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pH Dependency of the Reactions Catalyzed by Chorismate Mutase–Prephenate Dehydrogenase from *Escherichia coli*†

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ABSTRACT: The variation with pH of the kinetic parameters associated with the mutase and dehydrogenase reactions catalyzed by chorismate mutase–prephenate dehydrogenase has been determined with the aim of elucidating the role that ionizing amino acid residues play in binding and catalysis. The pH dependency of log V for the dehydrogenase reaction shows that the enzyme possesses a single ionizing group with a pK value of 6.5 that must be unprotonated for catalysis. This same group is observed in the $V/K_{prephenate}$, as well as in the V/K_{NAD}, profile. The $V/K_{prephenate}$ profile exhibits a second ionizing residue with a pK value of 8.4 that must be protonated for the binding of prephenate to the enzyme. For the mutase reaction, the $V/K_{chorismate}$ profile indicates the presence of three ionizing residues at the active site. Two of these residues, with similar pK values of about 7, must be protonated, while the third, with a pK value of 6.3, must be unprotonated. It can be concluded that all three groups are concerned with the binding of chorismate to the enzyme since the maximum velocity of the mutase reaction is essentially independent of pH. This conclusion is confirmed by the finding that the K_i profile for the competitive inhibitor, (3-*endo*,8-*exo*)-8-hydroxy-2-oxabicyclo[3.3]non-6-ene-3,5-dicarboxylic acid, shows the same three ionizing groups as observed in the $V/K_{chorismate}$ profile. By contrast, the K_i profile for carboxyethyldihydrobenzoate shows only one residue, with a pK value of 7.3, that must be protonated for binding of the inhibitor. On the basis of the aforementioned data, hypotheses are proposed for the chemical mechanisms of the prephenate dehydrogenase and chorismate mustase reactions. For the mutase reaction, the data suggest that the groups of chorismate required for the binding of substrate at the active site of the enzyme are the 4-hydroxyl, the ring carboxyl, and the oxygen moiety of the enolpyruvyl side chain. The group binding the latter moiety could also be involved with catalysis.

Chorismate mutase–prephenate dehydrogenase from *Escherichia coli* is a bifunctional enzyme that has a molecular weight of 78 000–88 000 and is composed of two identical subunits (SampathKumar & Morrison, 1982a; Hudson et al., 1984; Turnbull et al., 1990). Each subunit catalyzes two sequential reactions along the tyrosine biosynthetic pathway. Chorismate mutase (EC 5.4.99.5) catalyzes the rearrangement of chorismate to prephenate, while the dehydrogenase (EC 1.3.1.12) is responsible, in the presence of NAD, for the oxidative decarboxylation of prephenate to 4-hydroxyphenylpyruvate. Evidence now suggests that the two reactions are catalyzed at separate active sites (Turnbull & Morrison, 1990). It has been established that the kinetic mechanism of the prephenate dehydrogenase reaction conforms to a rapid equilibrium, random mechanism with catalysis as the rate-limiting step (SampathKumar & Morrison, 1982b), while hydride transfer and decarboxylation occur in a concerted manner (Hermes et al., 1984). The isomerization of chorismate to prephenate, as catalyzed by chorismate mutase, occurs through a Claisen rearrangement, and stereochemical studies have shown that this rearrangement proceeds through an isomerization of chair-like geometry (Andrews et al., 1973; Sogo et al., 1984). In addition, the reaction mechanism has been probed further through the use of isotope effects (Addadi et al., 1983; Guilford et al., 1987).

To gain further information about the chemical mechanisms of the mutase and dehydrogenase, studies have been under-

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taken of the variation with pH of the kinetic parameters for substrates and inhibitors of the two reactions. The results draw attention to the differences in the structural charactersitics of the active sites of the mutase and dehydrogenase.

Experimental Procedures

Materials

The substrate analogue (carboxyethyl)-1,4-dihydrobenzoate (CEDB)[1] was a gift from Professor Glenn Berchtold of the Massachusetts Institute of Technology, while (3-*endo*,8-*exo*)-8-hydroxy-2-oxabicyclo[3.3.1]non-6-ene-3,5-dicarboxylic acid, bis(dicyclohexyl)ammonium salt (*endo*-oxabicyclic diacid) was a gift from Professor Paul Bartlett of the University of California at Berkeley. Chorismic acid and sodium prephenate were prepared as previously described (Gibson, 1968; Dudzinski & Morrison, 1976). NAD was obtained in free acid form from Boehringer Mannheim. All other chemicals were of the highest purity available commercially. Chorismate mutase–prephenate dehydrogenase was isolated from *E. coli* JFM30 as described by Turnbull et al. (1990). The specific activities of the mutase and dehydrogenase were 65 and 54, respectively.

