The Phylogenetic Origin of the Bifunctional Tyrosine-Pathway Protein in the Enteric Lineage of Bacteria[1,2]

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Because bifunctional enzymes are distinctive and highly conserved products of relatively infrequent gene-fusion events, they are particularly useful markers to identify clusters of organisms at different hierarchical levels of a phylogenetic tree. Within the subdivision of gram-negative bacteria known as superfamily B, there are two distinctive types of tyrosine-pathway dehydrogenases: (1) a broad-specificity dehydrogenase (recently termed cyclohexadienyl dehydrogenase [CDH]) that can utilize either prephenate or L-arogenate as alternative substrates and (2) a bifunctional CDH that also possesses chorismate mutase activity (T-protein). The bifunctional T-protein, thought to be encoded by fused ancestral genes for chorismate mutase and CDH, was found to be present in enteric bacteria (*Escherichia, Shigella, Salmonella, Citrobacter, Klebsiella, Erwinia, Serratia, Morganella, Cedecea, Kluyvera, Hafnia, Edwardsiella, Yersinia,* and *Proteus*) and in *Aeromonas* and *Alteromonas.* Outside of the latter "enteric lineage," the T-protein is absent in other major superfamily-B genera, such as *Pseudomonas* (rRNA homology group I), *Xanthomonas, Acinetobacter,* and *Oceanospirillum.* Hence, the T-protein must have evolved after the divergence of the enteric and *Oceanospirillum* lineages. 3-Deoxy-D-*arabino*-heptulosonate 7-phosphate synthase-phe, an early-pathway isozyme sensitive to feedback inhibition by L-phenylalanine, has been found in each member of the enteric lineage examined. The absence of both the T-protein and DAHP synthase-phe elsewhere in superfamily B indicates the emergence of these character states at approximately the same evolutionary time.

Introduction

With the advancement of molecular-genetic techniques, it is now possible to ascertain the phylogenetic positioning of microorganisms with some precision. The phylogenetic relationships, even among distantly related groups, can be determined using 16S rRNA sequences that exhibit a low rate of evolutionary change (Fox et al. 1980; Stackebrandt and Woese 1981). One benefit of this advance is that the evolutionary history of variable character states, including biochemical pathways, can be deduced within a set of organisms having established phylogenetic relationships (Jensen 1985). For example, the pathway for the biosynthesis of aromatic amino acids is

1. Key words: tyrosine biosynthesis, aromatic amino acids, enteric bacteria, phylogeny. Abbreviations: CM-F = chorismate mutase species free of any known association; CM-P = chorismate mutase component of the P-protein; CM-T = chorismate mutase component of the T-protein; DAHP = 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; DS-phe = phenylalanine-inhibited DAHP synthase isozyme; DS-trp = tryptophan-inhibited DAHP synthase isozyme; DS-tyr = tyrosine-inhibited DAHP synthase isozyme; P-protein = chorismate mutase-prephenate dehydratase; T-protein = chorismate mutase-cyclohexadienyl dehydrogenase; CDH = cyclohexadienyl dehydrogenase.

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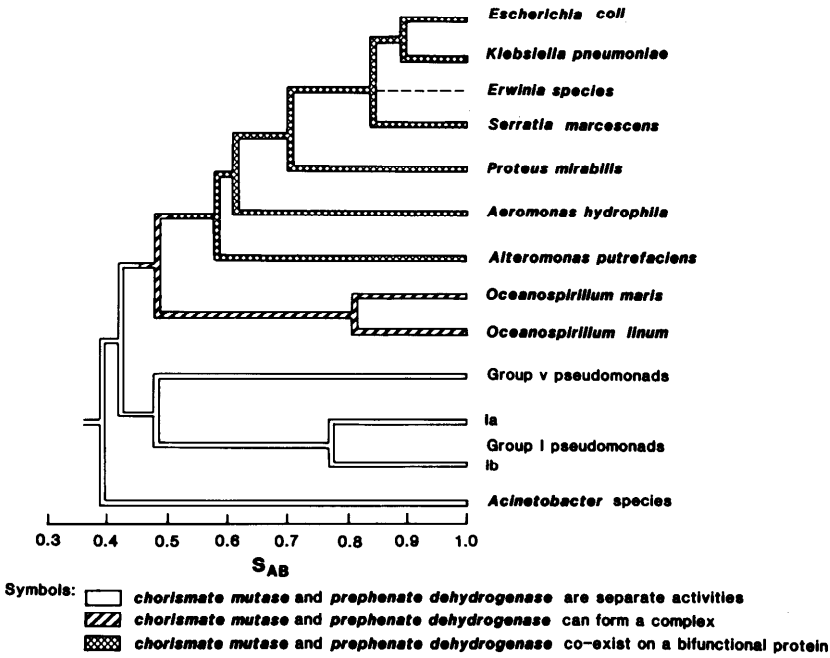

FIG. 1.—Dendrogram of purple sulfur bacteria (superfamily B) in relationship to the evolution of the bifunctional T-protein. The dendrogram is based on oligonucleotide-cataloging data compiled by Woese et al. (1985). Organisms named on the right were arbitrarily selected as representatives of the major lineages shown. The bifunctional T-protein is present only within the enteric lineage, defined as the cluster spanning *E. coli* and *Aeromonas putrefaciens*. The dashed line shown for *Erwinia* species indicates an approximate phylogenetic position, since an SAB value is not available. The rRNA homology group I pseudomonads, rRNA homology group V (*Xanthomonas*) pseudomonads, and *Acinetobacter* species lack the T-protein; they possess monofunctional species of chorismate mutase (CM-F) and prephenate dehydrogenase. The *Oceanospirillum* cluster, though lacking the T-protein, possesses an enzyme complex that may represent an intermediate stage in the evolution of the T-protein (Ahmad and Jensen 1986).

complex and exhibits a rich diversity of biochemical character states (Byng et al. 1982). Alternative biochemical steps, presence of distinct isozymes, different patterns of allosteric control, varied enzymic specificity for pyridine nucleotide cofactor requirement, and presence or absence of proteins with multifunctional capabilities exemplify character states that can be traced along the phylogenetic tree.

