Six of the eight mutant proteins generated from scanning mutagenesis did not exhibit kinetic properties that were remarkably different from wild-type enzyme. The 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 but still retained nearly wild-type mutase activity and Michaelis constants for chorismate, prephenate and NAD^+. Furthermore, H189N showed less than 0.01% the activity of 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).

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 3.3). For the dehydrogenase reaction, the Michaelis constant is a good estimate of the true dissociation constant of substrate from the enzyme, 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 3.4 shows that H197N and the wild-type enzyme eluted at a concentration of 0.55 M KCl, implying that both proteins

Figure 3.4 Elution patterns of wild-type and H197A mutase-dehydrogenase from Sepharose-AMP.

Wild-type (250 mg, O) and H197A (90 mg, •) were chromatographed separately on a 2.6 x 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.


Mutase activity (units/mL)
60
40
20
0
1.6
1.2
0.8
0.4
0
[KCl] (M)
60
80
Fraction Number

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. 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 fall-off 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 equation 3.2 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 $mM^{-1}s^{-1}$. The variation of log V with pH gives rise to a half-bell profile with slopes of +1 and 0. Fitting of the data to equation 3.3 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 3×10^{-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 (47, 48, 49, 50). Unfortunately but not surprisingly, 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 dehydrogenase from *E.coli*. We have identified two residues that are important for the activity of the enzyme--Lys37, which is critical for the mutase and His197, which is essential 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, trinitrobenzene sulfonate, 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, 17, 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*, Lys29 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 3.2), whereas the comparable mutant in the other mutases was well expressed. Such an observation may reflect the difference

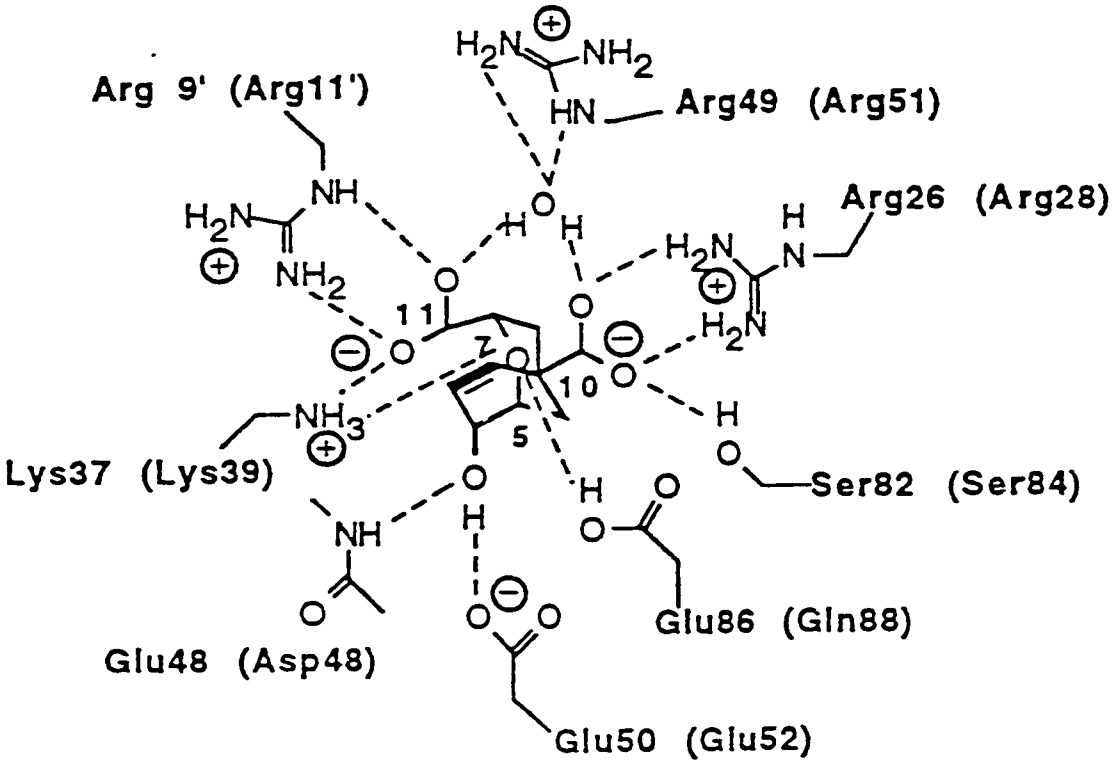
between the bifunctional enzyme, in the case of the mutase-dehydrogenase, versus the monofunctional form of the other mutases examined. From the crystal structure of the highly homologous mutase portion of mutase-dehydratase liganded to the transition state analogue *endo*-oxabicyclic diacid (10), it 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 (Figure 3.5). As concluded from previous findings with monofunctional mutases (17, 18, 19, 20, 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 additional assistance to catalysis by stabilizing the developing negative charge on the enolpyruvyl side chain initiated by C-5 - O-7 bond cleavage (51-53).

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.

Figure 3.5 Proposed active site of chorismate mutase from *E.coli* mutase-dehydrogenase bound to the transition state analogue 5.

Diagram adapted from Lee *et al.* (10) for the independently expressed mutase of mutase-dehydratase with its residues shown in brackets.


Arg 9' (Arg11')
Arg49 (Arg51)
Arg26 (Arg28)
Lys37 (Lys39)
Ser82 (Ser84)
Glu48 (Asp48)
Glu86 (Gln88)
Glu50 (Glu52)

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 3.3). 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 suggests that His197 is the essential catalytic residue in the dehydrogenase reaction. The H197N substitution eliminates totally and exclusively dehydrogenase activity (Table 3.3). Moreover, His197 does not play a role in the binding of prephenate or NAD^+ since the Michaelis constants for these two substrates in the mutant and wild-type enzymes are comparable (Table 3.3) as is the elution profile of both enzymes from an affinity column showing high specificity for

nucleotide-containing proteins (Figure 3.5). 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 3.6). It has been previously hypothesized (23, 24, 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 activation energy for hydride abstraction by NAD^+ and the concomitant decarboxylation (Figure 3.7).

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 3.8, His197 as well as His245 and His257 are conserved amongst six prephenate dehydrogenases, both bifunctional and monofunctional forms. While this is in accord with the kinetic results of H197N, in contrast, H245A 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, except at His189, yield mutant proteins that are effective catalysts. His131 is reasonably 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

Figure 3.6 Variation with pH of log V and log $(V/K)_{prephenate}$ for the reaction catalyzed by wild-type (A) and log V for H197N prephenate dehydrogenase (B).

NAD^+ concentrations were fixed at 3 mM. The points in (B) represent the average from three separate determinations. The units for V and V/K are s^{-1} and $M^{-1}s^{-1}$, respectively. The curves in panel A represent best fits of the data to equation 3.2 and 3.3, respectively. The values of the parameters used to draw the curves are given in the text.

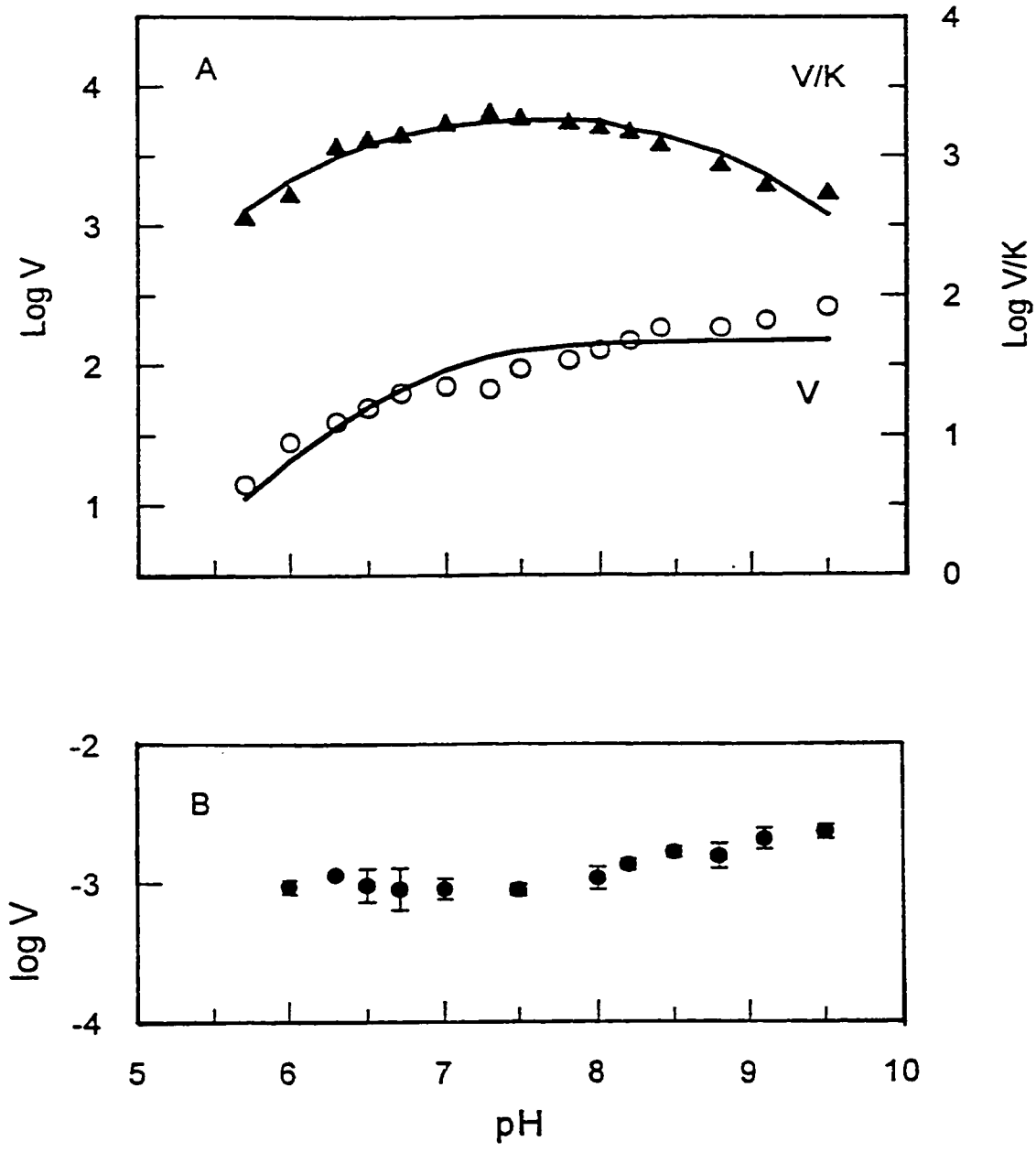

A
V/K
V
Log V
Log V/K
4
3
2
1
0
B
log V
-2
-3
-4
5
6
7
8
9
10
pH

Figure 3.7 Possible role of His197 in the concerted hydride transfer and decarboxylation of prephenate