Methods

Determination of Enzyme Activity. Mutase and dehydrogenase activities were measured at 30 °C in a three-component buffer of 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES), 0.51 M *N*-ethylmorpholine, and 0.051 M diethanolamine with 1 mM EDTA and 1 mM DTT. Over the pH range of 5.0–9.6, for which the initial velocities were measured, the ionic strength of this buffer mixture remained essentially constant (Ellis & Morrison, 1982). The conversion of chorismate to prephenate was followed at 273 nm, while the oxidative decarboxylation of prephenate in the presence of NAD was followed at 340 nm. Both reactions were monitored by using a Varian 219 spectrophotometer as described by Heyde & Morrison (1978). Maximum velocity (V) is expressed in units of reciprocal seconds, and the apparent second-order rate constant (V/KE_t) in units of M^{-1} s^{-1}.

The pH of the assay mixture was determined at the temperature of the assay before and after the reaction by using a Radiometer PHM 62 meter standardized with Beckman buffers. Protein concentration was estimated by the method of Bradford (1976).

Data Analysis. Initial velocity data obtained at each pH value by varying the concentration of prephenate, chorismate, or NAD (A) were fitted to eq 1 to yield values for the maximum velocity (V), the Michaelis constant (K), and the apparent first-order rate constant for the interaction of enzyme and substrate (V/K). When the concentration of inhibitor (I) was varied at a fixed concentration of substrate, the data at each pH value were fitted to eq 2 to obtain a value for an apparent inhibition constant ($K_{i(app)}$). The true inhibition constant (K_i) was determined by using the relationship given in eq 3, where A represents the fixed substrate concentration and K_a denotes the Michaelis constant for substrate. 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 the appropriate equations

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

$$v = \frac{V}{1 + (A/K_{i(app)})} \quad (2)$$

$$K_i = \frac{K_{i(app)}}{1 + (A/K_a)} \quad (3)$$

$$y = \frac{C}{1 + (H/K_A)} \quad (4)$$

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

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

$$y = \frac{C}{1 + (H/K_A) + (K_0/H^2)} \quad (7)$$

$$y = \frac{C}{[1 + (K_B/H)]^2} \quad (8)$$

$$y = \frac{Y_L + (Y_H K/H)}{1 + (K/H)} \quad (9)$$

In these equations, 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 parameter; K_A, K_B, and K_0 are acid dissociation constants; Y_L and Y_H denote limiting pH-independent values of the velocity at low and high pH, respectively; and H is the hydrogen ion concentration.

Data were fitted to the appropriate equation by using the computer programs of Cleland (1979). The values of V, V/K, and $1/K_i$ were weighted according to the inverse of their variance. Weighted means of values together with their standard errors were calculated by using the relationship given by Morrison and Uhr (1966).

Results

pH Dependency of the Prephenate Dehydrogenase Reaction. The effect of pH on the reaction catalyzed by prephenate dehydrogenase 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 results (Figure 1A) indicate that the $V/K_{prephenate}$ profile differs considerably from the V profile. The V/K profile is bell-shaped 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 eq 6 yielded pK values of 6.23 ± 0.08 and 8.42 ± 0.07 (Table I). By contrast, the variation of log V with pH gives rise to a half-bell profile with slopes of +1 and zero. Fitting of the data to eq 4 yielded a pK value of 6.57 ± 0.03 (Table I). These pK values are in agreement with those previously determined by Hermes et al. (1984).

With prephenate present at a saturating concentration of 40 μM at all pH values, both log V and log $(V/K)_{NAD}$ increase with increasing pH to yield half-bell profiles (Figure 1B). Both sets of data were fitted to eq 4 to give pK values of 6.52 ± 0.05 and 6.73 ± 0.04 from the V and V/K_{NAD} profiles, respectively. As expected, similar pK values were determined from the V profiles, irrespective of whether prephenate or NAD was the variable substrate. However, the maximum velocity of the reaction with prephenate as the variable substrate was 2-fold higher than that obtained with NAD as the variable substrate (Table I).

