Chorismate Mutase–Prephenate Dehydrogenase from *Aerobacter aerogenes*: Evidence That the Two Reactions Occur at One Active Site†

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ABSTRACT: The relationship between the sites for catalysis of two reactions by the bifunctional enzyme chorismate mutase–prephenate dehydrogenase has been investigated. The results are consistent with the occurrence of both reactions at one active site. Comparisons have been made between experimental data for the time course of the overall reaction and computer simulations according to various models for the relationship between the mutase and dehydrogenase sites. A model based on a single active site is consistent with the time course data if a minor proportion of the chorismate that reacts can be converted through to (hydroxyphenyl)pyruvate without the intermediate release of prephenate. Consistent with this requirement, some channeling of radioactivity from chorismate to (hydroxyphenyl)pyruvate has been detected. A model based on two separate sites has also been considered; the simulations show that if this model applies there is no need to postulate any channeling of the intermediate, prephenate, between the sites and there must be marked inhibition of the dehydrogenase reaction by chorismate. Since channeling has been observed and chorismate increases the dehydrogenase rate under all conditions, the two-site model appears unlikely. Consistent with the one-site model are the observations that a variety of inactivating conditions cause parallel loss of mutase and dehydrogenase activity and that identical protection against inactivation of both mutase and dehydrogenase by iodoacetamide is afforded by prephenate.

There is a growing number of known multifunctional enzymes composed of a single type of polypeptide chain but possessing multiple activities. In some cases the reactions appear to be sufficiently similar that they are likely to be catalyzed at a single active site by an enzyme of broad specificity, e.g. bis(phospho)glyceromutase–3-bis(phospho)-glycerate phosphatase–phosphoglyceromutase of erythrocytes (Ikura et al., 1976). However, there are several examples of enzymes that catalyze quite distinct reactions at active sites that appear to be on different parts of the same polypeptide chain. Some aspects of the field have been reviewed by Kirschner & Bisswanger (1976). Studies of the overall reactions for bifunctional enzymes catalyzing sequential reactions have not been extensive, although this approach could be useful in determining the relationship between the sites for catalysis of the two reactions.

Chorismate mutase–prephenate dehydrogenase from *Aerobacter aerogenes* catalyzes two sequential reactions on the tyrosine biosynthetic pathway:

$$\text{chorismate} \rightarrow \text{prephenate} \xrightarrow{NAD^+} \text{(4-hydroxyphenyl)pyruvate} + NADH + H^+$$

The enzyme contains two very similar or identical subunits in a molecule of molecular weight approximately 76000 (Koch et al., 1970a,b). Heyde & Morrison (1978a) have reported steady-state investigations of both the mutase and dehydrogenase reactions. The data indicate that the dehydrogenase reaction conforms to a rapid equilibrium random mechanism involving the formation of dead-end and enzyme–NADH–prephenate and enzyme–NAD–(hydroxyphenyl)pyruvate complexes and that the dehydrogenase reactants affect the mutase reaction and vice versa. Values were determined for the kinetic parameters associated with both reactions. It appeared that the enzyme could have either a single active site or two separate sites possessing similar kinetic properties.

The present studies are directed more specifically to the question of whether the mutase and dehydrogenase reactions occur at the same site. Time courses of each of the individual reactions have been successfully simulated by computer. Simulations have been made of the time course of the overall reaction, based on different models for the relationship between the mutase and dehydrogenase sites. The results are considered in conjunction with those of experiments to detect channeling of prephenate, as well as binding and inactivation studies. It appears that both reactions occur at the one active site.

Experimental Procedure

Materials

Iodoacetamide was purchased from Sigma, 5,5′-dithiobis(2-nitrobenzoic acid) and pyridoxal phosphate were from Calbiochem, urea was from Mallinckrodt, and carboxypeptidase A (DFP treated) was from Worthington. [U-^{14}C]Glucose (260 mCi/mmol), carrier free with 70% isotopic abundance in all carbon atoms, was obtained from the Radiochemical Centre, Amersham, as was the ammonium salt of nicotinamide [U-^{14}C]adenine dinucleotide (302 mCi/mmol). All reagents were of the highest grade commercially available. The source or method for preparation of other reagents and chorismate mutase–prephenate dehydrogenase from *A. aerogenes* was as described by Heyde & Morrison (1978a).

Methods

Measurement of Enzyme Activity. Assays were carried out at 30 °C using cells of 1-cm light path in either a Cary 118 or a Varian 635 spectrophotometer. As in the previous kinetic investigation (Heyde & Morrison, 1978a), reaction mixtures contained 100 mM Tris-HCl buffer (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, and reactants at various concentrations. For the mutase reaction, disappearance of chorismate was followed at 273 nm using a molar extinction coefficient of 2630; for the dehydrogenase reaction, the appearance of NADH was followed at 340 nm, using a molar extinction coefficient of 6400 for an equimolar solution of the dehydrogenase products, NADH and (4-hydroxyphenyl)pyruvate (Heyde & Morrison, 1978a). In experiments concerned with inactivation of the enzyme, the steady-state velocity of the mutase reaction was determined at a chorismate concentration

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of 0.125 mM, while that of the dehydrogenase was determined in the presence of 0.2 mM NAD and 0.1 mM prephenate.

When both reactions were observed in the one sample, monitoring was performed at both 340 and 281 nm, by using a Varian 635 spectrophotometer. The decrease in chorismate concentration, measured at the isosbestic point for NAD and NADH (281 nm), was calculated from the relationship

$$\Delta(\text{chorismate}) = \left(E_{281} - E_{340}\frac{1460}{6400}\right) \Big/ 2230$$

in which allowance is made for the determined absorption at 281 nm of (4-hydroxyphenyl)pyruvate produced in the dehydrogenase reaction (E_{281} = 1460). The molar extinction coefficient of chorismate at 281 nm was measured as 2230. The concentration of prephenate at any time t was calculated from the relationship

$$[\text{prephenate}]_t = [\text{chorismate}]_0 - [\text{chorismate}]_t - [\text{NADH}]_t$$

where the subscript zero denotes initial concentration.

Electrophoretic Separation of Chorismate, Prephenate, Phenylpyruvate, and (4-Hydroxyphenyl)pyruvate. These compounds were separated by high-voltage paper electrophoresis in a water-cooled flat-bed apparatus. Before application of samples to the paper, 10 μg of each of unlabeled carrier chorismate, prephenate, and (hydroxyphenyl)pyruvate was added to each sample. Measured volumes of up to 100 μL were then applied from a syringe in multiple applications to 2-cm strips on a 45 × 56 cm sheet of Whatman 3MM paper, being dried after each application with a stream of room-temperature air. Electrophoresis was carried out in 0.05 M sodium acetate buffer, pH 5.5, at 100 V/cm for 30–45 min, depending on the separation desired. The mobilities of chorismate, phenylpyruvate, and (4-hydroxyphenyl)pyruvate relative to prephenate were 0.98, 0.69, and 0.58, respectively. Thus, phenylpyruvate and (4-hydroxyphenyl)pyruvate could be well separated from each other and from chorismate and prephenate, although the latter two compounds were not satisfactorily separated from each other. After electrophoresis the paper was dried in a ventilated cupboard at 40 °C, and marker strips on each side of the paper were cut off. These were sprayed with a diazotized *p*-nitroaniline spray (Smith, 1960) which gives a color reaction with each of the above compounds. By use of the positions of the markers, 5 cm wide strips corresponding to the various compounds or combinations of compounds were cut from the electrophoresis sheet, pleated, and placed in scintillation vials. Counting was performed in a Packard Tri-Carb liquid scintillation spectrometer, using Bray's scintillant (1960).