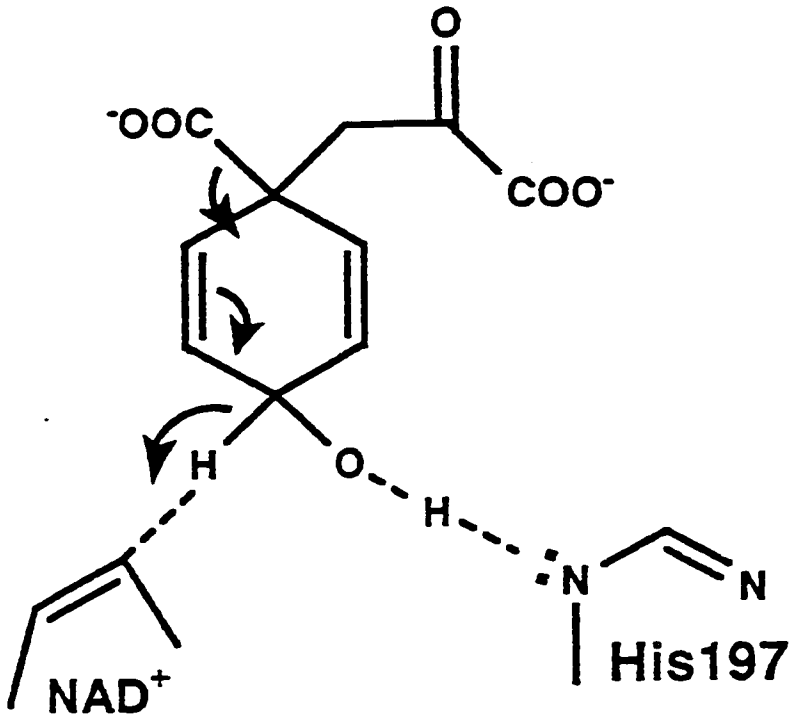

O
-OOC
COO-
H
O
H
N
N
NAD+
His197

Figure 3.8 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 invariant histidines. Sequences are from (a) *Escherichia coli* (B) (9), (b) *Erwinia herbicola* (B) (33), (c) *Haemophilus influenzae* (B) (35), (d) *Zymomonas mobilis* (M) (34), (e) *Bacullus subtilis* (M) (32), (f) *Lactococcus lactis* (M) (31).


(a) --MVAELTALRDQIDEVDKALLNLLAKRLELVAEVGEVKSRFGLPIYVPEREASMLASRRAEAEALGVPPDLIEDVLRRVMRESY 83
(b) --MVAELTALRDQIDSVDKALLDLLAKRLELVAEVGEVKSRYGLPIYVPEREASMLASRRKEAEALGVPPDLIEDVLRRVMRESY 83
(c) MSFMEALKDLRSEIDSLDRELIQLFAKRLELVSQVGKVKHQHGLPIYAPEREIAMLQARRLEAEKAGISADLIEDVLRRFMRESY 85
(d) ----------MTVFKHIAIIGLG--LIGSSAARAT-KAY 26
(e) ----------MNQMKDTILLAGLG--LIGGSIALAIKKNH 28
(f) ----------MKKILIIGLG--LIGSSIALGIKKAH 24
SSENDKGFKTLCPSLRPVVIVGGGGQMGRLFEKMLTLSGYQVRILEQHDWDRAADIVADAGMVIVSVPIHVTEQVIGKLPP--LP 166
TSENDKGFKTLCPELRPVVIVGGKGQMGRLFEKMLGLSGYTVKTLDKEDWPQAETLLSDAGMVIISVPIHLTEQVIAQLPP--LP 166
ANENQFGFKTINSDIIIKIVIVGGYGKLGGLFARYLRASGYPISILDREDWAVAESILANADVVIVSVPINLTLETIERLKPY-LT 169
(d) ----------CPD----VTVSLYD-KSEFVCDRARALNLGDNVTD--D---IQDAVREADLVLLCVPVRAMGIVAAAMAPA-LK 89
(e) ----------PG---KRIIG-----IDISDEQAVAALKLGVIDDRADS--FISGVKEAATVIIATPVEQTLVMLEELAHSGIE 81
(f) ----------PE---FEILG-----SDR-EEVENIAQKRGIIDSKVE---LVKGAQEADIIILAVPISVTLELLKQIATFDLK 85
KDCILVDLASVKN---GPLQAMLVAHDGPVLGLHPMFG-----PDSG-SLAKQ---VVVWCDGRKP-EAYQWFLEQIQVWGARLH 238
EDCILVDLASVKN---RPLQAMLAAHNGPVLGLHPMFG-----PDSG-SLAKQ---VVVWCDGRQP-EAYQWFLEQIQVWGARLH 238
ENMLLADLTSVKR---EPLAKMLEVHTGAVLGLHPMFG-----ADIA-SMAKQ---VVVRCDGRFP-ERYEWLLEQIQIWGAKIY 241
KDVIICDTGSVKVSVIKTLQDNLPNHI--IVPSHPLAGTENNGPDAGFAELFQDHPVILTPDAHTPAQAIAYIADYWEEIGGRIN 172
HELLITDVGSTKQKVVDYADQVLPSRY-QFVGGHPMAGSHKSGVAAAKEFLFENAFYILTPGQKTDKQAVEQLKNLLKGTNAHFV 165
DGLLITDAGSTKSEIVELANQLFSGTKVKFIGGHPMAGSHKSGVMAADLNLFENAYYVLTEE--S-----QELREFLKGLHAKFI 163
RISAVEHDQNMAFIQALRHFATFAYGLHLA-EENVQLEQLLALSSPIYRLELAMVGRLFAQDPQLYADIIMSSERNLALIKRYY 321
RISAVEHDQNMAFIQALRHFATFAYGLHLA-EENVNLDQLLALSSPIYRLELAMVGRLFAQDPQLYADIIMSSESNLALIKRYY 321
QTNATEHDHNMTYIQALRHFSTFANGLHLS-KQPINLANLLALSSPIYRLELAMIGRLFAQDAELYADIIMDKSENLAVIETLK 324
LMSAEHHDHVLALTSHLPHVIAYQLIGMVSGYEKKSRTPIMRYSAGSFRDAT----RVAASEPRLWQDIMLENAP--ALLPVLD 250
EMSPEEHDGVTSVISHFPHIVAASLVHQTH-HSENLYPLVKRFAAGGFRDIT----RIASSSPAMWRDILLHNKD--KILDRFD 242
ILDAKEHDKVTGQVSHFPHILASTLVWQSD-DYAKEHPLVKHLAAGGFRDLT----RIAEADSLMWTSVLLSNPE--ITLERIE 240
KRFGEAIELLEQG---DKQAFIDSFRKVEHWFGDYAQR--FQSESRVLLRQANDNRQ---------- 373
QRFGEAIALLEQG---DKQAFIASFNRVEQWFGDHAKR--FLVESRSLLRSANDSRP---------- 373
QTYDEALTFFENN---DRQGFIDAFHKVRDWFGDYSEQ--FLKESRQLLQQANDLKQG---------- 377
HFIADLKKLRTAI---ASQ---D-----EDYLLEHFKE--SQ-KARLALKTDHDIHP---------- 293
EWIREIDKIRTYVEQEDAENLFRYFKTAKDYRDGLPLRQKGAIPAFYDLYVDVPDHPGVISEITAILAAERISITNIRIIE 323
NFKKHLDEIALKITKRDSQAIEHFFEEGKKIRQAMEIH-KGALPNFYDLFISVPDEKGVVLRVLALL--QDFSITNIKINE 318

enzymes (9). The fact that the H131A mutant binds NAD^+ about one-third as well as the wild-type enzyme (Table 3.3) 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) which are conserved among prephenate dehydrogenases to determine if they play a role in prephenate binding.

