Regulation of Phenylalanine Ammonia Lyase in *Rhodotorula glutinis*

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In the red yeast *Rhodotorula glutinis*, phenylalanine ammonia lyase (PAL) was induced 10-fold during carbon starvation even in the absence of exogenous phenylalanine, although maximal induction occurred when phenylalanine was the nitrogen (40-fold) or carbon (100-fold) source. Apparent regulatory mutations that affected the expression of PAL were isolated by selecting mutants resistant to the analog p-fluoro-D,L-phenylalanine (PFP). One such mutant, designated FP1, could use phenylalanine as a nitrogen source but not as a carbon source. Similarly, FP1 failed to utilize intermediates of the phenylalanine degradative pathway, namely, benzoate, p-hydroxybenzoate, or 3,4-dihydroxybenzoate, as carbon sources. Although the PFP-resistant mutant contained a low level of PAL, no increase was found when it was grown with phenylalanine as the nitrogen source. A derivative of FP1, FP1a, was isolated that simultaneously regained an inducible PAL and the ability to use phenylalanine, benzoate, p-hydroxybenzoate, and 3,4-dihydroxybenzoate as carbon sources. In addition, when p-hydroxybenzoate was the carbon source, PAL was induced in the mutant FP1a but not in the PFP-sensitive parental strain. We propose that the mutation to PFP resistance occurred in a regulatory gene that controls the entire phenylalanine degradative pathway. Secondary mutations at this locus, as found in strain FP1a, not only restored expression of this pathway, but also altered the induction of PAL by metabolites of this pathway.

Phenylalanine ammonia lyase (PAL) catalyzes the nonoxidative deamination of phenylalanine, producing *trans*-cinnamate and NH_3. This enzyme, which has been found in *Streptomyces* sp. (6), yeasts (8–10, 14), fungi (3, 12), and plants (1, 2, 11), represents a key link between amino acid biosynthesis and degradation. In the latter case, PAL may function as the first enzyme in a catabolic pathway that generates NH_3 and energy or as the first enzyme leading to the synthesis of essential phenylpropanoids.

In the red yeast *Rhodotorula glutinis* this enzyme appears to serve a catabolic function. Several investigators (8–10, 14) report that PAL is induced only when the microorganism must degrade phenylalanine to obtain NH_3 or carbon or both. Gilbert and co-workers (8, 9) demonstrated in a convincing manner that PAL is synthesized de novo and that the PAL-specific mRNA is transcribed only in response to the presence of the inducer, phenylalanine. There is little information, however, on mutations that affect the expression of PAL or the entire phenylalanine catabolic sequence or both. In this report we describe the isolation and properties of a mutant that contains a regulatory defect in this catabolic pathway.

MATERIALS AND METHODS

Growth conditions and preparation of cell extracts. The red yeast *R. glutinis* was obtained from R. A. Jensen and was routinely grown at 30°C in a yeast nitrogen base medium (0392-15; Difco Laboratories) with 0.2% fructose as the carbon source. In experiments with other carbon sources the compounds were added to give a final concentration of 0.2%. When phenylalanine replaced fructose or $(NH_4)_2SO_4$ or both, it was used at a concentration of 0.2%.

Growth was monitored by measuring turbidity with a Gilford spectrophotometer at 600 nm or a Klett-Summerson colorimeter equipped with a green filter. Cultures were harvested at late log or early stationary phase of growth by centrifugation at 8,000 × g. The pellets were washed once with deionized water and suspended in the appropriate buffer. Cell extracts were prepared by passing the cell suspension through a French pressure cell at 18,000 lb/in² followed by centrifugation at 14,000 × g. The crude extract was used directly in the assay.

Assay for PAL. Cell extracts were prepared in either 50 mM Tris buffer, pH 8.5, or 50 mM KPO_4 buffer, pH 7.6. Activities were comparable in either buffer. Crude extract protein was added to a reaction mixture containing 15 mM phenylalanine and 50 mM Tris buffer (pH 8.5) at 37°C in a final volume of 1 ml. The increase in absorbance at 290 nm was monitored continuously for 5 min to determine initial velocities. A molar extinction coefficient of 10,000 was used to calculate the concentration of the product, cinnamate. The product was verified as cinnamate by high-pressure liquid chromatography. Activity is expressed as nanomoles of product formed per minute per milligram of protein. Protein concentrations were determined by the method of Bradford (4), with bovine serum albumin as a standard.

