Regulation of Phenylalanine Biosynthesis in *Rhodotorula glutinis*

MICHAEL J. FISKE AND JAMES F. KANE*

Corporate Research Laboratories, Monsanto Co., St. Louis, Missouri 63167

Received 17 May 1984/Accepted 17 August 1984

The phenylalanine biosynthetic pathway in the yeast *Rhodotorula glutinis* was examined, and the following results were obtained. (i) 3-Deoxy-d-arabinoheptulosonate-7-phosphate (DAHP) synthase in crude extracts was partially inhibited by tyrosine, tryptophan, or phenylalanine. In the presence of all three aromatic amino acids an additive pattern of enzyme inhibition was observed, suggesting the existence of three differentially regulated species of DAHP synthase. Two distinctly regulated isozymes inhibited by tyrosine or tryptophan and designated DAHP synthase-Tyr and DAHP synthase-Trp, respectively, were resolved by DEAE-Sephadex chromatography, along with a third labile activity inhibited by phenylalanine tentatively identified as DAHP synthase-Phe. The tyrosine and tryptophan isozymes were relatively stable and were inhibited 80 and 90% by 50 μM of the respective amino acids. DAHP synthase-Phe, however, proved to be an extremely labile activity, thereby preventing any detailed regulatory studies on the partially purified enzyme. (ii) Two species of chorismate mutase, designated CMI and CMII, were resolved in the same chromatographic step. The activity of CMI was inhibited by tyrosine and stimulated by tryptophan, whereas CMII appeared to be unregulated. (iii) Single species of prephenate dehydratase and phenylpyruvate aminotransferase were observed. Interestingly, the branch-point enzyme prephenate dehydratase was not inhibited by phenylalanine or affected by tyrosine, tryptophan, or both. (iv) The only site for control of phenylalanine biosynthesis appeared to be DAHP synthase-Phe. This is apparently sufficient since a spontaneous mutant, designated FP9, resistant to the growth-inhibitory phenylalanine analog p-fluorophenylalanine contained a feedback-resistant DAHP synthase-Phe and cross-fed a phenylalanine auxotroph of *Bacillus subtilis*.

The pathway for the biosynthesis of the aromatic amino acids has been previously shown to be a highly complex and diverse sequence of reactions in microorganisms (5) and is known to generate a variety of significant secondary metabolites (Fig. 1). Numerous studies have been documented on aromatic amino acid synthesis in yeasts (2, 3, 9, 11–13, 15); however, only a few reports exist that describe the loss of regulation of this pathway resulting in the overproduction of aromatic metabolites (1, 7, 17). In some cases there are sequential anabolic and catabolic pathways that must be appropriately controlled for the microorganism to compete successfully in its environment. The red yeast, *Rhodotorula glutinis*, for example, is capable of synthesizing phenylalanine as an essential amino acid, but it can also utilize phenylalanine as a source of carbon or nitrogen. Although there have been reports on factors that control the expression of phenylalanine ammonia-lyase (10, 14, 18, 22, 23), the first catabolic enzyme in the degradative pathway, there are essentially no data on the control of phenylalanine biosynthesis. The absence of this basic information makes it difficult to understand the physiological and regulatory relationships between these anabolic and catabolic sequences. It was our objective to elucidate the phenylalanine biosynthetic sequence, to study its regulation, and to determine the effects of regulatory mutations on the overproduction of phenylalanine.

**MATERIALS AND METHODS**

Strains and culture conditions. *R. glutinis* wild type was obtained from R. A. Jensen (14) and was the parent strain of analog-resistant mutants. Extracts were prepared from cells grown in Difco yeast nitrogen base without amino acids containing 1% (wt/vol) glucose unless otherwise indicated. Cultures were grown at 30°C under constant aeration, harvested during late-exponential growth by centrifugation, and stored at −70°C.

Isolation of analog-resistant mutants. Wild-type cultures were grown overnight at 30°C in a minimal salts medium (8) containing 2% (wt/vol) sorbitol. A 0.1-ml sample of a turbid culture was spread on the surface of minimal sorbitol agar plates. Crystals of p-fluorophenylalanine were placed on the surface of the plates, which were then incubated at 30°C. After several days resistant colonies appearing within the zone of growth inhibition were picked and purified on minimal sorbitol plates containing 20 μg of p-fluorophenylalanine ml⁻¹. Growth studies with the analog in liquid culture were also done, using minimal sorbitol media plus the indicated amount of p-fluorophenylalanine.

