Cyclohexadienyl dehydratase from *Pseudomonas aeruginosa* is a periplasmic protein

GENSHI ZHAO, TIANHUI XIA, HENRY ALDRICH and ROY A. JENSEN*

Department of Microbiology and Cell Science, 3103 McCarty Hall, University of Florida, Gainesville, FL 32611-0144, USA

(Received 9 September 1992; revised 2 December 1992; accepted 15 December 1992)

The gene encoding cyclohexadienyl dehydratase from *Pseudomonas aeruginosa*, designated *pheC*, was cloned in *Escherichia coli* and sequenced recently by Zhao *et al.* (*Journal of Biological Chemistry* 267, 2487–2493, 1992). N-Terminal sequencing of the purified cyclohexadienyl dehydratase yielded a run of 11 residues which matched perfectly with the deduced amino acid residues 26–36. This showed that a 25 residue peptide was cleaved from the N-terminus of a preprotein formed in *E. coli*. The amino acid composition of the 25 residue peptide was typical of signal sequences for periplasmic proteins. Most or all of the cyclohexadienyl dehydratase was released from *P. aeruginosa* and *E. coli* carrying the *pheC* gene following spheroplast formation, osmotic shock or chloroform treatment. The location of the enzyme in the periplasm of both *E. coli* and *P. aeruginosa* was confirmed by Western blotting analysis using antibody prepared against PheC. Electron microscopy using immunogold labelling showed an apparent localization of cyclohexadienyl dehydratase at the polar regions of the periplasmic space in *E. coli*.

Introduction

Cyclohexadienyl dehydratase catalyses two analogous aromatization reactions: either the conversion of prephenate to phenylpyruvate or the conversion of L-arogenate to L-phenylalanine (Jensen & Fischer, 1987). The enzyme was first described in *Pseudomonas aeruginosa*, an organism which possesses it as a component of one of the two separate pathways for L-phenylalanine biosynthesis (Patel *et al.*, 1977). The *P. aeruginosa* gene encoding cyclohexadienyl dehydratase, denoted *pheC*, has been cloned and sequenced recently, and the product expressed in *Escherichia coli* purified to electrophoretic homogeneity (Zhao *et al.*, 1992). Kinetic studies indicate that this enzyme probably possesses a single substrate-binding site which can utilize either prephenate or L-arogenate.

Although cyclohexadienyl dehydratase is widely distributed among Gram-negative prokaryotes (Ahmad *et al.*, 1990), its physiological role is unclear. Molecular genetic techniques have facilitated the production of *P. aeruginosa* PheC in large quantities by use of a recombinant *E. coli* strain (Zhao *et al.*, 1992). Comparison of its N-terminal amino acid sequence with that

deduced from the nucleotide sequence led to the unexpected finding that this protein is derived from a precursor with a cleavable leader sequence, and that the protein is localized in the periplasmic space of *P. aeruginosa*. Thus, cyclohexadienyl dehydratase is spatially separated from its prephenate dehydratase counterpart in the cytoplasm, which is dedicated to the classic phenylpyruvate pathway (Davis, 1953) of L-phenylalanine biosynthesis. This indicates that cyclohexadienyl dehydratase is involved in some unknown function in the periplasm.

Methods

Bacterial strains, plasmids and media. Bacterial strains and plasmids used in this study are listed in Table 1.

LB and M9 (Maniatis *et al.*, 1982) were used as enriched and minimal media, respectively. For induction of alkaline phosphatase activity in *P. aeruginosa*, the organic phosphate medium of Cheng *et al.* (1970) was used. Ampicillin (50 µg ml⁻¹), L-tyrosine (50 µg ml⁻¹) and thiamin (17 µg ml⁻¹) were added when appropriate.

