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A Single Cyclohexadienyl Dehydrogenase Specifies the Prephenate Dehydrogenase and Arogenate Dehydrogenase Components of the Dual Pathways to L-Tyrosine in *Pseudomonas aeruginosa**

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Dual biosynthetic pathways diverge from prephenate to L-tyrosine in *Pseudomonas aeruginosa*, with 4-hydroxyphenylpyruvate and L-arogenate being the unique intermediates of these pathways. Prephenate dehydrogenase and arogenate dehydrogenase activities could not be separated throughout fractionation steps yielding a purification of more than 200-fold, and the ratio of activities was constant throughout purification. Thus, the enzyme is a cyclohexadienyl dehydrogenase. The native enzyme has a molecular weight of 150,000 and is a hexamer made up of identical 25,500 subunits. The enzyme is specific for NAD^+ as an electron acceptor, and identical K_m values of 0.25 mM were obtained for NAD^+, regardless of whether activity was assayed as prephenate dehydrogenase or as arogenate dehydrogenase. K_m values of 0.07 mM and 0.17 mM were calculated for prephenate and L-arogenate, respectively. Inhibition by L-tyrosine was noncompetitive with respect to NAD^+, but was strictly competitive with respect to either prephenate or L-arogenate. With cyclohexadiene as variable substrate, similar K_i values for L-tyrosine of 0.06 mM (prephenate) and 0.05 mM (L-arogenate) were obtained. With NAD^+ as the variable substrate, similar K_i values for L-tyrosine of 0.26 mM (prephenate) and 0.28 mM (L-arogenate), respectively, were calculated. This is the first characterization of a purified, monofunctional cyclohexadienyl dehydrogenase.

Prephenate, a natural product having a carboxycyclohexadienyl moiety, is the initial precursor that is unique to the biosynthesis of L-tyrosine and L-phenylalanine in microorganisms and higher plants (1). The aromatization of prephenate to form the phenylalanine precursor (phenylpyruvate) via prephenate dehydratase, or to form the tyrosine precursor (4-hydroxyphenylpyruvate) via prephenate dehydrogenase, were assumed to be universal enzyme steps functioning in nature for some time. In 1974, an alternative fate of prephenate was established whereby it was utilized as a substrate by prephenate aminotransferase in cyanobacteria (2). The product of this reaction, initially named pretyrosine (2), is an amino acid that retains a carboxycyclohexadienyl structure. Pretyrosine was renamed L-arogenate (3) when its function as a precursor of L-phenylalanine in some organisms became apparent (literature background summarized in Ref. 4).

The diversity of aromatic amino acid biosynthesis in nature is substantial. Phenylpyruvate-to-phenylalanine/L-arogenate-to-tyrosine, L-arogenate-to-phenylalanine/4-hydroxyphenylpyruvate-to-tyrosine, and L-arogenate-to-phenylalanine/L-arogenate-to-tyrosine patterns (see Fig. 1) are widespread in addition to the classical phenylpyruvate-to-phenylalanine/4-hydroxyphenylpyruvate-to-tyrosine combination. It has also become apparent that alternative pathways to phenylalanine and/or tyrosine may exist in the same organism (4–6). The enzymological basis for a dual pathway to phenylalanine or tyrosine can be (i) the expression of two dehydratases (in the case of phenylalanine) or two dehydrogenases (in the case of tyrosine), or (ii) the expression of a single cyclohexadienyl dehydratase (in the case of phenylalanine) or cyclohexadienyl dehydrogenase (in the case of tyrosine), either having a degree of substrate ambiguity that accommodates catalysis with both prephenate and L-arogenate.

