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An extreme-halophile archaebacterium possesses the interlock type of prephenate dehydratase characteristic of the Gram-positive eubacteria

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Abstract. The focal point of phenylalanine biosynthesis is a dehydratase reaction which in different organisms may be prephenate dehydratase, arogenate dehydratase, or cyclohexadienyl dehydratase. Gram-positive, Gram-negative, and cyanobacterial divisions of the eubacterial kingdom exhibit different dehydratase patterns. A new extreme-halophile isolate, which grows on defined medium and is tentatively designated as *Halobacterium vallismortis* CH-1, possesses the interlock type of prephenate dehydratase present in Gram-positive bacteria. In addition to the conventional sensitivity to feedback inhibition by L-phenylalanine, the phenomenon of metabolic interlock was exemplified by the sensitivity of prephenate dehydratase to allosteric effects produced by extra-pathway (remote) effectors. Thus, L-tryptophan inhibited activity while L-tyrosine, L-methionine, L-leucine, and L-isoleucine activated the enzyme. L-Isoleucine and L-phenylalanine were effective at μM levels; other effectors operated at mM levels. A regulatory mutant selected for resistance to growth inhibition caused by β-2-thienylalanine possessed an altered prephenate dehydratase in which a phenomenon of disproportionately low activity at low enzyme concentration was abolished. Inhibition by L-tryptophan was also lost, and activation by allosteric activators was diminished. Not only was sensitivity to feedback inhibition by L-phenylalanine lost, but the mutant enzyme was now activated by this amino acid (a mutation type previously observed in *Bacillus subtilis*). It remains to be seen whether this type of prephenate dehydratase will prove to be characteristic of all archaebacteria or of some archaebacterial subgroup cluster.

Key words: Archaebacteria – Extreme halophiles – *Halobacterium vallismortis* – Phenylalanine biosynthesis – Regulatory mutants – Prephenate dehydratase – Metabolic interlock

The biochemical pathway employed in nature for biosynthesis of aromatic amino acids exhibits considerable diversity with respect to such character-state features as: alternative enzymic steps which may be used for phenylalanine and/or tyrosine synthesis, specificity of tyrosine-pathway dehydrogenases for NAD^+ or $NADP^+$, allosteric specificities, presence or absence of regulatory isozymes, presence or absence of multifunctional proteins, and genetic organization of pathway genes (Byng et al. 1982; Jensen and Fischer 1987; Ahmad and Jensen 1988). The diversity of character states present in contemporary organisms is ideal with respect to the objective of deducing the most probable ancestral state representing a phylogenetic cluster (Jensen 1985). Among the eubacteria, much information about aromatic biosynthesis and regulation is available about Gram-negative bacteria, Gram-positive bacteria and cyanobacteria. In contrast, little if any enzymological data about aromatic biosynthesis has been published for even a single archaebacterial organism.

An extreme halophile was of interest as the initial archaebacterial representative for study because: (i) a halobacterial strain became available that would grow in a defined medium and that would be amenable to the approach of mutant selection, and (ii) extremely halophilic archaebacteria have been argued by some (Lake et al. 1985) to be nearer to eubacteria than are any of the other archaebacteria.

The dehydratase reaction(s) of phenylalanine biosynthesis seemed an apt choice for initial focus in strain CH-1 because the three aforementioned eubacterial groups exhibit distinctive patterns of dehydratase evolution. Thus, cyanobacteria characteristically possess a prephenate-specific dehydratase which is subject to feedback inhibition by L-phenylalanine (Hall et al. 1982). Gram-positive bacteria so far studied (Berry et al. 1987) all possess a prephenate-specific dehydratase that is activated by "remote" effectors (hydrophobic amino acids not directly connected with aromatic biosynthesis). In Gram-negative prokaryotes (Ahmad and Jensen 1988) evolution of phenylalanine biosynthesis has been quite dynamic. These organisms may synthesize phenylalanine via a prephenate-specific dehydratase, an arogenate-specific dehydratase or a cyclohexadienyl dehydratase. Simultaneously present dehydratases are also common, particularly the combination of prephenate dehydratase and cyclohexadienyl dehydratase.