Preparation of [^{14}C]Chorismate and [^{14}C]Prephenate. [^{14}C]Chorismic acid was produced on a microscale by using *A. aerogenes* 62-1 and the method of Gibson (1968). Cells were suspended in 2 mL of medium B in which the glucose concentration was reduced from 18 to 1 mg/mL, including 1 mCi of carrier-free [U-^{14}C]glucose. The cells were incubated with shaking at 30 °C for 18 h and then centrifuged. The supernatant was divided in two, and the halves were treated separately to obtain labeled chorismate (a) or prephenate (b).

(a) Recrystallized chorismate (10 mg) was added as carrier to 1 mL of the above supernatant and the solution was applied at pH 8.1 and 4 °C to a 1 mL column of Dowex-1. After washing with water, chorismate was eluted with 1 M NH_4Cl (pH 8.5). The effluent (7 mL) was adjusted to pH 4.0 and extracted 3 times with 2 volumes of diethyl ether to remove contaminants such as 4-hydroxybenzoic acid. The solution was then adjusted to pH 1.5 and extracted with diethyl ether (24 mL). Chorismate was extracted from the ether with Tris-HCl buffer (pH 7.5; 6.5 mL; final concentration 0.034 M). The specific radioactivity of the product was 0.4 mCi/mmol, determined by measuring the counts that moved from the chorismate position to the (hydroxyphenyl)pyruvate position on a high-voltage electrophoretogram after treatment with excess chorismate mutase–prephenate dehydrogenase in the presence of excess NAD. The amount of labeled prephenate in the chorismate preparation was determined to be 2.4% by treatment of a sample with 0.5 N HCl for 10 min to convert the prephenate to phenylpyruvate, followed by neutralization, addition of all carriers, and high-voltage paper electrophoresis. Chorismate accounted for 46% of the radioactivity of the preparation, but the chemical concentrations of radioactive impurities would have been negligibly small.

(b) To the second milliliter of the above supernatant 1 unit of homogeneous chorismate mutase–prephenate dehydrogenase (sp act. 33) was added in 0.1 mL to convert the [^{14}C]chorismate produced into [^{14}C]prephenate. The solution was incubated at 20 °C for 1 h, and after cooling to 4 °C purified sodium prephenate (5 mg) was added as carrier. The labeled prephenate was precipitated by barium bromide in ethanol essentially by the method of Dudzinski & Morrison (1976). Barium phosphate was precipitated before barium prephenate, and extraction of the prephenate precipitate with 0.02 M Tris-HCl buffer (pH 7.5) dissolved the barium prephenate but left a small amount of insoluble barium phosphate and/or carbonate which was removed by centrifugation. This solution of barium prephenate was used in the experiments. The specific radioactivity of the product was 0.33 mCi/mmol, as determined from the movement of counts from the prephenate position to the (hydroxyphenyl)pyruvate position on a high-voltage electrophoretogram following treatment with excess NAD and chorismate mutase–prephenate dehydrogenase. Prephenate accounted for 42% of the total radioactivity of the preparation, but the chemical concentrations of radioactive impurities would have been negligibly small.

Transfer of Radioactivity from Chorismate to (Hydroxyphenyl)pyruvate. At intervals during the first few minutes after initiating the reaction in a spectrophotometer cuvette with enzyme, while the observed rates of both reactions were linear, 100-μL samples were withdrawn and added to 100 μL of ethanol on ice to stop the reaction. The reactants were then separated by high-voltage paper electrophoresis.

Binding Experiments. The binding of NAD, NADH, or prephenate to the enzyme was measured at 22 °C in volumes of 0.2 mL containing 0.1 M Tris-HCl buffer (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, a constant amount of radioactivity, and approximately 100 μg of enzyme. The method of Paulus (1969) was used in conjunction with Visking membranes instead of UM10 Diaflo membranes (Heyde, 1973). Counting of enzyme–ligand complex deposited on the membranes was performed in a Packard Tri-Carb liquid scintillation spectrometer. After subtraction of the appropriate blank value, data were converted to moles of ligand bound per mole of enzyme of molecular weight 76 000.

Analysis of Data. Binding data were fitted to an equation of the form

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

where v and V represent respectively the observed and maximum number of moles of ligand bound per mole of enzyme (Heyde, 1973) by means of two computer programs. The first (Cleland, 1963) assumes equal variance for determinations at different reactant concentrations, while the second (analogous) program fits to the same equation in logarithmic

form, thus assuming that variance is proportional to v. Steady-state velocity data for determining maximum velocities were fitted to eq 1 for the mutase reaction and to eq 2 for the dehydrogenase reaction

$$v = \frac{VA^2}{a + 2bA + A^2} \quad (2)$$

by using the computer programs of Cleland (1963). Equation 2 is of the form for a sequential reaction mechanism when the substrates are varied in constant ratio. Data for the inactivation of enzyme by iodoacetamide in the presence of varying concentrations of prephenate were plotted in a linear form and fitted to the equation for a straight line (Cleland, 1963). Replots from these data were fitted to the same equation. All data analysis was performed on a Univac 1100/42 computer.

Simulation of Reaction Time Courses. Simulations employed by a predictor–corrector method to solve simultaneous first-order differential equations (McCracken & Dorn, 1964; Kinns, 1975). The kinetic constants used in the simulations are given in Table I. In addition, it was necessary to determine the maximum velocities for both reactions, varying chorismate concentration or varying NAD^+ and prephenate in constant ratio, by using the same enzyme preparation that was used for the time courses. The method of Selwyn (1965) was used to check that there was no inactivation of the enzyme during the time for which the reactions were followed. Zero time for each time course was determined experimentally by comparing the change in chorismate concentration observed over the earliest possible time interval (0.2 min) with the steady-state mutase rate; a correction to the time of enzyme addition of 0.1 min or less was sometimes necessary. The simulations were performed on a PDP8 computer. Plotting of both simulations and experimental points for the figures was also done by means of the computer on a HP7200A plotter.

Theory

Simulation of Enzyme Reaction Time Courses. The models and equations used are given below. These are based on the results of previous steady-state kinetic investigations of the individual reactions (Heyde & Morrison, 1978a). Chorismate, prephenate, NAD^+, NADH, and (4-hydroxyphenyl)pyruvate are represented by A, B, C, P, and Q, respectively. The kinetic constants used are from Heyde and Morrison (1978a); definitions are given in Table I. V_M and V_{DH} are the maximum velocities for the mutase and dehydrogenase reactions, respectively.

(*A*) *Mutase Reaction Alone.* Equation 3 applies to an

$$v = \frac{-dA}{dt} = \frac{dB}{dt} = \frac{V_M A}{K_a\left(1 + \frac{B}{K_{ib}}\right) + A} \quad (3)$$

irreversible single substrate–single product reaction in the presence of both reactants.

(*B*) *Dehydrogenase Reaction Alone.* Equation 4 applies to

$$v = \frac{-dB}{dt} = \frac{-dC}{dt} = \frac{dP}{dt} = \frac{dQ}{dt} = V_{DH}BC \Big/ \left[K_{ib}K_c\left(1 + \frac{P}{K_{ip}} + \frac{Q}{K_{iq}} + \frac{PQ}{K_pK_{iq}}\right) + K_b\left(1 + \frac{Q}{K_{Iq}}\right)C + K_c\left(1 + \frac{P}{K_{Ip}}\right)B + BC\right] \quad (4)$$

an irreversible rapid equilibrium random reaction mechanism involving formation of dead-end ECQ and EBP complexes. For all the simulations the PQ term has been removed because an EPQ complex does not form to a detectable extent under the conditions used (Heyde & Morrison, 1978a).