Mutant isolation. Spontaneous mutants of *R. glutinis* resistant to p-fluoro-D,L-phenylalanine were obtained by spreading a lawn of cells on a minimal salt plate (5) containing 25 µg of p-fluoro-D,L-phenylalanine ml⁻¹. Plates were incubated at 30°C until resistant colonies appeared. A total of 18 isolated colonies were further purified by subsequent reisolation on the same medium. Five of 18 were unable to grow with phenylalanine as the carbon source.

Partial purification of PAL. Approximately 100 mg of protein from crude extracts of the parental strain, FP1, or FP1a were applied to a DEAE-Sephacel column (2 by 9 cm) equilibrated in 50 mM Tris-hydrochloride buffer (pH 8.5) at 4°C. The column was washed with 3 column volumes of starting buffer, and PAL was eluted with a 250-ml linear gradient of NaCl (0 to 0.5 M). Fractions (2 ml) containing PAL activity were pooled and concentrated on an Amicon PM-10 membrane.

Growth responses with different carbon sources. Benzoate, p-hydroxybenzoate, and 3,4-dihydroxybenzoate were tested as carbon sources on solid media. Yeast nitrogen base plates

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TABLE 1. Activity of PAL as a function of fructose concentration

Concn of fructose (%)	Time of harvest (h)	A_{600}[a]	Sp act[b]
0.05	15.5	0.73	1.7
	23.5	0.65	15.5
	39.5	0.50	13.1
	47.5	0.52	12.1
0.10	15.5	0.76	2.1
	23.5	1.00	6.8
	39.5	0.83	12.3
	47.5	0.91	13.1
0.20	15.5	0.72	0.7
	23.5	1.25	0.7
	39.5	1.57	4.9
	47.5	1.59	3.5

[a] A_{600}, Absorbance at 600 nm.
[b] Specific activity is expressed as nanomoles of cinnamate formed per minute per milligram of protein.

were prepared without a carbon source. A lawn of cells from the appropriate strain was inoculated onto the plate, and crystals (approximately 15 mg) of the compound to be tested were added to the center of the lawn. The plates were incubated at 30°C for 72 h before being examined for growth.

Chemicals. Phenylalanine, cinnamate, and p-fluoro-D,L-phenylalanine were purchased from Sigma Chemical Co. Cinnamate was recrystallized before use. All other chemicals were of the highest grade available.

RESULTS

Regulation of PAL in the parental strain of R. glutinis. R. glutinis was grown in yeast nitrogen base medium with increasing concentrations of fructose as carbon source. PAL levels increased as the concentration of carbon source decreased (Table 1). The activity of PAL remained relatively constant despite the fact that the cells were not growing. This observation contrasts sharply with that of Gilbert and Tully (9), who reported a 3-h half-life in stationary cells. In their case, however, cells were suspended in a medium lacking both carbon and nitrogen sources and, therefore, the environment in their experiment was physiologically distinct from that in our experiment. Marusich et al. (10) also reported that PAL activity remained relatively constant during stationary phase in the same growth medium as that used in our experiments.

The effect of phenylalanine on PAL synthesis is shown in Table 2. Maximal activity was found when phenylalanine served as either the carbon or the carbon and nitrogen source. A concentration of 0.4% gave us the best cell yield with the highest specific activity for PAL (Table 3). We attempted to induce PAL activity with several other com-

TABLE 2. Induction of PAL by phenylalanine[a]

Carbon source (%)	Nitrogen source (%)	Sp act[b]
Fructose (0.2)	$(NH_4)SO_4$ (0.5)	3.0
Fructose (0.2)	Phenylalanine (0.2)	31.0
Phenylalanine (0.2)	$(NH_4)SO_4$ (0.5)	70.0
Phenylalanine (0.2)	Phenylalanine (0.2)	90.0

[a] Cultures were grown in yeast nitrogen base media with the indicated carbon and nitrogen sources and harvested at the end of exponential growth.
[b] Specific activity is expressed as nanomoles of cinnamate formed per minute per milligram of protein.

TABLE 3. Effect of phenylalanine concentration on induction of PAL

Concn of phenylalanine (%)[a]	A_{600}[b]	Sp act[c]
0.0	1.3	6.0
0.2	1.5	60.0
0.4	4.1	53.0
0.8	4.8	35.0
1.6	4.9	29.0

[a] Cells were grown in yeast nitrogen base with the indicated concentration of phenylalanine. As a control, fructose (0.2%) was used as carbon source with 0% phenylalanine.
[b] A_{600}, Absorbance at 600 nm.
[c] Specific activity is expressed as nanomoles of cinnamate formed per minute per milligram of protein.

pounds. D-Phenylalanine and phenyllactate had a slight stimulatory effect, whereas aminooxyphenylpropionic acid, known to hyperinduce PAL in plants (1), was not effective. Aminooxyacetic acid and phenylpropiolic acid could not be tested since both compounds inhibited the growth of R. glutinis for at least 96 h.