Materials and methods

Isolation, growth and maintenance of strain CH-1. An extreme halophile, designated strain CH-1, was isolated from a saltern located in the San Francisco bay area by selection for ability to grow on a defined medium (below), and was clonally purified by single-colony isolation. Strain CH-1 was maintained by monthly transfer to a solidified minimal salts

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medium (HSM) modified from that originally described by Rodrigeuz-Valera et al. (1980). For 1 l of HSM basal salts, 200 g NaCl, 4 g KCl, 6 g Tris, and 36 g $MgSO_4 \cdot 7\,H_2O$ were dissolved in distilled water, adjusted to pH 7.2, and autoclaved. After cooling to 55° C, 0.0005% (w/v) $FeSO_4 \cdot 7\,H_2O$ [0.25% (w/v) in 0.001 N HCl]; 0.01% (w/v) K_2HPO_4 [5% (w/v) in distilled H_2O]; 0.1% (w/v) NH_4Cl [20% (w/v) in distilled H_2O]; 0.1% (w/v) $CaCl_2 \cdot 2\,H_2O$ [14.7% (w/v) in distilled H_2O]; and 0.5% (w/v) sodium pyruvate [12.5% (w/v) in basal salts] were added. All stock solutions were autoclaved except for pyruvate and ferrous sulfate which were filter-sterilized by passage through a Nalgene filtration apparatus (Nalge Co., Rochester, NY, USA). For solidified medium, 20 g of agar were added per liter of HSM basal salts. Isolated colonies were clearly visible on agar surfaces of sealed Petri plates following 6 days of incubation at 37° C.

Batch cultures and the preparation of crude extracts. A 25-ml volume of a turbid culture was used to inoculate 500 ml of HSM medium in a 2-l Erlenmeyer flask, and batch cultures were incubated at 37° C for 3–4 days with vigorous shaking until an optical density of 280 Klett units (using a no. 49 blue filter) was reached. Cells were collected by centrifugation at 7,000 rpm for 10 min at 4° C using a RC-5B preparative centrifuge (Sorvall Instruments, Wilmington, DE, USA) equipped with a GS-3 rotor. Alternatively, batch cultures of strain CH-1 (in 10 l of HSM medium) were grown at 37° C in a 15-l carboy and sparged vigorously with humidified air. Fermenter cultures were concentrated to 1 l by use of an Amicon cell-harvester (Amicon Corporation, Danvers, MA, USA), and cells were collected by centrifugation as above.

Cell pellets were resuspended in an equal volume of 4 M NaCl, 50 mM Tris-HCl (pH 7.2), and were disrupted by either sonicating for 30 s with a Lab-Line ultratip labsonic system (Lab-Line Instruments, Melrose Park, IL, USA) operating at 50% of maximum power, or by twice passing the resuspended pellet through a French pressure cell press (Aminco, SLM Instruments, Inc., Urbana, IL, USA) at 103,421 kN/m^2. If extracts were highly viscous, DNAse was added to a final concentration of 100 µg/ml followed by stirring for 1 h at 4° C. Extracts were then centrifuged at 45,000 rpm for 120 min at 4° C in a Beckman L5-65 ultracentrifuge equipped with a Ti50 rotor. Cell-free supernatants were carefully removed and either dialyzed or passed through a Sephadex G-25 column previously equilibrated with extraction buffer. The resulting crude extract, free of small molecular-weight contaminants, served as the source of protein for all experiments in this study. Enzyme extracts were routinely stored between 0–4° C.

Enzyme assays. Prephenate dehydratase was assayed according to Fischer and Jensen (1987a). Standard reaction mixtures contained 2.0 mM barium prephenate, 4 M NaCl, 50 mM Tris-HCl (pH 7.2), and enzyme in a final volume of 200 µl. Incubations were routinely carried out for 20 min at 37° C, and the reaction was terminated by addition of 800 µl of 2.5 N NaOH. Activities were quantified by relating phenylpyruvate absorbance at 320 nm to a molar extinction coefficient of 17,500 (Cotton and Gibson 1965). Protein concentrations were determined by the method of Bradford (1976) using standard curves constructed with fraction V bovine serum albumin as the reference protein.

Biochemicals. Barium prephenate preparations (74–92% pure and essentially free of organic impurities) were obtained from culture supernatants of a tyrosine auxotroph of *Salmonella typhimurium* (Dayan and Sprinson 1970). BSA (fraction V), amino acids, β-2-(D,L)-thienylalanine, and sodium pyruvate were obtained from Sigma Chemical Co., (St. Louis, MO, USA). Agar was obtained from Difco Laboratories (Detroit, MI, USA). All chemicals were reagent-grade or better.