(*C*) *Overall Reaction Starting from Chorismate and NAD^+ in the Absence of Prephenate.* The general forms of the differential equations are

$$\frac{dA}{dt} = -v_{mutase} \qquad \frac{dC}{dt} = -v_{dehydrogenase}$$

$$\frac{dB}{dt} = -\frac{dA}{dt} - \frac{dC}{dt} \qquad \frac{dP}{dt} = \frac{dQ}{dt} = -\frac{dC}{dt}$$

Several models of the overall reaction were simulated. The relevant rate equations for the models are given below.

(*1*) *Separate, Independent Sites for Mutase and Dehydrogenase.* This model allows for all known interactions but those between the sites and uses eq 3 and 4 for its rate equations except that an additional K_aQ/K_{iq_M} term is present in the denominator of eq 3.

(*2*) *Separate but Interacting Sites for Mutase and Dehydrogenase.* The rate equations for this model are eq 5 and

$$v_{mutase} = \left[V_M A\left[1 + 1.2\left(\frac{C}{K_{iac}} + \frac{P}{K_{iap}}\right)\right]\right] \Big/ \left[K_a\left[1 + \frac{P}{K_{ip_M}} + \frac{Q}{K_{iq_M}} + \frac{B}{K_{ib_M}} + \frac{B}{K_{ib_{DH}}}\left(\frac{C}{K_c} + \frac{P}{K_{Ip}}\right) + \frac{C}{K_{ic_M}}\left(1 + \frac{Q}{K_{Iq}}\right)\right] + A\left(1 + \frac{C}{K_{iac}} + \frac{P}{K_{iap}}\right)\right] \quad (5)$$

6. Allowance has been made in eq 5 for the effect of the coenzymes on chorismate combination (and vice versa) as well as on the mutase reaction rate. Equation 6 allows for the

$$v_{DH} = \left[V_{DH}BC\left(1 + \frac{A}{K_{ica}}\right)\right] \Big/ \left[K_{ib_{DH}}K_c\left[1 + \frac{P}{K_{ip_{DH}}} + \frac{Q}{K_{iq_{DH}}} + A\left(\frac{1}{K_a} + \frac{1}{K_{a'}}\right)\left(1 + \frac{C}{K_{iac}} + \frac{P}{K_{iap}}\right)\right] + K_b\left(1 + \frac{Q}{K_{Iq}}\right)C + K_c\left(1 + \frac{P}{K_{Ip}}\right)B + BC\left(1 + \frac{A}{K_{ica}}\right)\right] \quad (6)$$

enhancement of the combination of NAD^+ by chorismate. It has been assumed that (a) chorismate at the mutase site does not affect the velocity of the dehydrogenase reaction (since at high substrate concentrations the rate is not significantly affected by chorismate) or the combination of prephenate or (hydroxyphenyl)pyruvate at the dehydrogenase site (since there is no evidence for interactions between multiple sites for the combination of these reactants) and (b) chorismate can combine as an inhibitor at the dehydrogenase site with a dissociation constant $K_{a'}$.

(*3*) *One Active Site.* This model has one active site from which prephenate formed by the mutase reaction is released before it can take part in the dehydrogenase reaction, and uses eq 7 and 8.

$$v_{mutase} = \left[V_M A\left[1 + 1.2\left(\frac{C}{K_{iac}} + \frac{P}{K_{iap}}\right)\right]\right] \Big/ \left[K_a\left[1 + \frac{P}{K_{ip}} + \frac{Q}{K_{iq}} + \frac{B}{K_{ib}}\left(1 + \frac{C}{K_c} + \frac{P}{K_{Ip}}\right) + \frac{C}{K_{ic}}\left(1 + \frac{Q}{K_{Iq}}\right)\right] + A\left(1 + \frac{C}{K_{iac}} + \frac{P}{K_{iap}}\right)\right] \quad (7)$$

$$v_{\text{dehydrogenase}} = (V_{DH}BC)/\left[K_{ib}K_c\left[1 + \frac{P}{K_{ip}} + \frac{Q}{K_{iq}} + \frac{A}{K_a}\left(1 + \frac{C}{K_{iac}} + \frac{P}{K_{iap}} \right) \right] + K_bC\left(1 + \frac{Q}{K_{Iq}} \right) + K_cB\left(1 + \frac{P}{K_{Ip}} \right) + BC \right] \quad (8)$$

(*4*) *One Active Site.* At the one active site of this model, a proportion (X) of chorismate can be converted to (hydroxyphenyl)pyruvate without first being released as prephenate to equilibrate with the pool of free prephenate in the medium. The equations used are eq 7 and 8 except that an additional $V_{DH}[[(AC)(X)(K_{ib}K_c)]/(K_aK_{iac})]$ term is present in the numerator of eq 8.

Transfer of Radioactivity from Chorismate to Hydroxyphenylpyruvate. If there is full equilibration of the prephenate formed from chorismate with that in the bulk medium, the (hydroxyphenyl)pyruvate (Q) formed in any small time period (δt) will have the same specific radioactivity as the prephenate (B) in the medium at that time. The specific radioactivity (SRA) of (hydroxyphenyl)pyruvate at any time t will then be given by

$$(\text{SRA}_Q)_t = \frac{0.9\int_0^t (\text{SRA}_B)_{\delta t}\, dQ_{\delta t}}{Q_t} \quad (9)$$

where Q_t is the concentration of Q at time t, $(\text{SRA}_B)_{\delta t}$ is the specific radioactivity of B in the time interval δt, and the factor 0.9 allows for the fact that Q contains 9 carbon atoms compared with 10 for A and B. In eq 9

$$(\text{SRA}_B)_{\delta t} = \text{SRA}_A\left(\frac{B_t - B_0 + 0.024A_0}{B_t} \right)$$

where A_0 and B_0 are the initial concentrations of chorismate and prephenate, respectively, B_t is the concentration of prephenate at time t, and the constant, 0.024, is the proportion of the radioactivity in the chorismate preparation that is present as an impurity in the form of prephenate. The specific radioactivity of (hydroxyphenyl)pyruvate at time t while the initial steady-state rate of each reaction is maintained can therefore be expressed in the form

$$\alpha = (\text{SRA}_Q)_t = 0.9\left[\frac{(\text{SRA}_A)y}{Q_t} \right] \times \left\{ t - \frac{(B_0 - 0.024A_0)}{(x - y)} \ln\left[1 + \frac{(x - y)t}{B_0} \right] \right\}$$

where x and y are the rates of the mutase and dehydrogenase reactions, respectively.

By use of the observed specific radioactivity of (hydroxyphenyl)pyruvate ($\text{SRA}_{Q,obsd}$) and that calculated for full prephenate equilibration (α), calculation can be made of the proportion (γ) of (hydroxyphenyl)pyruvate arising from chorismate without equilibrating with the prephenate pool.

$$\gamma = \frac{\text{SRA}_{Q,obsd} - \alpha}{\text{SRA}_A - \alpha}$$

Results

Comparison of Experimental and Simulated Reaction Courses. (*1*) *Simulation of Individual Reactions.* Before simulation of the overall reaction, a test was made of the ability to simulate the mutase and dehydrogenase reactions individually. The values of kinetic constants used in the simulations are given in Table I. Figure 1 shows experimental points from four mutase reaction time courses over as wide a range of chorismate concentrations as could satisfactorily be used. It can be seen that the simulated time courses, shown by the lines on the figure, are in good agreement with the experimental data. A similar comparison of experimental and simulated data for the dehydrogenase reaction is shown in Figure 2, using a range of concentration of both NAD and prephenate. Again the overall agreement is good, although the simulation deviates from the experimental data to some extent at the lower NAD concentration. The sensitivity of the simulation to the various parameters associated with the dehydrogenase reaction was tested, and the degree of deviation seen in Figure 2 could be approximately accounted for by any one of the following: (a) an error of 10% in V_m, (b) an error of 50% in either Michaelis constant, or (c) an error of 100% in K_{ib}, K_{Ip}, or K_{Iq}. The simulation appeared to be relatively insensitive to values for K_{ip} and K_{iq}.

