A monofunctional prephenate dehydrogenase created by cleavage of the 5' 109 bp of the *tyrA* gene from *Erwinia herbicola*

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(Received 9 January 1992; revised 19 March 1992; accepted 3 April 1992)

A cohesive phylogenetic cluster that is limited to enteric bacteria and a few closely related genera possesses a bifunctional protein that is known as the T-protein and is encoded by *tyrA*. The T-protein carries catalytic domains for chorismate mutase and for cyclohexadienyl dehydrogenase. Cyclohexadienyl dehydrogenase can utilize prephenate or L-arogenate as alternative substrates. A portion of the *tyrA* gene cloned from *Erwinia herbicola* was deleted *in vitro* with exonuclease III and fused in-frame with a 5' portion of *lacZ* to yield a new gene, denoted *tyrA**, in which 37 N-terminal amino acids of the T-protein are replaced by 18 amino acids encoded by the polycloning site/5' portion of the *lacZ* α -peptide of pUC19. The TyrA* protein retained dehydrogenase activity but lacked mutase activity, thus demonstrating the separability of the two catalytic domains. While the K_m of the TyrA* dehydrogenase for NAD⁺ remained unaltered, the K_m for prephenate was fourfold greater and the V_{max} was almost twofold greater than observed for the parental T-protein dehydrogenase. Activity with L-arogenate, normally a relatively poor substrate, was reduced to a negligible level. The prephenate dehydrogenase activity encoded by *tyrA** was hypersensitive to feedback inhibition by L-tyrosine (a competitive inhibitor with respect to prephenate), partly because the affinity for prephenate was reduced and partly because the K_i value for L-tyrosine was decreased from 66 μ M to 14 μ M. Thus, excision of a portion of the chorismate mutase domain is shown to result in multiple extra-domain effects upon the cyclohexadienyl dehydrogenase domain of the bifunctional protein. These include alterations in apparent substrate specificity, isoelectric point, stability, catalytic properties and regulatory properties.

Introduction

The biochemical pathways which specify the biosynthesis of L-tyrosine and L-phenylalanine in enteric bacteria utilize two bifunctional proteins which are in competition for chorismate at a metabolic branchpoint. The P-protein encoded by *pheA* possesses catalytic domains for chorismate mutase (CM-P) and for prephenate dehydratase (converts prephenate to phenylpyruvate). The T-protein encoded by *tyrA* possesses catalytic domains for chorismate mutase (CM-T) and for prephenate dehydrogenase (converts prephenate to 4-hydroxy-

phenylpyruvate). This was originally shown in the landmark paper by Cotton & Gibson (1965) who studied *Escherichia coli* and *Klebsiella pneumoniae* (originally named *Aerobacter aerogenes*). The limited distribution of the T-protein in enteric bacteria – *Alteromonas* and *Aeromonas* – compared to the widespread presence of the P-protein throughout two of the three main subdivisions of Gram-negative bacteria, has been established more recently (Ahmad & Jensen, 1986). The T-protein dehydrogenase has proven to be a cyclohexadienyl dehydrogenase (able to use prephenate and L-arogenate as alternative substrates), in contrast to the highly specific P-protein dehydratase which cannot function as a cyclohexadienyl dehydratase (Ahmad & Jensen, 1987). Many enteric bacteria, but not *E. coli*, possess a third isoenzyme of chorismate mutase which is monofunctional (i.e. has one catalytic domain) and has been denoted CM-F (Ahmad *et al.*, 1990).

The *tyrA* and *pheA* genes are contiguous (tail-to-tail) in *E. coli*, and homologous chorismate mutase domains are indicated by the similarity of the N-terminal sequences

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Abbreviations: CM-F, a monofunctional chorismate mutase protein; CM-P, the chorismate mutase domain of the bifunctional P-protein; CM-T, the chorismate mutase domain of the bifunctional T-protein.