Results

Properties of the halobacterial isolate

Strain CH-1 proved to have the characteristics of the genus *Halobacterium*. The optimal salt concentration range for growth (20–30% NaCl) is typical (Bayley and Morton 1978) of extreme halophiles, and pink pigmentation is apparent in healthy cultures or colony populations. Colonies are round, regular and non-mucoid. Strain CH-1 lacks complex nutritional requirements and should prove to be a useful organism for genetic and physiological studies. Biochemical characterization done at American Type Culture Collection (R. Gherna, personal communication) and analysis of lipid composition (B. Tyndall, personal communication) suggest a tentative designation of strain CH-1 as a member of *Halobacterium vallismortis*. This culture has been deposited at ATCC as ATCC 34679.

Liquid cultures of strain CH-1 grown at 37° C in minimal salts medium with vigorous aeration exhibited a doubling time of about 10 h. The presence of aromatic amino acids, supplemented singly or in combination, did not affect the rate of growth. The phenylalanine analog, β-2-thienylalanine (Ta), was a very effective antimetabolite, with 50 µg/ml concentrations allowing only about one doubling in a growth experiment conducted as shown in Fig. 1. The exclusive action of Ta as an antimetabolite mimic of L-phenylalanine was demonstrated by the ability of L-phenylalanine to completely prevent growth inhibition by Ta.

The wild-type prephenate dehydratase

High salt concentrations (above 2.0 M NaCl) were needed for maximal stability of prephenate dehydratase (Table 1). Maximal enzyme activity required 3 M NaCl. Concentrations of 2 M and 1 M NaCl produced 80% and 55% of maximal activity, respectively. Enzyme extracts were usually prepared in 4 M NaCl, and assays were routinely carried out in the presence of 4 M NaCl. KCl was no more effective for catalysis than NaCl. The effects of allosteric effectors (see below) in the presence of NaCl were also compared to effects in the presence of KCl. Under conditions where maximal and half-maximal allosteric effects were obtained in the presence of NaCl, no differences exceeding the error of the assay were obtained in the presence of KCl. Strain CH-1 prephenate dehydratase possessed a broad pH optimum in the range of 7.0 to 7.5, and reaction mixtures were routinely buffered at pH 7.2. Activities at pH 6.2 and 9.0 were 86% and 65%, respectively, of that obtained at pH 7.2. No activity of arogenate dehydratase, a phenylalanine-pathway enzyme commonly found in eubacteria as either an arogenate-specific dehydratase or as a broad-specificity

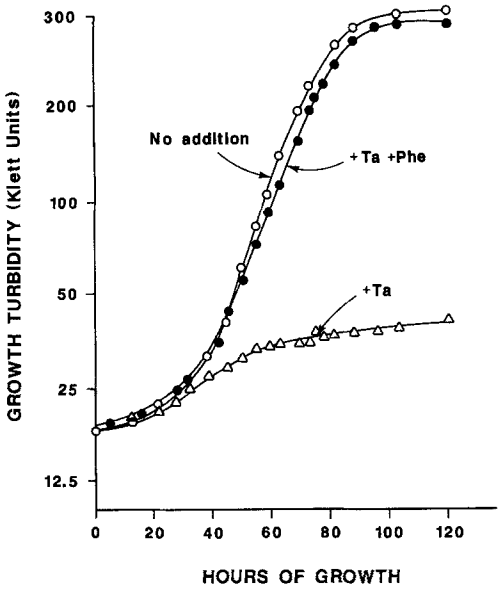

Fig. 1. Reversal by L-phenylalanine of Ta-mediated growth inhibition in strain CH-1. A starter culture was grown in HSM minimal salts to a turbidity of 330 Klett units and used as a common inoculum for four cultures. For clarity, data are not shown for growth of a culture supplemented with 50 µg/ml of L-phenylalanine; growth of this culture followed the same curves as the "*No addition*" control or the culture supplemented with 50 µg/ml of Ta plus 50 µg/ml of L-phenylalanine (*Phe*). Cultures were grown in volumes of 10 ml contained within 125-ml flasks fitted with sidearms made to fit the Klett aperture. All additions indicated were made at 50 µg/ml, and cultures were incubated in the dark at 37°C in a gyratory shaker operating at 300 rpm