Detailed studies on the aromatic biosynthetic pathway have been carried out on the gram-negative purple bacteria. This assemblage consists of three genealogical subdivisions referred to as superfamily A, superfamily B, and superfamily C (synonymous with the beta, gamma, and alpha subdivisions of Woese et al. [1985]). Interpretations about the evolution of aromatic amino acid biosynthesis are much more developed for superfamily-B organisms (fig. 1) than for any other phylogenetic grouping. Four major lineages (enteric bacteria, a cluster of *Oceanospirillum* species, rRNA homology groups I and V of pseudomonads, and species of *Acinetobacter*) have been the primary source of data used to deduce the most probable ancestral state of the pathway for superfamily-B organisms (Jensen 1985).

Two bifunctional proteins of phenylalanine and tyrosine biosynthesis (fig. 2) have evolved in superfamily-B organisms. The P-protein (chorismate mutase:prephenate dehydratase, E.C.5.4.99.5:4.2.1.51) of phenylalanine biosynthesis is of ancient

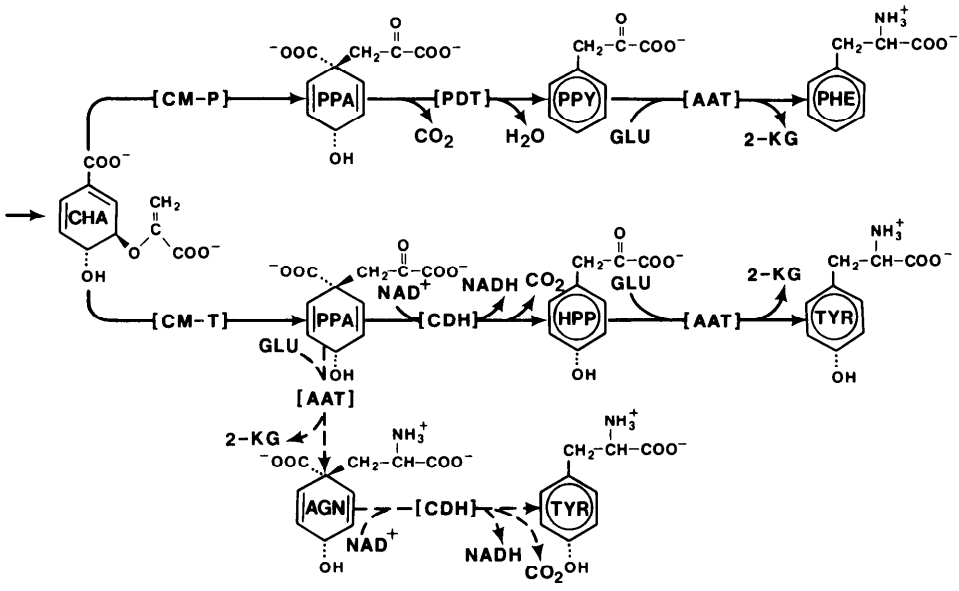

FIG. 2.—Operation of two bifunctional proteins in biosynthesis of phenylalanine (PHE) and tyrosine (TYR) in enteric bacteria. The bifunctional P-protein of the PHE branch consists of [CM-P] and prephenate dehydratase [PDT]. The bifunctional T-protein of the TYR branch consists of [CM-T] and [CDH]. [CM-P] and [CM-T] compete for chorismate (CHA) molecules, effectively making CHA the metabolic branchpoint of PHE and TYR biosynthesis. Phenylpyruvate (PPY), 4-hydroxyphenylpyruvate (HPP), and prephenate (PPA) are transaminated to PHE, TYR, and L-arogenate (AGN), respectively, in the presence of glutamate (GLU) via catalysis by a mixture of broad-specificity aromatic aminotransferases [AAT] able to transaminate PPY, HPP, and PPA (Jensen and Calhoun 1981). CDH is able to catalyze both reactions shown (prephenate dehydrogenase and arogenate dehydrogenase). In enteric bacteria the route to tyrosine via AGN (indicated by dashed arrows) is thought to be minor, mainly because of the spatial channeling of PPA molecules by the P- and T-proteins.

origin and is apparently distributed throughout all of both superfamily B and the connecting superfamily A (Ahmad and Jensen 1986). The prephenate dehydratase component of the bifunctional P-protein does not utilize L-arogenate as an alternative substrate (S. Ahmad, unpublished data). L-Arogenate is the last common intermediate in the biosynthesis of L-phenylalanine and/or L-tyrosine in many prokaryotic, plant, and other eukaryotic systems and is formed from prephenate by one or more aminotransferases (Jensen and Fischer 1987).

The bifunctional T-protein (chorismate mutase:prephenate dehydrogenase, E.C.5.4.99.5:1.3.1.12) of tyrosine biosynthesis, on the other hand, is of more recent origin. It has been identified in *E. coli* (Koch et al. 1971), *Salmonella typhimurium* (Dayan and Sprinson 1971), and *Klebsiella pneumoniae* (Koch et al. 1970) but not elsewhere in superfamily B. The prephenate dehydrogenase component of the bifunctional T-protein from *E. coli* and *K. pneumoniae* has recently been shown to utilize L-arogenate and other structurally related compounds as alternative substrates (Ahmad and Jensen 1987*b*), thus being a CDH. That majority of superfamily B that lacks the bifunctional T-protein possesses a monofunctional CDH (Patel et al. 1977; Byng et al. 1985; Ahmad and Jensen 1987*a*).

The rate of evolutionary change in the pathway of aromatic biosynthesis is not uniform, comparing groups whose phylogenetic span is similar as gauged by the amount of change in 16S rRNA. For example, the *Oceanospirillum* lineage includes species

whose 16S rRNA catalogs are related by a similarity coefficient of $S_{AB} = 0.59$ (Fox et al. 1980). The enteric lineage is defined by species of similar phylogenetic depth ($S_{AB} = 0.61$). Our studies indicate that, in contrast to the *Oceanospirillum* cluster in which the major character states of aromatic biosynthesis are monotonously similar, the enteric lineage has undergone evolutionarily dynamic changes in aromatic biosynthesis. The present paper presents data that pinpoint the evolution of the bifunctional T-protein within the enteric lineage of bacteria and notes the close correlation of this evolutionary event with the appearance of DS-phe (E.C.4.1.2.15), an early-pathway regulatory isozyme (Ahmad et al. 1987).

