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Kinetic Studies on the Reactions Catalyzed by Chorismate Mutase–Prephenate Dehydrogenase from *Aerobacter aerogenes*†

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ABSTRACT: Steady-state kinetic techniques have been used to investigate each of the reactions catalyzed by the bifunctional enzyme, chorismate mutase-prephenate dehydrogenase, from *Aerobacter aerogenes*. The results of steady-state velocity studies in the absence of products, as well as product and dead-end inhibition studies, suggest that the prephenate dehydrogenase reaction conforms to a rapid equilibrium random mechanism which involves the formation of two dead-end complexes, viz., enzyme-NADH-prephenate and enzyme-NAD^+-hydroxyphenylpyruvate. Chorismate functions as an activator of the dehydrogenase while both prephenate and hydroxyphenylpyruvate acted as competitive inhibitors in the mutase reaction. By contrast, both NAD^+ and NADH function as activators of the mutase. Values of the kinetic parameters associated with the mutase and dehydrogenase reactions have been determined and the results discussed in terms of possible relationships between the catalytic sites for the two reactions. The data appear to be consistent with the enzyme having either a single site at which both reactions occur or two separate sites which possess similar kinetic properties.

Chorismate mutase-prephenate dehydrogenase from *Aerobacter aerogenes* (Koch et al., 1970a) is a bifunctional enzyme which catalyzes the reactions:

$$\text{chorismate} \xrightarrow[(1)]{} \text{prephenate}$$

$$\xrightarrow[(2)]{NAD^+} \text{4-hydroxyphenylpyruvate} + NADH + CO_2$$

Reaction 1 is catalyzed by chorismate mutase and reaction 2 by prephenate dehydrogenase. Both reactions are essentially irreversible. The enzyme has been reported to contain two very similar or identical subunits and to have a molecular weight of approximately 76 000 (Koch et al., 1970a,b).

Up to the present time, no clear indications have emerged as to what, if any, advantages have been conferred on microorganisms as a result of their possessing bifunctional enzymes which are composed of a single type of polypeptide chain and which catalyze sequential reactions (cf. Kirschner & Bisswanger, 1976). Further, studies on this type of enzyme have not been sufficiently extensive to determine if such bifunctional enzymes possess one or two catalytic sites and if channeling occurs between two separate sites. Attempts have been made to elucidate the relationship between the sites responsible for the mutase and dehydrogenase activities of chorismate mutase-prephenate dehydrogenase from *A. aerogenes* and *Escherichia coli* (Koch et al., 1972) but the findings were not definitive.

It appeared that kinetic investigations to determine the mechanism of and parameters associated with each reaction catalyzed by the enzyme from *A. aerogenes* might yield information relevant to these problems. Thus, it seemed that an answer to the question of the number of active sites might come from a comparison of the magnitudes of those kinetic constants which could be determined from kinetic studies on both the mutase and dehydrogenase reactions. Toward this end, investigations have been made of the inhibition of the mutase by prephenate and hydroxyphenylpyruvate as well as its activation by NAD^+ and NADH.[1] In addition, the kinetic mechanism of the dehydrogenase reaction has been determined and shown to be of the rapid equilibrium random type. From a comparison of the dissociation constants for the combinations of prephenate and hydroxyphenylpyruvate with free enzyme, as determined from investigations on the mutase and dehydrogenase reactions, it is concluded that the enzyne possesses either a single site or two separate sites with kinetic similarities.

Experimental Procedure

Materials

Protamine sulfate, *p*-phenylmethylsulfonyl fluoride, and dithiothreitol were obtained from Calbiochem. Hydroxylapatite was from Bio-Rad and cyanogen bromide activated Sepharose 4B from Pharmacia. Tris(hydroxymethyl)aminomethane (Tris) from Sigma was used during the enzyme purification but that used for kinetic studies was from Merck. AMP, NAD^+, and NADH were supplied by P-L Biochemicals, and 4-hydroxyphenylpyruvic acid, crystallized and lyophilized bovine serum albumin, and *cis*-aconitic acid by Sigma. All other commercial reagents were of the highest quality available.

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[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced NAD; EDTA, (ethylenedinitrilo)tetraacetic acid.

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Methods

Preparation of Chorismate and Prephenate. Aerobacter aerogenes 62-1 was used to produce chorismic acid which was isolated and recrystallized by the method of Gibson (1968). Sodium prephenate was prepared from chorismate by the enzymatic action of chorismate mutase–prephenate dehydrogenase (Dudziński & Morrison, 1976).

Preparation of AMP–Sepharose Column. N^6-(6-Aminohexyl)AMP was prepared and coupled to cyanogen bromide activated Sepharose 4B in 0.1 M Na_2CO_3, pH 9.5, as reported by Trayer et al. (1974). The column was washed with two cycles of 0.1 M sodium acetate buffer, pH 4.0, containing 0.5 M NaCl followed by 0.1 M Tris-HCl buffer, pH 8.0, containing 0.5 M NaCl. Phosphate analyses indicated that the concentration of ligand coupled to the gel was approximately 4 μmol/mL of wet packed Sepharose.

Preparation of the Keto Form of 4-Hydroxyphenylpyruvate Solutions. Hydroxyphenylpyruvic acid was dissolved at room temperature in 0.02 M Tris-HCl buffer, initially at pH 7.5, and the pH was adjusted back to pH 7.5 with 0.01 N NaOH. Solutions were then frozen overnight at −15 °C after which they were thawed at room temperature, kept on ice, and used within 24 h. The hydroxyphenylpyruvate was present in the keto form as judged from its absorption spectrum (Knox & Pitt, 1957). The above procedure was adopted to avoid the decomposition of hydroxyphenylpyruvate under alkaline conditions (Doy, 1960) to hydroxybenzaldehyde as well as other unknown derivatives which were more inhibitory than hydroxyphenylpyruvate.