Table I: Kinetic Constants Used in Simulations of Time Courses for the Mutase and Dehydrogenase Reactions

reactant	enzyme form reacted with	determined by effect on	kinetic (dissociation) constant	value (mM)[a]
chorismate (A)	E	M	K_a	0.118 ± 0.012
	E-NAD	M	K_{ica}	0.016 ± 0.003
prephenate (B)	E	DH	$K_{ib_{DH}}$[b]	0.17 ± 0.02
	E	M	K_{ib_M}[b]	0.21 ± 0.01
	E-NAD	DH	K_b	0.030 ± 0.003
	E-NADH	DH	K_{ipb}	0.046 ± 0.010
NAD^+ (C)	E	DH	$K_{ic_{DH}}$[c]	0.72 ± 0.09
	E	M	K_{ic_M}[c]	0.60 ± 0.16
	E-prephenate	DH	K_c	0.11 ± 0.01
	E-chorismate	M	K_{iac}	0.096 ± 0.013
NADH (P)	E	DH	$K_{ip_{DH}}$[d]	0.086 ± 0.015
	E	M	K_{ip_M}[d]	0.17 ± 0.03
	E-prephenate	DH	K_{Ip}	0.023 ± 0.002
	E-chorismate	M	K_{iap}	0.059 ± 0.009
hydroxyphenyl-pyruvate (Q)	E	DH	$K_{iq_{DH}}$[e]	0.44 ± 0.07
	E	M	K_{iq_M}[e]	1.5 ± 0.2
	E-NAD	DH	K_{Iq}	0.032 ± 0.002

[a] The values are taken directly or calculated from Heyde & Morrison (1978a,b). M represents mutase and DH represents dehydrogenase. [b] Weighted mean of the two K_{ib} values is 0.20 ± 0.01 mM. [c] Weighted mean of the two K_{ic} values is 0.69 ± 0.08 mM. [d] Weighted mean of the two K_{ip} values is 0.10 ± 0.01 mM. [e] Weighted mean of the two K_{iq} values is 0.56 ± 0.07 mM.

(*2*) *Simulation of Overall Reaction.* After determining that the individual mutase and dehydrogenase reactions could be satisfactorily simulated, reaction time courses were recorded for the overall reaction. Because of the absorbance by both the pyridine nucleotides and chorismate at 281 nm, the usable concentration ranges of these compounds were limited. Two sets of conditions have been chosen for illustration, one with relatively high NAD^+ and low chorismate concentrations (Figure 3) and the other with relatively high chorismate and low NAD concentrations (Figure 4). In each case, the data are compared with simulations based on four different models, as described under Theory.

From Figures 3 and 4 it can be seen that the model with two independent sites does not fit the data in either case. When NAD is in excess over chorismate (Figure 3), the models for two interacting sites or a single site both appear in rea-

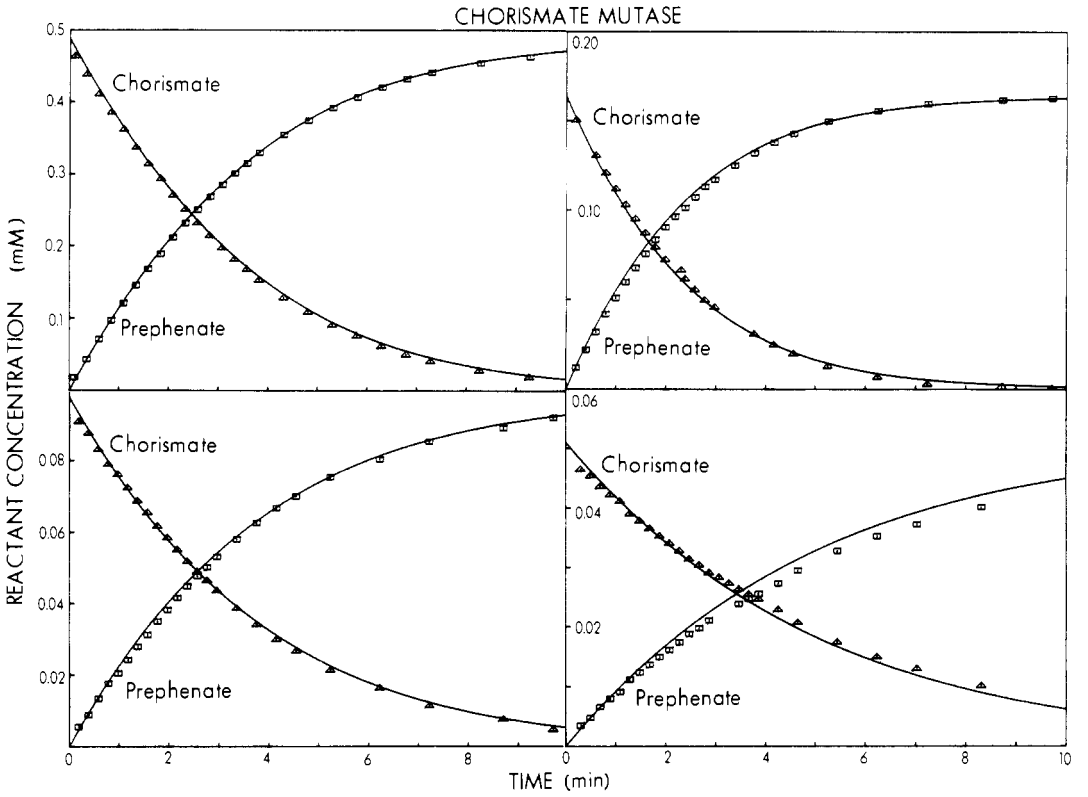

FIGURE 1: Comparison of simulated and experimental time courses for the mutase reaction. The curves are simulated according to eq 3, and the points are experimental data. For experiments in descending order of initial chorismate concentration, the amount of enzyme added per milliliter was 6.34, 3.17, 1.59, or 0.95 μg. The maximum velocity was 34.8 (μmol/min)/mg of enzyme.

