BIOSYNTHESIS OF PHENYLALANINE AND TYROSINE IN *PSEUDOMONAS AERUGINOSA* AND *ZYMOMONAS MOBILIS*: MOLECULAR CLONING OF THE GENES ENCODING CYCLOHEXADIENYL DEHYDRATASE AND CYCLOHEXADIENYL DEHYDROGENASE, AND CHARACTERIZATION OF THE GENE PRODUCTS

BY

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Dedicated to my father and my mother,
whose love and care gave me the encouragement
to complete this dissertation

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TABLE OF CONTENTS

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

BIOSYNTHESIS OF PHENYLALANINE AND TYROSINE IN PSEUDOMONAS AERUGINOSA AND ZYMOMONAS MOBILIS: MOLECULAR CLONING OF THE GENES ENCODING CYCLOHEXADIENYL DEHYDRATASE AND DEHYDROGENASE, AND CHARACTERIZATION OF THE GENE PRODUCTS

BY

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The gene encoding cyclohexadienyl dehydratase (CDT), denoted as pheC, was cloned from Pseudomonas aeruginosa. The pheC product produced in Escherichia coli was purified and showed identical physical and biochemical properties as those obtained for CDT purified from P. aeruginosa. The CDT exhibited K_m values of 0.42 mM for prephenate and 0.22 mM for arogenate, respectively. The pheC gene was 807 bp in length, predicating a protein of 30,480 Mr. This compares with a Mr value of 28,000 determined for the purified enzyme by SDS-PAGE. Comparison of the CDT sequence of P. aeruginosa with three P-proteins (chorismate mutase/prephenate dehydratase) did not establish obvious homology. The N-terminal sequencing revealed that the first 11 residues of the purified pheC gene

product matched the residues 26 to 36 from the translation start site deduced from the nucleotide sequence, indicating that a 25 residue amino peptide was cleaved in *E. coli*. The CDT from *P. aeruginosa* was then purified and exhibited an identical molecular weight and sequence as did the *pheC* product expressed in *E. coli*, thus showing that the cleavage of the amino-peptide occurred in *P. aeruginosa*. Chloroform and osmotic shock treatments released 95% of CDT activity from the periplasmic spaces of *P. aeruginosa* and *E. coli*, respectively, thus demonstrating that the CDT is a periplasmic protein.

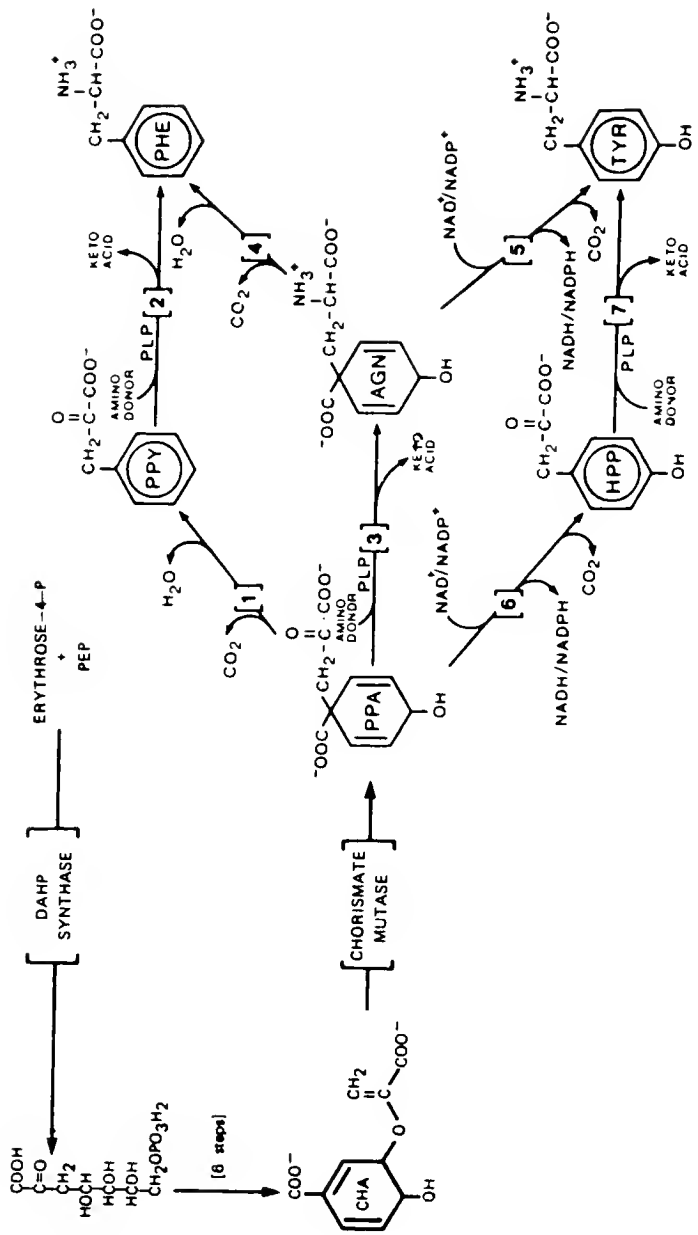
The cyclohexadienyl dehydrogenase (CDH) gene, denoted as *tyrC*, was cloned from *Zymomonas mobilis*. The *tyrC* gene was 882 bp in length, encoding a protein of 32,086 Mr. The *tyrC* product formed in *E. coli* was purified, and estimated to be 32,000 by SDS-PAGE. The activity ratios of arogenate dehydrogenase (ADH) to prephenate dehydrogenase (PDH) (3:1) remained constant throughout purification, indicating the two activities were inseparable. K_m values of 0.4 mM and 0.33 mM were obtained for prephenate and arogenate, respectively. A k_m value of 0.11 mM for NAD^+ was obtained when the enzyme was assayed as ADH or PDH. Unlike the CDHs of *E. coli* and *P. aeruginosa*, that of *Z. mobilis* is not sensitive to tyrosine inhibition. In pairwise alignments, the *Z. mobilis* CDH showed a partial identity with the PDHs of *Bacillus subtilis* (32%), *Saccharomyces cerevisiae* (19%), and the T-proteins (chorismate mutase/CDH) of *E. coli* (21%) and *Erwinia herbicola* (23%).

CHAPTER 1
LITERATURE REVIEW

The Aromatic Pathway

Biosynthesis of the aromatic amino acids begins with the condensation of erythrose-4-phosphate and phosphoenol pyruvate by 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase to form chorismate, the last intermediate common to all aromatic amino acids (Fig. 1-1). The pathway branches at chorismate to L-tryptophan, and L-phenylalanine and L-tyrosine. Chorismate, the major branchpoint metabolite, is then converted by chorismate mutase to prephenate, forming a unique precursor for L-phenylalanine and L-tyrosine. The conversion of prephenate to phenylpyruvate via prephenate dehydratase or to 4-hydroxyphenylpyruvate via prephenate dehydrogenase was assumed to be the universal enzymatic steps functioning in nature (Cotton and Gibson, 1965). In 1974, an alternative route to L-tyrosine was discovered in Cyanobacteria in which prephenate is transaminated to L-arogenate (Stenmark et al., 1974). L-Arogenate is then converted to L-tyrosine by arogenate dehydrogenase (Stenmark et al., 1974).

Fig. 1-1. Biochemical pathway for biosynthesis of aromatic amino acids. Erythrose-4-phosphate and phosphoenolpyruvate are first condensed to form 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) via the action of DAHP synthase. Chorismate (CHA) is the last intermediate common to all three aromatic amino acids. The post-prephenate pathways to phenylalanine and tyrosine, where considerable biochemical diversity has been found in nature, are shown as a composite scheme. Virtually every conceivable combination of the two dual pathway arrangements shown have been delineated in a variety of microorganisms and plants. Abbreviations: PPA, prephenate; AGN, L-arogenate; PPY, phenylpyruvate; HPP, 4-hydroxyphenylpyruvate; PHE, L-phenylalanine; TYR, L-tyrosine; PLP, pyridoxal-5-phosphate. Enzymes are numbered as follows: [1] prephenate dehydratase; [2] phenylpyruvate aminotransferase; [3] prephenate aminotransferase; [4] arogenate dehydratase; [5] arogenate dehydrogenase; [6] prephenate dehydrogenase; [7] 4-hydroxyphenylpyruvate aminotransferase. If a single dehydratase catalyzes both reactions 1 and 4, it is donated cyclohexadienyl dehydratase; if a single dehydrogenase catalyzes both reactions 5 and 6, it is denoted cyclohexadienyl dehydrogenase. The bifunctional P-protein catalyzes the sequential reactions of chorismate mutase and prephenate dehydratase. The bifunctional T-protein catalyzes the sequential reactions of chorismate mutase and prephenate dehydrogenase.


ERYTHROSE-4-P
+
PEP
DAHP SYNTHASE
[6 steps]
CHA
CHORISMATE MUTASE
PPA
AGN
PPY
HPP
PHE
TYR
PLP
AMINO DONOR
KETO ACID
NAD+/NADP+
NADH/NADPH
H2O
CO2
NH3+
[1]
[2]
[3]
[4]
[5]
[6]
[7]

L-Arogenate was initially identified as a unique precursor for L-tyrosine biosynthesis (Stenmark et al., 1974) in microorganisms, and now its role as a precursor for both L-tyrosine and L-phenylalanine has been established not only in microorganisms but also in higher plants (Byng et al., 1982).

The Dual Pathways to L-Phenylalanine and L-Tyrosine in Pseudomonas aeruginosa and Zymomonas mobilis

The biosynthetic pathways to L-phenylalanine and L-tyrosine have proven to be unexpectedly diverse in nature (Byng et al., 1982). This diversity generates a range of alternative character states that has greatly facilitated tracing the evolutionary history of aromatic biosynthesis within groups of phylogenetically related organisms (Jensen, 1985). P. aeruginosa is an opportunistic human pathogen which belongs to Superfamily B, a lineage where evolutionary history of aromatic biosynthesis has been studied in a greater detail than in any other phylogenetic grouping (Jensen, 1985). The disease of cystic fibrosis is the outcome of P. aeruginosa infection and a genetic defect in human chromosome (Rommens et al., 1989; Riordan et al., 1989; and Kerem et al., 1989).

Dual enzymatic routes to L-phenylalanine and L-tyrosine were first described in P. aeruginosa (Patel et al., 1977). A bifunctional P-protein leading to the biosynthesis of L-phenylalanine consists of chorismate mutase-P and prephenate dehydratase (Patel et al., 1977). The prephenate dehydratase

activity of the bifunctional P-protein is feedback inhibited by L- phenylalanine, but activated by L-tyrosine (Patel et al., 1977). In addition to the bifunctional P-protein, an unregulated route to L-phenylalanine is also present, which consists of monofunctional chorismate mutase-F, aromatic aminotransferase, and cyclohexadienyl dehydratase, whose catalytic reactions are often referred to as overflow pathway (Fiske et al., 1983). The cyclohexadienyl dehydratase utilizes prephenate and L-arogenate as alternative substrates (Patel et al., 1977, Zhao et al., 1992a). Thus, L-phenylalanine can be synthesized through the intermediate phenylpyruvate or L-arogenate. The dual pathways to L-phenylalanine are apparently due to substrate ambiguity of the cyclohexadienyl dehydratase.

Dual pathways to L-tyrosine biosynthesis also exist in P. aeruginosa. These consist of the chorismate mutase-F, aromatic aminotransferase, and cyclohexadienyl dehydrogenase (Patel et al., 1977). As noted above, the chorismate mutase-F, and the aminotransferase are also participating in L-phenylalanine over-flow pathway (Fiske et al., 1983). The cyclohexadienyl dehydrogenase catalyzes the conversion of prephenate to 4-hydroxyphenylpyruvate and arogenate to L- tyrosine (Patel at el., 1977). Thus, L-tyrosine can be synthesized via intermediates of arogenate and 4-hydroxyphenylpyruvate. Recently, the cyclohexadienyl dehydrogenase has been purified from P. aeruginosa. An identical K_m value was obtained regardless whether the enzyme was assayed as prephenate

dehydrogenase or as arogenate dehydrogenase, showing that a single NAD^+-linked cyclohexadienyl dehydrogenase exists which can utilize either arogenate or prephenate as substrate (Xia and Jensen, 1990). The enzyme is sensitive to feedback inhibition by L-tyrosine (Patel et al., 1977; and Xia and Jensen, 1990).

Zymomonas mobilis is a gram-negative bacterium and also a member of Superfamily C (Montenecourt, 1985). It exhibits high ethanol tolerance and is capable of rapid ethanol production, which makes it potentially useful for commercial production of ethanol (Montenecourt, 1985). The enzymic arrangement employed by this organism for the biosynthesis of L-phenylalanine and L-tyrosine is essentially unknown. Some studies (Bhatnagar and Jensen, unpublished) suggest that Z. mobilis possesses a chorismate mutase-F, prephenate dehydratase, arogenate dehydratase, and cyclohexadienyl dehydrogenase. Thus, the dual pathways to L-phenylalanine and L-tyrosine may also coexist. The presence of the cyclohexadienyl dehydrogenase in this organism has recently been established by Zhao et al. (1992b).

Evolutionary Scenario

The P-proteins from Escherichia coli (Davidson et al., 1972; and Gething and Davidson, 1976), Alcaligenes eutrophus (Friedrich et al., 1976), and Acinetobacter calcoaceticus (Ahmad et al., 1989) have been purified and characterized

thoroughly. The P-protein from E. coli has a native molecular weight of 85,000, and is composed of two identical subunits of 40,000 (Davidson et al., 1972; and Duggleby et al., 1978). The native form of enzyme tends to form tetramers at high protein concentration or in the presence of feedback inhibitor of phenylalanine (Dopheide et al., 1972). Evidence from chemical modification (Gething and Davidson, 1977a; and 1977b) and kinetic studies (Duggleby et al., 1978) strongly suggests that the P-protein has two separate catalytic sites for chorismate mutase and prephenate dehydratase. Both activities are feedback inhibited by L-phenylalanine (Gething and Davidson, 1977a; and 1977b). Mutant strains of E. coli which were defective in prephenate dehydratase, or chorismate mutase-P, or both activities, have been isolated (Baldwin and Davidson, 1981). These genetic studies further support the existence of independent catalytic sites for prephenate dehydratase and chorismate mutase-P.

Bifunctional T-proteins for L-tyrosine biosynthesis consist of chorismate mutase-T and cyclohexadienyl dehydrogenase. Bifunctional P-proteins are of ancient origin, and are apparently distributed throughout all of Superfamily B and the connecting Superfamily A (Ahmad and Jensen, 1986). On the other hand, bifunctional T-proteins are of more recent origin, and are distributed only in some species of Superfamily B (Ahmad and Jensen, 1986; and Jensen, 1985).

The T-proteins from E. coli (Hudson et al., 1984; and Koch, 1971) and Aerobacter arogenes (Koch, 1972) have been purified, and they consist of two identical subunits each with an estimated molecular weight of 39,000. Subsequently, kinetic, chemical modification, and mutational studies have suggested that the two reactions of the T-proteins occur at one active site or overlapping active sites (Hudson et al., 1984; Heyde, 1979; and Rood et al., 1982). Recently, in vitro separation of the two activities of T-protein from E. coli has been achieved genetically by Maruya et al. (1987), an indication that the T-protein also has two separate catalytic sites.

No P-proteins have been reported to utilize arogenate. However, the dehydrogenase component of T-proteins in enteric bacteria has been found to utilize both prephenate and arogenate (Ahmad and Jensen, 1987). The failure of the P-protein dehydratase to utilize arogenate might suggest that the dehydratase component of the P-proteins has lost the substrate ambiguity of the ancestral cyclohexadienyl dehydratase during evolution. The utilization of arogenate by T-proteins, on the other hand, may indicate that T-proteins did not have enough evolutionary time for the loss of recognition of arogenate as substrate since, compared with P-protein, T-protein is of relatively more recent origin.

The P-protein and T-protein genes from E. coli have been cloned and sequenced, and the two genes are contiguous

(Hudson and Davidson, 1984). Considerable similarity was observed between N-terminal portions of the two proteins, indicating that the components of chorismate mutase are located on their N-termini. Recently, the prephenate dehydratases from Corynebacterium glutamicum and Bacillus subtilis were found to share certain similarities with the C'-terminal portion of the P-proteins from E. coli and P. stutzeri (Fischer et al., 1992; Follettie and Sinskey, 1986; Hudson and Davidson, 1984; and Trach and Hoch, 1989), suggesting that the prephenate dehydratase component of P-protein resides on its C-terminus, and they may share a common evolutionary ancestor. More recently, it has shown that no sequence homology has been found between the chorismate mutase-F from Bacillus subtilis (Gray et al., 1991) and either P-protein or T-protein from E. coli (Hudson and Davidson, 1984). It would be interesting to see how the B. subtilis bifunctional chorismate mutase is related to the P-protein and T-protein from E. coli.

In view of all the evidence, it is reasonable to assume that T-protein evolved via the fusion of the gene encoding cyclohexadienyl dehydrogenase with one of two duplicates of the gene specifying chorismate mutase-F. Firstly, the dehydrogenase component of the T-proteins studied so far utilizes both prephenate and arogenate, functioning as a cyclohexadienyl dehydrogenase. Secondly, the presence of T-proteins in enteric bacteria is correlated with the absence of

cyclohexadienyl dehydrogenases. The presence of cyclohexadienyl dehydrogenases in Superfamilies B and C is also correlated with the absence of T-proteins. Finally, the dehydrogenase domain of T-proteins and many cyclohexadienyl dehydrogenases are feedback inhibited by L-tyrosine.

The scenario for the P-proteins is more complex. The P-proteins could have originated from chorismate mutase-F and cyclohexadienyl dehydratase. If so, the presence of cyclohexadienyl dehydratase in *P. aeruginosa* and many other members of Superfamily B suggests that the gene encoding this enzyme must have duplicated before gene fusion. However, the absence of cyclohexadienyl dehydratase in some members of Superfamily B suggests another possibility. Perhaps this gene was duplicated before fusion but then lost due to mutation. It is also very possible that P-proteins evolved from the fusion of the genes coding for prephenate dehydratase and chorismate mutase-F. Firstly, the presence of P-proteins in Superfamilies A and B is correlated with the absence of prephenate dehydratases. Secondly, the dehydratase component of P-proteins and many monofunctional prephenate dehydratases, but not cyclohexadienyl dehydratases, are feedback inhibited by L-phenylalanine, and some of them are allosterically activated by L-tyrosine. Thirdly, P-proteins examined so far possess prephenate dehydratase activity but not arogenate dehydratase activity.

One of the objectives of this study was to clone and characterize the genes coding for cyclohexadienyl dehydratase from P. aeruginosa and cyclohexadienyl dehydrogenase from Z. mobilis and products. Another objective was to test the proposed evolutionary hypotheses, one being that P-proteins evolved from the gene fusion of monofunctional chorismate mutase and cyclohexadienyl dehydratase, and another being that T-proteins evolved from the gene fusion of monofunctional chorismate mutase and cyclohexadienyl dehydrogenase.