[1] Abbreviations: CEDB, (carboxyethyl)-1,4-dihydrobenzoate; *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; NAD, oxidized form of nicotinamide adenine dinucleotide.

Table I: Values for pKs and pH-Independent Kinetic Parameters for the Reactions Catalyzed by Chorismate Mutase–Prephenate Dehydrogenase

reaction	conditions	parameter determined	pH-independent value of parameter	pK_A	pK_B	pK_0
dehydrogenase	prephenate varied (NAD saturating)[a]	V (s^{-1})	94 ± 3	6.57 ± 0.03		
		$V/K_{prephenate}$ (μM^{-1} s^{-1})	1.4 ± 0.19	6.23 ± 0.08	8.42 ± 0.07	
	NAD varied (prephenate saturating)	V (s^{-1})	49 ± 3	6.52 ± 0.05		
		V/K_{NAD} (μM^{-1} s^{-1})	0.28 ± 0.01	6.73 ± 0.04		
mutase	chorismate varied[b]	V (s^{-1})	18.0 ± 0.2 (Y_L) 60.0 ± 1.6 (Y_H)	7.08 ± 0.12		
		V/K (chorismate) (μM^{-1} s^{-1})	1.4 ± 0.1	6.26 ± 0.06		14.7 ± 0.1
	endo-oxabicyclic diacid varied	K_i (μM)	0.056 ± 0.025	6.63 ± 0.24		14.1 ± 0.2
	CEDB varied	K_i (μM)	540 ± 50		7.31 ± 0.08	
	prephenate varied	K_i (μM)	18 ± 7	6.02 ± 0.37	(7.44 ± 0.05)[c]	13.9 ± 0.2

[a] Weighted mean of two determinations. [b] Weighted mean of three determinations. [c] Value was determined by fitting data to eq 8.

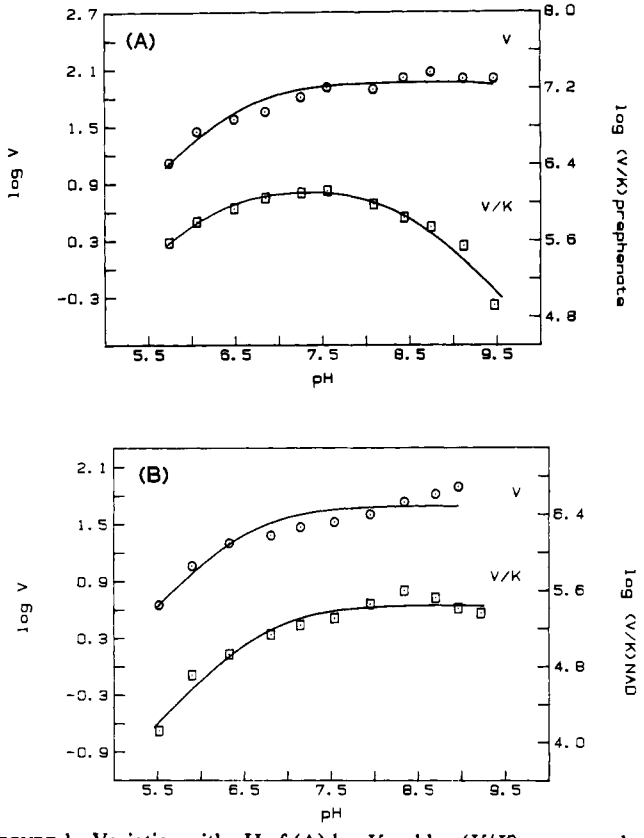

FIGURE 1: Variation with pH of (A) log V and log $(V/K)_{prephenate}$ and (B) log V and log $(V/K)_{NAD}$ for the reaction catalyzed by prephenate dehydrogenase. The units for V and V/K are s^{-1} and M^{-1} s^{-1}, respectively. The curves for V and V/K in panel A represent best fits of the data to eq 4 and 6, respectively, while the curves for V and V/K in panel B represent best fits of the data to eq 4. The values of the the parameters used to draw the curves are given in Table I.