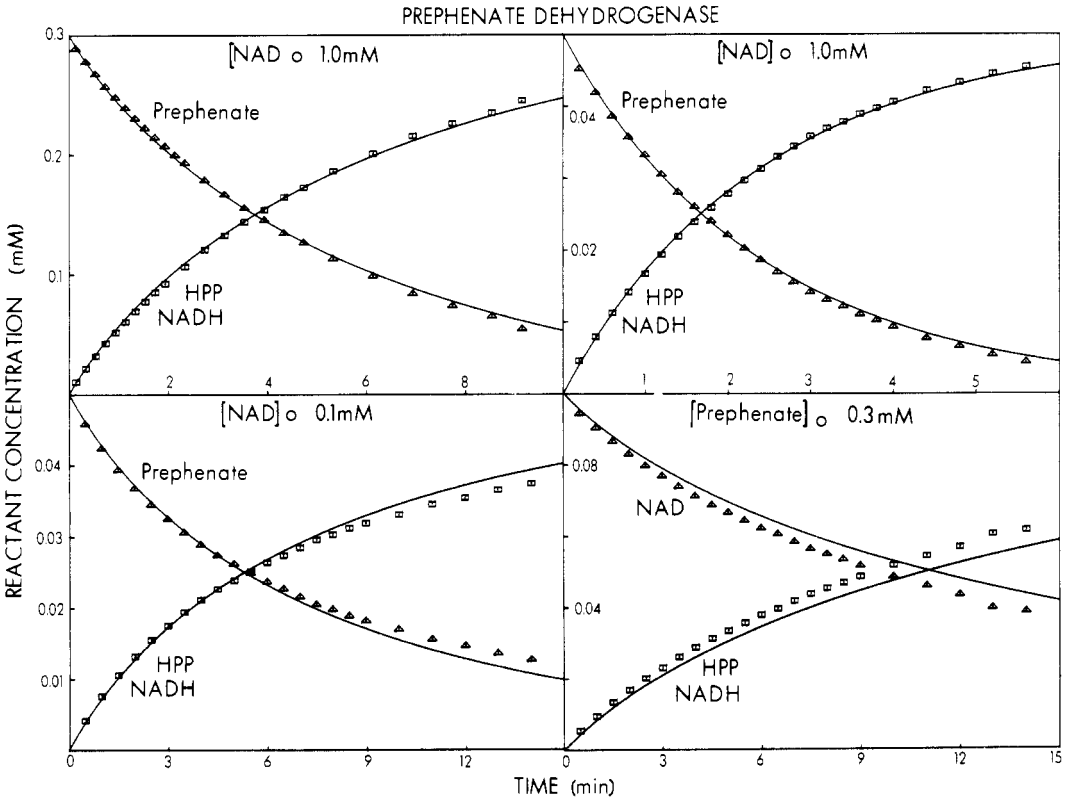

FIGURE 2: Comparison of simulated and experimental time courses for the dehydrogenase reaction. The curves are simulated according to eq 4, and the points are experimental data. The amount of enzyme added per milliliter was 3.17 μg for NAD 1.0 mM and prephenate 0.3 mM; 1.59 μg for NAD 1.0 mM and prephenate 0.05 mM; 1.90 μg for NAD 0.1 mM and prephenate 0.05 mM; and 0.95 μg for NAD 0.1 mM and prephenate 0.3 mM. The maximum velocity of the reaction was 28.1 (μmol/min)/mg of enzyme.

sonable agreement with the data. In the latter case, when there is no allowance for direct conversion of a proportion (X) of chorismate to (hydroxyphenyl)pyruvate without release as prephenate ($X = 0$), the simulated rate is too low. By substituting various values for X, in order to fit the initial part of the NADH simulation to the data, it was found that a value

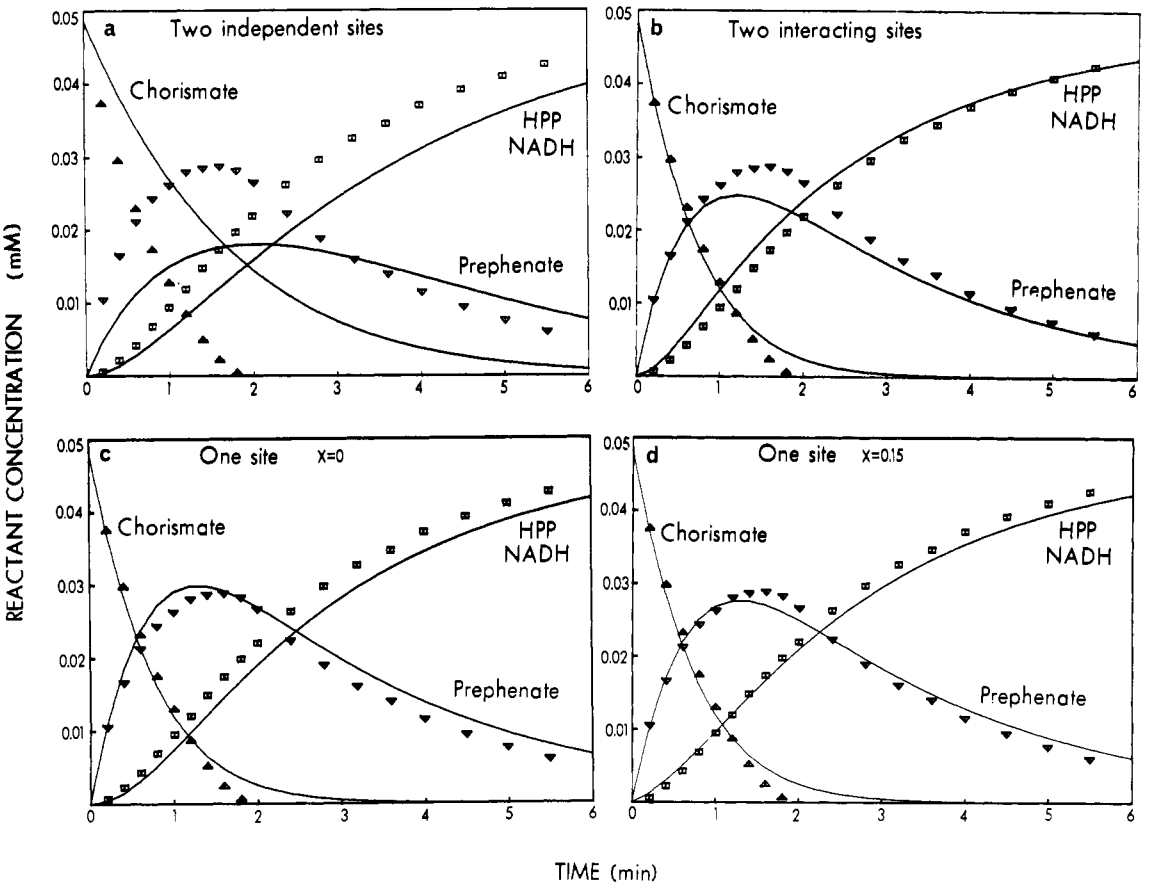

FIGURE 3: Comparison of experimental data for the time course of the overall reaction with simulations according to different models. The initial concentration of NAD, 0.336 mM, was in excess of chorismate concentration, and the change in NAD concentration is not shown. The equations for the simulations are given in the text. The values for K_{ib}, K_{ic}, K_{ip}, and K_{iq} used in the simulation of the one-site model were the weighted means of the pairs of values in Table I. Enzyme was added at 3.17 μg/mL. The maximum velocities of the mutase and dehydrogenase reactions were independently determined to be 29.2 and 23.3 (μmol/min)/mg of enzyme, respectively.

of 0.15 gave a precise fit at the 1-min time period. This corresponds to the conversion of 15% of the chorismate in such a way that the molecule could take part in the dehydrogenase reaction without first being released as prephenate.

When chorismate is present in excess over NAD (Figure 4), the model for two interacting sites predicts a rate of NADH production significantly in excess of that observed, although good agreement was observed between the simulated and experimental mutase time courses. In contrast, the model for one site without direct conversion of chorismate to (hydroxyphenyl)pyruvate ($X = 0$) predicts a rate which is significantly slower than that observed. A value of 0.33 for X gives an exact fit of the simulation to the data at the 1-min time period; however, the simulated rate in the later stages of the time course remains rather slower than the observed rate. In this connection, the simulations of the one-site model in Figures 3 and 4 were made by using the weighted mean value for each of those kinetic constants that were determined in more than one way (Table I); if instead the highest value is used in each case, a closer fit of the simulated and experimental curves is obtained.