Biochemicals. Restriction enzymes and T4 DNA ligase were obtained from BRL. Prephenate and L-arogenate were prepared from *Salmonella typhimurium* *tyrA19* (Nishioka *et al.*, 1967) by the procedures of Bonner *et al.* (1990). Sephadex G-200, DEAE-cellulose and hydroxylapatite were obtained from Sigma, Whatman and Bio-Rad, respectively. *M_r* standards for SDS-PAGE (α-lactalbumin, *M_r* 14400; soybean trypsin inhibitor, 20100; carbonic anhydrase, 30000; ovalbumin, 43000; bovine serum albumin, 67000; and phosphorylase b,

* Author for correspondence. Tel. (904) 392-9677; fax (904) 392-8479.

Table 1. *Bacterial strains and plasmids*

Strain or plasmid	Genotype or description	Source
<i>E. coli</i> K-12		
JM83	<i>ara</i> Δ (<i>lac-proAB</i>) ϕ 80d <i>lacZ</i> Δ M15	Gibco-BRL
JP2255	<i>rpsL</i> <i>aroF363 pheA361 pheO352 tyrA382</i> <i>thi strR712 lacY1 xyl5</i>	Baldwin & Davidson, (1981)
<i>P. aeruginosa</i>		
PAO1	Prototroph	Holloway, (1955)
PAT2	Prototroph	Holloway, (1955)
PAOH636	<i>oprF::</i> Ω	Woodruff & Hancock, (1988)
Plasmids		
pUC19	Ap ^r <i>lacZ</i>	Gibco-BRL
pJZ1g	1110 bp <i>SphI</i> - <i>SmaI</i> fragment containing the intact <i>pheC</i> gene cloned into pUC19	Zhao et al., (1992)
pJZpp	1477 bp <i>SphI</i> - <i>PstI</i> fragment containing the <i>P. stutzeri</i> <i>pheA</i> gene cloned into pUC19	This study; Fischer et al., (1991)

94000) and for gel-filtration (carbonic anhydrase, M_r 30000; bovine serum albumin, 67000; alcohol dehydrogenase, 150000; and β -amylase, 200000) were obtained from Pharmacia and Sigma, respectively. Other biochemicals were obtained from Sigma.

Expression construct of *P. stutzeri* *pheA*. The intact *pheA* gene encoding the bifunctional P-protein (chorismate mutase/prephenate dehydratase) from *P. stutzeri* (Fischer et al., 1991) was cloned from pJF1954 into pUC19 at the *SphI*/*PstI* restriction sites, and the resulting construct, designated pJZpp, was found to overexpress the P-protein in *E. coli*.

Purification of PheC. The cloned *pheC* gene product expressed in *E. coli* was purified as described previously (Zhao et al., 1992). The cyclohexadienyl dehydratase isolated directly from *P. aeruginosa* was purified in five steps as described previously (Zhao et al., 1992) with the following modifications. Ammonium sulphate precipitation was omitted. After the third step (Sephadex G-200 gel-filtration), the fractions possessing cyclohexadienyl dehydratase activity were pooled and applied to a hydroxylapatite column (1.5 $\times$ 30 cm) and then to a DEAE-cellulose column (1.5 $\times$ 30 cm) as described previously (Zhao et al., 1992). A sample (2.205 g) of crude extract from *P. aeruginosa*, possessing an initial specific activity of 3.71 nmol min⁻¹ (mg protein)⁻¹ when cyclohexadienyl dehydratase was assayed as arogenate dehydratase, was purified 1298-fold.

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.

M_r determinations. Native M_r values of the purified proteins were estimated by gel-filtration, and subunit M_r values were determined by SDS-PAGE (Laemmli, 1970).

Antibody preparation and Western blotting analysis. *P. aeruginosa* cyclohexadienyl dehydratase purified from *E. coli* (0.6 mg) was injected every 7 d into a New Zealand White rabbit (Hiebert et al., 1984), and antiserum was collected 1 week after a third injection. The Western blot procedure was done as described by Towbin et al. (1979).

