Chorismate Mutase–Prephenate Dehydrogenase from *Escherichia coli*. 2. Evidence for Two Different Active Sites†

Joanne Turnbull‡ and John F. Morrison*

Division of Biochemistry and Molecular Biology, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T. 2601, Australia

Received April 16, 1990; Revised Manuscript Received July 27, 1990

ABSTRACT: The inhibition of the bifunctional enzyme chorismate mutase–prephenate dehydrogenase by substrate analogues, by the end product, tyrosine, and by the protein modifying agent iodoacetate has been investigated. The purpose of the investigations was to determine if the two reactions catalyzed by the enzyme occur at a single active site or at two separate active sites. Evidence in support of the conclusion that the mutase and dehydrogenase reactions are catalyzed at two similar but distinct active sites comes from the following results: (1) A substrate analogue (*endo*-oxabicyclic diacid) that inhibits competitively the mutase reaction has no effect on the dehydrogenase reaction. (2) Malonic acid and several of its derivatives act as inhibitory analogues of chorismate in the mutase reaction and of prephenate in the dehydrogenase reaction. However, different dissociation constants for their interaction with the free enzyme are obtained from studies on the mutase and dehydrogenase reactions. (3) The kinetics of the inhibition by tyrosine of the mutase reaction in the presence of NAD differ from those of the dehydrogenase reaction. The results confirm that carboxymethylation with iodoacetate of one cysteine residue per subunit eliminates both mutase and dehydrogenase activities and show that the inactivation of the enzyme activities is due to iodoacetate functioning as an active site directed inhibitor.

Chorismate mutase–prephenate dehydrogenase (EC 5.4.99.5 and 1.3.1.12; 4-hydroxyphenylpyruvate synthase) 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.[1] The enzyme in *Escherichia coli* is reported to have a molecular weight from 78 000 to 88 000 and is a dimer composed of two identical subunits (SampathKumar & Morrison, 1982; Hudson et al., 1983; Turnbull et al., 1990). Tyrosine represses the synthesis of the enzyme (Mattern & Pittard, 1971; Brown & Somerville, 1971) and has recently been shown to modulate both mutase and dehydrogenase activities (Hudson et al., 1983; Christopherson, 1985), although the reports are conflicting as to the nature of the interaction.

Since the product of the first reaction is a substrate for the second reaction, considerable interest has centered on the spatial relationship between the sites at which the two reactions occur. But to data, the experimental data have not permitted a definitive conclusion to be reached about this relationship. Evidence in favor of a single combining site comes from studies on both the mutase and dehydrogenase activities of the enzymes from *E. coli* and *Aerobacter aerogenes* which can be lost coordinately under a variety of conditions. Thus treatment with heat and urea (Heyde, 1979) or the modification of one sulfhydryl group on each subunit with iodoacetamide has led to the loss of both activities (Heyde, 1979; Hudson et al., 1984). In addition, protection against inactivation of both activities is afforded by prephenate, NAD, and NAD plus tyrosine (Hudson et al., 1984). Kinetic data obtained by Heyde and Morrison (1978) with the enzyme from *A. aerogenes* were consistent with the two reactions occurring at a single site although the possibility of the involvement of two sites with similar kinetic properties could not be eliminated. The report by Heyde (1979) that some of the prephenate formed from chorismate is converted directly to (4-hydroxyphenyl)pyruvate did not permit a distinction between one active site and two catalytic sites in close proximity. Early attempts to obtain a mutant enzyme that possesses only prephenate dehydrogenase activity were unsuccessful, although proteins exhibiting only mutase activity or lacking both activities could be obtained (Rood et al., 1982). The results of investigations on the inhibition of mutase and dehydrogenase activities of the enzyme from *E. coli* by compounds that are clearly analogues of either prephenate or chorismate have also tended to indicate a common binding site (Christopherson et al., 1983). However, inhibition data obtained with some malonic acid derivatives prompted Christopherson et al. (1983) to suggest that the enzyme may contain two overlapping sites.

Earlier investigations on chorismate mutase–prephenate dehydrogenase have now been extened through the application of a wide range of kinetic techniques to studies on the mutase and dehydrogenase activities of the bifunctional enzyme from *E. coli*. The aim was to obtain definitive evidence which allowed determination of whether the two reactions occurred at one site or at two separate, closely related sites. The results indicate clearly that the mutase and dehydrogenase reactions occur at two distinct sites.

Experimental Procedures

Materials. Chorismate and prephenate were prepared as previously described (Gibson, 1968; Dudzinski & Morrison, 1976). NAD was obtained in free acid form from Boehringer Mannheim, while 5,5′-dithiobis(2-nitrobenzoate) (DTNB) was

† J.T. was in receipt of an Australian National University Scholarship during this work.

‡ Present address: Department of Molecular and Cell Biology, Wendell Stanley Hall, University of California, Berkeley, CA 94720.

[1] Abbreviations: CEDB, 1-(2-carboxyethyl)-1,4-dihydrobenzoate; DTNB, 5,5′-dithiobis(2-nitrobenzoate); *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, nicotinamide adenine dinucleotide; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/90/0429-10255$02.50/0

from Sigma. Iodoacetate was obtained from BDH chemicals, recrystallized from petroleum ether (80–100 °C bp), and decolorized with charcoal. Malonic acid was obtained from Calbiochem; ethyl- and diethylmalonic acids were supplied by Aldrich; methyl- and dimethylmalonic acids were from Ega-Chemie. Adamantane 1-phosphonate and 1-(2-carboxyethyl)-1,4-dihydrobenzoate (CEDB) were gifts from Professor Glenn Berchtold of the Massachusetts Institute of Technology; (3-*endo*,8-*exo*)-8-hydroxy-2-oxa[3.3.1]non-6-ene-3,5-dicarboxylic acid bis(dicyclohexylammonium) salt (*endo*-oxabicyclic diacid) was a gift from Professor Paul Bartlett of the University of California, Berkeley. All other chemicals were obtained commercially and were of the highest quality available.

