Arch Microbiol (1987) 149:87–94

Archives of Microbiology

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Original papers

Enzymic arrangement and allosteric regulation of the aromatic amino acid pathway in *Neisseria gonorrhoeae*

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Abstract. The pathway construction and allosteric regulation of phenylalanine and tyrosine biosynthesis was examined in *Neisseria gonorrhoeae*. A single 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthase enzyme sensitive to feedback inhibition by L-phenylalanine was found. Chorismate mutase and prephenate dehydratase appear to co-exist as catalytic components of a bifunctional enzyme, known to be present in related genera. The latter enzyme activities were both feedback inhibited by L-phenylalanine. Prephenate dehydratase was strongly activated by L-tyrosine. NAD^+-linked prephenate dehydrogenase and arogenate dehydrogenase activities coeluted following ion-exchange chromatography, suggesting their identity as catalytic properties of a single broad-specificity cyclohexadienyl dehydrogenase. Each dehydrogenase activity was inhibited by 4-hydroxyphenylpyruvate, but not by L-tyrosine. Two aromatic aminotransferases were resolved, one preferring the L-phenylalanine:2-ketoglutarate substrate combination and the other preferring the L-tyrosine: 2-ketoglutarate substrate combination. Each aminotransferase was also able to transaminate prephenate. The overall picture of regulation is one in which L-tyrosine modulates L-phenylalanine synthesis via activation of prephenate dehydratase. L-Phenylalanine in turn regulates early-pathway flow through inhibition of DAHP synthase. The recent phylogenetic positioning of *N. gonorrhoeae* makes it a key reference organism for emerging interpretations about aromatic-pathway evolution.

Key words: *Neisseria gonorrhoeae* – Biochemical evolution – Aromatic biosynthesis – Regulatory enzymes – Superfamily A – Bacterial phylogeny

Neisseria gonorrhoeae is an obligate human pathogen which can produce symptomatic or asymptomatic infections at a number of tissue sites. Catlin and co-workers developed a defined medium for growth of *N. gonorrhoeae*, and showed that the gonococci could be separated into groups on the criterion of different nutritional requirements that include amino acids, nucleic acid bases, or vitamins (Carifo and Catlin 1973; Catlin 1973). This procedure of auxotyping has provided a tool for epidemiological investigations, for example in the study of isolates from patients with disseminated gonococcal infection (Knapp and Holmes 1975) or inapparent male infection (Crawford et al. 1977). The biochemical and enzymological basis for most auxotype diversity is unknown. Another nutritional phenomenon of interest along these lines is that of metabolite sensitivity, a case in point being phenylalanine-mediated growth inhibition (Hendry and Dillon 1984). Basic enzymological data about aromatic biosynthesis in *N. gonorrhoeae* are of further interest. Since the mammalian host lacks a biochemical pathway for aromatic biosynthesis, potential enzyme targets of selective antimicrobial action exist in *N. gonorrhoeae*.

The enzymic arrangement and regulation of aromatic biosynthesis is highly variable in prokaryotes (Byng et al. 1982), and a sense of context would be provided if biochemical information were available about aromatic biosynthesis in *N. gonorrhoeae* and its close relatives. Fortunately, Woese (1987) has pinpointed the phylogenetic position of *N. gonorrhoeae* within one of three subgroups (subgroup β-3) making up the β-purple bacteria (Superfamily A). Some information about aromatic biosynthesis has been obtained for organisms within subgroup β-1 (e.g., *Pseudomonas testosteroni*, *P. acidovorans*) and for organisms within subgroup β-2 (e.g., *P. cepacia*, *Alcaligenes eutrophus*) as summarized by Byng et al. (1983). Companion members with *N. gonorrhoeae* in subgroup β-3 (*Spirillum*, *Nitrosomonas*, *Nitrosococcus* and *Nitrosolobus*) have not yet been characterized with respect to aromatic amino acid biosynthesis. This study establishes *N. gonorrhoeae* as a reference point for comparison with other subgroup β-3 members and for comparison with other members of the β-1 and β-2 subgroups.

Materials and methods

Strains and microbiological procedures

Neisseria gonorrhoeae ATCC 27630 exhibits a proline auxotype. The urogenital isolate HGH 154 (Hamilton General Hospital, Hamilton, Ontario) does not require any amino acids, but is sensitive to growth inhibition in the presence of L-phenylalanine. Neither isolate produces β-lactamase. ATCC 27630 and HGH 154 were grown confluently on defined agar medium containing 0.05% fatty acid-free bovine serum albumin (Hendry 1983). Duration of incubation in 5% CO_2 at 36° C was 18 h, after which the cell mass was removed from the agar surface, pooled in 1–3 g

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amounts on parafilm, sealed and frozen at −70°C immediately.