CHAPTER 2
CYCLOHEXADIENYL DEHYDRATASE FROM *PSEUDOMONAS AERUGINOSA*: MOLECULAR CLONING OF THE GENE AND CHARACTERIZATION OF THE GENE PRODUCT

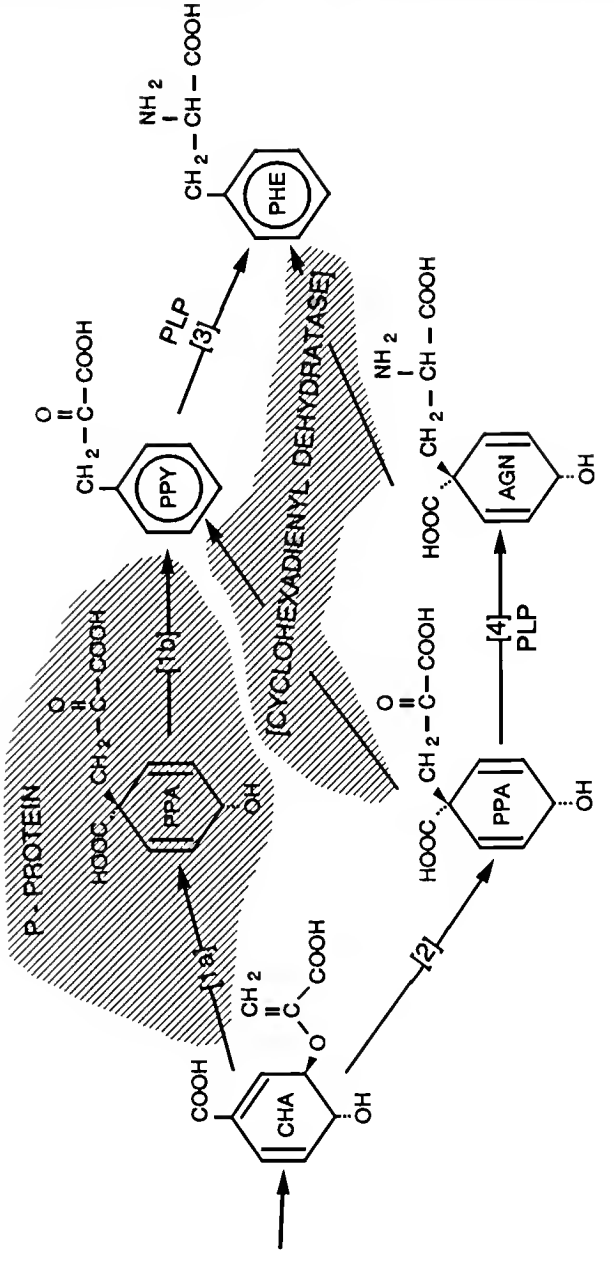
Introduction

Biosynthesis of the aromatic amino acids begins with the condensation of erythrose-4-phosphate and phosphoenolpyruvate by DAHP synthase to form chorismate, the last intermediate common to all aromatic amino acids (Weiss and Edwards, 1980). Chorismate is then converted to prephenate via chorismate mutase, forming an initial precursor that is unique for the biosynthesis of L-phenylalanine and L-tyrosine in microorganisms (Weiss and Edwards, 1980). The conversion of prephenate to phenylpyruvate via prephenate dehydratase or to 4-hydroxyphenylpyruvate via prephenate dehydrogenase was well established first in microorganisms and thought to be the universal enzyme steps functioning in nature (Cotton and Gibson, 1965). In 1974, an alternative route to L-tyrosine biosynthesis was established in cyanobacteria in which prephenate was converted to arogenate via prephenate aminotransferase rather than to 4-hydroxyphenylpyruvate via prephenate dehydrogenase (Stenmark et al., 1974). Arogenate was initially identified as a precursor of L-tyrosine, and now its function as a precursor of L-phenylalanine has become

apparent in both microorganisms and higher plants (Byng et al., 1982).

The pathway to phenylalanine biosynthesis in microorganisms has been demonstrated to be complex and diverse not only because of the presence of the alternative pathway but also because of the presence of the different combinations of enzymes that catalyze the overall reactions. For example, Escherichia coli and other Gram-negative bacteria synthesize L-phenylalanine via phenylpyruvate utilizing a bifunctional P-protein (Weiss and Edwards, 1980). The bifunctional P-protein catalyzes the overall conversion of chorismate to prephenate and prephenate to phenylpyruvate. On the other hand, other microorganisms such as Pseudomonas diminuta synthesize L-phenylalanine via arogenate using the alternative route (Zamir et al., 1985). In these organisms, three monofunctional enzymes, chorismate mutase-F, prephenate aminotransferase, and arogenate dehydratase, are utilized to carry out the conversion of chorismate to prephenate, prephenate to arogenate, and arogenate to L-phenylalanine. P. aeruginosa, however, was the first example of microorganisms possessing the dual pathways to L-phenylalanine biosynthesis (Patel et al., 1977) (Fig. 2-1). In addition to the bifunctional P-protein, P. aeruginosa possesses chorismate mutase-F, and cyclohexadienyl dehydratase which consist of the overflow pathway (Fiske et al., 1983). The cyclohexadienyl dehydratase of P. aeruginosa has a broad substrate specificity that

Fig. 2-1. Dual biosynthetic routes to L-phenylalanine in Pseudomonas aeruginosa. The bifunctional P-protein (denoted by shading) consists of chorismate mutase [1a] and prephenate dehydratase [1b] domains; The broad-specific cyclohexadienyl dehydratase catalyzes the two reactions indicated by shading: prephenate dehydratase or arogenate dehydratase. Enzyme [2] is the monofunctional chorismate mutase-F. Reactions [3] and [4] refer to a multiplicity of aminotransferase enzymes with overlapping specificities which transaminate phenylpyruvate and prephenate, respectively. Abbreviations: CHA, chorismate; PPA, prephenate; PPY, phenylpyruvate; PHE, L-phenylalanine; AGN, L-arogenate; PLP, pyridoxal 5'phosphate.


P - PROTEIN
CHA
PPA
PPY
PHE
AGN
PLP
[1a]
[1b]
[2]
[3]
[4]
[CYCLOHEXADIENYL DEHYDRATASE]

accommodates prephenate or arogenate as substrate for L-phenylalanine biosynthesis (Patel et al., 1977; and Zhao et al., 1992). The overflow pathway to L-phenylalanine biosynthesis is widely spread in Superfamily B microorganisms (Ahmad and Jensen, 1988a).

The physiological role of the overflow pathway in nature is essentially unknown, and the evolutionary relationship of the cyclohexadienyl dehydratase to the monofunctional prephenate dehydratases and the dehydratase domain of the bifunctional P-proteins is unclear. In this chapter, I describe the molecular cloning and nucleotide sequence of the cyclohexadienyl dehydratase gene from P. aeruginosa, and the purification, characterization, and expression of its gene product.

Materials and Methods

Strains, Plasmids, and Media

All bacterial strains and plasmids used in this study are listed in Table 2-1.

LB (Maniatis et al., 1982) was used as a rich growth medium, and M9 (Maniatis et al., 1982) was used as a minimal medium for E. coli and Pseudomonas aeruginosa. Ampicillin (50 ug/ml), tyrosine (50 ug/ml), and thiamine (17 ug/ml) were supplemented when appropriate. Agar was added at 15 g/liter for plates.

Table 2-1. Bacterial strains and plasmids

Strain/plasmid	Genotype or description	Source
E. coli K-12		
JM83	*ara* (*proAB-lac*) *rpsL* *thi*80 *dlac* ZM15	Yanisch-Perron
JP2255	*aroF*363 *pheA*361 *pheO*352 *tyrA*382 *thi* *strR*712 *lacY*1 *xyl*5	Baldwin & Davidson
KA197	*thi*1 *pheA*97 *relA*1 *spoT*1	CGSC 5243
P. aeruginosa		
PAO1	Prototroph	Holloway
Plasmids		
pUC18	Ap^r *lacZ*	
pUC19	AP^r *lacZ*	BRL
pJZ1	Original clone carrying *pheC* gene isolated from PAO1 library	This study
pJZ1a	2.0 kb derivative of pJZ1 generated by removal of a 3.8 kb EcoRI fragment	This study
pJZ1b	4.0 kb derivative of pJZ1 generated by removal of a 1.8 kb KpnI fragment	This study
pJZ1c	4.5 kb derivative of pJZ1 generated by removal of a 1259 bp SmaI-SmaI fragment	This study
pJZ1d-S	1.8 kb KpnI fragment of pJZ1 cloned into pUC18 with the same orientation as pJZ1	This study
pJZ1d-O	1.8 kb KpnI fragment of pJZ1 cloned into pUC18 with the opposite orientation of pJZ1	This study
pJZ1e	741 bp SmaI fragment of pJZ1d-S cloned into pUC18	This study
pJZ1f	515 bp SmaI fragment of pJZ1d-S cloned into pUC18	This study
pJZ1g	1110 bp SphI-SmaI fragment of pJZ1d-S cloned into pUC18	This study

Isolation of P. aeruginosa (PAO1) Chromosomal DNA and Construction of a Gene Library

Chromosomal DNA was isolated as described previously (Marmur, 1961). This DNA was partially digested with Sau3A, and fragments of 5 to 10 kilobases (kb) were isolated from agarose gels after electrophoresis. A library was constructed by ligation of these fragments into the dephosphorylated BamHI site of pUC18 (Yanisch-Perron et al., 1985). The ligation mixtures were transformed into E. coli JM83 (Yanisch-Perron et al., 1985). The transformants obtained on LB plates (supplemented with 50 ug/ ml ampicillin) were collected and stored in 50% glycerol at -75°C. Recombinant plasmids were purified from this library as described (Davis et al., 1980). The recombinant plasmids were used to transform E. coli pheA mutant of JP2255 (Baldwin and Davidson, 1981) in order to select for clones carrying the pheC gene.

DNA Manipulations

All restriction enzymes, T4 DNA ligase, and calf intestine phosphatase were obtained from Bethesda Research Laboratories and Promega, and were used according to the manufacturer instructions. Analyses of restriction sites and subcloning were conducted by standard methods (Maniatis et al., 1982). Southern blot hybridization, using biotinylated probes, was conducted following the instructions of Promega.

DNA Sequencing and Data Analysis

The subclones of pJZ1e, pJZ1f, and pJZ1g were first purified in a large scale by CsCl gradient centrifugation (Humphreys et al., 1975), and then sequenced in both directions as described (Prober et al., 1987) at the DNA Core Facility of the Univ. of Florida. Nucleotide sequence of the gene along with the deduced amino acid sequence was analyzed by using the Univ. of Wisconsin Genetics Computer Group (GCG) package (Devereux et al., 1984).

Crude Extract Preparation and Enzyme Assay

Cultures of E. coli JP2255 transformed by various clones were grown at 37°C in 450 ml LB supplemented with ampicillin, and harvested by centrifugation during late exponential phase of growth. The cells were suspended in 3 ml of 50 mM potassium phosphate buffer, pH 7.5, and disrupted by sonication. The resulting suspension was centrifuged at 150,000 g for 60 min at 4°C. The supernatant fraction collected was passed through a PD-10 Sephadex column to remove small molecules, and used for enzyme assay.

Prephenate dehydratase was assayed as described (Cotton and Gibson, 1965). A reaction mixture (200 ul) contained 1.0 mM prephenate, enzyme and 50 mM potassium phosphate (pH 7.5). After incubation at 37°C for 20 min, 0.8 ml of 2.5 N NaOH was added, and phenylpyruvate was measured spectrophotometrically at 320 nm. An extinction coefficient of 17,500 was used for calculation of phenylpyruvate formation (Cotton and Gibson,

1965). Arogenate dehydratase was assayed as described previously for the formation of phenylalanine using HPLC (high performance liquid chromatography) (Zamir et al., 1980). A reaction mixture of 200 ul contained 1.0 mM arogenate, enzyme and 50 mM potassium phosphate (pH 7.5). After incubation at 37°C for 20 min, the phenylalanine formed was measured using authentic phenylalanine as a standard. Protein was measured as described by Bradford (1976). One unit of enzyme activity was defined as the formation of 1 nanomole of phenylpyruvate or phenylalanine per min at 37°C.

Purification of The pheC Product from E. coli JP2255 and The Cyclohexadienyl Dehydratase from P. aeruginosa

The E. coli JP2255 carrying pJZ1g was grown in 1000 ml LB supplemented with 50 ug/ml of ampicillin at 37°C, and harvested by centrifugation during late exponential phase of growth. The cells were washed once with 20 mM potassium phosphate, 1 mM DTT, pH 8.5 (Buffer A), resuspended in the same buffer, and disrupted by sonication. The resulting suspension was centrifuged at 150,000 g for 60 min. The supernatant fraction collected was applied to a DEAE-cellulose column (2.5 x 30 cm) that was pre-equilibrated with Buffer A. The column was first washed with 100 ml of Buffer A, and then eluted with a 1,000 ml linear KCl gradient from 0 mM to 300 mM in Buffer A. The fractions of 2.8 ml were collected. The fractions showing high cyclohexadienyl dehydratase activity were pooled, and concentrated by using an Amicon-PM 10

membrane. The concentrated preparation was washed twice with 20 mM potassium phosphate, 1 mM DTT, pH 7.2 (Buffer B), and then applied to a hydroxylapatite column (1.5 x 15 cm) which was equilibrated with Buffer B. The column was eluted with a 600 ml linear gradient of phosphate from 20 mM to 300 mM. Fractions of 2.8 ml were collected, and those showing high cyclohexadienyl dehydratase activity were pooled. The pooled fractions were concentrated as described before, and then applied to a Sephadex G-200 column (2.5 x 98 cm) previously equilibrated with Buffer B. The column was eluted with Buffer B, and the fractions exhibiting cyclohexadienyl dehydratase activity were collected for further study.

P. aeruginosa PAO1 (Holloway, 1955) was grown in a M9 minimal medium (3,000 ml). The culture was harvested by centrifugation during late exponential phase of growth. The crude extract was prepared as described before. The supernatant fraction collected after ultra-centrifugation was adjusted to 40% ammonium sulfate saturation with solid salt. After stirring for 10 min, the pellet was removed by centrifugation. The supernatant fraction was collected and brought to 60% saturation. The pellet collected was dissolved in 20 ml of Buffer A, and dialyzed against several changes of 20 volumes of the same buffer. The dialyzed enzyme preparation was applied to a DEAE-cellulose column and purified as previously described under identical conditions.

Characterization of The pheC Product from E. coli and the Cyclohexadienyl Dehydratase from P. aeruginosa

The subunit molecular weight of the purified protein was determined by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). The molecular weight of the native proteins was determined by gel filtration (Sephadex G-200) as described above for enzyme purification.

Biochemicals and Chemicals

Prephenate was prepared from a tyrosine auxotroph of Salmonella typhimurium (Dayan and Sprinson, 1970), and arogenate was prepared from a triple auxotroph of Neurospora crassa (Zamir et al., 1980). Sephadex G-200 was obtained from Sigma Chemical Company, DEAE-cellulose was obtained from Whatman, and hydroxylapatite was obtained from Bio-Rad. Molecular weight standards for SDS-PAGE (alpha-lactalbumin, 14,400; soybean trypsin inhibitor, 20,100; carbonic anhydrase, 30,000; ovalbumin, 43,000; bovine serum albumin, 67,000; and phosphorylase, 94,000), and for gel filtration (carbonic anhydrase, 30,000; bovine serum albumin, 67,000; alcohol dehydrogenase, 150,000; and b-amylase, 200,000) were obtained from Pharmicia Fine Chemical and Sigma Chemical Company, respectively. All the chemicals were purchased from Sigma.

Results

Cloning of The Gene Encoding Cyclohexadienyl Dehydratase

Approximately 4000 recombinants were obtained after transformation of *E. coli* JM83. Purified plasmids from these recombinants were used to transform *E. coli* JP2255. The transformants of JP2255 were first allowed to grow in LB overnight at 37°C, washed twice with liquid M9 medium, and then plated on M9 plates which were supplemented with L-tyrosine and ampicillin. The plates were examined for the presence of colonies 4 days after incubation at 37°C. Two colonies were observed. Plasmids were purified from cultures derived from each of these transformants. The two plasmids isolated, designated as pJZ1 and pJZ2, respectively, were found to be able to transform JP2255 to L-phenylalanine prototropy. The transformants of JP2255 carrying the clones of pJZ1 and pJZ2 grew slowly on M9 plates. However, the transformation of pJZ1 or pJZ2 into KA197, a *pheA* mutant, resulted in a relatively faster growing phenotype, apparently due to the presence of chorismate mutase encoded by *tyrA*.

The presence of prephenate dehydratase and arogenate dehydratase activities was examined in the crude extracts of *E. coli* JP2255 carrying pJZ1 and pJZ2. A high level of both activities was observed in the crude extract of *E. coli* JP2255 carrying pJZ1 and pJZ2 while no enzyme activity was

detected in the crude extract of E. coli JP2255 carrying pUC18 (Table 2-2).

Digestion of pJZ1 and pJZ2 with HindIII, PstI, and KpnI showed that they carried two identical DNA fragments estimated to be 5.7 kb in length. The plasmid designated as pJZ1 was used for further study.

Southern blot hybridization analysis showed that, when the SmaI-EcoRI fragment of pJZ1 was biotinylated, it hybridized with a 4 kb fragment of P. aeruginosa chromosomal DNA which was completely digested with SmaI and EcoRI; however, it did not hybridize with E. coli chromosomal DNA which was also digested with SmaI and EcoRI (data not shown).

Localization of the P. aeruginosa pheC Gene and Expression of the pheC Gene in E. coli

Digestion and religation of pJZ1 with EcoRI, KpnI, and SmaI yielded subclones of pJZ1a, pJZ1b, and pJZ1c, respectively (Fig. 2-2a). These subclones were unable to complement E. coli JP2255 and E. coli KA197, and cyclohexadienyl dehydratase activity was not detected in crude extracts of the transformants carrying the subclones (Table 2-2), indicating that the gene may be located within the KpnI fragment. The KpnI fragment (Fig. 2-2a) was cloned into pUC18 at the KpnI site in both orientations. When the KpnI fragment was cloned in the same orientation as the original clone (designated as pJZ1d-S), the same high levels of enzyme activities conferred by pJZ1 were observed (Table 2-2).

Table 2-2. Expression of the *P. aeruginosa pheC* gene in *E. coli* JP2255

Plasmids in strain JP2255	Specific activity[+]	
	Prephenate dehydratase	Arogenate dehydratase
pJZ1	621	191
pJZ2	700	211
pJZ1a	0.0	0.0
pJZ1b	0.0	0.0
pJZ1c	0.0	0.0
pJZ1d-S	628	186
pJZ1d-O	5.6	1.7
pJZ1e	0.0	0.0
pJZ1f	0.0	0.0
pJZ1g	1314	387

[+]Specific activity is defined as nmole phenylpyruvate or phenylalanine formed per min per mg of protein. All the clones or subclones listed were first transformed into *E. coli* JP2255, and the crude extracts of the transformed *E. coli* JP2255 were used for enzyme assay.

However, when this fragment was cloned in the opposite orientation (designated as pJZ1d-O), relatively lower levels of the activities were observed (Table 2-2). These results showed that the over-expression of the enzyme activity of these clones was largely dependent on the LacZ promoter of the plasmid, and also indicated that the promoter of the cloned gene was functioning in E. coli although not efficiently.

Further localization of the gene was carried out by cloning the two SmaI fragments (released upon digestion of pJZ1d-S with SmaI) into pUC18 at SmaI site. The two resulting subclones, designated as pJZ1e, and pJZ1f (Fig. 2-2a), were unable to complement the defects of E. coli strains JP2255 and KA197, suggesting the gene was localized within the two SmaI fragments. In order to obtain an intact SphI-SmaI-SmaI fragment, the original clone was first completely digested with SphI, and then partially digested with SmaI. The SphI-SmaI-SmaI fragment was isolated, and cloned into pUC19, yielding a subclone denoted pJZ1g. The subclone pJZ1g complemented E. coli strains JP2255 and KA197, and exhibited a 2-fold increase in enzyme activities when compared with pJZ1d-S (Table 2-2). The increased enzyme activity was probably due to the decrease of the distance between lacZ promoter and the transcriptional start site of the gene.

