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Chorismate Mutase–Prephenate Dehydrogenase from *Escherichia coli*: Spatial Relationship of the Mutase and Dehydrogenase Sites†

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ABSTRACT: The inhibition of the bifunctional enzyme chorismate mutase–prephenate dehydrogenase (4-hydroxyphenylpyruvate synthase) by substrate analogues has been investigated at pH 6.0 with the aim of elucidating the spatial relationship that exists between the sites at which each reaction occurs. Several chorismate and adamantane derivatives, as well as 2-hydroxyphenyl acetate and diethyl malonate, act as linear competitive inhibitors with respect to chorismate in the mutase reaction and with respect to prephenate in the dehydrogenase reaction. The similarity of the dissociation constants for the interaction of these compounds with the free enzyme, as determined from the mutase and dehydrogenase reactions, indicates that the reaction of these inhibitors at a single site prevents the binding of both chorismate and prephenate. However, not all the groups on the enzyme, which are responsible for the binding of these two substrates, can be identical. At lower concentrations, citrate or malonate prevents reaction of the enzyme with prephenate, but not with chorismate. Nevertheless, the combining sites for chorismate and prephenate are in such close proximity that the diethyl derivative of malonate prevents the binding of both substrates. The results lead to the proposal that the sites at which chorismate and prephenate react on hydroxyphenylpyruvate synthase share common features and can be considered to overlap.

Chorismate mutase–prephenate dehydrogenase (EC 5.4.99.5 and EC 1.3.1.12; 4-hydroxyphenylpyruvate synthase) is a bifunctional enzyme that catalyzes the two sequential reactions along the tyrosine biosynthetic pathway which are shown in eq 1. The enzyme from *Escherichia coli* has a molecular

$$\text{chorismate} \rightarrow \text{prephenate} \xrightarrow{\text{NAD}} \text{4-hydroxyphenylpyruvate} + \text{NADH} + \text{H}^+ + \text{CO}_2 \quad (1)$$

weight of 88 000 and is composed of two apparently identical subunits (SampathKumar & Morrison, 1982a). The results of early studies by Koch et al. (1972) suggested that the sites for the mutase and dehydrogenase activities of the enzymes from *Aerobacter aerogenes* and *E. coli* were either contiguous or close to each other. The kinetic data obtained by Heyde & Morrison (1978) with the enzyme from *A. aerogenes* were consistent with the two reactions occurring at a single site or at two separate sites with similar kinetic properties. The idea of the reactions occurring at the same site or at two sites in close proximity was supported by the results of computer simulation of progress curves for the conversion of chorismate to hydroxyphenylpyruvate and by the finding that a small, but significant, proportion of prephenate formed from chorismate is converted directly to hydroxyphenylpyruvate (Heyde, 1979).

Further evidence that the mutase and dehydrogenase activities occur at the same or similar sites comes from the findings that the two activities are lost coordinately under a variety of inactivating conditions. The loss of each activity as a result of the reaction of iodoacetamide with a single sulfhydryl group on each subunit can be prevented by the addition of prephenate or of NAD plus tyrosine (Heyde, 1979; Hudson & Davidson, 1981). Attempts to obtain a mutant enzyme that possesses only prephenate dehydrogenase activity were unsuccessful although it was possible to obtain an enzyme that lacks dehydrogenase activity because of alteration at the NAD binding site. A protein lacking both activities could also be obtained (Rood et al., 1982).

The aforementioned results tend to indicate that the two catalytic activities of chorismate mutase–prephenate dehydrogenase do not take place at distinct sites. Thus, the enzyme differs from chorismate mutase–prephenate dehydratase which has been shown by a variety of procedures to have separate, noninteracting sites (Duggleby et al., 1978). The purpose of the present investigation was to gain additional information about the spatial relationship of the sites at which the mutase and dehydrogenase reactions occur by using as inhibitors analogues of both chorismate and prephenate. The

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0006-2960/83/0422-1650$01.50/0

results have led to the proposal that the sites at which chorismate and prephenate react on hydroxyphenylpyruvate synthase share common features and can be considered to overlap.

Experimental Procedures

Chemicals. *N*-Ethylmorpholine and (2-hydroxyphenyl)-acetic acid were obtained from Sigma. Diethyl malonate, adamantane-1-carboxylic acid, adamantane-1-acetic acid, and adamantane-1,3-diacetic acid were from Aldrich Chemical Co. Adamantane-1-phosphonic acid was a kind gift from Professor Glenn Berchtold of the Massachusetts Institute of Technology. Malonic acid and 2-(*N*-morpholino)ethanesulfonic acid (Mes) were supplied by Calbiochem. Citric acid was from Ajax Chemicals, diethanolamine was from Fluka, and NAD was from P-L Biochemicals. Chorismic acid and disodium prephenate were prepared as described by Gibson (1968) and Dudzinski & Morrison (1976), respectively.