Material and Methods

Organisms and Growth Conditions

Klebsiella pneumoniae ATCC 25304, *Erwinia carotovora* ATCC 15713, *E. herbicola* ATCC 33243, *E. amylovora* ATCC 15580, *Serratia marcescens* ATCC e113880, *Proteus mirabilis* ATCC 29906, *Aeromonas hydrophila* ATCC 14715, and *Alteromonas putrefaciens* ATCC 8071 were obtained from the American Type Culture Collection (Rockville, MD). *Klebsiella pneumoniae* (37 C), *E. carotovora* (26 C), *E. herbicola* (26 C), *S. marcescens* (30 C), and *A. hydrophila* (28 C) were grown on M9 medium according to a method described by Winkler and Stuckman (1979). *Proteus mirabilis* (37 C) was grown on M9 medium containing 1 mg/liter each of nicotinamide, *p*-aminobenzoate, calcium pantothenate, and thiamine. *Erwinia amylovora* was grown at 26 C as described by Miller and Schroth (1972). *Alteromonas putrefaciens* was grown at 30 C in basal medium containing half-strength artificial seawater and supplemented with 0.2% (w/v) glucose as described by Baumann et al. (1984). The organisms were grown to late exponential phase of growth, harvested by centrifugation, washed twice with 50 m*M* K phosphate buffer (pH 7.0) containing 1 m*M* dithiothreitol (DTT) (buffer A), and stored at −80 C until used.

Enzyme Assay and Preparation of Cell Extracts

Cell pellets were suspended in buffer A, disrupted by sonication, and centrifuged at 150,000 *g* for 1 h. The resulting supernatant was passed through a Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) column (1.5 × 20.0 cm) equilibrated either in buffer A or in buffer B (20 m*M* K phosphate, pH 7.0, containing 1 m*M* DTT) to remove small molecules. The protein-containing fractions were pooled and are termed crude extract.

Chorismate mutase was assayed by the method of Cotton and Gibson (1965). The reaction mixture in a final volume of 200 μl contained buffer A, 1 m*M* K chorismate, and a suitable amount of enzyme protein. Incubation at 37 C was carried out for 20 min, and then 100 μl of 1 *N* HCl was added and incubated at 37 C for 15 min to convert the prephenate formed to phenylpyruvate. Phenylpyruvate was measured at 320 nm after the addition of 0.7 ml of 2.5 *N* NaOH. A molar extinction coefficient of 17,500 was used for calculations (Cotton and Gibson 1965).

Prephenate dehydrogenase and arogenate dehydrogenase were assayed by following the appearance of NADH on a spectrophotofluorometer, according to a method described by Champney and Jensen (1970). The reaction mixtures in a final volume of 200 μl contained buffer A, 1 m*M* nicotinamide adenine dinucleotide (NAD^+), 1 m*M* K prephenate or 1 m*M* K arogenate, and a suitable amount of enzyme protein. The reaction was started by the addition of the substrate, and the continuous formation of nicotinamide adenine dinucleotide, reduced (NADH) was followed by use of an

Aminco-Bowman spectrophotofluorometer (excitation at 340 nm, emission at 460 nm).

Protein in the crude extract was estimated by the method of Bradford (1976) with bovine serum albumin as the standard protein.

DE52 Column Chromatography

Approximately 100 mg of crude extract protein was applied to a diethylaminoethyl-cellulose (DE52; Whatman Inc., Clifton, NJ) column (1.5 × 20.0 cm) equilibrated in buffer A or buffer B as indicated. The column was washed with two bed volumes of the equilibration buffer, and then the bound proteins were eluted with 300 ml of a linear gradient of KCl (0.0–0.35 *M*) contained in the equilibration buffer. Fractions of 2.2 ml were collected and assayed for absorbance at 280 nm and for chorismate mutase, prephenate dehydrogenase, and arogenate dehydrogenase activities. Fractions containing the enzyme activity were tested for sensitivity to inhibition by aromatic amino acids.

Hydroxylapatite Chromatography

Appropriate fractions eluted from the DE52 column were pooled, concentrated to 4 ml by ultrafiltration on a PM-10 membrane (Amicon Corp.), and dialyzed to 10 m*M* K phosphate (pH 7.0) containing 1 m*M* DTT (buffer C). The concentrated sample was applied to a Bio-Gel hydroxylapatite (HTP; 1.0 × 9.0 cm) column equilibrated with buffer C. After washing the column, the bound proteins were eluted with a 300-ml linear gradient of 0.01–0.3 *M* K phosphate (pH 7.0) containing 1 m*M* DTT. Fractions of 2.2 ml were collected and assayed for absorbance at 230 nm and enzyme activities as indicated.

Sephadex Gel Filtration

Appropriate fractions eluted from the DE52 column were pooled, concentrated to 2.0 ml by ultrafiltration on a PM-10 membrane, dialyzed against buffer A, loaded onto a Sephadex G-200 column (2.5 × 92 cm) equilibrated in buffer A, and eluted with the same buffer at a flow rate of 10 ml/h. Fractions of 2.2 ml were collected and assayed for absorbance at 280 nm and enzyme activities as indicated.

Biochemicals and Chemicals

Amino acids, NAD^+, nicotinamide adenine dinucleotide phosphate ($NADP^+$), bovine serum albumin, DTT, Sephadex G-25, and Sephadex G-200 were obtained from Sigma Chemical Co. (St. Louis). DE52 was purchased from Whatman. HTP was obtained from Bio-Rad Laboratories, (Richmond, Calif.). Prephenate was prepared as the barium salt from culture supernatants of a tyrosine auxotroph of *Salmonella typhimurium* (Dayan and Sprinson 1970) and was converted to the potassium salt before use. Chorismate was isolated from the accumulation medium of a triple auxotroph (62-1) of *K. pneumoniae* and purified as the free acid (Gibson 1964). L-Arogenate was prepared from a triple auxotroph of *Neurospora crassa* (ATCC 36373) according to a method described elsewhere (Zamir et al. 1980). All other chemicals were standard reagent grade.