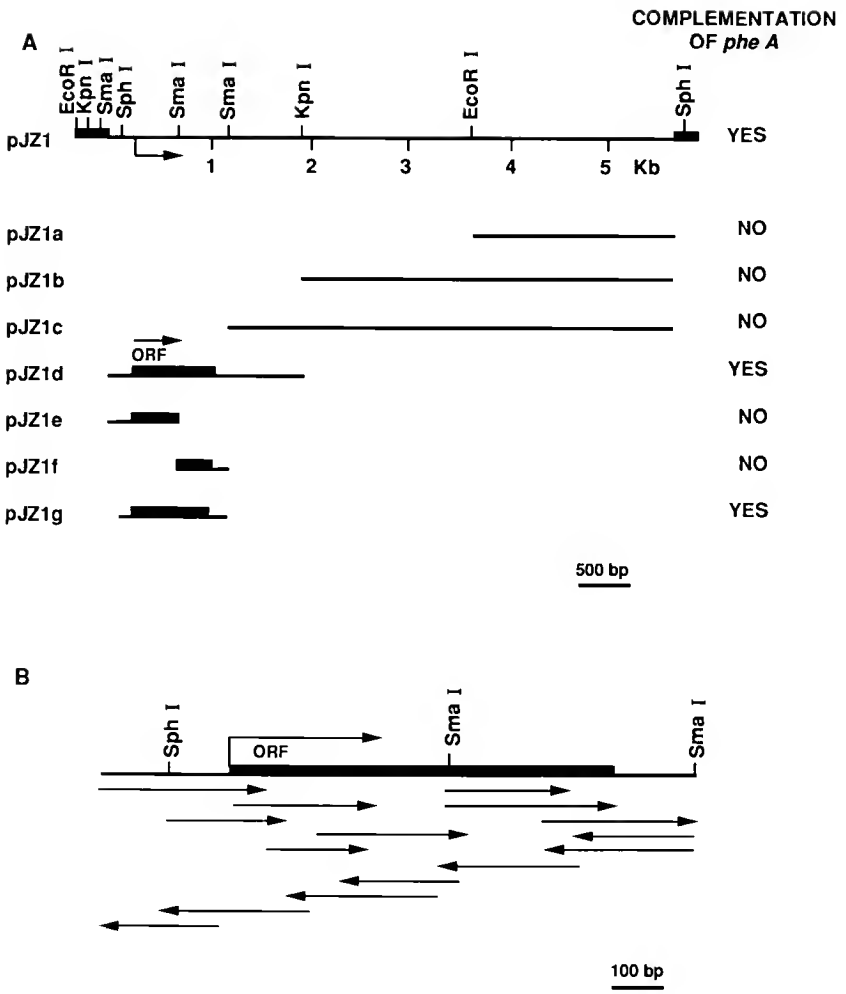


Fig. 2-2. A. Physical localization of the P. aeruginosa pheC gene. Linear maps of the clone and subclones are shown, and their ability to complement the pheA mutants of E. coli JP2255 and KA197 is also indicated. Restriction sites are labeled in the cloned P. aeruginosa DNA and in the flanking pUC18 multiple cloning site (depicted by heavy lines). Abbreviations: ORF, open reading frame. B. The sequencing strategy used for the pheC gene.

DAN Sequence of the P. aeruginosa pheC Gene and Its Flanking Regions

The complete nucleotide sequence of the 1.3 kb SmaI fragment (Fig. 2-2b) is presented in Fig. 2-3. The structural gene coding for cyclohexadienyl dehydratase was located within a single open reading frame, 807 bp in length. The deduced amino acid sequence presented in Fig. 2-3 indicates that the P. aeruginosa cyclohexadienyl dehydratase contained 268 residues with a molecular weight of 30,480. This compares with a value of 29,500 determined for the purified enzyme by SDS-PAGE (Fig. 2-4). The sequence AGGAG, located 6 bp upstream of the start cordon is presumably the ribosome binding site (Shine and Dalgarno, 1974). The open reading frame was terminated by a TAA codon.

The G+C content of the pheC gene was 65.6% which falls within the 60.6%-66.3% range for P. aeruginosa genome (West and Iglewski, 1988). The codon usage of the gene was typical of P. aeruginosa (West and Iglewski, 1988), exhibiting a striking preference for G or C in the third base position in 91.1% of the codons. As is the case for most P. aeruginosa genes, C (52.4%) was utilized more frequently than G (38.7%) in the third position.

A portion of an unidentified open reading frame encoding a truncated peptide of 74 residues (Fig. 2-3) was found upstream of the pheC gene. A search of Genbank sequences did not reveal obvious homology with any known protein sequences.

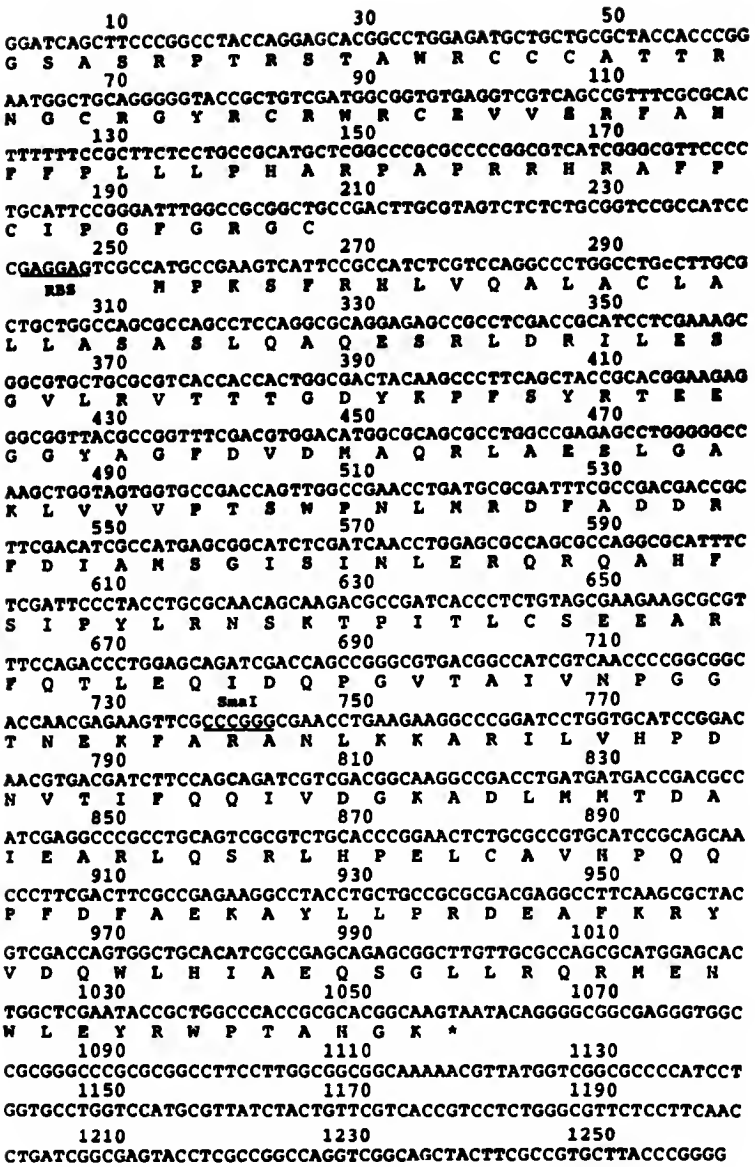


Fig. 2-3. Nucleotide sequence of the P. aeruginosa pheC gene and of its flanking regions. The deduced amino acid sequence of the gene along with its upstream flanking region is shown beneath the corresponding codons. The Shine-Dalgarno (1974) region is underlined and labeled (RBS). The SmaI site localized within the ORF of the pheC gene is also shown.

Characterization of the Cloned pheC Product Purified from E. coli JP2255 and the One Purified from P. aeruginosa

Purification of the cloned pheC product expressed in E. coli is summarized in Table 2-3. The ratio of 3:1 obtained for the activity of prephenate dehydratase compared to that of arogenate dehydratase remained constant throughout the process of purification. Only one major band was resolved by SDS-PAGE after the Sephadex G-200 column (Fig. 2-4). The subunit molecular weight of the cloned pheC product was 29,500 as determined by SDS-PAGE, and the molecular weight of the native product was 72,000 as determined by gel filtration on Sephadex G-200. Therefore, the native enzyme is made up of 2 identical subunits.

Purification of the cyclohexadienyl dehydratase from P. aeruginosa was essentially carried out under the identical conditions as those used for the cloned gene product. The cyclohexadienyl dehydratase from P. aeruginosa and the pheC product isolated from E. coli failed to bind to the DEAE-cellulose column at the pH value lower than 7.4, and they were found to be eluted into the equivalent fractions throughout the purification process. A specific activity ratio of prephenate dehydratase to that of arogenate dehydratase of 3:1 was also obtained and remained unaltered during the purification process (see Chapter 3). The native molecular weight of this enzyme was shown to be 72,000, a value identical to that obtained from the pheC product in E.

Table 2-3. Purification of the cloned *pheC* product expressed in *E. coli* JP2255(pJZ1g)

	Total Protein (mg)	Specific Activity (nmole/min/mg)		Ratio of PDT/ADT	Purification Factor
		PDT	ADT		
Crude Extract	1118	1319	381	3.46	1
DEAE-Cellulose	83.5	10616	3518	3.02	8.1
Hydroxyl apatite	4.83	87750	26340	3.32	67
Sephadex G-200	3.25	158242	48352	3.27	120

Abbreviations: PDT, prephenate dehydratase; ADT, arogenate dehydratase.

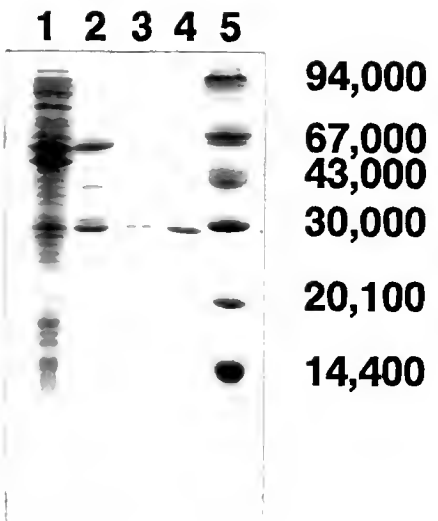


Fig. 2-4. SDS-polyacrylamide gel electrophoresis of the cloned pheC product expressed in E. coli JP2255. The protein samples were run on a 15% gel and stained with coomassie blue. From left to right: Lane 1, crude extract of E. coli JP2255 carrying the pheC gene; Lane 2, the collected fractions after DEAE-cellulose column; Lane 3, the collected fractions after Hydroxylapatite column; Lane 4, the collected fractions after Sephadex G-200 column; Lane 5, molecular weight standards.

coli. A subunit molecular weight of 28,000 was also obtained for the cyclohexadienyl dehydratase from P. aeruginosa (Chapter 3).

The K_m values of 0.42 mM for prephenate, and of 0.22 mM for L-arogenate were obtained for the cloned enzyme (Fig. 2-5). The corresponding values obtained for the cyclohexadienyl dehydratase directly isolated from P. aeruginosa were 0.40 mM and 0.19 mM, respectively. Vmax values of 307.7 umole/min/mg for prephenate and 102.8 umole/min/mg for L-arogenate were obtained for the cloned enzyme. Since the cyclohexadienyl dehydratase isolated from P. aeruginosa was not homogeneous, the Vmax values were not determined. Prephenate dehydratase activity of both preparations was competitively inhibited by L-arogenate with a Ki value of 0.20 mM, whereas arogenate dehydratase activity was competitively inhibited by prephenate with a Ki value of 0.40 mM. The P. aeruginosa cyclohexadienyl dehydratase was not subjected to allosteric regulation by L-phenylalanine, L-tyrosine and L-tryptophan.

Discussion

The Identity of the Cloned Gene and Its Product

This study has clearly demonstrated that the cloned gene coding for cyclohexadienyl dehydratase was obtained from P. aeruginosa. Southern blotting analysis showed that the cloned DNA fragment only hybridized with the P. aeruginosa chromosomal DNA. Analysis of the cloned DNA sequence by

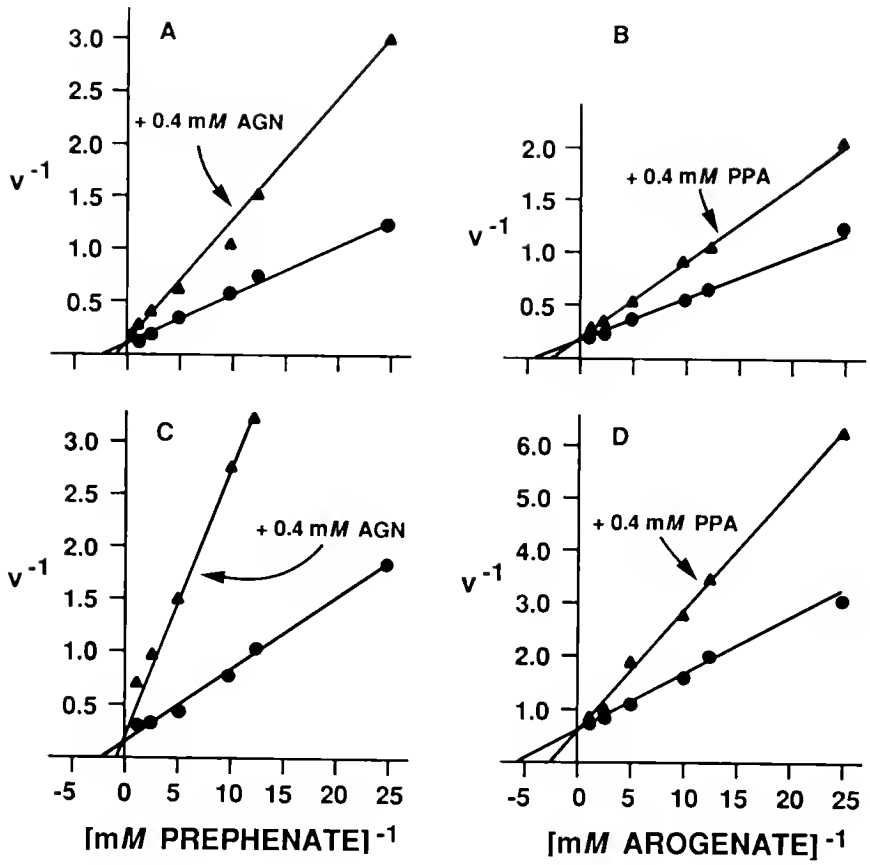


Fig. 2-5. Double reciprocal plots of the P. aeruginosa pheC product expressed in E. coli (panels A and B) and the cyclohexadienyl dehydratase purified from P. aeruginosa (panels C and D). When assayed as prephenate (PPA) dehydratase (panels A and C), v is expressed as nmoles of phenylpyruvate formed per min at 37°C in presence of () or absence () of 0.4 mM L-arogenate (AGN). When assayed as arogenate dehydratase (panels B and D), v was expressed as nmoles of L-phenylalanine formed per min at 37°C in the presence () or absence () of 0.4mM PPA.

Testcode has shown that the DNA sequence from 200 bp to 1000 bp corresponds to a coding region. An open reading frame (ORF), well situated within this region, codes for a protein with calculated molecular weight of 30,480. This is compatible with the value determined for the purified enzyme by SDS-PAGE. The SmaI restriction site was found to be localized within the ORF (Fig. 2-2). The interruption of this ORF by digestion with SmaI caused the simultaneous loss of both prephenate dehydratase and arogenate dehydratase activities. Subcloning analysis has shown that as long as the clones retained the SphI-SmaI-SmaI fragment, they complemented the pheA mutants of E. coli, JP2255 and KA197. The disruption of the integrity of the SphI-SmaI-SmaI fragment resulted in the loss of complementation of E. coli JP2255 and KA197.

Cyclohexadienyl dehydratase was first described in P. aeruginosa by Petal et al. (1977). In present study, a more extensive investigation has been carried out. The cyclohexadienyl dehydratase isolated from P. aeruginosa and the cloned gene product expressed in E. coli JP2255 have been purified to electrophoretic homogeneity. The extensive purification process was unable to separate the prephenate dehydratase activity from the arogenate dehydratase activity. The two activities were not regulated by either L-phenylalanine or L-tyrosine. The physical parameters obtained for the two purified preparations were essentially identical.

Therefore, the cloned gene product is a cyclohexadienyl dehydratase.

Unlike the bifunctional P-protein from P. aeruginosa, the cyclohexadienyl dehydratase is a monofunctional enzyme although possessing both prephenate dehydratase and arogenate dehydratase activities. The kinetic study has shown that prephenate competitively inhibited the arogenate dehydratase activity, and arogenate competitively inhibited prephenate dehydratase activity, an indication that the enzyme possessed only one single substrate binding site. Recently, the similar results have been obtained for the cyclohexadienyl dehydratase from Erwinia herbicola (Xia et al., 1991). The bifunctional P-protein from P. aeruginosa and those from many other Gram-negative bacteria possess two substrate binding sites, one for chorismate, and one for prephenate (Ahmad and Jensen, 1986; and Baldwin and Davidson, 1981).

The Role of the Cyclohexadienyl Dehydratase of P. aeruginosa

Auxotrophs of P. aeruginosa for L-phenylalanine and L-tyrosine have not been reported. The reluctant auxotrophy of P. aeruginosa for L-phenylalanine was explained as the consequence of independent dual pathways to L-phenylalanine (Patel et al., 1978). A mutant lacking the bifunctional P-protein has been identified (Berry et al., 1987), and the mutant exhibited a leaky requirement for L-phenylalanine, showing that the exclusive biosynthesis of L-phenylalanine via cyclohexadienyl dehydratase is rate-limiting to growth.

Another mutant of P. aeruginosa possessing a L-tyrosine insensitive DAHP synthase was found to excrete L-phenylalanine, presumably through the overflow pathway (Fiske et al., 1983).

L-Arogenate is generated by transamination of prephenate. Five aromatic aminotransferases capable of transamination of prephenate have been isolated from P. aeruginosa (Whitaker et al., 1982), and shown to have a relatively poor affinity for prephenate compared to the prephenate dehydratase component of the bifunctional P-protein. A possible candidate that may generate prephenate molecules for transamination is the monofunctional chorismate mutase. However, it has a poor affinity for chorismate compared to the chorismate mutase component of the bifunctional P-protein (unpublished results). As discussed earlier, the cyclohexadienyl dehydratase has relatively poor affinity for both L-arogenate and prephenate although slightly favoring L-arogenate over prephenate. Therefore, under normal growth conditions, most if not all L-phenylalanine is synthesized via the bifunctional P-protein rather than via the cyclohexadienyl dehydratase, and the L-phenylalanine synthesized is probably all derived from phenylpyruvate but not from L-arogenate. Thus, under ordinary growth conditions, the cyclohexadienyl dehydratase along with the monofunctional chorismate mutase probably does not play a significant role in terms of the contribution to L-phenylalanine biosynthesis. However, the overflow pathway

(the monofunctional chorismate mutase and the cyclohexadienyl dehydratase) could be well suited as a backup system for L-phenylalanine biosynthesis. It is interesting that P. stutzeri, a very close relative of P. aeruginosa, lacks the overflow pathway (Byng et al., 1983). The loss of the prephenate dehydratase activity of the bifunctional P-protein has yielded a tightly blocked L-phenylalanine auxotroph (Carlson et al., 1984), in contrast to the bradytrophy of the corresponding mutant of P. aeruginosa (Berry et al., 1987).

The physiological role of cyclohexadienyl dehydratase as discussed above is not well understood and is currently under investigation in our laboratory. To have a better understanding of the role of the cyclohexadienyl dehydratase in vivo, mutants lacking this enzyme activity would be desirable and such mutants now can be obtained by using the cloned cyclohexadienyl dehydratase gene to target the corresponding region of chromosome in P. aeruginosa through gene-scrambling mutagenesis (Mohr and Deretic, 1990).