Synthesis of Chorismate Derivatives. Chorismic acid 5,6-epoxide and 5,6-dihydrochorismic acid were synthesized by the procedures of Ife et al. (1976) with the following modifications. In the synthesis of the epoxide, the crude product was collected by centrifugation, and after decantation of the diethyl ether, the pellet was dried overnight at 4 °C under vacuum in a desiccator with paraffin chips and phosphorus pentoxide. The dry material was redissolved in a minimum volume of cold, anhydrous acetone, and then petroleum ether (bp 60–80 °C) was added until the solution became faintly cloudy. The opalescent solution was kept overnight at −20 °C, and the crystals were collected by centrifugation. The product, which was contaminated with about 1% (w/w) chorismic acid as judged by assay with hydroxyphenylpyruvate synthase, was obtained in pure form by recrystallization. Other contaminants of the crude epoxide preparation, as detected by thin-layer ion-exchange chromatography on poly(ethylenimine)–cellulose (Christopherson & Morrison, 1983), were an insoluble polymer, 5,6-dihydro-5,6-dihydroxychorismic acid, and possibly 3-chlorobenzoic acid with R_f values of 0, 0.51, and 0.88, respectively. No contaminants were detectable in the final preparation of chorismic acid 5,6-epoxide which had an R_f value of 0.65 and which was obtained in 5% yield. Epoxide solutions were made up freshly before use by dissolving the free acid in absolute ethanol and diluting the solution so that the final ethanol concentration was 25% (v/v). The NMR spectrum of chorismic acid 5,6-epoxide is shown in Figure 1 together with that for chorismic acid. The compounds were dissolved in deuterated acetone (acetone-d_6) and spectra recorded at 89.6 MHz by using a Jeol Model FX90Q NMR spectrometer in the Fourier transform mode.

5,6-Dihydro-5,6-dihydroxychorismic acid, which is a derivative not previously described, was formed by storing the epoxide in acetone-d_6 for 2 weeks at 4 °C. The opening of the epoxide ring to form the diol was monitored by conversion of the downfield triplet of the NMR spectrum, representing proton d of the epoxide, to a downfield doublet for proton d of the diol (Figure 1). The complete absence of the triplet in the diol spectrum indicates that this conversion was quantitative. A small quantity of insoluble material (presumably polymer) was removed by centrifugation, and the diol was recovered from acetone-d_6 by lyophilization. From the diol spectrum (Figure 1), the following chemical shifts and coupling constants have been assigned: δ_H [$(CD_3)_2CO$, 89.6 MHz] proton d, 7.04 (1 H, d, $J_{d,f}$ = 4.1 Hz), proton c, 5.60 (1 H, d, $J_{c,e}$ = 3.4 Hz), proton a, 5.49 (1 H, d, $J_{a,b}$ = 1.2 Hz), proton b, 5.03 (1 H, d, $J_{a,b}$ = 1.2 Hz), proton f, 4.58 (1 H, dd, $J_{d,f}$ = 4.0 Hz and $J_{f,g}$ = 8.9 Hz), proton e, 4.56 [1 H, t, $J_{c,e}$ = 3.4 Hz (from proton c) and $J_{e,g}$ = 4.2 Hz (from proton g)], proton g, 4.16 (1 H, dd, $J_{e,g}$ = 4.2 Hz and $J_{f,g}$ = 9.0 Hz), HOD, 3.32. Proton d appears as a triplet (t) in the spectrum of the epoxide because the peak is split, with approximately equal coupling constants, by proton c and by allylic coupling with proton f. Proton d of the diol is a doublet (d) because of the allylic coupling with proton f. The splitting of the proton d peak by proton c is very small and was detected only as fine structure in peak c ($J_{c,d}$ = 0.86 Hz).

Attempts to prepare crystals of 5,6-dihydrochorismic acid according to the procedure of Ife et al. (1976) were unsuccessful. When the ether was removed after extraction of the lyophilized product, a very hygroscopic oil was obtained. The product was prepared by dissolving the oil in acetone to give a concentrated solution, adding petroleum ether (bp 60–80 °C) until the solution became markedly opalescent, and then centrifuging the mixture. The NMR spectrum in acetone-d_6 was similar to that reported by Ife et al. (1976) but indicated the presence of impurities (Figure 1). Ion-exchange chromatography on poly(ethylenimine)–cellulose thin-layer plates (Christopherson & Morrison, 1983) showed that the product contained a major spot with an R_f value of 0.71 which absorbed ultraviolet light and which corresponded to dihydrochorismic acid. The product also contained a minor contaminant as well as 4-hydroxybenzoic acid with R_f values of 0.55 and 0.42, respectively. All three chorismic acid derivatives decomposed when chromatographed on columns of DEAE-Sephacel.

Purification of Hydroxyphenylpyruvate Synthase. The enzyme was obtained from a strain of *E. coli* (JFM 30) which produces very much higher levels of hydroxyphenylpyruvate synthase than the parent strain (Bhosale et al., 1982). The purification procedure was essentially that developed by these same authors except that all buffer solutions were degassed and bubbled with nitrogen. In addition, the final enzyme preparation was dialyzed again against buffer containing 0.1 M *N*-ethylmorpholine, 10% (v/v) glycerol, 21 mM citrate, 1 mM ethylenediaminetetraacetic acid (EDTA), and 20 mM dithiothreitol (pH 7.0).