Extract preparation

Frozen cell pellets were thawed at room temperature and suspended in 5−10 ml of extract buffer. The extract buffer used was 50 m*M* K phosphate, pH 7.0, containing 1.0 m*M* D,L-dithiothreitol (DTT) (Buffer A), except in the case of extracts used for analysis of DAHP synthase. The latter extracts were prepared in 100 m*M* K phosphate, pH 7.0, containing 1.0 m*M* DTT (Buffer B). Cell disruption was achieved by passing cell suspensions through an SLM-Aminco French pressure cell (three passages at 1,266 kg/cm^2, 4°C). Cell debris was removed by ultracentrifugation at 150,000 × *g* for 1 h at 4°C, and the resulting cell-free supernatants were then desalted by passage through a 1.5 × 20 cm column of Sephadex G-25-300, using the appropriate extract buffer (see above) for equilibration and elution. These desalted preparations (hereafter referred to as crude extracts) were maintained at 4°C and were either used directly for enzymological analyses or fractionated via ion-exchange chromatography.

Ion-exchange chromatography

Crude extracts, containing up to 86 mg protein, were fractionated using 1.5 × 20 cm columns of DEAE-cellulose (Whatman DE52). Buffer A was used for equilibration and elution in all cases except for fractionation of extracts for analysis of DAHP synthase, where Buffer B was substituted. Following sample application, columns were washed with 100 ml of equilibration buffer, and bound material was then eluted with linear gradients of KCl (0 to 0.5 *M*, prepared in equilibration buffer). Fractions of 2.2 ml were collected at 4°C.

Enzyme assays

Appropriate controls were included to account for possible interference by reaction-mixture components, and care was taken to ensure that reaction velocities were proportional to the variables of protein concentration and elapsed reaction time. Protein content of assay mixtures varied from 0.3−1.2 mg per 0.2 ml. Units of enzyme activity are all expressed as nmol per min. All reactions were carried out at 37°C, and the standard time of incubation was 20 min unless stated otherwise.

DAHP synthase was assayed according to Srinivasan and Sprinson (1959) as modified by Jensen and Nester (1966). Standard reaction mixtures contained 1.0 m*M* D-erythrose-4-phosphate (E4P), 1.0 m*M* phosphoenolpyruvate (PEP), 1.0 m*M* $MnSO_4$, Buffer B, and enzyme. Chorismate mutase and prephenate dehydratase were assayed as outlined by Patel et al. (1977). Standard reaction mixtures for both assays contained a 1.0 m*M* final concentration of the appropriate substrate, Buffer A, and enzyme. For prephenate dehydratase, reaction mixtures also contained 0.5 m*M* L-tyrosine, unless indicated otherwise. Arogenate dehydratase was assayed using the high performance liquid chromatography (HPLC) method described by Zamir et al. (1985). Reaction mixtures contained 1.0 m*M* L-arogenate, Buffer A, and enzyme. Reactions were allowed to proceed for 60 min prior to *o*-phthalaldehyde derivatization and subsequent HPLC analysis. Prephenate dehydrogenase and arogenate dehydrogenase were assayed by continuous monitoring of NADH formation, using a spectrophotofluorometric assay (Byng et al. 1980). Standard reaction mixtures contained 1.0 m*M* prephenate or L-arogenate, 0.5 m*M* NAD^+, Buffer A, and enzyme.

Aminotransferase activities, when measured using the L-phenylalanine:2-ketoglutarate or L-tyrosine:2-ketoglutarate substrate combinations, were assayed using a spectrophotometric method (Patel et al. 1977). Reaction mixtures contained 10 m*M* 2-ketoglutarate, 12.5 μ*M* pyridoxal-5′-phosphate, 2.0 m*M* L-tyrosine or 10 m*M* L-phenylalanine, Buffer A, and enzyme. Phenylalanine:2-ketoglutarate aminotransferase activity was quantified by relating the absorbance of phenylpyruvate at 320 nm in 2 *N* NaOH to a molar extinction coefficient of 17,500 (Gibson and Gibson 1964). Tyrosine:2-ketoglutarate aminotransferase activity was quantified by relating the absorbance of *p*-hydroxybenzaldehyde (formed from enzymatically generated *p*-hydroxyphenylpyruvate) at 331 nm in 2 *N* NaOH to a molar extinction coefficient of 19,900 (Diamondstone 1966).

Prephenate aminotransferase was assayed using the HPLC method described by Bonner and Jensen (1985). Reaction mixtures (0.1 ml) contained 2.0 m*M* prephenate, 2.0 m*M* L-glutamate, 25 μ*M* pyridoxal-5′-phosphate, Buffer A, and enzyme. The standard incubation time used was 30 min (at 37°C); both substrate blanks and enzyme blanks were included to ensure that the L-phenylalanine detected arose from aminotransferase activity and not from proteolysis or from L-arogenate that may contaminate some prephenate preparations.

Protein determinations

Protein concentrations were determined by the method of Bradford (1976) as outlined in the Bio-Rad Protein Assay Instruction Manual.