pH Dependency of the Mutase Reaction. The pH dependency of the values of V and $V/K_{chorismate}$ for the mutase reaction could be studied only over a limited range of pH values from 5.0 to 8.1. This was due to inactivation of the enzyme below pH 5.0 and to a dramatic increase in the Michaelis constant for chorismate above pH 7.8. The variation with pH of log $(V/K)_{chorismate}$ yielded a bell-shaped curve with limiting slopes of -2 on the alkaline side and +1 on the acid side (Figure 2). The data fitted well to eq 7 to yield pK values of 6.26 ± 0.06 and 14.7 ± 0.1. The latter represents the sum of the pK values for two ionizing groups with pK values that differ by less than two pH units. The maximum velocity of the mutase reaction did not show a 10-fold change per pH unit on either the acid or the alkaline side of the pH profile. Rather, the V profile is a shallow wave (Figure 2). Analysis

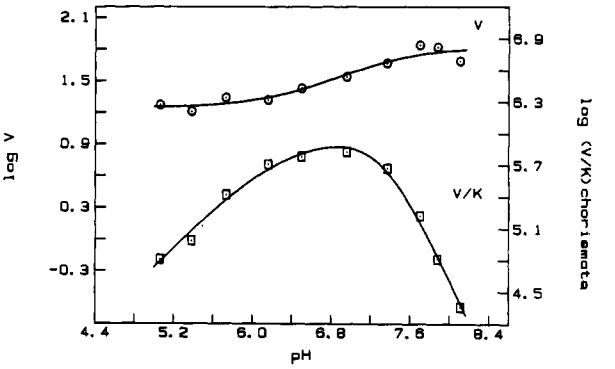

FIGURE 2: Variation with pH of log V and log $(V/K)_{chorismate}$ for the reaction catalyzed by chorismate mutase. The units for V and $(V/K)_{chorismate}$ are s^{-1} and M^{-1} s^{-1}, respectively. The curve for V represents the best fit of the data to eq 9, while the V/K curve illustrates the best fit of the data to eq 7. Values of the parameters used to draw the curves are given in Table I.

of the data by fitting to eq 9 showed that the limiting maximum velocity at alkaline pH values is about 3-fold higher than at acid values. The pK of the single ionizing group was determined to be 7.08 ± 0.12.

pH Dependence of the Binding of Mutase Inhibitors. To determine if the pK values observed in the $V/K_{chorismate}$ profile are true or apparent values, determinations were made of the effects of pH on the binding of *endo*-oxabicyclic diacid to the enzyme. This compound, which functions as a linear competitive inhibitor with respect to chorismate (Bartlett et al., 1988; Turnbull & Morrison, 1990), gave rise to a bell-shaped K_i profile with a limiting slope of +1 on the acid side and −2 on the alkaline side (Figure 3A). Thus the shape of this profile is similar to that for $V/K_{chorismate}$ (Figure 2). Fitting of the data to eq 7 yielded pK values of 6.63 ± 0.24 and 14.1 ± 0.2. It should be noted that these two values are comparable to those determined from the $V/K_{chorismate}$ profile (cf. Table I) and that the second value again represents the sum of the pK values for two ionizing groups with similar pK values. As prephenate, the product of the mutase reaction, also behaves as a linear competitive inhibitor with respect to chorismate (SampathKumar & Morrison, 1982b), the variation of its K_i value as a function of pH was also determined. However, it was not possible to obtain a complete K_i profile as plots of $1/v$ against prephenate, at a fixed concentration of chorismate, were parabolic at pH values below 5.6. Nevertheless, a plot of pK_i against pH showed that there were two ionizing groups on the alkaline side, and possibly one ionizing group on the acid side, of the profile (Figure 3B). This plot is similar to that obtained with the *endo*-oxabicyclic diacid (Figure 3A). Analysis of the data by fitting to eq 7 yielded a poorly determined pK value of 6.02 ± 0.37 for the ionizing group ob-