Pseudomonas aeruginosa was the first example of an organism possessing dual pathways to both phenylalanine and tyrosine (5). This pathway arrangement has proven to be quite common in Gram-negative bacteria (7). Since phenylalanine and tyrosine auxotrophs were not successfully isolated from *P. aeruginosa* by standard techniques, it appeared that multiple genes encoding alternative enzyme-step options explained this "reluctant auxotrophy" (8). Indeed, it was shown that the two pathways to phenylalanine utilize readily separated dehydratases, one a prephenate dehydratase and the other a cyclohexadienyl dehydratase (5). The leaky phenotype of a prephenate dehydratase mutant has recently been described (9).

With this background, by analogy with the established coexistence of two dehydratase enzymes for phenylalanine biosynthesis, it was assumed that the absence of tyrosine auxotrophs in *P. aeruginosa* would be explained on the basis of alternative dehydrogenase enzymes, either masking the absence of the other. This communication shows that, to the contrary, the enzymological basis of the pathway duality to tyrosine in *P. aeruginosa* is tied to the substrate ambiguity of a single cyclohexadienyl dehydrogenase.

MATERIALS AND METHODS[1]

RESULTS AND DISCUSSION

Physical and Catalytic Properties of Cyclohexadienyl Dehydrogenase—The subunit molecular weight of the enzyme was

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[1] Portions of this paper (including "Materials and Methods," Table I, and Figs. 1–5) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

25,500 as determined with 12.6% SDS[2]-polyacrylamide gel electrophoresis (Fig. 2), and the native enzyme molecular weight is about 150,000 as estimated by means of Sephadex G-200 gel filtration.

Both prephenate dehydrogenase and arogenate dehydrogenase require NAD^+ as cofactor, $NADP^+$ failing to be utilized. Identical K_m values of 0.25 mM were obtained for NAD^+, regardless of whether activity was assayed as prephenate dehydrogenase (prephenate was fixed at 0.25 mM) or as arogenate dehydrogenase (arogenate was fixed at 0.54 mM) (Fig. 5). When NAD^+ was fixed at a concentration of 0.5 mM in the assay system, the enzyme had a K_m value for prephenate of 0.07 mM, and a K_m value of 0.17 mM for L-arogenate (Fig. 4). Both prephenate dehydrogenase and arogenate dehydrogenase activities of cyclohexadienyl dehydrogenase were competitively inhibited by L-tyrosine (Fig. 4). Similar K_i values of 0.06 mM and 0.05 mM were calculated, with reference to prephenate and L-arogenate, respectively. Inhibition of cyclohexadienyl dehydrogenase assayed as either prephenate dehydrogenase or as arogenate dehydrogenase was noncompetitive with respect to NAD^+ (Fig. 5). Similar K_i values of 0.26 mM (prephenate) and 0.28 mM (L-arogenate) were calculated.

Biochemical Flow Route to Tyrosine in Vivo—Since *P. aeurginosa* possesses a cyclohexadienyl dehydrogenase that utilizes either prephenate or L-arogenate *in vitro*, the question arises as to what fraction of tyrosine derives from the two precursors *in vivo*. L-Arogenate molecules are generated by transamination of prephenate. Although five aromatic aminotransferase activities capable of prephenate transamination have been fractionated from *P. aeruginosa* (21), the substrate affinity for prephenate is poor compared to other systems (22). However, the monofunctional chorismate mutase (denoted CM-F) which provides prephenate molecules fated for transformation to tyrosine possesses low activity compared to CM-P (the chorismate mutase component of the bifunctional P-protein that channels (23) chorismate molecules to phenylalanine). Since the affinity of cyclohexadienyl dehydrogenase (0.07 mM) is greater than the affinity of any aromatic aminotransferase, preferential flow of prephenate to 4-hydroxyphenylpyruvate, rather than to L-arogenate, seems likely.

In the case of the dual pathways of phenylalanine biosynthesis, it has been shown that most or all phenylalanine is derived from phenylpyruvate, rather than from L-arogenate under ordinary conditions of wild-type growth (24). However, when endogenous prephenate levels were dramatically elevated by a mutation abolishing early pathway regulation, phenylalanine was heavily excreted due to the catalytic activities of the unregulated CM-F and cyclohexadienyl dehydratase components (20). In this mutant, it is still likely that more phenylalanine is derived from phenylpyruvate than from L-arogenate since cyclohexadienyl dehydratase favors prephenate over L-arogenate substantially.