The nucleotide sequence data reported in this paper have been submitted to EMBL and have been assigned the accession number X60420.

of the T-protein and the P-protein (Hudson & Davidson, 1984). It has been clear for some time that the two catalytic functions of the P-protein are largely independent, on the criterion that mutants lacking only CM-P activity or only prephenate dehydratase activity could be isolated (Baldwin & Davidson, 1981). A genetic construct derived from *pheA* has also been described (Stewart *et al.*, 1990) which encodes a monofunctional CM-P that lacks the prephenate dehydratase domain. The separability of the two T-protein functions had remained in doubt (Rood *et al.*, 1982) until recently, when molecular-genetic approaches established the genetic separability of functional CM-T and dehydrogenase components in *E. coli* (Maruya *et al.*, 1987). In the latter study, no enzymological characterizations of the new monofunctional enzymes were given.

Erwinia herbicola is an organism whose pathway of aromatic amino acid biosynthesis is more typical of enteric bacteria than is that of *E. coli* (Ahmad *et al.*, 1990). A DNA fragment carrying *pheA* and *tyrA* genes from *E. herbicola* has been cloned by functional complementation of *E. coli* (T. Xia and others, unpublished) as part of a project to compare gene organization in enteric bacteria. Subcloning procedures led fortuitously to the isolation of plasmids lacking a portion of the CM-T domain encoded by *tyrA*. The resulting monofunctional protein possessed multiple alterations of biocatalysis within the surviving dehydrogenase domain that are described here.

Methods

Media, strains and plasmids. Enriched growth medium (LB medium) and minimal medium (M9 salts) were prepared as described by Sambrook *et al.* (1989). Ampicillin ($50 \mu\text{g ml}^{-1}$) and vitamin B₁ ($17 \mu\text{g ml}^{-1}$) were added when required. Supplementation with L-phenylalanine or L-tyrosine, when appropriate, was at $50 \mu\text{g ml}^{-1}$. Agar (2%, w/v) was added for solid media. The description and origin of strains and plasmids are given in Table 1.

DNA manipulations. Plasmids pJX2, pJX3 and pJX4 were constructed from pJX1 (Fig. 1). Plasmid pJX1 carries the *E. herbicola* *tyrA* and *pheA* genes isolated from a *Sau3A* genomic library generated in pUC18 (Xia *et al.*, 1992). For deletion of nucleotides from the 5' portion of the *tyrA* gene, pJX1 DNA was linearized by digestion with *NcoI*, treated with exonuclease III as described by Henikoff (1984), and then mung bean nuclease was used to create blunt ends. After treatment with *EcoRI*, this DNA preparation was ligated to pUC19 which had been digested with *EcoRI* and *SmaI*. The ligation mixture was used to transform *E. coli* JP2255. Eleven transformants, able to grow on M9 medium lacking L-tyrosine and L-phenylalanine, were found to contain the truncated *tyrA* gene, and one of them (designated pJX2) was used for further study.

For construction of pJX3, pJX2 DNA was linearized by digestion with *SstII*, treated with mung bean nuclease to make blunt ends, and then digested with *HindIII*. A fragment of about 1.6 kb was ligated to pUC19 DNA that had been digested with *SmaI* and *HindIII*, and then used to transform *E. coli* JP2255. For construction of pJX4, the 2.7 kb

Table 1. *Bacterial strains and plasmids*

Strain or plasmid	Genotype or description	Source or reference
<i>Erwinia herbicola</i> ATCC 33243	Prototroph	ATCC
<i>Escherichia coli</i> JP2255	<i>aroF363 pheA361 pheO352 tyrA382 thi strR712 lacY1 xyl5</i>	Baldwin & Davidson (1981)
Plasmids		
pUC18	Ap ^r <i>lacI'POZ'</i>	Gibco-BRL
pUC19	Ap ^r <i>lacI'POZ'</i>	Gibco-BRL
pJX1	Recombinant of pUC19 carrying a DNA insert (3.4 kb) from <i>E. herbicola</i> containing both <i>tyrA</i> and <i>pheA</i>	T. Xia and others, unpublished
pJX2	Subclone derived from pJX1 carrying a 2.7 kb insert from <i>E. herbicola</i> which contains <i>pheA</i> and <i>tyrA*</i> . <i>TyrA*</i> is a fusion protein: $\phi(\text{lacZ}'\text{-}tyrA)$	This study
pJX3	Subclone derived from pJX2 carrying a 1.6 kb insert from <i>E. herbicola</i> which contains <i>tyrA*</i> and a defective <i>pheA</i>	This study
pJX4	Recombinant of pUC18 carrying the identical 2.7 kb insert (<i>pheA tyrA*</i>) of pJX2	This study