Isolation of periplasmic fractions from *E. coli*. *E. coli* JP2255 carrying plasmid pJZ1g (Table 2) or pJZpp (Table 3) was grown in 1.2 litres of

either LB medium supplemented with ampicillin, or M9 medium (data not shown) supplemented with ampicillin, L-tyrosine and thiamin. The cells were harvested by centrifugation during the late exponential phase of growth. Each culture was divided into four 300 ml parts. The cell pellet obtained from one part of the culture (1.13 g wet weight) was subjected to osmotic shock (Neu & Heppel, 1965). After centrifugation, the supernatant fluid was collected and referred to as the periplasmic fraction. The cell pellet remaining after osmotic shock was sonicated. The sonicated preparation was centrifuged at 150000 g for 60 min. The supernatant fraction was collected and then passed through a PD-10 Sephadex column. This preparation was designated the cytoplasmic fraction. A second part of the culture was used to obtain periplasmic and cytoplasmic fractions from spheroplast preparations (Birdsell & Cota-Robles, 1967). Chloroform treatment was carried out as described by Ames et al. (1984). The fourth part of the culture was sonicated, centrifuged and desalted as described above, but omitting the osmotic shock step. This preparation is referred to as the unfractionated extract.

Isolation of periplasmic fractions from *P. aeruginosa*. *P. aeruginosa* strains were grown in 800 ml of organic phosphate medium and harvested during the late exponential phase of growth. The cultures were divided equally into two 400 ml parts (1.5 g wet weight). One part of the culture was treated with chloroform (Ames et al., 1984), and the supernatant fluid recovered was designated the periplasmic fraction. The second part of the culture, which was not treated with chloroform, was sonicated, centrifuged and desalted as described above to yield the unfractionated extract.

Enzyme assay and protein determination. Prephenate dehydratase was assayed as described by Cotton & Gibson (1965). Arogenate dehydratase was assayed by measuring the formation of L-phenylalanine using HPLC (Fischer & Jensen, 1987). Alkaline phosphatase was assayed as detailed by Brickman & Beckwith (1975). Protein was measured using the procedure of Bradford (1976).

Electron microscopy. Exponential-phase populations of *E. coli* JP2255(pJZ1g) were fixed by use of 2.5% formaldehyde/0.5% glutaraldehyde and then embedded in Lowicryl K4M. After incubation with either antibody or pre-immune serum and gold-particle labelling, the thin sections were photographed under a JEOL 100-CX electron microscope.

Fig. 1. SDS-PAGE analysis of the cloned *P. aeruginosa pheC* gene product purified from *E. coli* and the cyclohexadienyl dehydratase purified directly from *P. aeruginosa*. The samples were run on a 15% polyacrylamide gel. Lane 1, M_r standards; lanes 2 and 4, cyclohexadienyl dehydratase purified from *P. aeruginosa* PAO1; lane 3, cyclohexadienyl dehydratase purified from *E. coli*.

Results

N-Terminal amino acid sequencing of purified cyclohexadienyl dehydratase

Purification of the cloned PheC was done as described previously (Zhao *et al.*, 1992). After the gel-filtration step, a single protein band was resolved by SDS-PAGE. The subunit M_r was 28000 (Fig. 1), in contrast to a value of 29500 reported earlier (Zhao *et al.*, 1992). The native M_r of the purified *pheC* product was estimated by gel-filtration to be 72000.

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. This sequence matched perfectly with the amino acids deduced at positions 26 to 36 (Zhao *et al.*, 1992). This indicated that an amino-terminal peptide (25 residues in length), a possible signal sequence, was cleaved in *E. coli* (Fig. 2).

In order to eliminate the possibility that this cleavage might be an artifact of cloning in *E. coli*, the cyclohexadienyl dehydratase was also purified directly from *P. aeruginosa*. The activities of prephenate dehydratase and aroenate dehydratase co-eluted, and the ratio of the two activities was constant throughout the purification process. After the five steps of purification used, the enzyme was homogeneous as judged by SDS-PAGE. The subunit M_r of 28000 (Fig. 1) was identical to that of the cloned *pheC* gene product. The native M_r of the purified enzyme was determined to be 72000 by gel-filtration.