Preparation of Chorismate Mutase–Prephenate Dehydrogenase. The enzyme was obtained from an overproducing strain of *E. coli* (JFM30) that carries a multicopy plasmid (Bhosale et al., 1982). It was purified to homogeneity by a procedure that involves affinity chromatography on Matrex Blue A and Sepharose–AMP (Turnbull et al., 1990). The specific activity of the mutase was 65 units/mg of protein while that of the dehydrogenase was 54 units/mg of protein. Protein concentration was estimated by the method of Bradford (1976).

Chemical Modification of Chorismate Mutase–Prephenate Dehydrogenase. Enzyme was dialyzed overnight against buffer containing 50 mM tris(hydroxymethyl)aminomethane (Tris) (pH 7.5), 1 mM EDTA, and 20 mM DTT. The protein solution was chromatographed on a column (1 × 10 cm) of Sephadex G-25 to remove the thiol reagent and then dialyzed against a second buffer containing 0.05 M *N*-ethylmorpholine, 0.05 M MES (pH 7.5), 1 mM EDTA, and 25% (v/v) glycerol. Enzyme (5 μM subunits) was incubated in the latter buffer with 0–0.3 M iodoacetate at 30 °C, in the absence or presence of protecting ligands, and nitrogen-purged and light-excluded reaction vessels. Aliquots were removed at various time intervals and assayed directly for mutase and dehydrogenase activities. Carboxymethylated derivatives were prepared by reacting the enzyme with 0.3 M iodoacetate at 30 °C. After 80 min, the reaction was terminated and excess iodoacetate removed by passage of the reaction mixture through a Sephadex G-25 column (1 × 10 cm) previously equilibrated with water. Protein factors were pooled and dialyzed for 3 h (Spectra/Por tubing, 12K–14K molecular weight exclusion) against 2 × 1 L of the aforementioned second buffer. The protein was concentrated with an Amicon Diaflow cell fitted with a UM-10 membrane. For modification with DTNB, native or carboxymethylated enzyme (2.5 μM) was reacted with 20 μM DTNB, and the reaction was followed as described by Gething and Davidson (1972).

Measurement of Enzyme Activities. Kinetic investigations on the mutase and dehydrogenase reactions were performed at 30 °C in the presence of a buffer containing 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES), 0.51 M *N*-ethylmorpholine, 0.51 M diethanolamine (pH 7.2), 1 mM EDTA, and 1 mM DTT. The total volume of reaction mixtures was 1.0 mL. Assays of mutase and dehydrogenase activities were performed as described by Heyde and Morrison (1978) on a Varian 219 spectrophotometer. In the presence of NAD and tyrosine, the disappearance of chorismate was followed at 290 nm because of the high absorbance of added ligands at 273 nm. When chorismate disappearance was followed in the presence of NAD, the change of absorbance at 340 nm was also measured so as to allow correction for the increase in absorbance at 290 nm due to (hydroxyphenyl)pyruvate formation (Heyde & Morrison, 1978). Maximum velocity (V) is expressed in units of reciprocal seconds.

Analysis of Kinetic Data. Data were first plotted graphically to check the linearity of double-reciprocal plots and to determine the pattern of the plots. An overall fit of each set of data was then made to the appropriate rate equation by one of the computer programs of Cleland (1979) in conjuction with a VAX 8700 computer. In the presence of an inhibitor (I), data conforming to linear competitive, linear noncompetitive, and hyperbolic noncompetitive inhibition were fitted to eqs 1–3, respectively.

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

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

$$v = \frac{VA\left(1 + \frac{I}{K_{id}}\right)}{K\left(1 + \frac{I}{K_{is}}\right) + A\left(1 + \frac{I}{K_{in}}\right)} \tag{3}$$

Data giving rise to curvilinear double-reciprocal plots of velocity as a function of prephenate concentrations at a fixed concentration of tyrosine were fitted to eq 4, while data obtained in the absence of tyrosine were fitted to eq 5. Steady-state velocity data obtained by varying the concentration of two inhibitors (I and J) were fitted to eq 6. Velocity

$$v = \frac{VA^2}{A^2 + 2bA + C} \tag{4}$$

$$v = \frac{VA}{K + A} \tag{5}$$

$$v = \frac{VA}{K\left(1 + \frac{I}{K_i} + \frac{J}{K_j}\right) + A} \tag{6}$$

data obtained for the inactivation of the mutase and dehydrogenase activities by iodoacetate, in the absence and presence of protecting ligands, were plotted in linear form as log (percent residual activity) against time. Data yielding linear plots were fitted to the equation for a straight line by linear regression analysis (Cleland, 1963). Data for the effect of substrates on the inactivation of enzyme activity by iodoacetate were fitted to eq 7, where K_{ib} represents the dissociation constant for the enzyme–ligand complex, k denotes the rate constant for enzyme inactivation, and E_t represents the total enzyme concentration.

$$k_{obs} = \frac{kE_t}{1 + B/K_{ib}} \tag{7}$$

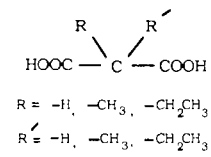
Results

Steady-State Velocity Patterns in the Presence of Malonic Acid and Its Derivatives. Investigations were undertaken on the inhibition of the mutase and dehydrogenase activities of the enzyme at pH 7.2 by malonic acid and the methyl, ethyl, dimethyl, and diethyl derivatives of malonic acid (Figure 1). For the range of concentrations over which the variable substrates were used, these inhibitors gave rise to linear compe-

