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Cyclohexadienyl dehydrogenase from *Pseudomonas stutzeri* exemplifies a widespread type of tyrosine-pathway dehydrogenase in the TyrA protein family☆

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Abstract

The uni-domain cyclohexadienyl dehydrogenases are able to use the alternative intermediates of tyrosine biosynthesis, prephenate or L-arogenate, as substrates. Members of this TyrA protein family have been generally considered to fall into two classes: sensitive or insensitive to feedback inhibition by L-tyrosine. A gene ($tyrA_c$) encoding a cyclohexadienyl dehydrogenase from *Pseudomonas stutzeri* JM300 was cloned, sequenced, and expressed at a high level in *Escherichia coli*. This is the first molecular-genetic and biochemical characterization of a purified protein representing the feedback-sensitive type of cyclohexadienyl dehydrogenase. The catalytic-efficiency constant k_{cat}/K_m for prephenate (7.0×10^7 M/s) was much better than that of L-arogenate (5.7×10^6 M/s). $TyrA_c$ was sensitive to feedback inhibition by either L-tyrosine or 4-hydroxyphenylpyruvate, competitively with respect to either prephenate or L-arogenate and non-competitively with respect to NAD^+. A variety of related compounds were tested as inhibitors, and the minimal inhibitor structure was found to require only the aromatic ring and a hydroxyl substituent. Analysis by multiple alignment was used to compare 17 protein sequences representing TyrA family members having catalytic domains that are independent or fused to other catalytic domains, that exhibit broad substrate specificity or narrow substrate specificity, and that possess or lack sensitivity to endproduct inhibitors. We propose that the entire TyrA protein family lacks a discrete allosteric domain and that inhibitors act competitively at the catalytic site of different family members which exhibit individuality in the range and extent of molecules recognized as substrate or inhibitor.

Keywords: *Pseudomonas stutzeri*; L-Tyrosine biosynthesis; Cyclohexadienyl dehydrogenase; Prephenate dehydrogenase; Arogenate dehydrogenase

☆ Terminology and abbreviations. In the new era of genomic and multiple molecular-genetic comparisons, it is no longer tenable to use acronyms whose meanings differ for given sets of orthologs or paralogs. A terminology dilemma is also posed by the existence of genes encoding functional protein domains which may exist separately (e.g. $aroQ_f$) or as fused components of multifunctional proteins (e.g. $aroQ_t$·$tyrA_c$). Table 1 provides a summary of the gene acronyms used in this paper and established by precedent in Gu et al. (1997) and Subramaniam et al. (1998). Differing substrate specificities of homologs are designated with subscripts. The convention of using a bullet to separate the potentially independent domains of a fusion protein is according to the precedent set by Crawford (1989).

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1. Introduction

Tyrosine biosynthesis in different organisms exhibits surprising individuality. The aromatization step may employ a specific prephenate dehydrogenase, a specific arogenate dehydrogenase, or a broad-specificity cyclohexadienyl dehydrogenase (Jensen and Fischer, 1987; Zhao et al., 1993). Cyclohexadienyl dehydrogenase was first described in *Pseudomonas aeruginosa*, where its broad specificity was accordingly recognized as the basis for the dual pathways to L-tyrosine that were known to exist (Patel et al., 1977). (A third pathway to L-tyrosine via phenylalanine hydroxylase was recently discovered (Zhao et al., 1994). Although this is a catabolic pathway, it does provide potential for genetic suppression of any biosynthetic-pathway deficiencies.) The prephenate dehydrogenase and arogenate dehydrogenase activities of a purified dehydrogenase preparation from *P. aeruginosa* were shown to be inseparable, the ratio of the two activities remaining constant throughout purification (Xia and Jensen, 1990). The existence of a broad-specificity cyclohexadienyl dehydrogenase was further indicated since the purified preparation yielded a single protein band following SDS–PAGE.

Extensive comparative enzymological surveys of cyclohexadienyl dehydrogenases have resulted in the recognition of two classes of enzyme, one sensitive to feedback inhibition by L-tyrosine and the other insensitive (Byng et al., 1980). This feature has been a reliable characteristic for separation of phylogenetic groups of pseudomonad bacteria (Byng et al., 1980). The tyrosine-insensitive class of enzyme is present in rRNA group III and group IV pseudomonads (Byng et al., 1980), and in *Zymomonas mobilis* (Zhao et al., 1993). Arogenate-specific dehydrogenases are frequently insensitive to inhibition by L-tyrosine, as exemplified by the large memberships of cyanobacteria (Hall et al., 1982), coryneform bacteria (Fazel and Jensen, 1979), and *Actinoplanes missouriensis* (Hund et al., 1989).

Of these two classes, only a representative of the feedback-insensitive class of cyclohexadienyl dehydrogenase has been studied at the molecular-genetic level. The cyclohexadienyl dehydrogenase gene from *Z. mobilis* was cloned, and the protein product was purified and characterized (Zhao et al., 1993). Amplification of the single open reading frame in an *Escherichia coli* background lacking any cyclohexadienyl dehydrogenase activity proved decisively that the two dehydrogenase activities can be attributed to a single broad-specificity enzyme. As expected, the ratio of the activity of arogenate dehydrogenase to that of prephenate dehydrogenase ($\sim$3:1) remained constant throughout purification, and the two activities were inseparable. It was concluded that both cyclohexadienyl substrates bind at a common catalytic site on the *Z. mobilis* enzyme, and therefore, that the enzyme possesses a single catalytic domain.

The dehydrogenase component of bifunctional T-proteins (proteins also possessing chorismate mutase activity) present in enteric bacteria (Ahmad and Jensen, 1986) has been shown to utilize L-arogenate (albeit poorly) in place of prephenate as substrate in vitro (Ahmad and Jensen, 1987). Therefore, in conflict with its current formal naming as prephenate dehydrogenase, the dehydrogenase components of all T-proteins are in fact cyclohexadienyl dehydrogenases (although prephenate is probably the exclusive substrate in vivo due to the combined impact of substrate preference and domain-domain channeling). Cyclohexadienyl dehydrogenase is ubiquitous throughout the three major superfamily divisions of Gram-negative bacteria (Jensen, 1985; Ahmad and Jensen, 1986, 1988). The recent bonanza of DNA sequences for a variety of aromatic-pathway dehydrogenases has provided ample information to confirm the proposed evolutionary relationship (Jensen, 1985; Ahmad and Jensen, 1987, 1988) between cyclohexadienyl dehydrogenase and the dehydrogenase domain of enteric T-proteins.

Pseudomonas stutzeri is a close relative of *P. aeruginosa* in rRNA homology group I (Palleroni, 1984). The enzymology of phenylalanine and tyrosine biosynthesis in this Gram-negative organism has been studied extensively (see Ref. Fischer et al., 1991 and references therein). More recently, molecular-genetic analysis of $aroQ_p{\cdot}pheA$, the key gene of phenylalanine biosynthesis, has been completed (Fischer et al., 1991; Gu et al., 1997). Molecular-genetic studies of the uni-domain cyclohexadienyl dehydrogenase present in *P. stutzeri*, reported herein, is the initial molecular-genetic characterization of a tyrosine-inhibited

type of cyclohexadienyl dehydrogenase. In this paper, we report the molecular cloning and sequencing of $tyrA_c$, the gene coding for cyclohexa dienyl dehydrogenase from *P. stutzeri* JM300, as well as the purification and characterization of the gene product.

2. Material and methods

2.1. Bacterial strains and media

Bacterial strains and plasmids utilized or constructed are described in Table 2. *P. stutzeri* was cultured as described by Carlson et al. (1983). *E. coli* strains were grown in M9 minimal medium (Miller, 1972) or LB medium (Davis et al., 1980a) at 37°C. Amino acids (50 μg/ml) and thiamin hydrochloride (17 μg/ml) were added as growth supplements when required. Antibiotics, when appropriate to provide selective pressure for plasmids, were added to the medium at standard concentrations (Maniatis et al., 1989). Agar was added at 20 g/l for preparation of solid medium. X-Gal (5-bromo-4-chloro-3-indoxyl β-D-galactopyranoside) was included at concentrations recommended by the supplier (Promega) when recombinants were screened in either pUC18 or pUC19 plasmids introduced into *E. coli* DH5α. T4 DNA ligase, DNA-modifying enzymes (New England Biolabs or Promega) and *Taq* DNA polymerase (Perkin–Elmer) were used as recommended by the suppliers.