Measurement of Enzyme Activity. The chorismate mutase activity of fractions obtained during the course of enzyme purification was determined at 30 °C by stopped-time assay. The prephenate formed from chorismate was converted with HCl to phenylpyruvate whose absorbance was measured under alkaline conditions at 320 nm (Koch et al., 1970a). Reaction mixtures contained, in 0.4 mL, 50 mM Tris-HCl buffer (pH 7.5), 2.5 mM chorismate, 0.5 mM EDTA, and 0.5 mM dithiothreitol. For the kinetic experiments with the mutase, the concentration of the Tris-HCl buffer was increased to 100 mM (pH 7.5) and the concentrations of both EDTA and dithiothreitol were increased to 1.0 mM. The disappearance of chorismate was measured spectrophotometrically at either 273 or 290 nm. The latter wavelength was used when pyridine nucleotides were present, because of their high absorbance at 273 nm. The molar extinction coefficients for chorismate were taken to be 2630 and 1850 at 273 and 290 nm, respectively.

Prephenate dehydrogenase activity was measured spectrophotometrically at 30 °C by following at 340 nm the formation of NADH from NAD^+ in the presence of prephenate. In calculating reaction velocities, allowance was made for the absorbance due to hydroxyphenylpyruvate by using a molar extinction coefficient of 6400 for the products, rather than 6220 for NADH alone. All reaction mixtures contained 100 mM Tris-HCl buffer (pH 7.5), 1.0 mM EDTA, and 1.0 mM dithiothreitol. The activities of fractions obtained during the course of enzyme purification were determined in the presence of 2.0 mM NAD^+ and 0.25 mM prephenate.

Kinetic studies were performed by using either a Cary 118 or a Varian 635 spectrophotometer. The latter was modified by Dr. K. J. Ellis so that a reaction could be monitored at two wavelengths with independent adjustable back-off and variable scale expansion at each wavelength. Thus, it was possible to follow in the same sample both the mutase and dehydrogenase reactions when chorismate and NAD^+ were present in reaction mixtures. Under these conditions, it was necessary to allow for the change in absorbance at 290 nm due to the dehydrogenase reaction, in order to calculate steady-state rates for the mutase reaction. The molecular extinction coefficient at 290 nm for the conversion of dehydrogenase substrates to products was determined to be 2530, and the changes in absorbance at 290 nm because of the mutase reaction were calculated from the relationship:

$$\Delta(\text{chorismate}) = -\Delta E_{290} + \Delta E_{340} \times \frac{2530}{6400}$$

One unit of activity is defined as the amount of enzyme that produces 1 μmol of product/min at 30 °C.

It should be noted that some results were obtained with enzyme preparations for which the specific activities were not maximal but which were free of interfering enzyme activities. Reaction velocities were proportional to enzyme concentration under all conditions.

Estimation of Protein. The protein concentrations of cell extracts and protamine sulfate fractions were estimated by the Biuret method using bovine serum albumin as a standard. The protein content of more highly purified fractions was determined from the absorbance of solutions at 280 nm. It was assumed that a solution containing protein at a concentration of 1 mg/mL has an absorbance of 1.0 in cells with 1-cm light path, and this relationship was confirmed for the purified enzyme using the Biuret method (cf. Koch et al., 1970a).

Analysis of Data. Data were first plotted graphically to check the linearity of double-reciprocal plots and to determine the patterns of the plots. An overall fit of each set of data was then made to the appropriate rate equation by using one of the computer programs of Cleland (1963) in conjunction with a Univac 1100/42 computer. In the absence of products, velocities were fitted to the equations:

$$v = \frac{VA}{K_a + A} \tag{1}$$

or

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \tag{2}$$

while competitive, noncompetitive, and uncompetitive inhibition data were fitted to eq 3, 4, and 5, respectively:

$$v = \frac{VA}{K(1 + I/K_i) + A} \tag{3}$$

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

$$v = \frac{VA}{K + A(1 + I/K_{ii})} \tag{5}$$

Data for slope-hyperbolic, intercept-hyperbolic activation were fitted to eq 6:

$$v = \frac{VA(1 + I/K_{id})}{K(1 + I/K_{is}) + A(1 + I/K_{in})} \tag{6}$$

The values of the parameters obtained from the computer analyses were used to draw the lines in the figures.

Organism and Growth of Cells. Aerobacter aerogenes (poly 3) is a multiple aromatic amino acid auxotroph (Koch et al., 1970a). Cells were grown in 40-L batches in glucose–mineral salts medium supplemented with tryptophan and phenylalanine, as well as limiting amounts of tyrosine (Koch et al., 1970a). Cells were harvested after 11.5 h, rather than after 16 h, because of the tendency for reversion to occur when the cells were kept for longer periods in the stationary phase.