The Basis for the pheC Gene to Complement the E. coli pheA Mutants of JP2255 and KA197

E. coli JP2255 was initially employed to select for the clones carrying the bifunctional P-protein gene, and KA197 was to be used for selection of the clones carrying the cyclohexadienyl dehydratase gene. Although a low leaky chorismate mutase activity was observed in E. coli JP2255 (Baldwin and Davidson, 1981), such outcome was not anticipated

because it was thought that such low activity was impossible to generate enough prephenate molecules for L-phenylalanine biosynthesis. Based on the slowness of growth, it was initially thought that the clones could carry a truncated bifunctional P-protein gene. This possibility has, however, been ruled out. First, no chorismate mutase activity was found to be associated with any clones. Second, extensive studies have shown that the bifunctional P-proteins are competitively inhibited by L-phenylalanine (Ahmad et al., 1989; Calhoun et al., 1973; Cotton and Gibson, 1965; and Friedrich et al., 1976). In case of the bifunctional P-protein of P. aeruginosa, the prephenate dehydratase component is inhibited by L-phenylalanine but activated by L-tyrosine (Calhoun et al., 1973; and Patel et al., 1977). Third, the P-proteins examined so far do not utilize L-arogenate as substrate (Ahmad et al., 1988). Finally, when the antibody prepared against the P-protein from Acinetobacter calcoaceticus (Ahmad et al., 1989) which cross-reacted with the P-protein from P. aeruginosa was added to and mixed with the crude extract of E. coli JP2255 carrying the clones, no immuno-inhibition of cyclohexadienyl dehydratase activity was observed (data not shown). Therefore, the complementation of E. coli JP2255 for L-phenylalanine requirement was due to the catalytic activities of the leaky chorismate mutase of the host and the cyclohexadienyl dehydratase of the clones. The slow growing phenotype of E. coli JP2255 transformants indicated that in vivo L-

phenylalanine was still limiting which was directly imposed by the low activity of the chorismate mutase.

E. coli KA197 was chosen to select for the cyclohexadienyl dehydratase gene. This was based on an assumption that the chorismate mutase component of the bifunctional T-protein would divert some prephenate for L-phenylalanine biosynthesis. Enzymological studies of the T-protein has suggested that chorismate molecules are tightly subjected to sequential enzymatic action (Heyde, 1979; Heyde and Morrison, 1978; and Rood et al., 1982). When the original clones and the resulting subclones were transformed into E. coli KA197, a relatively faster growing phenotype resulted, an indication that the limitation of L-phenylalanine in vivo was relieved due to the elevation of chorismate mutase activity. Since E. coli KA197, a pheA mutant lacking a bifunctional P-protein, still possesses an intact bifunctional T-protein (chorismate mutase/prephenate dehydrogenase), the prephenate molecules generated in vivo were probably derived from the catalytic activity of the chorismate mutase component of the T-protein. This study clearly indicates that the chorismate mutase component of the T-protein had the potential to participate in L-phenylalanine biosynthesis. Our recent study showed that the bifunctional P-protein was also participating in L-tyrosine biosynthesis (Zhao et al., 1992b).

Evolutionary Implications

The deduced sequence of the P. aeruginosa pheC product was pairwise aligned with those of the Corynebacterium glutamicum and Bacillus subtilis prephenate dehydratases (Follettie and Sinskey, 1986; and Trach and Hoch, 1989), and E. coli and P. stutzeri P-proteins (Fischer et al., 1992; and Hudson and Davidson, 1984). The P. aeruginosa enzyme was found to be marginally similar to these four proteins with an averaged 17.5% identity. The monofunctional prephenate dehydratases of C. glutamicum and B. subtilis have shown significant similarity to the two bifunctional P-proteins of E. coli and P. stutzeri (Fischer et al., 1992; Follettie and Sinskey, 1986; Hudson and Davidson, 1984; and Trach and Hoch, 1989), indicating that the prephenate dehydratase might share a common evolutionary origin. The marginal similarity of the P. aeruginosa cyclohexadienyl dehydratase to the prephenate dehydratases as well as the bifunctional P-proteins might suggest that the cyclohexadienyl dehydratase and the prephenate dehydratases evolved independently. However, a more detailed analysis, focusing on short, highly conserved sequence segments rather than the total peptide, revealed a conserved motif which includes the essential threonine residue demonstrated by Hudson and Davidson (1984) and a number of flanking residues. This motif, shown in Fig. 2-6, suggests residues (within boxes) that may prove to be common to all of dehydratases. It is interesting that all of the prephenate

dehydratases share the TRF sequence, whereas the cyclohexadienyl dehydratase sequence is TIF. It remains to be seen whether other cyclohexadienyl dehydratases will also possess TIF sequences. Note that in this alignment there was high degree of conservation of amino acid sequence between the peptides of the P. aeruginosa cyclohexadienyl dehydratase and the P. stutzeri P-protein. These organisms are very closely related.

Fig. 2-6. Multiple alignment of dehydratase sequences oriented to the sequence motif containing the threonine residue shown to be essential for catalytic activity (12, 35). Amino acid residues, beginning as numbered on the left, are compared for B. subtilis (Bs) prephenate dehydratase (PDT), Corynebacterium glutamicum (Cg, PDT), E. coli P-protein (Ec, PDT), P. stutzeri P-protein (Ps, PDT), and P. aeruginosa cyclohexadienyl dehydratase (Pa, CDT). Identities between the residues of one or more of the P. aeruginosa CDT with those of the other four dehydratases are shown by shading. Residues invariant in all sequences are represented by boxing.

Bs PDT:	166	R	D	I	Q	D	Y	R	D	N	H	T	R	F	V	I	L	S	P	D	E	N
Cg PDT:	173	D	D	V	A	D	V	R	G	A	R	T	R	F	V	A	V	Q	A	Q	A	A
EC PDT:	268	R	I	E	A	N	Q	R	Q	N	F	T	R	F	V	V	L	A	R	K	A	I
Ps PDT:	255	E	K	I	E	D	R	P	D	N	S	T	R	F	L	I	I	G	S	Q	E	V
Pa CDT:	169	A	R	I	L	V	H	P	D	N	V	T	I	F	Q	Q	I	V	D	G	K	A

CHAPTER 3
THE CYCLOHEXADIENYL DEHYDRATASE FROM PSEUDOMONAS AERUGINOSA: EVIDENCE FOR A PERIPLASMIC PROTEIN

Introduction

Cyclohexadienyl dehydratase catalyzes either the conversion of prephenate to phenylpyruvate or the conversion of L-arogenate to L-phenylalanine. The enzyme was first described in Pseudomonas aeruginosa, an organism which possesses dual pathways to L-phenylalanine biosynthesis (Patel et al., 1977). The gene encoding cyclohexadienyl dehydratase, denoted as pheC, has been cloned and sequenced recently from P. aeruginosa (Zhao et al., 1992a). The cloning and sequencing analyses have shown that the two catalytic activities, prephenate dehydratase and arogenate dehydratase, were apparently encoded by the single gene, pheC. The pheC gene product expressed in Escherichia coli was purified to electrophoretic homogeneity. Results of kinetic studies are consistent with this enzyme possessing one single substrate binding site. Thus, the dual pathways to L-phenylalanine in P. aeruginosa were partly due to the substrate ambiguity of the enzyme.

Cyclohexadienyl dehydratase is widely distributed among Superfamily B microorganisms (Ahmad et al., 1988a). However,

its physiological role in nature remains to be elucidated. The pheC gene product of P. aeruginosa was expressed to a large quantity in E. coli. The sequence of the protein in comparison with the deduced sequence has predicated a processing event and a possible location in the periplasmic space of P. aeruginosa. In this chapter, I report the results of the biochemical and immunological studies.

Materials and Methods

Bacterial Strains, Plasmids, and Media

Bacterial strains and plasmids used in this study are listed in Table 3-1.

LB and M9 (Maniatis et al., 1982) were used for E. coli and P. aeruginosa strains. Ampicillin (50 mg/ml), L-tyrosine (50 ug/ml), and thiamine (17 mg/ml) were supplemented when appropriate.

Biochemicals

Restriction enzymes and T4 DNA ligase were obtained from BRL. Prephenate was prepared from Salmonella typhimurium (Dayan and Sprinson, 1970), and L-arogenate was prepared from Neurospora crassa (Zamir et al., 1980). Sephadex G-200, DEAE-cellulose, and hydroxylapatite were obtained from Sigma Chemical Company, Whatman, and Bio-Rad, respectively. Molecular weight standards for SDS-polyacrylamide gel electrophoresis (a-lactalbumin, 14,400; soybean trypsin inhibitor, 20,100; carbonic anhydrase, 30,000; ovalbumin,

Table 3-1. Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Source
E. coli K-12		
JM83	*ara* (*lac-proAB*) *rpsL* 80 *lacZ* M15	BRL
JP2255	*aroF*363 *pheA*361 *pheo*352 *tyrA*382 *thi* *strR*712 *lacY*1 *xyl*5	1*
P. aeruginosa		
PAO1	Prototroph	2*
PAT1051	*pheA*	3*
Plasmids		
pUC19	Ap^r *lacZ*	BRL
pJZ1g	1110-bp SphI-SmaI fragment containing the intact *pheC* gene cloned into pUC19	4*
pJZpp	1477-bp SphI-PstI fragment containing the intact *pheA* gene cloned into pUC19	This study

*: 1, Baldwin and Davidson (1981); 2, Holloway (1955); 3, Berry et al. (1987); 4, Zhao et al. (1992a).

43,000; bovine serum albumin, 67,000; and phosphorylase, 94,000) and for gel filtration (carbonic anhydrase, 30,000; bovine serum albumin, 67,000; alcohol dehydrogenase, 150,000; and b-amylase, 200,000) were obtained from Pharmacia Fine Chemicals and Sigma Chemical Company, respectively. Other biochemicals were obtained from Sigma Chemical Company.

Expression Construct of The P. stutzeri pheA

The intact pheA gene coding for bifunctional P-protein from P. stutzeri (Fischer et al., 1991) was cloned from pJF1954 into pUC19 at SphI/PstI sites, and the resulting construct, designated as pJZpp was found to over-express the P-protein in E. coli.

Purification of The Cloned pheC Product from E. coli and The Cyclohexadienyl Dehydratase from P. aeruginosa

The cloned pheC product expressed in E. coli was purified as previously described (Zhao et al., 1992a). The cyclohexadienyl dehydratase from P. aeruginosa was purified as described before (Zhao et al., 1992a) with modifications. Ammonium sulfate precipitation was omitted. After the Sephadex G-200 column, the fractions showing cyclohexadienyl dehydratase activity were pooled and applied to a small hydroxylapatite column (1.5 cm x 30 cm) and then to a small DEAE-cellulose column (1.5 cm x 30 cm) as described before (Zhao et al., 1992a).

N-terminal Amino Acid Sequencing

The N-terminal sequences of the purified proteins were determined by using an Applied Biosystems 470A Protein Sequencer with On-Line 120A PTH-Analyzer at the Protein Core Facility of the University of Florida.

Molecular Weight Determinations

The native molecular weight of the purified proteins was estimated by gel filtration as described above for enzyme purification, and the subunit molecular weight of the proteins was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970).

Antibody Preparation and Immunological Analysis

The *P. aeruginosa pheC* gene product purified from *E. coli* (0.6 mg) was injected into a New Zealand white rabbit (Hiebert et al., 1984), and the antiserum was collected one week after the third injection. The Western blot was performed as described by Hiebter et al. (1984).

Immunological localization of the the *pheC* gene product in *E. coli* was carried out as described below. *E. coli* JP2255 carrying pJZ1g (*pheC* gene) was grown in LB supplemented with 50 ug/ml of ampicillin at 37°C, harvested during the late exponential phase of growth, fixed in cacodylate-buffered (pH 7.2) 2.5% formaldehyde/0.5% glutaraldehyde for 10 min on ice, dehydrated, and embedded in Lowicryl K4M (Carlemalm and Villiger, 1989). The resin was polymerized with UV at -20°C for 48 hr. Sections were allowed to react with the antibody

prepared against the purified pheC gene product, and then lebeled with gold particles (Carlemalm and Villiger, 1989).

Osmotic Shock and Chloroform Treatment

E. coli JP2255 carrying pJZ1g and pJZpp was grown in either M9 medium (1.2 liters) supplemented with ampicillin (50 ug/ml), L-tyrosine (50 ug/ml), and thiamine (17 ug/ml), or in LB medium (1.2 liters) supplemented with ampicillin (50 ug/ml). The cells were harvested by centrifugation during the late exponential phase of growth. Each culture was divided equally into three parts (400 ml each). One part of the culture (400 ml, 1.5 g) was subjected to osmotic shock (Neu and Heppel, 1965). After each centrifugation, supernatant fractions were collected and referred to as periplasmic fractions. For preparation of the cytoplasmic fractions, the cell pellet recovered after osmotic shock was sonicated. This sonicated preparation was centrifuged at 150,000 g for 60 min, and the resulting supernatant fraction was collected and then passed through a PD-10 Sephadex column. This preparation is referred to as cytoplasmic fraction. The second part of the culture was subjected to chloroform treatment as described by Ames et al. (1984) except that the culture mixed with chloroform was incubated for 45 min. The supernatant fraction collected after incubation is referred to as periplasmic fraction. The third part of the culture was treated as described above for the preparation of cytoplasmic fraction, and this preparation is referred to as crude extract.

P. aeruginosa PAO1 (Holloway, 1955), and PAT1051 (Berry et al., 1987) were grown in M9 medium (1.2 liters) and harvested during late exponential phase of growth. The cultures were also divided equally into three parts (400 ml each, 1.5 g). One part of the culture was treated with chloroform (Ames et al., 1984) as described above, and one part was suspended in Tris buffer (20 mM, pH 8.0) and shaken at 4°C for 45 min (Beacham, 1979). The pellets recovered after Tris treatment and the third part of the culture were treated as described above for the preparation of cytoplasmic fraction, and the resulting preparations were referred as to cytoplasmic fraction and crude extract, respectively.

Enzyme Assay and Protein Determination

Prephenate dehydratase was assayed as described by Cotton and Gibson (1965). Arogenate dehydratase was assayed as described by Zamir et al. (1985). Alkaline phosphatase was assayed by the procedure of Brickman and Beckwith (1975). Specific activity is defined as nmoles of phenylpyruvate, L-phenylalanine, or nitrophenyl formed per mg protein per min. Protein was measured according to Bradford (1976).

Results

Purification of The Cloned pheC Product from E. coli and The Cyclohexadienyl Dehydratase from P. aeruginosa and The N-terminal Amino Acid Sequencing

Purification of the cloned pheC was carried out under the identical conditions as previously described (Zhao et al.,

1992a). After the Sephadex G-200 column chromatography, only one single protein band was resolved by SDS-PAGE. The subunit molecular weight of the pheC product was determined to be 28,000 (Fig. 3-1) in comparison with a value of 29,500 obtained earlier (Zhao et al., 1992a). The native molecular weight of the purified pheC product was estimated to be 72,000, identical to the value determined previously (Zhao et al., 1992a).

The N-terminal sequence of the purified pheC gene product was determined to be Gln-Glu-Ser-Arg-Leu-Asp-Arg-Ile-Leu-Glu-Ser. The sequence determined herein is found to match with the deduced amino acid sequence starting from the residues 26 to 36 (Zhao et al., 1992a). This indicates that an amino-terminal peptide (25 residues in length), a possible signal sequence was cleaved in E. coli (Fig. 3-2). In order to establish if this cleavage also occurred in P. aeruginosa, the cyclohexadienyl dehydratase from this organism was also purified.

Purification of the cyclohexadienyl dehydratase from P. aeruginosa is summarized in Table 3-2. The activities of prephenate dehydratase and arogenate dehydratase were found to coelute into the same fractions, and the ratio of the two activities was constant throughout purification. After the five steps of purification, the enzyme was homogenous as judged by SDS-PAGE and found to have the same molecular weight (28,000) (Fig. 3-1) as the cloned pheC gene product. The

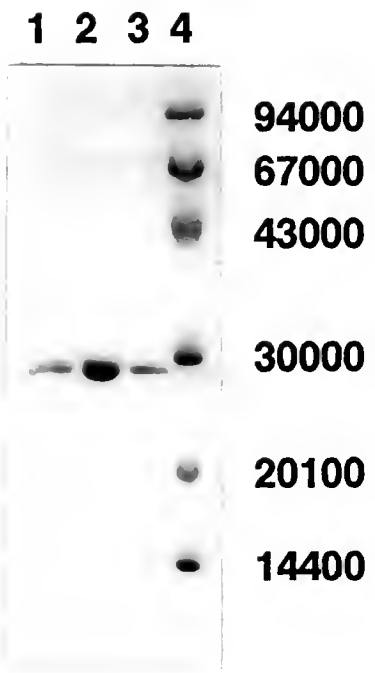


Fig. 3-1. SDS-PAGE analysis of the cloned pheC gene product purified from E. coli and the cyclohexadienyl dehydratase purified from P. aeruginosa. The samples were run on a 15% polyacrylamide gel. From left to right: Lane 1 and 3, the purified cyclohexadienyl dehydratase from P. aerugiosa; Lane 2, the purified pheC gene product from E. coli; and Lane 4, the molecular weight standards.

A.

N'-Gln-Glu-Ser-Arg-Leu-Asp-Arg-Ile-Leu-Glu-Ser....C'

B.

N'-Gln-Glu-Ser-Arg-Leu-Asp-Arg-Ile-Leu-Glu-Ser....C'

C.

```
5'-ATG CCG AAG TCA TTC CGC CAT CTC GTC CAG GCC CTG GCC
   Met Pro Lys Ser Phe Arg His Leu Val Gln Ala Leu Ala

   TGC CTT GCG CTG CTG GCC AGC GCC AGC CTC CAG GCG CAG
   Cys Leu Ala Leu Leu Ala Ser Ala Ser Leu Gln Ala Gln

   GAG AGC CGC CTC GAC CGC ATC CTC GAA AGC GGC GTG..-3'
   Glu Ser Arg Leu Asp Arg Ile Leu Glu Ser Gly Val..
```

Fig. 3-2. A and B, The N-terminal amino acid sequences of the pheC product purified from E. coli and the cyclohexadienyl dehydratase purified from P. aeruginosa, respectively; and C, the deduced N-terminal amino acid sequence for the cloned pheC gene product. The underlined sequence starting from the positions of 26 to 36 was identical to those of A and B.

native molecular weight of the purified enzyme was determined to be 72,000 by gel filtration, a value identical to the one previously determined for both the cloned pheC gene product and the cyclohexadienyl dehydratase directly isolated from P. aeruginosa (Zhao et al., 1992a).

The purified cyclohexadienyl dehydratase was also subjected to N-terminal sequencing. The 11 residues sequenced were found to be identical to those determined for the cloned pheC gene product purified from E. coli and to match with the deduced sequence starting from residues 26 to 36, thus showing the cleavage of the amino-terminal peptide also occurred in P. aeruginosa.

The amino peptide sequence of the cyclohexadienyl dehydratase from P. aeruginosa is shown in Fig. 3-2 and found to share several features with the known signal sequences for periplasmic proteins (Oliver, 1986). For example, the P. aeruginosa amino peptide contained a basic amino terminus (Lys, Arg, and His), a hydrophobic core region (Ala, Leu, Val, and Ser), and a putative processing site (Leu-Gln-Ala). The length of this amino peptide also falls within the range for the periplasmic signal sequences (Oliver, 1986).

Osmotic Shock and Chloroform Treatment

The results of osmotic shock and chloroform treatment of E. coli carrying pJZ1g and pJZpp are summarized in Table 3-3. Over 90% of cyclohexadienyl dehydratase and alkaline phosphatase activities was recovered in the periplasmic

Table 3-2. Purification of the cyclohexadienyl dehydratase of P. aeruginosa

	Total protein (mg)	Specific activity (nmol/mg/min)		Ratio of PDT/ADT	Purification ratio
		PDT	ADT		
Crude extract	2205	15[+]	3.7	4.1	
DEAE-cellulose	406	48.5	12.5	3.9	3.2
Hydroxylapatite	34.4	403	107	3.8	26
Sephadex G-200	6.0	1996	537	3.7	130
Hydroxylapatite	0.95	10301	2918	3.5	672
DEAE-cellulose	0.32	19882	5598	3.5	1298

[+] prephenate dehydratase was assayed in presence of 4.0 mM phenylalanine. Abbreviations: PDT, prephenate dehydratase; ADT, arogenate dehydratase.

fractions when E. coli JP2255(pJZ1g) was subjected to osmotic shock.