2.2. DNA manipulation and genetic procedures

Standard molecular biology procedures were performed as described by Maniatis et al. (1989) unless otherwise indicated. Chromosomal DNA was purified by the method of Yuan and Lin (1982). DNA for small-scale plasmid preparations was isolated according to the protocol of Davis et al. (1980b). *E. coli* strains were transformed by use of a $CaCl_2$ method (Dagert and Ehrlich, 1979). Restriction enzymes, ligase, and calf intestine alkaline phosphatase were purchased from New England Biolabs or Promega, and were used according to manufacturer instructions. DNA fragments were purified from agarose gel with a Geneclean kit (Bio101). *Taq* DNA polymerase (Perkin–Elmer), and *Vent* DNA polymerase (New England Biolabs) were used as recommended by the suppliers. Analyses of restriction sites and subcloning were conducted by standard methods (Maniatis et al., 1989).

2.3. DNA sequencing and data analysis

Plasmid subclones were purified by the method recommended in User Bulletin 18 offered by Applied Biosystems. Double-stranded plasmid DNA was sequenced in both directions at the DNA sequencing laboratory of the Department of Microbiology and Cell Science at the University of Florida. The nucleotide sequence and deduced amino acid sequence were analyzed using the updated version (Version 8.0, 1994) of the sequence

Table 1
Guide to gene designations

Homology group	Homology-group members	Corresponding gene products	Prior gene designations
AroQ	$aroQ_t$·	Chorismate mutase domain (CM-T) of T-protein	*tyrA* (*E. coli*)
	$aroQ_p$·	Chorismate mutase domain (CM-P) of P-protein	*pheA* (*E. coli*)
	$AroQ_f$	Monofunctional chorismate mutase (CM-F)	*chmU* (*Erwinia herbicola*)
TyrA	$tyrA_c$	Cyclohexadienyl dehydrogenase (CDH)	*tyrC* (*Z. mobilis*)
	$tyrA_p$	Prephenate dehydrogenase (PDH)	*tyrA* (*E. coli*)
	$tyrA_a$	Arogenate dehydrogenase (ADH)	
	$tyrA_x$	Substrate specificity unknown	
Fused genes		Protein domain designations	Prior gene designations
$aroQ_p$·*pheA*		Chorismate mutase·prephenate dehydratase (P-protein)	*pheA* (*E. coli*)
$aroQ_t$·$tyrA_c$		Chorismate mutase·cyclohexadienyl dehydrogenase (T-protein)	*TyrA* (*E. coli*)

Table 2
Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or description	Source or reference
E. coli		
BL21 (DE3)	F$^-$ *ompT hsdS$_B$* ($r_B^- m_B^-$) *gal dcm*; with DE3, a λ prophage carrying the T_7 RNA polymerase gene	Novagen
AT2471	*thi-1 tyrA4* relA1l$^-$ *spoT*	CGSC[a] 4510
JP2255	*AroF363 pheA361 pheO352 tyrA382 thi-1 strR712 lacY1 xy1-15*	Baldwin and Davidson (1981)
P. stutzeri		
JM300	Prototroph	Carlson et al. (1983)
Cosmids		
pHC79	Ap^r Tc^r cos	Hohn and Collins (1980)
pJF958	Phe$^+$ clone from PHC79: *PstI* gene bank of JM300	Carlson et al. (1983)
Plasmids		
pUC18	Ap^r, lacI′*IPOZ*′	Yanisch-Perron et al. (1985)
pJF9586	Phe$^+$ 4.35-kb *PstI* fragment subcloned from pJF958 into PhC79	Fischer et al. (1991)
pET24b(+)	*T7lac* promoter, *lac*I$^+$ Kan^r, His·Tag sequence, T7·Tag Sequence	Novagen
pETtyrA$_c$	PET24b(+) carrying tyrA$_c$ translation fusion at ATG start site and C-terminal His·Tag sequence	This study
pJX1670	1670-bp *Eco*RI-*Pst*I fragment subcloned from pJF9586 into pUC18	This study
pJX0800	870-bp *Eco*RI-*Sph*I fragment subcloned from pJX1670 into pUC18	This study
pJX0520	500-bp *Sph*I-*Sma*I fragment subcloned from pJX1670 into pUC18	This study

[a] CGSC, *E. coli* Genetic Stock Center, Yale University.

analysis software package offered by Genetics Computer Group (Devereux et al., 1984).

2.4. Analysis of raw DNA sequence data

Raw *P. aeruginosa* DNA sequence available from the database (www.pseudomonas.com) was analyzed by using the built-in BLAST service. DNA sequences from GenBank and protein sequences from SWISS-PROT and PIR were used as query entries. WS_FTP was used to fetch raw sequences. The WWW BLAST 2.0 (Altschul et al., 1997) and WWW ORF Finder offered by the National Center for Biotechnology Information were used to locate open reading frames and to confirm the similarity search result of the raw sequence. The updated version (Version 8.0, 1994) of the sequence analysis software package offered by Genetics Computer Group (Devereux et al., 1984) was used for detailed sequence analyses. The Codon preference and PILEUP programs were used to locate likely frameshift errors. The *P. aeruginosa* codon usage table was retrieved from ftp.ebi.ac.uk. The Segedt program was used to correct sequence errors.

The nucleotide sequence reported in this paper has been submitted to the GenBank Data Bank with accession number AF038578.

2.5. Crude extract preparation from E. coli AT2471(pJX1670)

Cell cultures of *E. coli* AT2471(pJX1670) were grown at 37°C in 450 ml of LB broth containing ampicillin at 50 μg/ml. Growth rates were determined by measuring turbidity of exponentially growing cultures at 600 nm in a Perkin-Elmer model 35 spectrophotometer. 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 s using a Lab-Line Ultratip Labsonic System (Lab-Line Instruments, 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 is denoted as crude extract. Protein concentration was determined by the method of (Bradford (1976)).

2.6. Enzyme assays

Arogenate dehydrogenase (Bonner and Jensen, 1987a) and prephenate dehydrogenase (Bonner and Jensen, 1987b) activities were assayed by using a spectrophotofluorometer (excitation at 340 nm and emission at 460 nm) to follow the appearance of NADH. Unless indicated otherwise, saturating concentrations of substrates were used in standard assays at 25°C: 1 mM for prephenate, 2 mM for L-arogenate, and 1 mM for NAD^+. Assays were initiated by addition of enzyme to otherwise complete reaction mixtures. In experiments where it was desirable to assay activity at the pH optimum of 7.1, this is indicated. For routine assays (such as monitoring column eluates), it was more convenient to use 50 mM potassium phosphate at pH 7.5 owing to concern for the high acid lability of prephenate and L-arogenate.

2.7. DEAE-cellulose chromatography

A 125-ml amount of crude extract (15.1 mg/ml of protein) was dialyzed overnight (with one change of buffer) against 2000 ml of buffer A, the formulation of which is 50 mM potassium phosphate (pH 7.5), 1 mM DTT, and 20% (v/v) glycerol. A 930-mg amount of protein was applied to a 2.5 × 30-cm DE-52 column equilibrated in buffer A. The column was washed with 400 ml of buffer, and bound protein was then eluted with a 1000-ml linear salt gradient (0–1.0 M KCl) prepared in the same buffer. Fractions (4 ml) were collected and assayed for cyclohexadienyl dehydrogenase activity.

2.8. Hydroxylapatite chromatography

A 45-mg amount of protein eluate recovered from DE-52 chromatography was loaded onto the bed of a 1.5 × 20-cm column of Bio-Gel HTP previously equilibrated with buffer A. The column was washed with 100 ml of buffer A and then eluted by application of a 700-ml gradient between 5 and 400 mM of potassium phosphate containing the other supplements of buffer A. Fractions (2 ml) were collected, and cyclohexadienyl dehydrogenase activity was assayed.

2.9. Construction of a $TyrA_c$ expression vector

For maximal expression of $TyrA_c$ protein, the T7 expression system in *E. coli* was employed (Novagen). The $tyrA_c$ coding region was cloned into a translational fusion vector pET24b(+). Polymerase chain reaction (PCR) was used to amplify the $tyrA_c$ gene. Since there is a NdeI restriction site within the $tyrA_c$ coding region, a modified strategy was necessary to create the $TyrA_c$ overexpression plasmid, pET$tyrA_c$ (Fig. 1A). The upper primer (5′-GATA AAT↓TA**ATG**AAACCTATCATCGATCGCCTG GTG-3′) was made with a built-in *Vsp*I restriction site (underlined), which allows fusion of $tyrA_c$ with the NdeI restriction site (CA↓TATG) of pET24b(+) at the translational start site (ATG in bold). The lower primer (5′-GACTTC↓TCGAGGGCTCTGCGAGCCAGTAT TTTG-3′) was made with a built-in XhoI restriction site (underlined) which allows fusion of the C-terminal codon of $tyrA_c$ with the leucine and glutamine codons which precede the His·Tag of pET24b(+). The $tyrA_c$ fragment was amplified by PCR (Perkin–Elmer Cetus, Norwalk, CT) and doubly-digested with VspI and XhoI. The 882-bp $tyrA_c$ fragment was ligated into pET24b(+) which had been digested by NdeI and XhoI. Thus, $tyrA_c$ was placed under control of a T7 promoter and a well-designed Shine-Delgarno sequence.

