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Chorismate Mutase–Prephenate Dehydrogenase from *Escherichia coli*. 1. Kinetic Characterization of the Dehydrogenase Reaction by Use of Alternative Substrates

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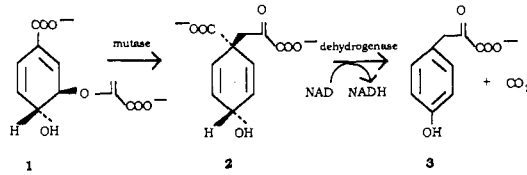
ABSTRACT: The bifunctional enzyme involved in tyrosine biosynthesis, chorismate mutase–prephenate dehydrogenase, has been isolated from extracts of a plasmid-containing strain of *Escherichia coli* K12 and purified to homogeneity by a modified procedure that involves chromatography on both Matrex Blue A and Sepharose–AMP. Detailed studies of the dehydrogenase reaction have been undertaken with analogues of prephenate that act as substrates. The analogues, which included two of the four possible diastereoisomers of 1-carboxy-4-hydroxy-2-cyclohexene-1-propanoate (deoxodihydroprephenate) as well as D- and L-arogenate, were synthesized chemically. As judged by their V/K values, all analogues were poorer substrates than prephenate. The order of their effectiveness as substrates is prephenate > one isomer of 1-carboxy-4-hydroxy-2-cyclohexene-1-propanoate > L-arogenate > other isomer of 1-carboxy-4-hydroxy-2-cyclohexene-1-propanoate > D-arogenate. Thus the dehydrogenase activity is dependent on the degree and position of unsaturation in the ring structure of prephenate as well as on the type of substitution on the pyruvyl side chain. With prephenate as a substrate, the reaction is irreversible because it involves oxidative decarboxylation. By contrast, 1-carboxy-4-hydroxy-2-cyclohexene-1-propanoate undergoes only a simple oxidation, and thus, with this substrate, the reaction is reversible. Steady-state velocity data, obtained by varying substrates over a range of higher concentrations, suggest that the dehydrogenase reaction conforms to a rapid equilibrium, random mechanism with 1-carboxy-4-hydroxy-2-cyclohexene-1-propanoate as a substrate in the forward reaction or with the corresponding ketone derivative as a substrate in the reverse direction. The initial velocity patterns obtained by varying prephenate or 1-carboxy-4-hydroxy-2-cyclohexene-1-propanoate over a range of lower concentrations, at different fixed concentrations of NAD, were nonlinear and consistent with a unique model that is described by a velocity equation which is the ratio of quadratic polynomials. An equilibrium constant of 1.4×10^{-7} M for the reaction in the presence of 1-carboxy-4-hydroxy-2-cyclohexene-1-propanoate indicates that the equilibrium lies very much in favor of ketone production.

Chorismate mutase–prephenate dehydrogenase [chorismate pyruvatemutase (EC 5.4.99.5)–prephenate:NAD oxidoreductase (decarboxylating) (EC 1.3.1.12)] is a bifunctional enzyme that catalyzes two sequential reactions in the tyrosine biosynthetic pathway of *Escherichia coli* and other organisms (Cotton & Gibson, 1965; Koch et al., 1971a). These reactions (Scheme I) involve the rearrangement of chorismate (**1**) to prephenate (**2**) and, in the presence of NAD,[1] the oxidative decarboxylation of prephenate to (4-hydroxyphenyl)pyruvate (**3**). Through the action of an aromatic aminotransferase, the product (**3**) undergoes conversion to tyrosine, which acts as an end-product inhibitor of chorismate mutase–prephenate dehydrogenase (Koch et al., 1971a).

Scheme I



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[1] Abbreviations: AMP, adenosine monophosphate; CHCP, 1-carboxy-4-hydroxy-2-cyclohexene-1-propanoate (deoxodihydroprephenate); COCP, 1-carboxy-4-oxo-2-cyclohexene-1-propanoate; DTT, dithiothreitol; MES, (*N*-morpholino)ethanesulfonic acid; NAD, nicotinamide adenine dinucleotide; NADH, reduced form of NAD; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane.

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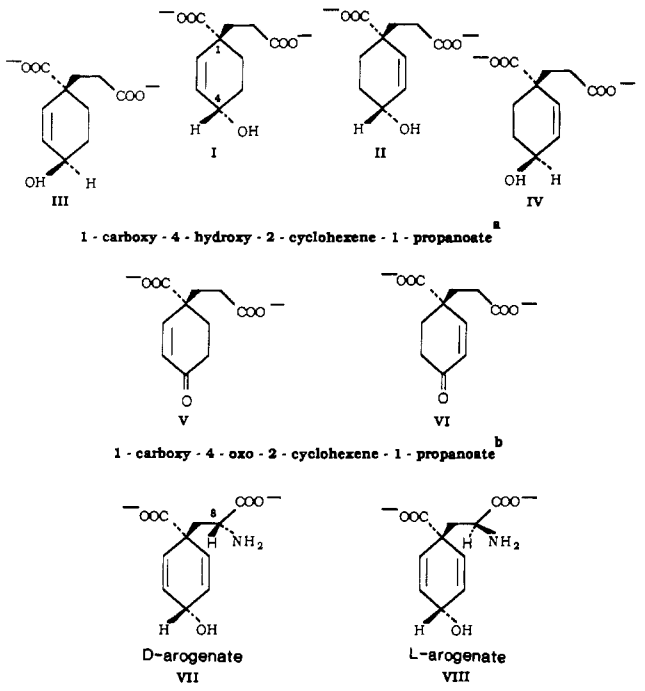


FIGURE 1: Structure of prephenate analogues prepared by chemical synthesis. Abbreviations used in the text are (a) CHCP and (b) COCP.

Since the mutase reaction is essentially irreversible and as NAD is required for dehydrogenase activity, each of the reactions can be studied independently. Steady-state kinetic investigations, at relatively high concentrations of chorismate or prephenate, have indicated that the mutase and dehydrogenase reactions conform to Michaelis–Menten kinetics (SampathKumar & Morrison, 1982b; Hudson et al., 1984), while the mechanism of the dehydrogenase reaction is of the rapid equilibrium, random type (SampathKumar & Morrison, 1982b). Hermes et al. (1984) have reported that the oxidative decarboxylation of prephenate to (4-hydroxyphenyl)pyruvate occurs via a concerted mechanism. At lower concentrations of substrates nonlinear kinetics suggestive of positive cooperativity have been reported for both reactions (Christopherson & Morrison, 1985).

The first investigation of the substrate specificity of prephenate dehydrogenase was undertaken recently by Hermes et al. (1984). These authors prepared derivatives of prephenate which did not have a carbonyl group in the side chain and which contained either one or no double bond. The preparation of the derivative with a single double bond contained two pairs of enantiomers of 1-carboxy-4-hydroxy-2-cyclohexene-1-propanoate (CHCP) which are illustrated in Figure 1 as I and II and as III and IV. The pair with the same stereochemistry as prephenate at carbons 1 and 4 (I and II, Figure 1) were active as substrates although one of the enantiomers is a poorer substrate than the other. These substrates differ from prephenate in that the oxidation products do not undergo decarboxylation, and thus, the reactions are reversible. Another potential substrate analogue for the dehydrogenase is L-arogenate [β-(1-carboxy-4-hydroxy-2,5-cyclohexadien-1-yl)alanine; Figure 1, VIII] which undergoes oxidative decarboxylation in several organisms to form tyrosine (Bonner & Jensen, 1987). However, this compound has not been examined for its ability to act as a substrate for prephenate dehydrogenase.