insert of pJX2 was excised with *EcoRI* and *HindIII* and ligated with an *EcoRI/HindIII* digest of pUC18 DNA.

All other DNA manipulations were performed by standard methods (Sambrook *et al.*, 1989) or by following manufacturers' instructions.

DNA sequencing. Caesium chloride/ethidium bromide buoyant density gradient centrifugation (Humphreys *et al.*, 1975) was used for large-scale purification of the recombinant plasmid pJX2. The first 300 base pairs were sequenced from the T7/T3 α sequencing primer by a methodology using fluorescent chain-terminating dideoxy nucleotides (Prober *et al.*, 1987) at the DNA Core Facility of the University of Florida. The nucleotide and deduced peptide sequences were analysed by using the University of Wisconsin Genetics Computer Group (GCG) package (Devereux *et al.*, 1984).

Amino acid sequencing. The sequencing of N-terminal amino acids was carried out at the Protein Chemistry Core Facility at the University of Florida.

Preparation of crude extracts. *E. coli* cells were grown at 37 °C in LB medium and harvested by centrifugation during the exponential growth phase. Cell pellets were resuspended in Buffer A (20 mM-potassium phosphate and 1 mM-DTT, pH 7.2), and disrupted by sonication. Cell debris was removed by ultracentrifugation at 150000 g at 4 °C for 60 min, and the clear supernatant was saved.

Enzyme assays. Prephenate dehydrogenase and arogenate dehydrogenase were assayed as described previously (Byng *et al.*, 1980). The continuous formation of NADH was determined spectrophotofluorometrically (excitation wavelength 340 nm and emission wavelength 460 nm): 1 nmol NADH produced corresponds to 20 fluorescence units. One unit (U) of dehydrogenase activity forms 1 nmol NADH min⁻¹ at 37 °C. Chorismate mutase and prephenate dehydratase were assayed as described previously (Ahmad & Jensen, 1988).

Protein concentrations of extracts were quantified by the procedure of Bradford (1976).

Purification of TyrA* from *E. coli* JP2255(pJX2) and TyrA from *E. coli* JP2255(pJX1). Column chromatographic steps of DEAE-cellulose (DE52), hydroxylapatite and Sephadex G-200 were carried out for TyrA* purification, while DE52 and hydroxylapatite chromatography were used for purification of TyrA. Crude extract (250 mg protein) was applied to a DE52 column (2.5 × 25 cm) equilibrated with Buffer A, and then eluted with a 600 ml linear KCl gradient (0–0.4 M) in Buffer A. Fractions of 3.5 ml were collected, and those showing high prephenate dehydrogenase activity were pooled and concentrated by use of an Amicon PM-10 membrane. The concentrated preparation was washed twice with Buffer A and applied to a hydroxylapatite column (1.5 × 20 cm). The column was eluted with 600 ml of a linear gradient (20–300 mM) of potassium phosphate buffer (pH 7.2) containing 1 mM-DTT. Fractions of 3.5 ml were collected, and those having high enzyme activity were saved (in the case of TyrA), or pooled for the next step (in the case of TyrA*). The pooled TyrA* eluate fractions were concentrated to 1.5 ml by use of a PM-10 membrane and applied to a Sephadex G-200 column (2.5 × 98 cm). Protein was eluted with Buffer A, and fractions of 3 ml were collected.