Materials

Sephadex G-25-300 and all biochemicals and standard reagent-grade chemicals, unless stated otherwise below, were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Barium prephenate was isolated from a tyrosine auxotroph of *Salmonella typhimurium* (Dayan and Sprinson 1970) and was converted to the potassium salt with an excess of K_2SO_4 before use. Chorismate was isolated from the accumulation medium of the triply auxotrophic *Klebsiella pneumoniae* 62-1, and was purified as the free acid (Gibson 1970). L-Arogenate was prepared according to Zamir et al. (1983). Protein assay reagent was obtained from Bio-Rad (Richmond, CA, USA).

Results

Activities of aromatic amino acid pathway enzymes in N. gonorrhoeae

Crude extracts of *Neisseria gonorrhoeae* HGH 154 and ATCC 27630 were examined for activities of aromatic-pathway enzymes (Table 1). DAHP synthase activity from both isolates HGH 154 and ATCC 27630 was stimulated by manganese (and to a lesser extent, cobalt) but was not

affected by magnesium. The maximum degree of activation observed (using a 1.0 m*M* final concentration of $MnSO_4$ in the standard reaction mixtures) was 6.9-fold and 4-fold for isolates HGH 154 and ATCC 27630, respectively (data not shown). The last enzymatic step common to both phenylalanine and tyrosine synthesis, chorismate mutase (see Fig. 1), was also detected in crude extracts of both *N. gonorrhoeae* isolates (Table 1).

Phenylalanine synthesis occurs primarily, if not exclusively, through the phenylpyruvate route rather than via L-arogenate (see Fig. 1). Activity of arogenate dehydratase was absent or below the level of background error (about 0.1 units/mg of crude-extract protein). Prephenate dehydratase was strongly activated by L-tyrosine, activation of enzyme from HGH 154 and ATCC 27630 being 18-fold and 28-fold, respectively.

Table 1. Specific activities of aromatic amino acid pathway enzymes in crude extracts of *Neisseria gonorrhoeae*

Enzyme[b]	Specific activity[a]	
	HGH 154[c]	ATCC 27630[c]
DAHP synthase	128.5	155.2
Chorismate mutase	26.3	45.4
Prephenate dehydratase	1.9/33.2[d]	1.4/38.6[d]
Prephenate dehydrogenase (NAD^+)	23.5	22.5
Arogenate dehydrogenase (NAD^+)	5.8	1.7
Phenylpyruvate aminotransferase	421.6	925.7
4-Hydroxyphenylpyruvate aminotransferase	71.1	363.5
Prephenate aminotransferase	193.0	360.0

[a] Expressed as nmol of product formed per minute per mg protein

[b] All assays were carried out using the conditions and standard reaction mixtures outlined in Methods. Aminotransferase activities given for each substrate represent the combined activities of multiple aminotransferase species having broad substrate specificities (see text and Fig. 2)

[c] Isolates HGH 154 (phenylalanine-sensitive), and ATCC 27630 (phenylalanine-resistant), are described in Methods

[d] Prephenate dehydratase from both strains was strongly activated by L-tyrosine. Specific activity values were obtained in the absence (left number) or presence (right number) of 0.5 m*M* L-tyrosine

Prephenate dehydrogenase and arogenate dehydrogenase activities (see Fig. 1) were both detected in crude extracts of both *N. gonorrhoeae* isolates. The ratio of these activities (Table 1) indicates that tyrosine synthesis occurs primarily via 4-hydroxyphenylpyruvate. Both dehydrogenase activities were specific for NAD^+ as cofactor; $NADP^+$ would not substitute.

Aminotransferase activities were measured when either prephenate, phenylpyruvate, or 4-hydroxyphenylpyruvate was utilized as co-substrate with L-glutamate as amino-group donor (Table 1). Aminotransferases having sufficiently broad substrate specificities to transaminate prephenate, phenylpyruvate, and 4-hydroxyphenylpyruvate (see Fig. 1) are common in nature (Jensen and Calhoun 1981). For example, in *Escherichia coli* the products of three genes (denoted *aspC*, *ilvE*, and *tyrB*, and encoding aspartate aminotransferase, branched-chain amino acid aminotransferase, and aromatic amino acid aminotransferase, respectively), all exhibit overlapping function in the three major metabolic pathways indicated by their genetic denotations (Gelfand and Steinberg 1977). In *Pseudomonas aeruginosa* five separate aminotransferases have been resolved, each capable of transaminating phenylpyruvate, 4-hydroxyphenylpyruvate, and prephenate (Whitaker et al. 1982).

