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Biosynthesis of L-Phenylalanine and L-Tyrosine in the Actinomycete *Amycolatopsis methanolica*

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Auxotrophic mutants of the actinomycete *Amycolatopsis methanolica* requiring L-Phe or L-Tyr were isolated and identified as strains lacking prephenate dehydratase (strain GH71) or arogenate dehydrogenase (strain GH70), respectively. *A. methanolica* thus employs single pathways only for the biosynthesis of these aromatic amino acids. Anion-exchange chromatography of extracts revealed two peaks with Phe as well as Tyr aminotransferase (AT) activity (Phe/Tyr ATI and Phe/Tyr ATII) and three peaks with prephenate AT activity (Ppa ATI to Ppa ATIII). Phe/Tyr ATI and Ppa ATI coeluted and appear to function as the *A. methanolica* branched-chain amino acid AT. Ppa ATII probably functions as the aspartate AT. Mutant studies showed that Phe/Tyr ATII is the dominant AT in L-Phe biosynthesis and in L-Tyr catabolism but not in L-Tyr biosynthesis. Biochemical studies showed that Ppa ATIII is highly specific for prephenate and provided evidence that Ppa ATIII is the dominant AT in L-Tyr biosynthesis.

The aromatic amino acids L-Phe and L-Tyr are both derived from chorismate, the end product of the shikimate pathway (11). The last common step in their biosynthesis is catalyzed by chorismate mutase, yielding prephenate from chorismate. Prephenate may be converted into phenylpyruvate (by prephenate dehydratase), 4-hydroxyphenylpyruvate (by prephenate dehydrogenase), or L-arogenate (by prephenate aminotransferase [Ppa AT]). L-Arogenate may be converted into L-Phe (by arogenate dehydratase) or L-Tyr (by arogenate dehydrogenase). Phenylpyruvate is subsequently transaminated to L-Phe and 4-hydroxyphenylpyruvate is transaminated to L-Tyr (6, 19). Aromatic amino acid AT (aro AT) enzymes have been studied in a number of gram-negative bacteria but in relatively few gram-positive bacteria. They usually possess a broad substrate specificity, for instance also accepting L-Asp and branched-chain amino acids as alternative substrates (4, 16, 30, 33, 37).

No information is currently available about the properties of aro AT enzymes in actinomycetes. These gram-positive bacteria are well-known producers of secondary metabolites. Many industrially important secondary metabolites are derived from intermediates of the aromatic amino acid biosynthetic pathway (2, 11). We are currently engaged in an analysis of the enzymology and regulation of aromatic amino acid metabolism in the methanol-utilizing nocardioform actinomycete *Amycolatopsis methanolica* (8, 12, 13, 15). Here we report the results of studies on the pathways of L-Phe and L-Tyr biosynthesis and the identity and properties of the aro AT enzymes involved.

MATERIALS AND METHODS

Microorganisms and cultivation. *A. methanolica* wild-type strain (NCIB 11946) (8), the plasmid pMEA300-deficient strain WV2 (34a), and auxotrophic mutants derived from strain WV2 (this study) were used. The procedures followed for cultivation in batch cultures, harvesting of cells, and measurements of growth have been described previously (9). Tyrosine (80 mM) and glucose (1 M) were heat sterilized, and other amino acid supplements were filter sterilized.

Mutant isolation. Mutants blocked in aromatic amino acid biosynthesis were isolated by either diepoxyoctane or UV irradiation treatment (9, 13, 14). In the screening procedure, glucose mineral medium was supplemented with aromatic amino acids to a final concentration of 1 mg · liter^{-1} each. Pinpoint colonies were selected for further characterization, using amino acid supplements at 50 mg · liter^{-1} each.

Preparation of cell extracts and enzyme assays. Washed cell suspensions were disrupted in a French pressure cell at 1,000 MPa. Unbroken cells and debris were removed by centrifugation at 40,000 × *g* for 20 min at 4°C, and the supernatant, containing 10 to 20 mg of protein · ml^{-1}, was used for enzyme assays performed at 37°C. When necessary, reactions were stopped by incubation for 1 min at 100°C. Precipitated proteins were removed by centrifugation. Amino acids formed were measured as described under "Analytical methods" below.