Specific Activity of Enzymes. Enzyme activity was determined at pH 7.5 in the presence of 100 mM Tris-HCl buffer containing 1 mM EDTA and 1 mM dithiothreitol. Mutase activity was measured by using 0.7 mM chorismate while dehydrogenase activity was determined in the presence of 0.2 mM prephenate and 1.0 mM NAD. Protein concentration was obtained by estimating the lysine, arginine, and histidine content of samples with a Beckman Model 120 B amino acid analyzer and using the values given by SampathKumar & Morrison (1982a) for residues of basic amino acids per 43 000-dalton subunit. The specific activity for mutase was 102 units/mg of protein, and that for dehydrogenase was 80 units/mg of protein. These values are higher than those reported previously (Bhosale et al., 1982), and this is attributable, at least in part, to the increase in activity that occurs on increasing the concentration of dithiothreitol in stock enzyme solutions from 1 to 20 mM.

Measurement of Enzyme Activities. Kinetic investigations on the mutase and dehydrogenase reactions were performed at 30 °C in the presence of a buffer containing 0.05 M Mes, 0.025 M *N*-ethylmorpholine, and 0.025 M diethanolamine, which was adjusted to pH 6.0 with acetic acid, as well as 1 mM EDTA and 1 mM dithiothreitol. The total volume of reaction mixtures was 1.0 mL. Reactions were started by the addition of enzyme and followed continuously by using a Cary 118 spectrophotometer. Spectral overlays for the conversion of chorismate to hydroxyphenylpyruvate in the presence of NAD were obtained with a Hewlett-Packard Model 8450A

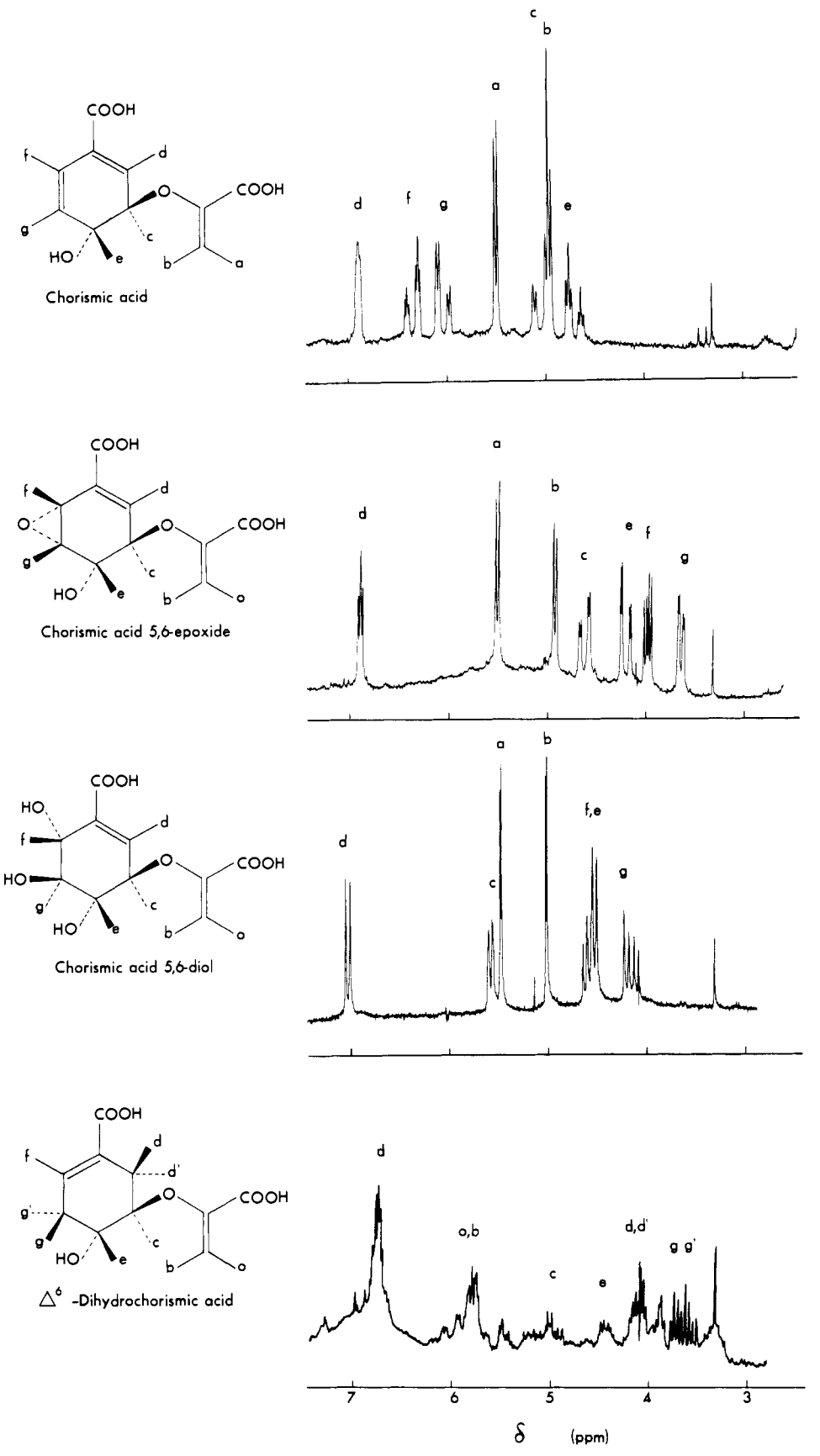

FIGURE 1: Proton nuclear magnetic resonance spectra for chorismic acid and its derivatives. Nonexchangeable hydrogen atoms are labeled alphabetically on the structures, and peak assignments are made on the spectra. Details are given under Experimental Procedures.