Fractionation of aromatic-pathway enzymes from N. gonorrhoeae

Crude extracts of both *N. gonorrhoeae* isolates were fractionated via ion-exchange chromatography to determine if any of the enzyme activities detected in crude extracts repre-

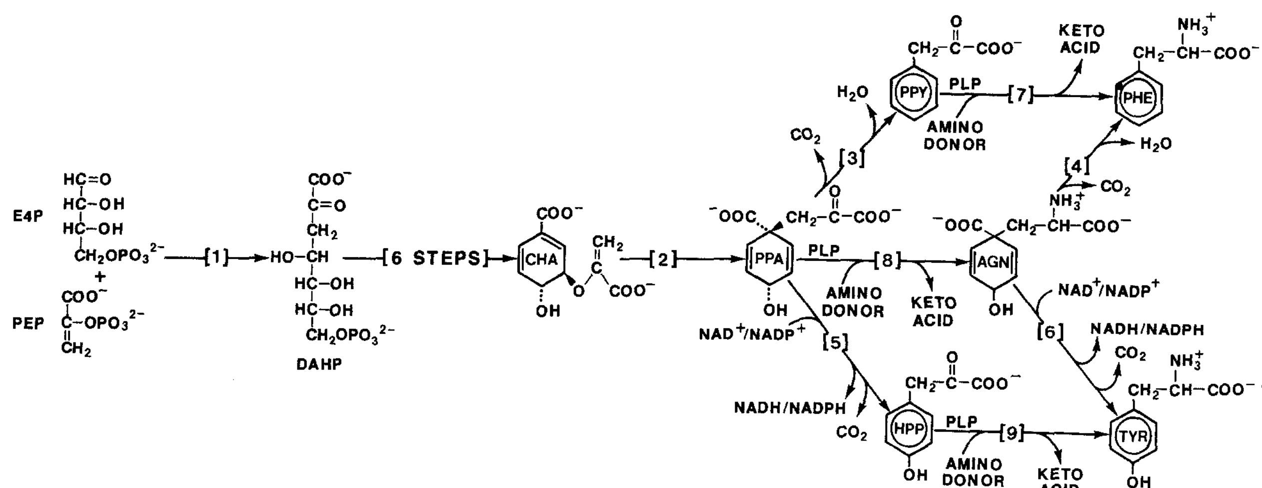

Fig. 1. Variant enzymic routings that exist for phenylalanine and tyrosine biosynthesis in nature. *Abbreviations: E4P*, D-erythrose-4-phosphate; *PEP*, phosphoenolpyruvate; *DAHP*, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; *CHA*, chorismate; *PPA*, prephenate; *AGN*, L-arogenate; *PHE*, L-phenylalanine; *PPY*, phenylpyruvate; *HPP*, 4-hydroxyphenylpyruvate; *TYR*, L-tyrosine; *PLP*, pyridoxal 5′-phosphate. Enzymes are numbered as follows. [1], DAHP synthase; [2], chorismate mutase; [3], prephenate dehydratase; [4], arogenate dehydratase; [5], prephenate dehydrogenase; [6], arogenate dehydrogenase; [7], phenylpyruvate aminotransferase; [8], prephenate aminotransferase; [9], 4-hydroxyphenylpyruvate aminotransferase. Enzymes [2] and [3] exist as a bifunctional protein (P-protein) in a large number of Gram-negative eubacteria (Ahmad and Jensen 1986). Aromatic aminotransferases (steps 7–9) often exhibit overlapping substrate specificities that allow utilization of all three of the aromatic-pathway intermediates: prephenate, phenylpyruvate, and 4-hydroxyphenylpyruvate (Jensen and Calhoun 1981)