Arogenate dehydrogenase (EC 1.3.1.43) was assayed by monitoring the formation of L-Tyr. The assay mixture (60 μl) contained 1.5 mM L-arogenate, 1 mM NAD(P)$^+$, 5 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.5), and cell extract.

Arogenate dehydratase was assayed by measuring the formation of L-Phe (18).

Prephenate dehydratase (EC 4.2.1.51) was assayed by monitoring the formation of phenylpyruvate, which was measured in 1 M NaOH at 320 nm (31).

Prephenate dehydrogenase (NAD$^+$ dependent; EC 1.3.1.12) (NADP$^+$ dependent; EC 1.3.1.13) was assayed by measuring the formation of NADH or NADPH (18).

Aspartate AT (Asp AT; EC 2.6.11) was assayed by monitoring the formation of oxaloacetate, using malate dehydrogenase as the coupling enzyme and measuring NADH consumption at 340 nm. The reaction mixture (1 ml) contained 50 mM Tris-HCl (pH 7.5), 30 μl of 1% Triton X-100, 10 mM α-ketoglutarate, 0.1 mM pyridoxal phosphate, 0.15 mM NADH, 6 U of malate dehydrogenase, 10 mM L-Asp, and cell extract.

Branched-chain amino acid AT (Leu AT; EC 2.6.1.42) was assayed by monitoring the formation of L-Leu. The assay mixture (0.1 ml) contained 50 mM Tris-HCl (pH 7.5), 5 mM α-ketoisocaproate, 20 mM L-Glu, 0.1 mM pyridoxal phosphate, and cell extract.

Phenylalanine/tyrosine AT (Phe/Tyr AT; EC 2.6.1.57; biosynthetic direction) was assayed by monitoring the formation of L-Phe or L-Tyr. The assay mixture (0.1 ml) contained 50 mM Tris-HCl (pH 7.5), 5 mM (4-hydroxy)phenylpyruvate, 20 mM L-Glu, 0.1 mM pyridoxal phosphate, and cell extract.

Phe/Tyr AT activity in the reverse direction was assayed by monitoring the formation of phenylpyruvate (Phe AT) or 4-hydroxyphenylpyruvate (Tyr AT), which was measured in 1 M NaOH at 320 or 330 nm, respectively (7).

Ppa AT was assayed by monitoring the formation of L-arogenate. The assay mixture (0.1 ml) contained 50 mM Tris-HCl (pH 7.5), 2 mM prephenate, 20 mM L-Glu, 0.5 mM pyridoxal phosphate, and cell extract.

AT activities were visualized after gel electrophoresis under nondenaturating conditions by amino acid-dependent tetrazolium activity staining (32). Protein was loaded on 0.75-mm polyacrylamide gels (running gel, 7% polyacrylamide in 375 mM Tris-HCl [pH 8.8]; stacking gel, 3% polyacrylamide in 125 mM Tris-HCl [pH 6.8]). Following electrophoresis in buffer containing 25 mM Tris and 32 mM glycine (100 V, 20 mA, 4°C), the gels were rinsed with water and stained in staining buffer at 37°C in the dark. Staining buffer contained 2.5 mM NAD$^+$, 0.03 mg of phenazine methosulfate · ml^{-1}, 0.5 mg of 3-(4,5-dimethyl-2-thiazolyl)-2,5-

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diphenyl-2H-tetrazolium bromide · ml^{-1}, 0.1 U of glutamate dehydrogenase · ml^{-1}, 20 mM α-ketoglutarate, 5 mM amino acid, and 0.1 mM pyridoxal phosphate.

Separation of amino acid ATs. Cell extracts (30 to 40 mg of protein) from glucose-grown cells of *A. methanolica* WV2 and GH141 were applied on a Mono Q HR 5/5 anion-exchange column previously equilibrated with 25 mM Tris-HCl (pH 7.5) containing 1 mM dithiothreitol. Bound protein was eluted with a 30-ml increasing linear gradient from 0 to 0.5 M NaCl in 25 mM Tris-HCl (pH 7.5) containing 1 mM dithiothreitol (flow rate, 1 ml · min^{-1}; fractions, 1 ml).