In the present investigation chorismate mutase–prephenate dehydrogenase has been prepared by a modified procedure and used to extend the earlier investigations of Hermes et al. (1984) on the substrate specificity of the dehydrogenase reaction in both the forward and the reverse directions. For this purpose, modified methods have been developed for the synthesis of CHCP, as well as for the corresponding oxidized product, 1-carboxy-4-oxo-2-cyclohexene-1-propanoate (COCP). In addition, a new procedure has been utilized for the preparation of DL-arogenate from prephenate. The results indicate that the reactions with CHCP in the forward direction and with COCP in the reverse direction can conform to rapid equilibrium, random mechanisms. At lower concentrations of prephenate and CHCP, the initial velocity patterns are nonlinear and can be described by a unique kinetic model.

EXPERIMENTAL PROCEDURES

Materials

Chorismate and prephenate were prepared as previously described (Gibson, 1968; Dudzinski & Morrison, 1976). N^6-(6-aminohexyl)-AMP was prepared by Dr. D. Magrath according to the method of Trayer et al. (1974). The purified product was reacted with cyanogen bromide activated Sepharose 4B as described by Cuatrecasas et al. (1968). Silica gel 60 thin-layer plates with F-254 fluorescent indicator and 0.25-mm layer thickness were obtained from Merck. D_2O (99.9 atom % D and low in paramagnetic impurities) was from The Australian Atomic Energy Commission, Lucas Heights. All other chemicals were obtained commercially and were of the highest purity available.

Methods

Bacterial Strains and Growth Conditions. *E. coli* strain JFM30, constructed by Bhosale et al. (1982), was derived from strain AT2273 (*F⁻ tyrA352*) carrying the multicopy plasmid pKB45 (Tc^r *pheA*$^+$ *aroK*$^+$ *tyrA*$^+$) (Zurawski et al., 1978). The strain yielded an amount of chorismate mutase–prephenate dehydrogenase that was 5000-fold higher than that yielded by the wild-type organism. Lawn cultures were grown overnight at 30 °C on agar plates containing mineral salts medium 56 (Monod et al., 1951) supplemented with 50 mM glucose, 0.7 mM arginine, 0.1 mM histidine, 1.5 μM thiamin, and 20 μM tetracycline and then used to innoculate three 1-L flasks of supplemented mineral media. The cells were grown for 6 h at 37 °C with shaking and then used to innoculate six 10-L fermenters containing the same media. After overnight growth under aerobic conditions to the stationary phase, the cells were harvested with a yield of 3.0–3.5 g wet wt/L of culture. The paste was stored at −20 °C for up to 1 year without loss of enzyme activity.

Buffers. Buffers used in the purification procedure were as follows: buffer A, 0.1 M *N*-ethylmorpholine (pH 7.5), 1 mM EDTA, 1 mM DTT, and 10% (v/v) glycerol; buffer B, same as buffer A plus 1 mM sodium citrate; buffer C, 0.1 M *N*-ethylmorpholine, 0.046 M 2-(*N*-morpholino)ethanesulfonic acid (MES, pH 7.5), 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol, and 20 mM KH_2PO_4; buffer D, 0.1 M *N*-ethylmorpholine (pH 7.0), 1 mM EDTA, 1 mM DTT, 21 mM sodium citrate, and 10% (v/v) glycerol. Sodium citrate and glycerol were added to the buffers following reports of improved stability of the enzyme during its purification (SampathKumar & Morrison 1982a; Hudson et al., 1984).

Purification of Chorismate Mutase–Prephenate Dehydrogenase. The procedure used for purification of the enzyme is a modification of those reported previously by SampathKumar and Morrison (1982a) and Hudson et al. (1984). All steps were performed at 0–4 °C. Frozen cells (80 g) were thawed and suspended in 6 volumes of buffer A containing 0.1 mM phenylmethanesulfonyl fluoride (PMSF), disrupted in a Ribi cell fractionator at 20 000 psi, and centrifuged at

14000*g* for 15 min. A solution of streptomycin sulfate (40% w/v) in buffer A was then added dropwise with stirring to the supernatant solution until a final concentration of 6% was reached. The suspension was stirred for a further 30 min before being centrifuged at 23500*g* for 30 min. An ammonium sulfate fraction (32–50%) was prepared, by adding slowly and with stirring 0.18 g of solid ammonium sulfate per milliliter of supernatant solution. The suspension was centrifuged at 23500*g* for 30 min before the addition of a further 0.121 g of ammonium sulfate per 100 mL of supernatant solution. The suspension was stirred for 1 h and then centrifuged for 20 min at 23500*g*. (The pellet could be stored at −20 °C for several months without loss of either enzyme activity.) The pellet was dissolved in a minimum volume of buffer B and then dialyzed for 15 h against the same buffer before being clarified by centrifugation for 10 min at 17000*g*. The supernatant was applied at about 1 mL/min to a column (2.6 × 19 cm) of Matrex Gel Blue A equilibrated with buffer B. The column was washed with 3 volumes of buffer B and then with 3 volumes of buffer B plus 1 mM NAD. The enzyme was eluted by washing the column with 6 volumes of buffer B (adjusted to pH 8.0) plus 1 mM NAD and 0.5 mM tyrosine. Enzymatically active fractions were pooled, concentrated to approximately 50 mL on an Amicon UM-10 membrane, and then dialyzed for 15 h against two changes of buffer C. The pooled fractions were adjusted to pH 6.0 with acetate buffer (5 M acetic acid adjusted to pH 4.0 with NaOH) and then applied at 1 mL/min to a column (2.6 × 18 cm) of Sepharose–AMP equilibrated with buffer C at pH 6.0. The column was washed with 4 volumes of buffer C (without KH_2PO_4) before the enzyme was eluted at 0.55 M salt with a gradient of KCl (0–1 M) in buffer B (500 mL total). Active fractions were pooled, concentrated to about 6 mg of protein/mL on an UM-10 membrane, and then dialyzed against two changes of buffer D with 20 mM DTT. In this form, the enzyme was stable at −20 °C for several months.

Molecular Weight Analysis. The native molecular weight of the enzyme was determined by molecular sieve chromatography on a column of Superose-12 HR 10/30 in combination with a fast protein liquid chromatography system (FPLC). Molecular weight marker proteins and enzyme were eluted from the column with a buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, and 0.1 M NaCl at a flow rate of 0.4 mL/min. The subunit molecular weight of the enzyme was determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS–PAGE) according to the method of Laemmli (1970). Duplicate samples of the purified enzyme were electrophoresed with one sample dialyzed against buffer containing 5% β-mercaptoethanol.