Plasmid pET$tyrA_c$ was introduced into *E. coli* BL21(DE3) by transformation. A loopfull of culture from the transformed plate was inoculated into 10 ml of LB medium containing kanamycin (30 μg/ml) and shaken at 25°C until the OD_{600} reached 0.73. IPTG was added to a final concentration of 1 mM, and the incubation was continued for 3 h. The resulting culture could be checked directly for $tyrA_c$ overexpression by SDS–PAGE.

2.10. SDS–PAGE

A Mini-Protean II Cell (Bio-Rad) was used for SDS–PAGE (12% gel) by the method of Laemmli

(1970). Samples of exponential-phase cells were collected by centrifugation, and the cell pellets were suspended in gel-loading buffer and heated at 100°C for 10 min. Samples of 5–10 μl were loaded onto SDS-polyacrylamide gels. After separation of the proteins by electrophoresis, the gel was stained with Coomassie blue.

2.11. Purification of *P. stutzeri* $TyrA_c$

E. coli BL21(DE3) carrying plasmid pET$tyrA_c$ was grown at 25°C in 200 ml LB medium supplemented with kanamycin (30 μg/ml) in a gyratory shaker until the OD_{600} reached 0.73. IPTG was added to a final concentration of 1 mM, and the

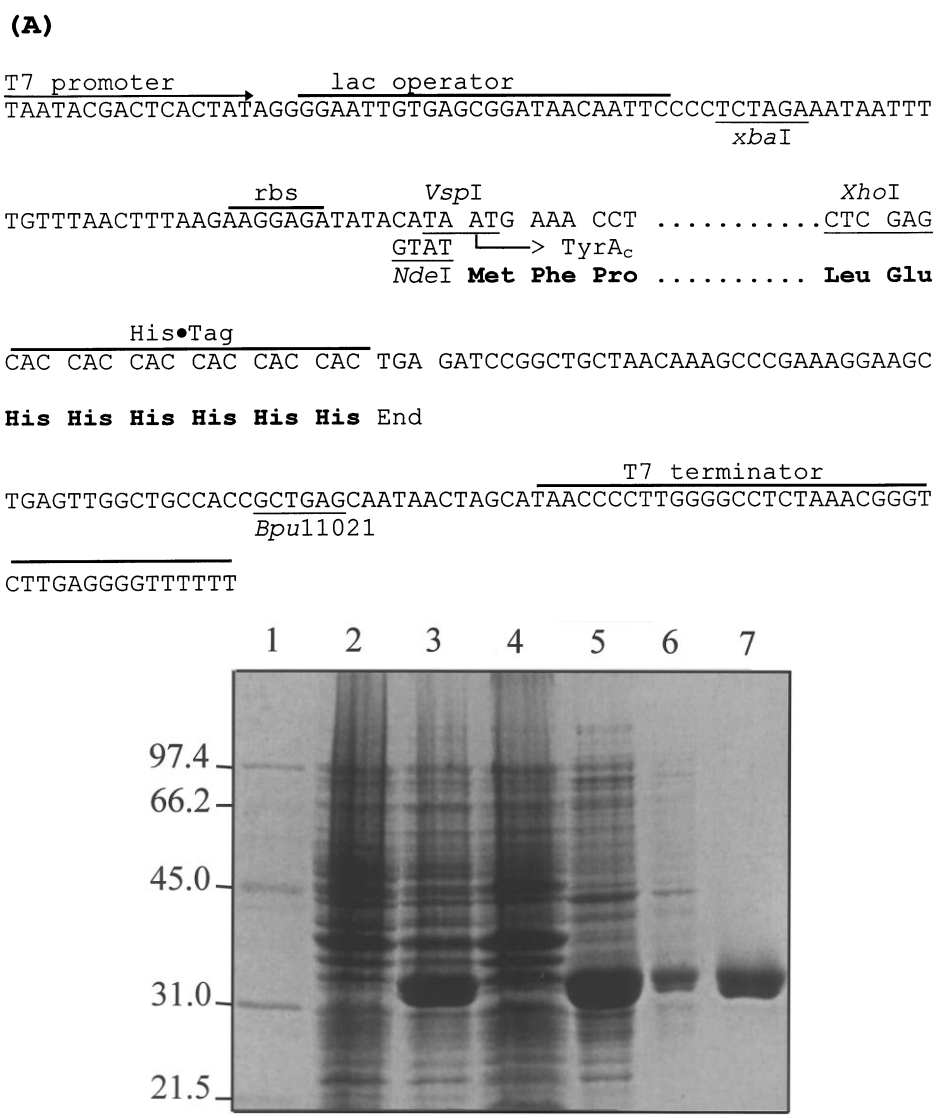

Fig. 1. Overexpression of $TyrA_c$ using a T7 expression vector in *E. coli*. (A) Construction of $tyrA_c$ overexpression vector pET$tyrA_c$ (with T7-ϕ10 translational signals). (B) SDS–PAGE analysis of fractionation samples obtained during purification of $TyrA_c$ protein. Lane 1, molecular-weight standards; lane 2, whole cell lysate of BL21(DE3) harboring pET$tyrA_c$ before 1 mM IPTG induction; lanes 3–5, BL21(DE3) harboring pET*tyrAc* after 1 mM IPTG induction for 3 h at 25°C: whole cell lysate (lane 3), crude extract from insoluble cell fraction (lane 4), and crude extract from soluble cell fraction (lane 5); lane 6, the fraction collected after DEAE cellulose chromatography; lane 7, the fraction collected after His·Bind affinity chromatography.

incubation was continued for 3 h at 25°C. The cells were harvested by centrifugation, and the pellet was resuspended in 50 mM potassium phosphate buffer (pH 7.1) containing 1 mM DTT and 20% (v/v) glycerol (buffer B). The cells were disrupted by sonication and centrifuged at 150 000 × *g* for 65 min at 4°C. The crude extract was dialyzed against 2 l of buffer B overnight with one change of buffer and then was applied to a 3 × 30-cm DE-52 column equilibrated with buffer B. The column was washed with 400 ml of buffer B, and bound protein was then eluted with 1 l of linear salt gradient (0–1.0 M KCl) prepared in buffer B. Fractions (3 ml) were collected, and those showing high cyclohexadienyl dehydrogenase activity were pooled, applied to a gel filtration column (Bio-Gel PD-10), and eluted with 4 ml of ice-cold 1 × binding buffer (5 mM imidazole, 0.5 M NaCl and 20 mM Tris–HCl, pH 7.9). The protein eluate was applied to a His·Tag affinity column which had been equilibrated with 1 × binding buffer. The flow rate was kept at 25 ml (ten bed volumes) per hour. The column was first washed with 25 ml of 1 × binding buffer, then washed with 15 ml of 1 × wash buffer (60 mM imidazole, 0.5 M NaCl and 20 mM Tris–HCl, pH 7.9), and then eluted with 15 ml of 1 × eluate buffer (1 M imidazole, 0.5 M NaCl and 20 mM Tris–HCl, pH 7.9). Fractions of 2.0 ml were collected, and those showing high cyclohexadienyl dehydrogenase activity were saved and used for further study.

2.12. N-Terminal amino acid sequencing of $TyrA_c$

The purified enzyme preparation was denatured by sodium dodecyl sulfate (SDS), and a single band was localized after SDS–PAGE. The $TyrA_c$ band then was transferred to a polvinylidene difluoride membrane (Bio-Rad) by a protein miniblotting apparatus. After staining with Coomassie brilliant blue R-250, the band corresponding to $TyrA_c$ was excised and sequenced at the N-terminus by using an Applied Biosystems model 407A protein sequencer with an online 120A phenylthiohydantoin analyzer by the Protein Core Facility at the University of Florida.