Purification of Phe/Tyr ATI and Phe/Tyr ATII. Enzyme purification was performed at room temperature unless stated otherwise. Centrifugation steps were performed at 4°C. All chromatographic steps were carried out with a System Prep 10 liquid chromatography system (Pharmacia LKB Biotechnology Inc.).

(i) Step 1. Glucose-grown cells (25 g [wet weight]) were harvested in the late exponential phase of growth. Cell extract was prepared as described above. DNase I was added to the cell extract and incubated for 10 min at room temperature.

(ii) Step 2. The material was applied onto a column of Q-Sepharose fast flow (16 by 2.6 cm) equilibrated in 50 mM Tris-HCl (pH 7.5) containing 1 mM dithiothreitol (buffer A). Bound protein was eluted with a 912-ml increasing linear gradient from 0 to 0.4 M NaCl in buffer A (flow rate, 5 ml · min^{-1}; fractions, 12 ml). Fractions containing Phe/Tyr ATI or Phe/Tyr ATII activity were pooled separately.

(iii) Step 3. Ammonium sulfate precipitation. Solid $(NH_4)_2SO_4$ was slowly added to the pooled fractions of step 2 to a final concentration of 35% saturation (Phe/Tyr ATI) or 50% saturation (Phe/Tyr ATII). The mixtures were stirred for 30 min at 4°C and centrifuged for 30 min at 40,000 × *g*. The supernatants were decanted and slowly adjusted to 80% (Phe/Tyr ATI) or 75% (Phe/Tyr ATII) $(NH_4)_2SO_4$. The mixtures were stirred for 30 min at 4°C and centrifuged for 20 min at 40,000 × *g*. The supernatants were discarded, and the pellets were dissolved in buffer A.

(iv) Step 4. Gel filtration chromatography. Protein from step 3 was applied onto a Superdex 200 column (1.6 by 60 cm) equilibrated in buffer A (flow rate, 1 ml · min^{-1}; fractions, 2 ml). Pooled Phe/Tyr ATI fractions were stored at −20°C in 20% (vol/vol) glycerol. Pooled Phe/Tyr ATII fractions were subjected to a further purification step.

(v) Step 5. Anion-exchange chromatography. Protein from step 4 was applied onto a Mono Q HR 5/5 anion-exchange column equilibrated in buffer A. Bound protein was eluted with a 30-ml increasing linear gradient from 0 to 0.5 M NaCl in buffer A (flow rate, 1 ml · min^{-1}; fractions, 1 ml). Fractions containing Phe/Tyr ATII activity were pooled and stored at −20°C in 20% (vol/vol) glycerol.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed by the method of Laemmli and Favre (26) with the Combithek calibration protein kit (Boehringer Mannheim Biochemical). Gels were stained with Coomassie brilliant blue R250.

Estimation of molecular mass. The molecular mass was estimated by using a Superdex 200 column and Bio-Rad gel filtration standards (Bio-Rad, Richmond, Calif.).

Kinetic studies with ATs. Kinetic parameters were determined at 37°C in 50 mM Tris-HCl (pH 7.5) and 0.1 mM pyridoxal phosphate. Substrate concentrations were as shown in the individual experiments. The kinetic parameters were determined with the curve-fitting option of Sigmaplot 1.1 for Windows (Jandell Scientific Software) by using a direct fit with the Michaelis-Menten equation.

Analytical methods. The amount of glucose was estimated by the GOD-Perid method (Boehringer, Mannheim, Federal Republic of Germany). Protein levels were determined with the protein determination kit from Bio-Rad, using bovine serum albumin as the standard (5). Amino acid concentrations were determined by high-pressure liquid chromatographic analysis by the method of Euverink et al. (15).

Biochemicals. Enzymes and biochemicals were obtained from Boehringer. L-Arogenate was isolated from a multiple auxotroph of *Neurospora crassa* ATCC 36373 (22, 38). All other chemicals were of analytical grade and commercially available.