Analysis of Chemically Synthesized Products. Proton NMR spectra were obtained on a Joel Model FX90Q spectrometer (at 89.6 MHz) and a Model XL-300 spectrometer (300 MHz) in the Fourier transform mode. Chemical shifts are reported as δ values with respect to tetramethylsilane as an internal standard. UV spectra were obtained to monitor the purity of compounds and to determine extinction coefficients by use of a Cary 219 spectrophotometer linked to an Apple II Plus computer. Scans were controlled and recorded with an Automatic Master Scan Program (Varian). For the determination of λ_{max} and extinction coefficients of the disodium salt and free acid forms of COCP, compounds were diluted in either water or 0.1 N HCl (pH 1.1). The absorbance was then recorded as a function of wavelength by scanning from 600 to 200 nm. The formation of COCP was monitored

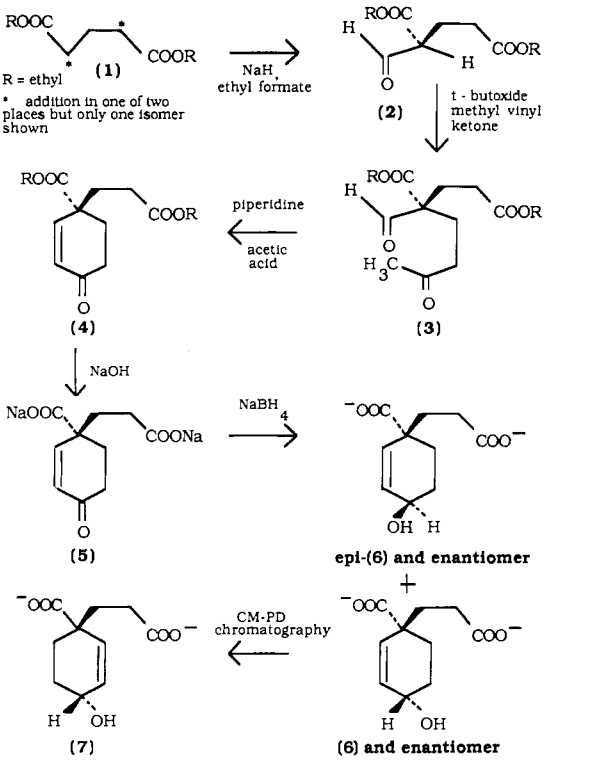


FIGURE 2: Overall scheme for the preparation of prephenate analogues. The compounds are denoted as (**1**) diethyl glutarate, (**2**) diethyl formylglutarate, (**3**) 4-formyl-4-carboxy-7-oxooctanoic diester, (**4**) COCP diester, (**5**) COCP disodium salt, (**6**) fast and slow enantiomers of CHCP that function as enzyme substrates, and (**7**) assumed structure of slow enantiomer of CHCP. CM–PD, chorismate mutase–prephenate dehydrogenase.

by thin-layer chromatography (TLC) on silica gel 60 (F-254) plates (20 × 60 cm) with an elution solvent of toluene–ethyl acetate (2:1). The products were visualized by fluorescence quenching of UV light or by staining with iodine vapors. The four enantiomers of CHCP could not be visualized with this system. Arogenate, prephenate, and phenylalanine were also separated on silica gel plates with an elution system of ethanol–chloroform–ammonium hydroxide (20:4:6). Products were visualized under UV light. Mobility (R_f) of the compounds on TLC was calculated as the distance traveled by the sample divided by the distance traveled by the solvent.

Synthesis of Prephenate Analogues. The steps involved in the chemical synthesis of prephenate analogues are shown in Figure 2, and individual compounds are identified numerically from **1** to **7**. It should be noted that the abbreviation CHCP can refer to any one of the four enantiomers that are produced in the synthesis while the abbreviation COCP can refer to either of the two keto derivatives that are produced in the synthesis or to either of the two keto derivatives that can be obtained enzymatically as a result of the action of prephenate dehydrogenase on CHCP.

1-Carboxy-4-oxo-2-cyclohexene-1-propanoate Disodium Salt (COCP, **5**). The synthesis of COCP as a racemic mixture of two enantiomers in the diethyl ester or disodium salt form followed the procedure outlined by Hermes et al. (1984) (Figure 2, **1**–**5**). The purity of the intermediates at each stage of the synthesis was monitored by proton NMR (FX 90Q-FT) and infrared spectroscopy (Unicam SP 1050 spectrometer). Briefly, the synthesis involves the condensation of diethyl glutarate (**1**) and ethyl formate to form diethyl formylglutarate (**2**). The components of the ring structure are generated by a Michael reaction involving the addition of methyl vinyl ketone to **2**. The resulting octanoic acid diester (**3**) is converted

to the carboxyoxocyclohexeneproponate diester (**4**) which, in turn, is saponified to produce the disodium salt (**5**). The yield of the disodium salt of COCP from diethyl glutarate was 17%, and the results of proton NMR spectroscopy were in agreement with those reported previously by Hermes et al. (1984). UV λ_{max}: (water, pH 7.0) 237 nm (ϵ = 6500); (water, pH 2.0) 228 nm (ϵ = 8000).

The free acid form of COCP could be prepared from the disodium salt by passage of an aqueous solution of the salt through a column of Dowex 50-W (50–100 mesh, H^+) cation exchanger. The free acid was detected by monitoring the eluant at 237 nm. Fractions showing significant absorbance readings were pooled and freeze-dried to obtain a yellow oil (50% yield). Both the disodium salt and free acid form of COCP could be separated on silica gel 60 and detected as a sharp fluorescing spot with an R_f of 0.4. Proton NMR (D_2O) showed the following: δ 1.85–2.4 (m, 8 H, methylene protons), 6.10 (d, 1 H, CH=C*H*C=O), 7.05 (d, 1 H, C*H*=CHC=O). The assignments were in agreement with those of the disodium salt.

1-Carboxy-4-hydroxy-2-cyclohexene-1-propanoate (CHCP, **6**). A total of 0.517 g (2.0 mmol) of the disodium salt of COCP (**5**) was dissolved with stirring in 20 mL of ice-cold water. To the stirred solution was added, over a period of 1 h, 20 mL of a 1 M solution of sodium borohydride (20 mmol). The pH of the reaction was maintained between 7 and 8 by the addition to the reaction mixture of dry ice chips. A total of 20 mL of washed Dowex 50-W resin (50–100 mesh, H^+) was added; then the mixture was gently swirled and vacuum filtered. The filtrate was acidified to pH 2.0 with 5 N H_2SO_4 and then extracted three times with 50 mL of diethyl ether. The extracts were pooled, dried with $MgSO_4$, and concentrated under reduced pressure to yield a clear oil. The sample was reconstituted in 1.0 mL of water and neutralized by the addition of 1 M *N*-ethylmorpholine, before being stored at 4 °C. The amount of CHCP which could function as substrate for prephenate dehydrogenase was determined enzymatically to be 0.78 mmol or 78% of the expected yield of enzymatically active isomers. For proton NMR analysis, the sample as a clear oil was reconstituted in D_2O, neutralized by the addition of 0.1 N NaOD, and lyophilized. The residue was reconstituted in D_2O and again lyophilized. This procedure was repeated twice, before the sample was taken up in 0.5 mL of D_2O. Proton NMR showed the following: δ 1.2–2.0 (m, 8 H, methylene protons), 3.1–3.7 (m, 1 H, C*H*OH), 5.8 (d, 2 H, C*H*=CH). The spectrum was in agreement with that obtained for CHCP by Hermes et al. (1984), who used a different synthetic procedure.

Preparation of the Enzymatically Active Slow Enantiomer of 1-Carboxy-4-hydroxy-2-cyclohexene-1-propanoate (CHCP, **7**). The pair of enantiomers of CHCP, which function as substrates for prephenate dehydrogenase, react at different rates. Thus they can be referred to as the fast and slow isomers. Such a difference in rate also permits the preparation of solutions that are enriched with the slow isomer, and this was achieved by the following procedure. To a reaction mixture (2.0 mL) containing 0.1 M MES/0.05 M *N*-ethylmorpholine/0.051 M diethanolamine (pH 8.0), 1 mM EDTA, 1 mM DTT, 10 mM CHCP (10 μmol of each of the fast and slow isomers, as well as the corresponding inactive epi isomers), and 4 mM NAD was added 1.0 mg of chorismate mutase–prephenate dehydrogenase. The mixture was incubated at 30 °C, and the progress of the reaction was followed by monitoring the absorbance change at 340 nm of appropriately diluted aliquots of the reaction mixture. The reaction was allowed to proceed to approximately 85% completion (1.5 h) and stopped by the addition of ice-cold methanol. The sample was stored overnight at −20 °C, after which the precipitated protein was removed by centrifugation. Concentration of the supernatant under nitrogen yielded a yellow-orange oil which was reconstituted in 1.0 mL of water (pH 7.8). The yield of the slow isomer of CHCP, as determined enzymatically, was 3.0 μmol. The ketone product of the fast isomer was removed from the product by a procedure that was based on the findings of Hermes et al. (1984).