The relationship between the two activities

The results of this investigation are consistent with the involvement of 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 (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 mM 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 channelling 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 poorly to the mutase portion of K37Q. *Endo*-oxabicyclic diacid, an analogue which mimics the transition 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 colleagues who reported the separate expression and purification of fully active, monofunctional proteins (10, 54, 55); 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 (56) that the mutase and dehydratase sites are distinct and non-interacting. To date, the molecular-genetic approach has not been demonstrated

as elegantly for the mutase-dehydrogenase. As steps towards this goal however, Mayuya *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 which 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 *E.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 (57). Moreover, we have demonstrated in the present study that substitutions of amino acid clearly in the dehydrogenase portion of the enzyme (H189N, H238N, H245N) can affect mutase activity. We are currently investigating 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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CHAPTER 4

Identification of Groups Involved in Prephenate Binding in the Mechanism of Prephenate Dehydrogenase from *E.coli*

SUMMARY

Site-directed mutagenesis was initiated on the *E.coli* bifunctional enzyme chorismate mutase-prephenate dehydrogenase to investigate the importance of three conserved positively charged residues, Lys178, Arg286 and Arg294, in the binding of prephenate to the dehydrogenase. To date, definite conclusions can only be drawn concerning the role of Arg294. Evaluation of the kinetic parameters and denaturation profile of R294Q compared to the wild-type enzyme indicates that the mutant protein is stable and that the substitution affects specifically the dehydrogenase reaction by reducing the affinity of the enzyme for prephenate. However, the dissociation constants for a series of inhibitory substrate analogues missing the ring carboxylate at C-1 were similar for the wild-type enzyme and R294Q. This implies that Arg294 interacts with the ring carboxylate of prephenate. The pH dependence of the $(V/K)_{prephenate}$ and the K_i of the inhibitor hydroxyphenyllactate (HPlactate) for the reaction catalyzed by the wild-type dehydrogenase indicated that a protonated group with pK of 8.8 is important for binding prephenate to the enzyme-NAD^+ complex. The same binding residue is observed in the V/K profile for R294Q implying that this protonated residue is not Arg294. The cationic residue titrating in the pH profile most likely interacts with the pyruvyl side chain carboxylate group of prephenate since the pH dependence of the binding of HPlactate and deoxoprephenate are identical. Both substrate analogues mentioned above possess the carboxyl groups on the side chain but are missing either the ring carboxylate (HPlactate) or the side chain carbonyl group (deoxoprephenate). Studies on Lys178 indicate that arginine cannot replace lysine at this position. The K178R is a poor catalyst of both reactions. Moreover, the mutant is conformationally less stable than wild-type enzyme as

determined by Gdn-HCl denaturation studies. Thus, structural changes may account for the altered kinetic properties of K178R mutase and dehydrogenase. More studies must be conducted on mutant proteins of Lys178 and Arg286 to determine if these two cationic residues play a role in prephenate binding.

INTRODUCTION

Chorismate mutase-prephenate dehydrogenase (EC 5.4.995/1.3.1.12) is a bifunctional enzyme that catalyzes the conversion of chorismate to prephenate as well as the oxidative decarboxylation of prephenate to (4-hydroxyphenyl)-pyruvate in the presence of NAD^+. The two reactions are essential for the biosynthesis of tyrosine in bacteria and other microorganisms, yeast and plants (1, 2, 3, 4, 5). The enzyme in *E.coli* is a homodimer with a molecular weight of 84 kDa (6, 7, 8). Tyrosine, the end product of this pathway, modulates both mutase and dehydrogenase activities (2, 7, 9, 10). Studies on the *E.coli* enzyme indicate that distinct enzymic groups participate in the transition states of the two reactions (11, 12) and that the two sites are separate (13, 14, 15).

The kinetic mechanism of prephenate dehydrogenase conforms to a rapid equilibrium, random mechanism with catalysis as the rate-limiting step (16), while hydride transfer and decarboxylation occur in a concerted manner (17). Studies on the pH dependencies of the kinetic parameters V and V/K for the dehydrogenase reaction have indicated that ionizable groups are involved in the chemical mechanism (10, 17). These studies have shown that a deprotonated group with a pK value of about 6.5 is required for catalysis, while a protonated group with a pK value of 8.7, is likely involved in the binding of prephenate to the enzyme-NAD^+ complex. The findings from temperature and solvent perturbation studies (17) and from chemical modification of the bifunctional enzyme with DEPC (11) indicated that a histidine may be the catalytic residue involved in the dehydrogenase reaction. 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 (12). These findings have led to the proposal in which a histidine, His197, polarizes the 4-hydroxyl group of prephenate, facilitating hydride transfer to NAD^+ and concomitant decarboxylation (17, 12).

Until recently, amino acid sequences from only a few prephenate dehydrogenase 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 and their alignments (Figure 4.1) indicate that His197, the putative catalytic base, and positively charged residues, Lys178, Arg286 and Arg294 of *E.coli* mutase-dehydrogenase are conserved amongst all prephenate dehydrogenases compared. Since a protonated group on the dehydrogenase is required for prephenate binding, we hypothesized that the conserved cationic residues may play an important role in the interactions with C-1 of prephenate through its ring carboxylate or side chain pyruvyl group (Figure 4.2).

In the work presented here, we have conducted site-directed mutagenesis on Lys178, Arg286 and Arg294 and have compared the kinetic properties of the mutant proteins to those of the wild-type enzyme. In addition, we have examined the pH dependency of the reactions catalyzed by the wild-type and R294Q dehydrogenase and have compared the ability of these enzymes to bind a series of prephenate analogues modified at C-1 of the substrate. Our findings revealed that the ring carboxylate and the carbonyl group on the pyruvyl side chain of prephenate are important, but not critical for substrate binding.

Moreover, Arg294 provides an ionic partner to the ring carboxylate of prephenate that is expelled during the decarboxylation reaction catalyzed by prephenate dehydrogenase.

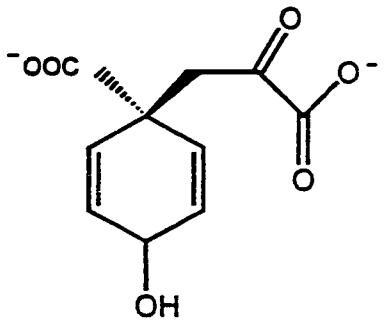
Figure 4.1. Amino acid sequence alignment of *E.coli* chorismate mutase-prephenate dehydrogenase with other known bacterial bifunctional mutase-dehydrogenases (B) and monofunctional prephenate dehydrogenases (M).

Invariant and nearly invariant residues are noted by stars and colons, respectively. Lys178, Arg286 and Arg294 are denoted by triangles. Sequences are from (a) *Escherichia coli (B)*(159), (b) *Erwinia herbicola (B)*(160), (c) *Haemophilus influenza (B)*(161), (d) *Aquifex aeolicus (M)(162),* (e) *Helicobacter pylori (M)*(163), (f) *Bacillus subtilis (M)*(164), (g) *Lactococcus lactis (M)*(165), (h) *Synechocystis sp. (M)*(166) *and* (i) *Zymomonas mobilis (M)*(167).

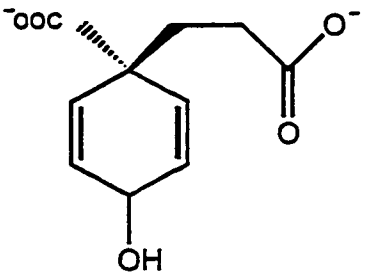
```
(a)  VKNGPLQAMLVAHDG---PVLGLHPMFGPDSGSLAKQVVVWCDGR-------KPEAYQWFLEQIQVWGAR 236
(b)  VKNRPLQAMLAAHNG---PVLGLHPMFGPDSGSLAKQVVVWCDGR-------QPEAYQWFLEQIQVWGAR 199
(c   VKREPLAKMLEVHTG---AVLGLHPMFGADIASMAKQVVVRCDGR-------FPERYEWLLEQIQIWGAK 233
(d)  VKGKLVYDLENILGK---RFVGGHPIAGTEKSGVEYSLDNLYEGKKVILTPTKKTDKKRLKLVKRVWEDV 193
(e)  AKAQIIRNIPKSIRK---NFIAAHPMCGTEFYGPKASVKGLYENALVILCDLEDSGTEQVEIAKEIFLGV 149
(f)  TKQKVVDYADQVLPSR-YQFVGGHPMAGSHKSGVAAAKEFLFENAFYILTPGQKTDKQAVEQLKNLLKGT 170
(g)  TKSEIVELANQLFSGTKVKFIGGHPMAGSHKSGVMAADLNLFENA-------YYVLTEESQELRELLKGL 158
(h)  VKTAIAEPASQLWSG----FIGGHPMAGTAAQGIDGAEENLFVNAPYVLTPTEYTDPEQLACLRSVLEPL 158
(i)  VKVSVIKTLQDNLPN--HIIVPSHPLAGTENNGPDAGFAELFQDHPVILTPDAHTPAQAIAYIADYWEEI 167
     :▲                  ::  **: *:   :          :              :
(a)  ---LHRISAVEHDQNMAFIQALRHFATFAYGLHLAEENVQLEQLLALSSP--IYRLELAMVGRLFAQDPQ 301
(b)  ---LHRISAVEHDQNMAFIQALRHFATFAYGLHLAEENVNLDQLLALSSP--IYRLELAMVGRLFAQDPQ 264
(c)  ---IYQTNATEHDHNMTYIQALRHFSTFANGLHLSKQPINLANLLALSSP--IYRLELAMIGRLFAQDAE 304
(d)  GGVVEYMSPELHDYVFGVVSHLPHAVAFALVDTLIHMSTPEVDLFKYPGG--GFKDF----TRIAKSDPI 256
(e)  KARLIKMKSNEHDTHVAYISHLPHVLSYALANSVLKQNDPEMILSLAGG---GFRDM----SRLSKSSPL 213
(f)  NAHFVEMSPEEHDGVTSVISHFPHIVAASLVHQTHHSENLYPLVKRFAAG--GFRDI----TRIASSSPA 233
(g)  HAKFIILDAKEHDKVTGQVSHFPHILASTLVWQSDDYAKEHPLVKHLAAG--GFRDL----TRIAEDSLM 221
(h)  GVKIYLCTPADHDQAVAWISHLPVMVSAALIQACAGEKDGDILKLAQNLASSGFRD----TSRVGGNPEL 225
(i)  GGRINLMSAEHHDHVLALTSHLPHVIAYQLIGMVSGYEKKSRTPIMRYSA-GSFRDA----TRVAAEPRL 233
        :   :  **      : :    :                                ▲       ▲:  :
```

Figure 4.2. Structure of substrates and inhibitory substrate analogues of prephenate dehydrogenase reaction.

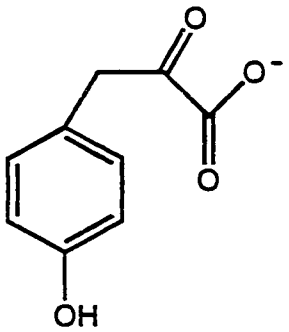
Substrates include prephenate and deoxoprephenate. Inhibitory substrate analogues include hydroxyphenylpyruvate (HPpyruvate), hydroxyphenyllactate (HPlactate) hydrophenylpropionate (HPpropionate) and ρ-hydroxybenzoate (Hbenzoate).