The cyclohexadienyl dehydratase purified directly from *P. aeruginosa* was sequenced at the amino-terminus. The 11 amino acid residues sequenced were identical to those determined for the cloned *pheC* gene product purified from *E. coli*. The matching sequence corresponded to the deduced sequence spanning residues 26 to 36, thus showing that cleavage of the amino-terminal peptide also occurred in *P. aeruginosa*.

The amino-terminal peptide of the cyclohexadienyl dehydratase from *P. aeruginosa* shown in Fig. 2 exhibits features typical of known signal sequences for periplasmic proteins (Oliver, 1986). Thus, the *P. aeruginosa* amino-terminal peptide possesses a basic amino terminus (Lys, Arg and His), a hydrophobic core region, and a processing-site motif (Leu-Gln-Ala). The length of this amino-terminal peptide also falls within the range for periplasmic signal sequences (Oliver, 1986).

Spheroplast preparation, osmotic shock and chloroform treatment

The evidence for N-terminal processing suggested that the mature cyclohexadienyl dehydratase might be located in the periplasm. The results obtained following application of different techniques for releasing peri-

- (a) N-Gln-Glu-Ser-Arg-Leu-Asp-Arg-Ile-Leu-Glu-Ser....C
- (b) 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. 2. (a) N-Terminal amino acid sequence of the *pheC* product purified from *E. coli*; (b) deduced N-terminal amino acid sequence for the cloned *pheC* gene product. Shading indicates identical residues.

Table 2. Release of cyclohexadienyl dehydratase from the periplasmic compartment of *E. coli* JP2255(pJZ1g)

Treatment	Fraction	Total protein (mg)	AP (units)*	CDT (units)†
None	Unfractionated	106	31	117
Spheroplast	Periplasmic	18	23	95
	Cytoplasmic	91	7	22
Osmotic shock	Periplasmic	23	34	106
	Cytoplasmic	85	0.3	3
Chloroform	Periplasmic	19	32	101

* 1 unit of alkaline phosphatase (AP) equals an amount of enzyme producing one A_{240} increment per min at 37 °C.

† 1 unit of cyclohexadienyl dehydratase (CDT) equals an amount of enzyme producing 1 μ mol of L-phenylalanine from L-arogenate per min at 37 °C.

Table 3. Retention of prephenate dehydratase by *E. coli* JP2255(pJZpp) under conditions which release periplasmic proteins

Treatment	Fraction	Total protein (mg)	AP (units)*	PDT (units)†
None	Unfractionated	115	35	86
Spheroplast	Periplasmic	12	26	0.1
	Cytoplasmic	101	10	87
Osmotic shock	Periplasmic	18	32	0.6
	Cytoplasmic	98	5	81
Chloroform	Periplasmic	18	35	0.2

* 1 unit of alkaline phosphatase (AP) equals an amount of enzyme producing one A_{240} increment per min⁻¹ at 37 °C.

† 1 unit of prephenate dehydratase (PDT) equals an amount of enzyme producing 1 μ mol of phenylpyruvate per min at 37 °C.

plasmic proteins from *E. coli* strains carrying pJZ1g and pJZpp are summarized in Tables 2 and 3. The known periplasmic protein, alkaline phosphatase, was monitored as a positive control. The efficiency of different techniques for release of cyclohexadienyl dehydratase (Table 2) was in the order: osmotic shock (91%), chloroform treatment (86%), and spheroplast preparation (81%).

Parallel studies were carried out with the JP2255(pJZpp) construct, because in this strain the *pheA* gene of *P. stutzeri* is highly expressed, but the gene product is a cytoplasmic protein. Thus, it served as a negative control in this study. In marked contrast to results obtained with alkaline phosphatase, less than 1% of the prephenate dehydratase activity was found in the periplasmic fractions when *E. coli* JP2255(pJZpp) was subjected to spheroplast preparation, osmotic shock or chloroform treatment.