TABLE I: Yields and Specific Activities of Fractions Obtained during the Course of Purification of Chorismate Mutase-Prephenate Dehydrogenase.[a]

Purification step	Vol (mL)	Protein (mg)	Total act. (units)	Sp act. (units/mg of protein)	Yield (%)	Act. ratio (mutase/dehydrogenase)
(1) Crude cell extract	400	8760	1132	0.13	100	0.82
(2) Protamine sulfate treated extract	455	5960	1037	0.17	92	0.81
(3) Reverse $(NH_4)_2SO_4$ fractionation & dialysis	90	900	543	0.60	48	0.91
(4) AMP-Sepharose col	42	40	270	6.8	24	0.78
(5) Hydroxylapatite col	33	5.3	175	33	15	0.81

[a] Weight of cells was 100 g. Activity is given as units of mutase activity.

Results

Purification of Enzyme. The purification of chorismate mutase-prephenate dehydrogenase has been reported previously (Koch et al., 1970a), but in our hands the procedure gave a preparation which had a specific activity of only 3-4 units/mg of protein and which was markedly heterogeneous on polyacrylamide gel electrophoresis. Thus, it was necessary to develop a new purification procedure, and details of the method are given below.

Extraction of Cells. Cells (100 g) which had been stored at −15 °C were thawed and suspended in 400 mL of 100 mM Tris-HCl buffer (pH 7.5) containing 1.0 mM EDTA, 1.0 mM dithiothreitol, and 0.1 mM phenylmethanesulfonyl fluoride. The latter compound was dissolved in 2-propanol to a concentration of 10 mg/mL, and an aliquot of 2 μL/mL of buffer mixture was added immediately before use. The cells were disrupted in a Ribi cell fractionator at 20 000 psi and cell debris was removed by centrifuging for 30 min at 23 000*g*. All further steps were performed at 4 °C.

Removal of Nucleic Acid. To 4 volumes of extract was added 1 volume of protamine sulfate (2%, w/v) in 100 mM Tris-HCl buffer (pH 7.5) containing 1.0 mM EDTA and 1.0 mM dithiothreitol. The addition was made dropwise and with stirring, after which stirring was continued for a further 30 min. The precipitate was removed by centrifuging for 20 min at 23 000*g*.

Reverse Ammonium Sulfate Fractionation. Solid ammonium sulfate (32.8 g/100 mL) was added with stirring to the supernatant from the previous step over a period of 15 min. The suspension was stirred for a further 30 min and then centrifuged for 20 min at 10 000*g*. The precipitate was repeatedly extracted with solutions containing 0.1 M Tris-HCl (pH 7.5), 1.0 mM EDTA, 1.0 mM dithiothreitol, and decreasing proportions of a saturated solution of ammonium sulfate. The latter solution was prepared at room temperature (20 °C), and before use, 1 M NH_4OH was added until the directly measured pH was 7.5. Extraction was made firstly with 25 mL of buffer solution containing 40% ammonium sulfate (v/v) and the precipitate was collected by centrifuging for 15 min at 17 000*g* and then reextracted successively with 10 mL of buffer solution containing 37.5, 35, 30, 27.5, and 25% (v/v) ammonium sulfate. Fractions containing enzyme with a specific activity greater than 0.3 units/mg of protein (usually the 35-27.5% ammonium sulfate extracts) were pooled, and the enzyme was completely precipitated by the addition over 5 min of solid ammonium sulfate (17 g/100 mL). The precipitate was collected by centrifugation and stored at −15 °C where it was stable for many weeks.

Chromatography on AMP-Sepharose. The precipitate from the previous step was dissolved in 70 mL of 0.1 M Tris-acetate buffer (pH 6.0) containing 1.0 mM EDTA and 1.0 mM dithiothreitol, and the same buffer was used throughout this chromatographic fractionation. After dialysis of the enzyme solution overnight against 3 L of buffer, it was clarified by centrifuging for 10 min at 12 000*g*, diluted with buffer to a protein concentration of 10 mg/mL, and applied to a column (2.6 × 13 cm) of AMP-Sepharose which had been equilibrated with buffer. The column was washed with 70 mL of buffer and the enzyme eluted with a linear NaCl gradient from 0 to 0.5 M. The gradient was prepared in buffer and had a total volume of 300 mL. The enzyme was eluted at about 0.3 M NaCl, and those fractions with a specific activity greater than 3 units/mg of protein were pooled.

Chromatography on Hydroxylapatite. The pooled enzyme from the previous step was applied to a column (2.6 × 12 cm) of hydroxylapatite which had been equilibrated with 0.1 M Tris-acetate buffer (pH 6.0) containing 1.0 mM EDTA, 1.0 mM dithiothreitol, and 0.3 M NaCl. After washing the column with 70 mL of the same buffer, the enzyme was eluted with a linear phosphate gradient from 0.03 to 0.1 M. The gradient was prepared in 0.1 M Tris-HCl buffer containing 1.0 mM EDTA and 1.0 mM dithiothreitol, and the pH of each solution (200 mL) was 7.5. Enzyme was eluted at about 0.05 M phosphate. A summary of the yields and specific activities of fractions obtained during the course of enzyme purification is given in Table I.

Storage of Enzyme. After the addition of sodium azide to a concentration of 0.02%, aliquots of the purified enzyme were placed in vials, snap frozen in liquid nitrogen, and stored at −15 °C. At least 1 day before use enzyme solutions were thawed rapidly in a water bath (30 °C) and then kept at 4 °C. Subsequently, it was found that the addition of glycerol (50%, v/v) stabilized the enzyme during storage at 4 °C.

The stability of the enzyme was poor if the protein concentration was less than 0.1 mg/mL. However, concentration of the enzyme could be achieved by reapplying the enzyme to a small column (0.9 × 3.0 cm) of AMP-Sepharose under the conditions described above (pH 6.0) and eluting with 0.1 M Tris-HCl buffer (pH 7.5) containing 1.0 mM EDTA and 1.0 mM dithiothreitol.