RESULTS

Enzymes involved in L-Phe and L-Tyr biosynthesis. Prephenate dehydratase (10, 15), Phe AT, Tyr AT, Ppa AT, and arogenate dehydrogenase activities were readily detected in cell extracts of glucose-grown cells of *A. methanolica* WV2 (Table 1). However, $NAD(P)^+$-dependent prephenate dehydrogenase and arogenate dehydratase could not be detected, suggesting the involvement of single pathways from prephenate to L-Phe and to L-Tyr (Fig. 1). Arogenate dehydrogenase could be measured with either NAD^+ or $NADP^+$. The apparent K_m value of the enzyme for L-arogenate (measured with NAD^+) was estimated as 0.4 mM. Arogenate dehydrogenase activity was not inhibited by aromatic amino acids (1 mM concentrations, added separately or in various combinations).

TABLE 1. Specific activities of enzymes involved in L-Phe and L-Tyr biosynthesis in glucose-grown cells of *A. methanolica* WV2 and mutants derived from WV2.

Enzyme	Sp act (mU · mg of $protein^{-1}$) in strain:				
	WV2	GH70	GH71	GH141	GH148
Prephenate dehydratase	19	18	0	17	17
Arogenate dehydrogenase (NAD^+)[a]	2	0.1	3	2	3
Phe AT	51	45	48	3	5
Tyr AT	44	40	42	4	3
Ppa AT	1	1	2	2	ND[b]
Asp AT	569	ND	ND	411	ND
Leu AT	3	ND	ND	ND	ND

[a] 35% lower activities were measured with $NADP^+$.
[b] ND, not done.

Mutants of strain WV2 blocked in L-Phe or L-Tyr biosynthesis. Mutants requiring either L-Phe or L-Tyr for growth could be isolated in a straightforward manner. However, among the tightly blocked mutants tested, only arogenate dehydrogenase- (10 mutants; e.g., strain GH70, L-Tyr auxotroph) and prephenate dehydratase (2 mutants; e.g. strain GH71, L-Phe auxotroph)-negative mutants were identified (Table 1; Fig. 1). These results confirmed the presence of single pathways for L-Phe and L-Tyr biosynthesis. Mutant strains GH70 and GH71 could also grow in media supplemented with 4-hydroxyphenylpyruvate and phenylpyruvate (50 mg · $liter^{-1}$), respectively. Tyr AT apparently converts sufficient 4-hydroxyphenylpyruvate into L-Tyr to allow growth to occur (Fig. 1). Similar Tyr AT activity levels were observed (Table 1) in glucose- and in L-Tyr-grown cells (data not shown). Toward the end of growth, mutant strains GH70 and GH71 excreted minor amounts of L-Phe and L-Tyr (ca. 1 mM), respectively.

Two leaky L-Phe auxotrophic mutants were subsequently identified as strains affected in Phe/Tyr AT. Mutant strains GH141 and GH148 were still able to grow on glucose mineral medium without supplements, albeit at reduced growth rates (doubling times increased from 2.5 to 6 h). In the presence of L-Phe (50 mg · $liter^{-1}$), wild-type growth rates were observed, showing that L-Phe biosynthesis, but not L-Tyr biosynthesis,

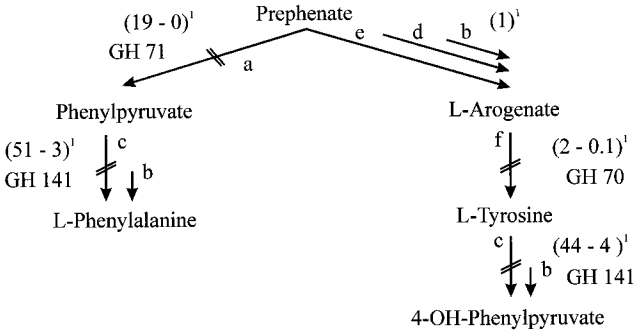

FIG. 1. Biosynthetic pathways for the conversion of prephenate into L-Phe and L-Tyr in *A. methanolica*. Arrows of different lengths are used to indicate the relative importance of isoenzymes detected for some of the steps in strain WV2 (see Fig. 2A). Labeled arrows: a, prephenate dehydratase; b and c, Phe AT (Phe ATI and -II, respectively); b, d, and e, (Ppa ATI, -II, and -III, respectively); f, arogenate dehydrogenase; b and c, tyrosine AT (Tyr ATI and II, respectively). Examples of mutants identified in the various steps are indicated. Specific enzyme activities (in milliunits · milligram of $protein^{-1}$) of strain WV2 and auxotrophic mutant strains derived from WV2 are shown in the parentheses with superscript one (see also Table 1).