spectrophotometer. The traces (Figure 2) indicate that the optimum wavelengths for monitoring the disappearance of chorismate and the appearance of NAD are 290 and 340 nm, respectively. The respective molecular extinctions are 1.85 and 6.40 mM^{-1} cm^{-1}. It will be noted in Figure 2 that there is an isosbestic point at 300 nm.

Analysis of Data. Kinetic data were plotted in double-reciprocal form to determine the pattern of the lines and then

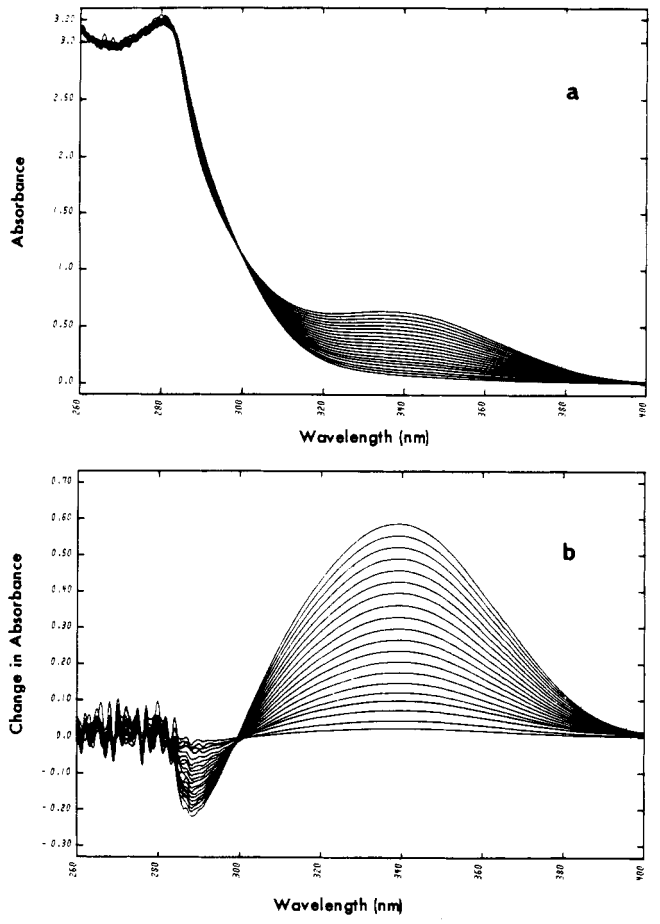

FIGURE 2: Spectral overlays of the overall mutase–dehydrogenase reaction, with 1.0 mM chorismate and 1.0 mM NAD as the starting substrates. Spectra were recorded at 15-s intervals with a 1-s dwell time starting 10 s after the addition of 0.21 μg of hydroxyphenylpyruvate synthase. Other details of the assay are described under Experimental Procedures. (a) Absorbance spectra; (b) difference spectra computed from (a) by subtracting the initial absorbance spectrum from subsequent spectra.

fitted to the appropriate rate equation by using one of the computer programs of Cleland (1979). The values for the kinetic parameters were used to draw the lines of Figure 3. Data conforming to competitive, parabolic competitive, noncompetitive, and hyperbolic, noncompetitive inhibition were fitted to eq 2, 3, 4, and 5, respectively. Data giving rise to linear plots of $1/v$ against inhibitor concentration were fitted to eq 6, and the true K_i value was calculated by using eq 7.

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

$$v = \frac{VA}{K_a(1 + I/K_{i1} + I^2/K_{i2}) + A} \quad (3)$$

$$v = \frac{VA}{K_a(1 + I/K_{is}) + A(1 + I/K_{ii})} \quad (4)$$

$$v = \frac{VA(1 + I/K_{id})}{K_a(1 + I/K_{is}) + A(1 + I/K_{in})} \quad (5)$$

$$v = \frac{V}{1 + I/K_{i,app}} \quad (6)$$

$$K_i = K_{i,app}/(1 + A/K_a) \quad (7)$$

Results

Inhibition of the Enzyme by Substrate Analogues. Investigations were undertaken on the inhibition of the mutase and dehydrogenase activities of hydroxyphenylpyruvate synthase