Properties of the Enzyme. The ratio of the mutase and dehydrogenase activities remained essentially constant throughout a single preparation (Table I), but the ratio varied from 0.8 to 1.2 between preparations. On polyacrylamide gel electrophoresis the final product showed a single, rather diffuse

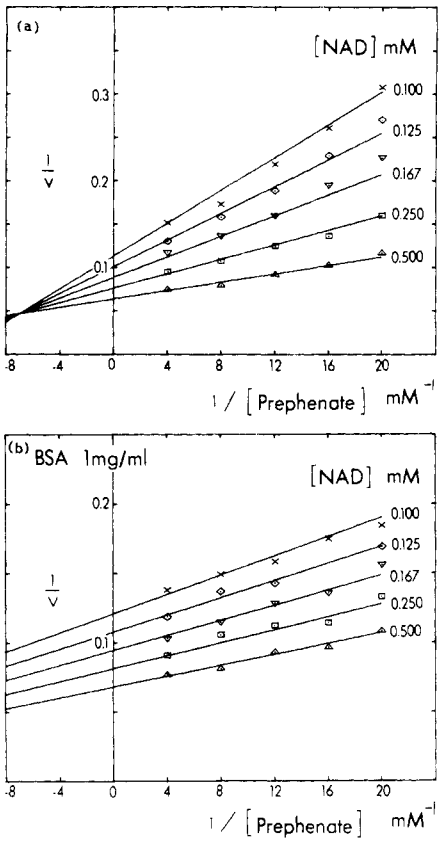

FIGURE 1: Effect of the concentrations of NAD^+ and prephenate on the steady-state velocity of the prephenate dehydrogenase reaction in the absence (a) and presence (b) of bovine serum albumin (BSA, 1 mg/mL). The data were fitted to eq 2. Velocities are expressed as micromoles of NADH formed per milligram of enzyme per minute.

TABLE II: Kinetic Constants for the Interaction of Substrates with Chorismate Mutase–Prephenate Dehydrogenase.[a]

Substrate	Kinetic constant	Value[b] (mM)	Reaction
Prephenate (B)	K_{ib}	0.17 ± 0.02[c] (0.028 ± 0.009)[d]	E + B
	K_b	0.030 ± 0.003 (0.029 ± 0.005)	EC + B
NAD^+ (C)	K_{ic}	0.72 ± 0.09 (0.12 ± 0.05)	E + C
	K_c	0.11 ± 0.01 (0.12 ± 0.01)	EB + C
Chorismate (A)	K_a	0.12 ± 0.01	E + A

[a] The values of the parameters for prephenate and NAD^+ were obtained by fitting steady-state velocity data, such as that of Figure 1, to eq 2. That for chorismate was obtained by fitting data to eq 1. [b] Values for prephenate and NAD^+ are the weighted means of values from five experiments. [c] Assuming that $(K_{ic}K_b)/K_c = K_{ib}$ for each experiment. [d] Values in parentheses were obtained from an experiment performed in the presence of bovine serum albumin (Figure 1b).

band which exhibited dehydrogenase activity (Koch et al., 1970a). A single band was also observed on isoelectric focusing and the p*I* value was 5.6. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Weber et al., 1972) revealed one major band, with a molecular weight of approximately 40 000, as well as a number (about nine) of minor bands exhibiting mobilities which were both faster and slower than the major band.

Kinetic Studies on the Dehydrogenase Reaction. The steady-state velocity pattern obtained in the absence of products is of the intersecting type (Figure 1a) and thus the reaction has a sequential mechanism. In the presence of bovine serum albumin, which functions as an activator of the dehydrogenase reaction, the kinetic mechanism is also sequential, but the lines intersect much further to the left of the vertical ordinate (Figure 1b). The maximum velocity of the reaction is not affected by albumin. Values for the kinetic parameters in the absence and presence of albumin are given in Table II, and it will be noted that activation arises through the lowering of the K_i values for the substrates while the Michaelis constants are not significantly affected. The mutase reaction was also activated by albumin, which caused a lower Michaelis constant

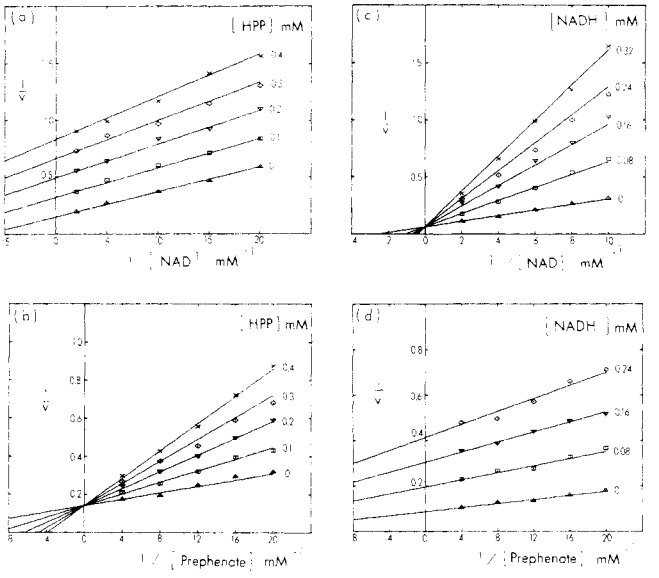

FIGURE 2: Product inhibition of the dehydrogenase reaction by 4-hydroxyphenylpyruvate (HPP) (a, b) and NADH (c, d): (a, c) NAD^+ varied and the prephenate concentration fixed at 0.05 mM; (b) prephenate varied and the NAD^+ concentration fixed at 0.1 mM; (d) prephenate varied and the NAD^+ concentration fixed at 0.2 mM. Velocities are expressed as micromoles of NADH formed per milligram of enzyme per minute.