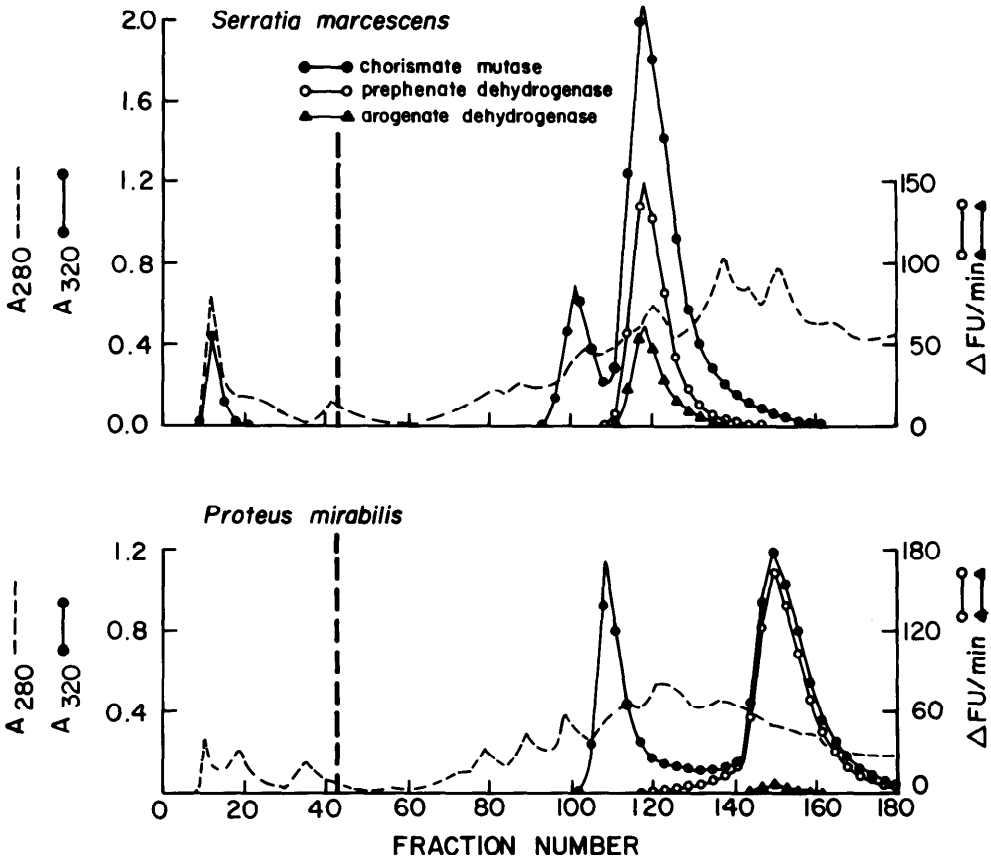

FIG. 3.—Elution profiles of chorismate mutase, prephenate dehydrogenase, and arogenate dehydrogenase activities following DE52 column chromatography of crude extracts prepared from *Serratia marcescens* (top panel) and *Proteus mirabilis* (bottom panel). The DE52 column was equilibrated and developed in buffer B. The vertical dashed lines indicate the onset point of gradient elution. Chorismate mutase activity is expressed as phenylpyruvate absorbance in base at 320 nm. An A_{320} of 1.0 corresponds to 2.9 nmol phenylpyruvate formed/min. The dehydrogenase activities are expressed as change in fluorescence units per min (ΔFU/min). A change of 100 FU/min corresponds to 1.9 nmol NADH formed/min. The distribution of proteins (as monitored by A_{280}) is shown by dashed lines.

Results

Tyrosine-Branch Enzymes

The elution profiles of the enzyme activities participating in the biosynthesis of L-tyrosine from *Serratia marcescens* (top panel) and *Proteus mirabilis* (bottom panel), following DE52 chromatography of the crude extracts, are shown in figure 3. Three bands of chorismate mutase eluted from *S. marcescens,* one of which (CM-T) coeluted with prephenate dehydrogenase (fig. 3, top panel). The early-eluting and middle peaks of chorismate mutase were identified as CM-F (F = free of association with another enzyme activity) and CM-P (P = association with the phenylalanine-pathway prephenate dehydratase), respectively. The prephenate dehydrogenase enzyme was able to use L-arogenate as an alternative cyclohexadienyl substrate, thus indicating its identity as CDH (fig. 2). DE52 fractions obtained from *P. mirabilis* contained two peaks of chorismate mutase activity, one of which (CM-T) again coeluted exactly with the

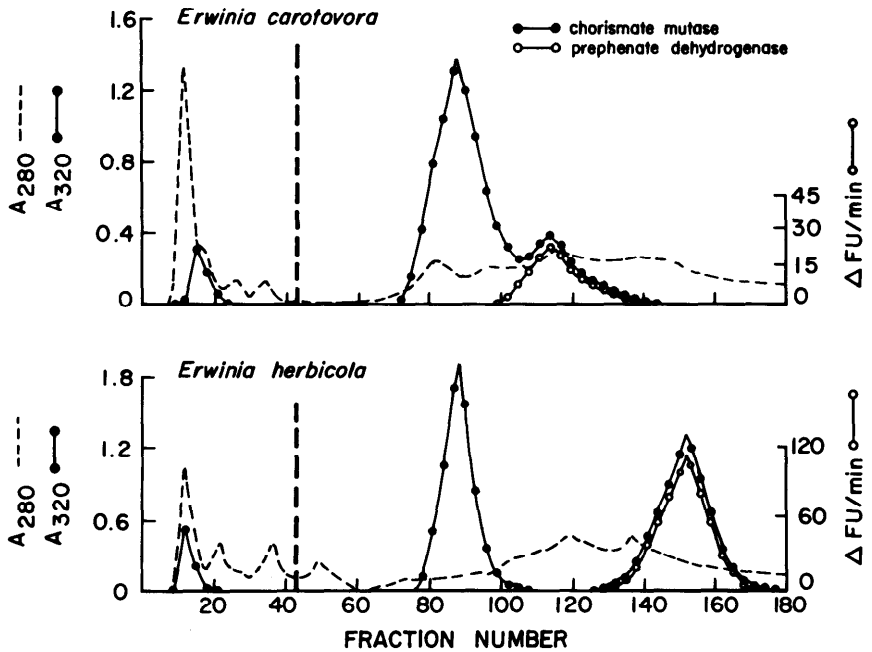

FIG. 4.—Elution profiles of chorismate mutase and prephenate dehydrogenase following DE52 column chromatography of crude extracts prepared from *Erwinia carotovora* (top panel) and *E. herbicola* (bottom panel). The DE52 column was equilibrated and developed in buffer A. Symbols are as in fig. 3.

prephenate dehydrogenase and arogenate dehydrogenase activities (fig. 3, bottom panel). The other isozyme was identified as CM-P. The CDH utilized L-arogenate relatively poorly as a substrate, compared with the *S. marcescens* CDH.