Table 1. Dependence of prephenate dehydratase stability upon salt concentration in strain CH-1

Storage duration (h)	Relative activity as a function of NaCl concentration during storage over ice		
	2.0 M NaCl	0.1 M NaCl	No NaCl
0	100	100	98
5	100	77	60
10	100	51	34
20	100	24	11
40	100	5	1

Crude extracts were prepared as described under Methods. NaCl was removed by gel filtration (Sephadex G-25). This preparation was split into three portions and equal volumes of buffer, appropriately made up to give the final concentration of NaCl indicated, were added to each. A relative activity of 100 equals a specific activity or 16 nmol/min · mg of extract protein. Enzyme activity was assayed under standard assay conditions as detailed in Methods with 60 µg of extract protein used in the presence of 4 M NaCl

cyclohexadienyl dehydratase (Fischer and Jensen 1987b), was observed.

All three aromatic amino acids (L-phenylalanine, L-tyrosine, and L-tryptophan) and L-isoleucine, L-leucine and L-methionine proved to be allosteric effectors for strain CH-1 prephenate dehydratase (Fig. 2). None of the remaining common amino acids affected the activity of prephenate

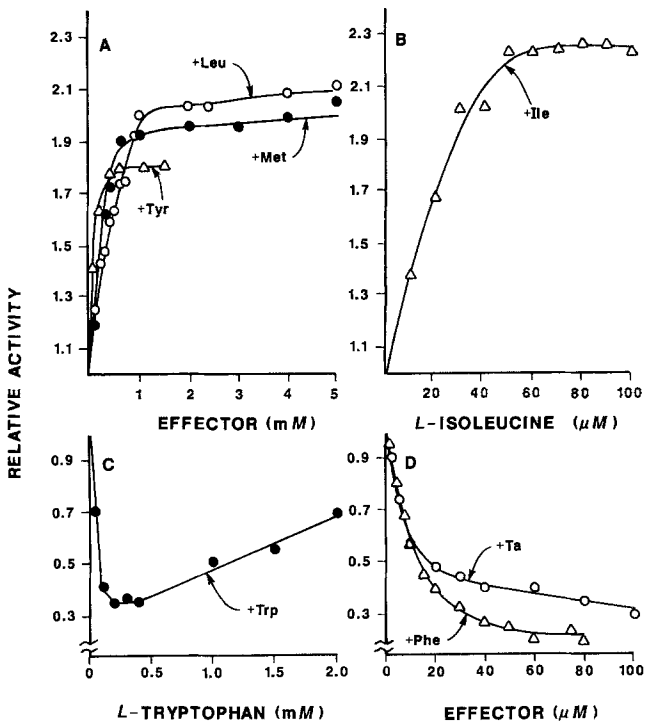

Fig. 2A–D. Allosteric activation **A** and **B** and inhibition **C** and **D** of prephenate dehydratase from wild-type strain CH-1. Reaction mixtures (200 µl) contained 4 M NaCl, 50 mM Tris-HCl buffer at pH 7.2, 2 mM prephenate, and effector molecules as indicated. Reactions were initiated by the addition of 50 µg of crude-extract protein and incubated at 37°C for 20 min. Each curve represents the results of separate experiments where the activities shown are relative to the specific activity measured in the absence of effector molecules. A relative activity of 1.0 is equal to a specific activity of 14.0 nmol/min · mg crude-extract protein. Additional controls included reaction mixtures lacking substrate, and complete-assay mixtures where the extract was added following the addition of NaOH. *Abbreviations:* L-leucine, *Leu*; L-methionine, *Met*; L-tyrosine, *Tyr*; L-isoleucine, *Ileu*; L-tryptophan, *Trp*; β-2-thienylalanine, *Ta*; and L-phenylalanine, *Phe*

dehydratase. The most potent allosteric activator was L-isoleucine (panel B), highly effective at µM levels. The next most effective activator molecule was L-tyrosine (panel A), although the maximal effect (1.8-fold at saturating substrate concentration) was less than that found for any of the other activators. L-Methionine and L-leucine were required at mM concentrations to produce maximal activation (panel B), L-methionine being somewhat more effective than L-leucine at lower concentrations. L-Tryptophan produced a complex curve (panel C) which indicates a mixture of activating and inhibitory effects. An inhibition of about 65% was observed at 0.2 mM L-tryptophan. At higher concentrations of L-tryptophan progressively less inhibition was obtained. This suggests that tryptophan binds to both inhibitor and activator sites, higher concentrations being required to saturate the activator site(s) than the inhibitor site(s). Both L-phenylalanine and Ta were inhibitors of prephenate dehydratase, being effective at µM concentrations (panel D).