The ability of cyclohexadienyl dehydrogenase to utilize L-arogenate may be an evolutionary relic. In this connection, it is interesting that the prephenate dehydrogenase catalytic component of the bifunctional T-protein in *E. coli* and all of its enteric relatives is actually a cyclohexadienyl dehydrogenase (25). The bifunctional T-protein probably originated from fusion of ancestral genes encoding CM-F and cyclohexadienyl dehydrogenase (26). This channeling arrangement effectively removes prephenate molecules from accessibility to prephenate aminotransferase. Hence, L-arogenate molecules must rarely or never be precursors for L-tyrosine biosynthesis under ordinary laboratory growth conditions in organisms like *E. coli*, even though the enzymological potential is there.

Since the ability to utilize L-arogenate has persisted so extensively, one wonders if some selective advantage exists. For example, the ability to scavenge exogenous L-arogenate, a widespread amino acid of plants and microorganisms, may spare the substantial biochemical expense for biosynthesis of tyrosine molecules in natural environments.

Acquisition of Tyrosine Auxotrophs—The reluctant auxotrophy of *P. aeruginosa* for phenylalanine was explained as the consequence of independent dual pathways to phenylalanine (8). A mutant lacking dehydratase exhibited a leaky requirement for phenylalanine, showing that the exclusive biosynthesis of phenylalanine via cyclohexadienyl dehydratase is rate-limiting to growth. If deficient mutants lacking cyclohexadienyl dehydrogenase activity would eliminate both the 4-hydroxyphenylpyruvate and L-arogenate flow routes, why have tyrosine auxotrophs not been isolated? Tyrosine (but not phenylalanine) is an excellent carbon source for *P. aeruginosa*, being catabolized to fumarate and acetoacetate. Catabolic depletion of tyrosine is not abolished in the presence of glucose or fructose. Since mutant hunts were carried out on glucose-based or fructose-based medium, it now seems likely that tyrosine catabolism would account for failure to select tyrosine auxotrophs. It is possible that growth of *P. aeruginosa* on a trichloroacetic acid cycle intermediate such as malate or fumarate would abolish catabolite depletion of tyrosine, thus providing conditions allowing positive selection of tyrosine auxotrophs.

Acknowledgments—We gratefully acknowledge the receipt of a sample of (+)-2,3-dihydro-2,3-dihydroxybenzoate from Frank Rusnak and Christopher Walsh of Harvard Medical School. George D. Hegeman generously supplied a culture of *Alcaligenes eutrophus* mutant strain B9, used to accumulate (−)-3,5-cyclohexadiene-1,2-diol-1-carboxylic acid.

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[2] The abbreviation used is: SDS, sodium dodecyl sulfate.

Continued on next page.

SUPPLEMENTARY MATERIAL TO

A Single Cyclohexadienyl Dehydrogenase Specifies the Prephenate Dehydrogenase and Arogenate Dehydrogenase Components of the Dual Pathways to L-Tyrosine in *Pseudomonas aeruginosa*

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MATERIALS AND METHODS

Microbiological Aspects -- The PAO-1 strain of *P. aeruginosa* (wild-type prototroph) was originally obtained from B. W. Holloway (10). Cultures were grown at 37°C in a minimal salts medium (11) containing 0.5% glucose (autoclaved separately). The cells were harvested by centrifugation during the late exponential phase of growth.