The pheA construct of P. stutzeri was chosen for this study because this gene was over-expressed in E. coli and the gene product is a cytoplasmic protein. Thus, it was served as a negative control in this study.

Less than 1% of the bifunctional P-protein activity, but over 90% of the alkaline phosphatase activity was found in the periplasmic fractions when E. coli JP2255(pJZpp) was subjected to osmotic shock. However, over 95% of the P-protein activity was detected in the cytoplasmic fraction.

When E. coli carrying pJZ1g and pJZpp were treated with chloroform, similar results were obtained (Table 3-3) except that the chloroform treatment was only 30-50% as efficient as osmotic shock based on the amount of activity released.

Similar results were also obtained when E. coli JP2255 carrying pJZ1g or pJZpp was grown in minimal medium (Table 3-4). Since the alkaline phosphatase activity was repressed to a very low level by phosphate present in the medium, its activity was not determined.

The results of chloroform and Tris treatments of P. aeruginosa strains are summarized in Table 3-5. Approximately, 50% of cyclohexadienyl dehydratase activity was recovered in periplasmic fraction of the strain PAT1051, but less than 10% of the activity was recovered in the periplasmic fraction of the strain PAO1 when the cells of these strains were subjected

Table 3-3. Release of the cyclohexadienyl dehydratase expressed in E. coli JP2255 by osmotic shock and chloroform treatment when the cells were grown in rich medium

	Total protein (mg)	AP (OD/min)	PDT --(umol/min)-- + Phe	PDT --(umol/min)-- - Phe	ADT
pJZ1g (CDT)					
Periplasmic fraction (Osmotic shock)	63	90	1168	1174	318
Periplasmic fraction (Chloroform treatment)	30	46	480	486	318
Cytoplasmic fraction (Osmotic shock)	250	1	25	26	7
Crude extract (Intact cells)	316	93	1231	1257	354
pJZpp (P-protein)					
Periplasmic fraction (Osmotic shock)	35	63	0.0	1.1	0.0
Periplasmic fraction (chloroform treatment)	12	26	0.0	0.09	0.0
Cytoplasmic fraction (Osmotic shock)	196	6	1.9	162	0.0
Crude extract (Intact cells)	231	71	2	172	0.0

Abbreviations: AP, alkaline phosphatase; PDT, prephenate dehydratase; ADT, arogenate dehydratase; CDT, cyclohexadienyl dehydratase; Phe, phenylalanine.

Table 3-4. Release of the cyclohexadienyl dehydratase expressed in E. coli JP2255 by osmotic shock and chloroform treatment when the cells were grown in minimal medium

	Total protein (mg)	PDT (umol/min) + Phe	PDT (umol/min) - Phe	ADT (umol/min)
pJZ1g (CDT)				
Periplasmic fraction (Osmotic shock)	33	444	444	120
Periplasmic fraction (Chloroform treatment)	10	219	219	58
Cytoplasmic fraction (Osmotic shock)	79	4	4	1
Crude extract (Intact cells)	116	462	462	122
pJZpp (P-protein)				
Periplasmic fraction (Osmotic shock)	37	0.0	0.4	0.0
Periplasmic fraction (Chloroform treatment)	11	0.03	19	0.0
Cytoplasmic fraction (Osmotic shock)	70	0.11	40	0.0
Crude extract (Intact cells)	108	0.14	42	0.0

Abbreviations: PDT, prephenate dehydratase; ADT, arogenate dehydratase; CDT, cyclohexadienyl dehydratase; Phe, phenylalanine

Table 3-5. Release of cyclohexadienyl dehydratase of P. aeruginosa PAO1 and PAT1051 by chloroform and Tris treatments when cells were grown in minimal medium

	Total protein (mg)	PDT (nmole/min) + Phe	PDT (nmole/min) - Phe	ADT (nmole/min)
PAO1				
Periplasmic fraction (Tris treatment)	7.5	198.7	218.2	56.7
Periplasmic fraction (Chloroform treatment)	11.4	2349.4	2475.3	647.1
Cytoplasmic fraction (Tris treatment)	155.8	2153.3	5754.3	581.9
Crude extract (Intact cells)	166.7	2435.9	6489.0	667.4
PAT1051				
Periplasmic fraction (Tris treatment)	4.06	317.2	306.2	88.1
Periplasmic fraction (Chloroform treatment)	17.8	150.3	148.7	40.1
Cytoplasmic fraction (Tris treatment)	170.4	267.9	255.6	78.5
Crude extract (Intact cells)	181.3	608.3	580.9	164.3

Abbreviations: PDT, prephenate dehydratase; ADT, arogenate dehydratase.

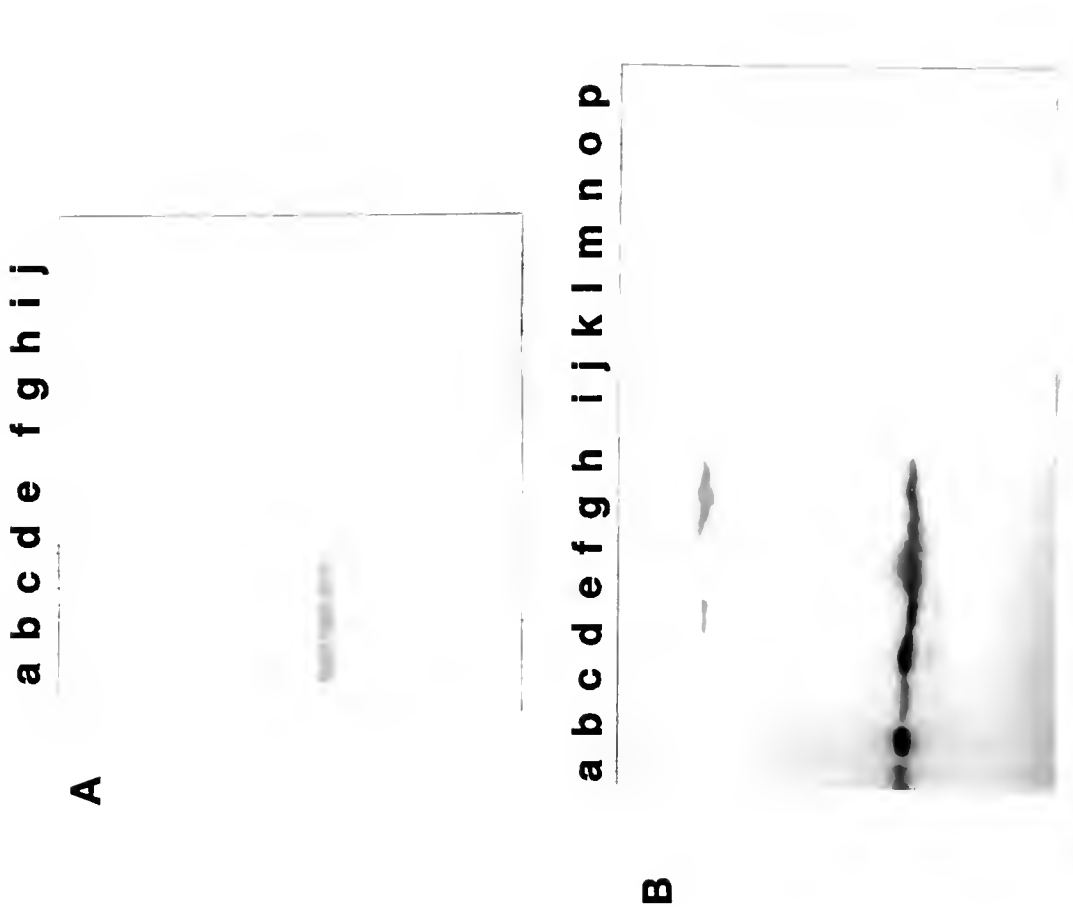
to Tris treatment. The fact that the cyclohexadienyl dehydratase rather than the bifunctional P-protein was present in the periplasmic fractions was established by lack of feedback inhibition by L-phenylalanine and capability to utilize L-arogenate, which are all the characteristics of the cyclohexadienyl dehydratase but not of the bifunctional P-protein (Patel et al., 1977, Zhao et al., 1992a; and Berry et al., 1987). Furthermore, P. aeruginosa PAT1051 does not possess the P-protein activities (Berry et al., 1987).

The chloroform treatment yielded a better result for the strain PAO1 since over 85% of the activity was released from periplasmic space. On the other hand, chloroform treatment released less than 40% of the activity from the periplasmic space of the strain PAT1051. Since cells of P. aeruginosa are known to undergo massive lysis in the presence of EDTA, osmotic shock could not be used for this study.

Immunological Localization of The Cyclohexadienyl Dehydratase in E. coli and P. aeruginosa

All the periplasmic fractions collected were first concentrated using a PM-10 membrane and then subjected to SDS-PAGE along with the crude extracts. The proteins were then transferred to a nitrocellulose membrane and probed with a preimmune serum and the antibody prepared against the purified pheC product. The presence of the cyclohexadienyl dehydratase was detected in the periplasmic fractions of JP2255(pJZ1g), PAO1, and PAT1051 (Fig. 3-3). The antibody prepared against

Fig. 3-3. Western blotting analysis of the cyclohexadienyl dehydratase of P. aeruginosa and the pheC product expressed in E. coli JP2255. Protein samples were run on a 15% polyacrylamide gel and transferred to a nitrocellulose membrane. A: Lane a + f, and b + g, periplasmic fractions collected when E. coli JP2255 carrying pJZ1g was subjected to osmotic shock and chloroform treatment, respectively; Lane c + h, d + i, and e + j, the crude extracts of E. coli JP2255 carrying pJZ1g, pUC19, and pJZpp, respectively. Lane a, b, c, d, and e were probed with the antibody prepared against the purified pheC product produced in E. coli. Lane f, g, h, i, and j were probed with preimmune serum. B: Lane a + i, the purified pheC product formed in E. coli; Lane b + j, the purified cyclohexadienyl dehydratase from P. aeruginosa PAO1; Lane c + k, and d + l, the periplasmic fractions collected when P. aeruginosa PAO1 was subjected to Tris treatment and chloroform treatment, respectively; Lane e + m, the crude extract of P. aeruginosa PAO1; Lane f + n and g + o, the periplasmic fractions collected when P. aeruginosa PAT1051 was subjected to Tris treatment and chloroform treatment, respectively; Lane h + p, the crude extract of P. aeruginosa PAT1051. Lane a, b, c, d, e, f, g, and h were probed with the antibody prepared against the pheC product, and lane i, j, k, l, m, n, o, and p were probed with preimmune serum.


A
a b c d e f g h i j
B
a b c d e f g h i j k l m n o p

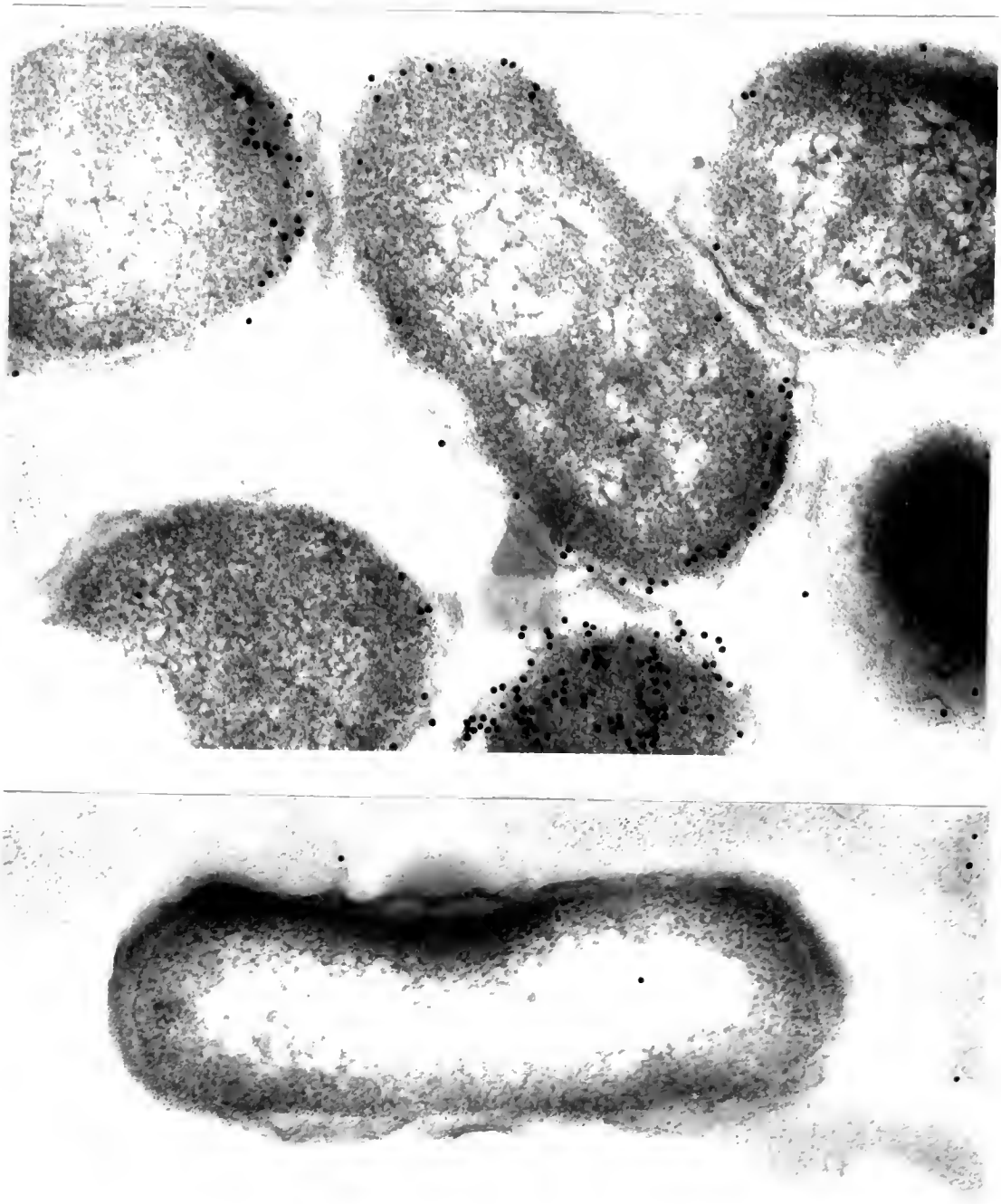


Fig. 3-4. Immunological localization of the pheC gene product formed in E. coli. Cells of E. coli JP2255 (pJZ1g) were fixed by 2.5% formaldehyde/0.5%glutaraldehyde, and embedded in Lowicryl K4M. Thin sections of these cells were incubated with the antibody prepared against the purified pheC gene product (upper panel) or preimmune serum (lower panel), labeled with gold-particles, and examined with a JEOL 100-CX electron microscope (52,000 x).

the pheC product did not react with the bifunctional P-protein apparently because of lack of a significant identity.

The localization of the cyclohexadienyl dehydratase in the periplasmic space of E. coli was clearly established by electron microscopy (Fig. 3-4). Interestingly, the cyclohexadienyl dehydratase expressed in E. coli was only found in the periplasmic space of the two polar regions (Fig. 3-4).

Discussion

Cyclohexadienyl Dehydratase from P. aeruginosa: A Periplasmic Enzyme

Several lines of evidence in this study have led to a conclusion that the cyclohexadienyl dehydratase from P. aeruginosa is a periplasmic protein. The sequencing of the pheC product expressed in E. coli indicated that an amino peptide, 25 residues in length was cleaved. This could be due to some proteases which acted on the expressed pheC product in E. coli. However, this possibility was ruled out since the enzyme purified directly from P. aeruginosa exhibited the identical molecular weight and sequence, and was shown to possess no methionine residue at its N-terminus. A substantial amount of the cyclohexadienyl dehydratase activity was released from the periplasmic spaces when the cells were subjected to either osmotic shock or chloroform treatment, an indication of a periplasmic protein. This contention was

confirmed by Western blotting analysis of the periplasmic fractions of P. aeruginosa and E coli and by immunological localization study.

The cloned pheC product was recovered in the periplasmic fractions when expressed in E. coli, indicating that the E. coli machinery was able to recognize the signal of the protein, to process and translocate it to its correct destiny within the cell. Similar results have often been obtained not only for microorganisms and but also for eukaryotes (Benson et al., 1985; and Talmadge et al., 1980).

Cyclohexadienyl Dehydratase from P. aeruginosa: Its In Vivo Function

In P. aeruginosa, L-phenylalanine can be synthesized either through the bifunctional P-protein or through the monofunctional chorismate mutase and the cyclohexadienyl dehydratase. The latter two enzymes are referred to as overflow pathway (Fiske et al., 1983). The presence of the overflow pathway in microorganisms may confer some physiological advantages. A mutant of P. aeruginosa lacking the bifunctional P-protein exhibited a leaky requirement for L-phenylalanine, showing the exclusive biosynthesis of phenylalanine through the overflow pathway is rate-limiting to growth (Berry et al., 1987). On the other hand, a mutant of P. stutzeri, a close relative of P. aeruginosa, defective in the bifunctional P-protein activity, yielded a tight phenylalanine auxotroph (Carlson et al., 1984). Therefore, the overflow

pathway may be used as a backup system for phenylalanine biosynthesis (Zhao et al., 1992a).