Results similar to those given in Tables 2 and 3 were obtained when *E. coli* JP2255 carrying pJZ1g or pJZpp was grown in M9 minimal medium, except that alkaline

Table 4. Release of cyclohexadienyl dehydratase from the periplasmic compartment of *P. aeruginosa*

Strain	Treatment	Periplasmic AP (%)	Periplasmic CDT (%)
PAO1	Chloroform	100	90
	MgCl ₂	100	<1
PAT2	Chloroform	47	56
PAOH636	None	22	21

The ranges of total protein, total units AP (alkaline phosphatase), and total units CDT (cyclohexadienyl dehydratase) in various experiments were 42–56 mg, 37–67 units AP and 232–347 units CDT, respectively. For mutant PAOH636 the values given equal the percentage of total enzyme released into the culture medium.

phosphatase activities were repressed to very low levels by the phosphate content of the medium.

The results of chloroform treatment of *P. aeruginosa* PAO1 are given in Table 4. The fact that the cyclohexadienyl dehydratase rather than the *pheA*-encoded prephenate dehydratase was present in the periplasmic

Fig. 3. Immunocytochemical localization of cyclohexadienyl dehydratase. (a) Exponential-phase cells of *E. coli* JP2255(pJZ1g) were prepared as described under Methods. Thin sections of these cells were incubated with antibody prepared against purified PheC, labelled with gold particles, and examined under the electron microscope. Cyclohexadienyl dehydratase molecules are visualized as black dots in the thin sections shown. Arrowheads indicate accumulation of gold particles in the polar region of the periplasm. (b) Control cells were treated with pre-immune serum instead of specific antibody and then handled as described in (a). Bars, 0.1 μ m.

fractions was established by its insensitivity to feedback inhibition by L-phenylalanine and by its ability to utilize L-arogenate as substrate, both of which are characteristics of the cyclohexadienyl dehydratase but not of the bifunctional P-protein (Patel *et al.*, 1977; Xia *et al.*, 1991; Zhao *et al.*, 1992).

Since cells of *P. aeruginosa* undergo massive lysis during the osmotic shock process due to their sensitivity to EDTA (Wilkinson, 1967), this procedure could not be used. The chloroform treatment method resulted in the release of 90% of the activity from the periplasmic space of *P. aeruginosa* PAO1, but only 56% from another commonly used strain, PAT2. This strain difference is not specific for cyclohexadienyl dehydratase, as shown by the parallel results obtained with alkaline phosphatase.

We confirmed the results of Cheng *et al.* (1970), who found that simple washing in 0.2 M-Mg²⁺ effectively releases 100% of the PAO1 alkaline phosphatase activity. Interestingly, this treatment was completely ineffective for release of periplasmic cyclohexadienyl dehydratase.

Mutant PAOH636 is deficient in porin protein F and leaks periplasmic proteins into the growth medium (Woodruff & Hancock, 1988). Table 4 shows that over

20% of both alkaline phosphatase and cyclohexadienyl dehydratase were lost into the growth medium. Under comparable growth conditions neither of the periplasmic enzyme activities were found in the culture media of strains PAO1 or PAT2. The data given in Tables 2 to 4 are representative of results obtained from at least three and as many as five repetitions of experiments. The values obtained in different experiments were within 10% of one another.

Western blotting analysis of the cyclohexadienyl dehydratase in the periplasmic fractions

Periplasmic fractions collected following osmotic shock in *E. coli* or chloroform treatment of *P. aeruginosa* PAO1 were first concentrated using a PM-10 membrane and then subjected to SDS-PAGE. The proteins were then transferred to a nitrocellulose membrane and probed with antibody prepared against the purified *pheC* product. One control lane was also probed with pre-immune serum. Cyclohexadienyl dehydratase was readily detected in the periplasmic fractions of *E. coli* JP2255(pJZ1g) and *P. aeruginosa* PAO1 (data not shown). Antibody prepared against the *pheC* product did not cross-react with the bifunctional P-protein.

Immunogold labelling

Incubation of ultra-thin sections of *E. coli* JP2255(pJZ1g) with gold-labelled antibody resulted in the electron microscopic visualization of the subcellular localization shown in Fig. 3. The majority of the antibody, as judged by the location of gold particles on the section, bound to the polar regions of the periplasm. Little or no antibody was bound to the remaining periplasmic regions, or to the cytoplasmic space. Control preparations were practically devoid of label.