Molecular mass estimations by gel-filtration. The molecular masses of both purified TyrA and TyrA* were estimated by gel-filtration on a Sephadex G-200 column (see above) in the presence or absence of the inhibitor L-tyrosine. The elution buffer was Buffer A plus 100 µM-L-tyrosine when enzyme was run in the presence of the inhibitor. Carbonic anhydrase (30 kDa), bovine serum albumin (67 kDa), alcohol dehydrogenase (150 kDa) and β -amylase (200 kDa) were used as molecular mass standards.

SDS-PAGE. This was done by the procedure of Laemmli (1970), using a 10% (w/v) polyacrylamide gel with the following molecular mass standards: α -lactalbumin (14.4 kDa), soybean trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa) and phosphorylase (94 kDa).

Biochemicals and chemicals. Restriction enzymes, exonuclease III, mung bean nuclease and T4 DNA ligase were obtained from Promega, Gibco-BRL, or New England Biolabs. Protein standards for SDS-PAGE were from Pharmacia. L-Tyrosine, NAD⁺, DTT, Sephadex G-200 and protein standards for gel-filtration were from Sigma. DEAE-cellulose (DE52) was from Whatman and hydroxylapatite was from Bio-Rad. Prephenate was prepared from culture supernatants of a tyrosine auxotroph of *Salmonella typhimurium* (Dayan & Sprinson, 1970), L-arogenate was isolated from the accumulation medium of the triple auxotroph of *Neurospora crassa* (Zamir *et al.*, 1980), and chorismate was prepared from *Klebsiella pneumoniae* 62-1 (Gibson, 1964).

Results

Isolation of *tyrA**

As depicted in Fig. 1, the *tyrA** construct (pJX2) was obtained from pJX1 by utilizing exonuclease III to delete a portion of the 5' end of the *E. herbicola tyrA*. Eleven colonies were obtained by selection on M9 medium containing vitamin B₁, each growing slowly in comparison with *E. coli* JP2255(pJX1). Plasmids isolated from each of the eleven clones were 5.4 kb in size, and were capable of transforming JP2255 to slow-growing strains

in the absence of L-tyrosine. Given the 2.7 kb size of pUC19, the size of each insert is about 2.7 kb. One of these plasmids, designated pJX2, was selected for further study.

Sequence comparison of *tyrA* and *tyrA**

When the nucleotide sequence of *tyrA* (T. Xia and others, unpublished) was compared with that of *tyrA**, it was found that 109 bp had been deleted from the 5' portion of *tyrA* by the exonuclease III/mung bean nuclease treatment used to generate *tyrA**. Fig. 2 shows a comparison of the 5' regions of *tyrA* and *tyrA**.

The 5' portion of *tyrA** was identified as the site of a gene fusion, whereby the residual *tyrA* sequence was joined to the *lac* α -peptide/polycoding region in pUC19 by replacement of the *Sma*I/*Eco*RI fragment. Fig. 2 shows that the sequence of *tyrA** downstream from bp 55 matches exactly with the sequence of *tyrA* beginning with bp 110. A total of 55 bp intervenes between the start codon of the *lac* α -peptide gene and the axis of dyad symmetry for the *Sma*I cleavage site (Yanisch-Perron *et al.*, 1985). Thus, the first 37 codons of *tyrA* were replaced by the first 18 codons of the *lac* α -peptide/polycoding site, and a recombinant codon (CAG) was created. The calculated size of TyrA* is 1934 Da less than the size of TyrA.

Confirmation of the foregoing conclusions was obtained by sequencing the N-terminal 19 amino acid residues of TyrA*. The sequence obtained matched exactly the first 19 amino acid residues (underlined in Fig. 2) predicted on the basis of the sequence obtained for the 5' terminus of *tyrA**.

Expression of *tyrA**

In contrast to control extracts prepared from JP2255(pUC19) or JP2255(pUC18), crude extracts prepared from cultures of JP2255(pJX1) exhibited substantial activities for chorismate mutase, prephenate dehydrogenase and prephenate dehydratase (Table 2). The low level of chorismate mutase present in JP2255(pJX2) in comparison to that of JP2255(pJX1) indicated the probable absence of one of the two chorismate mutase isoenzymes.