The ability of a combination of inhibitor (L-phenylalanine) and activator (L-methionine) to antagonize one another was evaluated in an experiment (Fig. 3) where activity was followed as a function of reaction time for 20 min. L-Methionine was added at a concentration producing half

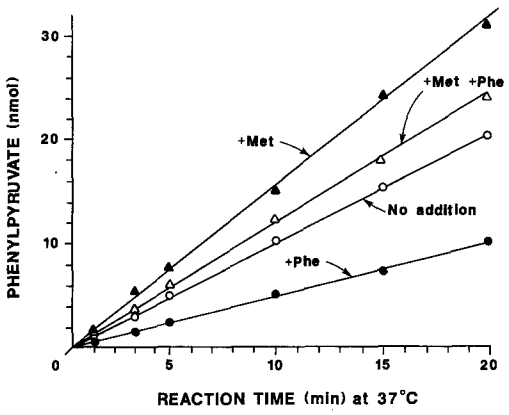

Fig. 3. Progress curves obtained in the presence or absence of allosteric activators or inhibitors. Reaction mixtures in 10 × 100 mm borosilicate tubes contained in a volume of 1.6 ml: 2 mM prephenate, 4 M NaCl, 50 mM Tris-HCl buffer at pH 7.2, and (where indicated) L-phenylalanine (*Phe*) at 12.5 µM and L-methionine at 0.25 mM. Reactions were initiated by addition of 640 µg of protein (at 37° C) to a mixture of the other components previously brought to 37° C. At the sampling times indicated, 0.2 ml of reaction mixture was added to 0.8 ml of 2.5 N NaOH and absorbance of phenylpyruvate at 320 nm was recorded. Controls lacking substrate or enzyme were monitored in parallel with each sample

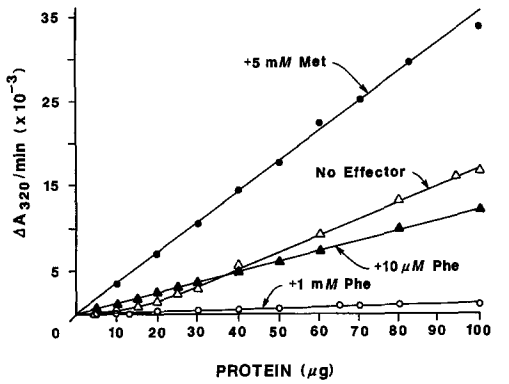

Fig. 4. Effect of allosteric effectors upon disproportional relationship of reaction velocity (*ordinate*) and enzyme concentration (*abscissa*) for prephenate dehydratase from wild-type strain CH-1. Assay mixtures (200 µl) contained 4 M NaCl, 50 mM Tris-HCl buffer at pH 7.2, 2 mM prephenate, and crude-extract protein as indicated. Reactions were initiated by addition of substrate and incubated at 37° C for 20 min. An absorbance of 1.0 at 320 nm corresponds to 57.1 nmol of phenylpyruvate

maximal activation, while L-phenylalanine was present at a concentration producing 50% inhibition (see Fig. 2). Under these conditions the mixture produced a progress curve exhibiting an overall activation of about 10%. The progress curves obtained in the absence or presence of either inhibitor or activator molecules were uncomplicated, linear progressions.

Reaction velocity was a proportional function of enzyme concentration only when reaction mixtures contained more than about 40 µg of crude-extract protein (Fig. 4). Addition of bovine serum albumin did not alter this non-proportionality. However, activator molecules produced linear relationships between reaction velocity and protein concentration as illustrated with L-methionine in Fig. 4. Effectively, activation becomes dramatic if low enzyme concentration is used. Unexpectedly, the inhibitor molecule, L-phenylalanine, also abolished non-proportionality. This was sufficiently effective that at low phenylalanine concentrations, activation rather than inhibition could be demonstrated, i.e., under these conditions the positive impact of abolishing non-proportionality more than offset the extent of feedback inhibition. In contrast to other effectors, L-tryptophan failed to alter the sigmoid shape of the velocity vs. enzyme concentration curve.