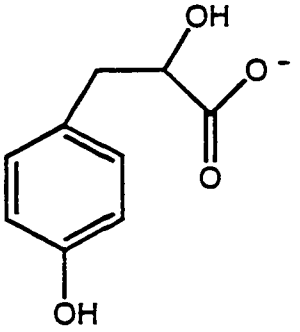
Prephenate



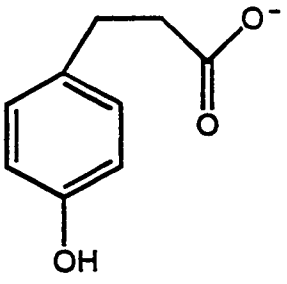
Deoxoprephenate



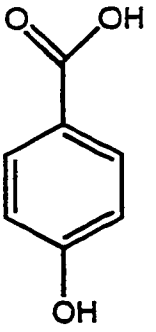
Hydroxyphenylpyruvate
(HPpyruvate)



Hydroxyphenyllactate
(HPlactate)



Hydroxyphenylpropionate
(HPpropionate)



p-Hydroxybenzoate
(Hbenzoate)

EXPERIMENTAL PROCEDURES

Materials

Chorismate was isolated from *Klebsiella pneumonia* 62-1 as described by Rieger and Turnbull (27) with a slight modification. Increasing the tryptophan concentration to 60 mg per L of accumulation media resulted in 3-fold improvement in the yield of chorismate from this organism. Sodium prephenate was prepared as previously described (28). NAD^+ was obtained in free acid form from Boehringer Mannheim. Hydroxyphenylpropionate (HPpropionate), hydroxyphenyllactate (HPlactate), hydroxyphenylpyruvate (HPpyruvate) and ρ-hydroxybenzoate (Hbenzoate) were purchased from Sigma-Aldrich chemicals. Oligonucleotides were obtained from BioCorp Inc. Restriction enzymes, T4 DNA ligase and T4 DNA polymerase were purchased from Pharmacia Biotech or M.B.I. Fermentas. All other chemicals were of the highest purity available commercially. Chorismate mutase-prephenate dehydrogenase was isolated from *E.coli* strain XL2-Blue carrying the plasmid pVIV1 in wild-type or mutant form as described by Christendat *et al.* (12). The specific activities of the purified wild-type mutase and dehydrogenase were 37 and 40, respectively. These values are in good agreement to those previously reported for this expression system (12).

Site-Directed mutagenesis

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 (29) as outlined in Christendat *et al.*, (12). 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, using Sequenase® version 2 DNA sequencing kit (U.S.Biochemicals) by the method of Sanger *et al.*, (30). The following synthetic oligonucleotides were used for mutagenesis: U.S.E, 5'- CAT GTA CAG AGC **G**CG AGA AGT AC- 3'; K178R, 5' – GCA TCA GTG **CGC** AAT GGG CCA TTA C- 3'; R294K, 5'- GCG ATG GTC GGG **AAA** CTG TTT GCT C- 3'; R286Q, 5'- CG ATT TAC **CAA** CTT GAG CTG G- 3'; R286K, 5'- CG ATT TAC **AAA** CTT GAG CTG G- 3'; R294Q, 5'- GCG ATG GTC GGG **CAA** CTG TTT GCT C- 3'. The most frequently used codons in *E.coli* (31) were used and are underlined. The U.S.E. oligonucleotide eliminates a unique *Xho*I site in the multiple cloning region of pSE380.

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 5.5 – 9.5, for which the initial velocity were measured, the ionic strength of this buffer remained essentially constant (32). 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 Pelltier-Effect temperature controlled cuvette holder. The pH of the assay mixture was determined at 30°C before and after the reaction. Protein concentration was estimated using the ESL protein determination kit (Boehringer Mannheim) with bovine serum albumin as a

standard and was compared to recording the absorbance obtained at 205 nm (33). The results from these two methods were in good agreement.

Data Analysis

Kinetic data were fitted to the appropriate rate equation using GraFit (Erathicus Software) or the program of Cleland (34). Initial velocity data obtained by varying the concentration of chorismate, prephenate or NAD^+ (A) were fitted to equation 4.1 to yield values for the maximal velocity (V), the Michaelis constant (K), and the apparent first-order rate constant for the interaction of enzyme with substrate (V/K).

$$v = \frac{VA}{K + A} \tag{4.1}$$

When a saturation curve was difficult to obtain due to the poor binding of prephenate to the enzyme, the kinetic parameter, V/K was calculated from the linear portion of a plot of velocity versus prephenate concentration. To examine the effect of substrate analogues on enzyme activity, the data were first plotted to check for linearity of the double reciprocal plots and to determine the pattern of the plots. In the presence of inhibitor (I), data conforming to linear competitive inhibition were fitted to equation 4.2 to yield the true inhibition constant K_{is}.

$$v = \frac{VA}{K(1 + \frac{I}{K_{is}}) + A} \tag{4.2}$$

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 equation 4.3 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 equation 4.4, where A represents the fixed substrate concentration and K denotes the Michaelis constant for substrate.

$$v = \frac{V}{1+(A/K_{i(app)})} \tag{4.3}$$

$$K_i = \frac{K_{i(app)}}{1+(A/K)} \tag{4.4}$$

The variation of the values for V, V/K, or $1/K_i$ as a function of pH were fitted to the log form of equation 4.5 or equation 4.6.

$$y = \frac{C}{1+(H/K_A)+(K_B/H)} \tag{4.5}$$

$$y = \frac{C}{1+(H/K_A)} \qquad (4.6)$$

Y represents the value of *V*, *V/K*, 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.

Denaturation by Guanidine-HCl

Mutase-dehydrogenase was dialyzed for 12 h in 50 mM phosphate buffer (2 X 2 L changes). A stock of 8 M Gdn-HCl solution was prepared in 50 mM phosphate buffer and the pH adjusted to 7.4 with NaOH. This stock solution was diluted with 50 mM phosphate buffer, pH 7.4, to prepare samples of different concentrations of Gdn-HCl containing 0.1 mg/ml (2.2 μM) mutase-dehydrogenase. The protein samples were then incubated at room temperature for 24 h. Denaturation of mutase-dehydrogenase was monitored by circular dichroism using a Jasco J-710 spectropolarimeter. Spectra were recorded from 190 to 300 nm in a 0.1 cm quartz cuvette at ambient temperature following the change in the α-helical signal of the protein at 222 nm as a function of denaturant concentration. Measurements were corrected for background signal from the buffer. To verify that the denaturation was a reversible process, mutase-dehydrogenase (2.2 μM final concentration) was incubated in 6 M Gdn-HCl for 24 h at room temperature. Following incubation, the sample was dialyzed against 0 M, 1 M, 2 M, 3 M, 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, then renaturation was followed under conditions as described above for the unfolding. The unfolding of mutase-dehydrogenase monitored by circular dichroism appears to follow a two-state transition from native (N) to unfolded protein (U) shown in equation 4.7 and hence was analyzed according to the method of Pace *et al.*, (35).

$$N_2 \rightleftharpoons 2U \tag{4.7}$$

The equilibrium constant for the unfolding process is given by equation 4.8,

$$Keq = \frac{[U]^2}{[N_2]} = 2P_t\left[\frac{F_U{}^2}{1-F_U}\right] \tag{4.8}$$

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. F_u was calculated at each denaturant concentration from the observed ellipticity using equation 4.9,

$$F_U = \frac{[\theta]_N - [\theta]_{obs}}{[\theta]_N - [\theta]_U} \tag{4.9}$$

K_{eq} is related to the Gibbs free energy of denaturation (ΔG_d) by equation 4.10,

$$\Delta G_d = -RT \ln K_{eq} \tag{4.10}$$

where R is the universal gas constant and T is the ambient temperature in Kelvin. The denaturation of mutase-dehydrogenase was fitted to a two-state transition model (equation 4.10), using a nonlinear regression program with Sigma Plot (36). ΔG_d and K_{eq} depend on the denaturation concentration of denaturant according to equation 4.11,

$$\Delta G_d = \Delta G_{d,aq} - m[denaturant] \qquad (4.11)$$

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 with denaturant concentration.

RESULTS

Kinetic Parameters of Wild-Type and Mutant Mutase-Dehydrogenase

On the basis of the amino acid sequence alignments from nine bacterial prephenate dehydrogenases (Figure 4.1), we targeted the conserved cationic residues Lys178, Arg286 and Arg294 for site-directed mutagenesis to determine if they played a role in prephenate binding. The mutant proteins K178R and R294K/Q were purified and fully characterized while R286K/Q were only preliminarily characterized.

The K178R mutant was poorly expressed as judged by SDS-PAGE (data not shown) and reasonably inefficient at catalyzing both mutase and dehydrogenase reactions. Values of k_{cat}/K_m obtained with either chorismate, prephenate or NAD^+ as the variable substrate were at least two orders of magnitude less than the wild-type protein (Table 4.1). 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. Surprisingly, k_{cat} for the mutase reaction was reduced by a factor of 30, while maximum dehydrogenase activity was decreased by a factor of only 6.

The R294K mutant protein was also poorly expressed and was inactive. In contrast, R294Q protein was obtained in high yield and exhibited kinetic parameters for the mutase reaction similar to wild-type enzyme (Table 4.1). The dehydrogenase was also active, but only in the presence of high concentrations of prephenate. Analysis of the

Table 4.1 Summary of kinetic data for wild-type and mutant mutase-dehydrogenase.