Analytical Procedures -- Prephenate dehydrogenase and arogenate dehydrogenase were assayed as previously described (12). The reaction mixture (400 µl) contained 0.5 m*M* NAD^+, enzyme, and 0.4 m*M* prephenate or L-arogenate in 50 m*M* potassium phosphate buffer, pH 7.5. The continuous formation of NADH was determined spectrophotofluorimetrically with a Shimadzu RF500U spectrophotofluorimeter, excitation wavelength 340 nm and emission wavelength 460 nm. One nanomole of NADH produced corresponds to 20 fluorescence units. One unit of activity forms 1 micromole of NADH per min at 37°C.

Protein concentration was determined by the method of Bradford (13). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the procedure of Laemmli (14). For subunit molecular weight determination, 12.6% polyacrylamide gel was employed with the following molecular-weight standards: α-lactalbumin (14,400), soybean trypsin inhibitor (20,100), carbonic anhydrase (30,000), ovalbumin (43,000), bovine serum albumin (67,000) and phosphorylase (94,000).

Sephadex G-200 gel filtration was carried out for the molecular-weight determination of native enzyme. The method was the same as that employed in the following purification precedure. The molecular-weight standards were carbonic anhydrase (30,000), bovine serum albumin (67,000), alcohol dehydrogenase (150,000) and β-amylase (200,000).

Biochemicals -- Protein standards for molecular-weight determination were obtained from Pharmacia Fine Chemicals. L-Tyrosine, NAD^+, dithiothreitol, and Sephadex G-200 were obtained from Sigma. DEAE-cellulose (DE52) was acquired from Whatman, and hydroxylapatite was obtained from Bio-Rad. Barium prephenate was prepared from *Salmonella typhimurium* (15) and converted to the potassium salt with excess K_2SO_4 prior to use. L-Arogenate was prepared from *Neurospora crassa* (16). All chemicals were of the best grade commercially available. (+)-2,3-Dihydro-2,3-dihydroxybenzoate was a gift from Dr. Frank Rusnak and Dr. Christopher Walsh. (-)-3,5-Cyclohexadiene-1,2-diol-1-carboxylic acid was accumulated in the culture medium of a mutant of *Alcaligenes eutrophus*, strain B9 (17).

Purification of Prephenate Dehydrogenase-Arogenate Dehydrogenase -- All steps were performed at 4°C, and the centrifugations were at 36,000 x *g* for 30 min unless otherwise stated. The Buffer A solution contained 20 m*M* potassium phosphate and 1 m*M* dithiothreitol, pH 7.2.

(I) Preparation of extract. Whole cell pellets (30 grams) were suspended in Buffer A, and disrupted by sonication, using a Labline Ultratip sonicator. Cellular debris was removed by centrifugation, ultracentrifugation at 150,000 x *g* was then carried out for 60 min, and the clear supernant was collected.

(II) Ammonium sulfate precipitation. The clear extract was adjusted to 40% $(NH_4)_2SO_4$ saturation with solid salt. After stirring for 10 min, the pellet was removed by centrifugation. The supernatant was brought to 60% saturation with $(NH_4)_2SO_4$. After stirring and centrifugation, the precipitate was dissolved in a minimal amount of Buffer A and dialyzed against several changes of 20 volumes of the same buffer.

(III) DEAE-cellulose chromatography. The dialyzed enzyme solution was applied to a DE52 column (2.5 x 30 cm) equilibrated in Buffer A. After washing with 100 ml of buffer, the column was eluted with a linear salt gradient of KCl, one reservoir containing 300 ml of Buffer A and the other 300 ml of Buffer A plus 0.5 *M* KCl. Fractions of 2.8 ml were collected, and appropriate fractions were pooled.

(IV) Hydroxylapatite chromatography. The pooled fractions were concentrated to 5 ml by means of an Amicon-PM10 membrane. Then 50 ml of Buffer A was added, mixed and concentration to 5 ml was again carried out. The concentrated enzyme was applied to a column (1.5 x 15 cm) of hydroxylapatite previously equilibrated with Buffer A, and the column was then eluted with a 600-ml linear gradient of 20 m*M* to 250 m*M* potassium phosphate buffer, plus 1 m*M* dithiothreitol (pH 7.2). Fractions of 3.2 ml volume were collected, and those containing most of the enzyme activities were pooled.