Separation of Enzymatically Active Slow Enantiomer of CHCP. The slow isomer of CHCP was separated from any remaining fast isomer, the ketone corresponding to the fast isomer, and the enzymatically inactive epi isomers of CHCP by the following procedure. A solution (3.0 mL) containing 10 μmol of the slow isomer of CHCP was diluted in 9 mL of 1 M morpholine (pH 9.5), incubated at 40 °C for 1 h, and then passed through a column of AG1-X8 (bed volume 5 mL) equilibrated previously with 1 M morpholine (pH 9.5). The column was washed with 5 mL of equilibration buffer and then with 5 mL each of 100 mM KCl and 350 mM KCl in 1 M morpholine (pH 9.5). The wash and eluants from the two salt fractions were acidified with 2 N H_2SO_4 and extracted with diethyl ether. The organic phases were then dried with $MgSO_4$ and concentrated under reduced pressure. Each sample was reconstituted in 0.5 mL of water and adjusted to pH 7.0 with 1 M *N*-ethylmorpholine. The fractions were assayed for the presence of enzymatically active isomers of CHCP, and the products in each fraction were monitored by TLC. The yield of the slow isomer of CHCP was 3.7 μmol. Although the separation procedure resulted in only 37% recovery of the slow isomer, the preparation was essentially free of the inhibitory COCP.

Synthesis and Purification of DL-*Arogenate.* Prephenate was converted to arogenate by reductive amination according to the procedure adapted from Borch et al. (1971) for the synthesis of amino acids from α-keto acids. To a solution of sodium prephenate (0.134 g, 0.5 mmol) in 0.5 mL of water were added 1.0 mL of methanol and a mixture containing ammonium bromide (0.245 g, 2.5 mmol) and sodium cyanoborohydride (0.0615 g, 1.0 mmol) in 8.5 mL of methanol. The reaction mixture was covered and stirred for 15 h at 25 °C. The pH of the solution was maintained at 8.0 throughout the reaction by the addition of methanolic NH_3. The volatile components were removed under reduced pressure, and the residue was taken up in 2 mL of water. The solution was adjusted to pH 8.5 with 1 M NaOH and was stored at 4 °C. The yield of DL-arogenate, as determined both chemically and enzymatically, was 0.12 mmol (24%).

To separate DL-arogenate from phenylalanine and prephenate, 1.5 mL of a solution containing 90 μmol of DL-arogenate was chromatographed over a column of Sephadex G-10 (0.8 × 30 cm) previously equilibrated with water. Fractions (2 mL) were collected, and their absorbance was monitored at 254 nm. Fractions with the highest absorbance readings were collected and pooled before adjustment of the pH to 9.0 with ammonia. The eluate (18 mL, 76 μmol of DL-arogenate) was passed through an anion exchange column (1.5 × 20 cm) of AG1-X8 (200–400 mesh, Cl^- form) which had been equilibrated previously with 0.1% (v/v) *N*-ethylmorpholine hydrochloride (pH 9.0). The column was washed with 80 mL of equilibration buffer at 0.5 mL/min and then treated with a linear gradient prepared from 60 mL of the equilibration buffer and 60 mL of the same buffer containing 1 M ammonium carbonate. Fractions (4 mL) were collected, and the

presence of arogenate and phenylalanine was determined chemically while prephenate was estimated enzymatically. DL-Arogenate (43 μmol), which eluted at 0.22 M ammonium carbonate, was lyophilized, reconstituted in methanol, and solubilized by the addition of 1 M NaOH. Analysis by TLC revealed a single fluorescent spot with an R_f value of 0.38, which is similar to that reported by Zamir et al. (1982). There was minor contamination of the product by phenylpyruvate and prephenate.

Enzyme Assays and Protein Concentration. Chorismate mutase activity was determined at 30 °C, in reaction mixtures (0.4 mL) containing 1.0 mM chorismate, 50 mM tris(hydroxymethyl)aminomethane (Tris, pH 7.5), 1 mM EDTA, and 1 mM DTT, by the stopped-time assay of Koch et al. (1970). Prephenate dehydrogenase activity was measured at 30 °C by continuously monitoring the formation of NADH from NAD at 340 nm. An extinction coefficient of 6400 was used to calculate initial velocities (Heyde & Morrison, 1978a). Reaction mixtures contained 0.25 mM prephenate and 2 mM NAD in the same buffer as used to determine mutase activity. Kinetic studies were performed in the presence of a three-component buffer system (Ellis & Morrison, 1982) of 0.1 M MES/0.051 M *N*-ethylmorpholine/0.051 M diethanolamine (pH 7.2), 1 mM EDTA, and 1 mM DTT. Prephenate dehydrogenase activities were measured spectrophotometrically with the continuous assay described above while chorismate mutase activites were determined by following the disappearance of chorismate at 273 nm (Heyde & Morrison, 1978a). When analogues of prephenate were employed as substrates in the dehydrogenase reaction, an extinction coefficient of 6200 was used to calculate initial velocities. A unit of enzyme is defined as the amount of enzyme required to produce 1 μmol of product per minute at 30 °C. Specific activity of the enzyme is reported as units per milligram of protein. Reaction velocities are reported in reciprocal seconds. A subunit molecular weight of 44 000 was used to calculate the turnover number. Protein was estimated by the method of Bradford (1976).

Determination of Equilibration Constant. The equilibrium constant for the dehydrogenase reaction with CHCP as substrate was determined at pH 8.0 and 25 °C by following the appearance of NADH. Reaction mixtures contained the three-component buffer system (pH 8.0) as well as 0.10 mM NAD, 0.10 mM CHCP (fast and slow isomers), and 0.6 mg of purified chorismate mutase–prephenate dehydrogenase. The sample cuvette was matched against a reference cuvette containing all the components of the assay mixture except enzyme.

Determination of Substrate Concentrations. The concentrations of prephenate, CHCP (fast and slow isomers), DL-arogenate, and chorismate were determined enzymatically in the presence of 2 mM NAD by following the time course of the dehydrogenase reaction as described above. The concentration of the fast isomer of CHCP was assumed to be half that of the total concentration of the active isomers. Prephenate concentrations were further confirmed by measuring the acid-catalyzed hydrolysis of prephenate to phenylpyruvate as described in the stopped-time assay for chorismate mutase. Concentrations of DL-arogenate were determined by incubating solutions with 1 N HCl for 1 h at 25 °C and monitoring the production of phenylalanine at 254 nm in water (ϵ = 195). Concentrations of NAD and NADH were routinely determined by measuring the absorbance at 259 nm and by use of an extinction coefficient of 17 800 and 16 900, respectively.

Analysis of Kinetic Data. Initial velocity data were first plotted graphically in double-reciprocal form to observe any departures from linearity. An overall fit of each set of data was then made to the appropriate rate equation with one of the computer programs of Cleland (1979) in conjunction with a VAX 8700 computer. In the absence of products and when double-reciprocal plots were linear, a single set of velocity data varying one substrate (A) was fitted to eq 1, while a single set of velocity data resulting in a parabolic velocity pattern was fitted to eq 2. Velocity data for the dehydrogenase reaction that gave rise to linear intersecting initial velocity patterns were fitted to either eq 3 or 4 while data that yielded a curvilinear initial velocity pattern were fitted to eq 5. Inhibition data conforming to linear noncompetitive inhibition were fitted to eq 6. Data obtained from progress curves generated in the presence of one or both enzymatically active isomers of the prephenate analogues were analyzed with eq 7 or 8 to obtain V/K values and the ratio of the amplitudes for the two reactions. The weighted means of the kinetic constants and the standard errors of the means were calculated as described by Morrison and Uhr (1966).