Cross-feeding. Analog-resistant isolates were screened for phenylalanine excretion by their ability to cross-feed a phenylalanine auxotroph of *Bacillus subtilis* (BGSG 1A96) and on plates containing Spizizen minimal medium (8), 1% (wt/vol) glucose, and 50 μg of L-tryptophan ml⁻¹. Cross-feeding could be detected after 2 days of incubation at 30°C. The wild type did not cross-feed under these conditions.

Preparation of crude extracts. Whole-cell pellets were suspended in 50 mM potassium phosphate buffer (pH 7.0), passed through an Amino French pressure cell twice at 12,000 psi, and centrifuged at 15,000 × g to remove cellular debris.

Analytical procedures. 3-Deoxy-d-arabinoheptulosonate-7-phosphate (DAHP) synthase was assayed by the method of Calhoun et al. (6). Since this enzyme is often stimulated by divalent metal ions (19), CoSO₄, MnSO₄, and MgSO₄ were tested for stimulatory effects. A typical reaction mixture (200 μl) contained 2 mM phosphoenolpyruvate, 1 mM erythrose-4-phosphate, 1 mM metal ion, 50 mM potassium phosphate buffer (pH 7.0), enzyme, and any specified compounds tested as effectors. All assays were performed at 37°C and included appropriate controls to ensure against interference.

* Corresponding author.
of the chemical assay by the effector molecules (20). The concentration of DAHP formed was calculated with a molar extinction coefficient of 45,000 at 549 nm.

Chorismate mutase and prephenate dehydratase were assayed by the method of Patel et al. (16). For both enzyme activities, the appearance of phenylpyruvate was monitored by incubating at 37°C a reaction mixture (200 μl) containing 1.0 mM of either chorismate or prephenate, 50 mM potassium phosphate buffer (pH 7.0), enzyme, and the indicated concentrations of effector molecules. The concentration of phenylpyruvate produced was estimated with a molar extinction coefficient of 17,500 at 320 nm.

Aromatic aminotransferase activity was assayed by the method of Whitaker et al. (21). Reaction mixtures (200 μl) containing 10 mM l-phenylalanine, 2.5 mM α-ketoglutarate, 12.5 mM pyridoxal 5'-phosphate, 50 mM potassium phosphate buffer (pH 7.0), and enzyme were incubated at 37°C. The absorbance of phenylpyruvate was monitored at 320 nm and quantitated with a molar extinction coefficient of 17,500.

Protein concentrations were determined by the method of Bradford (4) with bovine serum albumin as a standard.

**DEAE-Sephadex chromatography.** Crude extract protein (50 mg) from the wild-type strain was applied to a DEAE-Sephadex column (1.5 by 7 cm) equilibrated in 50 mM potassium phosphate buffer (pH 7.0) at 4°C. Protein was loaded and washed onto the column with 50 ml of starting buffer. Proteins were eluted with a 180-ml linear 0 to 0.5 M gradient of KCl. Fractions of 20 ml were collected.

**Biochemicals.** Amino acids, p-fluorophenylalanine, erythro 4-phosphate, phosphoenolpyruvate, prephenate, chorismate, aminoxyacetic acid, α-ketoglutarate, pyridoxal 5'-phosphate, cobalt sulfate, and manganese sulfate were obtained from Sigma Chemical Co. DEAE-Sephadex was obtained from Pharmacia Fine Chemicals, Inc. Sodium arogenate was a generous gift from the laboratory of R. A. Jensen. All chemicals were of the best grade commercially available.

**RESULTS**

**Enzymatic profile of unfractionated crude extract.** Activities for DAHP synthase, chorismate mutase, and prephenate dehydratase were measured in crude extracts of prototrophic R. glutinis (Table 1). The first enzyme of the aromatic amino acid pathway, DAHP synthase, was stimulated 1.5- to 2.0-fold by the addition of either 1 mM Co²⁺ or Mn²⁺; however, the same concentration of Mg²⁺ had no effect. Tyrosine, tryptophan, and phenylalanine, either individually or together, inhibited DAHP synthase in an additive manner, suggesting the presence of isozymic species.

Phenylalanine, tyrosine, and tryptophan at 50 μg ml⁻¹ were added separately and in combination to growth medium (yeast nitrogen base without amino acids plus 1% glucose) to determine whether the aromatic amino acids repressed the synthesis of DAHP synthase. Crude extracts were prepared from these cells, and DAHP synthase was assayed. The specific activities in each case were comparable to that found in cells grown without the addition of end products (data not shown). Thus, repression control of DAHP synthase does not appear to be a significant control mechanism in R. glutinis.