A comparable experiment with *P. aeruginosa* PAO1 did not result in visualization of the cyclohexadienyl dehydratase location, presumably due to the low quantities of the enzyme.

Discussion

The periplasmic location of cyclohexadienyl dehydratase in *P. aeruginosa* has been established. Although cyclohexadienyl dehydratase has variable properties [e.g. *Erwinia herbicola* cyclohexadienyl dehydratase is a homo-tetramer made up of M_r 18000 subunits (Xia *et al.*, 1991)], while *P. aeruginosa* cyclohexadienyl dehydratase is a dimer of M_r 28000 subunits (Zhao *et al.*, 1992), cyclohexadienyl dehydratase from *E. herbicola* and other Gram-negative bacteria can be released from the periplasmic space by osmotic shock (unpublished data).

Cyclohexadienyl dehydratase is an enzyme component of one of two separate pathways for phenylalanine biosynthesis, an arrangement which is common in Gram-negative bacteria (Byng *et al.*, 1982). Its insensitivity to feedback inhibition is the basis for reference to this pathway as an 'overflow' pathway of phenylalanine biosynthesis in *P. aeruginosa* (Fiske *et al.*, 1983). This overflow pathway is not thought to function for primary phenylalanine biosynthesis in wild-type cells under most physiological conditions, where intracellular prephenate is maintained at low levels. However, it can partially suppress a *pheA* defect of the primary biosynthetic pathway, resulting in leaky growth (Berry *et al.*, 1987). When the major point of early-pathway control is eliminated in a 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase-tyr mutant (in which allosteric control has been abolished), the unregulated cyclohexadienyl dehydratase permits an unrestrained flow of excess precursor molecules to L-phenylalanine. In view of the periplasmic location of cyclohexadienyl dehydratase, it appears that in the foregoing situations a fractional amount of an aromatic-pathway intermediate (such as prephenate or L-arogenate) must be diverted to the periplasm. In the case of the *pheA* leaky auxotroph, the phenylpyruvate or L-phenylalanine product must be

transported back to the cytoplasm. A monofunctional species of chorismate mutase denoted CM-F (Xia & Jensen, 1992) exhibits a similar phylogenetic distribution as does cyclohexadienyl dehydratase (Ahmad & Jensen, 1988), and CM-F might also prove to be a periplasmic enzyme. If so, the point of pathway diversion with respect to compartmentation would be at the level of chorismate.

The location of cyclohexadienyl dehydratase in the periplasm with various scavenging enzymes suggests an interactive role with the immediate environment of the cell. One possibility relates to the ability of cyclohexadienyl dehydratase to convert L-arogenate to L-phenylalanine. Since higher plants use L-arogenate as an exclusive precursor of L-phenylalanine (Jung *et al.*, 1986), the diversion of plant L-arogenate to the microbial pool of L-phenylalanine would undermine the precursor resources of plant phenylalanine needed for phytoalexin synthesis. Because non-pathogenic relatives of plant pathogenic bacteria (e.g. *P. aeruginosa*) often possess cyclohexadienyl dehydratase, this ability to undermine the formation of plant defence compounds may also exist in saprophytic or opportunistic species of bacteria associated with plants. In this context, it is interesting that cyclohexadienyl dehydratase is absent in genera such as *Escherichia*, *Shigella*, *Salmonella* and *Proteus*, but is present in genera such as *Klebsiella*, *Erwinia* and *Serratia*. Species in the latter genera are known to associate with plant surfaces in a pathogenic or opportunistic mode. The ability to scavenge L-arogenate as an immediate precursor of L-phenylalanine may confer a metabolic advantage upon the bacterium since biosynthesis of aromatic amino acids is extremely expensive in energetic terms.