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

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

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \tag{3}$$

$$v = \frac{VAB}{K_{ia}K_b + K_bA + AB} \tag{4}$$

$$v = \frac{V\left(1 + \frac{K_b}{B}\right)}{\left(1 + \frac{K_a}{A}\right)\left(1 + 2\frac{K_b}{B}\right) + \frac{K_b^2}{B^2}\left(1 + \alpha\frac{K_a}{A}\right)} \tag{5}$$

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

$$y = Ae^{-kt} + C \tag{7}$$

$$y = Ae^{-k_1t} + De^{-k_2t} + C \tag{8}$$

Results

Purification of Chorismate Mutase–Prephenate Dehydrogenase. The method described under Experimental Procedures yielded a 23-fold purification of the enzyme from cell extracts of *E. coli* with an overall yield of 25% (Table I). The enzyme was homogeneous on both native PAGE and SDS–PAGE and had specific activities of 65 for chorismate mutase and 54 for prephenate dehydrogenase. These values are comparable to those reported by Hudson et al. (1984) for the enzyme purified from the regulatory mutant JP2319. But they are almost twice the values reported for the enzyme isolated from JFM30 (Bhosale et al., 1982) and from the regulatory mutant JP2312 (SampathKumar & Morrison, 1982a). The use of a Matrex Blue A column represented the major purification step as judged by the 12-fold increase in specific activities over that of the ammonium sulfate fraction. The optimum binding of the enzyme to the blue dye column occurred at neutral pH, and elution was facilitated by raising the pH to 8.0 in the presence of 1 mM NAD plus 0.5 mM tyrosine. The subsequent chromatography on Sepharose–AMP produced only a slight improvement in the specific activity but served to remove soluble NADH oxidases. The removal of

Table I: Purification of Chorismate Mutase–Prephenate Dehydrogenase from *E. coli* JFM30[a]

purification step	vol (mL)	protein (mg)	mutase activity total (units)	mutase activity SA (units/mg)	dehydrogenase activity total (units)	dehydrogenase activity SA (units/mg)	mutase/dehydrogenase ratio	purification (x-fold)	yield (%)
cell-free extract	430	5466	15305	2.8	ND[b]	ND	ND	1	100
streptomycin sulfate treated extract	500	4725	13703	2.9	ND	ND	ND	1.0	90
ammonium sulfate fractionation	112	2267	10247	4.5	8684	3.8	1.2	1.6	67
chromatography on Matrex Blue A	90	78	4108	53	3541	46	1.2	19	27
chromatography on Sepharose–AMP	76	59	3820	65	3159	54	1.2	23	25

[a] Weight of cells was 76 g. [b] ND, not accurately determined due to contamination by NADH oxidases.

such oxidases was essential for the studies with prephenate analogues as alternate substrates because of the need to use much higher concentrations of enzyme.

Properties of Purified Chorismate Mutase–Prephenate Dehydrogenase. Purified enzyme eluted from a column of Superose-12 as a single symmetrical peak with an elution volume corresponding to a molecular weight of 84000, which compares favorably with previously reported values that range from 78000 to 88000 (Koch et al., 1971a,b; SampathKumar & Morrison, 1982a; Hudson et al., 1984). Electrophoresis in the presence of SDS revealed a single band with a molecular weight of 41500. Treatment of enzyme samples with β-mercaptoethanol had no effect on the electrophoresis pattern, and thus the subunits are not joined by disulfide bridges.

Preparation of Analogues of Prephenate. Earlier work by Hermes et al. (1984) had demonstrated that prephenate dehydrogenase is able to utilize as substrates derivatives of prephenate with a methylene group replacing the keto group of the pyruvyl side chain and with the ring having two, one, or no double bonds. The preparation by these authors of the derivatives with a single double bond yielded two pairs of enantiomers (I and II and III and IV, Figure 1). Parenthetically, it should be mentioned that the systematic name for each of the four isomers is 1-carboxy-4-hydroxy-2-cyclohexene-1-propanoate, and thus all are represented by the single abbreviation CHCP. One pair of isomers with the same stereochemistry at the C-1 and C-4 carbons, but with the double bond on opposite sides of the ring, was shown by Hermes et al. (1984) to act as substrates for prephenate dehydrogenase, although at different rates. Further, it was established that the ketone products of the oxidation reactions do not undergo decarboxylation. However, the isomers of CHCP were not separated, and no studies were undertaken of the reduction by prephenate dehydrogenase of the ketone reaction products in the presence of NADH.

One of the basic aims of the present investigation was to prepare each of the four isomers of CHCP so as to obtain detailed kinetic information about the two that undergo reaction and to determine the action of the other two isomers, with the incorrect stereochemistry at the C-4 carbon, as inhibitors of the enzyme. In this connection, a new method was developed for the synthesis of CHCP which involved the use of sodium borohydride to reduce 1-carboxy-4-oxo-2-cyclohexene-1-propanoate (COCP, **5**; Figure 2). The procedure offered an advantage over the synthesis reported by Hermes et al. (1984) in that it was not necessary to subject the reaction products to ester hydrolysis and saponification prior to use in kinetic experiments. But it was important to separate CHCP from borate, which is a byproduct of the reaction and which appears to act as a competitive inhibitor in the dehydrogenase reaction with respect to NAD. Presumably, the inhibition is due to the reduction in the concentration of free NAD because of the interaction of borate with the nucleotide. Efforts were made to separate the two enantiomers of CHCP that act as substrates by separating the two isomers of the ketone (**5**; Figure 2) before reduction with borohydride. Thus the acid form of **5** was reacted with a range of optically active bases, and attempts were made to fractionally crystallize the racemic salt mixture. The approach was unsuccessful. But it was possible to enrich a preparation of CHCP containing all four isomers with the one that behaves as the slower substrate. This was done enzymatically with prephenate dehydrogenase as described below. The ketone product of the CHCP enantiomer that reacts more rapidly with the enzyme was shown to function as an inhibitor of the dehydrogenase. Thus, before kinetic studies were undertaken with the slow isomer of CHCP, the ketone was removed as described under Experimental Procedures.

Preparation of DL-*Arogenate.* A relatively fast and easy method was developed for the synthesis of DL-arogenate by reductive amination of prephenate. This procedure circumvented the problems associated with the total synthesis of L-arogenate (Danishefsky et al., 1981) and with the presence of contaminants when arogenate is produced by use of extracts of *Neurospora crassa* and bacteria (Jensen et al., 1977; Zamir et al., 1980, 1982; Mayer et al., 1985). The synthesis of the racemic mixture of D- and L-arogenate was achieved through the use of an achiral reducing agent, sodium cyanoborohydride. The concentration of arogenate in the product (70 mM), as estimated chemically, was in agreement with that determined enzymatically. Thus, it follows that both isomers of arogenate can act as substrates for prephenate dehydrogenase.

In an attempt to characterize the D and L isomers of arogenate according to their relative V/K values, the time course of the dehydrogenase reaction was followed with DL-arogenate as a substrate. However, the analysis was complicated because the product of the reaction, L-tyrosine, is a very potent inhibitor of the dehydrogenase and not only affected its own synthesis but also the rate of conversion of D-arogenate to D-tyrosine. As the observed rate constants for the two reactions undergo continuous change, accurate V/K values could not be determined. The data obtained by following NADH production as a function of time were fitted to eq 8 to obtain estimates of apparent V/K values for the L and D forms of arogenate. The values differed by a factor of 64.