The second key enzyme, chorismate mutase, was inhibited 50% by 0.5 mM tyrosine and stimulated 1.4-fold by 0.5 mM tryptophan. Phenylalanine had no effect on this activity.

Phenylalanine can be produced by either of two routes, prephenate dehydratase or arogenate dehydratase (5, 16). Although prephenate dehydratase was readily detected in crude extracts, no dehydratase was found that used arogenate as a substrate for phenylalanine biosynthesis. Surprisingly, however, prephenate dehydratase was not inhibited by phenylalanine, and activity was not affected by tyrosine, tryptophan, or cinnamate (a product of phenylalanine ammonia-lyase) (Table 1). It has been observed that phenylalanine inhibition of prephenate dehydratase may be masked in crude extracts by an aromatic transaminase capable of converting phenylalanine to phenylpyruvate, the measured product of the prephenate dehydratase reaction (R. A. Jensen, personal communication). We found that the addition of 1.0 mM aminoxyacetic acid to the reaction mixtures inhibited the aromatic transaminase activity by 99%, whereas the prephenate dehydratase activity was reduced only 15%. Even after elimination of the contaminating transaminase activity, the addition of l-phenylalanine up to 2.5 mM had no effect on the prephenate dehydratase activity.

**Partial purification of key aromatic biosynthetic enzymes.** Key aromatic biosynthetic enzymes were fractionated by ion-exchange chromatography on DEAE-Sephadex. When the fractions were assayed for DAHP synthase, three distinct activities were found. An extremely labile, low-activity enzyme was observed in the column wash. Two separable and relatively stable DAHP synthases eluted in the gradient

**TABLE 1. Enzymatic profile in crude extracts of R. glutinis**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Relative activity in presence of effector:</th>
<th>None</th>
<th>Pheny-</th>
<th>Tyro-</th>
<th>Trypto-</th>
<th>Aro²</th>
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<td>DAHP synthase</td>
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<td>0.76</td>
<td>0.58</td>
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<td>Prephenate dehydratase</td>
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<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>ND²</td>
</tr>
</tbody>
</table>

² The final concentration of the individual effectors was maintained at 0.5 mM.

² The value of 1.00 represents specific activities (nanomoles of product formed min⁻¹ mg of protein⁻¹) of 15.1, 22.6, and 28.2 for DAHP synthase, chorismate mutase, and prephenate dehydratase, respectively.

² Aro represents 0.5 mM each of phenylalanine, tyrosine, and tryptophan.

² ND, Not determined.

**FIG. 1.** Aromatic amino acid biosynthetic pathway. Symbols: 1, DAHP synthase-Phe; 2, DAHP synthase-Tyr; 3, DAHP synthase-Trp; 4, CM1; 5, CMII; 6, prephenate dehydratase; 7, anthranilate synthase. Abbreviations: E4P, erythrose-4-phosphate; PEP, phosphoenolpyruvate; CHA, chorismate; PPA, prephenate; PYY, phenylpyruvate; HPP, hydroxyphenylpyruvate; ANT, anthranilate; PHE, phenylalanine; TYR, tyrosine; TRP, tryptophan. Only enzymes referred to in this report are labeled in the figure.
Each species of enzyme was tested for sensitivity to feedback inhibition by phenylalanine, tyrosine, and tryptophan. The first isozyme eluted from the gradient was inhibited by tyrosine only (Fig. 3) and was designated DAHP synthase-Tyr. Tyrosine at 17 μM inhibited activity 50%, with a maximal inhibition of 90% at 70 μM. This activity was stimulated by both Mn²⁺ (2.5-fold with 1.0 mM) and Co²⁺ (1.7-fold with 1.0 mM). The second isozyme to elute was inhibited only by tryptophan (Fig. 3) and was designated DAHP synthase-Trp. This enzyme was also very sensitive to feedback inhibition (50% with 8 μM tryptophan) and was stimulated by Mn²⁺ (1.5-fold with 1.0 mM) but not by Co²⁺, which slightly inhibited activity (25% inhibition by 1.0 mM). The isozyme which eluted in the wash proved extremely sensitive to feedback inhibition by tryptophan.