was affected. The specific Phe AT activity in mutant strains GH141 and GH148, reduced to 3 to 5 mU · mg of protein^{-1} (Table 1), apparently is sufficiently high to support slow growth in glucose mineral medium. Mutant strains GH141 and GH148 had also lost most of the Tyr AT activity (Table 1), resulting in the complete failure to grow on L-Tyr as a carbon source. Growth on 4-hydroxyphenylpyruvate (5 mM) as a carbon source remained possible but required the addition of L-Phe to the growth medium for optimal growth. Prototrophic revertants of mutant strain GH141 selected on media containing glucose as the sole carbon source regained both Phe AT and Tyr AT activities and were also able to grow on L-Tyr as a carbon source. Revertants selected on media containing L-Tyr as the sole carbon source also regained both Phe AT and Tyr AT activities and were also able to grow normally in glucose mineral medium. These results indicate that the final step in L-Phe biosynthesis and the initial step in L-Tyr catabolism are catalyzed by one and the same Phe/Tyr AT enzyme. Further evidence supporting this hypothesis was obtained in biochemical studies (see below).

Presence of AT isoenzymes. Failure to isolate tightly blocked Phe/Tyr AT- and Ppa AT-negative mutants of *A. methanolica* could be due to the presence of multiple aro AT enzymes with overlapping substrate specificities. Fractionation of extracts of strain WV2 by Mono Q anion-exchange chromatography yielded two peaks with Phe AT as well as Tyr AT activity, and three peaks with Ppa AT activity (Fig. 2A). The Phe/Tyr ATII peak contained more than 90% of the total Phe AT and Tyr AT activities. In extracts of mutant strains GH141 (Fig. 2B) and GH148 (not shown), the level of Phe/Tyr ATI was normal, but the level of Phe/Tyr ATII was strongly reduced. Most of the total Ppa AT activity was contributed by the Ppa ATII and Ppa ATIII peaks (Fig. 2A). Similar Ppa AT profiles were observed with mutant strains GH141 (Fig. 2B) and GH148 (not shown).

The possible synonymy of aro AT activities with branched-chain amino acid AT and/or Asp AT enzymes was investigated (Table 1; Fig. 2C). A single Leu AT activity peak was observed, coeluting with Phe/Tyr ATI and Ppa ATI. This result suggests that the branched-chain amino acid AT of *A. methanolica* can use prephenate, L-Phe, and L-Tyr as substrates. Also a single peak with Asp AT activity was observed, coeluting with Phe/Tyr ATII and Ppa ATII (Fig. 2B and C). The Asp AT and Phe/Tyr ATII activity peaks show considerable overlap in strain WV2; Asp AT is unaffected in mutant strain GH141, however (Table 1 and Fig. 2). Asp AT of *A. methanolica* thus may be able to use prephenate, but not L-Phe or L-Tyr as a substrate.

Properties of ATs. The dominant Phe/Tyr AT enzyme (Fig. 2A) was purified 134-fold with a yield of approximately 32% (Table 2). A similar ratio of activities was obtained with either L-Phe or L-Tyr as the substrate for activity assays throughout purification; the final Tyr AT specific activity was 3.8 U · mg of protein^{-1}. The final preparation obtained still showed several bands on SDS-polyacrylamide gels. Further purification attempts, involving among others hydrophobic-interaction chromatography, resulted in severe activity losses and failed to yield a more purified, active preparation. Activity stains in nondenaturating polyacrylamide gels revealed single Phe AT and Tyr AT bands with the same mobility. Further characterization of Phe/Tyr ATII showed that L-Glu could not be replaced by any other amino acid as the amino-group donor. The addition of pyridoxal phosphate was not required for optimal activity. By gel filtration chromatography, a molecular mass of 80 kDa was estimated for the native enzyme. The temperature and pH optima for activity were 55°C and pH 8.0. The apparent K_m values for L-Phe, L-Tyr, phenylpyruvate, L-Glu, and α-ketoglutarate, were estimated (Table 3).