This study has shown that the cyclohexadienyl dehydratase of P. aeruginosa is a periplasmic enzyme rather than a cytoplasmic one as previously assumed. Thus, its function in vivo may be also different. Most of bacterial proteins localized in the periplasm are either binding proteins or scavenging enzymes (Oliver, 1986). It is possible that the cyclohexadienyl dehydratase of P. aeruginosa is also a (phenylalanine) scavenging enzyme. Many bacteria such as P. aeruginosa, P. syringae, Xanthomonas campestris, and Erwinia herbicola that possess cyclohexadienyl dehydratases are plant pathogens. It is known that higher plants synthesize L-phenylalanine through an intermediate, L-arogenate (Jensen, 1986). Cyclohexadienyl dehydratase in microorganisms may be involved in scavenging L-arogenate of plants. Upon binding with L-arogenate, the enzyme converts L-arogenate to L-phenylalanine that is then transported into bacterial cells. Two lines of evidence to some extent support this hypothesis. The two cyclohexadienyl dehydratases, one from P. aeruginosa (Zhao et al., 1992a), and one from E. herbicola (Xia et al., 1991) were characterized and found to have a higher affinity for L-arogenate than for prephenate. A search of Genbank has revealed that the cyclohexadienyl dehydratase from P. aeruginosa shared some sequence similarity with the products of hisJ, argT and glnQ which are known to code for the amino

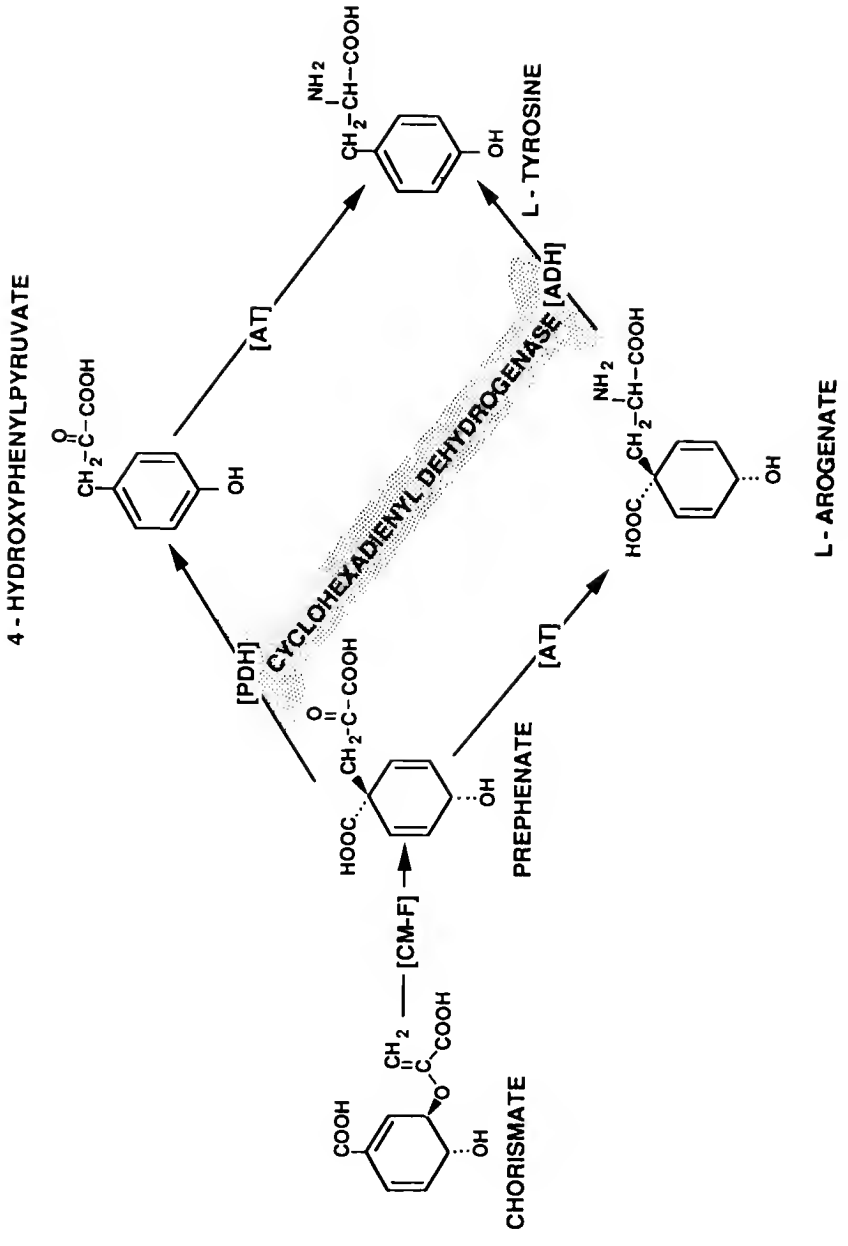
acid binding proteins and permease (Higgins et al., 1982, Nonet et al., 1987). In order to study the function of the cyclohexadienyl dehydratase, the *pheC* mutants of *P. aeruginosa* will be needed. Experiments designed to selectively inactivate the *P. aeruginosa pheC* gene *in vivo* (Mohr and Deretic, 1991) are currently in progress.

CHAPTER 4
CYCLOHEXADIENYL DEHYDROGENASE FROM ZYMOMONAS MOBILIS:MOLECULAR CLONING OF THE GENE AND CHARACTERIZATION OF THE GENE PRODUCT

Introduction

Cyclohexadienyl dehydrogenase catalyzes either the conversion of prephenate to 4-hydroxyphenylpyruvate or the conversion of L-arogenate to L-tyrosine (Patel et al., 1977; and Xia and Jensen, 1990) (Fig. 4-1). Thus, this enzyme may function as either prephenate dehydrogenase or as arogenate dehydrogenase (Fig. 4-1). Cyclohexadienyl dehydrogenase was first described in Pseudomonas aeruginosa, an organism that possesses dual pathways to L-tyrosine (Patel et al., 1977). The dual pathways to L-tyrosine biosynthesis appear to result from substrate ambiguity of the enzyme (Patel et al., 1977; 1978). The prephenate dehydrogenase and arogenate dehydrogenase activities of the purified cyclohexadienyl dehydrogenase from P. aeruginosa were inseparable, and the ratio of the two activities remained constant throughout purification (Xia and Jensen, 1990). A single protein band was resolved by sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis (Xia and Jensen, 1990). Evidence was obtained in support of the conclusion that both cyclohexadienyl substrates bind at a common catalytic site,

FIG. 4-1. The pathways to L-tyrosine biosynthesis in Zymomonas mobilis. Abbreviations: CM-F, monofunctional chorismate mutase; PDH, prephenate dehydrogenase; AT, aminotransferase; ADH, arogenate dehydrogenase. The shading denotes the ability of cyclohexadienyl dehydrogenase to function as either PDH or ADH.


4 - HYDROXYPHENYLPYRUVATE
[AT]
L - TYROSINE
[PDH]
CYCLOHEXADIENYL DEHYDROGENASE
[ADH]
L - AROGENATE
[AT]
PREPHENATE
[CM-F]
CHORISMATE

and therefore, the enzyme is a monofunctional protein having multiple activities.

The dehydrogenase component of bifunctional T-proteins (named as chorismate mutase/prephenate dehydrogenase) present in enteric bacteria (Ahmad and Jensen, 1986) has recently been shown to utilize L-arogenate in addition to prephenate as substrate (Ahmad and Jensen, 1987). Therefore, the named prephenate dehydrogenase component of all T-proteins is in fact a cyclohexadienyl dehydrogenase (Ahmad and Jensen, 1987). Cyclohexadienyl dehydrogenase is ubiquitous throughout Superfamilies A, B, and C of gram-negative bacteria (Ahmad and Jensen, 1986; 1988b; and Jensen, 1985). An evolutionary relationship between cyclohexadienyl dehydrogenase and the T-protein has been proposed (Ahmad and Jensen, 1986; 1988; and Jensen, 1985).

Z. mobilis is a gram-negative member of rRNA Superfamily C. Genes and enzymes of the glycolytic pathway in this organism have been studied extensively (Montenecourt, 1985). However, the pathway arrangement for tyrosine and phenylalanine biosynthesis is essentially unknown in this organism and indeed within the entire Superfamily to which it belongs. In this chapter, I describe the molecular cloning and sequencing of tyrC, the gene coding for cyclohexadienyl dehydrogenase from Z. mobilis CP4, as well as the purification and characterization of the gene product.

Materials and Methods

Bacterial Strains, Plasmids and Media

Bacterial strains and plasmids used in this study are described in Table 4-1. LB medium was used as an enriched medium, and the M9 recipe (Maniatis et al., 1982) was used to prepare minimal medium for *E. coli* strains. Ampicillin was supplemented to media where indicated at 50 μg/ml, and thiamine was added at 17 μg/ml. A complex medium was used for *Z. mobilis* (Osman et al., 1987). Agar was added to media at 15 g/liter for solid medium.

Isolation of Z. mobilis Chromosomal DNA and Construction of A Gene Library

Chromosomal DNA was isolated from *Z. mobilis* CP4 as described previously (Clark-Curtiss et al., 1985). The DNA was partially digested with Sau3A, and fragments of 5 to 7 kilobases (kb) were isolated by using a sucrose gradient (Clark-Curtiss et al., 1985). Libraries of these fragments were constructed by ligation into the dephosphorylated BamHI site of pUC18 (Yanisch-Perron et al., 1985). The ligation mixtures were transformed into *E. coli* DH5α. Transformants were collected from LB plates, and recombinant plasmids were purified. The purified recombinant plasmids were then used to transform *E. coli* AT2471 to independence of the tyrosine requirement.

Table 4-1. Bacterial strains and plasmids

Genotype or description	Strain/plasmid Source
E. coli K-12	
JM83 *ara*Δ (*proAB-lac*)*rpsL* φ80 *lacZ*ΔM15	Gibco-BRL
DH5a 480d*lacZ*ΔM15Δ(*lacZYA-argF*) U169*recA1 hsdR*17 (r_k^-,mk^+) *SupE*44λ$^-$*thi-1 gyrA relA1*	BRL
AT2471 *thi*1 *tyrA*	CGSC
JP2255 *aroF*363 *pheA*361 *pheO*352 *tyrA*382 *thi strR*712 *lacY*1 *xyl*5	CGSC
Z. mobilis (CP4) Prototroph	Ingram
Plasmids	
pUC18 *lacZ* Ap^r	
pUC19 *lacZ* Ap^r	BRL
pGEM-5Zf(+) *lacZ* Ap^r	Promega
pJZ5 Original clone of cyclohexadienyl dehydrogenase gene isolated from CP4 library	This study
pJZ5a A derivative of pJZ5 generated by removal of a 1-kb EcoRI-EcoRI fragment	This study
pJZ5b A derivative of pJZ5 generated by removal of a 3.2-kb HindIII fragment	This study
pJZ5c A derivative of pJZ5 generated by removal of a 4.2-kb SphI fragment	This study
pJZ5d A derivative of pJZ5 generated by removal of a 5.3-kb EcoRV-PstI fragment	This study
pJZ5e A derivative of pJZ5 generated by removal of a 6.7-kb SstII-PstI fragment	This study

Table 4-1 (continued)

pJZ5f	2.5-kb NcoI fragment of pJZ5 subcloned into pGEM-5Zf(+) at NcoI site	This study
pJZ5g-1	1.1-kb NsiI-(SstII) HindIII fragment of pJZ5f subcloned into pUC18 at PstI-HindIII sites	This study
pJZ5g-2	1.1-kb NsiI-(SstII) HindIII fragment of pJZ5f subcloned into pUC19 at PstI-HindIII sites	This study
pJZ5h	0.6-kb StuI fragment of pJZ5f subcloned into pUC18 at SmaI site	This study
pJZ5i	0.8-kb StuI-PstI fragment of pJZ5f subcloned into pUC18 at SmaI-PstI sites	This study
pJZ5j	1.4-kb StuI-StuI-(SstII) PstI fragment of pJZ5f subcloned into pUC18 at SmaI-PstI sites	This study

DNA Manipulations

All restriction endonuclease enzymes, T4 DNA ligase, and calf intestine phosphatase were obtained from Gibco-BRL or Promega and were used according to manufacturer instructions. Subcloning was conducted by standard methods (Maniatis et al., 1982). Southern blot hybridization, using a biotinylated probe, was carried out under stringent conditions according to the instructions of Promega.

DNA Sequencing and Data Analysis

Subclones pJZ5g, pJZ5h, and pJZ5i were purified by use of a CsCl gradient (Humphreys et al., 1975), and sequenced in both directions (Prober et al., 1987) at the DNA Core Facility of the University of Florida. The nucleotide sequence and the deduced amino acid sequence were analyzed by using the University of Wisconsin Genetics Computer Group (GCG) package (Devereux et al., 1984).

Crude Extract Preparation and Enzyme Assay

Cell cultures were grown at 37°C in 450 ml of LB broth containing ampicillin at 50 μg/ml. The cells were harvested by centrifugation during the late exponential phase of growth, suspended in 3 ml of 50 mM potassium phosphate buffer (pH 7.5), and sonicated for 30 sec using a Lab-Line Ultratip Labsonic System (Lab-Line Instruments, Inc., Melrose Park, IL). The resulting suspension was centrifuged at 150,000 g for 60 min at 4°C. The supernatant fraction was collected and passed through a PD-10 Sephadex column to remove small

molecules. This preparation was used to assay for enzyme activity.

Arogenate dehydrogenase and prephenate dehydrogenase activities were assayed by following the appearance of NADH on a spectrophotofluorometer (excitation at 340 nm and emission at 460 nm) (Byng et al., 1982). For kinetic studies, arogenate dehydrogenase was assayed by following L-tyrosine formation using HPLC (Zamir et al., 1985). One unit of enzyme activity was defined as formation of 1 nanomole of L-tyrosine or NADH per min at 37°C. Protein concentration was determined by the method of Bradford (1976).

Purification of The Cloned Cyclohexadienyl Dehydrogenase from E. coli AT2471

E. coli AT2471 carrying the subclone pJZ5f was grown at 37°C in 3 liters of minimal medium supplemented with 50 μg/ml of ampicillin and harvested during the late exponential phase of growth. The cells were washed with 50 ml of 20 mM potassium phosphate containing 1 mM DTT (pH 7.4), resuspended in the same buffer, and disrupted by sonication. After centrifugation (150,000 g for 60 min at 4°C), the supernatant was applied to a DEAE-cellulose column (2.5 x 30 cm) equilibrated with the same buffer. The column was first washed with 100 ml of the buffer and then eluted with 800 ml of buffer containing a linear gradient from 0-to-400 mM KCl. Fractions of 3 ml were collected, and those showing high cyclohexadienyl dehydrogenase activity were pooled and concentrated by use of

an Amicon PM-30 membrane. The concentrated preparation was washed twice with the phosphate buffer, and then applied to a hydroxylapatite column (2.5 x 30 cm) which was equilibrated with the phosphate buffer. The column was first washed with 100 ml of the buffer and then eluted with a 700-ml linear gradient of phosphate from 20-to-400 mM. Fractions of 3.0 ml were collected, and those showing high cyclohexadienyl dehydrogenase activity were pooled. The pooled fractions were concentrated as described before and then applied to a Sephadex G-200 column (2.5 x 98 cm) previously equilibrated with the phosphate buffer. The column was eluted with the buffer, and fractions exhibiting cyclohexadienyl dehydrogenase activity were combined. The preparation was then loaded on a hydroxylapatite column (1 x 30 cm), and the column was eluted as described before. Fractions containing cyclohexadienyl dehydrogenase were collected and used for further study.

Amino Acid Sequencing of The Cloned Cyclohexadienyl Dehydrogenase

The purified enzyme preparation was denatured by SDS and then subjected to polyacrylamide gel electrophoresis (Laemmli, 1970). The protein was then transferred to a polyvinylidene difluoride membrane and sequenced using an Applied Biosystems 470A Protein Sequencer with On-line 120A PTH-Analyzer at the Protein Core Facility of the University of Florida.

Molecular Weight Determinations

The molecular weight of the native enzyme was estimated by gel filtration (Sephadex G-200) as described above for

enzyme purification. The subunit molecular weight of the cloned cyclohexadienyl dehydrogenase was determined by SDS-PAGE (Laemmli, 1970). In order to confirm further the identity of the protein band resolved by SDS-PAGE, the purified enzyme preparation was first subjected to electrophoresis on a native gel which was performed as described for SDS-PAGE (Laemmli, 1970) except that SDS was omitted. After electrophoresis, a part of the gel was stained in 1 mM tetrazolium blue, 1 mM prephenate, and 1 mM NAD^+, a part of the gel was stained in 1 mM tetrazolium blue, and 1 mM NAD^+, and a part of the gel was stained in Coomassie blue.

Biochemicals and Chemicals

Prephenate was prepared from Salmonella typhimurium (Dayan and Sprinson, 1970), and L-arogenate was prepared from Neurospora crassa (Zamir et al., 1980). DEAE-cellulose was obtained from Whatman; Sephadex G-200, ampicillin, thiamine and amino acids were obtained from Sigma Chemical Company; and hydroxylapatite was obtained from Bio-Rad. Molecular weight standards for SDS-PAGE (α-lactabumin, 14,400; soybean trypsin inhibitor, 20,100; carbonic anhydrase, 30,000; ovalbumin, 43,000; bovine serum albumin, 67,000; and phosphorylase, 94,000), and for gel filtration (carbonic anhydrase, 29,000; bovine serum albumin, 67,000; alcohol dehydrogenase, 150,000; and ß-amylase, 200,000) were obtained from Pharmacia Fine Chemicals and Sigma Chemical Company, respectively. LB medium and agar were purchased from Difco.

Results

Cloning of The Gene Encoding Z. mobilis Cyclohexadienyl Dehydrogenase

E. coli AT2471 was employed to select clones carrying the cyclohexadienyl dehydrogenase gene. E. coli AT2471 was first transformed with the recombinant plasmids purified from gene libraries prepared from Z. mobilis, and the ampicillin-resistant transformants were then allowed to grow in LB medium for 3 hr, 6 hr, and 16 hr. The transformants were washed twice with growth-volume amounts of liquid minimal medium, and plated out on agar plates of minimal medium supplemented with ampicillin. No colonies were observed 2 days after incubation at 37°C when the transformants were first grown in LB medium for 3 hr. However, 4 and 16 colonies were observed when the transformants were grown in LB medium for 6 hr, and 16 hr, respectively. Plasmids were purified from the 20 colonies obtained, and all were able to transform E. coli AT2471 to tyrosine independence.

All 12 of the 20 clones which were examined showed cyclohexadienyl dehydrogenase activity in crude extracts (data not shown). Restriction endonuclease cleavage analysis showed that the 12 clones shared a 2.5-kb NcoI fragment. One of the 12 clones, designated as pJZ5, was chosen for further study (Table 4-2). This clone carried a DNA fragment from Z. mobilis estimated to be 10 kb in size (Fig. 4-2).

Southern blot hybridization showed that a labeled 6-kb EcoRI-HindIII fragment of pJZ5 (Fig. 4-2) hybridized with a 6-kb fragment of Z. mobilis chromosomal DNA digested with EcoRI and HindIII, but did not hybridize with E. coli chromosomal DNA digested with PstI (data not shown).

Subcloning and Expression of The Cyclohexadienyl Dehydrogenase Gene in E. coli

The subcloning strategy used for the clone pJZ5 is shown in Fig. 4-2. Subclones denoted pJZ5a, pJZ5b, and pJZ5c, pJZ5d, and pJZ5e expressed cyclohexadienyl dehydrogenase activity at a similar level (Table 4-2). The gene was therefore localized within the NcoI fragment. When this fragment was cloned into pGEM-5Zf(+) at the NcoI site to give pJZ5f, a relatively high level of cyclohexadienyl dehydrogenase activity was observed (Table 4-2). The increase of the activity may be due to the decrease of the distance between the vector promoter and the transcriptional start site of the gene.

In order to localize the gene precisely, the NsiI-(SstII)HindIII fragment was cloned into pUC18 and pUC19 by replacement of the PstI/HindIII fragment, and the resulting subclones, designated as pJZ5g-1, and pJZ5g-2, respectively, were transformed into E. coli AT2471. Only strain AT2471(pJZ5g-1) exhibited cyclohexadienyl dehydrogenase activity in crude extract, whereas strain AT2471(pJZ5g-2) did not yield detectable enzyme activity, indicating that the

FIG. 4-2. Localization of the cyclohexadienyl dehydrogenase gene. The linear maps of the original clone and derivative subclones are shown, and their ability to complement (+) or not to complement (-) the *tyrA* deficiency of *E. coli* AT2471 is also indicated at the far right. The position of restriction sites in the cloned *Z. mobilis* DNA and in part of the pUC18 multiple cloning site (depicted by bold line) is shown at the top.