Protein[a]	Mutase Activity			Dehydrogenase Activity					
	Chorismate			Prephenate			NAD^+		
	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 ($M^{-1}s^{-1}$)
Wild-type	45 ± 7	27 ± 1	6×10^5	43.6 ± 8.5	27 ± 1	6.2×10^5	103 ± 11	27 ± 1	2.6×10^5
K178R	366 ± 80	0.93 ± 0.09	2.5×10^3	1660 ± 467	4.8 ± 0.44	2.8×10^3	1630 ± 230	5.3 ± 0.3	3.3×10^3
R294Q	68 ± 7	26 ± 1	3.8×10^5	5700 ± 550[b]	25 ± 1[b]	4.3×10^3	380 ± 50[b]	25 ± 1[b]	6.7×10^4

[a] data shown only for those mutants that were significantly expressed [b] linear initial velocity data were obtained by varying prephenate from 0.4 to 16 mM and NAD^+ from 0.05 to 1.0 mM and the data were fitted to the equation $v = VAB/(K_{ia}K_b + K_aB + K_bA + AB)$.

data obtained by varying both prephenate and NAD^+ yielded the values shown in Table 4.2. Compared to the wild-type dehydrogenase, the Michaelis constant for prephenate for R294Q was increased about 150-fold, while that for NAD^+ was essentially unaffected.

Mutants at position 286, R286K and/or Q, were identified preliminarily through screening colonies by restriction analysis (29). *In vitro* mutase and dehydrogenase assays conducted on crude protein extract derived from these colonies indicated that the mutant proteins were inactive. These results implied that either the lysine and/or glutamine replacements generated proteins that were unstable. No further work was conducted on these mutants at this time.

pH Dependence of the Dehydrogenase Reaction

The pH dependency of the values of V and $(V/K)_{prephenate}$ for wild-type dehydrogenase was studied from pH 5.7 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 profile was a half-bell with slopes of +1 and zero (Figure 4.3) indicating that a group must be unprotonated to achieve maximum activity. Fitting the data to equation 4.6 yielded a pK of 6.63 ± 0.05 for this group (Table 4.2). In contrast, the $(V/K)_{prephenate}$ profile was bell shaped showing the ionization of two groups associated with prephenate and/or the enzyme-NAD^+ complex (Figure 4.3A). Fitting the data to equation 4.5 yielded pK values of 6.31 ± 0.06 and 8.72 ± 0.18 on the acidic and basic limbs, respectively (Table 4.2). As the ionization of the group of pK 8.8 is not seen in the V profile, this

FIGURE 4.3. pH dependency of the dehydrogenase reaction.

Variation with pH of (A) log V (•) and log $(V/K)_{prephenate}$ (◆) for the reaction catalyzed by wild-type prephenate dehydrogenase and of log $(V/K)_{prephenate}$ (▲) for the reaction catalyzed by R294Q prephenate dehydrogenase at 30°C and pH 7.2. Variation with pH of (B) pK_i (O) for the reaction catalyzed by wild-type prephenate dehydrogenase in the presence of hydroxyphenyllactic acid. The units for V, V/K and K_i are s^{-1}, $M^{-1}s^{-1}$ and M^{-1} respectively. The curves for V/K and V in panel A represent best fits of the data to equation 4.4 and 4.5 respectively, while the curve for panel B represent best fit of the data to equation 4.4. The values of the parameters used to draw the curves are given in Table 4.2.

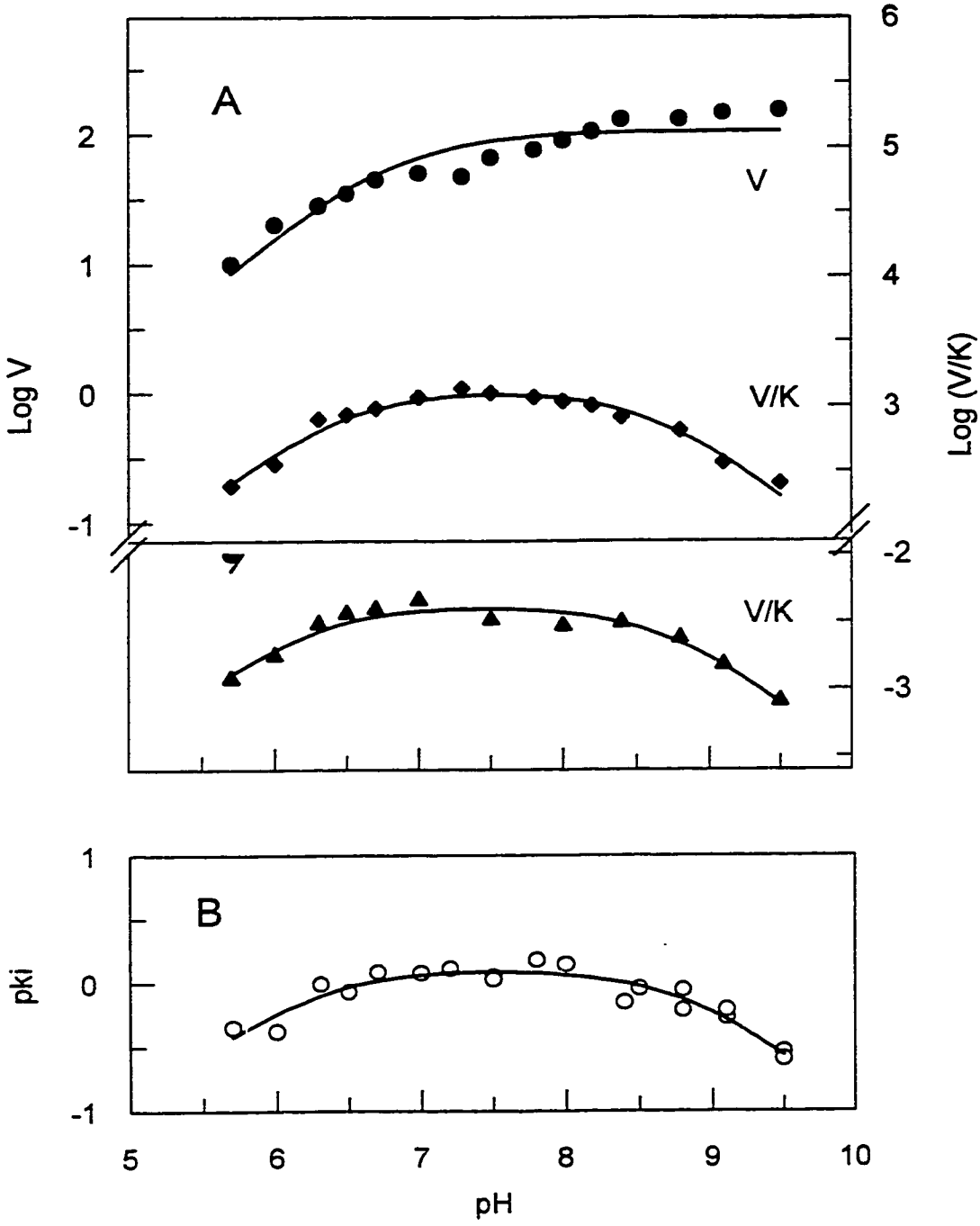

A
V
V/K
V/K
Log V
Log (V/K)
2
1
0
-1
6
5
4
3
-2
-3
B
pki
1
0
-1
5
6
7
8
9
10
pH

Table 4.2 Values for p*K*s and pH-Independent Kinetic Parameters for the Reaction Catalyzed by Wild-Type and R294Q Prephenate Dehydrogenase

Protein	Conditions	Parameters determined	pH-independent value of parameter	pK_A	pK_B
WT	Prephenate Varied	$V/K_{prephenate}$ (μM^{-1} s^{-1})	1.4 ± 0.1	6.31 ± 0.06	8.72 ± 0.18
		V (s^{-1})	105 ± 5	6.63 ± 0.05	-
WT	Hplactate Varied	K_I (mM)	0.75 ± 0.05	6.11 ± 0.08	8.86 ± 0.06
R294Q	Prephenate Varied	$V/K_{prephenate}$ (μM^{-1} s^{-1})	4.4×10^{-3} ± 3.5×10^{-4}	6.14 ± 0.06	8.82 ± 0.07

protonated residue must be involved in binding prephenate to the enzyme-NAD^+ complex. The results are in agreement with those previously reported (10, 12).

To determine if the pK values observed in the V/K profile are true or apparent values and if the ionizable groups play a role in substrate binding, we determined the effect of pH on the binding of HPlactate to the enzyme. HPlactate structurally resembles the product of the dehydrogenase reaction HPpyruvate, 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 4.3B). Fitting the data to equation 4.5 yielded pK values of 6.11 ± 0.06 on the acidic limb and 8.86 ± 0.07 on the basic limb (Table 4.2). These values were comparable to those determined from the (V/K) profile.

The pH dependency of the $(V/K)_{prephenate}$ for the dehydrogenase reaction catalyzed by R294Q was studied. The pH dependence of V was not examined since high concentrations of prephenate were required to monitor saturation kinetics with this mutant protein. Initial velocity data were obtained by varying prephenate at three concentrations one-fifth its Michaelis constant and lower, while keeping NAD^+ saturating. The V/K value at each pH was derived from the slope of the linear fits. The mutant protein also gave rise to a bell-shaped (V/K) profile (Figure 4.3A) with pK values similar to those obtained for the wild-type enzyme (Table 4.2).

Steady State Velocity Patterns in the Presence of Substrate Analogues

Investigations were undertaken on the inhibition of the activity associated with wild-type and R294Q prephenate dehydrogenase by analogues of prephenate. This provided information concerning the importance of the ring carboxylate and the side chain carbonyl group of prephenate in substrate binding. All substrate analogues possess an aromatic ring instead of the 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 lacks the carbonyl group on the side chain of prephenate, while the carbonyl group is replaced with an alcohol in HPlactate (Figure 4.2). For the range of concentration over which prephenate was used, these inhibitors gave rise to linear competitive inhibition with respect to prephenate (data not shown). Analysis of the data yielded the true values of the dissociation constants for the interaction of the enzyme-NAD+ complex with inhibitor shown in Table 4.3.