Isolation of regulatory mutants

Since Ta behaves as a specific antimetabolite of phenylalanine and mimics phenylalanine as a feedback inhibitor of prephenate dehydratase, a convenient basis was available for selection of analog-resistant mutants – some of which were expected to be regulatory mutants having altered allostery or altered levels of prephenate dehydratase. About 0.1 ml of a fully grown liquid culture (turbidity about 300 Klett units) was spread on the surface of a Petri plate containing 40 ml of solidified HSM medium to produce a uniform confluent cell lawn. When the agar surfaces were sufficiently dry, a small portion of Ta crystals was applied to the center of the agar surface. The Petri plates were sealed and incubated in the dark at 37° C. The gradient of diffusing Ta produced inhibition of growth throughout the plated area. Each plate eventually produced a number of circular, slightly convex and glistening colonies of about 1 mm diameter. Some of these were surrounded by a halo of background growth. The latter type of mutant proved to be phenylalanine excretors which could confer resistance to the surrounding halo of wild-type cells by cross-feeding. A number of such colonies were picked and transferred to 1 ml of HSM medium containing 50 µg/ml of Ta and were aerated with vigorous shaking at 37° C for 3 days. Then 0.1 ml of each culture was serially diluted prior to plating out on solid HSM medium containing 50 µg/ml of Ta. Four additional rounds of single-colony isolation under selective conditions were carried out to ensure clonal purity.

One mutant, labelled Ta-75, was the most dramatic excretor of phenylalanine as judged by cross-feeding ability and was chosen for further study. The growth rate of mutant Ta-75 was similar to that of wild type as shown in Fig. 5. The growth curve obtained for mutant Ta-75 in the presence of Ta was similar to that obtained in the absence of Ta, at least in early-exponential growth. A progressive decline in growth rate appeared to occur later. This may reflect an effect of Ta on some target other than prephenate dehydratase, e.g., incorporation of Ta into "false" proteins.

Prephenate dehydratase of mutant Ta-75 differed from wild type in a number of properties. (i) The level of enzyme (specific activity) was about 2-fold higher than in crude extracts from wild type. (ii) The relationship of reaction velocity to enzyme concentration was proportional, even in the low-protein concentration range (see Fig. 6 in comparison with Fig. 4). (iii) Although activator molecules retained ability to stimulate activity, the magnitude of the effect was less as illustrated with L-leucine in Fig. 6. (iv) Not only did L-phenylalanine fail to inhibit, but it activated the Ta-75 enzyme modestly (about 14%). Such mutants having altered prephenate dehydratase enzyme subject to activation by L-phenylalanine rather than inhibition have been described by Coats and Nester (1967) in *Bacillus subtilis*. (v)

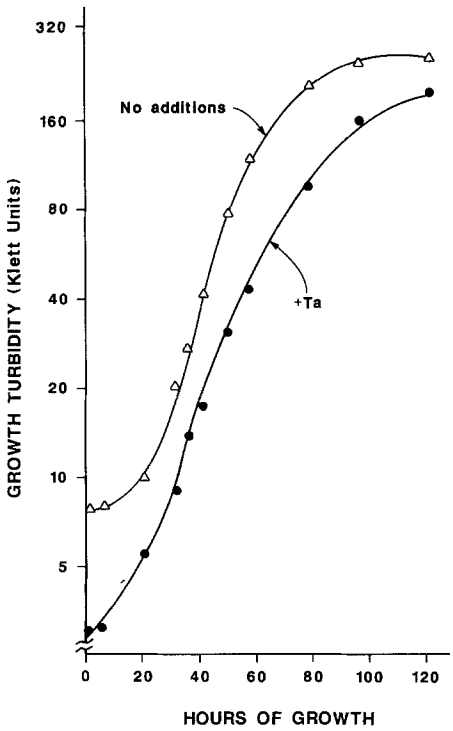

Fig. 5. Resistance of mutant Ta-75 to growth inhibition by Ta in liquid medium. Starter cultures at about 300 Klett units were used to inoculate two 10-ml cultures contained in a 125-ml flask fitted with sidearm tubes. Ta was present at a final concentration of 50 μg/ml in one culture as indicated. Other conditions are specified in the legend of Fig. 1