Initial Velocity Studies with Prephenate and NAD. Initial velocity patterns for the dehydrogenase reaction obtained by varying the concentrations of prephenate (40–200 μM) and NAD (100–1000 μM) were linear and intersected to the left of the vertical axis. Thus when the substrates are varied over a range of higher concentrations, the reaction conforms to a simple sequential mechanism. Values for the kinetic parameters (Table II) are similar to those reported previously (Heyde & Morrison, 1978a; SampathKumar & Morrison, 1982b; Hudson et al., 1984; Christopherson & Morrison, 1985). However, when the prephenate concentrations ranged from 5 to 100 μM, double-reciprocal plots were concave up (Figure 3A). Such a result is indicative of positive cooperativity for

Table II: Kinetic Parameters for the Interaction of Substrates with Chorismate Mutase–Prephenate Dehydrogenase at pH 7.2 and 30 °C

substrate	K_m for substrate (μM)	K_i for substrate (μM)	K_m for nucleotide (μM)[a]	K_i for nucleotide (μM)	V (s^{-1})	relative V	relative V/K
chorismate[b]	64 ± 11				55 ± 3		
prephenate[c]	44 ± 7	50 ± 7	150 ± 20	170 ± 30	36 ± 3	100	100
COCP from fast isomer of CHCP	170 ± 30[f]	490 ± 130[e]	16 ± 5	47 ± 6[d]	0.013 ± 0.0006	0.036	0.009
fast isomer of CHCP[g]	110 ± 10	400 ± 110	119 ± 23	420 ± 80	1.9 ± 0.1	5.3	2.1
slow isomer of CHCP	460 ± 80				0.81 ± 0.07	2.3	0.21[h]
L-arogenate	420 ± 90				4.2 ± 0.2	20	1.2
D-arogenate							0.018[i]

[a] The nucleotide was NAD when prephenate and CHCP were substrates and NADH when COCP was substrate. [b] Initial velocities were determined at chorismate concentrations ranging from 50 to 250 μM, and the data were fitted to eq 1. Reported values are weighted means of values from five experiments. [c] Linear initial velocity data obtained at high concentrations of NAD and prephenate were fitted to eq 3. Values represent the weighted means of values from three experiments. [d] Determined from noncompetitive inhibition by NADH with respect to prephenate at fixed NAD concentration. [e] Determined from noncompetitie inhibition by COCP with respect to NAD at fixed prephenate concentration. [f] Determined from the relationship $K_i(COCP)K_m(NADH) = K_m(COCP)K_i(NADH) = 7950 \pm 860\ \mu M^2$. [g] Determined from linear double-reciprocal plot obtained at higher concentrations of substrates. [h] Ratio of V/K values for fast and slow isomers of CHCP of 10 was compared with a value of 16 as obtained from analysis of biphasic progress curve for NADH production. [i] Ratio of V/K values as obtained from biphasic progress curve for NADH production in the presence of DL-arogenate.

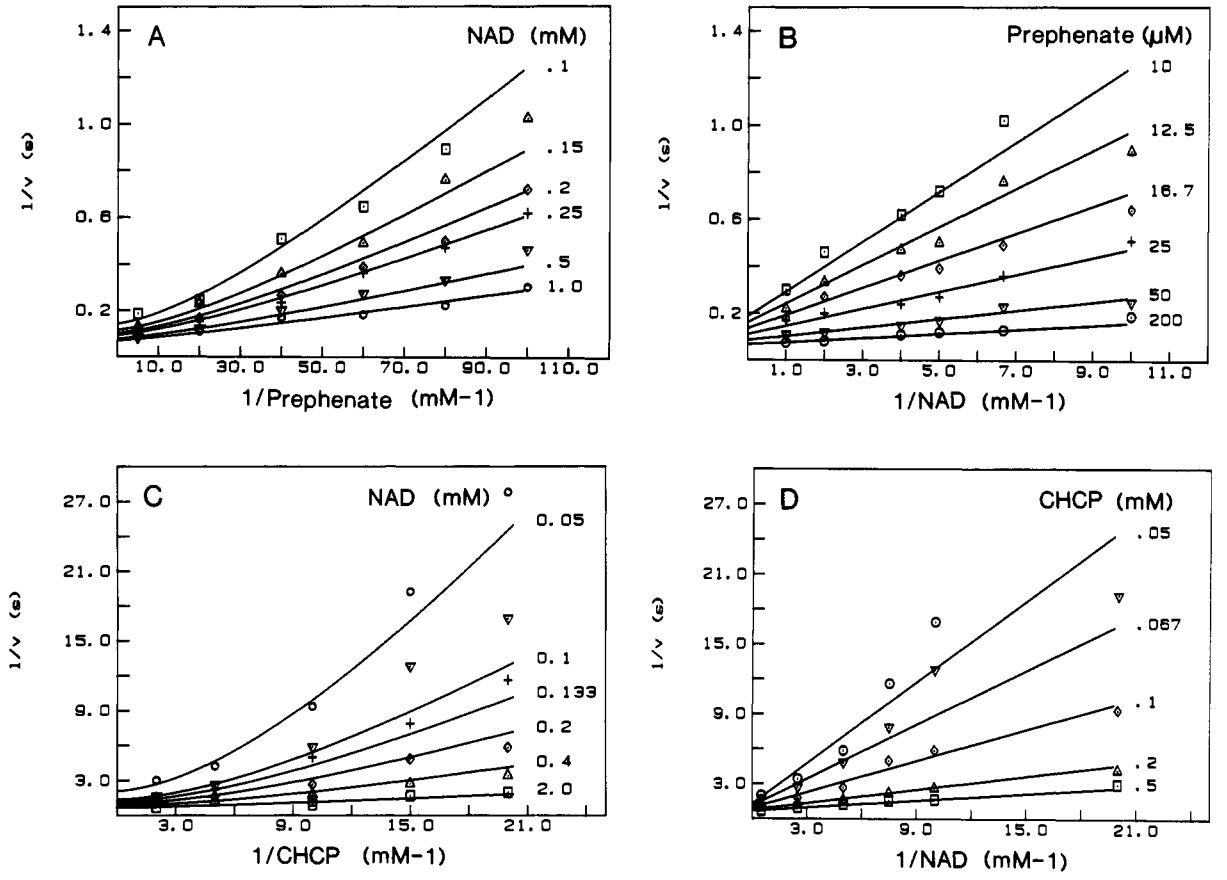


FIGURE 3: Variation of initial velocity of dehydrogenase reaction as a function of the concentrations of prephenate and NAD (A and B) and of the fast isomer of CHCP and NAD (C and D). The data were fitted to eq 5.

the interaction of prephenate with the enzyme. The curvature was abolished when the concentration of NAD was increased to 1 mM. The data fitted well to a 2/1 function described by eq 5 and yielded parameter values of $V = 16 \pm 1\ s^{-1}$, $K_{prephenate} = 20 \pm 3\ \mu M$, $K_{NAD} = 130 \pm 10\ \mu M$, and $\alpha = 8.4 \pm 2$. A replot of the data with NAD as the variable substrate gave rise to a linear double-reciprocal plot (Figure 3B). Thus there are no cooperative interactions for the interaction of NAD with the enzyme. A curvilinear double-reciprocal plot is also observed when chorismate is used over a range of concentrations below 10 μM for the mutase reaction (data not shown). This finding confirms the results of Christopherson and Morrison (1985) and indicates that positive cooperativity is also associated with the mutase reaction. The limited number of data points did not permit a fit to the general equation for a 2/1 function (Cleland, 1979). The turnover numbers (per monomer) at 30 °C for the mutase and dehydrogenase reactions were 55 and 36 s^{-1}, respectively (Table II). These values are comparable to those previously reported by Hermes et al. (1984) after allowance for the difference in temperatures.