2.13. Molecular mass determination

SDS–PAGE was carried out to estimate the subunit molecular mass of the enzyme. Lysozyme (M_r, 14 400), soybean trypsin inhibitor (M_r, 21 500), carbonic anhydrase (M_r, 31 000), ovalbumin (M_r, 66 000), and phosphorylase b (M_r, 97 400), were used as molecular mass standards.

The molecular mass of the native enzyme was estimated by FPLC using a Superdex 75 HR 10/30 column equilibrated with 50 mM potassium phosphate buffer at pH 7.4 containing 0.15 M NaCl and 20% (v/v) glycerol (buffer C). The enzyme applied (250 μl of enzyme recovered from the DEAE-cellulose step) was eluted with buffer C at a flow rate of 0.5 ml/min. Standards used to calibrate the column were alcohol dehydrogenase (M_r, 150 000), bovine serum albumin dimer (M_r, 132 000) bovine serum albumin monomer (M_r, 66 000), carbonic anhydrase (M_r, 30 000) and cytochrome C (M_r, 12 300).

2.14. Biochemicals and chemicals

Barium prephenate (92% pure) and a 31-mM stock solution of potassium L-arogenate were prepared from *Salmonella typhimurium tyr*A19 (Dayan and Sprinson, 1970), as described by Bonner et al. (1990). Liquid stocks of potassium prephenate were prepared by treating barium prephenate with a twofold molarity excess of K_2SO_4. Care was taken to use substrate preparations uncontaminated with spiro-arogenate (Zamir et al., 1983) or D-prephenyllactate (Zamir et al., 1988), compounds which might complicate kinetic studies. DEAE-cellulose was obtained from Whatman; ampicillin, kanamycin, thiamine and amino acids were obtained from Sigma. Molecular mass standards for gel filtration and for SDS–PAGE were from Sigma and Bio-Rad, respectively. Luria-Bertani medium and agar were purchased from Difco. Other biochemicals were purchased from Sigma. Inorganic chemicals (analytical grade) were from Fisher Scientific.

3. Results

3.1. Cloning of the P. stutzeri $tyrA_c$ gene in E. coli

The original *P. stutzeri* · *aro*Q_p·*phe*A clone (pJF9586) isolated by Fischer et al. (1991) contained an intact $tyrA_c$ gene, since it was successfully used for functional complementation of the tyrosine deficiencies of both *E. coli* AT2471 and *E. coli* JP2255. Cosmids purified from the tetracy-

cline-resistant transformants had the same size and banding pattern (supercoiled, circular and linear) as pJF958 on agarose gel, and a second round of transformation of *E. coli* AT2471 and JP2255 with these purified cosmids again conferred tyrosine prototrophy.

Appropriate restriction sites were utilized to construct a set of subclones from pJF9586 in pUC18 for nucleotide sequencing (Table 2). A 1.67-bp *Eco*RI-*Pst*I fragment cloned into *Eco*RI-*Pst*I sites (within the multiple cloning region) of pUC18 complemented the *aro*Q_t·*tyrA*$_c$ mutation in *E. coli* AT2471, but not the *aro*Q_p · *phe*A mutation in *E. coli* JP2255. Disruption of the *Eco*RI-*Pst*I fragment of plasmid pJX1670, with *Sph*I (yielding pJX0800) or with *Sph*I and *Sma*I (yielding pJX0520) resulted in loss of complementation of the *tyrA*$_c$ defect. The presence of prephenate dehydrogenase and arogenate dehydrogenase activities was sought in crude extracts of *E. coli* AT2471 carrying plasmid pJX1670. Both activities (107 nmol min/mg and 68 nmol min/mg, respectively) were evident, whereas no enzyme activity was detected in the crude extracts of *E. coli* AT2471 carrying pUC18.

3.2. DNA sequence of the *P. stutzeri tyrA*$_c$ gene and its flanking regions

The complete nucleotide sequence of *P. stutzeri* *tyr*A_c along with the flanking upstream and downstream regions was determined. The structural gene, 885 bp in length, begins at codon ATG and stops at codon TGA. No match for a prokaryotic factor-independent RNA polymerase terminator was detected.

The translational start site could not be confirmed by N-terminal sequencing because of the failure to purify the enzyme sufficiently from an *E. coli* clone harboring a construct possessing the native upstream translational signals of *P. stutzeri* (Section 3.3). However, the designated ATG start site appears to be the only possibility because the nearest upstream possibility is within the coding region of *hisH*, while the nearest downstream possibility (a GTG codon) would result in truncation of the NAD-binding domain. The excellent positioning of *P. stutzeri* $TyrA_c$ within the multiple alignment (Section 4) also strongly supports the translational start site indicated.

3.3. Expression of *P. stutzeri tyrA*$_c$ in *E. coli*

Crude extracts containing soluble $TyrA_c$ were prepared from AT2471(pJX1670). The $TyrA_C$ enzyme was unstable, exhibiting a half-life ($t_{1/2}$) of $\sim$4 days at 4°C. Purification of the protein led to unacceptable losses of activity after the first step of several different fractionation protocols attempted. Under various conditions where instability was encountered, both arogenate dehydrogenase and prephenate dehydrogenase activities were affected equally. A 50 mM K-phosphate buffer at pH 7.5 containing 20% (v/v) glycerol was found to protect $TyrA_c$ activity significantly during storage and purification, and $t_{1/2}$ was increased to 6–7 days.

The specific activity of $TyrA_C$ in crude extracts of *P. stutzeri* was difficult to gauge accurately because of the presence of an interfering activity noted earlier (Byng et al., 1980). Assuming the activity level to be similar to that of the closely related *P. aeruginosa* (Xia and Jensen, 1990), the *P. stutzeri* $TyrA_c$ activity, expressed in *E. coli*, was estimated to be only about twofold greater than in the extracts from wildtype *P. stutzeri*. Sequential fractionation steps employing DEAE-cellulose chromatography, hydroxylapatite chromatography, and Superdex 75 HR 10/30 column chromatography and using glycerol-containing buffers produced an overall purification of $\sim$50-fold. However, SDS–PAGE analysis showed multiple bands, and the preparation was too unstable for further studies.

In order to obtain a purified preparation by exploiting the advantage of greater initial expression, a T7 overexpression system was employed. $TyrA_c$ protein was produced at a very high level in *E. coli* BL21(DE3) as detailed in Section 2. PCR was used to generate a *tyrA*$_c$ insert for placement into the translation overexpression vector pET-24b(+). This construct (pET-*tyrA*$_c$) lacked all *P. stutzeri* DNA upstream of the ATG start codon (Fig. 1A). pET*tyrA*$_c$ was transformed into *E. coli* BL21(DE3), and the culture was supplemented with IPTG to induce the chromosomally encoded (λ prophage) T7 RNA polymerase. Whole-cell lysates stained before and after IPTG induction were analyzed by SDS–PAGE. Overproduction of a 32-kDa protein was observed (Fig. 1). The enzyme activity assayed in the crude extract fractions was extremely high, more than 400-fold greater than that previously obtained with the pJX1670 construct.

3.4. Purification and physical properties of the cloned $TyrA_c$

The crude extract was fractionated on a DEAE-cellulose matrix first, and then the $TyrA_c$ fraction recovered was applied to a His·Tag affinity column. Pure enzyme (0.66 mg/ml) was stable at −70°C, as were aliquots diluted 10-fold into 50 mM K-phosphate buffer at pH 7.5 containing 20% (v/v) glycerol. An additional 100-fold dilution into the same buffer yielded enzyme activity in a range suitable for enzyme assays, but this was quite labile. Therefore, additives were tested that might protect $tyrA_c$ (1 mM DTT, 0.2 mM tyrosine, 1 mM NAD^+, 0.2 mM prephenate, 0.2 mg/ml BSA). Among these, prephenate exhibited marginal protection, whereas BSA stablilized activity for at least 12 h. Pure enzyme was aliquoted and for enzyme assays was routinely diluted into BSA to give a final concentration of 0.2 mg/ml.

A comparison of the activity of arogenate dehydrogenase to that of prephenate dehydrogenase gave a ratio of 3:4 under standard assay conditions using purified $TyrA_c$. This ratio remained constant throughout purification, and the two enzyme activities eluted with identical profiles from chromatography columns. A single protein band with an estimated molecular mass of 33 kDa was visualized by SDS–PAGE of the purified enzyme from the His·Tag affinity column fraction (Fig. 1B). Thus, the product of the cloned gene is a broad-specificity cyclohexadienyl dehydrogenase. The deduced amino acid sequence encoded by $tyrA_c$ predicts a subunit M_r of 31 951 Da; the deduced amino acid sequence of the His•tag $TyrA_C$ is 33 016 Da. When the purified His•tag $TyrA_C$ preparation was subjected to SDS–PAGE, a subunit molecular mass of 33 kDa was estimated.