Figure 4 shows that three chorismate mutase isozymes were resolved from *Erwinia carotovora* (top panel) and *E. herbicola* (bottom panel) following DE52 chromatography of crude extracts. The CM-T isozyme from both *E. carotovora* and *E. herbicola* was readily identified by its coelution with the CDH activity. The dehydrogenase activities from both *E. carotovora* and *E. herbicola* were CDH enzymes, since they were also able to utilize L-arogenate as substrate (data not shown). Similar results were obtained from *E. amylovora* (data not shown).

Two peaks of activity for chorismate mutase (species CM-P and CM-T) eluted in column fractions following DE52 chromatography of the crude extracts from both *Alteromonas putrefaciens* (fig. 5a) and from *Aeromonas hydrophila* (fig. 6a). In each case isozyme CM-T was readily identified by the exact coincidence of its elution position with prephenate dehydrogenase and arogenate dehydrogenase activities. Since *A. putrefaciens* and *A. hydrophila* represent lineages that are the most divergent from *E. coli* within the enteric cluster, the apparent inseparability of CM-T and CDH (i.e., the presence of a bifunctional T-protein) was pursued further in these species.

The fractions (124–137) containing the chorismate mutase and CDH activities after DE52 chromatography of the crude extract from *A. putrefaciens* (fig. 5a) were pooled, concentrated, dialyzed, and chromatographed on both a Sephadex G-200 column and a hydroxylapatite column. The results given in figures 5b and 5c, respectively, show that the two activities coeluted. The ratio of the two activities remained constant during further fractionation steps following DE52 chromatography of the

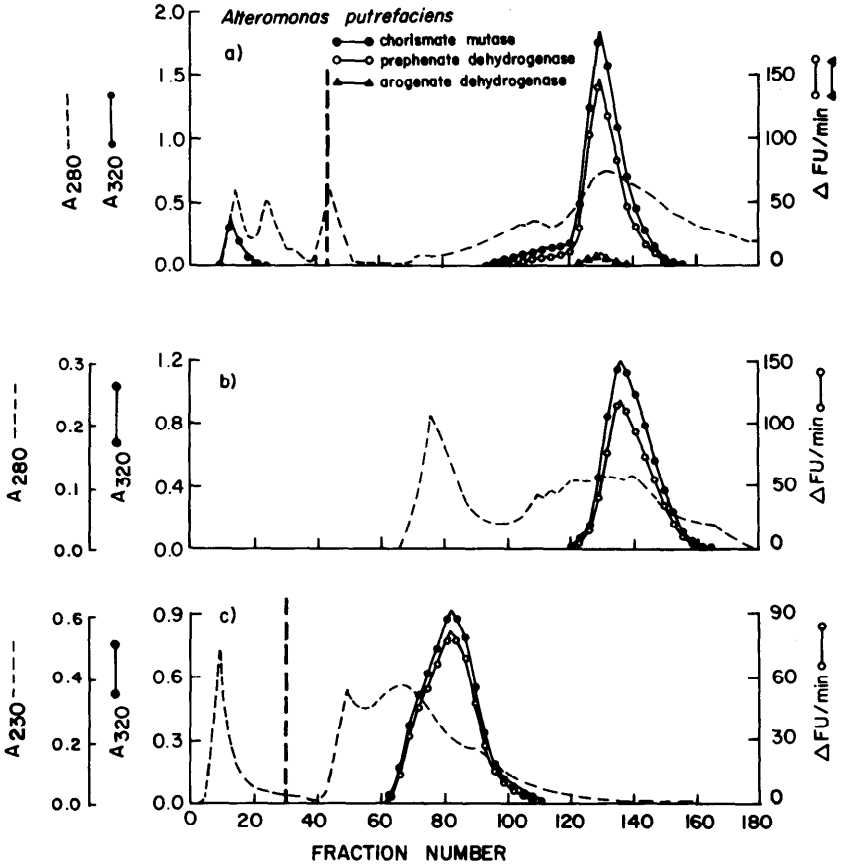

FIG. 5.—Integrity of the T-protein from *Alteromonas putrefaciens*. Symbols are as in fig. 3. Panel a), Elution profiles of chorismate mutase, prephenate dehydrogenase, and arogenate dehydrogenase following DE52 column chromatography of crude extract. The column was equilibrated and developed in buffer A. Panel b), Partially purified T-protein recovered from the DE52 column (fractions 124–137, panel a) was chromatographed on a Sephadex G-200 column as described in Material and Methods; the elution profiles of chorismate mutase and prephenate dehydrogenase are shown. These T-protein activities eluted at a molecular-weight position of 83,000, as determined on the basis of molecular-weight calibration standards. Panel c), Partially purified T-protein from *A. putrefaciens*, recovered from the DE52 column (fractions 124–137, panel a) was chromatographed on a hydroxylapatite column as described in Material and Methods. The elution profiles of chorismate mutase and prephenate dehydrogenase are shown. The distribution of protein was monitored at 230 nm.

crude extracts. The CDH dehydrogenase activity eluting from both columns also retained the original ratio of activities with prephenate and arogenate.

To explore the possible existence of a complex between chorismate mutase and prephenate dehydrogenase activities in *A. hydrophila,* as has been reported for *Oceanospirillum* species (Ahmad and Jensen 1986), efforts were made to disrupt such a hypothetical complex by use of a nonionic detergent. The effect of Triton X-100 on the CM-T and CDH activities obtained from the DE52 column were studied. Triton X-100 inhibited both enzyme activities, the inhibition increasing with increasing Triton X-100 concentration (table 1). Since concentrations >0.05% (v/v) interfered with the chorismate mutase assay, a concentration of 0.04% was used in experiments shown in figure 6.