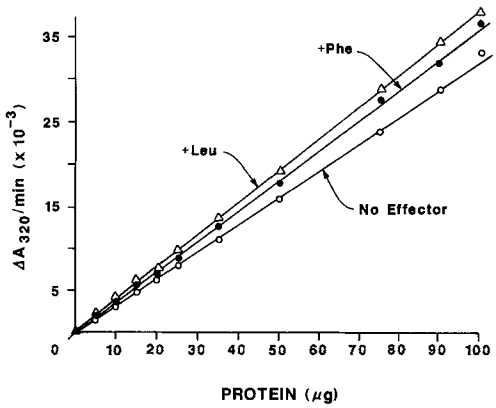

Fig. 6. Relationship of reaction velocity (*ordinate*) and enzyme concentration (*abscissa*) in the presence and absence of allosteric effectors, L-leucine (*Leu*) and L-phenylalanine (*Phe*) for prephenate dehydratase from mutant Ta-75. See legend of Fig. 4 for details of the assay protocol followed. An absorbance of 1.0 at 320 nm corresponds to 57.1 nmol of phenylpyruvate

Allosteric effects of L-tryptophan were completely abolished.

Discussion

Metabolic interlock

Interlocking relationships of allosteric regulation (metabolic interlock) exist as a supra-pathway level of control whereby metabolite molecules outside a given pathway exert allosteric effects of either inhibition or activation. The prephenate dehydratase of strain CH-1, like the typical microbial prephenate dehydratase, binds the endproduct L-phenylalanine as a potent feedback inhibitor. Such effectors have been termed primary effectors to distinguish them from remote effectors (allosteric agents of metabolic interlock which are formed from seemingly unconnected pathways). The inhibition of prephenate dehydratase by L-tryptophan and the allosteric activation produced by either L-methionine or L-leucine in *Bacillus subtilis* was one of several examples of inter-pathway regulation originally described and named metabolic interlock (Jensen 1969). Detailed enzymological studies have proceeded with the *B. subtilis* enzyme (Rebello and Jensen 1970; Pierson and Jensen 1974; Riepel and Glover 1979). Extra-pathway effectors were shown to act in vivo as well as in vitro by Jensen (1969), and it is interesting to note that the inhibitory effect of L-tryptophan upon L-phenylalanine synthesis has played a key role in biotechnological strategies employed recently to isolate superior tryptophan excretors in *B. subtilis* (Kurahashi et al. 1986). The molecular-weight interconversion mediated by remote effectors has also played an important role in ingenious methodologies developed to purify the enzyme (Riepl and Glover 1978).

Regulatory relationships of metabolic interlock have been interpreted in terms of a mechanism that maintains a balance of hydrophobic amino acids for optimal protein synthesis (Jensen 1969). In addition to *Bacillus*, the closely related *Staphylococcus* and *Streptococcus* genera possess the interlock type of prephenate dehydratase (Jensen, unpublished data). Fazel and Jensen (1980) showed that Gram-positive coryneform bacteria such as *Brevibacterium* possess the interlock type of prephenate dehydratase. Recently Berry et al. (1987) showed that mycoplasma (*Acholeplasma laidlawii*) also possesses an interlock type of prephenate dehydratase. These divergent genera of Gram-positive bacteria differ significantly from one another in the enzymic arrangement for L-tyrosine synthesis as well as in the pattern of allosteric control employed for DAHP synthase. Thus, it would appear that interlock control of prephenate dehydratase has been highly conserved (implying selective value) and at a deep hierarchical level (implying ancient origin).

Prephenate dehydratase in a phylogenetic context

Woese (1973), Ycas (1974) and Jensen (1976) have discussed the rudimentary translation machinery that primitive cells must have possessed, and the vulnerability of this machinery to errors. In this connection it is noteworthy that erroneous substitution of hydrophobic amino acids (especially L-leucine and L-isoleucine) for L-phenylalanine is the most common example of translation infidelity in eubacteria. Similar results have been demonstrated by White and Bayley (1972) for extreme-halophile systems. If archaebacteria generally prove to possess the interlock type of prephenate dehydratase, this may reflect the conservation of an ancient substrate-balancing mechanism (for protein synthesis). If so, the presence of this character state in the Gram-positive eubacteria may indicate that the Gram-positive division diverged earliest of the eubacterial divisions. Perhaps a common ancestor of the latter divisions lost the primitive balancing mechanism in the aftermath of other fine-tuning