Denaturation of Wild-Type and R294Q Mutase-Dehydrogenase

The stability of wild-type and R294Q mutase-dehydrogenase was assessed using circular dichroism at 222 nm by determining the pattern of unfolding of each enzyme in Gdn-HCl (Figure 4.4) and comparing the unfolding transition midpoints (Table 4.4). The pattern of unfolding of wild-type enzyme and R294Q were superimposable in contrast to the more shallow pattern obtained for K178R. The arginine mutation destabilized the protein as indicated by the decrease of the unfolding transition midpoint from 2.70 to 2.45 M Gdn-HCl. Proteins refolded by dialyzing with Gdn-HCl solutions of 0 M, 1 M, 2 M, 3 M, and

Table 4.3 Kinetic Constants for the Interaction of Substrate Analogues with Wild-Type and R294Q Prephenate Dehydrogenase[a]

Protein	Inhibitor	K_I (E-NAD^+ + I) (mM)	K_M Prephenate (mM)
Wild-type	HPpyruvate	0.18 ± 0.03	0.053 ± 0.006
	HPpropionate	1.8 ± 0.2	0.070 ± 0.006
	HPlactate[b]	0.17 ± 0.03	0.067 ± 0.008
R294Q	HPpyruvate	0.23 ± 0.08	6.6 ± 0.5
	HPpropionate	1.72 ± 0.24	5.5 ± 0.6
	HPlactate[b]	0.33 ± 0.04	5.3 ± 0.5

[a] data were obtained with six different substrate concentrations and six different inhibitor concentrations. NAD^+ concentration was held constant at 3 mM with prephenate as the varied substrate. The constants were determined by fitting the data to equation 4.2. [b] HPlactate is prepared as a racemic mixture, therefore, the dissociation constants were multiplied by one-half to reflect the binding of the active conformer.

Table 4.4 Thermodynamic Parameters for the Unfolding of Wild-Type and Mutant Mutase-Dehydrogenase

Protein	$\Delta G_{d,aq}$[a] (Kcal mol^{-1})	m[b] (Kcal mol^{-1} M^{-1})	$[D]_{1/2}$[c] (M)
Wild-type	12.36 ± 0.55	1.72 ± 0.19	2.70
K178R	10.49 ± 0.31	1.16 ± 0.09	2.45
R294Q	12.06 ± 0.22	1.63 ± 0.07	2.74

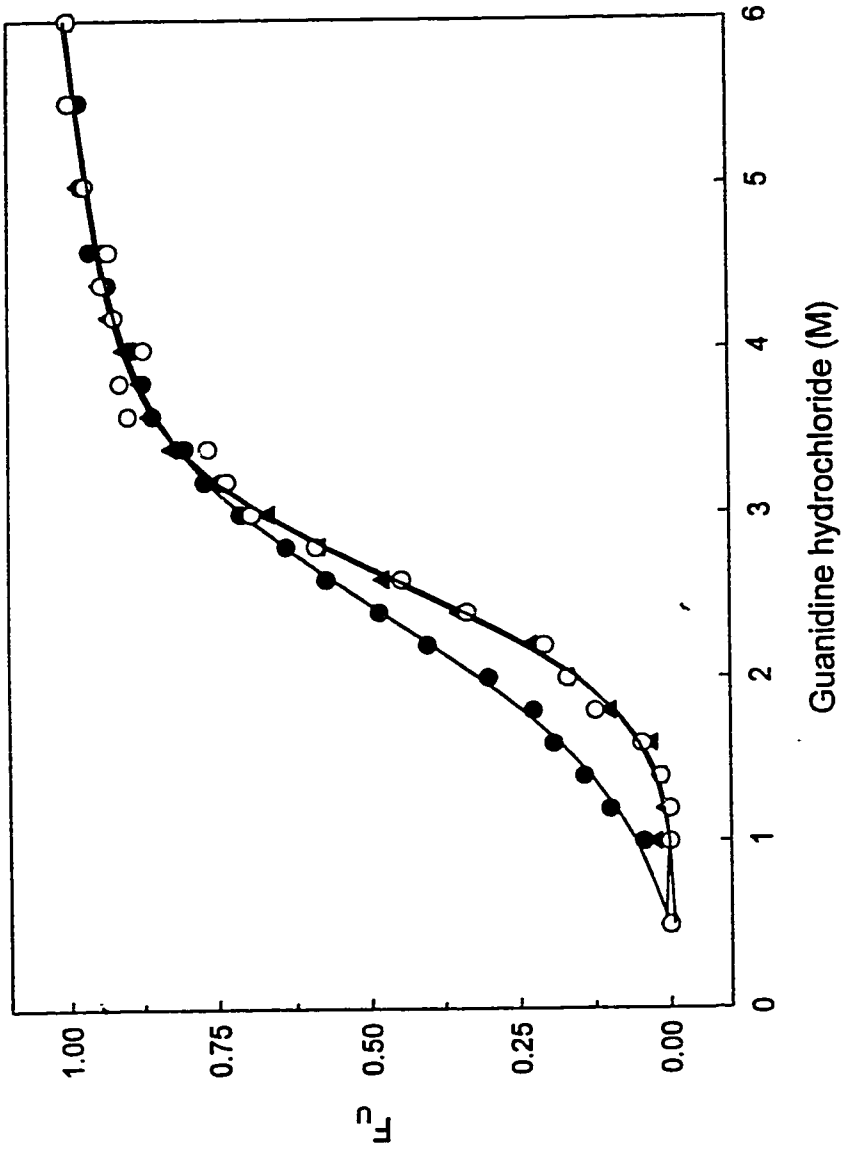
[a] $\Delta G_{d,aq}$ represents the free energy in 0 M denaturant.

[b] m represents the linear dependence of $\Delta G_{d,aq}$ on denaturant concentration.

[c] Concentration of denaturant at half maximum ellipticity.

FIGURE 4.4. Gdn-HCl 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 by nonlinear least-square fitting of the data by method of Hughson and Baldwin (36).


1.00
0.75
0.50
0.25
0.00
F_D
0
1
2
3
4
5
6
Guanidine hydrochloride (M)

4 M yielded similar signals to those obtained at the same concentration during the unfolding process (data not shown). This indicated that denaturation was a reversible process. This being the case, data from the unfolding curves could be analyzed to yield two other parameters: the value of *m*, which reflects the slope of the unfolding curve in the transition region; and $\Delta G_{d,aq}$, which represents the difference in free energy between the native and the denatured state of the protein extrapolated to 0 M Gdn-HCl in aqueous solution. The thermodynamic parameters listed in Table 4.4, indicated that K178R is conformationally less stable than wild-type or R294Q.

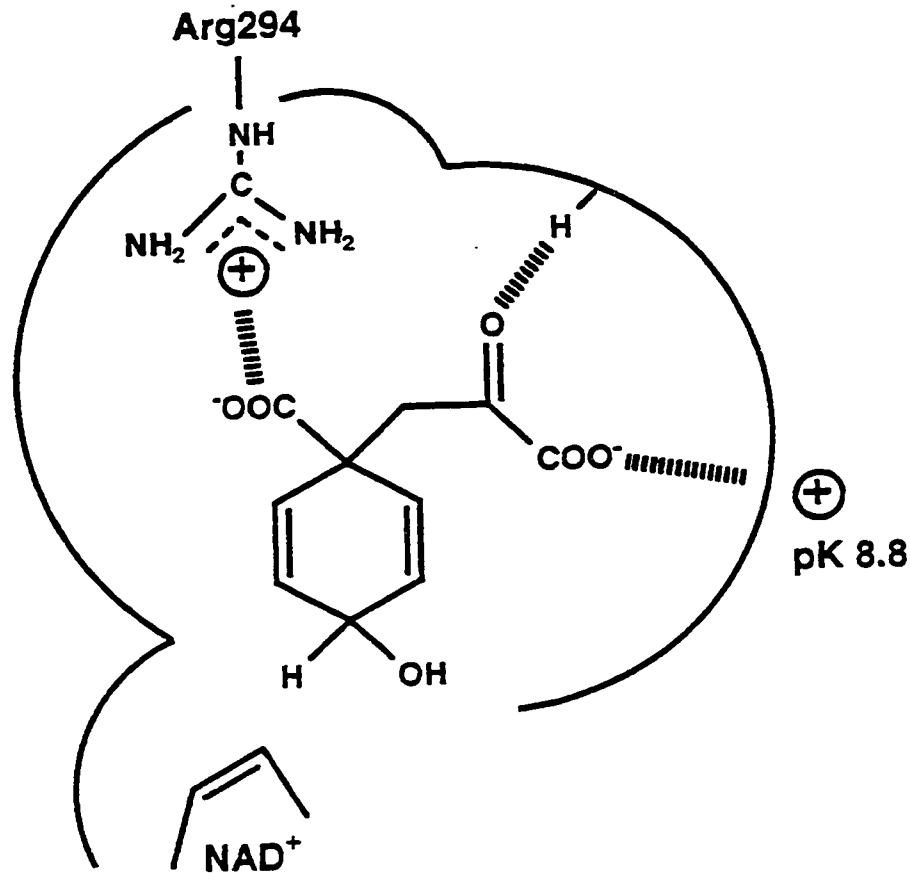
DISCUSSION

The purpose of this investigation was to identify groups associated with *E.coli* prephenate dehydrogenase and with the substrate prephenate that are essential for binding prephenate in the enzyme active site. Towards this goal, we conducted site-directed mutagenesis on three cationic residues, Lys178, Arg286, and Arg294, that are absolutely conserved amongst nine mono and bifunctional prephenate dehydrogenases (Figure 4.1) and 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 findings are summarized in Figure 4.5. Arg294 interacts electrostatically with the ring carboxylate of prephenate. A protonated residue in the dehydrogenase with a p*K* value of about 8.8 interacts with the carboxyl group on the pyruvyl side chain of prephenate. This residue is not Arg294 but the other conserved residues have not been ruled out. Moreover, the carbonyl group on the pyruvyl side chain is important but not critical for prephenate binding and likely hydrogen bonds to an enzymic group. We also show that an arginine substitution at position 178 affects the kinetic parameters of both the mutase and the dehydrogenase reactions as well as the stability of the enzyme.