**FIG. 2.** Fractionation of key aromatic pathway enzymes after DEAE-Sephacel chromatography. Crude extract protein (50 mg) was applied to a DEAE-Sephacel column as described in the text. The vertical dashed lines mark the boundary between the end of the wash eluate and the beginning of the salt gradient.

**FIG. 3.** Sensitivity of DAHP synthase-Tyr and DAHP synthase-Trp to end product inhibition. Partially purified DAHP synthase-Tyr (○) (specific activity = 38.1 nm min⁻¹ mg of protein⁻¹) and DAHP synthase-Trp (□) (specific activity = 30.2 nm min⁻¹ mg of protein⁻¹) were obtained from DEAE-Sephacel chromatography. Percent inhibition of enzyme activity is plotted as a function of effector concentration.

**FIG. 4.** Regulation of CMI. Partially purified CMI (specific activity = 1,500 nm min⁻¹ mg of protein⁻¹) was obtained from DEAE-Sephacel chromatography. Relative activity is plotted as a function of the effector concentration. Abbreviations: TRP, tryptophan; TYR, tyrosine.
labile, and although several attempts (such as addition of reducing agents, protease inhibitors, and divalent metal ions to the extract buffers) were made to stabilize this activity, no additional studies could be carried out. We propose, however, that this activity represents the phenylalanine-sensitive isozyme.

Two separable species of chorismate mutase were eluted from the DEAE-Sephasel column (Fig. 2). The major isozymic species eluted first and was designated CMI, and the minor isozyme which eluted with higher salt was called CMI1. CMI was found to be feedback inhibited by tyrosine and stimulated by tryptophan (Fig. 4). A concentration of 750 μM tyrosine was required for maximal inhibition (70%), whereas 50 μM tryptophan gave a twofold stimulation of activity. Phenylalanine did not affect the activity of CMI. The second isozyme, CMI1, was unaffected by the addition of phenylalanine, tyrosine, or tryptophan at concentrations as high as 2.5 mM.

Prephenate dehydratase eluted as a single species (Fig. 2). As we found with the crude extract preparations, the partially purified enzyme was unaffected by the addition of phenylalanine at concentrations up to 2.5 mM. Since the prephenylpyruvate transaminase was separated from prephenate dehydratase, the lack of inhibition by phenylalanine cannot be masked by the conversion of phenylalanine to prephenylpyruvate. As expected, neither tyrosine nor tryptophan affected prephenate dehydratase activity, and no argenate dehydratase activity was found in the peak fraction.

**Growth inhibition by PFP and isolation of regulatory mutants.** We isolated mutants resistant to the phenylalanine analog p-fluoro-DL-phenylalanine (PFP) to gain insight into the regulation of phenylalanine biosynthesis. PFP serves as a potent growth inhibitor of *R. glutinis* in minimal salts media containing glucose, fructose, or sorbitol as the carbon source. The growth of wild-type *R. glutinis* was completely inhibited by 20 μg of PFP ml⁻¹ in a minimal sorbitol medium (Fig. 5). The addition of 500 μg of phenylalanine ml⁻¹ partially reversed the inhibitory effects of the analog. Similarly, 500 μg of tryptophan ml⁻¹ partially reversed the growth inhibition. This observation is consistent with the tryptophan-dependent activation of CMI resulting in an increased flux of carbon through the unregulated phenylalanine branchlet. On the other hand, 500 μg of tyrosine ml⁻¹ allowed only two doublings in mass before growth ceased, and the addition of similar concentrations of shikimic acid had no effect (data not shown).

Spontaneous resistant mutants were selected on PFP-containing media, and regulatory mutants were screened based upon their ability to cross-feed a phenylalanine auxotroph of *B. subtilis*. One such mutant, designated FP9, was completely resistant to growth inhibition by PFP (Fig. 6). The biochemical basis for this resistance resided at the level of DAHP synthase. Whereas the DAHP synthase in crude extracts of the parental strain was inhibited ca. 45% by either 0.5 mM phenylalanine or PFP, this enzyme from FP9 was virtually unaffected (Fig. 7). The inhibition of DAHP synthase-Tyr and DAHP synthase-Trp by their respective amino acids was not affected, however, in the mutant FP9. There was essentially no change in the specific activity of DAHP synthase in the crude extracts of FP9, and furthermore, the levels of chorismate mutase and prephenate dehydratase and their sensitivities to effectors were comparable to the wild type.