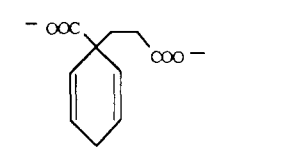
Table I: Kinetic Constants for the Interaction of Malonic Acid and Its Derivatives with Chorismate Mutase–Prephenate Dehydrogenase[a]

compd	mutase[b] K_i(E + I) (mM)	dehydrogenase[c] K_i(E + I) (mM)	dehydrogenase[c] K_i(E-NAD + I) (mM)
malonic acid	240 ± 26	93 ± 15	82 ± 13
methyl	86 ± 8	18 ± 4	14 ± 4
ethyl	38 ± 4	9.2 ● 2.6	7.4 ± 2.2
dimethyl	28 ± 2	12 ± 2	13 ± 3
diethyl	2.6 ± 0.4	3.7 ± 0.6	5.0 ± 0.9

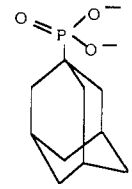
[a] Data were obtained with five different substrate concentrations and five different inhibitor concentrations. True dissociation constants were calculated from the determined apparent values by the procedure outlined by Smith and Morrison (1971). For the dehydrogenase reaction, NAD concentration was held constant at 2 mM with prephenate as the varied substrate while the fixed concentration of prephenate was 80 μM with NAD as the variable sustrate. [b] These constants were determined by fitting data to eq 1. [c] These constants were determined with the apparent values obtained by fitting data to eq 2.



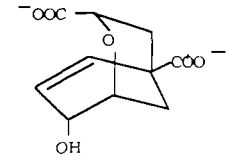
malonic acid derivatives



(carboxyethyl)-1,4-dihydrobenzoate (CEDB)



adamantane-1-phosphonate



endo -oxabicyclic diacid

FIGURE 1: Structure of inhibitory substrate analogues of chorismate mutase–prephenate dehydrogenase.

titive inhibition with respect to both chorismate and prephenate and linear noncompetitive inhibition with respect to NAD (data not shown). Analysis of the data yielded the true values of the dissociation constants for the interaction of free enzyme with inhibitor from studies of both the mutase and dehydrogenase reactions and for the interaction of the enzyme–NAD complex with inhibitor from studies of the dehydrogenase reaction (Table I).

Steady-State Velocity Patterns in the Presence of Substrate Analogues. Inhibition studies on the mutase and dehydrogenase reactions were performed with CEDB, a compound that is clearly an analogue of prephenate, and with an *endo*-oxabicyclic diacid that has been reported to structurally mimic a proposed transition-state intermediate in the mutase reaction (Bartlett & Johnson, 1985). The structures of these compounds are shown in Figure 1. CEDB gave rise to linear competitive inhibition with respect to prephenate and linear noncompetitive inhibition with respect to NAD in the dehydrogenase reaction. CEDB also functions as a linear competitive inhibitor with respect to chorismate in the mutase reaction. Further, analysis of the data, as obtained from studies of each reaction, yielded similar values of the dissociation constants for the interaction of CEDB and free enzyme in both reactions (Table II). The inhibition of the mutase reaction by the *endo*-oxabicyclic diacid was clearly linear competitive with respect to chorismate. The dissociation constant for the interaction of the inhibitory analogue with the free enzyme was 0.11 ± 0.01 μM, which is in agreement with the value obtained by Bartlett et al. (1988) (Table II). When assayed over the pH range from 5.6 to 9.2 in the presence of 40 μM prephenate and 2 mM NAD, the *endo*-oxabicyclic diacid failed to show any inhibition of the dehydrogenase reaction, even up to a concentration of 200 μM.

Steady-State Velocity Patterns in the Presence of Two Inhibitors. To obtain information concerning the geometry of the mutase and dehydrogenase sites, the kinetics of both reactions were examined in the presence of two structurally dissimilar but competitive inhibitors, CEDB and adamantane 1-phosphonate. The latter compound has been shown previously to act as a linear competitive inhibitor with respect to chorismate and prephenate in the mutase and dehydrogenase reactions, respectively, and as a linear noncompetitive inhibitor with respect to NAD in the dehydrogenase reaction (Christopherson et al., 1983). CEDB was varied at different, fixed concentrations of adamantane 1-phosphonate while the chorismate concentration (mutase reaction) or the concentrations of prephenate and NAD (dehydrogenase reaction) were held constant. The resulting patterns of parallel straight lines (Figure 2) suggest that there may be mutually exclusive binding of the two inhibitors at different active sites for the mutase and dehydrogenase reactions. The data were fitted to eq 6 to yield values for the apparent inhibition constants, $K_i(1 + A/K)$ and $K_j(1 + A/K)$. True dissociation constants for the interaction of free enzyme with each inhibitor were calculated from the apparent values together with the concentration of the fixed substrate (A) and its Michaelis constant (K). Values for the true dissociation constants, K_i and K_j, are listed in Table II.