(V) Sephadex G-200 gel filtration. The enzyme solution was concentrated to 1.5 ml with an Amicon-PM10 membrane and applied to a Sephadex G-200 column (2.5 x 98 cm) equilibrated with Buffer A. Protein was eluted with the same buffer at a rate of 6 ml/hr. Fractions of 3.0 ml were collected, and those having high enzyme activities were pooled.

One major protein band was detected when fractions of step V having the highest activities of both prephenate dehydrogenase and arogenate dehydrogenase were examined by SDS-polyacrylamide gel electrophoresis (Fig. 2). Table I shows that the two dehydrogenase activities co-purified more than 200-fold, and the activity ratio was nearly constant during purification. The elution profiles following fractionation steps of DEAE-cellulose chromatography, hydroxylapatite chromatography and Sephadex G-200 gel filtration showed co-elution of the two activities (Fig. 3).

Substrate Ambiguity of CDH -- 2,3-Dihydro-2,3-dihydroxybenzoate is the substrate for a dehydrogenase that is the gene product of *entA* in *E. coli*. The aromatic catecholic product of this reaction, 2,3-dihydroxybenzoate, is a precursor of enterobactin, a potent siderophore (19). CDH was unable to use this compound as substrate.

Another carboxycyclohexadienyl compound, 3,5-cyclohexadiene-1,2-diol-1-carboxylic acid is a substrate for a dehydrogenase enzyme catalyzing a step in the metabolism of benzoate and mandelate in organisms such as *Alcaligenes eutrophus* (17), *Pseudomonas putida* (20), and *Acinetobacter calcoaceticus* (20). Although this reaction not only aromatizes but also decarboxylates the substrate, similar to the CDH-catalyzed reaction, CDH was unable to utilize 3,5-cyclohexadiene-1,2-diol-1-carboxylic acid as an alternative substrate.

TABLE I

Purification of cyclohexadienyl dehydrogenase from *P. aeruginosa*

Purification step	Total protein	Prephenate dehydrogenase (PDH)			Arogenate dehydrogenase (ADH)			Ratio PDH:ADH
		total	specific activity	purification	total	specific activity	purification	
	mg	units	units/mg	fold	units	units/mg	fold	
Crude extract								
36,000 x *g*	1550	85.6	0.06	1.0	53.5	0.04	1.0	1.60
150,000 x *g*	1125	82.5	0.07	1.2	52.5	0.05	1.3	1.57
Ammonium sulfate precipitation	485	63.1	0.13	2.2	41.5	0.09	2.3	1.52
DEAE-cellulose chromatography	51	49.8	0.98	16	30.6	0.60	15	1.63
Hydroxyapatite chromatography	7.0	27.4	3.91	65	16.1	2.30	58	1.70
Sephadex G-200 gel filtration	1.1	13.7	12.5	208	8.9	8.09	202	1.54

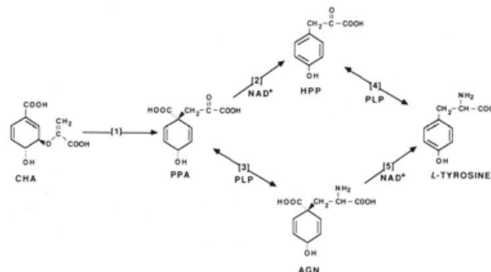

Fig. 1. Dual biosynthetic routes to L-tyrosine in *P. aeruginosa*. Enzyme steps: 1, chorismate (CHA) mutase; 2, prephenate (PPA) dehydrogenase; 3, prephenate aminotransferase; 4, 4-hydroxyphenylpyruvate (HPP) aminotransferase; 5, arogenate (AGN) dehydrogenase. Enzyme steps 2 and 5 are catalyzed by a single enzyme called cyclohexadienyl dehydrogenase (CDH). NAD^+, nicotinamide adenine dinucleotide; PLP, pyridoxal 5'-phosphate.