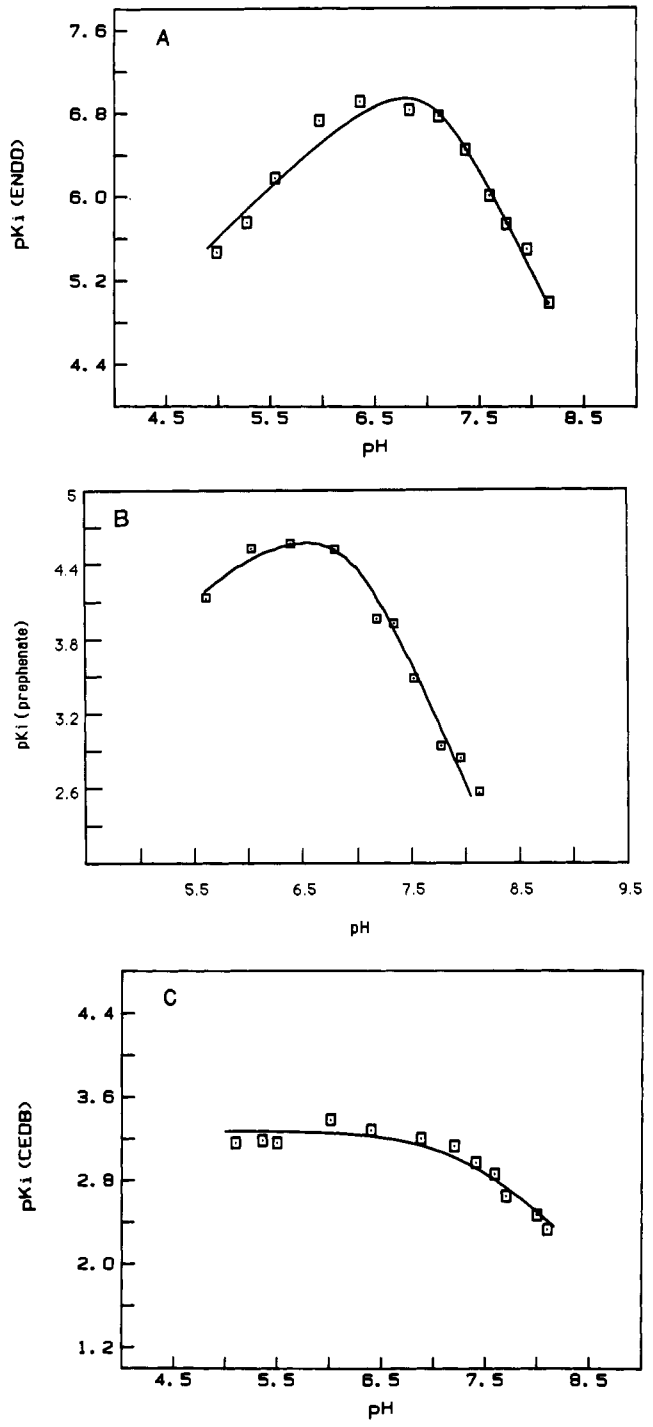

FIGURE 3: Variation with pH of pK_i for the reaction catalyzed by chorismate mutase in the presence of *endo*-oxabicyclic diacid (A), prephenate (B), and carboxyethylidhydrobenzoate (C). The units of K_i are M^{-1}. The curves for A and B represent the best fit of the data to eq 7, while the curve for C represents the best fit of the data to eq 5. Curves were drawn by using the parameter values given in Table I.

served on the acid side, and a well-determined value of 13.9 ± 0.2 for the two ionizing residues on the alkaline side, of the pH profile. The effect of pH on the binding of CEDB was also determined as it behaves as a competitive inhibitor with respect to chorismate (Turnbull & Morrison, 1990). By contrast with the aforementioned results, this competitive inhibitor yielded a simple half-bell profile with slopes of zero and −1 (Figure 3C). A pK_a value of 7.31 ± 0.08 for the single ionizing group was determined by fitting the data of Figure 3C to eq 5.