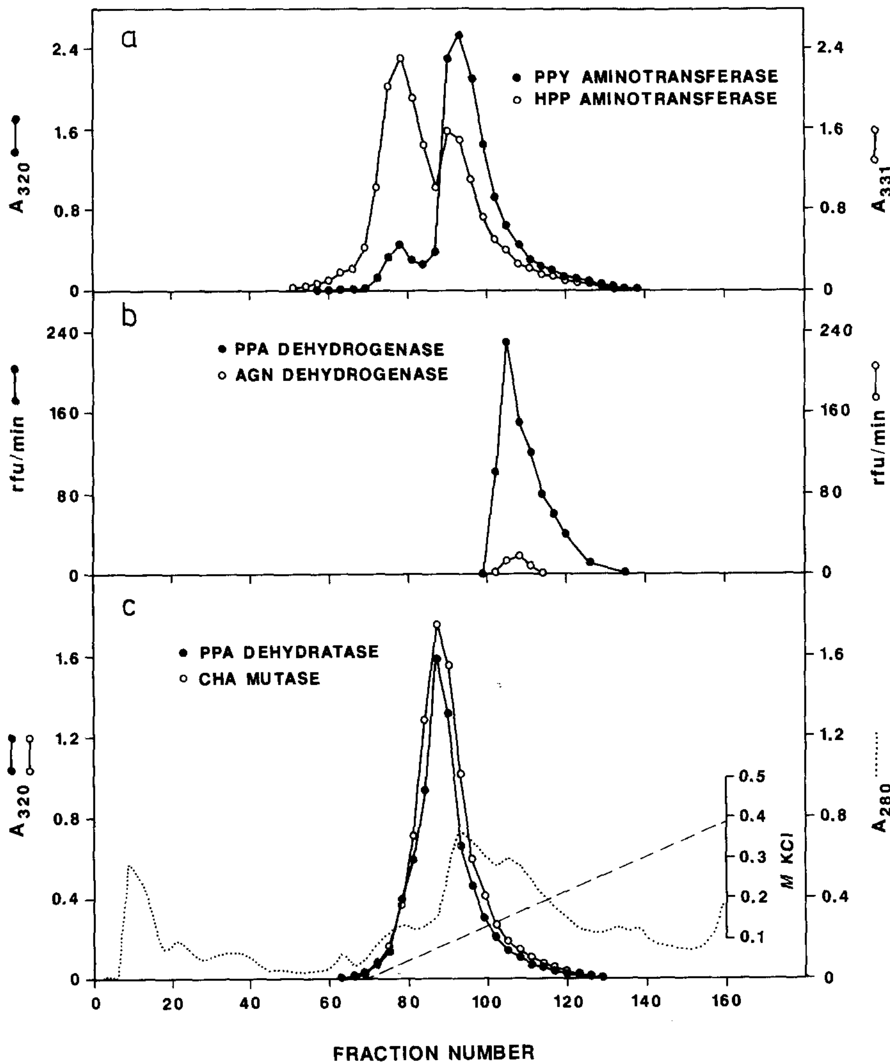

Fig. 2a–c Elution profiles of aromatic-pathway enzymes from *Neisseria gonorrhoeae* HGH 154. Essentially identical results were obtained with ATCC 27630. Fractionation of crude extract via DEAE-cellulose chromatography, as well as standard assay procedures, were described in Methods. **a** Elution profile of aromatic aminotransferases. Phenylpyruvate (PPY) aminotransferase activity was assayed by using the L-phenylalanine:2-ketoglutarate substrate combination and determining the concentration of phenylpyruvate formed (measured in the assay protocol as A_{320}). 4-Hydroxyphenylpyruvate aminotransferase activity was assayed by using the L-tyrosine:2-ketoglutarate substrate combination and determining the concentration of 4-hydroxyphenylpyruvate formed (measured in the assay protocol as absorbance at 331 nm). **b** Elution profile of prephenate (*PPA*) dehydrogenase and arogenate (*AGN*) dehydrogenase activities. Dehydrogenase activities were determined by following the continuous formation of NADH [measured as relative fluorescence units (rfu) per minute]. **c** Elution profiles of prephenate (PPA) dehydratase and chorismate (CHA) mutase. Both activities were measured in the assay protocol as A_{320}. The *dotted line* represents the distribution of protein (measured as A_{280}) while the *dashed line* represents the linear KCl gradient

sented the combined activities of isozymes. The results obtained for both strains were essentially identical, and data obtained from HGH 154 are shown. Figure 2 shows the elution patterns of enzymes of the post-chorismate portion of the aromatic amino acid pathway (refer to Fig. 1) in *N. gonorrhoeae*.

Two species of aromatic aminotransferase were resolved (Fig. 2a). With the use of the L-phenylalanine:2-ketoglutarate assay, two peaks of aminotransferase activity were detected in the gradient fractions. A minor peak of activity eluting in fractions 70–85 was followed by a major peak of activity eluting in fractions 87–120. When the L-tyrosine:2-ketoglutarate substrate combination was used, two peaks of aminotransferase activity occupying the same elution positions were again recovered. However, the ratio of the two peaks of activity, comparing activity with phenylalanine to activity with tyrosine, was opposite. Thus, the leading-peak enzyme prefers transamination between 4-hydroxyphenylpyruvate and tyrosine, while the trailing-peak enzyme prefers transamination between phenylpyruvate and phenylalanine. Each aminotransferase species was able to utilize prephenate (with L-glutamate as amino donor). The specific activities of peak fractions 78 and 93 (with prephenate as substrate) were 228.6 and 320.5 nmol/min·mg, respectively. When the ratio of the abilities of the two aminotransferase enzymes to utilize prephenate compared to the favored substrate were examined, the trailing-peak enzyme was more than twice as effective as the leading-peak enzyme for prephenate utilization. Because the prephenate aminotransferase assay used requires HPLC fractionation of each reaction mixture (Bonner and Jensen 1985), only those fractions initially found to contain L-phenylalanine:2-ketoglutarate and L-tyrosine:2-ketoglutarate activity were screened for prephenate aminotransferase activity.

Prephenate dehydrogenase and arogenate dehydrogenase coeluted in the gradient fractions (Fig. 2b). This is characteristic of many Gram-negative organisms (Jensen and Fischer 1986) which apparently possess a single cyclohexadienyl dehydrogenase (having activity with both prephenate and L-arogenate).

Prephenate dehydratase and chorismate mutase eluted with exact coincidence in the gradient fractions (Fig. 2c), suggesting the presence of a bifunctional protein. Additional peaks of chorismate mutase activity were not found.