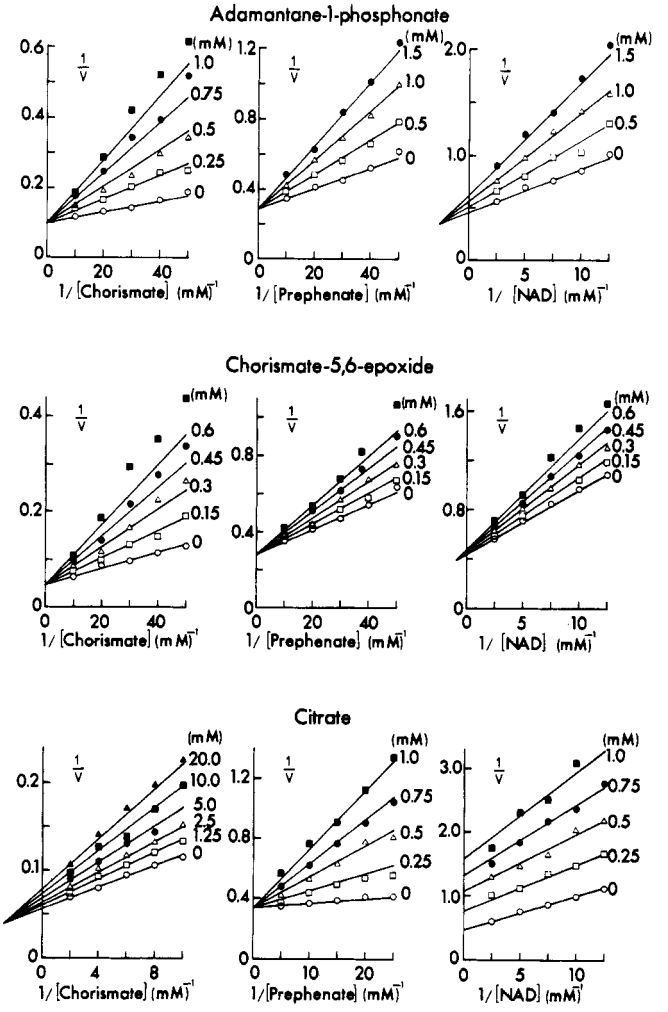

FIGURE 3: Inhibition patterns for adamantane-1-phosphonate, chorismate 5,6-epoxide, and citrate with respect to chorismate in the mutase reaction and both prephenate and NAD^+ in the dehydrogenase reaction. The data for the competitive inhibition of the mutase reaction by adamantane-1-phosphonate and chorismate 5,6-epoxide were fitted to eq 2; data for the hyperbolic noncompetitive inhibition of the mutase reaction by citrate were fitted to eq 4; data for the competitive inhibition of the dehydrogenase reaction by all compounds with respect to prephenate were fitted to eq 2; data for the noncompetitive inhibition of the dehydrogenase reaction by all compounds with respect to NAD were fitted to eq 3. Assays were initiated with 0.63 μg of synthase for the mutase and 0.31 μg for the dehydrogenase as described under Experimental Procedures. Reaction velocity units are micromolar per minute.

with the aim of determining if specific inhibitory analogues of each activity could be found. It was reasoned that the action of such analogues may well permit definitive conclusions to be reached about the spatial relationship that exists between the sites for the mutase and dehydrogenase activities. The data of Figure 3 show that, for the range over which the variable substrates were used, adamantane-1-phosphonate and chorismate 5,6-epoxide act as linear competitive inhibitors with respect to both chorismate and prephenate and as linear noncompetitive inhibitors with respect to NAD. The data obtained with these compounds as inhibitors of the mutase reaction were also fitted to eq 3. The results of F tests showed that, at the 5% level, the fits to eq 3 were not significantly better than those to eq 2. Results similar to those given by adamantane-1-phosphonate and chorismate 5,6-epoxide were obtained with 2-hydroxyphenylacetate, adamantane-1-carboxylate, adamantane-1-acetate, adamantane-1,3-diacetate, and diethyl malonate. Analysis of the data yielded the true

Table I: Kinetic Constants for the Interaction of Substrate Analogues with Hydroxyphenylpyruvate Synthase[a]

compound	mutase K_i(E + I) (μM)	dehydrogenase K_i(E + I) (μM)	dehydrogenase K_I(E-NAD + I) (μM)
2-hydroxyphenyl acetate	320 ± 40	170 ± 30	1200 ± 170
adamantane-1-carboxylate	430 ± 40	310 ± 140	570 ± 160
adamantane-1-acetate	220 ± 60	130 ± 30	840 ± 80
adamantane-1,3-diacetate	340 ± 30	150 ± 50	330 ± 90
adamantane-1-phosphonate	200 ± 20	140 ± 20	720 ± 60
chorismate 5,6-epoxide	230 ± 30	130 ± 20	620 ± 90
5,6-dihydro-5,6-dihydroxychorismate[b]	410[c]	110[c]	3100[c]
Δ^6-dihydrochorismate	430[c]	590[c]	1900[c]
citrate	3100 ± 600 (9200 ± 2100)[d]	110 ± 40	150 ± 30
malonate	51000 ± 9000 (93000 ± 18000)[d]	4500 ± 1500	9100 ± 2000
diethyl malonate	1700 ± 100	1900 ± 600	2600 ± 400