The Destabilization of Mutase-Dehydrogenase by K178R.

Our kinetic studies on K178R indicated that this mutation reduced the binding of all three substrates-- prephenate and NAD^+ in the dehydrogenase reaction and chorismate in the mutase reaction. However, the interaction of prephenate with the dehydrogenase was the

Figure 4.5. Possible interactions of prephenate with prephenate dehydrogenase.



most affected, reduced to one-fortieth that of the wild-type enzyme. The k_{cat} for the mutase reaction was more affected by this mutation; only 3% mutase activity was retained compared to 20% dehydrogenase activity. 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 (36). Subtle structural changes may likely displace these strategically placed residues, thereby reducing the catalytic efficiency of the enzyme. This hypothesis was confirmed by circular dichroism spectroscopy. The far-UV CD spectra of K178R and wild-type enzyme were shown to be very similar but not overlapping (data not shown) indicating that this mutation caused small global secondary structural changes in the protein. These destabilizing effects were reflected in the thermodynamic parameters of unfolding by Gdn-HCl. K178R unfolds at a Gdn-HCl concentration 11% lower than the wild-type enzyme. Moreover, the values for $\Delta G_{d,aq}$ suggest that the arginine mutation destabilizes the native protein by about 1.3 kcal/mol. Finally, a decrease in the slope of the transition curves from folded to unfolded, as reflected in the value of *m*, indicates that the overall fold of the native enzyme is looser and therefore tends to unfold over a wider range of denaturant concentrations compared to wild-type enzyme (37). The finding that K178R was poorly expressed is also consistent with these results. Regrettably, we cannot draw any definite conclusions as to the importance of Lys178 in prephenate binding until other mutant proteins carrying amino acid substitutions other than an arginine at this position are characterized.

The Role of Arg294 in Prephenate Binding

In order to address the importance of R294 in prephenate binding, two mutant proteins R294K and R294Q were isolated and characterized. The R294K mutant was inactive and poorly expressed. This result was not too surprising given our findings that K178R was also poorly expressed and results from previous studies where we show that *E.coli* mutase-dehydrogenase can be destabilized by "conservative" point mutations (12). 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 the interaction of R294Q with NAD^+ in the dehydrogenase reaction and chorismate in the mutase reaction remained essentially unchanged. This implies that Arg294 plays an important role in prephenate binding. In order 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 was conducted for K178R. As expected, the pattern of unfolding (Figure 4.4) and the thermodynamic parameters of unfolding for R294Q (Table 4.4) were very similar 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 substrate analogues HPpyruvate, HPlactate, and HPpropionate for both wild-type enzyme and R294Q mutant. The results in Table 4.3 show that the Michaelis constant of prephenate for R294Q is markedly reduced while this mutant's interactions with 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 ionic bond between enzyme and substrate (38).

It may also be that by removing this positively charged residue, an environment is created that does not favorably accommodate this negatively charged carboxylate group. Hermes *et al.*, (17) have shown that the oxidative decarboxylation of prephenate to HPpyruvate proceeds with hydride transfer and decarboxylation occurring in the same transition state. They hypothesized that these steps are concerted because the ring carboxyl group lies in a hydrophobic pocket that promotes decarboxylation. We speculate that Arg294 with its long non-polar side chain 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 4.5). Indeed, eight residues in the primary sequence surrounding Arg294 are hydrophobic (LAMVGRLFA). We were unable to isolate an active mutant by replacing Arg294 with a lysine residue by site-directed mutagenesis. 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. These properties may somehow cause lysine to interact unfavorably with its environment. In contrast, the glutamine residue did not affect enzyme stability; as an uncharged residue, it might interact more favorably with the other residues in a hydrophobic region. Moreover, in contrast to the case for wild-type enzyme, prephenate analogues lacking the ring carboxyl

group bind significantly better than does the substrate to R294Q since neither electrostatic partner (Arg294 or the carboxyl group on the inhibitor) are present.

The pH Dependency of the Dehydrogenase Reaction

The pH dependence of the kinetic parameters V and V/K for the dehydrogenase reaction as performed in this study and others (10, 12, 17) indicate that a protonated group with a pK 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 indicate that this protonated group is not Arg294. The $(V/K)_{prephenate}$ profile for wild-type enzyme and R294Q are identical (Figure 4.3), indicating that the residue titrating with a pK of 8.8 is still present when this arginine is missing. Thus, the pK value of Arg294 is likely greater than 9.5 and outside the experimentally accessible pH range of the dehydrogenase reaction. We await further characterization of mutants at position 178 and 286 to determine if either of these residues is this group.

The pH dependence of the K_i for HPlactate, an inhibitory substrate analogue of prephenate, is identical to the V/K profile for the dehydrogenase reaction catalyzed by wild-type enzyme and R294Q (Figure 4.3). As HPlactate can only be involved in binding, it follows that the group with a pK value of 8.8 observed in the V/K profiles is also a binding group. Moreover, pK values derived from V/K profile can be displaced upwards or downwards from their true values if the binding of the substrate to the

enzyme is not at equilibrium relative to the catalytic step. According to Cleland (40) the pH dependence of pK_i for a competitive inhibitor of the substrate will yield the true pK value of ionizing residues in the V/K profile. The V/K profile for prephenate and the K_i for the binding of HPlactate were identical, yielding a pK value on the basic limb of 8.8 (Table 4.2). Therefore, we can conclude that the pK of this binding residue observed in the V/K profile represents a true value.

Both the V and V/K 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/K) with a pK of about 6.3, and in the ternary complex (V) with a pK of about 6.6 (8, 12, 17). Surprisingly, the K_i profile for HPlactate presented in this study indicates that an unprotonated group with a pK of about 6.1 is also involved in binding the inhibitor, and by inference, prephenate to the enzyme-NAD^+ complex. It may be that the catalytic group is also involved in prephenate binding. However, our previous studies (12) do not support this premise, since removal of the putative catalytic base by site-directed mutagenesis (H197N) does not markedly alter prephenate binding. Our data suggest instead, that there are two ionizable groups titrating on the acidic limb in the V/K profile and possessing similar pK values-- one group involved in binding and the other solely in catalysis. Regrettably, it was not possible to obtain more data points below pH 6.0 in the V/K profile to yield a good fit to a slope of +2 on the acidic limb because the enzyme is unstable below this pH (6)

What group on prephenate interacts with the enzymic residue possessing a pK 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.*, (17) showed however, that an alternate substrate in the dehydrogenase reaction, deoxoprephenate, which lacks the carbonyl on the side chain but retains the ring carboxylate (Figure 4.2), also yields a bell-shaped V/K profile similar to that for prephenate. Together, these findings implicate the pyruvyl carboxylate as an electrostatic partner with the enzymic cationic residue of pK about 8.8. These findings can only be confirmed by additional site-directed mutagenesis studies, the synthesis of other inhibitory prephenate analogues and/or by the elucidation of the crystal structure of *E.coli* mutase-dehydrogenase.

The Binding of Substrate Analogues

We have used the dissociation constants for the interaction of the dehydrogenase with inhibitory substrate analogues shown in Figure 4.2, to formulate 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. This observation is consistent with other studies (15) showing that some short chain dicarboxylic acids bind with low mM 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 is only one-third the Michaelis constant for prephenate, corresponding to a ΔG of about 0.3 kcal/mol. Since HPpyruvate no longer possesses the ring carboxylate which 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 (39). 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 μM concentrations (7, 9, 10) although it remains to be established conclusively if tyrosine binding occurs at the prephenate binding site or 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. This change translates to a ΔG of about 1.5 kcal/mol and 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). The results of Hermes *et al.*, (17) 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 and 12-fold, respectively.

Summary

Our work suggests that Arg294 is important for prephenate binding by providing an electrostatic partner for the ring carboxyl group of prephenate. However, neither the ring carboxyl nor the carbonyl group on the pyruvyl side chain of prephenate is critical for substrate binding. An unidentified cationic group with a pK value of 8.8 most likely interacts with the side chain carboxylate of prephenate.

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CHAPTER 5

Summary

The *E.coli* bifunctional enzyme chorismate mutase-prephenate dehydrogenase is involved in the biosynthesis of L-tyrosine. Chorismate mutase catalyzes the Claisen rearrangement of chorismate to prephenate via a chair-like transition state (1, 2). Crystal structures of monofunctional chorismate mutase from B.subtilis and an engineered "minimutase" from the N-terminal 109 amino acids of E.coli chorismate mutase-prephenate dehydratase complexed with the transition state analogue endo-oxabicyclic diacid have indicated that this enzyme may catalyze the rearrangement of chorismate through electrostatic interactions. We conducted chemical modification studies on mutase-dehydrogenase with diethylpyrocarbonate (DEPC) (Chapter 2), a reagent that reacts reasonably specifically with histidine residues, but also modifies primary amines such as lysines (3). This modification resulted in the rapid loss of both mutase and dehydrogenase activity. However, treatment of the modified enzyme with hydroxylamine, a reagent that rapidly reverses the carbethoxylation of histidine, did not restore chorismate mutase activity. This result was consistent with the idea that the mutase was inactivated by the reaction of DEPC with a lysine, rather than a histidine residue. Further modification of mutase-dehydrogenase with trinitrobenzene sulfonate, a reagent that is highly selective towards lysine resulted in over 95% inactivation of mutase activity. We proposed that trinitrobenzene sulfonate modified a lysine residue resulting in the inactivation of chorismate mutase.