**DISCUSSION**

In *R. glutinis*, only two steps in the pathway for phenylalanine biosynthesis were regulated. The first was DAHP synthase, and this activity was found in three isozymes, each of which was subjected to feedback inhibition by a single aromatic amino acid. A similar isozymic pattern has been reported in *Neurospora crassa* (11, 15), whereas other yeasts, such as *Saccharomyces cerevisiae* (9) and *Hansenula henricii* (3), contain only two isozymes controlled by tyrosine and phenylalanine. The major enzyme species in *R. glutinis* appeared to be DAHP synthase-Phe, since phenylalanine inhibited activity in crude extracts by nearly 50%.

**FIG. 5.** PFP inhibition of growth in wild-type *R. glutinis*. PFP was added at a final concentration of 20 μg ml⁻¹, whereas L-phenylalanine (PHE) and L-tryptophan (TRP) were supplied at 500 μg ml⁻¹.

**FIG. 6.** Growth of mutant FP9 in the presence of PFP. The PFP-resistant mutant FP9 was grown in the presence and absence of 20 μg of PFP ml⁻¹.
The second site of control, chorismate mutase represents the branch-point enzyme leading to the synthesis of phenylalanine and tyrosine. Two isozymes of chorismate mutase were isolated. One species, CMI, was not regulated by the aromatic amino acids, whereas the other, CMI, was controlled negatively by tyrosine and positively by tryptophan. Regulation of chorismate mutase by aromatic end products has also been observed in other yeasts. In S. cerevisiae, crude extract preparations of chorismate mutase have been found to be inhibited by tyrosine and activated by tryptophan. Chromatography of the S. cerevisiae chorismate mutase has resulted in the separation of two isozymic species, both inhibited by tyrosine (12). No mention was made of the effects of tryptophan. In contrast, a single chorismate mutase has been isolated from Hansenula polymorpha that is activated by tryptophan but unaffected by tyrosine (2).

Prephenate dehydratase, the first committed step of phenylalanine biosynthesis, has been recognized as a conventional site for tight feedback control by the end product phenylalanine. This enzyme is often under the additional control of more remote effectors, exhibiting the phenomenon of metabolic interlock. This has been well documented in procaryotic organisms (5); however, little information exists on the regulatory control of prephenate dehydratase in eucaryotes. It has been shown previously that S. cerevisiae possesses a prephenate dehydratase that is subject to feedback inhibition by phenylalanine (13). Surprisingly, this enzyme from R. glutinis proved to be insensitive to inhibition by phenylalanine and was not affected by tyrosine or tryptophan.

As a result of this type of metabolic control, we predict the following. First, loss of feedback control of DAHP synthase-Phe would lead to overproduction of phenylalanine. Second, tryptophan would stimulate the synthesis of phenylalanine. The physiological support for these predictions was found in studies with the effect of the analog p-fluorophenylalanine, a potent growth inhibitor (20 μg ml⁻¹ prevented growth for 72 h) of R. glutinis. A mutant of R. glutinis, FP9, resistant to the inhibitory effects of PFP contained a feedback-resistant DAHP synthase-Phe isozyme (Fig. 7). As a result, this mutant cross-fed a phenylalanine auxotroph (prephenate dehydratase-deficient pheA) of B. subtilis. Thus the loss of control at DAHP synthase led to the overproduction of the end product. Curiously, attempts to identify and quantitate phenylalanine as the excreted product in liquid broths were not successful. It is possible that the phenylalanine excreted into the liquid medium was subsequently reabsorbed and degraded by a low level of phenylalanine ammonia-lyase. Perhaps the diffusion of the amino acid into the agar and away from the yeast allowed us to observe at least the potential for overproduction of this end product.

Furthermore, we found that tryptophan alleviated, to some degree, the inhibitory effects of PFP, presumably through the stimulation of CMI, whereas tyrosine and shikimic acid had little or no effect. As expected, phenylalanine more completely reversed the growth-inhibitory effects of PFP (Fig. 5). We propose that neither tryptophan nor phenylalanine was totally effective in restoring normal growth rates and yields because PFP was deaminated by phenylalanine ammonia-lyase to p-fluorotranscinnamate (Fiske, unpublished data). Since low levels of cinnamate are toxic to R. glutinis, it seems reasonable to suppose that p-fluorocinnamate is also toxic. Whereas the endogenously generated phenylalanine in strain FP9 apparently prevented the formation of p-fluorocinnamate, exogenously added phenylalanine was not as effective. The reason for this observation is not clear but may reflect differential compartmentation of the two sources of phenylalanine.

LITERATURE CITED