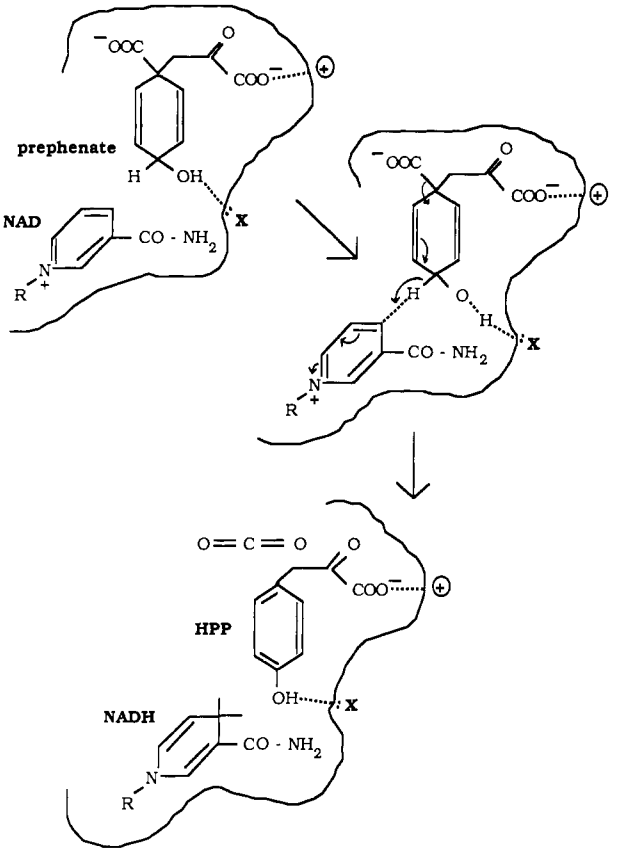

FIGURE 4: Possible mechanism for the prephenate dehydrogenase reaction that involves ionizing amino acid residues at the active site. A base (X) serves to polarize the hydroxyl oxygen of prephenate through hydrogen bonding that facilitates hydride abstraction by NAD and decarboxylation.

Discussion

Investigations have been made of the role played by ionizing amino acid residues in the chemical mechanisms of the reactions catalyzed by the bifunctional enzyme, chorismate mutase–prephenate dehydrogenase. The investigations have involved determination of the variation with pH of the two fundamental kinetic parameters associated with the reactions and of inhibition constants for substrate analogues. The results for the dehydrogenase reaction (Figure 1) indicate that a single ionizing group with a pK value of about 6.5 (Table I) must be unprotonated to achieve maximum velocity. This same ionizing group is observed in the $V/K_{prephenate}$ and V/K_{NAD} profiles, and thus it can be concluded that this group is involved with catalysis. The $V/K_{prephenate}$ profile exhibits a second ionizing residue, with a pK value of 8.42 (Table I), that must be protonated for reaction. As this pK is not observed in the V profiles and as prephenate does not possess a group with a pK in this region, it follows that the group is involved with the binding of prephenate to the enzyme–NAD complex. The identity of the amino acid residue that is of importance for the dehydrogenase reaction has been explored by Hermes et al. (1984) by temperature and solvent perturbation studies with dimethyl sulfoxide. As this organic solvent has no significant effect on the observed pK value that is seen in the $V/K_{prephenate}$ profile, and as the enthalpy of ionization is low, it was suggested that the catalytic group is a cationic acid that may well be the imidazole moiety of a histidine residue. The pK values determined from the $V/K_{prephenate}$ profile by Hermes et al. (1984) are similar to those listed in Table I, but it has not been established that they are intrinsic values.