The fractionation protocol used above was unsuitable for analysis of DAHP synthase (this enzyme activity eluting partially in the wash eluate, and tailing into the gradient fractions). Thus, the conditions used for ion-exchange chromatography were modified slightly (see Methods) for the

Table 2. Allosteric control of DAHP synthase in *N. gonorrhoeae* strains

Isolate	% Inhibition (0.5 m*M* effector)[a]											
	PHE	TYR	TRP	PHE TYR	PHE TRP	TYR TRP	PHE TYR TRP	CHA	PPA	PPY	HPP	AGN
HGH 154	80	0	0	72	72	0	76	0	0	0	0	0
ATCC 27630	66	0	0	64	39	0	53	0	2	2	0	0

[a] Per cent inhibition of activity relative to controls lacking effectors. Abbreviations: PHE, L-phenylalanine; TYR, L-tyrosine; TRP, L-tryptophan; CHA, chorismate; PPA, prephenate; PPY, phenylpyruvate; HPP, 4-hydroxyphenylpyruvate; AGN, L-arogenate. Assays were carried out using partially-purified enzyme preparations recovered following DEAE-cellulose and Sephadex G-75 chromatography. Sub-saturating concentrations of substrates were also used to facilitate the recognition of possible weak competitive inhibition

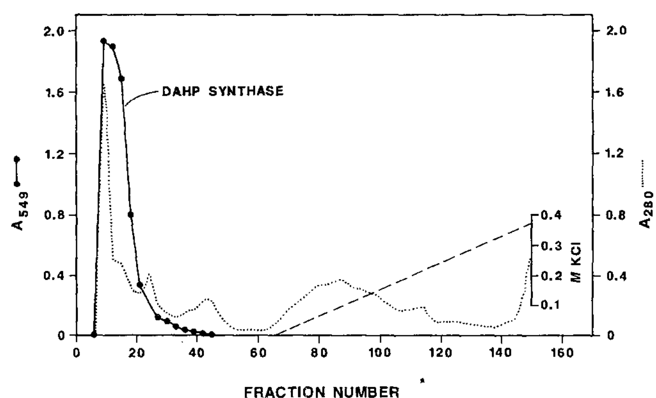

Fig. 3. Elution profile of DAHP synthase from *N. gonorrhoeae* HGH 154. Essentially identical results were obtained with ATCC 27630. DAHP synthase activity was measured in the assay protocol as A_{549}. The *dotted line* represents the distribution of protein (measured as A_{280}) while the *dashed line* represents the linear KCl gradient. Further fractionation of DAHP synthase via Sephadex G-75 chromatography again yielded a single peak of activity (data not shown)

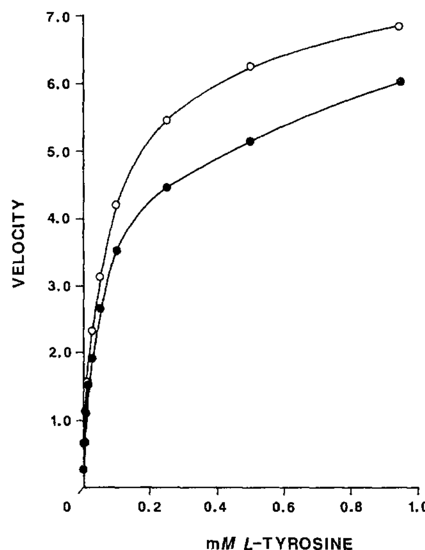

Fig. 4. L-Tyrosine-mediated activation of prephenate dehydratase from *N. gonorrhoeae* HGH 154 (●) and ATCC 27630 (○). Assays were performed with crude-extract preparations, using standard reaction mixtures (see Methods) containing the indicated final concentrations of L-tyrosine

study of DAHP synthase. Figure 3 shows the elution profile for DAHP synthase from HGH 154. A single peak of activity passed unretarded through the DEAE-cellulose column. When fractions containing DAHP synthase activity were pooled, concentrated, and re-chromatographed on a Sephadex G-75 column, a single peak of DAHP synthase activity was again recovered (data not shown). Essentially identical results were obtained for ATCC 27630.

Allosteric control of aromatic-pathway enzymes in N. gonorrhoeae

The data in Tables 2–4 show the allosteric control specificities of aromatic-pathway enzymes in *N. gonorrhoeae*. DAHP synthase from both *N. gonorrhoeae* strains examined was subject to inhibition by L-phenylalanine (Table 2). L-Tyrosine and L-tryptophan (singly or in combination), as well as aromatic-pathway intermediates tested had no effect on DAHP synthase activity.

Prephenate dehydratase from both *N. gonorrhoeae* isolates examined was activated by L-tyrosine (see Table 1). Figure 4 shows the activation curves obtained for prephenate dehydratase. Prephenate dehydratase was inhibited by L-phenylalanine (Table 3). Experiments in which both L-phenylalanine and L-tyrosine were present simultaneously in reaction mixtures revealed that L-phenylalanine-mediated inhibition dominates over allosteric activation by L-tyrosine. Chorismate mutase from both *N. gonorrhoeae* isolates was also inhibited by L-phenylalanine (Table 3). In addition, chorismate mutase was product inhibited by prephenate.