Initial Rate of NADH Production from Chorismate and NAD. Under a variety of conditions used in studying the overall reaction, the initial rate of NADH production was always significant. Higher concentrations of chorismate and NAD^+ than those in Figures 3 and 4 could be used if the dehydrogenase reaction alone was to be observed. The initial rate at 1 mM chorismate and 1 mM NAD^+ was apparently linear for at least 2 min, showing a slight increase after this period, and the recording could be linearly extrapolated back to the time at which enzyme was added. From Table II it can be seen that 1 mM chorismate in the absence of prephenate gives rise to an initial dehydrogenase rate approximately two-thirds of that observed with 0.25 mM prephenate in the absence of chorismate. Calculation and experimentation demonstrate that the initial rate with 1 mM chorismate is approximately the same as that given by 0.1 mM prephenate. However, the concentration of prephenate produced from chorismate in the first 10 s of the mutase reaction could only be one-fiftieth, approximately, of this level. Table II also shows that the addition of 1 mM chorismate in the presence of 0.25 mM prephenate does not inhibit the dehydrogenase rate; rather, a small increase is observed. A similar increase resulted when the assays were conducted in the presence of 1 mg/mL bovine serum albumin, under which conditions the concentrations of all three reactants were approximately 1 order of

Table II: Initial Velocity of NADH Production with High Concentrations of Chorismate and/or Prephenate[a]

reactant concn (mM)		reaction velocity
chorismate	prephenate	[(μmol/min)/mg]
0.0	0.25	14.4
1.0	0.0	10.9
1.0	0.25	15.6
0.0	0.25[b]	20.5
1.0	0.25[b]	22.3

[a] Reactions were initiated with 0.67 μg of enzyme and followed by the increase in absorbance at 340 nm within 8 s of enzyme addition. The concentration of NAD was 1 mM. [b] Bovine serum albumin (1 mg/mL) was added.

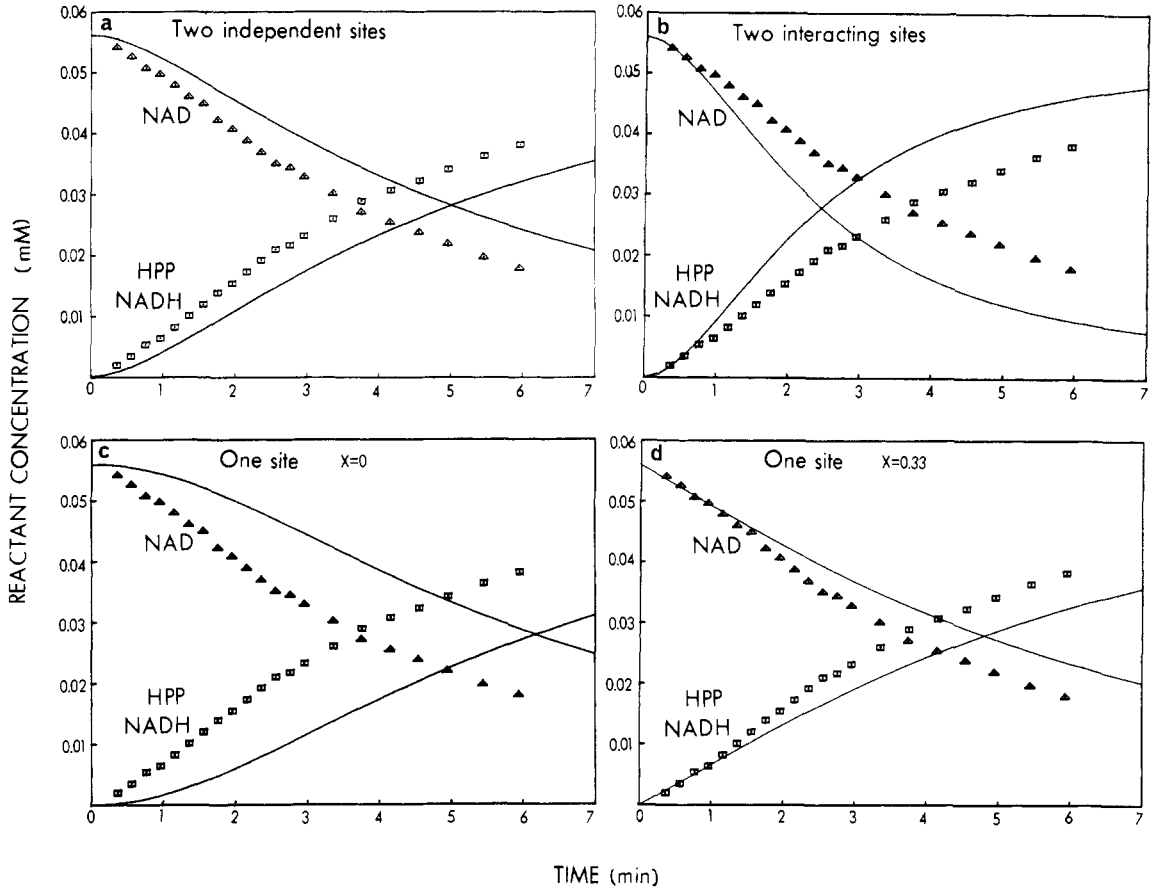

FIGURE 4: Comparison of experimental data for the time course of the overall reaction with simulations according to different models. The initial concentration of chorismate, 0.203 mM, was in excess of NAD concentration, and the change in chorismate concentration is not shown. The equations for the simulations are given in the text. The value of $K_{a'}$ was taken as 0.118 mM, equal to K_a. The values for K_{ib}, K_{ic}, K_{ip}, and K_{iq} used in the simulation of the one-site model were the weighted means of the pairs of values in Table I. Enzyme was added at 3.17 μg/mL. The maximum velocities of the mutase and dehydrogenase reactions were independently determined to be 29.2 and 23.3 (μmol/min)/mg of enzyme, respectively.

Table III: Transfer of Radioactivity from Chorismate to (Hydroxyphenyl)pyruvate[a]

expt	time (min)	reactant concn (μM)			cpm/mL in HPP		sp radioactivity of HPP (cpm/nmoL)	
		chorismate	HPP	prephenate	obsd	calcd	obsd	calcd
I	2.2	87.4	1.6	11.0	1434	1452		
II	2.2	91.6	5.3	103.1	546	172	103	32
	4.4	83.9	10.4	105.7	995	459	96	44

[a] Reaction mixtures initially contained 0.1 mM chorismate (specific radioactivity 900 cpm/nmol) (experiments I and II), 0.2 mM NAD^+, and, when present, 0.1 mM prephenate (experiment II). Reactions were initiated with 0.3 μg of enzyme. The calculated values for radioactivity in (hydroxyphenyl)pyruvate (HPP) are based on the assumption of full equilibration of the prephenate formed from chorismate with the bulk medium before it takes part in the dehydrogenase reaction. Calculations were performed as described under Theory.

magnitude greater than their dissociation or Michaelis constants (Heyde & Morrison, 1978a).

Transfer of Radioactivity from Chorismate to (Hydroxyphenyl)pyruvate. Experiments were designed to follow the movement of a ^{14}C label from chorismate to (hydroxyphenyl)pyruvate in the absence and presence of added unlabeled prephenate. The aim was to determine whether the prephenate formed from chorismate via the mutase reaction equilibrates fully with the pool of prephenate in the bulk medium before taking part in the dehydrogenase reaction. The counts appearing in (hydroxyphenyl)pyruvate are compared in Table III with values expected for full equilibration of prephenate with the medium; the initial velocities of the mutase and dehydrogenase reactions, directly measured on the same reaction mixture and used in the calculations, were 3.82 and 2.41 nmol/min, respectively. The counts transferred to (hydroxyphenyl)pyruvate in the presence of 0.1 mM prephenate are approximately twice those calculated for full equilibration of the first product, prephenate, with the bulk medium. The proportion (γ) of (hydroxyphenyl)pyruvate arising from chorismate without equilibrating with the prephenate pool was calculated, as described under Theory, to be 8% at 2.2 min and 6% at 4.4 min. Over three experiments conducted under similar conditions, the value of γ ranged between 6 and 11%.