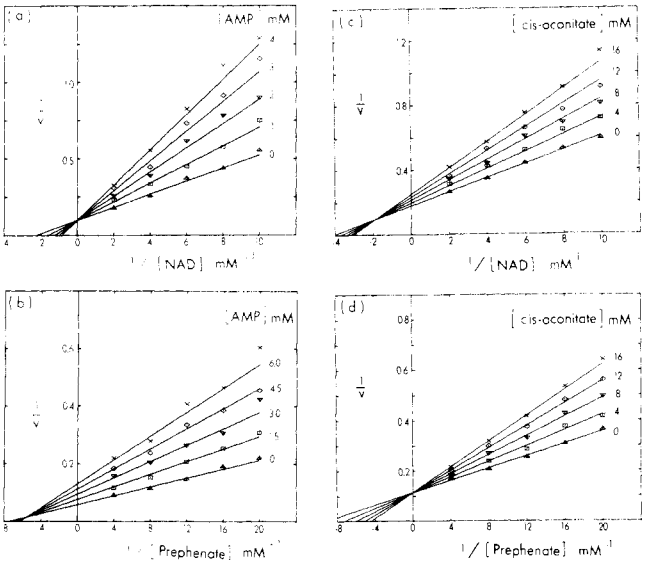

FIGURE 3: Dead-end inhibition of the dehydrogenase reaction by AMP (a, b) and *cis*-aconitate (c, d): (a, c) NAD^+ varied and the prephenate concentration fixed at 0.05 mM; (b) prephenate varied and the NAD^+ concentration fixed at 0.2 mM; (d) prephenate varied and the NAD^+ concentration fixed at 0.1 mM. Velocities are expressed as micromoles of NADH formed per milligram of enzyme per minute.

TABLE III: Inhibition of Prephenate Dehydrogenase by Products and Substrate Analogues.

Inhibitor	Substrate		Apparent K_i (mM)[a]		Kinetic dissoc. constant[c]	Value (mM)	React.
	Varied	Fixed	Slope	Intercept			
NADH (P)	Prephenate (B)	NAD^+ (C) (0.2 mM)	0.110 ± 0.019		K_{ip}	0.086 ± 0.015	E + P
				0.065 ± 0.004	K_{Ip}	0.023 ± 0.002	EB + P
	NAD^+ (C)	Prephenate (B) (0.05 mM)	0.059 ± 0.003 (0.053 ± 0.008)[b]				
4-Hydroxyphenylpyruvate (Q)	Prephenate (B)	NAD^+ (C) (0.1 mM)	0.12 ± 0.01 (0.17 ± 0.02)[b]				
	NAD^+ (C)	Prephenate (B) (0.05 mM)	0.57 ± 0.09		K_{iq}	0.44 ± 0.07	E + Q
				0.085 ± 0.003	K_{Iq}	0.032 ± 0.002	EC + Q
AMP (C*)	Prephenate (B)	NAD^+ (C) (0.2 mM)	3.6 ± 0.5		K_i	2.8 ± 0.4	E + C*
				4.9 ± 0.8	K_I	1.7 ± 0.3	EB + C*
	NAD^+ (C)	Prephenate (B) (0.05 mM)	2.3 ± 0.2 (2.4 ± 0.4)[b]				
cis-Aconitate (B*)	Prephenate (B)	NAD^+ (C) (0.1 mM)	15 ± 1 (12 ± 2)[b]				
	NAD^+ (C)	Prephenate (B) (0.05 mM)	16 ± 2		K_i	12.1 ± 1.3	E + B*
				33 ± 5	K_I	12.4 ± 2.1	EC + B*

[a] Values for the apparent inhibition constants were obtained by fitting the data to eq 3 or 4 according to whether the inhibition was competitive or noncompetitive. The values listed are the weighted mean of values from two experiments. [b] Values given in parentheses are apparent inhibition constants which were calculated for comparison with the directly determined value for the apparent K_i. Calculations were performed assuming a rapid equilibrium random reaction mechanism (eq 7) using the true dissociation constants obtained from the noncompetitive inhibitions with respect to the other substrate (values given in column 7 of this table) and the fixed concentration of the nonvaried substrate (cf. Morrison & James, 1965). [c] True values for the product inhibition and dead-end inhibition constants were determined from the apparent values by using the concentration of the nonvaried substrate and the relationships given by Morrison & James (1965) and Smith & Morrison (1969).

for chorismate but no change in the maximum velocity. Bovine serum albumin was not added routinely to reaction mixtures because of the intention to compare kinetically and thermodynamically determined dissociation constants. The latter values could well be difficult to obtain in the presence of albumin.

From the product-inhibition patterns (Figure 2), it is apparent that NADH acts as a linear competitive inhibitor with respect to NAD and as a linear noncompetitive inhibitor in relation to prephenate. Hydroxyphenylpyruvate causes linear competitive inhibition with respect to prephenate and linear noncompetitive inhibition with respect to NAD^+. A double-inhibition experiment, varying NADH (0–0.25 mM) at a number of fixed concentrations of hydroxyphenylpyruvate (0–0.3 mM), showed intercept variation but no slope variation. The product inhibition by bicarbonate was so weak that it was not feasible to use sufficiently high concentrations (above 0.2 M) to determine the inhibition patterns for CO_2.