The identity of the ionizing residue, which has a pK value

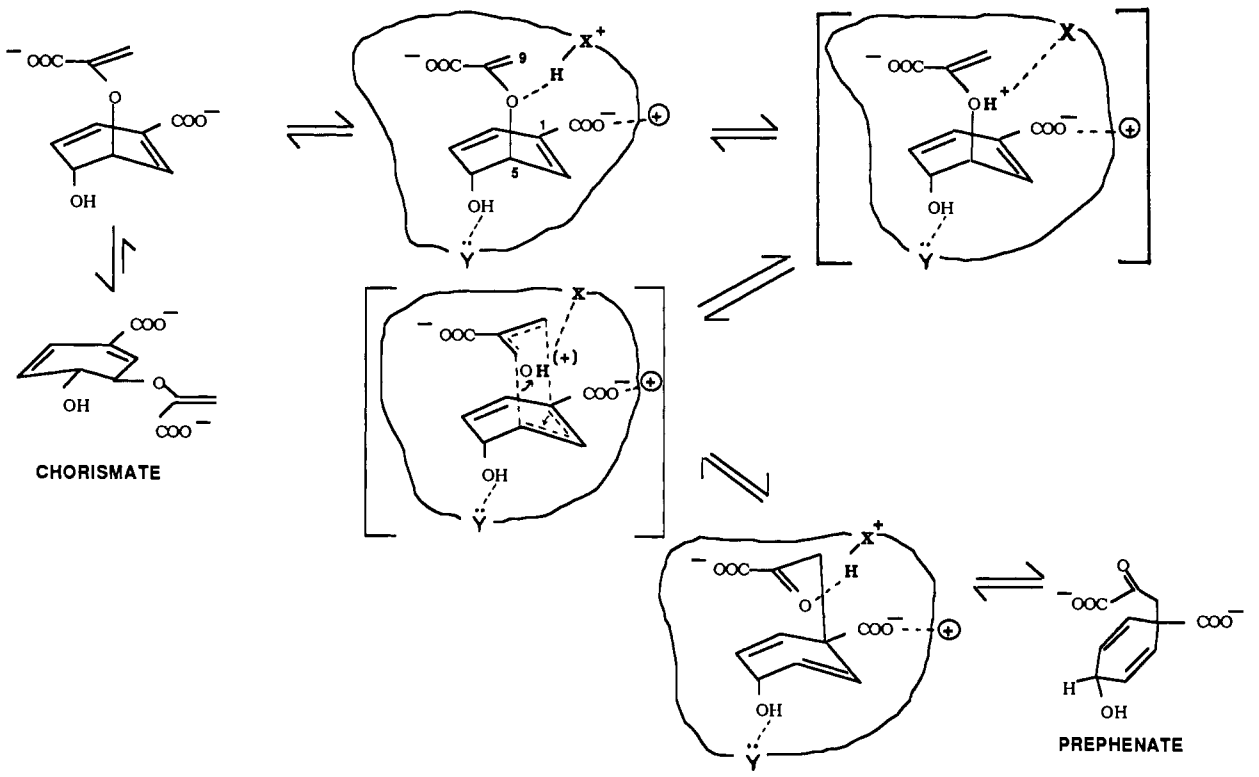

FIGURE 5: Possible mechanism for the chorismate mutase reaction. The enzyme binds the pseudodiaxial conformer of chorismate. Rearrangement to prephenate is facilitated by the protonation of the ether oxygen that causes cleavage of the C_5–O bond and subsequent formation of the C_9–C_1 covalent bond through a transition-state complex.

of 8.42 and which is involved in the binding of prephenate, has yet to be determined. Chemical modification studies with iodoacetate (Hudson et al, 1984; Turnbull & Morrison, 1990) have demonstrated that a single cysteine residue is essential for dehydrogenase activity. However, its role in substrate binding is problematic as cysteine does not form hydrogen bonds. It seems more likely that the group would be a lysine residue. A possible mechanism for the prephenate dehydrogenase reaction can be postulated (Figure 4). It is envisioned that a protonated amino acid residue, such as lysine, is of importance for binding through its interaction with one of the negatively charged carboxyl groups of prephenate. Further, it can be considered that the group with a pK value of 6.23 might well be the imidazole moiety of a histidine residue and that such a group plays an important role in catalysis as the group shown as **X** in Figure 4. The formation of a hydrogen bond between the lone pair of electrons of this residue and the 4-hydroxyl group of prephenate would facilitate hydride transfer to NAD and the concomitant decarboxylation to yield hydroxyphenylpyruvate. It should be noted that the rates of interaction of prephenate with the enzyme–NAD complex and of NAD with the enzyme–prephenate complex are well below the diffusion rate (Table I).

The $V/K_{chorismate}$ profile (Figure 2) indicates that three ionizing groups are of important for the mutase reaction. Two of these groups, which have pK values that differ by less than two pH units, must be protonated, while the third ionizing residue must be unprotonated for reaction. It is apparent that all three groups are concerned with the binding of chorismate as the same three ionizing residues are observed in the K_i profile for the inhibitory analogue of chorismate, *endo*-oxabicyclic diacid (Figure 3). As the same pK values are obtained from analysis of the V/K and the K_i profiles, it follows that the values of Table I represent intrinsic pK values. By contrast with the bell-shaped $V/K_{chorismate}$ profile and the 10- or 100-fold fall off in the value of $V/K_{chorismate}$ for each pH unit, the V profile is wave shaped (Figure 2) with the maximum velocity differing only by a factor of three to four at low and high pH (Table I). Thus the reaction is not absolutely dependent on the group that exhibits a pK value of about 7.1, although that group can influence the chemistry of the reaction and/or the rate of product release.