Tyrosine Inhibition of the Mutase and Dehydrogenase Reactions. There was a marked difference in the inhibition of the mutase and dehydrogenase reactions by the end-product, tyrosine (Figure 3). Increasing the concentration of tyrosine up to 0.8 mM resulted in about 90% inhibition of the de-

Table II: Kinetic Constants for the Interaction of Substrate Analogues of Chorismate Mutase–Prephenate Dehydrogenase

compd	kinetic constant	value (mM)	reaction
endo-oxabicyclic diacid[a]	K_i	$1.1 \times 10^{-4} \pm 0.1 \times 10^{-6}$	E + endo (M)
CEDB[a]	K_i	1.4 ± 0.1	E + CEDB (M)
		2.0 ± 0.4	E + CEDB (D)
	K_i	1.3 ± 0.2	E–NAD + CEDB (D)
CEDB (J)[b]/adamantane 1-phosphonate (I)	K_i	0.022 ± 0.002	E + adamantane (M)
	K_j	1.0 ± 0.1	E + CEDB (M)
	K_i	0.096 ± 0.006	E + adamantane (D)
	K_j	1.0 ± 0.1	E + CEDB (D)

[a] Data were fitted to eq 1 to obtain values for the mutase (M) reaction. For the dehydrogenase (D) reaction, the NAD concentration was fixed at 2 mM when prephenate was the variable substrate while prephenate was held constant at 80 μM with NAD as the variable substrate. Data were fitted to eq 2 to obtain values for the apparent constants. These values were used together with the relationship outlined by Smith and Morrison (1971) to determine true dissociation constants. [b] In the mutase reaction, chorismate was held constant at 0.25 mM while the concentration of the two inhibitors was varied. In the dehydrogenase reaction, prephenate and NAD were fixed at 0.04 mM and 0.2 mM, respectively. Apparent dissociation constants were determined by fitting the data to eq 6 and were used to calculate the true dissociation constants with the relationship outlined in the text.

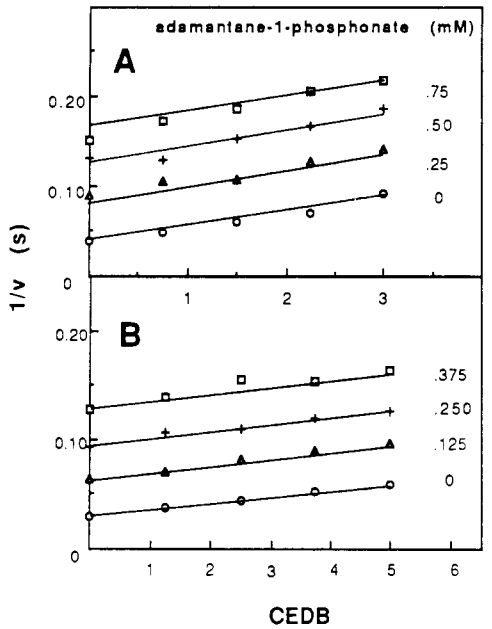


FIGURE 2: Double inhibition of the prephenate dehydrogenase (A) and chorismate mutase (B) reactions by 1-(2-carboxyethyl)dihydrobenzoate (CEDB) and adamantane 1-phosphonate. The concentrations of prephenate and NAD for the dehydrogenase reaction were 0.04 mM and 2.0 mM, respectively, while the concentration of chorismate for the mutase reaction was 0.25 mM. Both sets of data were fitted to eq 6.

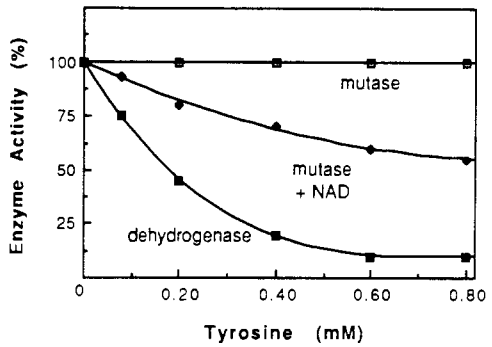


FIGURE 3: Inhibition of chorismate mutase–prephenate dehydrogenase by tyrosine. For the chorismate mutase reaction, chorismate was held constant at 0.25 mM either in the presence or in the absence of 2 mM NAD. For the prephenate dehydrogenase reaction, the concentrations of prephenate and NAD were maintained constant at 0.16 mM and 2 mM, respectively. Loss of enzyme activity is expressed as a percentage of the activity in the absence of tyrosine but in the presence of NAD.

hydrogenase activity, while in the absence of NAD there was no inhibition of the mutase reaction. However, in the presence of 2 mM NAD, tyrosine caused inhibition of the mutase reaction which was clearly partial with a limiting value of about 50% [cf. Hudson et al. (1984)]. Inhibition of the dehydrogenase reaction by tyrosine with prephenate as the variable substrate yielded double-reciprocal plots which were concave upward (Figure 4A) and which are indicative of allosteric inhibition. Similar results have been reported by Hudson et al. (1983) and Christopherson (1985). With NAD as the variable substrate, the inhibition by tyrosine yielded an unusual double-reciprocal plot in relation to the slopes of the curves (Figure 4B). The results contrast with those of Christopherson (1985), who reported that no inhibition by tyrosine is observed with NAD as the variable substrate.

By contrast with its behavior in the dehydrogenase reaction, tyrosine appeared to act as a noncompetitive inhibitor with respect to chorismate in the mutase reaction (Figure 4C). The

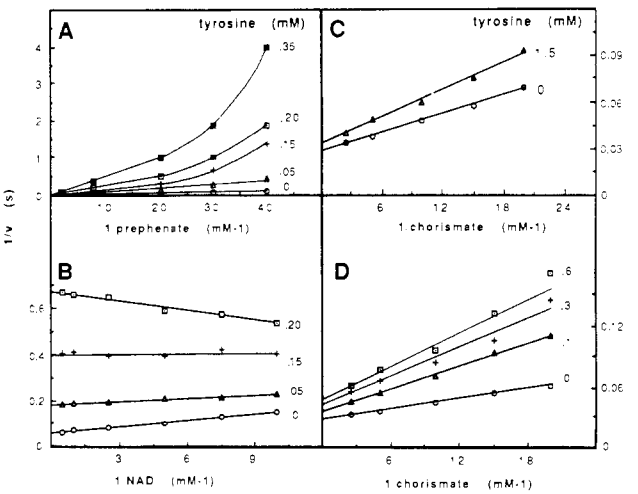


FIGURE 4: Double-reciprocal plots of the inhibition of chorismate mutase–prehenate dehydrogenase by tyrosine. (A) Prephenate varied with the concentration of NAD fixed at 2 mM. Curvilinear data for each of the lines were fitted to eq 4 while the data obtained in the absence of tyrosine were fitted to eq 5. (B) NAD varied at a fixed prephenate concentration of 40 μM. Data for each of the lines were fitted to eq 5. (C) Chorismate varied in the absence of NAD. Data were fitted to eq 2. (D) Chorismate varied in the presence of 1 mM NAD. Data were fitted to eq 3.