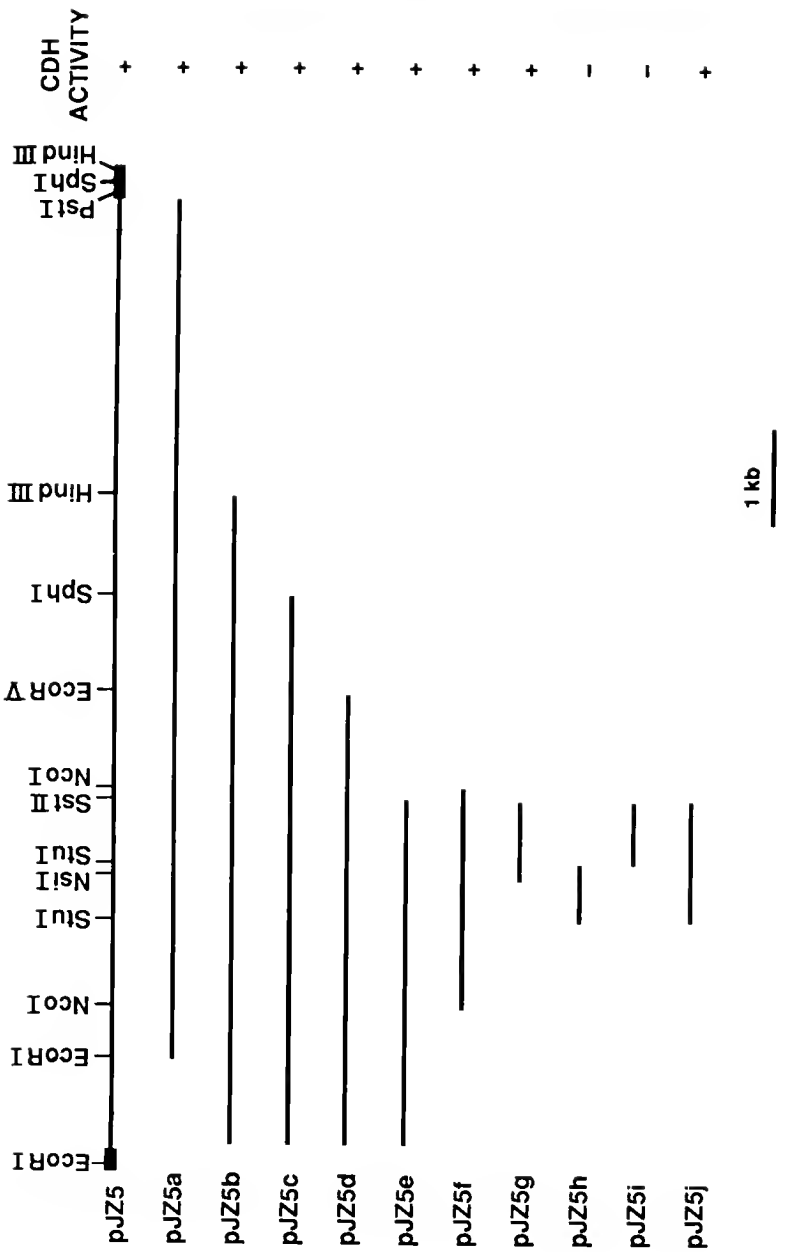

CDH ACTIVITY
+ + + + + + + + - - +
HindIII
SphI
PstI
HindIII
SphI
EcoRV
NcoI
SstII
StuI
NsiI
StuI
NcoI
EcoRI
EcoRI
1 kb
pJZ5
pJZ5a
pJZ5b
pJZ5c
pJZ5d
pJZ5e
pJZ5f
pJZ5g
pJZ5h
pJZ5i
pJZ5j

Table 4-2. Expression of the *Z. mobilis* cyclohexadienyl dehydrogenase gene in *E. coli*

	Specific activity[a]	
Plasmid	ADH	PDH
pJZ5	15.2	4.8
pJZ5a	16.5	5.9
pJZ5b	15.7	5.1
pJZ5c	16.0	5.1
pJZ5d	14.7	4.9
pJZ5e	17.3	6.1
pJZ5f	37.8	12.9
pJZ5g-1	40.4	14.6
pJZ5g-2	-0-	-0-
pJZ5h	-0-	-0-
pJZ5i	-0-	-0-
pJZ5j	35.5	12.6
pUC18	-0-	-0-
pGEM-5Zf(+)	-0-	-0-

[a]Specific activity is defined as nmole NADH formed per min per mg of protein.
Abbreviations: ADH, arogenate dehydrogenase; PDH, prephenate dehydrogenase.
The plasmids were first transformed into *E. coli* AT2471, and the crude extracts of the transformed AT2471 were used for enzyme activity assay.

transcription of the gene proceeded from the same direction as lacZ of pUC18. The promoter of this Z. mobilis gene is apparently destroyed or removed by digestion with NsiI. Thus, expression of this Z. mobilis gene in pJZ5g-1 depended upon the lacZ promoter of the plasmid. Consistent with the enzymological results, the subclone pJZ5g-1 was able to complement E. coli AT2471, while pJZ5g-2 failed to do so.

When the StuI-(SstII)PstI fragment was cloned into pUC18 at SmaI-PstI sites, the resulting subclone, designated as pJZ5i, was unable to complement E. coli AT2471. The transformants of E. coli AT2471 carrying pJZ5i did not show detectable activity of either arogenate dehydrogenase or prephenate dehydrogenase. This loss of cyclohexadienyl dehydrogenase activity suggested that the cleavage of pJZ5g by StuI disrupted the integrity of the structural gene, a supposition which was later confirmed by the sequencing data.

Nucleotide Sequence of The Cyclohexadienyl Dehydrogenase Gene

The complete nucleotide sequence of the cyclohexadienyl dehydrogenase gene along with its flanking regions is shown in Fig. 4-3. The structural gene was located within a single open reading frame (ORF), 882 bp in length. The deduced amino acid sequence yields a protein containing 293 residues with a molecular weight of 32,086. This agreed well with the value of 32,000 determined for the purified enzyme by SDS-PAGE (Fig. 4-4). The ORF was started at codon GTG, and terminated at

codon TAA. The sequence AGGCAGG, located 8 bp upstream of the start codon may serve as the ribosome binding site (Pond et al., 1989; and Shine and Dalgarno, 1974).

The G+C content of the gene was 44.44% which falls into the range for the Z. mobilis genome (Montenecourt, 1985). The codon usage of the gene was typical of Z. mobilis (Pond et al., 1989), showing a preference for A+T. However, the codon usage of this gene (data not shown) was less biased than that of highly expressed genes (Pond et al., 1989).

Purification and Identification of The Cloned Dehydrogenase

A ratio of 3:1 was obtained in comparison of the activity of arogenate dehydrogenase to that of prephenate dehydrogenase (Table 4-3). This ratio remained constant throughout purification, and the two activities of the enzyme co-eluted from chromatography columns with identical profiles. A single protein band with an estimated molecular weight of 32,000 was visualized by SDS-PAGE of the purified enzyme (Fig. 4-4). Thus, the product of the cloned gene is a cyclohexadienyl dehydrogenase.

When the purified enzyme preparation was run on a native gel, and then stained in Coomassie blue or in tetrazolium blue, prephenate and NAD^+, a single blue band or a single purple band was observed, respectively. The blue and purple bands were located at the exactly the same position on the gel. When the gel was stained in tetrazolium blue and NAD^+, no

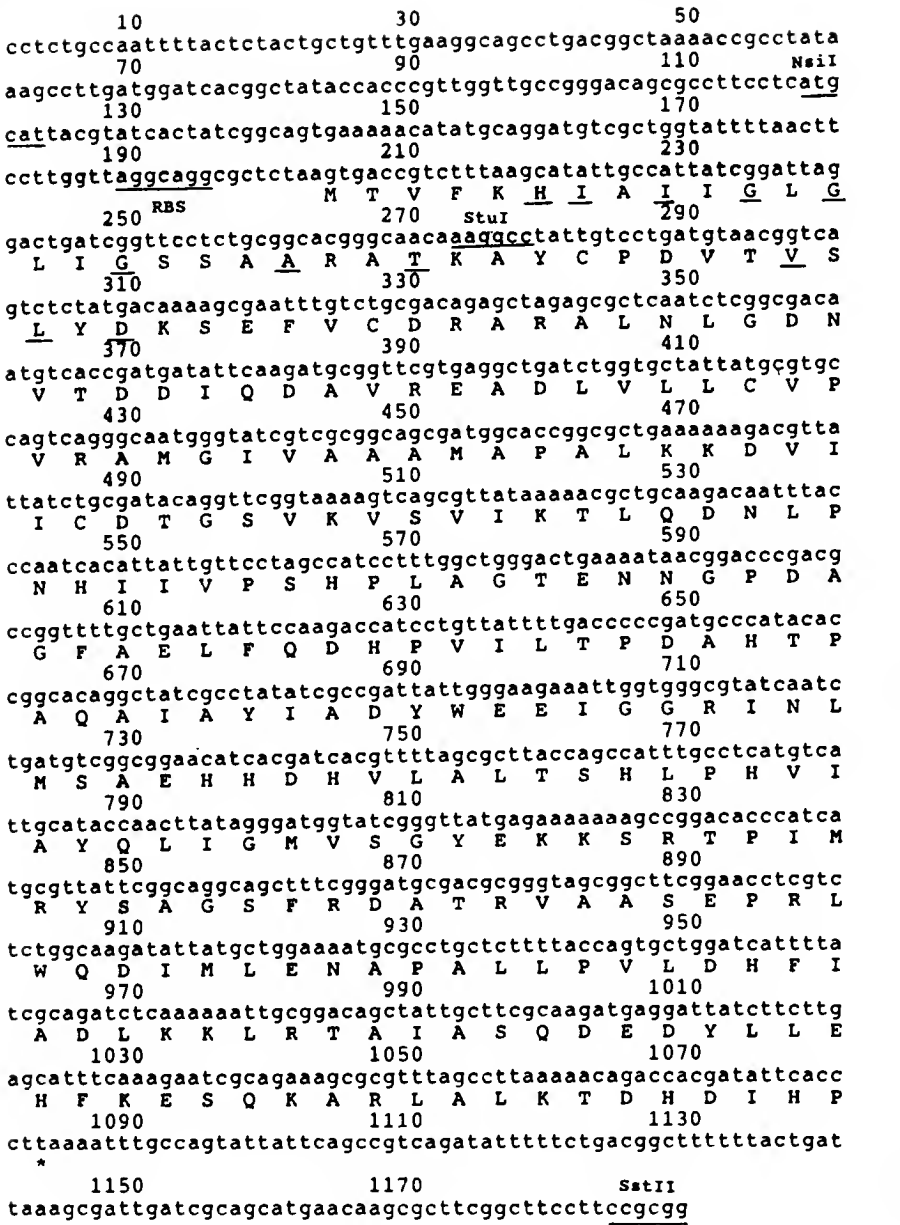


FIG. 4-3. Nucleotide sequence of the cyclohexadienyl dehydrogenase gene along with its flanking regions. The deduced amino acid sequence of the gene is shown beneath the corresponding codons. The Shine-Dalgarno (S-D) region and the restriction sites are underlined and labeled. The residues which correspond to the NAD^+ binding domain are also underlined.

Table 4-3. Purification of the cloned cyclohexadienyl dehydrogenase from *E. coli tyrA* mutant of AT2471

	Total protein (mg)	SA[a] (nmol/mg/min)		ADH/PDH Ratio	Purification factor
		ADH	PDH		
Crude extract	908	42	14	3	1
DEAE-cellulose	216	150	53	2.9	3.7
Hydroxyl apatite	19	1188	409	2.9	29
Sephadex G-200	3.6	2357	3929	3.2	277
Hydroxyl apatite	1.2	18825	6618	3.0	466

[a]Specific activity is defined as nmol NADH formed per min per mg of protein.
Abbreviations: SA, specific activity; ADH, arogenate dehydrogenase; PDH, prephenate dehydrogenase.

detectable band was observed in the absence of prephenate. When the region corresponding to the purple band was excised (Hiebert et al., 1984) from the unstained gel and run on SDS-PAGE, the protein migrated to a molecular weight position of 32,000. Thus, the subunit molecular weight of the cloned cyclohexadienyl dehydrogenase was 32,000. The molecular weight of the native enzyme was 76,000 as determined by gel filtration on Sephadex G-200. Therefore, the native enzyme is probably a homodimer.

N-terminal Amino Acid Sequence of The Cyclohexadienyl Dehydrogenase

In order to confirm that the ORF was started at the GTG codon, the N-terminal portion of the cyclohexadienyl dehydrogenase was sequenced. The 11 residues determined were shown to be Met-Thr-Val-Phe-Lys-His-Ile-Ala-Ile-Ile-Gly, a sequence which was identical to that deduced from the nucleotide sequence (Fig. 4-3).

Kinetic and Regulatory Properties of The Cyclohexadienyl Dehydrogenase

Both arogenate dehydrogenase and prephenate dehydrogenase activities of the purified enzyme required NAD^+ as a cofactor and failed to utilize $NADP^+$. Uncomplicated, first-order substrate saturation curves were obtained. A K_m value of 0.11 m<u>M</u> was obtained for NAD^+, regardless of whether the enzyme was assayed as arogenate dehydrogenase (Fig. 4-5A) or as prephenate dehydrogenase (Fig. 4-5B). The cyclohexadienyl

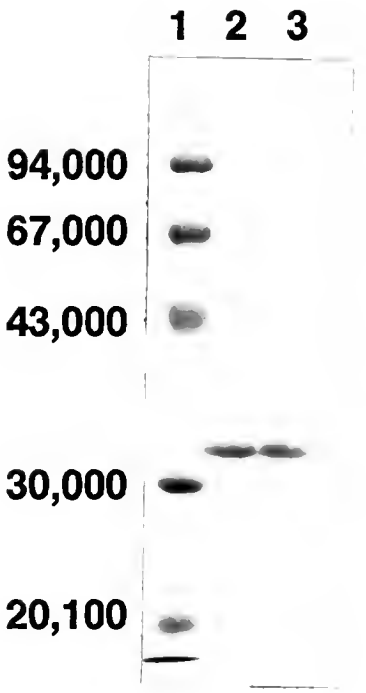


FIG. 4-4. The analysis of the cloned cyclohexadienyl dehydrogenase purified from *E. coli* AT2471 by SDS-polyacrylamide gel electrophoresis. The protein samples were run on a 12% gel and stained with Coomassie blue. From left to right: Lane 1, the protein markers; Lane 2, the purified cyclohexadienyl dehydrogenase preparation after the final step of hydroxyapatite chromatography; Lane 3, a different fraction of the purified enzyme preparation after the final step of hydroxyapatite chromatography.

dehydrogenase had a K_m value of 0.40 mM for L-arogenate (Fig. 4-5A) and a K_m value of 0.33 mM for prephenate (Fig. 4-5B).

Cyclohexadienyl dehydrogenase was not sensitive to feedback inhibition by 1 mM L-tyrosine. The enzyme was assayed at various substrate concentrations of prephenate or of L-arogenate, as low as 0.04 mM, in the presence of 1 mM L-tyrosine in order to detect any possible weak competitive inhibition. L-Phenylalanine and L-tryptophan were also tested as possible allosteric agents, but no effects upon activity were found.

Discussion

Identity of The Cloned Gene and Its Gene Product

The conclusion that we have cloned and sequenced the structural gene coding for cyclohexadienyl dehydrogenase from *Z. mobilis* is amply supported. This gene complemented the *tyrA* defect of *E. coli* AT2471. *E. coli* transformants produced cyclohexadienyl dehydrogenase having the properties of the *Z. mobilis* (unpublished, Jensen) enzyme rather than those of the cyclohexadienyl dehydrogenase component of the *E. coli* T-protein. Southern blotting analysis has established that the *tyrC* gene hybridized to *Z. mobilis* DNA but not *E. coli* one. Complementation of the *tyrA* mutant of *E. coli* required a NsiI-SstII insert. The disruption of the physical integrity of this fragment in subclone derivatives abolished functional complementation, and resulted in the simultaneous loss of

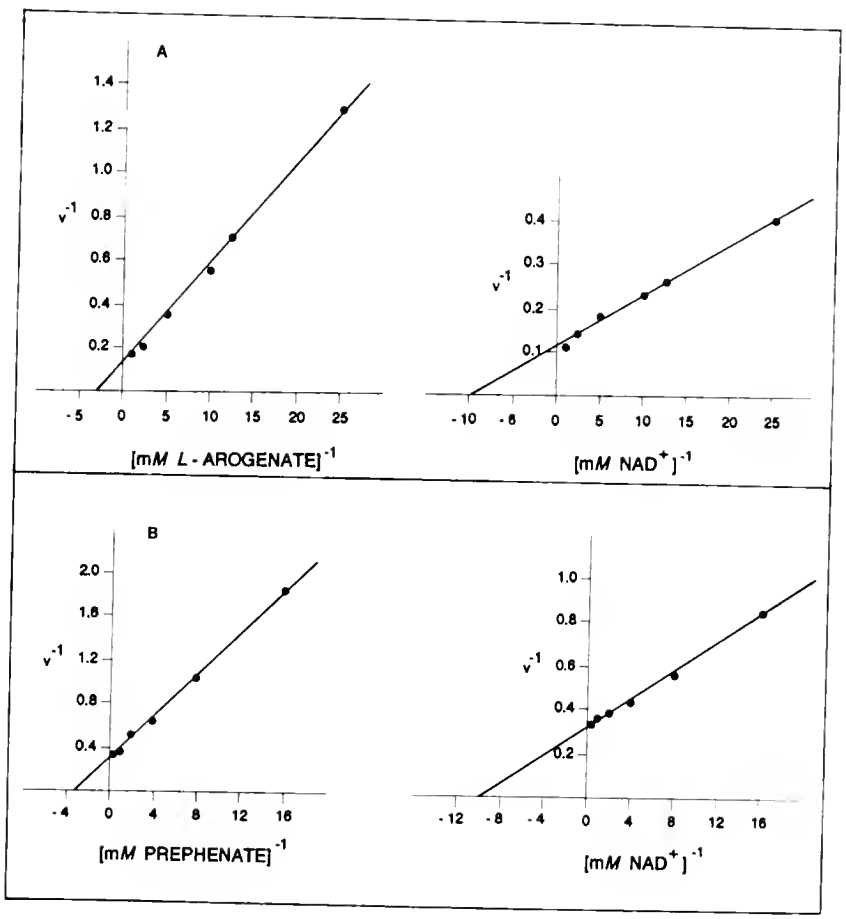


FIG. 4-5. Double-reciprocal plots of purified cyclohexadienyl dehydrogenase assayed as *L*-arogenate dehydrogenase **(A)**, or as prephenate dehydrogenase **(B)**. **A.** Velocities on the ordinate scale are expressed as nmoles min^{-1} of *L*-tyrosine formed at 37°C. A 1.0 m*M* concentration of NAD^+ (left side) and a 1.6 m*M* concentration of *L*-arogenate (right side) was used as the fixed substrate. **B.** Velocities on the ordinate scale are expressed as nmoles min^{-1} of NADH formed at 37°C. A 1.0 m*M* concentration of NAD^+ (left side) and a 1.0 m*M* concentration of prephenate (right side) was used as the fixed substrate.

arogenate and prephenate dehydrogenase activities in crude extracts of the transformants.

Only one ORF was localized within the NsiI-SstII fragment. This ORF would encode a protein with a calculated molecular weight of 32,086, a value which agrees well with the subunit size determined for the purified enzyme by SDS-PAGE. A StuI restriction site was located in the 5'-terminus of the ORF in a region coding for the NAD^+ binding site. A subclone retaining the StuI-SstII fragment was unable to complement the tyrosine auxotroph of *E. coli* since most of the coding region for NAD^+ binding domain was cleaved upon digestion by StuI.

Synonymy of Prephenate Dehydrogenase and Arogenate Dehydrogenase

Biochemical studies with cyclohexadienyl dehydrogenase from *P. aeruginosa* (Xia and Jensen, 1990) have indicated that prephenate and L-arogenate are cyclohexadienyl substrates utilized at a common catalytic site. Similarly, the *Z. mobilis* enzyme cloned in *E. coli* exhibited a constant ratio of prephenate dehydrogenase:arogenate dehydrogenase during purification to electrophoretic homogeneity. Strictly competitive inhibition produced by each cyclohexadienyl substrate in the presence of the other, as well as an identical Km value for NAD^+ regardless of which cosubstrate was present indicated a monofunctional protein exhibiting substrate ambiguity. The genetic evidence presented here definitively establishes that prephenate dehydrogenase and

arogenate dehydrogenase are activities common to a single protein (i.e., cyclohexadienyl dehydrogenase) in Z. mobilis.

Expression of The Gene in E. coli

The cyclohexadienyl dehydrogenase gene was poorly expressed in E. coli. The original insert of the gene cloned was relatively large in size, and subcloning failed to improve expression of the gene in E. coli significantly. The rare use of GTG, rather than ATG, as the start codon was indicated by sequencing analysis and confirmed by amino acid sequencing. Neither a typical ribosome binding site nor any E. coli-like promoter sequences upstream of the start site were found. The codon usage of the gene also deviated from that of highly expressed Z. mobilis genes (Pond et al., 1989). Therefore, the inefficient expression of the gene in E. coli is probably due to some combination of disadvantageous factors related to the ribosome binding site, promoter sequences, the use of GTG as a start codon, and codon usage.