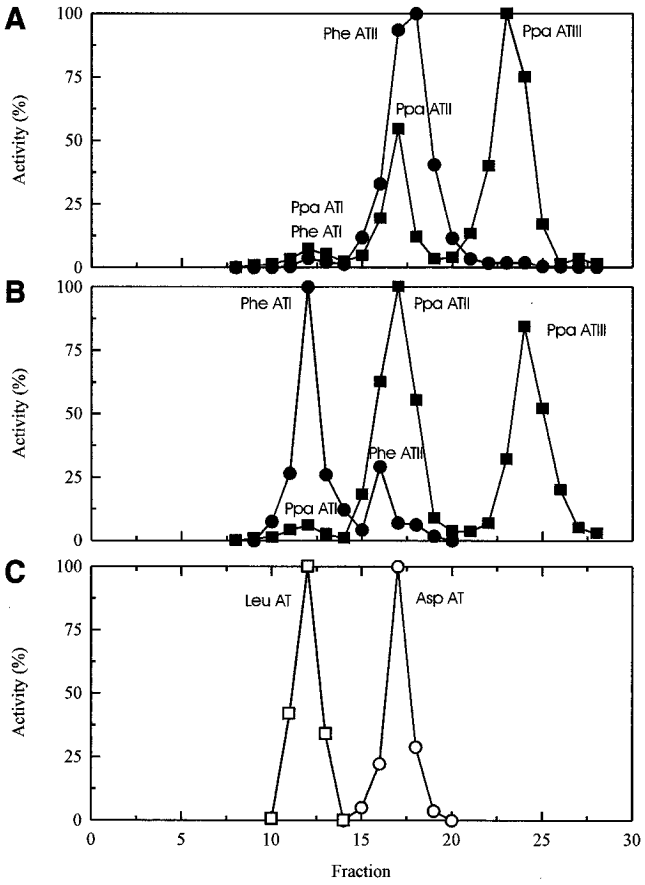

FIG. 2. Aro AT activity profiles obtained by Mono Q anion-exchange chromatography of cell extracts of *A. methanolica* WV2 (A) and mutant strain GH141 (B and C). Relative activities (100% activities are given in parentheses and corrected for the amount of protein loaded on the column) of Phe AT (●, 6.5 mU · ml^{-1} · mg^{-1} in panel A; 0.14 mU · ml^{-1} · mg^{-1} in panel B), Ppa AT (■, 1.4 mU · ml^{-1} · mg^{-1} in panel A; 1.3 mU · ml^{-1} · mg^{-1} in panel B), Leu AT (□, 1.9 mU · ml^{-1} · mg^{-1}), and Asp AT (○, 325 mU · ml^{-1} · mg^{-1} in panel C). A similar plot for Leu AT and Asp AT was obtained with strain WV2.

The minor Phe/Tyr AT enzyme (Fig. 2A) was partially purified (not shown) from glucose-grown cells of strain WV2, using steps 1 to 4 of the purification protocol for Phe/Tyr ATII (see Materials and Methods and Table 2). The Phe AT and Tyr AT specific activities of the final preparation were 0.32 and 0.25 U · mg of protein^{-1}. When assuming a Phe AT specific activity of 3 mU · mg of protein^{-1} for Phe/Tyr ATI in wild-type

TABLE 2. Purification of aro AT (Phe/Tyr ATII) from glucose-grown cells of *A. methanolica* WV2[a]

Step	Amt (mg) of protein	Sp act (U · mg^{-1})	Total activity (U)	Purification (fold)	Yield (%)
Extract	1,263	0.037	46.6	1	100
Q-Sepharose	135	0.26	35.4	7.1	76
$(NH_4)_2SO_4$ precipitation	68	0.34	22.8	9.1	49
Superdex 200	10.6	1.72	18.3	46.6	39
Mono Q	3.0	4.94	14.9	133.8	32

[a] Activities were measured with L-Phe as the substrate.