Table III: Effect of Tyrosine and NAD on the Activity of Chorismate Mutase

effectors	kinetic constant[a]	value (mM)[b]	reaction
tyrosine (C)	K_{ic}	30 ± 0.6	E + tyrosine
	K_{dc}	11 ± 3	E–chorismate + tyrosine
tyrosine (C)	K_{ibc}	0.046 ± 0.006	E–NAD + tyrosine
+1 mM NAD (B)	K_{dbc}	0.080 ± 0.010	E–NAD–chorismate + tyrosine

[a] Chorismate is denoted as "d". [b] The values for K_{ic} and K_{dc} were obtained by fitting velocity data shown in Figure 4C to eq 2, while the values for K_{ibc} and K_{dbc} were obained by fitting data shown in Figure 4D to eq 3. In eq 3, K_{is} represents the dissociation constant for the interaction of tyrosine with the E–NAD complex, K_{in} denotes the dissociation constant for the interaction of tyrosine with the E–NAD–chorismate complex, and K_{in}/K_{id} represents the ratio of the maximum velocities attained in the presence and absence of a saturating concentration of tyrosine.

data are limited because of the relatively low solubility of tyrosine and its weak binding to both the free enzyme and the enzyme–chorismate complex (cf. Table III). In the presence of NAD, tyrosine causes slope hyperbolic–intercept hyperbolic noncompetitive inhibition (Figure 4D). This result indicates that, when tyrosine is bound to the enzyme–NAD–chorismate complex, it still gives rise to prephenate although the rate of product formation is reduced. The fitting of the data of Figure 4D to eq 3 yielded dissociation constants for the binding of tyrosine to the enzyme–NAD and enzyme–NAD–chorismate complexes (Table III). A comparison of the results obtained for tyrosine inhibition in the absence and presence of NAD shows that NAD has considerable ability to enhance the binding of tyrosine to the enzyme. It should be noted that tyrosine does not introduce curvature into the lines of double-reciprocal plots for the mutase reaction.

Reaction of Enzyme with Thiol Reagents. Reaction of the enzyme with DTNB under native and denaturing (6 M guanidine hydrochloride) conditions indicated that, while the enzyme contains three sulfhydryl groups per subunit, only two of these are modified in the native enzyme. One of the two sulfhydryl groups reacts more rapidly with DTNB than the

Table IV: Kinetic Constants for the Interaction of NAD and Prephenate with Chorismate Mutase–Prephenate Dehydrogenase

substrate	kinetic constant	value (mM) from steady-state velocity data	value (mM) from protection experiments	reaction
prephenate (A)	K_{ia}	0.11 ± 0.01[a]	0.16 ± 0.03	E + A (D)
		0.15 ± 0.02[b]	0.12 ± 0.02	E + A (M)
NAD (B)	K_{ib}	0.20 ± 0.03[a]	0.21 ± 0.03	E + B (D)

[a] For the dehydrogenase (D) reaction, values were determined from fitting linear initial velocity data of varying prephenate from 0.04 to 0.2 mM and NAD from 0.1 to 1.0 mM to the equation $v = VAB/(K_{ia}K_b + K_aB + K_bA + AB)$. [b] For the mutase (M) reaction, value was determined by fitting velocity data of varying chorismate at five concentrations of prephenate to eq 1.

other, and it is only the faster reacting group that is modified by iodoacetate. The thiol group that does not undergo carboxymethylation can still react with DTNB (data not shown). Carboxymethylation of a single sulfhydryl group by iodoacetate causes loss of both mutase and dehydrogenase activities. The loss of each activity occurs as a first-order reaction with the rate increasing as a function of the iodoacetate concentration (data not shown). Secondary plots of the data for the mutase and dehydrogenase reactions were hyperbolic. Thus it may be concluded that iodoacetate behaves as an active site directed inhibitor which reacts rapidly with the enzyme at one or the other of the active sites prior to bringing about the slower irreversible alkylation of a nearby sulfhydryl group.

Substrate Protection against Alkylation by Iodoacetate. Protection against inactivation by iodoacetate of both the mutase and dehydrogenase activities of the enzyme could be achieved by the addition of either prephenate or NAD (data not shown). Data for the protection of the dehydrogenase activity were fitted to eq 7 to obtain values of the dissociation constants for the interaction of prephenate and NAD with the free enzyme. The resulting values (Table IV) are similar to those obtained from the analysis of steady-state kinetic data as expected for a reaction that conforms to a rapid equilibrium, random mechanism (SampathKumar & Morrison, 1982). NAD protects against inactivation of the dehydrogenase by binding at its subsite while prephenate protects against inactivation by binding at either the prephenate subsite of the dehydrogenase or by binding as a product at the active site of the mutase. NAD was also observed to protect against the inactivation of mutase by iodoacetate. Tyrosine did not prevent the inactivation of the enzyme but rather enhanced the protective effect of NAD in both reactions. This was due presumably to its ability to increase the binding of the nucleotide. The results are similar to those of Hudson et al. (1984), who showed that the inactivation of both the mutase and dehydrogenase by DTNB could be reduced by prephenate or by tyrosine plus NAD.