Alternatively, cyclohexadienyl dehydratase could be one component of a periplasm-localized arm of biosynthesis which is dedicated to the provision of phenylalanine to the periplasmic compartment for some as yet unidentified function. Such alternative roles of cyclohexadienyl dehydratase are not mutually exclusive. Inactivation of the *pheC* gene by molecular genetic techniques may illuminate the role(s) of cyclohexadienyl dehydratase *in vivo*.

Periplasmic proteins undoubtedly exhibit different localizations within the periplasmic compartment (Oliver, 1986). *P. aeruginosa* alkaline phosphatase has been suggested to be linked to the exocytoplasmic region through divalent Mg^{2+} bridges (Cheng *et al.*, 1970). Results obtained with Mg^{2+} washing show that *P. aeruginosa* cyclohexadienyl dehydratase does not share this feature, an indication that the spatial organization of the two enzymes is different. The apparent restriction of cyclohexadienyl dehydratase to the polar regions of the periplasm may be an important clue to the function of

this enzyme. Experiments to confirm this spatial location and to determine the mechanism accomplishing it are in progress.

This work was supported by grant DK38309 from the US Public Health Service. Robert E. W. Hancock generously provided the porin-deficient mutant PAOH636. This paper is Florida Agricultural Station Journal Series no. R-02681.