[a] Data were obtained by using five different substrate concentrations and four to six different inhibitor concentrations. True dissociation constants were calculated from the determined apparent values by using the procedures outlined by Smith & Morrison (1971). For the dehydrogenase reaction, NAD concentration was held constant at 1.0 mM with prephenate as the varied substrate while the fixed concentration of prephenate was 80 μM with NAD as the variable substrate. [b] Dissociation constants were determined from Dixon plots by using 5,6-dihydro-5,6-dihydroxychorismate concentrations of up to 1 mM. [c] These values were determined by using the apparent values obtained by fitting data to eq 4. Because of the limited number of data points and the relative complexity of the calculations, standard errors of the values were not calculated. [d] Values in parentheses are dissociation constants for the interaction of the enzyme-chorismate complex with the inhibitor: the ratios of the maximum velocities for the mutase reaction in the presence and absence of citrate and malonate were 0.66 ± 0.07 and 0.42 ± 0.05, respectively.

values for the dissociation constants that are listed in Table I. Because of the limited amounts of 5,6-dihydro-5,6-dihydroxychorismate and 5,6-dihydrochorismate that were available, inhibition of the two enzyme activities by these compounds was determined at fixed substrate concentrations. Both these chorismate derivatives inhibited the mutase and dehydrogenase reactions, and plots of the reciprocal initial velocity against inhibitor concentration were linear. Values for the dissociation constants of the enzyme–inhibitor complexes (Table I) were calculated on the assumption that each of the compounds functions as a linear competitive inhibitor with respect to chorismate and prephenate.

In contrast to the linear competitive inhibition given by the aforementioned compounds, citrate and malonate function as hyperbolic noncompetitive inhibitors of the mutase. However, they still act as linear competitive inhibitors with respect to prephenate and as linear noncompetitive inhibitors in relation to NAD. The inhibition constants associated with these inhibitors are given in Table I.

Discussion

The results of the present investigation indicate that the compounds which function as inhibitors of the mutase activity also act as inhibitors of the dehydrogenase activity of hydroxyphenylpyruvate synthase (Table I). It is of particular interest that the compounds which are structurally similar to chorismate, because either they are derived from this substrate or they are adamantane derivatives which are regarded as transition-state analogues for the mutase reaction (Andrews et al., 1977), also act as inhibitory analogues of prephenate. The type of inhibition that is observed varies with the structure of the inhibitor. Derivatives of chorismate and adamantane, as well as 2-hydroxyphenyl acetate and diethyl malonate, act as linear competitive inhibitors for the mutase reaction, while for the dehydrogenase reaction, they give rise to inhibitions that are linear competitive with respect to prephenate and linear noncompetitive in relation to NAD. The latter dead-end inhibition pattern is as expected for a reaction that conforms to a rapid equilibrium, random mechanism (SampathKumar & Morrison, 1982b).

The similarity of the dissociation constants for the interaction of the aforementioned compounds with the free enzyme (Table I), as determined from steady-state velocity studies on the mutase and dehydrogenase reactions, suggests strongly that only a single site of interaction for each inhibitor is involved. Thus, combination of these inhibitors with the enzyme excludes the binding of both chorismate and prephenate. The result also suggests that the same groups on the enzyme are involved with the interaction of the two substrates or that there are common groups for the binding of chorismate and prephenate. It should be noted that the presence of NAD on the enzyme can markedly hinder the binding of the inhibitors under discussion (Table I).

Citrate and malonate also cause inhibition of the mutase and dehydrogenase activities of the enzyme, but the observed types of inhibition are different from those obtained with the chorismate and adamantane derivatives (Table I). While citrate and malonate act as linear competitive inhibitors with respect to prephenate and as linear noncompetitive inhibitors in relation to NAD, they function as hyperbolic noncompetitive inhibitors with respect to chorismate. Qualitatively this finding is in accord with the idea that the binding of citrate (or malonate) and prephenate is mutually exclusive whereas citrate (or malonate) and chorismate can be present on the enzyme at the same time. However, inspection of the values for the binding of these inhibitors to the free form of enzyme (Table I) shows that they differ by at least 1 order of magnitude as determined from studies with the mutase and dehydrogenase reactions. Thus, there must be two sites on the enzyme at which citrate (or malonate) can bind. When either of these inhibitors occupies the site at which they are bound more strongly, the binding of prephenate is precluded. With the higher concentrations of citrate (or malonate) required for the inhibition of the mutase reaction, both binding sites would be occupied, and as chorismate binding can still occur, it follows that chorismate and prephenate cannot have identical binding sites. No interaction between the binding of the two molecules of inhibitor is observed, and this may well be due to the large difference in magnitude of the two dissociation constants.