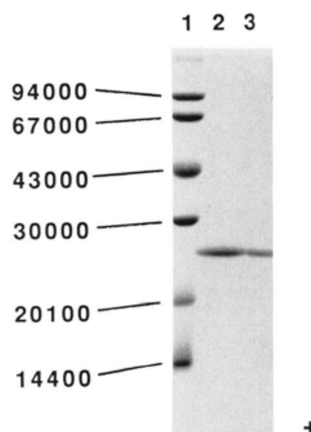

Fig. 2. SDS-polyacrylamide gel electrophoresis of purified CDH. Protein samples were separated on a 12.6% polyacrylamide gel and stained with coomassie blue. Lane 1, molecular-weight standards; lane 2 and lane 3, purified enzyme.

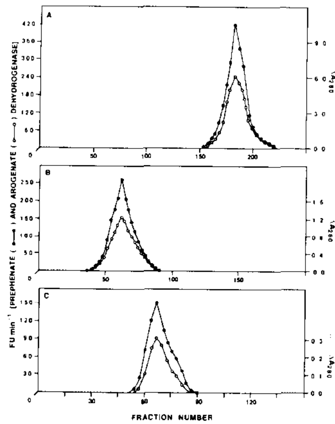

Fig. 3. Elution profiles of CDH assayed as prephenate dehydrogenase and arogenate dehydrogenase. Activities of both prephenate dehydrogenase and arogenate dehydrogenase were assayed immediately after fractionation steps of (A) DEAE-cellulose chromatography, (B) hydroxylapatite chromatography and (C) Sephadex G-200 gel filtration. The activities are expressed as fluorescence units, and the distribution of protein was monitored at 280 nm.

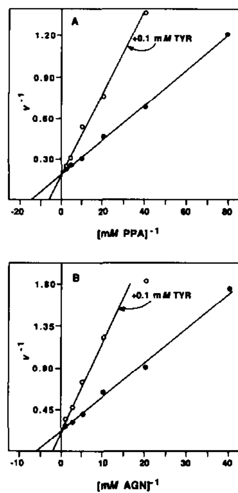

Fig. 4. Double-reciprocal plot for kinetic analysis of CDH assayed as prephenate dehydrogenase (A) or as arogenate dehydrogenase (B). Velocity was measured as NADH appearance at 37°C as described under "Materials and Methods". Reaction mixtures having a total volume of 0.4 ml contained 0.5 mM NAD^+, the concentration of prephenate or L-arogenate indicated on the abscissa scales and 0.3 µg of purified enzyme. Symbols: No L-tyrosine present, ● – ●; L-tyrosine present in reaction mixtures at a fixed concentration of 0.1 mM, o – o.

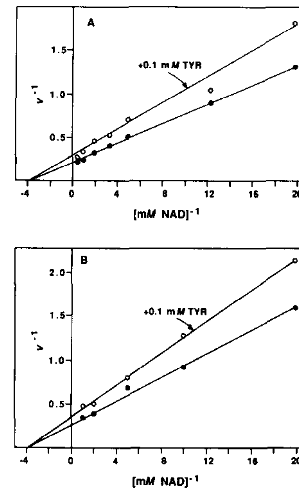

Fig. 5. Double-reciprocal plot for kinetic analysis of prephenate dehydrogenase (A) and arogenate dehydrogenase (B) with NAD^+ as the variable substrate. Velocity was measured as NADH formation as described under "Materials and Methods". Reaction mixtures having a total volume of 0.4 ml contained 0.25 mM prephenate (A) or 0.54 mM L-arogenate (B), the concentration of NAD^+ indicated on the abscissa scales and 0.3 µg of purified enzyme. Symbols: No L-tyrosine present, ● – ●; L-tyrosine present in reaction mixtures at a fixed concentration of 0.1 mM, o – o.