Kinetic Characterization of the Dehydrogenase Reaction with CHCP as Substrate. The progress curve for NADH formation by prephenate dehydrogenase with the preparation of CHCP that contains two pairs of enantiomers was biphasic. The V/K values obtained by fitting the data to eq 8 indicated a 16-fold difference in reaction rate for the fast and slow enantiomers. This value can be compared with the 23-fold difference reported by Hermes et al. (1984). The difference in rates is sufficiently great to allow detailed kinetic investi-

Table III: Inhibition of Prephenate Dehydrogenase by NADH and COCP and of Chorismate Mutase by COCP

inhibitor	varied substrate	fixed substrate	type of inhibition	apparent K_i (μM)[a] slope	apparent K_i (μM)[a] intercept	value of kinetic constant (μM)[b]	reaction
COCP[c]	NAD	prephenate (20 μM)	NC[d]	670 ± 20		490 ± 130	E + ketone
					340 ± 40	220 ± 30	E–NAD + ketone
NADH	prephenate	NAD (75 μM)	NC	68 ± 8		47 ± 6	E + NADH
					28 ± 2	19 ± 2	E–prephenate + NADH
COCP	prephenate	NAD (2 mM)	NC	240 ± 30		18 ± 5	E–NAD + COCP
					770 ± 140	53 ± 7	E–prephenate–NAD + COCP
COCP	chorismate		NC	1200 ± 300		1200 ± 300	E + COCP
					7500 ± 200	7500 ± 200	E–chorismate + COCP

[a] Values for the apparent inhibition constants were obtained by fitting the data to eq 7. [b] True dissociation constants were calculated from the determined apparent values by use of the concentrations of fixed substrate together with values of the appropriate kinetic parameter (Table II) by the procedure outlined by Smith and Morrison (1971). [c] COCP was derived from the fast isomer of CHCP. [d] NC denotes noncompetitive inhibition.

gations with the fast isomer of CHCP without interference by the slow isomer.

Initial velocity data obtained for the dehydrogenase reaction by varying NAD and the fast isomer of CHCP, over relatively high concentration ranges, gave rise to double-reciprocal plots that were linear and of the intersecting type (data not shown). Values for the kinetic parameters are shown in Table II. However, over a range of lower concentrations of CHCP, reciprocal plots of velocity as a function of the CHCP concentration were concave up (Figure 3C), while a plot of the same data with NAD as the variable substrate was linear (Figure 3D). The data could be fitted to the 2/1 function described by eq 5 and yielded parameter values of $V = 1.58 \pm 0.17$ s^{-1}, $K_{CHCP} = 49 \pm 19$ μM, $K_{NAD} = 117 \pm 22$ μM, and $\alpha = 29 \pm 19$. Because the maximum velocity of the reaction with the fast isomer of CHCP is much less than that with prephenate, it could be demonstrated readily that this isomer acts as a linear competitive inhibitor with respect to prephenate (data not shown). Similar initial velocity experiments with the purified slow isomer of CHCP yielded the data listed in Table II. It will be noted that by comparison with the data for the fast isomer there is a 4-fold increase in the value of the Michaelis constant and about a 2.5-fold decrease in the maximum velocity (cf. Table II).

Kinetic Characterization of the Dehydrogenase Reaction with COCP as Substrate. Initial velocity studies of the dehydrogenase reaction in the reverse direction, in the presence of NADH and the ketone derived from the fast isomer of CHCP, yielded a family of straight lines that intersected on the vertical axis (data not shown). The data fitted poorly the equations that describe sequential (eq 3) and equilibrium ordered (eq 4) mechanisms. However, kinetic constants were well determined when the data were fitted to the equation for a sequential mechanism (eq 3) from which the terms K_aB and K_bA are omitted. These terms become kinetically negligible when both substrates are varied over a range of concentrations that is high relative to their Michaelis constants. It should also be noted that if the reaction conforms to a rapid equilibrium, random mechanism, $K_{ia}K_b = K_aK_{ib}$. The values obtained for V and $K_{ia}K_b$ (or K_aK_{ib}.) were 0.013 s^{-1} and 7950 μM^2, respectively. To determine the Michaelis constant for COCP (K_a), the value of K_{ib}, the dissociation constant for the reaction of NADH with free enzyme, was determined independently from a study of the noncompetitive inhibition of the forward reaction by NADH with respect to prephenate (Table III). The true value of K_{ib} was determined to be 47 ± 6 μM, and thus the value for K_a is 170 ± 30 μM (Table II). Similarly, the value for K_{ia}, the dissociation constant for the interaction of COCP with the free enzyme, was obtained from studies of the noncompetitive inhibition of the same forward reaction by COCP with respect to NAD. The resulting value of 490 ± 130 μM (Table III) was used to calculate a value of 16 ± 5 μM for the Michaelis constant of NADH (Table II).

Inhibition of Mutase and Dehydrogenase Reactions by COCP. COCP, derived from the fast isomer of CHCP, was tested as an inhibitor of the dehydrogenase reaction by varying prephenate in the presence of a saturating concentration of NAD and as an inhibitor of the mutase reaction by varying chorismate. The initial velocity data indicated that the inhibitor was noncompetitive in each reaction, and values for the dissociation constants are listed in Table III.

pH-Independent Equilibrium Constant. Since the dehydrogenase reaction is reversible with the fast and slow isomers of CHCP as substrates, determinations were made of the equilibrium constant for the reaction by use of a preparation that contained both isomers. The pH-independent value so obtained at pH 8.0 and 25 °C was 1.42×10^{-7} M. The value for the constant is in the range of 10^{-7}–10^{-8} M as obtained with alcohol dehydrogenase when cyclohexenol is used as the substrate (unpublished observations of W.W.C.).

Kinetic Characterization of the Dehydrogenase Reaction with DL-*Arogenate as Substrate.* The kinetic parameters for the dehydrogenase reaction when purified DL-arogenate was used as a substrate are listed in Table II. Naturally occurring L-arogenate is a poor substrate for prephenate dehydrogenase from *E. coli*, as indicated by a V/K value which is about 1% that of prephenate. The maximum velocity of the dehydrogenase reaction with L-arogenate is reduced about 10-fold while the Michaelis constant for this substrate is elevated about 10-fold relative to that of prephenate. The value for the Michaelis constant may be compared with values of 20–340 μM reported for arogenate dehydrogenases isolated from a variety of sources (Bonner & Jensen, 1987).

Discussion

The present investigation has yielded insights into the ability of prephenate dehydrogenase to utilize substrates other than the naturally occurring prephenate. Analogues of prephenate have been prepared by new or improved chemical syntheses and used to undertake detailed kinetic investigations on the mechanism of the dehydrogenase reaction. For these studies, the bifunctional enzyme chorismate mutase–prephenate dehydrogenase has been isolated in pure form by a modified procedure. In addition, the inhibitor effect of an analogue of prephenate on chorismate mutase activity has been determined.