Discussion

The results of the present investigation provide definitive support for the concept that the chorismate mutase and prephenate dehydrogenase reactions of chorismate mutase–prephenate dehydrogenase are catalyzed at two distinct sites. The most conclusive piece of evidence stems from the observation that the *endo*-oxabicyclic diacid is a strong competitive inhibitor of the chorismate mutase activity without having any ability to inhibit the dehydrogenase reaction. This is the first time that kinetic studies have been able to show such a convincing distinction between the chorismate and prephenate binding sites of the enzyme. A possible reason for the selective binding comes from studies on the chemical mechanism of the mutase reaction. The intramolecular Claisen rearrangement of chorismate to prephenate in both the uncatalyzed (Copley & Knowles, 1987) and the enzyme-catalyzed reaction (Andrews et al., 1973, 1977; Sogo et al., 1984; Asano et al., 1985)

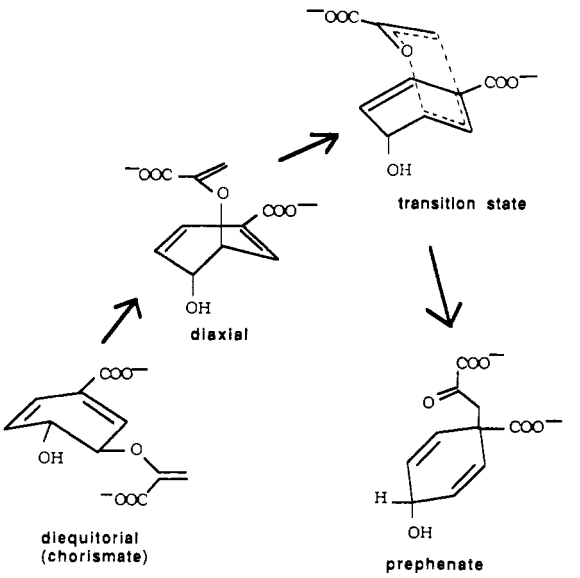


FIGURE 5: Proposed rearrangement of chorismate to prephenate through a transition-state complex.

is believed to proceed through a chair-like transition state as depicted in Figure 5. Thus, the approach to the design of inhibitors of the mutase has been to mimic this bicyclic structure (Andrews et al., 1973, 1977; Chao & Berchtold, 1982; Bartlett & Johnson, 1985; Bartlett et al., 1988). The inhibition constant for the *endo*-oxabicyclic diacid (Figure 1), synthesized as a racemate, is about 300-fold lower than the Michaelis constant for chorismate in the mutase reaction. Thus, if the enzymic rate acceleration is reflected in the enhanced binding of this inhibitor in comparison to that of chorismate, then it is clear that this compound captures at least part of the additional binding affinity expected for a good intermediate-state analogue. The binding affinity of this analogue is greatly reduced by the removal of the ether oxygen and the positioning of the bridged carboxyl away from the unsaturated ring (exo conformation) (Bartlett & Johnson, 1985; Bartlett et al., 1988). This observation highlights the importance that these two groups from the enolpyruvyl side chain of chorismate play in substrate binding.

Additional evidence supporting the concept of two distinct active centers stems from the marked differences in the inhibition constants for malonic acid and several of its derivatives as determined from studies of both reactions (Table I). If a single active site were responsible for the two catalytic activities, the true inhibition constant for a substrate analogue would be independent of which activity was measured. But as malonic acid and its derivatives inhibit both activities, the two active sites must have features in common. It is also apparent that the presence of NAD on the enzyme does not markedly alter the binding of the inhibitors under discussion (Table I). In contrast to the results obtained in the present study at pH 7.2, the inhibition studies performed by Christopherson et al. (1983) at pH 6.0 indicated that the binding

of malonic acid and prephenate was mutually exclusive but that malonic acid and chorismate could be on the enzyme at the same time. Since the pK value of one carboxyl group on malonic acid is about 6.0 (Dawson et al., 1986), the contrast in the inhibition patterns for the two studies may reflect the difference in the ionization states of the inhibitor at the two pH values. The present findings are in accord with the postulate of Christopherson et al. (1983) that the enzyme possesses two active sites but not with the proposal that the sites overlap.

Results of the double inhibition studies obtained with the structurally dissimilar substrate analogues, adamantane 1-phosphonate and CEDB (Figure 2), indicate that the inhibitors are binding to the same region of each active center. This again suggests that there are similar groups on the enzyme involved in the binding of both chorismate and prephenate. In spite of its structural similarity to prephenate, CEDB binds equally well to the mutase and dehydrogenase sites (Table II). However, the interaction of adamantane 1-phosphonate at the chorismate binding site was about 5 times greater than at the binding site for prephenate (Table II). The tighter binding of adamantane 1-phosphonate may reflect, in part, its structural similarities to the proposed transition state of the mutase reaction (Andrews et al., 1973, 1977; Chao & Berchtold, 1982).

Further evidence to support the concept that the mutase and dehydrogenase reactions are catalyzed at two distinct sites comes from the differential inhibition of the two reactions by the end-product inhibitor, tyrosine. Even in the presence of NAD, which enhances the binding of tyrosine (Table III; Heyde & Morrison, 1978; Hudson et al., 1984), the dehydrogenase reaction is more sensitive than the mutase reaction to tyrosine inhibition. In addition, the mechanism of inhibition is different for the two reactions. The concave upward kinetics produced by tyrosine, while prephenate is varied at saturating NAD concentrations, has been interpreted in terms of two models: an interconversion between the dimeric form of the enzyme, which has a lower affinity for tyrosine, and a high-affinity tetrameric form (Hudson et al., 1983); or as a result of positive cooperativity in the binding of tyrosine with interactions between the two subunits, as observed with the binding of prephenate and chorismate (Christopherson, 1985; Turnbull et al., 1990).