Table 2 also shows the total lack of chorismate mutase activity together with the presence of high dehydrogenase activity in JP2255 carrying a plasmid construct (pJX3) where *tyrA** is present on a 1.6 kb fragment lacking most of *pheA*.

When the *pheA tyrA** insert carried by pJX2 was placed in the opposite orientation of pUC18, this strain [JP2255(pJX4)] expressed the CM-P and prephenate

Fig. 1. Construction of plasmids pJX1, pJX2, pJX3 and pJX4. The thin line represents vector DNA. □, β -Lactamase and *lac* genes of pUC18 and pUC19; ▨, flanking *E. herbicola* DNA; ▩, *E. herbicola pheA*; ▤, *E. herbicola tyrA*. Ap^r, ampicillin resistance.

dehydratase activities encoded by *pheA*, but failed to express TyrA*. This shows that *tyrA** is expressed from the *lac* promoter of pUC19, whereas *pheA* can be expressed from its own promoter.

DEAE-cellulose chromatography of crude extract from JP2255(pJX2) revealed a normal peak of chorismate mutase (CM-P) eluting in exact coincidence with prephenate dehydratase, as expected for the bifunctional

<i>tyrA*</i>	5'ATG ACC ATG ATT ACG <u>Met Thr Met Ile Thr</u>	15
<i>tyrA</i>	5'ATG GTG GCT GAA CTG ACC GCG TTA CGC GAT CAA ATT GAC AGT GTA GAT AAA GCG CTG CTG GAT CTG CTG Met Val Ala Glu Leu Thr Ala Leu Arg Asp Gln Ile Asp Ser Val Asp Lys Ala Leu Leu Asp Leu Leu	69
<i>tyrA*</i>	CCA AGC TTG CAT GCC TGC AGG TCG ACT CTA GAG GAT CCC CAG AGC CGT TAC GGC CTG CCT ATC TAT GTG ... 3' <u>Pro Ser Leu His Ala Cys Arg Ser Thr Leu Glu Asp Pro Gln Ser Arg Tyr Gly Leu Pro Ile Tyr Val</u>	84
<i>tyrA</i>	GCT AAG CGA CTG GAA CTG GTG GCC GAG GTA GGT GAG GTG AAG AGC CGT TAC GGC CTG CCT ATC TAT GTG ... 3' Ala Lys Arg Leu Glu Leu Val Ala Glu Val Gly Glu Val Lys <u>Ser Arg Tyr Gly Leu Pro Ile Tyr Val</u>	138

Fig. 2. Comparison of the nucleotide and deduced amino acid sequences of the 5' regions of *tyrA* and *tyrA**. The shading which begins at bp positions 110 in *tyrA* and 56 in *tyrA** shows the fusion point in *tyrA** where the sequence is identical with *tyrA*. The N-terminal amino acids of the *tyrA** gene product, which have been confirmed by protein sequencing, are underlined. The numerical positions of the nucleotide sequences are given at the upper right of each sequence.

Table 2. Expression of *Erwinia herbicola* genes in *E. coli* JP2255

Plasmid in strain JP2255	Specific activity of:		
	Chorismate mutase*	Prephenate dehydratase*	Prephenate dehydrogenase†
pUC19	0	0	0
pUC18	0	0	0
pJX1	5.54	0.44	4.71
pJX2	0.64	0.62	2.44
pJX3	0	0	3.63
pJX4	0.39	0.36	0

* Expressed as μmol phenylpyruvate produced min^{-1} (mg protein) $^{-1}$.

† Expressed as μmol NADH produced min^{-1} (mg protein) $^{-1}$.