TABLE 3. Kinetic properties of partially purified aro AT enzymes from *A. methanolica* WV2 (Phe/Tyr ATI and Phe/Tyr ATII) and mutant strain GH141 (Ppa ATII and Ppa ATIII)

Direction	Substrate	K_m (mM)			
		Phe/Tyr ATI	Phe/Tyr ATII	Ppa ATII	Ppa ATIII
Forward	Ppy[a]	0.13	0.14	—[b]	—
	L-Glu	15.3	11.49	—	—
Reverse	L-Phe	1.01	0.79	—	—
	α-Kg[c]	2.72	2.75	—	—
Reverse	L-Tyr	ND[d]	0.87	—	—
	α-Kg	ND	1.6	—	—
Forward	Ppa	ND	—	2.0	0.29

[a] Ppy, phenylpyruvate.
[b] —, no activity.
[c] α-Kg, α-ketoglutarate.
[d] ND, not done.

cells (see strain GH141 in Table 1), a 107-fold overall purification was achieved. The Leu AT specific activity in this preparation was 375 mU · mg of protein^{-1}, an 125-fold increase. Activity stains in nondenaturating polyacrylamide gels revealed a single Phe AT band with a slightly higher mobility than Phe/Tyr ATII. Omitting pyridoxal phosphate did not affect Phe/Tyr ATI activity. By gel filtration chromatography, a molecular mass of 70 kDa was estimated for the native enzyme. The temperature and pH optima for activity were 60°C and pH 8.5. The apparent K_m values for L-Phe, phenylpyruvate, L-Glu, and α-ketoglutarate, were estimated (Table 3).

Ppa ATII and Ppa ATIII both appeared rather unstable during purification attempts. Some properties of both enzymes therefore were estimated from freshly prepared Mono Q anion-exchange chromatography peak fractions of mutant strain GH141 (Fig. 2B). Ppa ATII overlapped completely with Asp AT. Ppa ATIII possessed a very high substrate specificity. L-Glu could not be replaced by any other amino acid as amino-group donor in the reaction with prephenate. In the reverse reaction, with α-ketoglutarate as the amino-group acceptor, no activity was observed with any amino acid other than L-arogenate. Omitting pyridoxal phosphate strongly affected Ppa ATIII (38% reduction in specific activity), but not Ppa ATII. The apparent K_m values of both enzymes for prephenate were estimated (Table 3).

DISCUSSION

The biosynthesis of L-Phe in bacteria may proceed via dual pathways involving prephenate dehydratase, cyclohexadienyl dehydratase, or arogenate dehydratase (19). The isolation of a L-Phe auxotrophic mutant of *A. methanolica* blocked in prephenate dehydratase (strain GH71) showed that a single pathway for the conversion of prephenate into L-Phe is present in this organism, involving phenylpyruvate as an intermediate. Recently we have purified prephenate dehydratase from *A. methanolica* to homogeneity and characterized its kinetic properties (15).

L-Tyr biosynthesis may also proceed via dual pathways involving prephenate dehydrogenase, cyclohexadienyl dehydrogenase, or arogenate dehydrogenase (6). The isolation of a L-Tyr auxotrophic mutant of *A. methanolica* blocked in arogenate dehydrogenase (strain GH70) showed that a single pathway toward L-Tyr is present in this organism, involving L-arogenate as an intermediate. L-Tyr biosynthesis was not affected in strains with a strongly reduced Phe/Tyr ATII activity (GH141 and GH148 [Table 1]). The use of arogenate dehydrogenase activity for L-Tyr biosynthesis has also been reported in some other gram-positive bacteria: *Streptomycetes* (23), sporeforming members of the *Actinomycetales* (21), and coryneform bacteria (17, 23). The actinomycete *Microtetraspora glauca* was found to possess a single enzyme active with both prephenate and L-arogenate (cyclohexadienyl dehydrogenase) (34), differing from the situation reported here for *A. methanolica*. While the alternative L-Tyr biosynthetic pathway with prephenate dehydrogenase is generally present in gram-negative bacteria, it has been reported in relatively few of the gram-positive bacteria studied, e.g., *Bacillus subtilis* (20) and *Nocardia mediterranei* (36). The previously reported presence of prephenate dehydrogenase in crude extracts of *A. methanolica* (10) now appears due to the combined Ppa AT and arogenate dehydrogenase activities in the assay system (NADH formation) used.