It is clear that no essential group, which is free to undergo ionization in the presence of substrate, is required for the conversion of chorismate to prephenate (Figure 2). The result might appear to be consistent with the conclusion that the mutase reaction does not involve any chemistry and that the enzyme simply accelerates the rearrangement of chorismate to prephenate by selectively binding the diaxial form of chorismate, which is in equilibrium with the kinetically and thermodynamically more stable pseudo-diequatorial form (Andrews et al., 1973; Andrews & Haddon, 1979). However, the conclusion cannot be correct as the pH-independent V/K value of 1.4 μM^{-1} s^{-1} is significantly less than the diffusion rate for small molecules. Further, it has been shown by Copley and Knowles (1987) that there is a significant amount (10–20%) of the diaxial conformation of chorismate in solution and that the diaxial and diequatorial forms differ only slightly in their values for the energy of activation. It seems more likely that the conversion of chorismate to prephenate, as catalyzed by chorismate mutase, would be limited by the rate of rearrangement or by the rate of prephenate release from the enzyme.

The V profile for the mutase reaction would be pH-independent if chorismate were able to bind only to the correctly protonated form of the enzyme. Under these circumstances, the pK for any amino acid residue involved with catalysis would be seen only in the V/K profile (Plaut & Knowles, 1972; Cook & Cleland, 1981a,b; Kiick et al. 1986). However, the same three ionizing groups are seen in both the $V/K_{chorismate}$ profile

and the K_i profile with *endo*-oxabicyclic diacid. Thus, no ionizing group in the V/K profile can be concerned solely with catalysis. Of course, one or more of these groups, with a required protonation state for binding, could also play a role in catalysis.

The interaction of chorismate and the *endo*-oxabicyclic diacid at the mutase binding site of chorismate mutase–prephenate dehydrogenase requires that two amino acid residues be protonated (Figures 2 and 3) and that a third amino acid residue be ionized. These findings can be interpreted in terms of the scheme illustrated in Figure 5, which allows for groups involved with binding to participate also in catalysis. It is proposed that one of the two groups, which must be protonated for the binding of chorismate, forms an ion pair with the negatively charged ring carboxyl group at position 1, which is essential for mutase activity (Ife et al., 1976; Pawlak et al., 1989), while the other is involved with the formation of a hydrogen bond involving the bridge oxygen at position 6. This residue (X in Figure 5) should also participate in catalysis by promoting the protonation of the bridge oxygen. Such protonation would facilitate the cleavage of the C–O ether bond at position 5 as well as a nucleophilic attack on the C-1 carbon by the electron pair on the methylene group of the endolpyruvyl side chain of chorismate. This hypothesis is supported by the findings of Bartlett et al. (1988), who have shown that the *endo*-oxabicyclic diacid containing the bridging oxygen binds 250-fold more tightly than the corresponding compound in which this atom is replaced by a methylene group. It is also proposed that the group at the active site of chorismate mutase, which must be ionized for binding, is involved with hydrogen bonding of the 4-hydroxyl group of chorismate (Y in Figure 5).

The two ionizing residues that must be protonated for chorismate binding must also be protonated for the binding of prephenate at the active site of the mutase. Thus it seems likely that these groups are concerned with the formation of an ion pair with the ring carboxyl and a hydrogen bond with the carbonyl group of the enolpyruvyl side chain of prephenate. It might be expected that, as with chorismate and the *endo*-oxabicyclic diacid (Figure 5), the 4-hydroxyl group of prephenate would be involved with binding. But definitive evidence on this point was not obtained (Figure 3B). The differences in the pH profiles for the binding of prephenate as a substrate in the dehydrogenase reaction (Figure 1A) and as a product inhibitor in the mutase reaction (Figure 3B) are in accord with the conclusion that the mutase and dehydrogenase reactions occur at distinct sites (Turnbull & Morrison, 1990). Only a single ionizing residue, in the protonated form, is required for the binding of CEDB, which can function as an inhibitory analogue of chorismate (Turnbull & Morrison, 1990). The result is consistent with the facts that the compound possesses neither a 4-hydroxyl group nor a side-chain carbonyl group and with the finding that CEDB exhibits a very higher dissociation constant than does the *endo*-oxabicyclic diacid for reaction at the mutase active site.

An alternative mechanism for the reaction catalyzed by chorismate mutase has been proposed by Guilford et al. (1987) from the results of secondary tritium isotope effects at the C-4 position of chorismate and of solvent deuterium isotope effects. They concluded that the data were consistent with a reaction pathway along which the rate-limiting step was not catalysis but rather the heterolytic cleavage of the chorismate ether bond. Further, it was suggested that the cleavage is assisted by the attack of an enzymatic nucleophile at the C-5 position. It has yet to be determined which of the two models that have been discussed describes more correctly the chemical mechanism for the reaction catalyzed by chorismate mutase.

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