AMP functions as a dead-end inhibitor, giving inhibitions which are linear competitive with respect to NAD and linear noncompetitive in relation to prephenate (Figure 3). Various compounds were tested as potential analogues of prephenate, but most caused double-reciprocal plots to become nonlinear with NAD^+, though not prephenate, as the variable substrate. Such compounds included dihydroxy derivatives of benzoic acid with substitutions in the 3,4, or 2,3, or 2,4 positions, phenylpyruvate, phenyllactate, as well as the 1-acetic, 1-carboxylic, and 1,3-diacetic derivatives of adamantane (cf. Andrews et al., 1977). By contrast, *cis*-aconitate behaves as a weak inhibitory analogue of prephenate, giving rise to dead-end inhibitions that are linear competitive in relation to this substrate and linear noncompetitive with respect to NAD^+. The kinetic constants associated with the product and dead-end inhibitions are listed in Table III.

Kinetic Studies on the Mutase Reaction. Double-reciprocal plots of the steady-state velocity as a function of the chorismate concentration were linear. However, with different preparations there was a twofold variation from 0.12 to 0.25 mM in the values obtained for the Michaelis constant of chorismate. The value of 0.12 ± 0.01 mM given in Table II appeared to be the most reliable. Product inhibition of the mutase reaction by prephenate was linear competitive as expected for a uni-uni reaction, and the value for the inhibition constant is listed in Table IV. Both hydroxyphenylpyruvate and *cis*-aconitate inhibited the mutase reaction, but the inhibition was weak. Indeed, it was possible to obtain only an indication of the magnitude of the inhibition constants by using a single concentration of each inhibitor and assuming that the inhibitions were linear competitive (cf. Table VI).

Activation of the Mutase by NAD^+ and NADH. Both pyridine nucleotides function as activators of the mutase (Figure 4) and the data fit well to eq 5 which describes slope-hyperbolic, intercept-hyperbolic activation. However, it will be noted that there is only a small variation in the intercepts of Figure 4, while both pyridine nucleotides cause considerable decreases in the slopes of the lines. The values of the kinetic constants derived from the data of Figure 4 are included in Table IV. The ratio of the maximum velocity attained in the presence of pyridine nucleotide to that in its absence was 1.2 ± 0.2 for NAD^+ and 1.3 ± 0.2 for NADH.

Effect of Chorismate on Dehydrogenase Activity. With prephenate at a concentration of 0.25 mM, which is about three times greater than its apparent Michaelis constant, and with NAD^+ at 0.2 mM, the addition of 0.4 mM chorismate had no effect on the reaction velocity (Table V). However, when prephenate was present at a concentration which is less than its apparent Michaelis constant, the reaction velocity was increased 50% by the addition of the same concentration of chorismate. From the simultaneous measurement of the mutase reaction velocity, it was estimated that the maximum

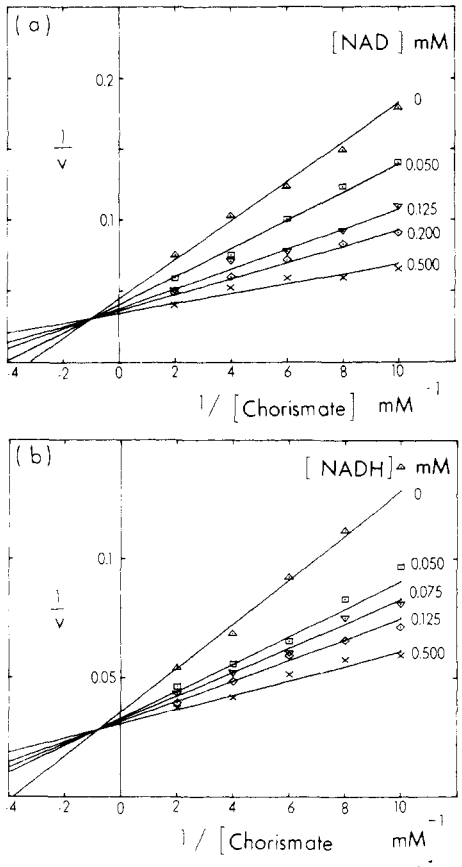

FIGURE 4: Activation of the mutase reaction by NAD^+ (a) and NADH (b). Velocities in the presence of NAD^+ were calculated from rate measurements at both 290 and 340 nm as described under Methods; those in the presence of NADH were calculated from measurements at 290 nm. In both experiments velocities are expressed as micromoles of chorismate converted per milligram of enzyme per minute.

TABLE IV: Effect of the Reactants for Prephenate Dehydrogenase on the Activity of Chorismate Mutase.

Reactant	Effect	Kinetic constant	Value[a] (mM)	React.
Prephenate (B)	Inhib	K_{ib}	0.21 ± 0.01	E + B
4-Hydroxyphenyl-pyruvate (Q)	Inhib	K_{iq}	1.5 ± 0.2	E + Q
NAD^+ (C)	Activ	K_{ic}	0.60 ± 0.16	E + C
		K_{IaC}	0.096 ± 0.013	EA + C
NADH (P)	Activ	K_{ip}	0.17 ± 0.03	E + P
		K_{IaP}	0.059 ± 0.009	EA + P

[a] The value for K_{ib} was obtained by fitting data to eq 3. The data for the competitive inhibition by 4-hydroxyphenylpyruvate (0.8 mM) with respect to chorismate were fitted to the same equation to yield a value for K_{iq}. Values for the kinetic parameters associated with NAD^+ and NADH are weighted mean values from two experiments, including those illustrated in Figure 4. They were obtained by fitting the data to eq 6, in which K_{is} is equivalent to the dissociation constant for the interaction of pyridine nucleotide (I) with free enzyme, K_{in} to the dissociation constant for the interaction with the enzyme-chorismate complex, and K_{in}/K_{id} to the ratio of the maximum velocity attained in the presence of the pyridine nucleotide to that in its absence. The value of this ratio is given in the text.