Substrate Specificity of Prephenate Dehydrogenase. The effectiveness of prephenate analogues as substrates for the prephenate dehydrogenase reaction in the forward direction can best be expressed in terms of V/K values. Such values represent apparent first-order rate constants for the interaction of substrates with the enzyme–NAD complex. The results

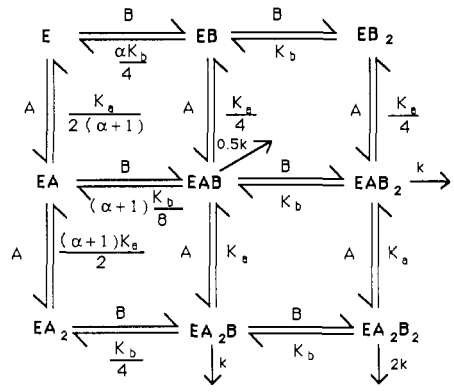
(Table II) indicate that, in relation to prephenate, the fast isomer of CHCP is a poor substrate with a V/K value only 2% of that obtained with prephenate. However, it is a better substrate than either L-arogenate or the slow isomer of CHCP. Because of the reversibility of the reaction with CHCP, it was possible to determine the equilibrium constant (K_{eq}) for the reaction and to study the reverse reaction with COCP and NADH as substrates. The K_{eq} value of 1.4×10^{-7} M shows that the equilibrium of the reaction lies very much toward ketone (COCP) formation. COCP derived from the fast isomer of CHCP also proved to be a very poor substrate with a V/K value that is only 0.4% of that observed with CHCP in the forward direction. This result is in accord with the finding that the maximum velocity of the reverse reaction is some 150 times slower than that of the forward reaction. However, the Michaelis constants for the fast isomer of CHCP and COCP are comparable and only 4-fold higher than that for prephenate (Table II).

The studies with DL-arogenate establish for the first time that L-arogenate can act as a substrate for prephenate dehydrogenase from *E. coli*. An earlier report by Byng et al. (1985) with the dehydrogenase from *Acinetobacter calcoaceticus* listed Michaelis constants of 350 μM and 33 μM for L-arogenate and prephenate, respectively. However, these authors used only a partly purified preparation of the enzyme. While the value given for the Michaelis constant of L-arogenate by Byng et al. (1985) is similar to that recorded in Table II, a significantly lower value of 90 μM has been determined for L-arogenate with a purified arogenate-specific dehydrogenase (Mayer et al., 1985).

Structural Requirements for Substrates of Prephenate Dehydrogenase. From the results of the present study and those of Hermes et al. (1984), conclusions can be drawn about the structural features that determine whether or not derivatives of prephenate function as good substrates for prephenate dehydrogenase. It is clear that both the carbonyl group of the pyruvyl moiety and the degree of unsaturation of the cyclohexadiene ring of prephenate influence the kinetic characteristics of the compounds that act as substrates. The more marked changes are associated with alterations in the degree of saturation of the ring. Thus, the absence of a carbonyl group, as in 1-carboxy-4-hydroxy-2,5-cyclohexadiene-1-propanoate (deoxoprephenate), has little effect on catalysis but does result in a 4-fold increase in the value of the Michaelis constant. Saturation of one double bond of deoxoprephenate to produce the fast isomer of CHCP has relatively little additional effect on the value of the Michaelis constant for the substrate but does cause a further 15-fold decrease in the maximum velocity of the reaction (cf. Table II). An even more marked 1000-fold drop in the maximum velocity occurs when the ring is completely saturated. While there is also an elevation in the Michaelis constant, the increase is only in the vicinity of 10-fold (Hermes et al., 1984). The most marked effect of saturation of one double bond of prephenate is the production of compounds that, unlike prephenate, do not undergo decarboxylation. The position of the double bond in the ring structure is also critical as exemplified by a 10-fold difference in the relative V/K values for the fast and slow isomers of CHCP. This difference arises because of the 4-fold elevation of the Michaelis constant and the 2.5-fold reduction in the maximum velocity when the position of the double bond is shifted from one side of the ring to the other.

Although removal of the carbonyl group of the pyruvyl moiety of prephenate has relatively small effects on the K_m and V values, the change of the carbonyl to an amino group, to produce arogenate, causes a 10-fold increase in K_m and a 10-fold reduction in V. L-Arogenate and the slow isomer of CHCP exhibit similar K_m values, although there is a 5-fold greater maximum velocity for the reaction with L-arogenate.

Scheme II



Kinetic Mechanism of the Dehydrogenase Reaction with Prephenate and CHCP as Substrates. Linear initial velocity patterns are obtained for the dehydrogenase reaction in the forward direction when prephenate and the fast isomer of CHCP are varied over a range of higher substrate concentrations. Under these conditions, the reaction with prephenate or CHCP over a range of lower concentrations in the presence of different, fixed concentrations of NAD (Figure 3) can be accounted for by the mechanism illustrated in Scheme II. In this scheme, E represents the dimeric chorismate mutase–prephenate dehydrogenase with one active site on each of the subunits. Thus two molecules of NAD (A) and two molecules of prephenate or CHCP (B) can be present on the enzyme at the same time. It is assumed that, when the two substrates for the reaction are bound at one of the active sites, the rate of product formation is not influenced by the binding of substrate at the other site. Under these conditions, the central complexes EA_2B_2, EA_2B, EAB_2, and EAB give rise to products in the ratio 2:1:1:0.5. Such a ratio is determined by the distribution of substrates between the two active sites. (Product formation is indicated simply by arrows in Scheme II.) It is also assumed, on the basis of the aforementioned arguments, that catalysis is rate-limiting so that K_a and K_b represent dissociation constants for the reactions with the enzyme of NAD and prephenate (or CHCP), respectively. However, there must be associated with the dissociation constants for each step of the reaction sequence a statistical factor which is determined by the number of reactions that can give rise to a particular complex. Further, an interaction factor, α, must be included to allow for the variation in the binding of substrate to different enzyme forms.

The initial rate equation for the proposed mechanism with B as the variable substrate can be expressed in double-reciprocal form as eq 9. The equation has the form of a 2/1

$$\frac{1}{v} = \frac{1}{V}\left[\frac{1 + \frac{K_a}{2A} + K_b\left(1 + \frac{K_a}{2A}\right)\frac{1}{B} + \frac{K_b^2}{4}\left(1 + \frac{\alpha K_a}{2A}\right)\left(\frac{1}{B}\right)^2}{\left(1 + \frac{K_b}{2}\right)\frac{1}{B}}\right] \quad (9)$$

function (Cleland, 1979) and predicts that double-reciprocal plots of velocity as a function of substrate concentration at nonsaturating concentrations of NAD would be curvilinear

as shown in panels A and C of Figure 3. As indicated in the text, the data of these two figures fitted well to the equation for a 2/1 function. Therefore, the data are both qualitatively and quantitatively in accord with the predictions of Scheme II. In the presence of high concentrations of NAD (A) or either prephenate or CHCP (B), eq 9 reduces to general equations for simple Michaelis–Menten reactions in which $K_a/2$ and $K_b/2$ denote the Michaelis constants for NAD and the non-nucleotide substrates, respectively. The data of Figure 3 are in agreement with these predictions. Rearrangement of eq 9 with NAD (A) as the variable substrate yields eq 10,

$$\frac{1}{v} = \frac{K_a}{V}\left[\frac{\frac{1}{2}\left(1 + \frac{K_b}{B}\right) + \frac{K_b^2}{4B^2}}{1 + \frac{K_b}{2B}}\right]\frac{1}{A} + \frac{1}{V}\left(1 + \frac{K_b}{2B}\right) \qquad (10)$$

which predicts that double-reciprocal plots of velocity as a function of the concentration of NAD will be linear irrespective of the fixed concentration of prephenate (or CHCP). The data of Figure 3B,D confirm this prediction. Thus all the kinetic data obtained with either prephenate or CHCP in the presence of NAD can be described by the unique kinetic mechanism illustrated in Scheme II.

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