The molecular mass of the active species of purified $TyrA_C$ was determined by FPLC size-exclusion chromatography (data not shown). At high protein concentration (2.1 mg/ml) a mixture of dimer (60–65%) and tetramer (35–40%) was eluted at positions corresponding to 66 and 132 kDa, respectively. At $TyrA_c$ concentrations of 0.6 mg/ml and lower, only the dimer was present.

3.5. N-terminal amino acid sequence of P. stutzeri $TyrA_C$

To confirm the translational start site expected, the N-terminal portion of the purified cyclohexadienyl dehydrogenase was sequenced. The 15 residues determined were shown to be MKPIIDRLVVIGLGL.

3.6. Catalytic properties of the cyclohexadienyl dehydrogenase

Both the arogenate dehydrogenase and prephenate dehydrogenase activities of cyclohexadienyl dehydrogenase were tested for optimal pH by measuring initial rates of catalysis over a pH range of 6.0–8.0 at 25°C. A broad pH profile was obtained, with the optimal pH for each activity being 7.0–7.1 in 50 mM potassium phosphate.

All kinetic determinations were made in the presence of BSA at a final concentration of 0.2 mg/ml. Apparent sigmoid substrate saturation curves were the result of enzyme lability and the stabilizing effect of substrate at higher concentrations. In the presence of BSA hyperbolic substrate saturation curves were obtained for *P. stutzeri* $TyrA_c$ using either L-arogenate or prephenate as the variable substrate in combination with NAD^+ as the fixed substrate. Use of NAD^+ as the variable substrate in combination with either of the other alternative substrates also yielded hyperbolic substrate saturation curves. Double-reciprocal plots of the substrate saturation data were obtained at three progressively greater concentrations of fixed substrate, and used to construct secondary intercept plots (data not shown). Under standard assay conditions, enzyme activity was below the level of detection when $NADP^+$ was substituted for NAD^+. When a great excess of $TyrA_c$ protein was used, a weak activity was measured with $NADP^+$. This was estimated to be 0.25% of the activity measured with NAD^+. The K_m value calculated for NAD^+ was 0.14 mM (Table 3).

The kinetic parameters of *P. stutzeri* $TyrA_c$ are summarized in Table 3. The apparent K_m value calculated for prephenate (0.07 mM) was more than sevenfold lower than for L-arogenate (0.56 mM). This preference for prephenate over L-arogenate as substrate was also reflected by the better catalytic efficiency for prephenate that is indicated by the k_{cat}/K_m values shown in Table 3. *P. stutzeri* $TyrA_c$ has a value of k_{cat}/K_m for prephenate (7.0×10^7 M/s) which is relatively close to the diffusion-controlled limit of $\sim 10^9$ M/s.

Table 3
Kinetic parameters calculated for purified *P. stutzeri* cyclohexadienyl dehydrogenases at pH 7.1 and 25°C[a]

Kinetic constant	Substrate			Inhibitor	
	Prephenate	L-Arogenate	NAD^+	L-Tyrosine	4-Hydroxylphenyl-pyruvate
K_m (mM)	0.07	0.56	0.14		
k_{cat} (s)	4.9×10^3	3.2×10^3			
k_{cat}/K_m (M/s)	7.0×10^7	5.7×10^6			
K_i (mM)				0.26	0.23

[a] The values given are the means of data obtained from two independent experiments using three determinations for each data point. S.D. ranged from 4 to 8%.

3.7. Regulatory properties of *P. stutzeri* $TyrA_c$

Both L-tyrosine (immediate product of the arogenate dehydrogenase reaction) and 4-hydroxyphenylpyruvate (immediate product of the prephenate dehydrogenase reaction) were effective inhibitors. Double-reciprocal plots demonstrated that each inhibitor inhibits activity competitively with respect to either prephenate or L-arogenate and non-competitively with respect to NAD^+. 4-Hydroxyphenylpyruvate and L-tyrosine were similarly effective as inhibitors, judging from K_i values calculated (Table 3). A variety of analog structures were tested as inhibitors. Concentrations of test inhibitor (2 mM) and substrate (0.2 mM prephenate) were used which produced ~ 50% inhibition when either L-tyrosine (49%) or 4-hydroxyphenylpyruvate (57%) was used. The D-isomer of tyrosine was nearly as effective (41% inhibition) as L-tyrosine. 4-Hydroxy compounds having a variety of sidechain substituents at the 1-position were effective inhibitors (data summarized in Table 4). Of the 3-carbon sidechains, the lactyl sidechain was even more effective than the alanyl and pyruvyl sidechains. The 2-carbon acetyl sidechain was almost as effective as the 3-carbon pyruvyl sidechain. Even 4-hydroxybenzoate produced significant inhibition. In fact, the minimal structural requirement for effective inhibition appeared to be that of an aromatic ring having a hydroxyl substituent (phenol). Compounds possessing a hydrogen at the 4-position of the ring (e.g. L-phenylalanine, phenylpyruvate, or phenyllactate) were not inhibitors, in contrast to the corresponding 4-hydroxy compounds. However, 4-amino-L-phenylalanine was an even better inhibitor than L-tyrosine. An hydroxy substituent at the 3-position blocked inhibition capability substantially, as revealed by the weak inhibition produced by 3,4-dihydroxy-phenylalanine in comparison with L-tyrosine. Thus, $TyrA_c$ from *P. stutzeri* recognizes an inhibitor structure which has an aromatic ring with a 4-hydroxy or a 4-amino group. It can tolerate many sidechain variations at the 1-position. Although replacement of the hydrogen at the 3-position by fluorine yields a highly effective inhibitor, a 3-hydroxy substituent is not tolerated (compare *m*-fluoro-DL-tyrosine and 3, 4-dihydroxyphenylalanine).

Table 4
Compounds tested as inhibitors of prephenate dehydrogenase

Compound (2 mM)	Relative activity[a]	% Inhibition
L-Tyrosine	0.51	49
D-Tyrosine	0.59	41
4-Hydroxyphenylpyruvate	0.43	57
DL-4-Hydroxyphenyllactate	0.44	56
4-Hydroxyphenylacetate	0.58	42
4-Hydroxybenzoate	0.62	38
4-Amino-L-phenylalanine	0.43	57
m-Fluoro-DL-tyrosine	0.21	79
3,4-Dihydroxyphenylalanine	0.97	3
Phenol	0.62	38
L-Phenylalanine	0.98	2
Phenylpyruvate	1.02	0
Phenyllactate	1.00	0
Benzoate	1.08	0

[a] Activity in the absence of inhibitor was assigned a relative value of 1.00; the control activity was 1.98 nmol/min. The reaction mixture contained 13.2 ng of pure protein and BSA at a concentration of 0.2 mg/ml. Activity determinations obtained in three independent experiments were averaged.

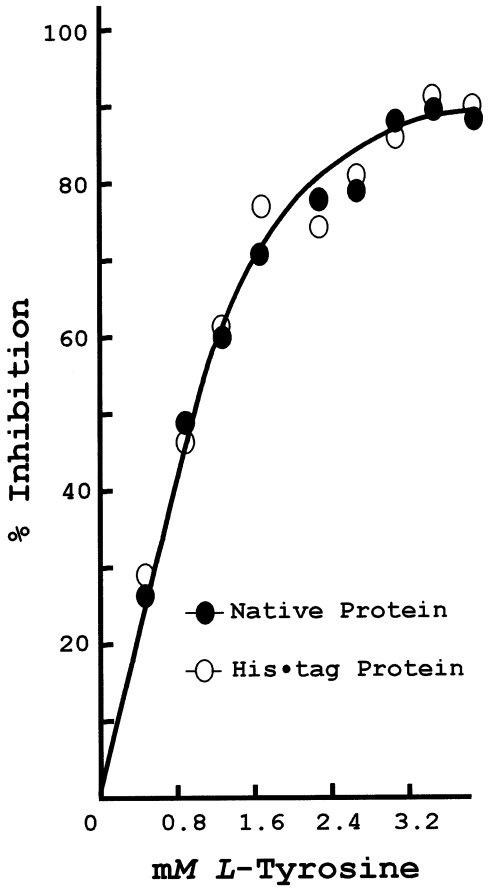

Fig. 2. Feedback inhibition of cyclohexadienyl dehydrogenase by L-tyrosine. Reaction mixtures contained 1 mM NAD^+, 1 mM prephenate, 0.02 μg protein (purified by His·Bind chromatography), and the indicated concentration of L-tyrosine in a total volume of 0.4 ml. NADH appearance was measured fluorometrically as described in Section 2. The activity of a crude extract (6.1 μg) prepared from *E. coli* AT 2471(pJX1670) (◆) was 1.09 nmol/min, and the activity of purified His·Tag $TyrA_c$ (13.2 ng) from pET*tyrA*$_c$ (●) was 1.276 nmol/min.