Next, we carried out differential peptide mapping by modifying mutase-dehydrogenase with trinitrobenzene sulfonate in the presence and absence of DEPC and isolated a

peptide containing the reactive lysine residue by high pressure liquid chromatography. Mass spectrometry of the purified peptide resulted in the identification of Lys37 as the essential group in the chorismate mutase catalyzed reaction. Site-directed mutagenesis of Lys37 to glutamine yielded an inactive chorismate mutase and a fully functional prephenate dehydrogenase with kinetic properties similar to the wild-type enzyme (Chapter 3). These findings were consistent with our hypothesis that Lys37 is required for chorismate mutase activity. Our conclusion was confirmed by the emergence of crystal structures for chorismate mutases form *B.subtilis* and *E.coli* "minimutase" complexed with the *endo*-oxabicyclic diacid. These structures revealed that a cationic residue analogous to Lys37 in *E.coli* mutase-dehydrogenase makes important interactions with the ether oxygen of this transition state analogue.

Prephenate dehydrogenase catalyzes the oxidative decarboxylation of prephenate to hydroxyphenylpyruvate in the presence of NAD^+. Studies on the pH dependence of the kinetic parameters V and V/K for prephenate with the enzyme-NAD^+ complex have determined that ionizable groups are involved in binding and catalysis. The variation of V with pH resulted in a half-bell profile and indicated that an unprotonated group is essential for catalysis. The results of temperature and solvent perturbation studies by Hermes *et. al.*, (4) implied that this catalytic group is a cationic acid, possibly a histidine residue. In order to identify the catalytic group required for prephenate dehydrogenase activity, we chemically modified the enzyme with DEPC and observed a time-dependent loss of enzyme activity that was linearly dependent on concentration of the modifying reagent (Chapter 2). Exposure of the carbethoxylated mutase-dehydrogenase to

hydroxylamine, a reagent that rapidly reverses DEPC modification of histidine, resulted in rapid restoration of more than 80% of the prephenate dehydrogenase activity. This indicated that a carbethoxylation of a histidine resulted in the inactivation of prephenate dehydrogenase. Moreover, the reactivity of this essential histidine residue increased with pH and revealed the participation of an ionizable group with pK of 7.0. The pK value of this group is similar to that observed in the V profile for the dehydrogenase. We observed that prephenate protected the dehydrogenase against DEPC-mediated inactivation and the protection followed saturation kinetics. These results supported the idea that the modified histidine was in or close to the active site. Differential peptide mapping of carbethoxylated mutase-dehydrogenase in the presence and absence the protecting ligand prephenate, resulted in the isolation of a peptide containing the reactive histidine. Mass spectrometry of the purified peptide resulted in the identification of histidine at position 131.

Site-directed mutagenesis of His131 to alanine followed by kinetic characterization of the mutant protein showed that H131A retained about 10% mutase and dehydrogenase activity. Hence, His131 was not essential for prephenate dehydrogenase activity (Chapter 3). We conducted additional modification studies with DEPC but this time using H131A, and observed that the 10% residual dehydrogenase activity was rapidly lost upon exposure of the enzyme to the reagent. Moreover, the rate of inactivation of H131A was significantly faster compared to wild-type dehydrogenase. These results implied that a histidine other than His131 was modified and was required for prephenate dehydrogenase activity. We concluded that although His131 was not required for prephenate

dehydrogenase activity, it might be exposed for easy carbethoxylation by DEPC and positioned such that its modification hindered access to the prephenate site.

To identify this other reactive group, we replaced the remaining eight histidines located in the proposed prephenate dehydrogenase portion of mutase-dehydrogenase with alanine or, if the alanine mutant was unstable, with asparagine. The mutagenesis scan resulted in the isolation and characterization of two interesting mutant proteins, H189N and H197N. Both the mutase and dehydrogenase of H189N were inactive which tended to indicate that the enzyme had undergone global conformational changes. The H197N mutant, by contrast, retained only chorismate mutase activity. The dehydrogenase portion of H197N may have been inactivated by either the disruption of NAD^+ binding, prephenate binding, or removal of a catalytic residue from the enzyme. We observed that both wild-type mutase-dehydrogenase and H197N bound to Sepharose-AMP and eluted with similar salt concentrations, indicating that His197 was not involved in NAD^+ binding. Moreover, H197N had a similar Michaelis constant for prephenate to that of the wild-type enzyme. Therefore, the removal of H197 did not affect the binding of prephenate to mutase-dehydrogenase. Comparison of pH profiles for wild-type and H197N prephenate dehydrogenase revealed that the unprotonated group with pK of about 6.8 observed in the V profile for wild-type dehydrogenase was absent in H197N. Hence, His197 likely functions as the key catalytic residue in the dehydrogenase and polarizes the proton on the hydroxyl group of prephenate facilitating hydride transfer to NAD^+ and concomitant decarboxylation.

The initiation of genome sequencing projects resulted in the emergence of nucleic acid and predicted amino acid sequences of prephenate dehydrogenases from many organisms. This allowed us to carry out multiple sequence alignments of prephenate dehydrogenases. These alignments have indicated that three positively charged residues, Lys178, Arg286 and Arg294 in *E.coli* mutase-dehydrogenase are conserved. We hypothesized that these cationic residues may make important electrostatic interactions with the two carboxylate groups of prephenate (Chapter 4).

Site-directed mutagenesis of Lys178 to arginine resulted in decreased mutase and dehydrogenase activities and also a 10-fold reduction in the binding of both chorismate and NAD^+. However, the interaction of prephenate with the dehydrogenase site was reduced by over 40-fold. Biophysical characterization of this mutant by CD spectroscopy revealed that the loss of enzyme activity and the reduced substrate binding could be attributed to conformational changes in the protein.

We attempted to replace Arg286 with glutamine and lysine. However, we were unable to isolate an active mutant through preliminary *in vitro* screening of crude extracts for mutase and dehydrogenase activity. We are currently investigating the role of this conserved arginine residue by random mutagenesis.

Site-directed mutagenesis of Arg294 successfully produced a lysine and a glutamine mutant at this position. R294K was poorly expressed and did not retain any detectable mutase or dehydrogenase activity, whereas R294Q was well expressed. R294Q

dehydrogenase exhibited a turnover number and Michaelis constant for NAD^+ similar to the wild-type enzyme. However, the binding of prephenate was reduced by about 120-fold (ΔG of about 3 kcal/mol) which is within the range expected for the loss of an ion pair between the enzyme and the substrate.

The pH dependence of the kinetic parameters V, $(V/K)_{prephenate}$ and K_i for the binding of a competitive inhibitor, HPlactate, indicated that a protonated group with a pK of about 8.8 was involved in binding prephenate to the enzyme-NAD^+ complex. This same group was also observed in the V/K profile for the Arg294 mutant implying that the binding residue was not Arg294. While Arg294 may be involved in prephenate binding, its pK value is probably > 9.5 and outside the experimentally accessible pH range. The group on prephenate interacting with the binding group (pK 8.8) was identified by examining the pH dependence of the interaction of wild-type mutase-dehydrogenase with prephenate, HPlactate or deoxoprephenate. These compounds differed from each other at C-1 of the ring except for the presence of a side chain carboxylate. As identical pH profiles were noted for the binding of all three compounds it followed that the group on prephenate interacting with the enzyme (pK 8.8) must be the side chain carboxylate of the substrate.

Our studies with the inhibitory substrate analogues indicated that the side chain carbonyl group is also important for substrate binding. Replacing the carbonyl group with an alcohol (HPpyruvate to HPlactate) did not alter the enzyme's affinity for the inhibitor, but replacing the carbonyl group with a methylene group (HPpropionate) reduced the binding

by 10-fold (ΔG about 1.5 kcal/mol). This free energy change is constant with the loss of a hydrogen bond between enzyme and substrate.

FUTURE STUDIES

The work discussed in this thesis contributed a great deal to the understanding of the catalytic mechanism of *E.coli* chorismate mutase-prephenate dehydrogenase. Studies on chorismate mutase revealed that Lys37 is required for proper positioning of the enolpyruvyl side-chain of chorismate in the enzyme-catalyzed reaction. Our studies on the dehydrogenase have implicated His197 in the polarization of the 4-hydroxyl group of prephenate in the hydride transfer to NAD^+. Through site-directed mutagenesis, mutant proteins were generated that selectively lost either mutase or the dehydrogenase activity, thus providing additional information about the genetic separability of both domains. There are no reports of the successful expression and characterization of the mutase and dehydrogenase domains from this bifunctional enzyme. It would be of interest to construct fusion proteins which may help in the proper expression and folding of each domain. These studies have already been initiated in our lab. Although the mutase domain was significantly expressed, it retained very low levels of catalytic activity. Other fusions with the mutase and/or dehydrogenase must be made and tested for activity.

In the elucidation of the catalytic mechanism of prephenate dehydrogenase a mutant H189N was isolated that did not retain either mutase or dehydrogenase activity. Random mutagenesis at this position produced an active mutant, H189Q. It would be of interest to characterize this mutant kinetically and biophysically to determine the role of His189 in the mutase-dehydrogenase.

In chapter 4, pH profiles for the dehydrogenase reaction revealed that a protonated residue (pK of 8.8) was involved in prephenate binding. It is of interest to carry out additional mutagenesis studies to identify this ionizable group in the dehydrogenase domain.

A battery of mutant proteins have been generated ready for further characterization with respect to their ability to bind tyrosine, kinetic mechanism and changes in the rate-limiting step of the reaction. Such studies would help elucidate the mechanism by which the two activities are regulated by the end-product of the pathway and provide clues as to the role of particular residues in the chemistry (i.e. hydride transfer versus decarboxylation) of the dehydrogenase reaction.

There are no crystal structures for any bifunctional mutase-dehydrogenase or for any prephenate dehydrogenases. Attempts to obtain crystals for the *E.coli* bifunctional enzyme have failed. Successful construction of active monofunctional domains from the bifunctional enzyme should yield proteins that produce diffraction quality crystals. Alternatively, purification and crystallization of related monofunctional prephenate dehydrogenases may help in elucidating the mechanism of the *E.coli* bifunctional dehydrogenase.

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