The difference in the types of inhibition observed with malonate and diethyl malonate is of particular interest. While malonate prevents the binding of prephenate but not chorismate, diethyl malonate does not allow either substrate to bind. Clearly this is due to the addition of the bulky ethyl groups

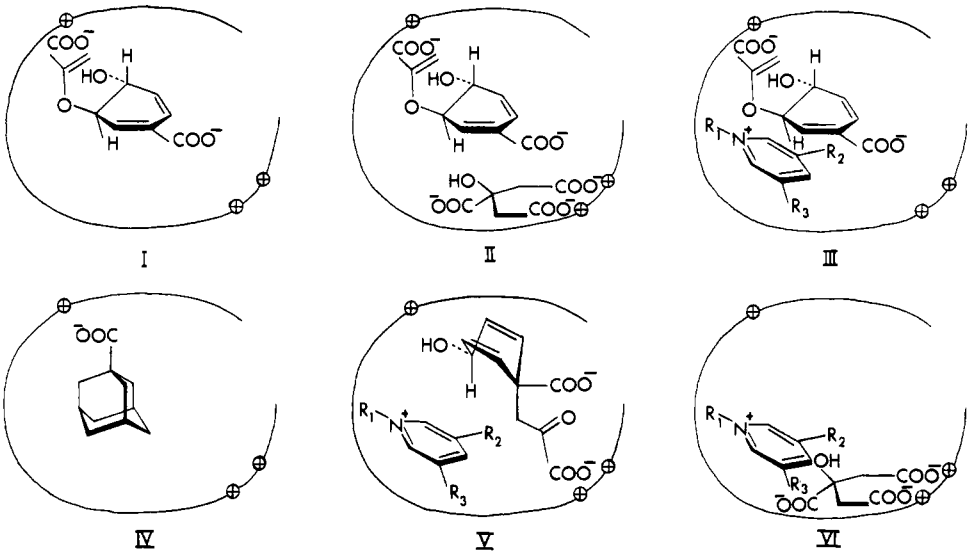

FIGURE 4: Proposed spatial relationships between substrates and inhibitors of hydroxyphenylpyruvate synthase. The interaction of the second molecule of citrate, which occurs at higher concentrations, is not illustrated.

to the α-carbon of malonate. Since the same dissociation constant for diethyl malonate is obtained from studies on each reaction, the combination of this inhibitor at one site on the enzyme is sufficient to prevent the interaction of both substrates. Such a result draws attention to the closeness of the substrate binding sites, although no conclusions can be reached as to which of the two malonate sites is involved with the binding of diethyl malonate.

The results obtained with the range of substrate analogues indicate that although the sites for the two reactions are not identical, a close relationship must exist between them. A final answer to whether the sites are contiguous or overlapping may well come from knowledge of the three-dimensional structure of the enzyme, but attempts to obtain enzyme crystals have been unsuccessful. Nevertheless, it is possible to draw some conclusions about the way in which substrates and inhibitors interact with hydroxyphenylpyruvate synthase. It is postulated (Figure 4, I) that chorismate in the ground state is bound with the carboxyl group of the enolpyruvyl side chain interacting with a positively charged group on the enzyme. Chorismate may also be bound close to the NAD binding site as pyridine nucleotides enhance the interaction of this substrate with the enzyme, possibly by delocalization of π electrons between the nicotinamide ring and the conjugated double bonds of chorismate (Figure 4, III; Heyde & Morrison, 1978). By contrast, NAD hinders the interaction of chorismate analogues with the enzyme (Table I), and this may be due to loss of the conjugated cyclohexadiene structure for the chorismate derivatives (Figure 1) and the absence of similar delocalized electrons for the adamantanes. The same positively charged group on the enzyme that interacts with the enolpyruvyl side chain of chorismate (Figure 4, I) can be considered to react with the carboxyl group of 2-hydroxyphenyl acetate while the phenyl ring lies in the same plane as the cyclohexadiene ring of chorismate. As 3- and 4-hydroxyphenyl acetate are less inhibitory than the 2-hydroxy derivative, it appears that a hydroxyl group ortho to the side chain of chorismate may well be involved with substrate binding. A negatively charged group is also of importance for the binding of adamantane derivatives (Table I; Figure 4, IV) since adamantane-1-phosphonate, adamantane-1-carboxylate, adamantane-1-acetate, and adamantane-1,3-diacetate bind far more strongly than unsubstituted adamantane. The presence of the additional carboxyl group in adamantane-1,3-diacetate does not enhance the binding. It would appear that citrate and malonate function as linear competitive inhibitors with respect to prephenate in the dehydrogenase reaction (Table I) because of their structural resemblance to the pyruvyl side chain of the substrate. The interaction can be envisaged as involving two positively charged groups on the enzyme that also react with the pyruvyl side chain of prephenate (Figure 4, II, V, and VI). Such an interaction is distinct from the additional reaction which occurs at higher concentrations of citrate and malonate (Table I) and which hinders, but does not prevent, the binding of chorismate.

The spatial relationship between the mutase and dehydrogenase sites established here is in contrast to the distinct chorismate mutase and prephenate dehydratase sites of phenylpyruvate synthase (Duggleby et al., 1978). Since these two bifunctional enzymes catalyze similar reactions, have the same dimeric structure, and contain subunits of similar molecular weight, it might be argued that they are closely related. The different spatial arrangement of their catalytic sites now suggests that this is not the case.

Acknowledgments

We are grateful to Dr. David Fenn for running the NMR spectra, to Dr. Wilfred Armarego for assigning the proton nuclear magnetic resonance peaks, and to Leah Matthews for competent technical assistance. We thank Hewlett-Packard for the use of their Model 8450A spectrophotometer.