3.8. Effect of the C-terminal His·Tag

Constructs which produce a C-terminal His·Tag are claimed to affect enzyme properties only rarely (Novagen), and this seems to be the case for *P. stutzeri* $TyrA_c$. Kinetic data similar to that reported in this paper for pure His·Tag enzyme were obtained with extracts of enzyme prepared from AT2471(pJX1670). Secondly, the purified His·Tag protein and unpurified $TyrA_c$ from AT2471(pJX1670) both migrated similarly during passage through a size-exclusion matrix during FPLC chromatography. Thirdly, sensitivity to inhibition by L-tyrosine was nearly identical for the two preparations, as illustrated in Fig. 2 by the inhibition curves obtained in the presence of L-tyrosine. Inhibition data with the other compounds shown in Table 4 for pure His·Tag enzyme was also nearly identical when native *P. stutzeri* enzyme was used.

3.9. $TyrA_c$ from P. aeruginosa

As described in Section 2, it was possible to assemble the $tyrA_c$ gene sequence from appropriate contigs in the unfinished genome sequence project for *P. aeruginosa*. The deduced amino acid sequences of *P. stutzeri* and *P. aeruginosa* exhibited 67% identity. The two proteins have predicted isoelectric points of 6.44 and 7.57, respectively. They have equal lengths of 294 residues. There were no significant differences in overall amino acid composition.

4. Discussion

4.1. Identity of the cloned gene and its gene product

The conclusion that the structural gene coding for cyclohexadienyl dehydrogenase from *P. stutzeri* has been cloned and sequenced is amply supported. This gene complemented the *aro*Q_t·*tyr*A_c defect of *E. coli* AT2471. *E. coli* transformants produced cyclohexadienyl dehydrogenase having the properties of the *P. stutzeri* enzyme rather than those of the cyclohexadienyl dehydrogenase component of the *E. coli* T-protein.

The single ORF localized within a 1.67-kb *Eco*RI-*Pst*I fragment was sufficient for functional complementation. This ORF would encode a protein with a calculated molecular mass of 31 951 Da, a value which agrees well with the subunit size determined for the purified enzyme by SDS–PAGE. *P. stutzeri tyrA*$_c$ was not well expressed in the plasmid construct carried by *E. coli*, presumably because the native *P. stutzeri* ribosome binding site is poor. In the native organism the upstream region lies within the coding region of a flanking gene (*hisH*). HisH is a species of aromatic aminotransferase. Only 24 bp separate *hisH* and *tyrA*$_c$. Ribosomes completing *hisH* translation in *P. stutzeri* might disrupt some secondary mRNA structure in this region and unmask translation inhibition of $TyrA_C$.

4.2. In the absence of an $aroQ_t$ domain, how does *P. stutzeri* $tyrA_c$ complement the *E. coli* $aroQ_p{\cdot}tyrA_c$ deficiency?

An *E. coli* $aroQ_t{\cdot}tyrA_c$ 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. Precedents supporting this expectation are the successful complementation results achieved when a truncated $\cdot tyrQ_c$ gene (lacking the chorismate mutase domain) from *E. herbicola* (Xia et al., 1992) or the $tyrA_c$ gene from *Z. mobilis* (Zhao et al., 1993) were transformed into an *E. coli* $aroQ_t{\cdot}tyrA_c$ mutant. 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.

4.3. NAD^+-binding domain

The NAD^+-binding domain of dehydrogenases is strongly conserved in nature (Wierenga et al., 1986). Based on the analysis developed by Wierenga et al. (1986), the NAD^+-binding site of the cloned cyclohexadienyl dehydrogenase from *P. stutzeri* was located on the N-terminus of the polypeptide (Fig. 3). Similarly, the NAD^+ binding domains of the prephenate dehydrogenases of *B. subtilis* (Henner et al., 1986) and *S. cerevisiae* (Mannhaupt et al., 1989) and several other organisms 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 (spanning residues 1–100); the NAD^+ binding site was located adjacent to the chorismate mutase domain (from residue 101 to 132), but at the extreme 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 an evolutionary scenario (Jensen, 1985; Ahmad and Jensen, 1986) whereby the gene encoding a multidomain T-protein originated by a fusion which joined a chorismate mutase domain to the N-terminus of a cyclohexadienyl dehydrogenase. The *P. stutzeri* cyclohexadienyl dehydrogenase matches the Wierenga fingerprint (Wierenga et al., 1986) at ten of the 11 positions.

The amino acid residue at position 32 is always L-aspartate (or L-glutamate) for NAD^+-specific dehydrogenases. Each member of the thus-far constituted TyrA homology family possesses an aspartate residue, except for the cyanobacterial representative (Ssp $TyrA_a$). This is, in fact, the single family member identified as an $NADP^+$-specific enzyme (Stenmark et al., 1974; Hall et al., 1982). Consistent with precedents that $NADP^+$-specific dehydrogenases cannot tolerate a negatively charged residue at position 32 (Fan et al., 1991; Feeney et al., 1990), Ssp $TyrA_a$ possesses a serine residue in this position.

Griffin and Gasson (1995) provided evidence that *L. lactis* $TyrA_x$ is a secreted protein, and noted that its N-terminus generally conforms with the requirements of a cleavable signal peptide. While P-Sort analysis also predicts cleavable signal peptides for Zmo $TyrA_a$ and Bsu $TyrA_p$, such processing seems improbable since the essential NAD^+-binding domain would be disrupted. However, given the unlikely possibility that outbound translocation of such proteins does occur, perhaps the truncated proteins could be secondarily engaged as binding proteins for transport of L-tyrosine and/or related metabolites.

4.4. The TyrA dehydrogenase superfamily

Members of the TyrA superfamily of dehydrogenases are quite divergent and in Fig. 3 have been separated into four families. TyrA individuals belonging to different families share only $\sim$ 20% amino acid identity (Fig. 3). Beyond the residues within the NAD^+ binding region, eight residues are conserved in at least 16 of the 17 sequences (Fig. 4). One of these corresponds to His197 of *E. coli* which has been indicated as an essential catalytic residue for the dehydrogenase reaction (Christendat et al., 1998). If the most divergent yeast sequences are excluded, three additional residues are conserved in all of the remaining sequences.

Members of the TyrA superfamily vary in substrate specificity, and they vary in sensitivity to