TABLE V: Effect of Chorismate on the Rate of the Dehydrogenase Reaction.[a]

[Chorismate] (mM)	[Prephenate] (mM)	Rate (μmol min^{-1} mg^{-1}) Dehydrogenase	Mutase
0.0	0.25	6.0	
	0.05	2.6	
	0.056	2.9[b]	
0.4	0.25	6.0	8.8
	0.05	3.8	12.2

[a] The concentration of NAD^+ was 0.2 mM. [b] Value calculated using five steady-state velocities obtained over a range of prephenate concentrations from 0.05 to 0.25 mM.

amount of prephenate formed from chorismate over the period of measurement would raise the concentration of prephenate only from 0.05 to 0.056 mM. Further, it was calculated that the increase in prephenate concentration would raise the steady-state rate of the dehydrogenase reaction from 2.6 to 2.9 μmol min^{-1} mg^{-1}, whereas the experimentally determined value was 3.8 μmol min^{-1} mg^{-1}.

Discussion

The steady-state velocity patterns in the absence of products as well as the product and dead-end inhibition patterns for the prephenate dehydrogenase reaction are qualitatively consistent with a kinetic mechanism which involves the random addition of substrates and the formation of two dead-end complexes, viz., enzyme-NADH-prephenate and enzyme-NAD^+-hydroxyphenylpyruvate. In this respect, prephenate dehydrogenase is similar to IMP dehydrogenase (Heyde et al., 1976) and isocitrate dehydrogenase (Uhr et al., 1974).

Conclusions about the order of product release are limited because it was not possible to determine the inhibition patterns with bicarbonate (CO_2). Possibilities are a completely random release of the three products or release of CO_2 first, followed by random dissociation of NADH and hydroxyphenylpyruvate. However, the experiment on double inhibition by NADH and hydroxyphenylpyruvate revealed that an enzyme-NADH-hydroxyphenylpyruvate complex does not form to a significant extent under the conditions used.

It should also be noted that the product inhibitions are qualitatively consistent with ordered mechanisms of the Theorell-Chance type in which (a) prephenate adds before NAD^+ and products are released in the order NADH, CO_2, and hydroxyphenylpyruvate or (b) NAD^+ adds before prephenate and products dissociate in the order hydroxyphenylpyruvate, CO_2, and NADH. However, quantitative aspects of the product inhibition data and the dead-end inhibition patterns obtained with AMP and *cis*-aconitate are not in accord with either Theorell-Chance mechanism. It follows from the discussion by Fromm (1975) that if a two substrate reaction which is essentially irreversible conforms to a Theorell-Chance mechanism the steady-state velocity pattern in the absence of products will consist of a family of parallel straight lines, provided that the irreversibility of the reaction is due to the negligible rate of release of the first substrate to add in the forward direction. However, it should be noted that if the reaction is not reversible because of an irreversible isomerization of a transitory enzyme form on the release side of the reaction sequence an intersecting steady-state velocity pattern will be obtained. Thus, Theorell-Chance mechanisms for irreversible reactions cannot be ruled out simply because the steady-state velocity pattern is of the intersecting type.

When it is assumed that the random addition of prephenate (B) and NAD^+ (C) occurs under rapid equilibrium conditions with the products hydroxyphenylpyruvate (P) and NADH (Q) combining in the same way as inhibitory analogues of pre-

TABLE VI: Summary of the Dissociation Constants for the Interaction of Reactants with Free Enzyme.

Reactant	Dissociation constant (mM)	
	Dehydrogenase	Mutase
NAD^+	0.72 ± 0.09	0.60 ± 0.16
NADH	0.09 ± 0.02	0.17 ± 0.03
Prephenate	0.17 ± 0.02	0.21 ± 0.01
4-Hydroxyphenylpyruvate	0.44 ± 0.07	1.5 ± 0.2
cis-Aconitate	12 ± 1	~40

phenate and NAD, respectively, the rate equation may be expressed as:

$$v = \frac{V_1[B][C] - V_2[P][Q](K_{ib}K_c/K_pK_{iq})}{K_{ib}K_c\left(1 + \frac{[P]}{K_{ip}} + \frac{[Q]}{K_{iq}} + \frac{[P][Q]}{K_pK_{iq}}\right) + K_c[B]\left(1 + \frac{[P]}{K_{Ip}}\right) + K_b[C]\left(1 + \frac{[Q]}{K_{Iq}}\right) + [B][C]}$$

The Michaelis constants K_b, K_c, K_p, and K_q are dissociation constants for the interaction of B, C, P, and Q with EC, EB, EQ, and EP, respectively, while K_{ib}, K_{ic}, K_{ip}, and K_{iq} represent dissociation constants for the interaction of free enzyme (E) with B, C, P, and Q, respectively. K_{Iq} and K_{Ip} denote dissociation constants for the combination of Q with EC and P with EB, respectively. Analysis of the data on the basis of a rapid equilibrium random mechanism demonstrates that the data are in quantitative agreement with this model, Thus, in each of the four competitive inhibition experiments (Table III) the directly determined values for each of the apparent inhibition constants are in good agreement with the corresponding calculated values which were obtained as described in the legend to Table III. The data (Tables II and III) also indicate that the presence of prephenate on the enzyme causes considerable enhancement of the combination of NAD^+ and NADH, while prephenate and hydroxyphenylpyruvate bind more strongly to the E-NAD^+ complex than they do to the free enzyme. By contrast, there is no significant synergistic effect of complementary substrates in the reaction of the inhibitory analogues, AMP and *cis*-aconitate, with the enzyme.