P-protein (data not shown). However, a second isoenzyme of chorismate mutase (CM-T), one catalytic component of the bifunctional T-protein, was totally absent in the expected elution position coincident with the cyclohexadienyl dehydrogenase profile (assayed as prephenate dehydrogenase). Fig. 3 shows a comparison of the dehydrogenase elution profiles following DEAE-cellulose chromatography of crude extract from JP2255(pJX2) containing TyrA* and extract from JP2255(pJX1) containing TyrA. Fig. 3(b) shows the co-elution of the CM-T and cyclohexadienyl dehydrogenase activities of TyrA at about 0.21 M-KCl. In contrast, TyrA* exhibited no CM-T activity, but did express a dehydrogenase activity having an altered elution position at about 0.17 M-KCl. Thus, TyrA* is less

negatively charged than is the native TyrA. This is consistent with information obtained with the GCG program showing that the isoelectric point of TyrA* is 6.08, compared to 5.55 for TyrA.

Characterization of TyrA*

The overproduction of TyrA in *E. coli* JP2255(pJX1) and TyrA* in *E. coli* JP2255(pJX2) facilitated their purification. Two chromatographic steps and three chromatographic steps were sufficient for purification to homogeneity of TyrA and TyrA*, respectively, as judged from SDS-PAGE. Fig. 4 shows that the subunit molecular mass of TyrA is about 41 kDa, while that of TyrA* is about 39 kDa. The size is consistent with the difference expected on the basis of sequence comparison (1.9 kDa).

Sephadex G-200 gel-filtration indicated that the native molecular mass of TyrA was ~81 kDa, while that of TyrA* was ~75 kDa. Thus, both TyrA and TyrA* are dimers. In the presence of L-tyrosine during gel-filtration, the same molecular mass values were obtained for TyrA and for TyrA*.

TyrA* was found to be hypersensitive to feedback inhibition by L-tyrosine compared to the dehydrogenase component of the T-protein. The inhibition curves shown in Fig. 5 indicate that 50% inhibition of the TyrA prephenate dehydrogenase activity requires 700 μM -L-tyrosine, whereas 50% inhibition of the TyrA* prephenate dehydrogenase activity requires only 87 μM -L-tyrosine. At 0.5 mM-L-tyrosine, TyrA* was completely inhibited, in comparison to 40% inhibition of TyrA.

Kinetic evaluation of the TyrA* and TyrA dehydrogenases showed Michaelis-Menten kinetics of substrate

Fig. 3. Comparison of elution profiles of TyrA* (a) and TyrA (b) following DE52 column chromatography of crude extracts prepared from *E. coli* JP2255 carrying pJX2 and pJX1, respectively. Approximately 15 mg of crude extract were loaded onto a DE52 column (1.5 × 20 cm) equilibrated with Buffer A (see Methods). The column was washed with 50 ml of the same buffer, and then the bound proteins were eluted with 400 ml of a linear gradient of KCl (0–0.4 M) in Buffer A. Fractions of 2.5 ml were collected and assayed for prephenate dehydrogenase (●) and chorismate mutase (○) activities at 37 °C using 5 µl of eluate. ---, KCl gradient; ···, protein (A_{280}).

Fig. 4. SDS-PAGE of purified TyrA* from *E. coli* JP2255(pJX2) (lane 1) and TyrA from *E. coli* JP2255(pJX1) (lane 2). Bands were visualized with Coomassie blue. Lane 3 was run with molecular mass standards (see Methods).

Fig. 5. Inhibition curves comparing the sensitivities of prephenate dehydrogenase activity from TyrA or from TyrA* to feedback inhibition by L-tyrosine. Activity determinations were carried out as described in Methods. A relative activity of 100% corresponds to 27 units of activity.

saturation and competitive inhibition by L-tyrosine with respect to prephenate utilization (data not shown). Both TyrA and TyrA* exhibited identical K_m values of 0.07 mM for NAD⁺. However, TyrA* had a K_m value for prephenate of 0.25 mM, four times greater than that of TyrA (0.06 mM). The affinity of TyrA* for L-tyrosine ($K_i = 14 \mu\text{M}$) was about fivefold greater than that of TyrA

($K_i = 66 \mu\text{M}$). The apparent $V_{\max}$ of TyrA* for prephenate was about twofold greater than that of TyrA. The $V_{\max}$ of TyrA was $1.3 \times 10^5 \text{ U min}^{-1} \text{ mg}^{-1}$, and the $V_{\max}$ of TyrA* was $2.4 \times 10^5 \text{ U min}^{-1} \text{ mg}^{-1}$.