Binding Studies. Each of the substrates prephenate and NAD^+ was bound to the enzyme in the absence of the other. The binding of NAD^+ was weak, with a dissociation constant of the order of 1 mM, and at the concentrations of enzyme obtainable (5–8 μM) could not be well determined. Labeled NAD^+ was converted in binding reaction mixtures to labeled NADH by the enzyme in the presence of 1 mM excess prephenate; under these conditions the binding of NADH to form a dead-end enzyme–prephenate–NADH complex was

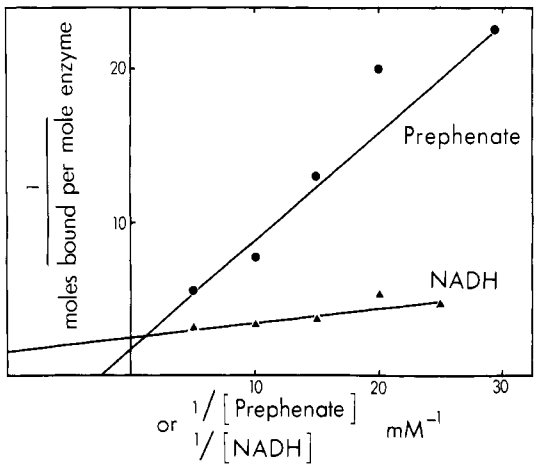

FIGURE 5: Binding of prephenate and NADH to chorismate mutase–prephenate dehydrogenase. Determinations were made by using 0.88 nmol of enzyme of specific activity 26 mutase units. Equal variance was assumed for fitting the data. The binding of NADH was measured in the presence of 1 mM prephenate.

measured. The weighted mean of four experimental estimates of the dissociation constant was 0.040 ± 0.009 mM, which is in reasonable agreement with the kinetic dissociation constant for the same reaction, 0.023 ± 0.002 mM (Table I).

The binding of prephenate to free enzyme was stronger than that of NAD^+ but weaker than that of NADH. The weighted mean of four experimental estimates of the dissociation constant for prephenate was 0.40 ± 0.06 mM, which is comparable with the kinetic dissociation constant (Table I).

From pairs of binding experiments, each pair performed at the same time with the same enzyme preparation, a comparison can be made of the number of moles of prephenate and NADH bound per mole of enzyme (M_r 76 000). The weighted means of four values for each reactant, obtained by fitting data to eq 1 by assuming equal variance, were 0.39 ± 0.03 mol of NADH and 0.55 ± 0.07 mol of prephenate per mol of enzyme. A typical pair of plots is shown in Figure 5. If the data were analyzed without assuming equal variance, the standard errors of the parameters for prephenate increased by a factor of approximately 3, although those for NADH were not significantly affected. The specific activities of the enzyme preparations used were approximately 25 (μmol/min)/mg of enzyme in the standard mutase assay.

Inactivation Studies. The effects on the enzyme under a variety of inactivating conditions are shown in Table IV. It can be seen that the mutase and dehydrogenase activities disappear in parallel under all conditions tested. Prephenate protects the enzyme against inactivation by iodoacetamide, and the dissociation constant for the enzyme–prephenate complex has been determined from the protective effect. In the absence or presence of prephenate, the inactivation of the enzyme by iodoacetamide is an apparent first-order reaction, and the results of Figure 1 show that the effects on the mutase and dehydrogenase reactions are indistinguishable. The apparent first-order rate constants for the inactivation reaction at different concentrations of prephenate (k') are given by the slopes of the lines in Figure 6. Assuming that the rate of inactivation is proportional to the concentration of free enzyme

$$k' = kE_t/(1 + B/K_{ib})$$

where E_t is the total enzyme concentration, B is the concentration of prephenate, and K_{ib} is the dissociation constant for the enzyme–prephenate complex. A value of 0.32 ± 0.07 mM for K_{ib} was obtained from the horizontal intercept of a plot of $1/k'$ vs. the concentration of prephenate. This estimate

Table IV: Inactivation of Chorismate Mutase–Prephenate Dehydrogenase[a]

inactivating conditions		% original act. remaining	
		mutase	dehydrogenase
pH 2.2	20 °C, 15 min	35	38
heat (45 °C)	20 min	58	60
urea (3 M)	0 °C, 2 h	20	26
pyridoxal phosphate (1 mM)[b]	30 °C, 30 min	59	68
carboxypeptidase A (40 μg/mL)	37 °C, 1 h	74	64
iodoacetamide (1 mM)[c]	30 °C, 30 min	23	24
dithio(bisnitrobenzene) (6 μM)[c,d]	30 °C	5	5

[a] The concentration of enzyme in each of the inactivating reaction mixtures was approximately 3 μM. Assays were initiated with up to 20 μL of partially inactivated enzyme and performed as described under Methods. [b] All parts of this experiment were performed in 0.1 M *N*-ethylmorpholine buffer, pH 7.5, instead of Tris-HCl. [c] Inactivation and assay were performed in the absence of dithiothreitol. [d] The reaction with the enzyme was monitored at 410 nm until complete, before assaying.

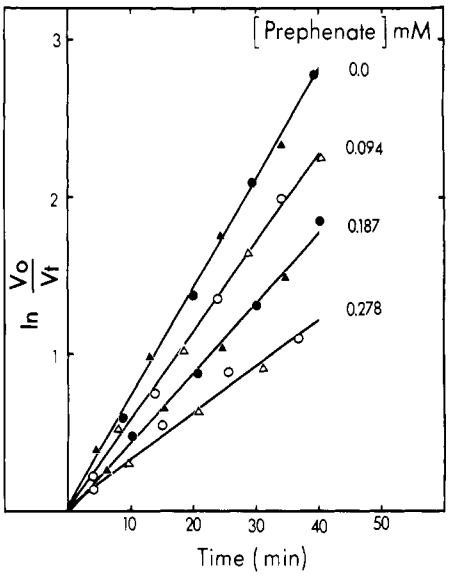

FIGURE 6: Protection by prephenate against inactivation of chorismate mutase–prephenate dehydrogenase by iodoacetamide. The concentration of enzyme in the incubation with 5 mM iodoacetamide at 30 °C was 246 μg/mL. Mutase (△) and dehydrogenase (○) activities were determined as described under Methods. V_0 and V_t are the enzyme reaction velocities at zero time and time t, respectively.

is similar to other estimates of K_{ib} from binding studies and steady-state kinetics (Table I).

Discussion

The results of Figures 1 and 2 demonstrate that it is possible to simulate each of the mutase and dehydrogenase reactions successfully by using models based on the reaction mechanisms determined by steady-state kinetic investigations.

In considering the fit of more complex simulations of the overall reaction to experimental time courses, it is clear that the model for two separate and independent sites does not fit the data (Figures 3 and 4). The lack of fit demonstrates a significant effect of having both activities vested in one protein, unlike the situation that appears to apply for chorismate mutase–prephenate dehydratase (Duggleby et al., 1978).