The ability of NAD^+ to enhance the binding to the enzyme of prephenate and hydroxyphenylpyruvate extends also to chorismate, and a similar effect is given by NADH (Figure 4, Table IV). The data of Figure 4, which were obtained from studies on the mutase reaction, yield values for the dissociation constants of the E-NAD^+ and E-NADH complexes which are in reasonable agreement with those calculated from the kinetic data for the dehydrogenase reaction (Table VI). Thus, it may be concluded that NADH causes inhibition of the dehydrogenase reaction and activation of the mutase reaction by combining at a single nucleotide-binding site, at which NAD^+ also reacts either as a substrate or an activator. The data of Table VI attest to the validity of the rapid-equilibrium assumption for the binding of NAD^+ to the enzyme.

A point of particular interest with regard to bifunctional enzymes is whether two sequential reactions occur at the same active site or at separate sites with transfer of the intermediate between them. In an attempt to gain information on this point, a comparison has been made of the dissociation constants for the reaction of prephenate, hydroxyphenylpyruvate, and *cis*-aconitate with the free form of enzyme as determined from kinetic investigations on the dehydrogenase and mutase reactions. On the basis that the dehydrogenase reaction conforms to a rapid equilibrium random mechanism, dissociation constants can be determined for the enzyme-prephenate and enzyme-hydroxyphenylpyruvate complexes. Further, when it is considered that the mutase reaction is irreversible because of the irreversibility of either the interconversion of the central complexes or of an isomerization of the E-prephenate complex, product-inhibition data yield a dissociation constant for the binding of prephenate to free enzyme rather than a Michaelis constant. The inhibition constants for any compounds which act as substrate analogues must, of course, be dissociation constants. The data of Table VI indicate that similar values are obtained for the combination of prephenate as a substrate for the dehydrogenases reaction and as a product inhibitor of the mutase reaction. This result would suggest that either a single site or two sites with similar properties are involved in the binding of prephenate for the two reactions. However, some caution is necessary in reaching this conclusion. The correct value of the dissociation constant for the interaction of prephenate and free enzyme is obtained from studies on the dehydrogenase reaction only if its reaction mechanism is truly rapid equilibrium random. From the apparent concavity of the upper three lines of Figure 1a it could be inferred that the rapid equilibrium condition does not hold. However, in the absence of albumin there was unusual variability in the velocity determinations at lower concentrations of both substrates, while in its presence there was no evidence of nonlinearity (Figure 1b). The results obtained with hydroxyphenylpyruvate and *cis*-aconitate do not provide support for the concept of a single site or two kinetically similar sites, as there is a threefold difference in the values of the dissociation constants associated with the dead-end inhibition of the two reactions by these substrate analogues. However, it must be noted that there were experimental difficulties in obtaining accurate inhibition constants from studies on the mutase reaction, because of high absorbance backgrounds with hydroxyphenylpyruvate and increases in ionic strength as well as absorbance with *cis*-aconitate.

It is clear that the enhanced rate of the dehydrogenase reaction in the presence of chorismate at lower concentrations of prephenate is not due to the increase of prephenate in the bulk medium because of the action of chorismate mutase (Table V). But these results do not permit a conclusion to be drawn about the number of catalytic sites. The increase in the rate of hydroxyphenylpyruvate formation on the addition of chorismate is consistent with the enzyme possessing a single site at which a proportion of the hydroxyphenylpyruvate can be produced from chorismate without the intermediate formation and release of prephenate from the enzyme. Alternatively, the results are in accord with the idea of separate mutase and dehydrogenase sites that interact, with chorismate enhancing the combination of NAD^+ (cf. Table IV); calculations show that it is not necessary to postulate any direct channeling of prephenate from the mutase to the dehydrogenase site in order to account for this effect. Computer simulations of the overall reaction from chorismate to hydroxyphenylpyruvate, according to these models, are to be reported separately.

From the results of the present investigation it is concluded that chorismate mutase-prephenate dehydrogenase possesses either a single active site or two active sites which exhibit kinetic similarities. However, in any two-site model, the sites cannot be independent. Attempts are now being made to use additional techniques to distinguish between the possible models.

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CORRECTION

Reaction of (Na-K)ATPase with 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole: Evidence for an Essential Tyrosine at the Active Site, by Lewis C. Cantley, Jr.,* Jeff Gelles, and Lee Josephson, Volume 17, Number 3, February 7, 1978, pages 418–425.

Equation 2 on page 422, column 2, should read:

$$A_{(\mathrm{PNPase})} = 0.3e^{-k_1 t} + 0.7e^{-k_2 t}$$

and eq 4 on page 422, column 2, should read:

$$\mathrm{OD}_{380} = a_{380}(2 - e^{-k_1 t} - e^{-k_2 t})$$

The constants in Table I were derived from these equations.