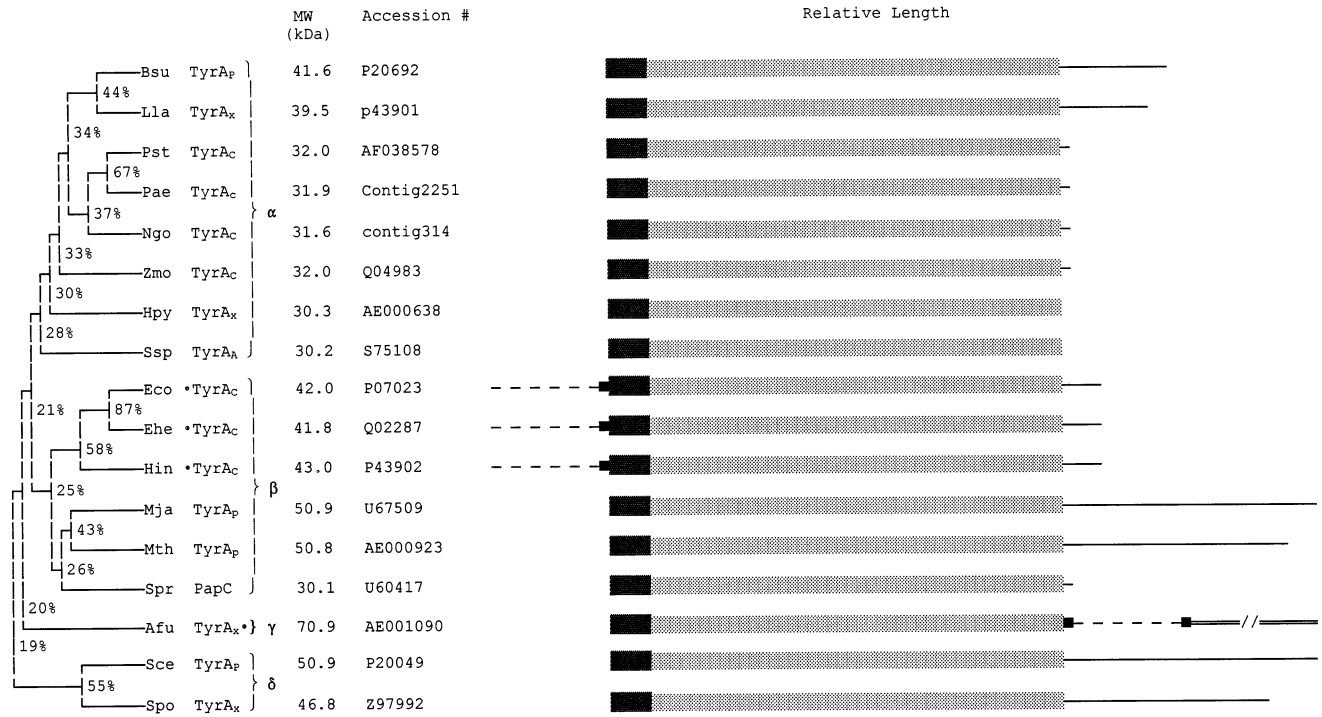

Fig. 3. Dendrogram (GCG PILEUP program) showing cyclohexadienyl dehydrogenase relationships. Representative percent of identities (GCG GAP program) of deduced amino acid sequence are shown at node positions. Bsu, *Bacillus subtilis*; Eco, *E. coli*; Ehe, *E. herbicola*; Hin, *Haemophilus influenzae*; Hpy, *Helicobacter pylori*; Lla, *Lactococcus lactis*; Mja, *Methanococcus jannaschii*; Mth, *Methanobacterium thermoautotrophicum*; Ngo, *N. gonorrhoeae*; Pae, *P. aeruginosa*; Pst, *P. stutzeri*; Sce, *Saccharomyces cerevisiae*; Spo, *Schizosaccharomyces pombe*; Spr, *Streptomyces pristubaespirakis*; Ssp, *Synechocystis* sp.; TyrA$_a$, arogenate dehydrogenase; TyrA$_c$, cyclohexadienyl dehydrogenase; TyrA$_P$, prephenate dehydrogenase; PapC, 4-amino 4-deoxyprephenate dehydrogenase; Zmo, *Z. mobilis*. Gene fusions are indicated with •. Fused chorismate mutase domains are indicated with dashed lines. The basic dehydrogenase catalytic regions are represented by wide shaded bars (block shaded dark is the NAD(P)-binding domain). A fused prephenate dehydratase domain (•*pheA*) for Afu TyrA$_x$ is indicated with a double line. Only the portion of the sequences shown as bars were used for data entry into the PILEUP and GAP programs to avoid extraneous alignment results.

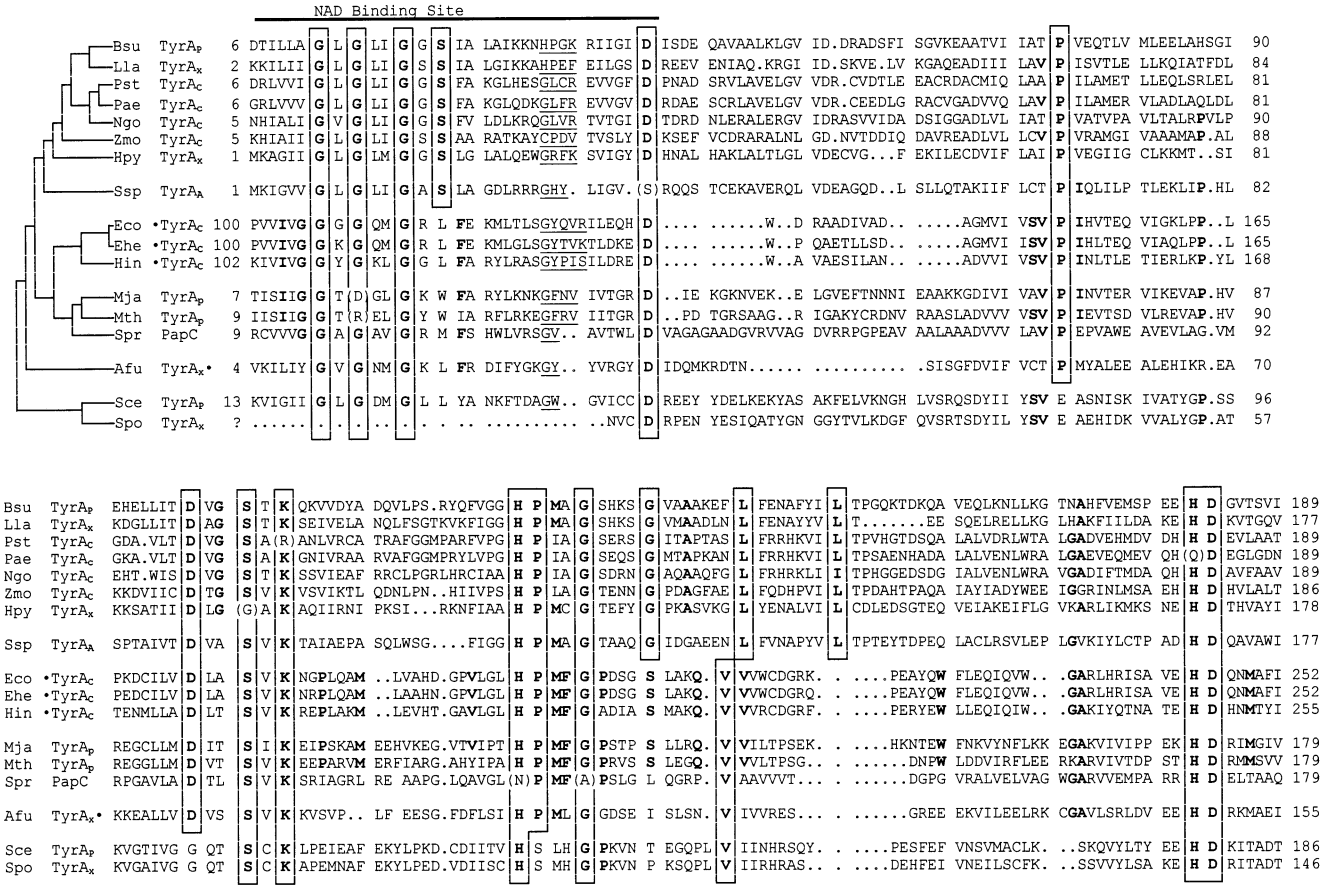

Fig. 4. Multiple alignment of deduced amino-acid sequences of $TyrA_c$, $TyrA_p$, $TyrA_a$ and PapC. The PILEUP software program of GCG was used to obtain the multiple alignment shown. The dendrogram generated is shown at the upper left. Immediately to the right of the dendrogram are individual designations, with beginning residue numbers shown in the multiple alignment. Ending residue numbers are shown at the right of each horizontal data block. At the far lower right is given the number of the final residue presented followed (slash) by the number of the final sequence residue. Residues that are highly conserved within the four groups compared are boxed, less highly residues are printed in boldface type. Center dots indicate gaps introduced to optimize the alignment. The initial sequences spanning the NAD-binding sites, ending at the conserved D residue, were aligned manually according to the Weierenga fingerprint. An error in which a downstream ATG (codon 10) was mistakenly annotated as the start codon of Hpy-$TyrA_x$ was corrected. An asterisk marks the position of residue 194 (a TAA stop codon) in the Pae $TyrA_c$ sequence which almost certainly is a sequencing error. Afu, *Archaeoglobus fulgidus*; Bsu, *B. subtilis*; Eco, *E. coli*; Ehe, *E. herbicola*; Hin, *H. influenzae*; Hpy, *H. pylori*; Lla, *L. lactis*; Mja, *M. jannaschii*; Mth, *M. thermoautotrophicum*; Ngo, *N. gonorrhoeae*; Pae, *P. aeruginosa*; PapC, 4-amino 4-deoxyprephenate dehydrogenase; Pst, *P. stutzeri*; Sce, *S. cerevisiae*; Spo, *S. pombe*; Spr, *S. pristubaespirakis*; Ssp, *Synechocystis* sp.; $TyrA_a$, arogenate dehydrogenase; $TyrA_c$, cyclohexadienyl dehydrogenase; $TyrA_P$, prephenate dehydrogenase; Zmo, *Z. mobilis*.