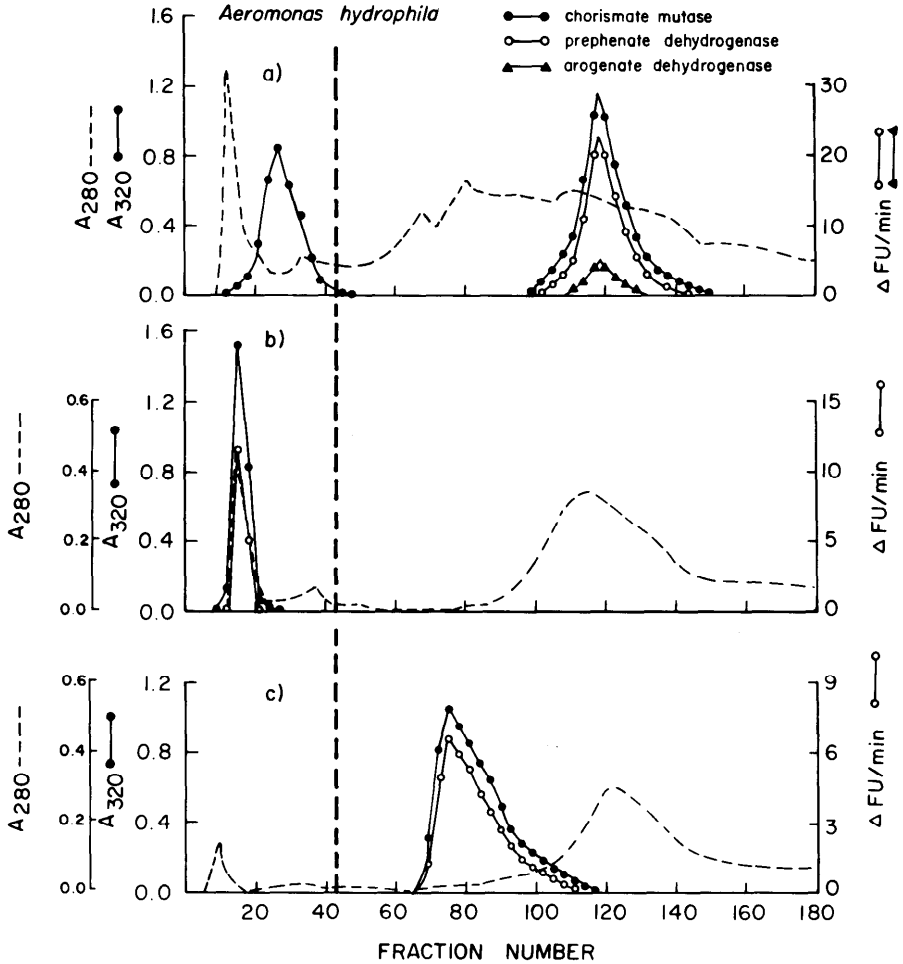

FIG. 6.—Integrity of the T-protein from *Aeromonas hydrophila*. Symbols are as in fig. 3. Panel a), Elution profiles of chorismate mutase, prephenate dehydrogenase, and arogenate dehydrogenase following DE52 column chromatography of crude extract. The column was equilibrated and developed in buffer A. Panel b), Partially purified T-protein recovered from the DE52 column (fractions 105–135, panel a) was chromatographed on a second DE52 column (1.5 × 20.0 cm), equilibrated, and developed in buffer A containing 0.04% (v/v) Triton X-100. The elution profiles of chorismate mutase and prephenate dehydrogenase are shown. Panel c), Partially purified T-protein recovered after the first DE52 column chromatography (fractions 110–130, panel a) was chromatographed on another DE52 column (1.5 × 20.0 cm), equilibrated, and developed in buffer A containing 0.04% (v/v) Triton X-100 and 0.5 m*M* L-tyrosine. The elution profiles of chorismate mutase and prephenate dehydrogenase are shown.

The fractions (105–135) containing CM-T and CDH activities (fig. 6a) following DE52 chromatography of the crude extract from *A. hydrophila* were pooled, concentrated, dialyzed against buffer A containing 0.04% Triton X-100, and chromatographed on a second DE52 column equilibrated in buffer A containing Triton X-100. Both the CM-T and CDH activities now failed to bind to the resin, washing through the column with exact coincidence (fig. 6b). Only the activity profile obtained with prephenate is shown, but the ratio of CDH activities assayed with its two substrates remained constant.

Table 1
Effect of Triton X-100 on Chorismate Mutase and Prephenate Dehydrogenase Activities from *Aeromonas hydrophila*

Triton X-100 Concentration (v/v)	Enzyme Activities	
	Chorismate Mutase	Prephenate Dehydrogenase
0.000	100	100
0.006	105	102
0.012	100	98
0.025	65	61
0.040	56	54
0.050	ND	49

NOTE.—Enzyme activities were recovered from DE52 column fractions (fraction 118) after chromatography of the crude extract from *A. hydrophila* (fig. 6a). The enzyme activities in the absence of Triton X-100 were assigned a relative value of 100. ND. = not determined owing to turbidity.

The fractions from the second DE52 column containing CM-T and CDH activities were again pooled, concentrated, dialyzed against buffer C containing 0.04% Triton X-100, and chromatographed on a hydroxylapatite column. The CM-T and CDH activities again washed through the column with exact coincidence (data not shown).

In another experiment the effect of Triton X-100 was studied in the presence of L-tyrosine because of the effect of tyrosine on complex formation in *Oceanospirillum* (Ahmad and Jensen 1986). The fractions (110–130) following the first DE52 chromatography of the crude extracts (fig. 6a) were pooled, concentrated, dialyzed against buffer A containing 0.04% Triton X-100 and 0.5 m*M* L-tyrosine, and again chromatographed on a DE52 column equilibrated in buffer A containing 0.04% Triton X-100 and 0.05 m*M* L-tyrosine. Although the elution position was altered, the two enzyme activities again eluted together (fig. 6c).

The ratio of the chorismate mutase activity to prephenate dehydrogenase or arogenate dehydrogenase activity after the first DE52-column chromatography step remained constant following further chromatographic fractionations (except, of course, when the column was run in the presence of L-tyrosine, a feedback inhibitor of CDH but not of CM-T).