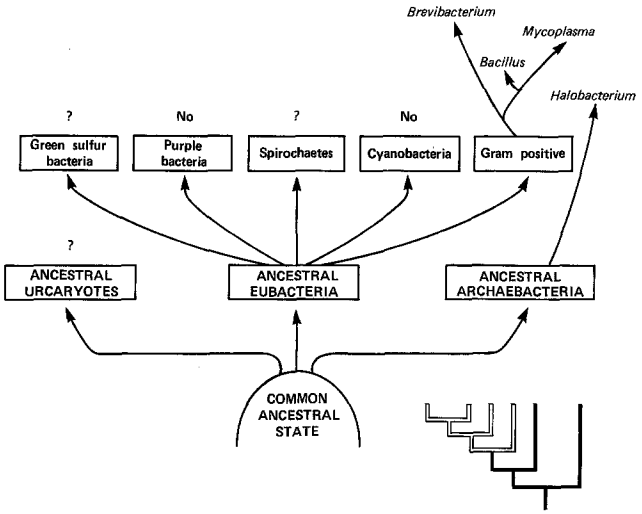

Fig. 7. The three phylogenetic kingdoms defined by Woese (1987). Extreme halophiles are members of the archaebacteria (but see Lake 1985). Five major divisions of contemporary eubacteria are shown. Of these the Gram-positive grouping possesses the interlock type of prephenate dehydratase. The cyanobacteria and Gram-negative purple bacteria do not possess the interlock type of prephenate dehydratase, and green sulfur bacteria and spirochaetes have not yet been studied. The dendrogram at the lower right illustrates a speculative order of branching for eubacterial divisions whereby Gram-positive bacteria diverged at the deepest position – closest to the archaebacterial lineage on the far right. Thus, the Gram-positive eubacterial and archaebacterial lineages are represented by solid lines

mechanisms in the evolution of a more precise translation mechanism. This scenario is indicated by the dendrogram inset at the lower right of Fig. 7.

It should be mentioned that Lake et al. (1985) have presented an evolutionary tree which places the halobacteria with the eubacteria, rather than with the archaebacteria. If this scheme is correct (although it does not appear to be generally accepted), it is intriguing that at least one major division of accepted eubacteria (Gram-positive bacteria) and halobacteria share the character state under discussion. The analysis of the distribution of the interlock type of prephenate dehydratase throughout the archaebacterial assemblage should be informative along these lines. In this context it is of interest that another very striking biochemical character state is shared by Gram-positive eubacteria and by extreme halophiles. *Halobacterium cutirubrum* has been shown to form glutaminyl-tRNA^{Gln} indirectly by amidation of glutamyl-tRNA^{Gln} as occurs in Gram-positive eubacteria (White and Bayley 1972).

Prospects for comparative analysis of interlock-type systems

Only the *B. subtilis* prephenate dehydratase has been purified and characterized in detail. In this system substrate (prephenate) and allosteric activators promote formation of an active octamer while inhibitors promote formation of an inactive dimer. Whether such molecular-weight interconversions occur in any of the other systems is not known, although the disproportional relationship of enzyme concentration and activity in strain CH-1 (together with proportionality in the presence of effector molecules) suggests the possibility of interconvertible forms of prephenate dehydratase.

Qualitative differences in the various interlock systems have been noted. For example, in *Bacillus subtilis* L-leucine activates as well as L-methionine (5-fold), while L-leucine failed to activate at all in both *A. laidlawii* and *Brevibacterium glutamicus*. In *Brevibacterium glutamicus* activation by L-methionine was labile to purification. In strain CH-1 L-isoleucine was an order-of-magnitude more effective than any other activators, but failed to activate at all in *Brevibacterium glutamicus*; L-isoleucine was only moderately effective in *Bacillus subtilis* and *Acholeplasma*. In *Brevibacterium glutamicus* L-tyrosine was absolutely essential for measurement of prephenate dehydratase activity. In strain CH-1 L-tyrosine was a fairly good activator, while it only activated the mycoplasma enzyme to a modest extent and produced no activation at all with the *Bacillus subtilis* enzyme. If the interlock relationships are meaningful in vivo at a general level as discussed above, it will be interesting to know the significance of particular differences in remote-effector effects with respect to their distribution in different genealogical lines of descent.

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