The coeluting prephenate dehydrogenase and arogenate dehydrogenase activities possessed an unusual pattern of allosteric control that has not previously been described. At saturating substrate concentrations each activity was inhibited by 4-hydroxyphenylpyruvate, but neither activity was inhibited by L-tyrosine (Table 4). L-Phenylalanine, L-tryptophan, and phenylpyruvate had no effect on either dehydrogenase activity.

Discussion

Routes and regulation of aromatic amino acid biosynthesis in Neisseria gonorrhoeae

N. gonorrhoeae possesses dual enzymatic routes to phenylalanine and to tyrosine (see Fig. 1). This biochemical complexity is actually quite common (Byng et al. 1982;

Table 3. Allosteric control of prephenate dehydratase and chorismate mutase in *N. gonorrhoeae*[a]

Isolate	% Inhibition (0.5 mM effector)[b]					
	Prephenate dehydratase[c]		Chorismate mutase			
	PHE	TRP	PHE	TYR	TRP	PPA
HGH 154	15 (18)	7 (4)	19	2	0	32
ATCC 27630	33 (33)	0 (3)	15	0	7	27

[a] Since no dehydratase activity was detected in the absence of L-tyrosine following fractionation of crude extracts by ion-exchange chromatography, assays were performed using crude-extract preparations in order to observe the effects of L-phenylalanine and L-tryptophan on prephenate dehydratase in the absence of the allosteric activator, L-tyrosine. Identical results were obtained when partially-purified preparations (see Fig. 2) were used (L-tyrosine being present at 0.5 mM concentration)
[b] Per cent inhibition versus controls lacking effectors. Abbreviations are as given in Table 2
[c] Prephenate dehydratase was screened for inhibition by L-phenylalanine and L-tryptophan in the absence and presence of activator (0.5 mM L-tyrosine). Numbers in parentheses were obtained when L-tyrosine was present in reaction mixtures

Table 4. Allosteric control of prephenate dehydrogenase and arogenate dehydrogenase activities[a] in *N. gonorrhoeae*

Isolate	Substrate[b]	% Inhibition (0.5 mM effector)[c]				
		PHE	TYR	TRP	PPY	HPP
HGH 154	Prephenate	0	7	0	0	29
	L-Arogenate	0	0	0	0	58
ATCC 27630	Prephenate	0	0	0	0	37
	L-Arogenate	0	0	7	14	64

[a] Prephenate dehydrogenase and arogenate dehydrogenase activities coeluted following fractionation of crude extracts via DEAE-cellulose chromatography (see Fig. 2). Allosteric studies were carried out using such partially-purified preparations. In all assays 0.5 mM NAD^+ was used
[b] Prephenate and L-arogenate were used at final concentrations of 1.0 mM
[c] Per cent inhibition of activity relative to controls lacking effectors. Abbreviations are as given in Table 2

Jensen and Fischer 1986). Although *N. gonorrhoeae* has the metabolic potential to utilize the L-arogenate route for tyrosine synthesis, we conclude that the phenylpyruvate route to L-phenylalanine and the 4-hydroxyphenylpyruvate route to L-tyrosine are the primary paths of synthesis. The latter conclusion is supported by comparison of catalytic properties of the appropriate enzymes as well as by the implications of certain features of regulation (see below).

The overall picture of the regulation of aromatic biosynthesis in *N. gonorrhoeae* is one in which L-tyrosine is the key regulatory molecule. Prephenate dehydratase is dependent on L-tyrosine for activation, and therefore L-tyrosine directly controls the synthesis of L-phenylalanine. L-Phenylalanine in turn governs overall aromatic biosynthesis by exerting allosteric control over DAHP synthase. L-Phenylalanine further regulates both its own synthesis (through inhibition of prephenate dehydratase) as well as the synthesis of prephenate (through inhibition of chorismate mutase). Thus, although L-phenylalanine plays a dominant role in regulating the synthesis of common-pathway intermediates, the synthesis of L-phenylalanine itself is dependent on the level of L-tyrosine.

The use of prephenate dehydratase as a focal point of allosteric control by both L-phenylalanine and L-tyrosine, and the use of 4-hydroxyphenylpyruvate as an inhibitor of prephenate dehydrogenase and arogenate dehydrogenase activities implicate roles of phenylpyruvate and 4-hydroxyphenylpyruvate as important intermediates of phenylalanine and tyrosine synthesis. The inhibition by 4-hydroxyphenylpyruvate of both dehydrogenase activities is consistent with expectations for a single cyclohexadienyl dehydrogenase capable of utilizing either prephenate or L-arogenate as substrate. This is further supported by the exact coelution of prephenate dehydrogenase and arogenate dehydrogenase activities following ion-exchange chromatography.