Registry No. Chorismic acid 5,6-epoxide, 61414-76-4; 5,6-dihydrochorismic acid, 61414-77-5; 5,6-dihydro-5,6-dihydroxychorismic acid, 84520-94-5; 2-hydroxyphenyl acetate, 2848-25-1; adamantane-1-carboxylate, 828-51-3; adamantane-1-acetate, 4942-47-6; adamantane-1,3-diacetate, 17768-28-4; adamantane-1-phosphonate, 23906-88-9; citrate, 77-92-9; malonate, 141-82-2; diethyl malonate, 105-53-3.

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Affinity Labeling of Nicotinamide Adenine Dinucleotide Dependent Isocitrate Dehydrogenase by the 2′,3′-Dialdehyde Derivative of Adenosine 5′-Diphosphate. Evidence for the Formation of an Unusual Reaction Product†

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ABSTRACT: Modification of the pig heart NAD-dependent isocitrate dehydrogenase by the 2′,3′-dialdehyde derivative of ADP (oADP) resulted in a time-dependent inactivation of the enzyme. Two kinetically distinct phases are observed for the loss in enzymatic activity with maximum rate constants of 0.38 and 0.023 min^{-1}, at saturating concentrations of oADP, at pH 7.0 and in the presence of 2.0 mM $MnSO_4$. The K_i values for both phases of the reaction are very similar; an average of 22.9 ± 4.6 μM for free oADP is obtained with constants determined in the presence of 0.2, 0.3, and 2.0 mM $MnSO_4$. At pH 7.0 and in the presence of Mn^{2+} ions, almost complete protection of isocitrate dehydrogenase from inactivation by oADP is provided by ADP and isocitrate, while only partial protection is afforded by NADH and ATP, and NAD is without effect. Only the protection by ADP is consistent with its directly determined binding constant which may indicate that isocitrate, NADH, and ATP exert allosteric effects on the inactivation by oADP, whereas ADP may compete with the analogue for the same nucleotide binding site. Affinity labeling of isocitrate dehydrogenase with [^{14}C]oADP results in radioactive labeling of the three distinct subunits. The incorporation of approximately 1 mol of [^{14}C]oADP/mol of average subunit corresponds to total inactivation of the enzyme. Inactivation of isocitrate dehydrogenase by oADP resulted in the formation of an enzyme–oADP product that was unaffected by subsequent reaction with sodium borohydride which suggests that the reaction product with this enzyme was not the generally expected Schiff base. Formation of the relatively stable product involved a loss of the pyrophosphoryl group of oADP as demonstrated by a comparison of the stoichiometry of the reaction determined with [^{14}C]oADP and [^{32}P]oADP. Further evidence obtained in this study is most consistent with the formation of a 4′,5′-didehydro-2′,3′-dihydroxymorpholino derivative between oADP and the ε-amino group of lysine on isocitrate dehydrogenase. The results obtained through affinity labeling of NAD-dependent isocitrate dehydrogenase by oADP suggest that an allosteric site for ADP is present on each type of subunit and that the structurally distinct subunits of this enzyme may be functionally similar.

NAD-dependent isocitrate dehydrogenase [*threo*-D_s-isocitrate:NAD^+ oxidoreductase (decarboxylating); EC 1.1.1.41] has an important role in the control of the mitochondrial oxidative decarboxylation of isocitrate to α-ketoglutarate (Plaut, 1970; Colman, 1975; Dalziel, 1980). The enzyme isolated from pig heart is composed of three distinct types of subunits in the approximate ratio 2α:1β:1γ (Ramachandran & Colman, 1980). Binding experiments indicated that the activators, Mn^{2+} and ADP, and the substrates, NAD and isocitrate, each have one binding site for every two subunits (Ehrlich & Colman, 1981). The stoichiometry of the ligand binding suggests either that isocitrate dehydrogenase has half the number of catalytic and ADP regulatory sites as it has subunits or that strong negative cooperativity exists in ligand binding. The latter assumption may be supported by the detection of enzymatically active α and β subunits by isoelectric focusing in the presence of 20% glycerol (Hayman & Colman, 1982) and by an initial report describing the isolation of catalytically active α and β subunits by chromatofocusing (Ehrlich & Colman, 1982a). Furthermore, modification of isocitrate dehydrogenase by 3-bromo-2-ketoglutarate, 3,4-didehydro-2-ketoglutarate, cyanate, and carbodiimide, which appear to label the substrate binding site, resulted in labeling of all types of subunits (Bednar & Colman, 1982). These results are consistent with the postulate that the structurally distinct subunits are functionally similar.

Isocitrate dehydrogenase is allosterically activated by ADP, which lowers the K_m for isocitrate (Cohen & Colman, 1972), as well as the K_m for Mn^{2+} (Cohen & Colman, 1974), but has no effect on the intrinsic maximum velocity. ADP appears

† From the Department of Chemistry, University of Delaware, Newark, Delaware 19711. *Received September 20, 1982.* Supported by U.S. Public Health Service Grant 2-R01-AM-17552.

0006-2960/83/0422-1656$01.50/0