Two isoenzymes with Phe/Tyr AT activity were (partially) purified from *A. methanolica*. The data obtained for Phe/Tyr ATI suggests that this protein actually is the branched-chain amino acid AT of *A. methanolica*. Characterization of branched-chain amino acid AT enzymes from other sources generally revealed a broad substrate specificity (7, 24, 30, 33, 35, 37). The characterization of mutant strain GH141 shows that Phe/Tyr ATII is the dominant AT in L-Phe biosynthesis. Quite surprisingly, the constitutive Phe/Tyr ATII also appears to catalyze the initial step in L-Tyr catabolism. The involvement of one and the same enzyme in amino acid biosynthetic as well as catabolic pathways is very unusual; another example observed in *A. methanolica* is 3-dehydroquinate dehydratase (13). Inducible enzymes normally are involved in the catabolism of aromatic amino acids. An inducible L-Phe dehydrogenase for instance catalyzes the initial step in L-Phe catabolism in *A. methanolica* (9). *Brevibacterium linens* 47 and other coryneform bacteria employ an inducible AT for growth on L-Phe (27–29). In *Saccharomyces cerevisiae*, L-Trp is degraded by an inducible AT (25). The situation observed in *A. methanolica* thus appears quite exceptional.

Ppa ATII coeluted with Asp AT during anion-exchange chromatography. The data obtained with mutant strain GH141 (Fig. 2) show that Asp AT of *A. methanolica* is able to use prephenate, but not aromatic amino acids, as a substrate. The high specific activity for Asp AT in cell extracts is unusual for biosynthetic enzymes. An Asp AT with similar properties has been reported in *Brevibacterium flavum* (33). The *A. methanolica* Asp AT enzyme has recently been purified to homogeneity (1).

Ppa ATIII of *A. methanolica* displayed a high affinity for prephenate (Table 3) and appeared highly specific for prephenate and L-Glu. To our knowledge, this is the first example of a specific Ppa AT from a bacterial source; such enzymes thus far have only been detected in plants (3). The high specificity and the relatively low K_m of Ppa ATIII for prephenate suggest that this enzyme plays an important physiological role in L-Tyr biosynthesis. Instability of Ppa ATIII prevented its more detailed characterization.

The danger of multiple mutations always exist in mutant studies. The clear effects of single medium supplements on growth of mutant strains GH70 (L-Tyr), GH71 (L-Phe), and GH141 and GH148 (both stimulated by L-Phe), the reversion studies carried out with mutant strain GH141, and the clear identification of specific enzyme lesions in the biochemical studies reported, however, provide clear evidence that the *A. methanolica* mutants characterized in this study carry single mutations only. From the results obtained, we conclude (Fig. 1) that *A. methanolica* utilizes the enzymes prephenate dehydratase and Phe/Tyr ATII (the dominant AT) for L-Phe bio-

synthesis. Phe/Tyr ATII also catalyzes the initial step in L-Tyr catabolism but is not involved in L-Tyr biosynthesis. Although Phe/Tyr ATI normally functions as a branched-chain amino acid AT, it can be forced to play a (minor) role in L-Phe biosynthesis, as indicated by the leaky phenotype of mutants lacking Phe/Tyr ATII in glucose mineral medium. Biosynthesis of L-Tyr involves the enzymes Ppa ATIII and arogenate dehydrogenase. The less efficient prephenate-utilizing capabilities of Asp AT (= Ppa ATII) and Leu AT (= Ppa ATI) may also contribute to the in vivo L-arogenate pool. This study thus provides detailed insights in aromatic amino acid biosynthesis from prephenate in the actinomycete *A. methanolica*. The data provide clear targets for the rational construction of strains of *A. methanolica* with improved capabilities to synthesize primary and secondary metabolites derived from prephenate (Fig. 1).

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