The Basis for Functional Complementation of E. coli tyrA by The Z. mobilis Cyclohexadienyl Dehydrogenase Gene

An E. coli tyrA mutant lacking the bifunctional T-protein activities (chorismate mutase and cyclohexadienyl dehydrogenase) was utilized for selection of transformants which were able to grow without L-tyrosine. This approach was based on the assumption that some of prephenate molecules formed by the chorismate mutase component of the bifunctional P-protein could be diverted to L-tyrosine biosynthesis if a

cyclohexadienyl dehydrogenase were successfully expressed. Our results do indeed indicate that in the absence of the chorismate mutase domain of the bifunctional T-protein, the chorismate mutase component of the bifunctional P-protein is able to provide prephenate not only for L-phenylalanine biosynthesis but also for L-tyrosine biosynthesis. Recently, similar results were obtained when a truncated tyrA gene (lacking the chorismate mutase domain) from Erwinia herbicola was transformed into an E. coli tyrA mutant (Xia et al., 1992a).

Route to L-tyrosine Biosynthesis in Z. mobilis

Three variant pathways exist in nature for L-tyrosine biosynthesis (Jensen and Pierson, 1975). Bacillus subtilis exemplifies organisms which synthesize L-tyrosine via 4-hydroxyphenylpyruvate (Jensen and Pierson, 1975; and Stenmark et al., 1974). Other organisms such as cyanobacteria and Brevibacterium synthesize L-tyrosine via L-arogenate (Jensen and Pierson, 1975; and Stenmark et al., 1974). P. aeruginosa can synthesize L-tyrosine by use of either 4-hydroxyphenylpyruvate or L-arogenate (Jensen and Pierson, 1975; and Patel et al., 1977). The key enzymes in these three systems are prephenate dehydrogenase, arogenate dehydrogenase, and cyclohexadienyl dehydrogenase, respectively. Our kinetic data showed that the cyclohexadienyl dehydrogenase of Z. mobilis had a slightly higher affinity for prephenate than for L-arogenate, but had a much greater Vmax for L-arogenate. It

is presently uncertain what the fractional utilization of the two alternative substrates might be *in vivo*.

Unlike the cyclohexadienyl dehydrogenase activities of *P. aeruginosa* and *E. coli*, the cyclohexadienyl dehydrogenase of *Z. mobilis* is insensitive to feedback inhibition by L-tyrosine. In other systems such as cyanobacteria and Brevibacterium (Jensen and Pierson, 1975) the tyrosine branch is controlled indirectly via other allosteric effects exercised at the level of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase and prephenate dehydratase. It will be of interest to determine the overall pattern of pathway regulation in *Z. mobilis*.

Evolutionary Implications

It is well known that the NAD^+-binding domain of dehydrogenases is strongly conserved among all organisms studied (Hudson and Davidson, 1984; and Wierenga et al., 1986). Based on the analysis developed by Wierenga *et al.* (Wierenga et al., 1986), the NAD^+ binding site of the cloned cyclohexadienyl dehydrogenase from *Z. mobilis* was located on the N-terminus of the polypeptide (Fig. 4-3). Similarly, the NAD^+ binding domains of the prephenate dehydrogenases of *Bacillus subtilis* (Henner et al., 1986) and *Saccharomyces cereevisae* (Mannhaupt et al., 1989) were also found to be localized on the N-terminus of their peptides (Mannhaupt et al., 1989). The chorismate mutase domain of the T-protein of *E. coli* is located on the N-terminus of the peptide (probably

spanning residues 1 to 100), and the NAD^+ binding site was located adjacent to the chorismate mutase domain (from residues 101 to 132) but within the amino-terminal portion of the dehydrogenase domain (Hudson and Davidson, 1984). The internal location of the NAD^+ binding domain in the T-protein is consistent with the evolutionary scenario (Jensen, 1985) whereby the gene encoding a monofunctional chorismate mutase fused to a gene encoding cyclohexadienyl dehydrogenase having a conventional 5′ NAD^+ binding domain. The Z. mobilis cyclohexadienyl dehydrogenase matches the Wierenga fingerprint at 10 of the 11 positions (Zhao et al., 1992b).

Since the bifunctional T-protein possesses enzymatic activities equivalent to cyclohexadienyl dehydrogenase and chorismate mutase-F, and also because the presence of the bifunctional T-protein correlates perfectly with the absence of the cyclohexadienyl dehydrogenase, it has been proposed that the T-protein evolved from two monofunctional enzyme species (chorismate mutase-F and cyclohexadienyl dehydrogenase) via gene duplication and fusion (Ahmad and Jensen, 1986; 1988b; and Jensen, 1985).

The pairwise alignment of the cyclohexadienyl dehydrogenase sequence with the C-terminal portion of the T-proteins of E. coli (Hudson and Davidson, 1984) and E. berbicola (Xia et al., 1992b), and prephenate dehydrogenases of B. subtilis (Henner et al., 1986) and S. cerevisae (Mannhaupt et al., 1989) was performed. The cyclohexadienyl

dehydrogenase showed 21% identity with the T-protein of E. coli, 23% with the T-protein of E. herbicola, and 19% with the prephenate dehydrogenase of S. cerevisae, but 32% identity with the prephenate dehydrogenase of B. subtilis. The significant identity of the Z. mobilis cyclohexadienyl dehydrogenase with the prephenate dehydrogenase of B. subtilis suggests a common evolutionary origin. The lack of obvious homology between the cyclohexadienyl dehydrogenase and the prephenate dehydrogenase of S. cerevisae might not be surprising since the two organisms are phylogenetically distant.

Clear evidence for homology between the Z. mobilis cyclohexadienyl dehydrogenase and the dehydrogenase domain of the E. coli and E. herbicola T-proteins was initially expected since all are cyclohexadienyl dehydrogenases. The conserved motif illustrated in Table 4-4 indicates residues which may be common to cyclohexadienyl dehydrogenase and those which may be common to both cyclohexadienyl dehydrogenases and prephenate dehydrogenases. The lack of obvious homology in a pairwise alignment of the Z. mobilis and E. coli dehydrogenases might be explained as a consequence of the gene fusion which generated the E. coli T-protein. The newly fused cyclohexadienyl dehydrogenase might have been subjected to intense selective pressure for mutant alterations to improve domain:domain interactions. Since the monofunctional cyclohexadienyl dehydrogenase would be free of this selective

pressure, rapid divergence could be accounted for in *E. coli*, thus resulting in a greater overall identity of the *Z. mobilis* gene with the more distant *B. subtilis* gene.

Table 4-4. Possible substrate-binding motif for tyrosine-pathway dehydrogenases

Enzyme[a]		Sequence[b]	
Zmo CDH	86	PALKKDVIICDTGSVK	101
Eco CDH	163	PPLPKDCILVDLASVK	178
Ehe CDH	163	PPLPEDCILVDLASVK	178
Sce PDH	94	PSSKVGTIVGGQTSCK	109
Bsu PDH	88	SGIEHELLITDVGSTK	103

[a]Abbreviations: Zmo CDH, Z. mobilis cyclohexadienyl dehydrogenase; Eco CDH, E. coli cyclohexadienyl dehydrogenase domain of the T-protein; Ehe CDH; E. herbicola cyclohexadienyl dehydrogenase domain of the T-protein; Sce PDH, S. crevisiae prephenate dehydrogenase; Bsu PDH, B. subtilis prephenate dehydrogenase.

[b]Single-letter amino acid code deduced for sequence spanning the 16 residues at positions given at the right and left of the sequences shown. Residues which are common to all three CDH enzymes are dotted.

SUMMARY

The gene coding for cyclohexadienyl dehydratase, designated as pheC was cloned from Pseudomonas aeruginosa by functional complementation of an E. coli pheA mutant. Subcloning analysis showed that arogenate dehydratase and prephenate dehydratase activities were encoded by a 1.3 kb SphI-SmaI fragment, and disruption of the integrity of this fragment resulted in the simultaneous loss of the two activities and functional complementation of the pheA mutant. These data suggests that the cyclohexadienyl dehydratase of P. aeruginosa is encoded by a single gene. This notion was first supported by results of Southern blotting analysis and then by DNA sequencing analysis. Only one single band was observed when the cloned DNA fragment containing the pheC gene was labeled and hybridized to the chromosomal DNA of P. aeruginosa. The SphI-SmaI fragment contained a single open reading frame (ORF), 807 bp in length, which had the potential to code for a protein having a molecular weight of 30,480. Identity of this ORF was later confirmed by sequencing of the N-terminus of the cloned pheC product.

The cloned pheC product produced in E. coli was purified to electrophoretic homogeneity by column chromatography. The

molecular weight of the purified protein was estimated to be 72,000 by gel filtration and 28,000 by SDS-PAGE. The latter value is slightly smaller than that predicted by the ORF. The native enzyme of the cyclohexadienyl dehydratase is probably a homodimer. The cyclohexadienyl dehydratase was then partially purified from P. aeruginosa. This partially purified enzyme exhibited identical molecular weights as compared with the cloned pheC product expressed in E. coli.

Purification and kinetic studies have indicated that the cyclohexadienyl dehydratase is a monofunctional enzyme. The activity ratio of prephenate dehydratase and arogenate dehydratase remained constant throughout purification, and the two activities were therefore inseparable, showing a single protein having two activities. The kinetic studies showed that prephenate dehydratase activity of the enzyme was competitively inhibited by arogenate, and arogenate dehydratase activity was competitively inhibited by prephenate. One substrate competitively inhibiting another suggested that the enzyme only had one single substrate binding site. Thus, it is monofunctional.

In pairwise alignment, no obvious homology was observed between the cyclohexadienyl dehydratase of P. aeruginosa and the prephenate dehydratases of Bacillus subtilis and Corynebacterium glutamicum, and the bifunctional P-proteins of E. coli and P. stutzeri. A more detailed analysis focusing on

short peptides rather than the overall identity indicated that these dehydratases are evolutionarily related.

In order to confirm if the ORF of the pheC gene started at the ATG codon as predicted, the N-terminal sequence of the cloned pheC product was determined. The 11 residues determined were found to match the deduced sequence starting from residues 26 to 36, indicating that a N-terminal peptide, 25 residues in length was cleaved in E. coli. The cyclohexadienyl dehydratase from P. aeruginosa was then purified to electrophoretic homogeneity, and its N-terminal sequence (11 residues determined) was identical to that of the cloned gene product, thus demonstrating that the cleavage of the 25 residue amino peptide also occurred in P. aeruginosa. Analysis of the cleaved amino peptide revealed several features unique to a periplasmic signal peptide. Osmotic shock and chloroform treatment then showed that the cyclohexadienyl dehydratase of P. aeruginosa was localized in the periplasmic space. The presence of cyclohexadienyl dehydratase in the periplasmic space was confirmed by enzyme assay, by Western blotting analysis, and by electron microscopic localization.

The gene coding for cyclohexadienyl dehydrogenase, designated as tyrC was cloned from Zymomonas mobilis by functional complementation of an E. coli tyrA mutant. The original clone of the tyrC gene was relatively large in size (over 10 kb). Subcloning analysis showed that the structural gene was localized within a EcoRI-NcoI fragment. When this

fragment was cloned into pUC18 and pUC19, the resulting constructs complemented the tyrA mutant, indicating that the promoter of the structural gene was functioning in E. coli. Further subcloning study showed that when a 1.3 kb NsiI-SstII fragment was cloned into pUC18 and pUC19, the pUC18 construct complemented the tyrA mutant, but the pUC19 construct failed to do so, suggesting that the NsiI-SstII fragment contained a promoterless tyrC structural gene. The promoter of tyrC is now known to be localized within the StuI-NsiI fragment, which is about 400 bp in length.

As long as clones contained the NsiI-SstII fragment, they complemented the tyrA mutant, and produced arogenate dehydrogenase and prephenate dehydrogenase activities. Disruption of this fragment resulted in the loss of complementation of the tyrA mutant and the loss of arogenate dehydrogenase and prephenate dehydrogenase activities. This suggests that the arogenate dehydrogenase and prephenate dehydrogenase activities of the enzyme were encoded by a single gene. This contention was supported by Southern blotting and DNA sequencing analyses.

The nucleotide sequence of the NsiI-SstII fragment was then determined. The tyrC structural gene was 882 bp in length, encoding a protein with a molecular weight of 32,086. The codon usage of the tyrC gene was less biased than that of highly expressed genes, an indication of a low abundance protein. This is consistent with the observation that the tyrC

gene was poorly expressed in *E. coli* even when cloned in pUC18, a high copy number plasmid carrying a *lacZ* promoter.

The cloned *tyrC* gene product was purified from *E. coli* to electrophoretic homogeneity, and estimated by SDS-PAGE to have a molecular weight of 32,000. The native enzyme had a molecular weight of 78,000 as determined by gel filtration, and probably consisted of two identical subunits. The purification and kinetic studies essentially established that the *Z. mobilis* cyclohexadienyl dehydrogenase was also a monofunctional protein. The activity ratio of arogenate dehydrogenase to prephenate dehydrogenase (3:1) was constant throughout purification, demonstrating that the two activities were inseparable. An identical K_m value was obtained regardless of whether the enzyme was assayed as arogenate dehydrogenase or prephenate dehydrogenase, indicating that the enzyme has a single NAD^+ binding site. This is also consistent with the sequencing results since only one single NAD^+ binding site was observed at the N-terminus. Similar findings were also obtained for two prephenate dehydrogenases. Therefore, this protein probably is also a monofunctional enzyme.

In pairwise alignments, the cyclohexadienyl dehydrogenase of *Z. mobilis* showed significant identity with the prephenate dehydrogenase of *Bacillus subtilis* (32%), but marginal identities with the prephenate dehydrogenase of *Saccharomyces cerevisiae* (19%), and the dehydrogenase domains of the T-proteins of *E. coli* (21%) and *Erwinia herbicola* (23%). The

internal location of the NAD^+ binding sites of the bifunctional T-proteins suggests that they evolved from the fusion of chorismate mutase-F and cyclohexadienyl dehydrogenase.

Future Directions

The Cyclohexadienyl Dehydratase of P. aeruginosa

The cyclohexadienyl dehydratase from P. aeruginosa has been proved to be a periplasmic protein, rather than a cytoplasmic one as previously assumed. This finding raises new possibilities about physiological functions of this protein. To pursue this finding further, the following studies can be done.

Generation of P. aeruginosa pheC Mutants

P. aeruginosa pheC mutants could be obtained through gene-scrambling mutagenesis or marker exchange techniques. Once a P. aeruginosa pheC mutant is generated, physiological studies could be performed to determine the growth characteristics of the mutant. Since P. aeruginosa is also a plant pathogen (although it is opportunistic), a simple infection test could be performed to see if the pheC product has any impact on infection. The pheC gene can also be inactivated in P. aeruginosa strain, PAT1051, a mutant lacking the bifunctional P-protein. This should yield a tightly

blocked phenylalanine auxotroph. Then, similar studies can be carried out along with those lines.

Mutational Analysis of the pheC Gene

Since the region encoding the signal peptide for the P. aeruginosa cyclohexadienyl dehydratase is known, it will be feasible to mutate this region by using PCR techniques to generate a series of mutants. One class of mutants which fail to be translocated to periplasmic space will be particularly interesting. This class of mutants, cytoplasmic in nature, should abolish the essential periplasmic function of the cyclohexadienyl dehydratase. When this mutation is introduced into the type strain PAO1, physiological effects, if any, will be determined. Similar studies can be carried out in the strain PAT1051. As anticipated, introduction of this mutation into PAT1051 should yield a relatively faster growing phenotype since in PAT1051 prephenate and arogenate, the substrates for the cyclohexadienyl dehydratase must be transported across the inner membrane to the periplasmic space first and the phenylalanine formed then has to be transported back to the cytoplasm.

Mutations which alter the signal peptide cleavage site should produce a full-length precursor protein. It will be interesting to see whether this altered precursor protein is enzymatically active. Detailed mutational analysis of the pheC structural gene should give insights about which regions are responsible for processing and translocation.

Gene Organization

Subcloning analysis showed that the promoter of the pheC gene was functional in E. coli. Thus, if the pheC gene forms an operon with other genes, it must be the first gene in the operon although we could not rule out the possibility that the promoter is an internal one of yet another operon. Nevertheless, it will be worthwhile to sequence the region downstream of the pheC gene and then to search the Genbank. This, in conjunction with transcriptional mapping should determine exactly where the pheC transcription starts and if the pheC forms an operon with other genes. Even if the pheC gene does not form any operon with other genes, the information about the pheC surrounding area is still useful because it might give us some clues about where the pheC gene is actually located on the chromosome.

Survey of Cyclohexadienyl Dehydratases from Other Organisms

Since the cyclohexadienyl dehydratase from P. aeruginosa is a periplasmic protein, it will be interesting to see if all the cyclohexadienyl dehydratases are localized in periplasmic space. I am currently working on this project. I have conclusive evidence showing that the cyclohexadienyl dehydratase from E. herbicola is also a periplasmic protein. I will purify the protein and then sequence its N-terminus. This result should tell us if the protein possesses the first Met residue. If not, this probably means that its signal peptide was cleaved. Next, I plan to examine some more

cyclohexadienyl dehydratases from different microorganisms so that we can have a general idea about how widespread this phenomenon is.

The Cyclohexadienyl Dehydrogenase of Z. mobilis

Probably one of the findings in this study is that the cyclohexadienyl dehydrogenase of Z. mobilis is a low abundance protein. One explanation for this is codon usage. It is known that the codon usage of the tyrC gene is less biased than that of highly expressed genes. The lack of a strong promoter can be another explanation. I am currently trying to localize the promoter region. Subcloning analysis showed that when the EcoRI-HindIII fragment (see Fig. 4-2, Chapter 4) was cloned into pUC18 and pUC19, the resulting constructs complemented the tyrA mutant, suggesting that the promoter of the tyrC gene was functioning in E. coli. The tyrC gene was previously localized within the NcoI fragment. When this fragment was cloned into pUC18 and pUC19, the resulting constructs complemented the tyrA mutant. Further subcloning analysis showed that the 1.4 kb StuI-SstII fragment also complemented the tyrA mutant when cloned into pUC18 and pUC19. However, when the NsiI-SstII fragment was cloned into pUC18 and pUC19, only the pUC18 construct complemented the tyrA mutant, thus showing that the NsiI-SstII fragment contained a promoterless tyrC structural gene. Therefore, the promoter must be localized within the StuI-NsiI fragment.

This study will also give us some insights about gene organization. It is possible that the tyrC gene forms a potential operon with other genes. This possibility can be tested by transcriptional mapping studies.

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BIOGRAPHICAL SKETCH

Genshi Zhao was born on June 24, 1961, in Shaanxi Province, China. He completed his elementary and high school education in Hu County, Shaanxi Province. In 1978, he attended the North-western University of Agriculture, where he majored in plant protection. He received his B.S. degree in 1982, and then worked for the Institute of Plant Protection, Shaanxi Academy of Agricultural Sciences. In 1985, he was awarded a scholarship by the Ministry of Agriculture of China, and the Institute of Plant Protection, and came to the Plant Pathology Department at the University of Florida for training in plant virology. In 1986, he was awarded a graduate research assistantship and then worked on legume viruses under the joint supervision of Dr. David D. Baltensperger, Dr. John R. Edwardson, Dr. Ernest Hiebert, and Dr. Dan E. Purcifull in the Departments of Agronomy and Plant Pathology. He joined Dr. Roy A. Jensen's group in August, 1988, before receiving his M.S. degree in December, 1988. He studied the biosynthesis of phenylalanine and tyrosine in bacteria under the supervision of Dr. Roy A. Jensen, and obtained his Ph.D. degree in December, 1991.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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