The model for two separate but interacting sites fits reasonably well when NAD concentration is high relative to chorismate concentration (Figure 3) but not when chorismate

concentration is high relative to NAD concentration (Figure 4). Allowance has been made in both instances for the directly determined enhancement of the combination of NAD by chorismate (Heyde & Morrison, 1978a) and also for a postulated inhibitory combination of chorismate at the prephenate-binding part of the dehydrogenase site, by analogy with the known inhibitory combination of prephenate at the mutase site (Heyde & Morrison, 1978a). A time course predicted for this model under the conditions of Figure 4 lies twice as far above the observed time course if the postulated inhibitory combination of chorismate does not occur. Furthermore, there is evidence that, if there are separate sites for the two reactions, prephenate is channeled between them. Thus, in the absence of prephenate the production of NADH from chorismate plus NAD proceeds at a linear rate much greater than can be accounted for by the prephenate produced from chorismate within the first few seconds of mutase reaction, and the experiments of Table III demonstrate channeling directly. Such channeling has not been allowed for in Figure 4b, and if it were, the simulated time course would lie even further above the data.

In theory, a better fit to the observed data in Figure 4b could be attained by varying the values of parameters used, e.g., by lowering inhibition constants and/or raising substrate constants, but such values would be quite inconsistent with those determined directly (Table I). The fit could also be adjusted by letting chorismate be more inhibitory at the dehydrogenase site, with K_i value 1 order of magnitude lower than its K_m value, although this might seem a rather unsatisfactory phenomenon for the organism.

Thus, if a two-site model applies, the simulations indicate that either (a) chorismate combines much more strongly as an inhibitor at the dehydrogenase site than it does as a substrate at the mutase site or (b) the rate of the dehydrogenase reaction is decreased by the presence of chorismate at the mutase site. In either case chorismate would be expected to cause inhibition of the dehydrogenase rate. However, it has not been possible to detect inhibition under any experimental conditions; the results of Table III show that the addition of chorismate always causes an increase in the dehydrogenase rate even when its concentration is high relative to prephenate. It therefore seems unlikely that the mutase and dehydrogenase reactions occur at two separate sites, although the possibility can not be definitely ruled out.

In the model for a single active site, chorismate would be converted to prephenate on the part of the dehydrogenase site that binds prephenate, adjacent to the NAD binding site. Figures 3c and 4c show that the observed rate for the production of NADH is faster than the rate simulated according to this model when no allowance is made for the conversion of chorismate in situ to a substrate for the dehydrogenase reaction which might either be released or converted to (hydroxyphenyl)pyruvate. Hence, it is clear that, if a one-site model applies, there must be some conversion of chorismate to (hydroxyphenyl)pyruvate without the intermediate release of prephenate, in agreement with the experiments of Tables II and III. Allowance for this (Figures 3d and 4d) both increases the simulation rate to a level comparable with the experimental data and eliminates the lag in NADH production, seen particularly in Figure 4c, which is not observed experimentally. The proportion of chorismate converted directly through to (hydroxyphenyl)pyruvate might be expected to change during the time course of the reaction with the concentration of NAD. Therefore, the values of 15–33% determined in Figures 3 and 4 to be necessary to produce a fit to the experimental data at a particular time period would only be approximate values. The magnitude of the directly observed channeling, 6–11%, is quantitatively consistent with the one site model simulations.

The results of inactivation studies (Table IV) show that the mutase and dehydrogenase activities disappear in parallel under a variety of conditions. This is consistent with a one-site hypothesis but could also be observed if there were two separate but similar sites. The protection afforded by prephenate against inactivation by iodoacetamide confirms earlier kinetic indications that the dissociation constants for the interactions of prephenate as an inhibitor of the mutase or as a substrate of the dehydrogenase are indistinguishable (Heyde & Morrison, 1978a). This is of course required by the one-site hypothesis, since the same interaction would be involved. The result is consistent with the demonstration by Koch et al. (1972) on chorismate mutase–prephenate dehydrogenase from *Escherichia coli* that a single group appears to be concerned with inactivation of both enzymic functions by iodoacetamide and their protection by prephenate.

The above discussion indicates that it is not necessary to postulate two separate sites for the mutase and dehydrogenase activities; indeed, there are several observations that appear inconsistent with a two-site model. It should be pointed out that there are also two observations that do not obviously concur with the one-site hypothesis and could be more simply explained in terms of two separate sites. First, although the values for the dissociation constants for NAD and prephenate are in agreement when determined by means of the mutase or dehydrogenase reactions, there is a threefold discrepancy between the corresponding values for (hydroxyphenyl)pyruvate (Table I). Second, the results of the present binding studies indicate that there could be more moles of prephenate bound per mole of enzyme than there are of NADH. Nevertheless, it is feasible to consider each of these discrepancies in terms of a one-site model.

The discrepancy between values for (hydroxyphenyl)pyruvate could arise because of its structural resemblance to the end-product inhibitor, tyrosine, which causes nonlinear double-reciprocal plots of kinetic data (Heyde & Morrison, 1978b). It has been noted previously (Heyde & Morrison, 1978a) that a number of compounds resembling tyrosine cause similar kinetic effects, particularly with NAD^+ as the varied substrate. Nonlinearity of such plots was not obvious in the presence of (hydroxyphenyl)pyruvate, but it is possible that the rate equation on which calculations of the inhibition constants for this product were based is an oversimplification. Thus, the value for the dissociation constant for (hydroxyphenyl)pyruvate, determined from the dehydrogenase reaction, may not represent the true constant. A similar resemblance between tyrosine and prephenate may be relevant to the interpretation of the binding results; more moles of prephenate than NAD^+ would be bound if prephenate binds at an allosteric site as well as at the active site. However, evidence for a kinetically irrelevant binding of prephenate with a dissociation constant of approximately 2 mM has come from fluorescence quenching studies on chorismate mutase–prephenate dehydrogenase from *E. coli* (E. Heyde, unpublished experiments). The absolute number of moles of all ligands bound per mole of enzyme was unexpectedly low, and it is possible that the enzyme preparations do not have maximal activity or binding properties.

Molecular orbital calculations (P. R. Andrews and R. D. Haddon, unpublished experiments) have shown that the transition states for the mutase and dehydrogenase reactions

are strikingly similar. Thus, if NAD is present adjacent to the site at which the mutase transition state is formed, the dehydrogenase reaction may occur by direct interaction with the transition state. However, only a minor proportion of the overall reaction would occur by this route, as deduced from the simulation studies.

The occurrence of significantly different reactions at the same active site is uncommon (Kirschner & Bisswanger, 1976), although ribulose-1,5-bisphosphate carboxylase–oxygenase appears to be an example (Jensen & Bahr, 1977). Other possible examples might more reasonably be considered to result from broad specificity rather than from the ability to catalyze two dissimilar reactions (Francis et al., 1973; Nordlie, 1974; Ikura et al., 1976). The evolution of multifunctional enzymes has been postulated to occur by a process of gene fusion, so that the different activities might be expected to occur at separate sites in discrete regions of the polypeptide chain. Such an arrangement might well be obligatory if the reactions catalyzed are complex and chemically dissimilar. However, the chorismate mutase reaction is not complex, but proceeds readily even without the enzyme. Andrews et al. (1973) have postulated, from a comparison of the enzyme enhancement of the reaction rate and the calculated energy difference between different conformations of chorismate, that the catalytic action of the enzyme may be due to selective binding of the diaxial conformation of chorismate, which rearranges spontaneously to form prephenate. Chorismate mutase–prephenate dehydrogenase would indeed be a protein of economical design if NAD were bound alongside the mutase site so that it were possible for dehydrogenation to occur simultaneously with the mutase reaction. At this stage the evidence for the enzyme from *A. aerogenes* appears to favor such a structure. Andrews & Heyde (1979) have recently proposed a theoretical model for the interaction of substrates and transition states at a common active site.

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