Similar data demonstrating the presence of the T-protein were obtained in representatives of virtually all other enteric bacteria. These included *Shigella, Citrobacter, Morganella, Cedecea, Kluyvera, Hafnia, Edwardsiella,* and *Yersinia.*

Sensitivity of Enzyme Activities to Inhibition by Aromatic Amino Acids

For each organism studied in this paper, the CM-T activity recognized by coelution with CDH activity was not affected by any of the aromatic amino acids or by any combination thereof (data not shown). In contrast to CM-T, both the prephenate dehydrogenase and the arogenate dehydrogenase activities of CDH were strongly inhibited by L-tyrosine in each organism studied (table 2). Although arogenate dehydrogenase exhibits an apparently greater sensitivity to inhibition by L-tyrosine than does prephenate dehydrogenase, this probably reflects an order-of-magnitude difference in K_m values for prephenate and L-arogenate since inhibition exerted by L-tyrosine is competitive. In-depth analysis of similar data for CDH from other superfamily-B

Table 2
Feedback Inhibition by L-Tyrosine of the Two Dehydrogenase Activities of CDH

Organism	Inhibition (%)	
	Prephenate Dehydrogenase	Arogenate Dehydrogenase
Serratia marcescens	66 (84)	100
Erwinia carotovora	92	100
E. herbicola	91	100
Proteus mirabilis	93	100
Aeromonas hydrophila	95	100
Alteromonas putrefaciens ...	100	100

NOTE.—CDH was recovered from DE52 column fractions (refer to column profiles shown in figs. 3, 4, 5a, and 6a). L-Tyrosine was used at a final concentration of 0.5 m*M*. The sensitivity of the dehydrogenases to L-tyrosine inhibition was studied at prephenate concentrations of 0.1 m*M*, while L-arogenate substrate concentrations for arogenate dehydrogenase were 1.0 m*M*. The value in parentheses was obtained at 1.0 m*M* L-tyrosine concentration.

organisms revealed identical K_i values for tyrosine with respect to prephenate or arogenate (Byng et al. 1985; Ahmad and Jensen 1987*a*).

The dehydrogenase activities of CDH occurred only with NAD^+ as cofactor, $NADP^+$ never satisfying the cofactor requirement.

Discussion

Significance of Bifunctional Proteins as Special Evolutionary Determinants

Bifunctional proteins undoubtedly arise in most cases via gene fusion. The efficiency afforded by channeling of an enzyme-bound intermediate and by the formation of perfectly constant ratios of sequentially acting catalytic centers supports the expectation of high selective value. The availability of phylogenetic trees now allows this expectation to be evaluated. The bifunctional P-protein is broadly distributed over two of the three gram-negative superfamilies of purple bacteria. So far, not a single instance of loss of this character state has been seen in a fairly comprehensive analysis (Ahmad and Jensen 1986). The same can be said for the more limited cluster within superfamily B that possesses the bifunctional T-protein. Within the enteric lineage a yet smaller subcluster including *Escherichia, Citrobacter, Salmonella, Klebsiella,* and *Enterobacter* possesses a gene fusion (trp[G]D) corresponding to gene products of the tryptophan pathway (Zalkin 1980). Thus, these three gene fusions can reliably place organisms in or out of clusters at three different phylogenetic levels. A gene-fusion event (bifunctional protein) can, in special cases, define order of phylogenetic branching more precisely than can the most sophisticated sequencing methods (Berry and Jensen 1988). Accordingly, multifunctional proteins will probably be among the most interesting and useful character states for tracing evolutionary histories of biochemical pathways and for defined markers of phylogenetic divergence. Additional bifunctional enzymes, for which almost no comparative data exist, are available for study. For example, *E. coli* possesses two bifunctional aspartokinase-homoserine dehydrogenase proteins (Cohen and Dautry-Varsat 1980). Whether these are of shallow or deep phylogenetic distribution is totally unknown.

The Parallel Phylogenetic Distribution of T-protein and DS-phe

Escherichia coli and *K. pneumoniae* have been known for some time to possess the T-protein (Koch et al. 1970, 1971), and we have demonstrated inevitable coincidence of elution profiles for CDH activity and CM-T activity. Since we showed (Ahmad and Jensen 1986) that a cluster of *Oceanospirillum* species, close in phylogenetic position to the enteric lineage (fig. 1), possesses separable CDH and CM-F activities that associate to form a complex under special conditions, we considered the possibility of apparent T-proteins within the enteric lineage that might in fact be dissociable complexes.

Within the enteric lineage *Alteromonas putrefaciens* and *Aeromonas hydrophila* were among the first to diverge within the enteric bacteria after the latter's divergence from the *Oceanospirillum* cluster (Woese et al. 1985). Thus, these two were the organisms of choice for in-depth studies to determine whether the two component activities could be separated either following additional chromatographic steps or by treatment with detergents. The results strongly supported the existence of the bifunctional T-protein in both *A. hydrophila* and *A. putrefaciens. Alteromonas putrefaciens* was originally classified as *Pseudomonas putrefaciens* but was later placed with other marine bacteria in the genus *Alteromonas* (Lee et al. 1977). The correctness of this assignment has been demonstrated by sequencing methodology (Woese et al. 1985) and by the presence of the bifunctional T-protein of tyrosine biosynthesis (in the present paper) and the presence of DAHP synthase-phe (Ahmad et al. 1987). These two character states are not found in any of the pseudomonad groupings (Byng et al. 1980; Whitaker et al. 1980) but are present throughout the enteric lineage.

Figure 1 shows the distribution of the bifunctional T-protein of L-tyrosine biosynthesis within major lineages of superfamily B. The T-protein is present throughout the enteric lineage and extends at least as far as *A. putrefaciens.* On the other hand, group I/group V pseudomonads and *Acinetobacter* species lack the T-protein and possess a monofunctional prephenate (cyclohexadienyl) dehydrogenase (Jensen 1985). *Oceanospirillum* species represent a seemingly intermediate state in the sense that separable CM-F and CDH enzymes can form a complex in the presence of L-tyrosine (Ahmad and Jensen 1986).

The enteric lineage differs from the *Oceanospirillum* cluster in the acquisition of DAHP synthase-phe and the formation of a bifunctional T-protein (Ahmad and Jensen 1986; Ahmad et al. 1986). The T-protein presumably evolved by the fusion of CDH and CM-F. This is supported by the observations that the dehydrogenase activity exhibits substrate recognition for prephenate, L-arogenate, and other structurally related compounds, irrespective of whether the dehydrogenase activity is associated with a monofunctional protein or with the bifunctional T-protein (Byng et al. 1985; Ahmad and Jensen 1987*a*, 1987*b*). Additionally, since both CM-F and CM-T are not controlled by feedback inhibition (in contrast to CM-P), the possibility that CM-T arose directly from CM-P is unlikely.