The phenylalanine-sensitive phenotype

The growth of isolate HGH 154 is inhibited in the presence of L-phenylalanine (Hendry and Dillon 1984). In the presence of exogenous L-phenylalanine the intracellular L-phenylalanine levels are no longer dictated by L-tyrosine synthesis (see above). One might then expect L-phenylalanine to inhibit growth by exercise of early-pathway regulation and subsequent starvation for L-tyrosine. Indeed, there seems to be a perfect correlation between the phenomenon of growth inhibition by L-phenylalanine in a variety of organisms and the presence of a single phenylalanine-inhibited species of DAHP synthase (Kelly 1969; Jensen et al. 1974; Hall and Jensen 1980). However, the explanation cannot be this simple because isolate ATCC 27630, which is resistant to growth inhibition in the presence of L-phenylalanine, exhibits overall enzyme levels and features of regulation that resemble those of isolate HGH 154. A difference at the level of phenylalanine transport is unlikely since isolate ATCC 27630 is sensitive to growth inhibition by analogues of L-phenylalanine, reasonably assumed to share the transport system used by L-phenylalanine (Hendry and Dillon 1984). It would appear that a single phenylalanine-sensitive species of DAHP synthase is necessary but not sufficient to account for inhibition of growth by L-phenylalanine. More detailed studies, highlighting the approach of isolating phenylalanine-resistant mutants from isolate HGH 154, are in progress.

Evolutionary implications

Jensen (1985) showed that the evolution of biochemical pathways in prokaryotes can be traced backward, provided that a suitable phylogenetic tree exists to guide the selection of organisms for study. Such phylogenetic trees based on oligonucleotide cataloging of 16S rRNA (Fox et al. 1980; Stackebrandt and Woese 1981) or on full sequencing (Woese 1987) are now firmly established and are being expanded at a rapid rate. The most comprehensive studies of the evolution of the aromatic amino acid pathway have focused on a large phylogenetic grouping of Gram-negative bacteria denoted Superfamily B (reviewed by Jensen 1985). Some information is available for the connecting Superfamily A, of which *N. gonorrhoeae* is a member (Woese 1987).

The bifunctional P-protein, which catalyzes the sequential reactions of chorismate mutase and prephenate dehydratase (see Fig. 1), is a highly conserved feature of aromatic biosynthesis that is present throughout Superfamily B (Ahmad and Jensen 1986). It was suggested (Ahmad and Jensen 1986) that the P-protein may be a character state present within Superfamily A as well as Superfamily B, implicating its origin in a common ancestor of the two Superfamilies. The existence of the bifunctional P-protein in *Alcaligenes eutrophus* of Superfamily A has been proven (Friedrich et al. 1976a), and our data suggest that *N. gonorrhoeae* also possesses a P-protein.

Regulatory isozymes of DAHP synthase exemplify evolutionarily dynamic character states of evolution within Superfamily B (Ahmad et al. 1986). Relatively little information is available about DAHP synthase isozymes within Superfamily-A bacteria at this time. The one-enzyme system of *N. gonorrhoeae* differs from the two-isozyme system of Group III pseudomonads. *Pseudomonas acidovorans*, a representative of the latter grouping, possesses phenylalanine-sensitive and tyrosine-sensitive isozymes (Berry et al. 1985).

Cofactor specificity of tyrosine-pathway dehydrogenases has proven to be a useful character state for recognition of phylogenetic clusters (Byng et al. 1980; Jensen 1985). Members of Superfamily A previously studied possess prephenate dehydrogenase and arogenate dehydrogenase activities that will utilize either NAD^+ or $NADP^+$ as cofactor (Jensen 1985). In contrast, these enzyme activities from *N. gonorrhoeae* are specific for NAD^+. One would anticipate that close phylogenetic neighbors of *N. gonorrhoeae* will also possess NAD^+-linked cyclohexadienyl dehydrogenases.

Byng et al. (1980) showed that in nearly all Superfamily-A pseudomonads and *Alcaligenes* species, prephenate dehydrogenase was insensitive to feedback inhibition by L-tyrosine. Since *N. gonorrhoeae* dehydrogenase activity was also insensitive to L-tyrosine, but was unexpectedly inhibited by 4-hydroxyphenylpyruvate, sensitivity of tyrosine-pathway cyclohexadienyl dehydrogenases to 4-hydroxyphenylpyruvate may prove to be a general characteristic of other Superfamily-A organisms. This prospect is strengthened by the fact that prephenate dehydrogenase from *A. eutrophus*, although subject to feedback inhibition by L-tyrosine, was also found to be inhibited by 4-hydroxyphenylpyruvate (Friedrich et al. 1976b).

Acknowledgements. This work was supported by grant AM-19447 (to RAJ) from the United States Public Health Service and by a grant (to ATH) from the Hamilton Civic Hospitals Research Fund (Hamilton, Ontario).

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Received April 28, 1987/Accepted September 3, 1987