References

- AHMAD, S. & JENSEN, R. A. (1988). New prospects for deducing the evolutionary history of metabolic pathways in prokaryotes: aromatic biosynthesis as a case-in-point. *Origins of Life and Evolution of the Biosphere* **18**, 41–57.
- AHMAD, S., WEISBURG, W. G. & JENSEN, R. A. (1990). Evolution of aromatic amino acid biosynthesis and application to the fine-tuned phylogenetic positioning of enteric bacteria. *Journal of Bacteriology* **172**, 1051–1061.
- AMES, G. F.-L., PRODY, C. & KUSTU, S. (1984). Simple, rapid, and quantitative release of periplasmic proteins by chloroform. *Journal of Bacteriology* **160**, 1181–1183.
- BALDWIN, G. S. & DAVIDSON, B. E. (1981). A kinetic and structural comparison of chorismate mutase/prephenate dehydratase from mutant strains of *E. coli* K-12 defective in the PheA gene. *Archives of Biochemistry and Biophysics* **211**, 66–75.
- BERRY, A., BHATNAGAR, R. K. & JENSEN, R. A. (1987). Enzymic basis for leakiness of auxotrophs for phenylalanine in *Pseudomonas aeruginosa*. *Journal of General Microbiology* **133**, 3257–3263.
- BIRDELL, D. C. & COTA-ROBLES, E. H. (1967). Production and ultra-structure of lysozyme and ethylene-diaminetetraacetate-lysozyme spheroplasts of *Escherichia coli*. *Journal of Bacteriology* **93**, 427–437.
- BONNER, C. A., FISCHER, R. S., AHMAD, S. & JENSEN, R. A. (1990). Remnants of an ancient pathway to L-phenylalanine and L-tyrosine in enteric bacteria: evolutionary implications and biotechnological impact. *Applied and Environmental Microbiology* **56**, 3741–3747.
- BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.
- BRICKMAN, E. & BECKWITH, J. (1975). Analysis of the regulation of *Escherichia coli* alkaline phosphatase synthesis using deletions and transducing phages. *Journal of Molecular Biology* **96**, 307–316.
- BYNG, G. S., KANE, J. F. & JENSEN, R. A. (1982). Diversity in the routing and regulation of complex biochemical pathways as indicators of microbial relatedness. *Critical Reviews in Microbiology* **9**, 227–252.
- CHENG, K.-J., INGRAM, J. M. & COSTERTON, J. W. (1970). Release of alkaline phosphatase from cells of *Pseudomonas aeruginosa* by manipulation of cation concentration and of pH. *Journal of Bacteriology* **104**, 748–753.
- COTTON, R. G. H. & GIBSON, F. (1965). The biosynthesis of phenylalanine and tyrosine: enzymes converting chorismic acid into prephenic acid and their relationships to prephenate dehydratase and prephenate dehydrogenase. *Biochimica et Biophysica Acta* **100**, 76–88.
- DAVIS, B. D. (1953). Autocatalytic growth of a mutant due to accumulation of an unstable phenylalanine precursor. *Science* **118**, 251–252.
- FISCHER, R. & JENSEN, R. A. (1987). Arogenate dehydratase. *Methods in Enzymology* **142**, 495–502.
- FISCHER, R. S., ZHAO, G. S. & JENSEN, R. A. (1991). Cloning, sequencing, and expression of the P-protein gene (*pheA*) of *Pseudomonas stutzeri* in *Escherichia coli*: implications for evolutionary relationships in phenylalanine biosynthesis. *Journal of General Microbiology* **137**, 1293–1301.
- FISKE, M. J., WHITAKER, R. J. & JENSEN, R. A. (1983). Hidden overflow pathway to L-phenylalanine in *Pseudomonas aeruginosa*. *Journal of Bacteriology* **154**, 623–631.
- HIEBERT, E., PURCIFULL, D. E. & CHRISTIE, R. G. (1984). Purification and immunological analysis of plant viral inclusion bodies. *Methods in Virology* **8**, 225–280.
- HOLLOWAY, B. W. (1955). Genetic recombination in *Pseudomonas aeruginosa*. *Journal of General Microbiology* **13**, 572–581.
- JENSEN, R. A. & FISCHER, R. S. (1987). The postprephenate biochemical pathways to L-phenylalanine and tyrosine: an overview. *Methods in Enzymology* **142**, 472–478.
- JUNG, E., ZAMIR, L. O. & JENSEN, R. A. (1986). Chloroplasts of higher plants synthesize L-phenylalanine via L-arogenate. *Proceedings of the National Academy of Sciences of the United States of America* **83**, 7231–7235.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- NEU, H. C. & HEPPLE, L. A. (1965). The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts. *Journal of Biological Chemistry* **240**, 3685–3692.
- NISHIOKA, M., DEMEREC, M. & EISENSTARCK, A. (1967). Genetic analysis of aromatic mutants of *Salmonella typhimurium*. *Genetics* **56**, 341–351.
- OLIVER, D. B. (1986). In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, pp. 56–69. Edited by F. C. Neidhardt and others. Washington, DC: American Society for Microbiology.
- PATEL, N., PIERSON, D. L. & JENSEN, R. A. (1977). Dual enzymatic routes to L-tyrosine and L-phenylalanine via pretyrosine in *Pseudomonas aeruginosa*. *Journal of Biological Chemistry* **252**, 5839–5846.
- TOWBIN, H., STAHELIN, T. & GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 4350–4354.
- WILKINSON, S. G. (1967). The sensitivity of pseudomonads to ethylenediaminetetraacetic acid. *Journal of General Microbiology* **47**, 67–76.
- WOODRUFF, W. A. & HANCOCK, R. E. W. (1988). Construction and characterization of *Pseudomonas aeruginosa* protein F-deficient mutants after *in vitro* and *in vivo* insertion mutagenesis of the cloned gene. *Journal of Bacteriology* **170**, 2592–2598.
- XIA, T. & JENSEN, R. A. (1992). Monofunctional chorismate mutase from *Serratia rubidaea*: a paradigm system for the three-isozyme gene family of enteric bacteria. *Archives of Biochemistry and Biophysics* **294**, 147–153.
- XIA, T., AHMAD, S., ZHAO, G. & JENSEN, R. A. (1991). A single cyclohexadienyl dehydratase specifies the prephenate dehydratase and arogenate dehydratase components of one of two independent pathways to L-phenylalanine in *Erwinia herbicola*. *Archives of Biochemistry and Biophysics* **286**, 461–465.
- ZHAO, G., XIA, T., FISCHER, R. S. & JENSEN, R. A. (1992). Cyclohexadienyl dehydratase from *Pseudomonas aeruginosa*: molecular cloning of the gene and characterization of the gene product. *Journal of Biological Chemistry* **267**, 2487–2493.