Possible Mechanisms for Origin of T-protein and DS-phe

A simple scenario for the evolution of the T-protein is the fusion of side-by-side genes encoding CM-F and CDH enzymes (Jensen 1985). This predicts the absence of CM-F in organisms having an evolved T-protein. However, the unexpected existence of CM-F in *Serratia, Salmonella,* and *Erwinia* species (organisms having both CM-P and CM-T) indicates that a third isozyme of chorismate mutase was generated prior

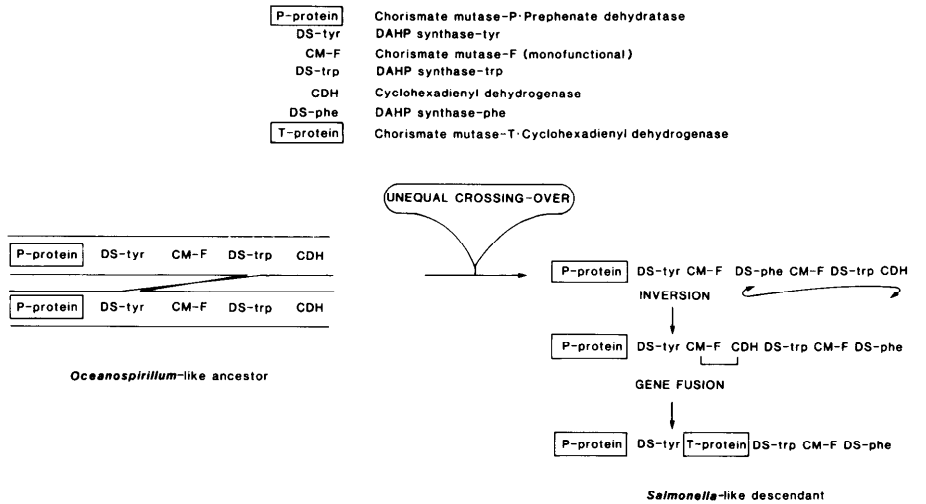

FIG. 7.—Hypothetical scheme accounting for the evolutionary origin of both the DAHP synthase-phe isozyme and the bifunctional T-protein within the enteric lineage. Enzymic gene products (upper right) are assigned the cistron designations shown on the upper left. Illustrated on the left of the arrow is a mechanism of unequal crossing-over (Riley 1984) that generates both a deletion lacking CM-T (not shown) and the gene duplication illustrated at the right of the arrow. The duplication results in the addition of both a hybrid gene (crossover between homologous genes specifying DS-trp and DS-tyr) now encoding the newly arisen DS-phe isozyme and a new gene encoding a second isozyme species of CM-F. After evolution of the T-protein, the remaining gene for CM-F, still expressed in *Serratia, Erwinia,* and *Salmonella,* has commonly been lost or become cryptic within the enteric lineage.

to (or at the time of) the gene-fusion event producing the T-protein. The fusion of cistrons for chorismate mutase and CDH via transposon migration of the chorismate mutase gene is an attractive mechanism to explain the simultaneous presence of CM-T and CM-F because gene duplication of CM-F, gene fusion (to give the CM-T component of the T-protein), and gene dispersal (to give CM-F) all could follow from a single molecular event (Riley 1984).

Another mechanism that could have simultaneously generated a third 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthase gene as well as a third chorismate mutase gene is that of unequal crossing-over. The hypothetical scheme shown in figure 7 begins with an arrangement of genes in an *Oceanospirillum*-like ancestor. *Oceanospirillum* species, a phylogenetic cluster close to the enteric lineage, lack both the bifunctional T-protein and phenylalanine-inhibited DAHP synthase isozyme (DS-phe). It seems likely that DS-phe arose after the relatively ancient tyrosine-inhibited DAHP synthase isozyme (DS-tyr) and tryptophan-inhibited DAHP synthase isozyme (DS-trp) isozymes had evolved their allosteric specificities. All three isozymes are homologous, but DS-phe exhibits a surprisingly greater similarity to DS-trp than to DS-tyr (Shultz et al. 1981; Hudson and Davidson 1984). In view of the close structural similarity of phenylalanine and tyrosine, it seems easier to produce DS-phe by duplicating the DS-tyr gene and modifying its allosteric site than by duplicating the DS-trp gene and modifying its allosteric site. This consideration can be addressed by considering the unequal crossing-over mechanism shown in figure 7. A newly evolved hybrid gene (DS-tyr/DS-trp) in which the major portion encoded the catalytic site originating from DS-trp and in which a minor portion encoded the regulatory site originating from DS-tyr could have yielded DS-phe in a single step. Fortuitous con-

formational alterations of the hybrid-gene enzyme (DS-phe) might even have enhanced binding and inhibition by L-phenylalanine in concert with decreased binding and inhibition by L-tyrosine, prior to further steps of selective change. The mechanism shown in figure 7 exemplifies an alternative to transposon migration of the CM-F cistron to account for the generation of a third chorismate mutase gene. The chromosomal organization for genes specifying the P-protein (*phe*A), DS-tyr (*aro*F), and T-protein (*tyr*A) is synonymous with the contiguous map positions of these genes demonstrated in *S. typhimurium* (Sanderson and Roth 1983). As the genetic-map positions of these genes become determined in other organisms on the phylogenetic tree, it will become possible to deduce the validity of these mechanisms or others.

The evolutionary origin of both the T-protein and DS-phe on a common lineage may represent a case of biochemical coevolution. In *Oceanospirillum* the preferential channeling of late-pathway intermediates to phenylalanine is apparently balanced by early-pathway control exerted by tyrosine (on DS-tyr). This ensures that favored endogenous metabolic flow to phenylalanine will not trigger starvation of precursors needed for tyrosine biosynthesis. With the eventual evolutionary symmetry of a bifunctional T-protein to compete equally with the bifunctional P-protein for chorismate, the coevolution of a third end product–sensitive isozyme of DAHP synthase (DS-phe) would promote balanced participation of each amino acid end product in early-pathway control.

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