The noncompetitive inhibition of the mutase reaction by tyrosine indicates that this compound does not simply bind at the chorismate site. The limited solubility of tyrosine recluded determination of the type of noncompetitive inhibition. But in the presence of NAD, which causes enhancement of tyrosine binding, it was possible to demonstrate that the inhibition was hyperbolic noncompetitive. Such a result indicates that chorismate and tyrosine can reside on the enzyme at the same time and provides support for the idea that the enzyme possesses an allosteric binding site for tyrosine. It is interesting that, in the presence of tyrosine, cooperative effects are observed for the dehydrogenase but not for the mutase reaction.

The kinetics of the inactivation of the enzyme by iodoacetate indicate that this alkylating reagent behaves as an active site directed inhibitor. Thus it forms a reversible enzyme–iodoacetate complex prior to the irreversible carboxymethylation of a single thiol group which results in the loss of both mutase and dehydrogenase activities. The results are, as expected, independent of the reaction that is monitored because the inactivation was allowed to proceed in the absence of substrate. The data do not permit a conclusion to be reached about whether iodoacetate binds at the mutase site or the prephenate site or both. Since acetate gives rise to parabolic competitive inhibition with respect to each of these substrates, it is likely that both sites could be involved.

The conclusion that the mutase and dehydrogenase reactions are catalyzed at two distinct sites is in accord with results of the kinetic and immunological investigations of SampathKumar (1978). This author found that the dissociation constants for the interaction of prephenate and (hydroxyphenyl)pyruvate with the free enzyme differed by 5-fold and 12-fold, respectively, according to the reaction used to study the inhibition. Further, she showed that antiserum prepared against the enzyme caused differential inhibition of the two reactions. Such a result implies that there are present in the antiserum two antibody populations for reaction with the different antigenic determinants of the catalytic sites. Determination of the nucleotide sequences of the TyrA gene, which codes for chorismate mutase–prepehenate dehydrogenase, and the PheA gene, which codes for chorismate mutase–prepehenate dehydratase, has led to the conclusion that one-third of each polypeptide chain at the N-terminal end specifies the active site for the mutase reaction, while the remainder of the amino acid sequence specifies either the dehydrogenase or the dehydratase activity (Hudson et al., 1984). Such a conclusion is in agreement with the finding of Baldwin and Davidson (1981) that each of the two encoded activities of chorismate mutase–prephenate dehydratase can be lost independently by mutations in the *pheA* gene and with the recent demonstration by Maruya et al., (1987) of the independent expression of chorismate mutase and prephenate dehydrogenase activity.

Evidence in support of two catalytic sites on chorismate mutase–prephenate dehydrogenase may indicate that the enzyme resembles the bifunctional enzyme, chorismate mutase–prephenate dehydratase, for which two distinct catalytic sites have been identified (Duggleby et al., 1978). Thus, the mutase and dehydrogenase (dehydratase) enzymes may have evolved by a process of gene fusion.

ACKNOWLEDGMENTS

We are grateful to Professors Paul Bartlett and Glenn Berchtold for the generous gifts of *endo*-oxabicyclic diacid, CEDB, and adamantane 1-phosphonate and to Mrs. Irene Davies for excellent technical assistance in the preparation of chorismate mutase–prephenate dehydrogenase. We thank Drs. W. W. Cleland and P. Bartlett for helpful discussions.

REFERENCES

Andrews, P. R., Smith, G. D., & Young, I. G. (1973) *Biochemistry 12*, 3492–3498.

Andrews, P. R., Cain, E. N., Rizzardo, E., & Smith, G. D. (1977) *Biochemistry 16*, 4848–4852.

Asano, Y., Lee, J. J., Shieh, T. L., Spreafico, F., Kowal, C., & Floss, H. G. (1985) *J. Am. Chem. Soc. 107*, 4314–4320.

Baldwin, G. S., & Davidson, B. E. (1981) *Arch. Biochem. Biophys. 211*, 66–75.

Bartlett, P. A., & Johnson, C. R. (1985) *J. Am. Chem. Soc. 107*, 7792–7793.

Bartlett, P. A., Nakagawa, Y., Johnson, C. R., Reich, S. H., & Luis, A. (1988) *J. Org. Chem. 53*, 3195–3210.

Bhosale, S. B., Rood, J. I., Sneddon, M. K., & Morrison, J. F. (1982) *Biochim. Biophys. Acta 717*, 6–11.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Brown, K. D., & Somerville, R. L. (1971) *J. Bacteriol. 108*, 386–399.

Chao, H. S. I., & Berchtold, G. A. (1982) *Biochemistry 21*, 2778–2781.

Christopherson, R. I. (1985) *Arch. Biochem. Biophys. 240*, 646–654.