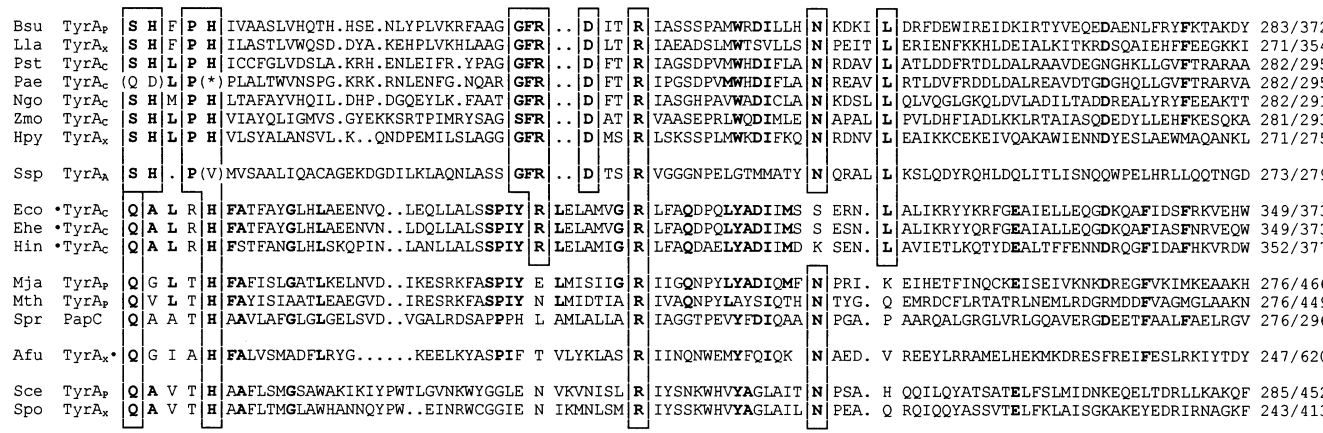

Fig. 4. (*Continued*)

feedback inhibition by L-tyrosine or to inhibition by other compounds. All of the dehydrogenase domains possess an N-terminal motif that is an established signature for NAD^+ binding, but the lengths of many *tyr*A coding regions exhibit unconserved extensions of variable length at the 3′ ends. In some cases presumed fusions have created bifunctional (e.g. Eco·$AroQ_t$·$TyrA_c$) or trifunctional (e.g. Afu $TyrA_x$·$AroQ_p$·PheA) proteins.

The α family of TyrA dehydrogenases contains prephenate-specific members (e.g. Bsu $TyrA_p$), arogenate-specific members (e.g. Ssp $TyrA_A$), or broad-specificity members (e.g. Pst $TyrA_c$). In the β family, a fourth class of specificity is represented by Spr PapC which utilizes 4-amino-4-deoxyprephenate for conversion to 4-aminophenylpyruvate (Blanc et al., 1997). The latter metabolite is an antibiotic precursor. The enteric TyrA proteins belong to the β family and are broad-specificity enzymes, although they exhibit marked preference for prephenate (Ahmad and Jensen, 1986). They possess an N-terminal extension which carries a domain for chorismate mutase ($AroQ_t$). Afu $TyrA_x$ is currently the sole member of the γ family. Its dehydrogenase-domain specificity is unknown, but it appears to co-exist with chorismate mutase ($AroQ_p$) and prephenate dehydratase (PheA) domains, being the N-terminal domain of a trifunctional protein. Confirmation of such a unique multi-domain protein would be worthwhile. Finally, the δ family consists of two yeast enzymes.

4.5. Comparison of the TyrA and PheA homology families

TyrA and PheA proteins have much in common in that they catalyze reactions which utilize similar or identical substrates (e.g. prephenate) and which result in a concerted aromatization process which is driven by decarboxylation. The PheA homology family includes only prephenate-specific enzymes (Gu et al., 1997). The single cyclohexadienyl dehydratase so far cloned and sequenced apparently belongs to a separate homology family (Zhao et al., 1992). An L-arogenate-specific dehydratase has yet to be cloned and sequenced. All PheA proteins are of similar length and each divides into a discrete N-terminal catalytic region and a C-terminal allosteric region. In contrast, the TyrA superfamily includes members having four classes of substrate specificity (five if NAD^+ vs. $NADP^+$ is included), and no discrete allosteric domain is evident. Some members lack endproduct control, while others are highly sensitive to feedback inhibition. The future availability of more sequences may reveal patterns of TyrA subfamily clustering in correlation with substrate specificity or with presence or absence of endproduct control, but no such correlations are evident at the family level defined in Fig. 3.

4.6. Endproduct control exerted directly at the catalytic site?

Endproduct control of biosynthetic pathways at the activity level is almost always exerted at allosteric sites which can influence the spatially distant catalytic region following conformational changes induced by small-molecule binding. The discrete C-terminal allosteric domains of PheA proteins alluded to above exemplify this.

In contrast to the latter, it seems quite possible that many or all members of the TyrA superfamily lack a spatially distinct allosteric region. The *Z. mobilis* $TyrA_c$ and the *Synechocystis* sp. $TyrA_a$ (insensitive to L-tyrosine-mediated feedback inhibition) are the same lengths as the feedback-sensitive $TyrA_c$ proteins of *P. stutzeri*, *P. aeruginosa*, and *Neisseria gonorrhoeae*. The Zmo $TyrA_c$ and Spp $TyrA_A$ proteins may require the catalytically crucial 1-carboxy substrate substituent for binding at the catalytic site. Thus, binding of a molecule like L-tyrosine would be minimized. The Ssp $TyrA_A$ protein additionally is specific for the alanyl sidechain of L-arogenate and will not accept the pyruvyl sidechain of prephenate. On the other hand, Pst $TyrA_c$, Pae $TyrA_c$ and Ngo $TyrA_c$ can bind substrate-related compounds such as L-tyrosine, which lack the 1-carboxy substituent, thereby exerting competitive inhibition. For Pst $TyrA_c$, a six-membered ring (cyclohexadienyl or aromatic) having a 4-hydroxy substituent appears to be the minimal structure required for binding.

B. subtilis $TyrA_p$ will not accept L-arogenate as a substrate, being a specific prephenate dehydrogenase (Jensen and Stenmark, 1975). L-Tyrosine ($K_i = 3$ μM), D-tyrosine ($K_i = 1.3$ mM), and L-phenylalanine ($K_i = 0.3$ mM) were competitive inhibitors with respect to prephenate. L-Tryptophan and 4-hydroxyphenylpyruvate were also effective inhibitors. In this case it appears that a 4-hydroxy substituent is unnecessary for effective binding (e.g. as with L-phenylalanine) and that an alanyl

sidechain can be accommodated provided that a 1-carboxy substituent is not simultaneously present (as in L-arogenate).

The proposed binding of inhibitor molecules directly at the catalytic site is consistent with the properties of a *B. subtilis* mutant selected for resistance to growth inhibition by D-tyrosine (Champney and Jensen, 1970). Not only was the mutant $TyrA_p$ enzyme insensitive to inhibition by D-tyrosine, but also to inhibition by L-tyrosine, L-phenylalanine, or L-tryptophan. The mutant enzyme retained catalytic competence, but exhibited a decreased affinity for prephenate (substrate) and decreased inhibition by 4-hydroxyphenylpyruvate.

In *P. aeruginosa m*-fluorotyrosine is an effective growth inhibitor in fructose-based medium, and this is reversed by L-tyrosine (Calhoun and Jensen, 1972). Mutants resistant to this analog are readily selected, but Berry and Jensen (unpublished results) could not isolate a class of tyrosine-excreting mutants having a feedback-resistant $TyrA_c$. It seems likely that mutations which abolished the binding of L-tyrosine simultaneously decreased prephenate binding, with consequent auxotrophy or partial auxotrophy. Indeed, a class of *m*-fluorotyrosine-resistant mutants were recovered which grew slowly in minimal medium (tyrosine bradytrophs) and which exhibited barely detectable $tyrA_c$ activity.

The yeast and Archeal TyrA proteins exhibit substantial carboxy extensions that are not present in other TyrA proteins. It is possible that these enzymes have acquired discrete allosteric domains. Analysis (BLAST) of these carboxy regions did not reveal any similarity with one another or with any other proteins in the database.

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