TyrA carries a cyclohexadienyl dehydrogenase domain that is able to utilize L-arogenate about 8% as well as prephenate at substrate saturation. Since the two cyclohexadienyl substrates bind at a common site, the decreased affinity for prephenate is accompanied by a reduction of the affinity for L-arogenate to a level near the limits of detection. In effect, the modification of the T-protein to produce TyrA* has resulted in the conversion of the CDH domain to a prephenate dehydrogenase.

TyrA* was also compared to TyrA with respect to thermal stability, protection conferred by DTT and pH optimum. The pH optimum for both dehydrogenase proteins was 8.0 when assayed in 50 mM-potassium phosphate buffer containing 1.0 mM-DTT. TyrA* exhibited an increased sensitivity to thermal inactivation. At 45 °C in the presence of DTT the half-life of TyrA was 8 min, compared to 4.5 min for TyrA*. Under the same conditions, but in the absence of DTT the half-life of TyrA was 4 min, compared to 3 min for TyrA*. Thus, the protection conferred by DTT upon TyrA was retained in TyrA*.

Discussion

It appears that a very limited number of cleavage sites exist in *tyrA* that will yield an operational monofunctional dehydrogenase. Although the exonuclease III digestion procedure carried out should have produced a size distribution of fragments, the inserts in all 11 independent recombinants cloned in pUC19 were of similar size. The point of cleavage was well within the CM-T portion of *tyrA* since only about 12% of the amino terminus of the T-protein was excised, whereas the region of homology with CM-P is at least 25% of the T-protein.

Objectives of biotechnology often include the systematic alteration of biocatalytic properties. In this context, some biocatalytic properties of the newly created monofunctional dehydrogenase were dramatically and pleiotropically altered by replacement of the amino-terminal fragment of the T-protein. The affinity for prephenate decreased fourfold, the affinity for NAD⁺ remained unchanged, and the affinity for L-tyrosine (feedback inhibitor) increased almost fivefold. The $V_{\max}$ for prephenate increased almost twofold. The decreased affinity for both cyclohexadienyl substrates effectively narrowed the substrate specificity, transforming the original cyclohexadienyl dehydrogenase to a prephenate dehydrogenase.

These multiple effects upon the dehydrogenase domain caused by a partial deletion of the mutase domain indicate intimate protein-protein relationships between the mutase and dehydrogenase components of the T-protein molecule. This is consistent with results of enzymological studies (Heyde, 1979; Heyde & Morrison, 1978; Koch *et al.*, 1972), which led to earlier speculations that *tyrA* might possess only a single, complex catalytic domain. The T-protein probably originated by fusion of genes encoding a monofunctional species of chorismate mutase and cyclohexadienyl dehydrogenase. If so, the catalytic domains must have co-evolved to develop domain-domain interactions.

The *tyrA pheA* auxotroph of *E. coli* possessing the *pheA* and *tyrA** genes from *Erwinia* demonstrates the physiological potential for CM-P to provide prephenate for tyrosine biosynthesis when CM-T is absent. The amplification of *pheA* on the high-copy-number plasmid pUC19 probably results in high intracellular levels of prephenate since prephenate dehydratase is vastly more sensitive to feedback inhibition by L-phenylalanine than is CM-P. Under these conditions prephenate availability to TyrA* would presumably be greatly increased. In this context, it is of interest that one small phylogenetic cluster of pseudomonads in nature has been reported to utilize the CM-P component of the P-protein for both phenylalanine and tyrosine biosynthesis in a 'channel-shuttle' mechanism (Ahmad & Jensen, 1988).

This publication is Florida Agricultural Experiment Station Journal Series no. R-02146.

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