Christopherson, R. I., Heyde, E., & Morrison, J. F. (1983) *Biochemistry 22*, 1650–1656.
Cleland, W. W. (1963) *Nature 198*, 463.
Cleland, W. W. (1979) *Methods Enzymol. 63*, 103–138.
Copley, S. D., & Knowles, J. R. (1987) *J. Am. Chem. Soc. 109*, 5008–5013.
Dawson, R. M. C., Elliot, D. C., Elliot, W. H., & Jones, K. M., Eds. (1986) *Data for Biochemical Research*, 3rd ed., p 46, Oxford University Press, London.
Dudzinski, P. K., & Morrison, J. F. (1976) *Prep. Biochem. 6*, 113–121.
Duggleby, R. G., Sneddon, M. K., & Morrison, J. F. (1978) *Biochemistry 17*, 1548–1554.
Gething, M. J. H., & Davidson, B. E. (1972) *Eur. J. Biochem. 30*, 352–353.
Gibson, F. (1968) *Biochem. Prep. 12*, 94–98.
Heyde, E. (1979) *Biochemistry 18*, 2766–2775.
Heyde, E., & Morrison, J. F. (1978) *Biochemistry 17*, 1573–1580.
Hudson, G. S., & Davidson, B. E. (1984) *J. Mol. Biol. 180*, 1023–1051.
Hudson, G. S., Howlett, G. J., & Davidson, B. E. (1983) *J. Biol. Chem. 258*, 3114–3120.
Hudson, G. S., Wong, V., & Davidson, B. E. (1984) *Biochemistry 23*, 6240–6249.
Maruya, A., O'Connor, M. J., & Bachman, K. (1987) *J. Bacteriol. 169*, 4852–4853.
Mattern, I. E., & Pittard, J. (1971) *J. Bacteriol. 107*, 8–15.
Rood, R. I., Perrot, B., Heyde, E., & Morrison, J. F. (1982) *Eur. J. Biochem. 124*, 513–519.
SampathKumar, P. (1978) Ph.D. Thesis, Australian National University.
SampathKumar, P., & Morrison, J. F. (1982) *Biochim. Biophys. Acta 702*, 204–211.
Smith, E., & Morrison, J. F. (1971) *J. Biol. Chem. 246*, 7764–7772.
Sogo, S. G., Widlanski, T. S., Hoare, J. H., Grimshaw, C. E., Berchtold, G. A., & Knowles, J. R. (1984) *J. Am. Chem. Soc. 106*, 2701–2703.
Turnbull, J., Cleland, W. W., & Morrison, J. F. (1990) *Biochemistry* (preceding paper in this issue).

Mechanisms of Activation of Tissue Procollagenase by Matrix Metalloproteinase 3 (Stromelysin)†

Ko Suzuki,‡ Jan J. Enghild,§ Tatsuhisa Morodomi,‡ Guy Salvesen,§ and Hideaki Nagase*,‡
Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, 39th and Rainbow Boulevard, Kansas City, Kansas 66103, and Department of Pathology, Duke University Medical Center, Durham, North Carolina 27710
Received May 14, 1990; Revised Manuscript Received July 20, 1990

ABSTRACT: The mechanism of activation of tissue procollagenase by matrix metalloproteinase 3 (MMP-3)/stromelysin was investigated by kinetic and sequence analyses. MMP-3 slowly activated procollagenase by cleavage of the Gln^{80}–Phe^{81} bond to generate a fully active collagenase of M_r = 41 000. The specific collagenolytic activity of this species was 27 000 units/mg (1 unit = 1 μg of collagen digested in 1 min at 37 °C). Treatment of procollagenase with plasmin or plasma kallikrein gave intermediates of M_r = 46 000. These intermediates underwent rapid autolytic activation, via cleaving the Thr^{64}–Leu^{65} bond, to give a collagenase species of M_r = 43 000 that exhibited only about 15% of the maximal specific activity. Similarly, (4-aminophenyl)mercuric acetate (APMA) activated procollagenase by intramolecular cleavage of the Val^{67}-Met^{68} bond to generate a collagenase species of M_r = 43 000, but with only about 25% of the maximal specific activity. Subsequent incubation of the 43 000-M_r species with MMP-3 resulted in rapid, full activation and generated the 41 000-M_r collagenase by cleaving the Gln^{80}–Phe^{81} bond. In the case of the proteinase-generated 43 000-M_r species, the action of MMP-3 was approximately 24 000 times faster than that on the native procollagenase. This indicates that the removal of a portion of the propeptide of procollagenase induces conformational changes around the Gln^{80}–Phe^{81} bond, rendering it readily susceptible to MMP-3 activation. Prolonged treatment of procollagenase with APMA in the absence of MMP-3 also generated a 41 000-M_r collagenase, but this species had only 40% of the full activity and contained Val^{82} and Leu^{83} as NH_2 termini. Thus, cleavage of the Gln^{80}–Phe^{81} bond by MMP-3 is crucial for the expression of full collagenase activity. These results suggest that the activation of procollagenase by MMP-3 is regulated by two pathways: one with direct, slow activation by MMP-3 and the other with rapid activation in conjunction with tissue and/or plasma proteinases. The latter event may explain an accelerated degradation of collagens under certain physiological and pathological conditions.

Mammalian tissue collagenases (matrix metalloproteinase 1) (E.C. 3.4.24.7) are metalloendopeptidases capable of degrading interstitial collagen types I, II, and III at specific sites to generate 3/4 and 1/4 fragments of the native molecules (Miller et al., 1976; Hofmann et al., 1978; Dixit et al., 1979). It has recently been shown that they also digest collagen types VII (Seltzer et al., 1989) and X (Schmid et al., 1986). The involvement of collagenase in pathological breakdown as well as normal remodeling of connective tissues has been proposed (Harris & Krane, 1974; Woolley & Evanson, 1980). The production of collagenase by connective tissue cells is greatly enhanced by monocyte-derived inflammatory mediators such

† This work was supported by National Institutes of Health Grants AR 39189 and CA 29589 and a grant from Speas Foundation.
* To whom correspondence should be addressed at the University of Kansas Medical Center.
‡ University of Kansas Medical Center.
§ Duke University Medical Center.

0006-2960/90/0429-10261$02.50/0