Mutants resistant to PFP. We observed that the analog p-fluorophenylalanine (PFP) was a potent inhibitor of growth of R. glutinis (Fig. 1). Concentrations as low as 2.5 µg ml^{-1} increased the doubling time from 2.5 to 7.5 h. At 20 µg ml^{-1} growth was essentially stopped, and the turbidity at 96 h was equal to that at 25 h. Since phenylalanine, but not tyrosine, reversed the inhibition of growth (data not shown), we propose that PFP affects only the phenylalanine biosynthetic sequence.

We used PFP to isolate mutants resistant to this analog. As expected, one class of mutants contained a feedback-re-
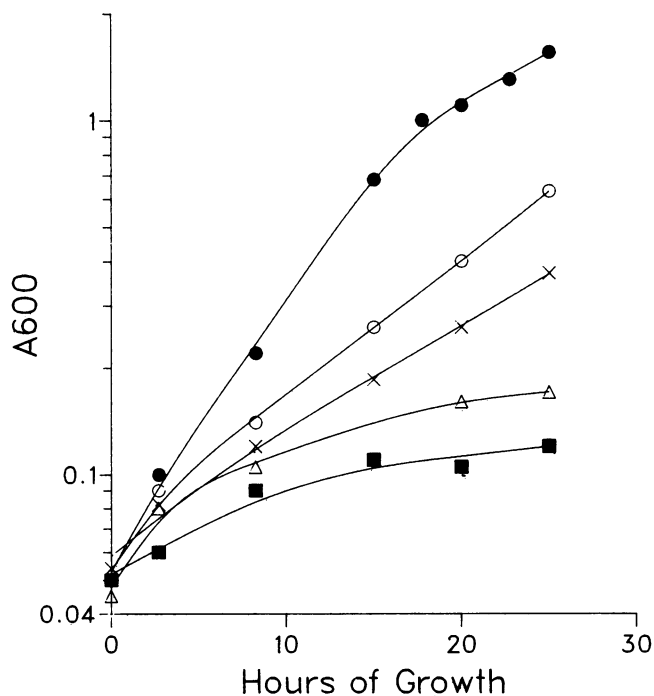

FIG. 1. Effect of PFP on growth of the parental strain of R. glutinis. R. glutinis was grown overnight in minimal salts medium containing 2% sorbitol and used to inoculate the same medium with: no PFP (●); 2.5 µg of PFP ml^{-1} (○); 5.0 µg of PFP ml^{-1} (×); 10 µg of PFP ml^{-1} (△); 20 µg of PFP ml^{-1} (■).

sistant isozyme of 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase, the first enzyme in the aromatic acid biosynthetic pathway (7; M. J. Fiske and J. F. Kane, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, K261) and the only phenylalanine regulated step in this sequence. Unexpectedly, we found a class of mutants represented by strain FP1. This mutant possessed an unusual phenotype; that is, FP1 cross-fed a phenylalanine auxotroph of *Bacillus subtilis*, contained three normally inhibitable DAHP synthase isozymes, and grew on phenylalanine as a nitrogen source but could not use phenylalanine as a carbon source. Therefore, we measured PAL activity in strain FP1 grown in a medium containing either fructose-$(NH_4)_2SO_4$ or fructose-phenylalanine. The specific activity in the former case was 4 nmol of cinnamate formed min^{-1} mg of $protein^{-1}$ and compares favorably with the PAL activity of the parental strain (Table 2, line 1). In the latter case, however, strain FP1 contained only one-fourth of the activity of the parent (8 versus 31 nmol of cinnamate formed min^{-1} mg of $protein^{-1}$). Thus, strain FP1 was unable to induce an active PAL and contained a mutation in either the structural gene or a regulatory gene. In an attempt to distinguish between these two alternatives, we partially purified PAL from the parental strain and the mutant FP1. The stability and apparent K_m for phenylalanine of PAL from both sources were essentially identical. Although these results do not rule out a structural gene mutation, it seems a less likely explanation.

Defects in regulation may be due to one of two possible causes. First, the enzyme may be superrepressed; that is, the mutation affects repression per se, not induction. Second, the enzyme may not be induced at all. The first possibility was tested by growing FP1 in fructose-NH_4^+ medium to mid-log phase. The culture was harvested, washed, and suspended in phenylalanine medium. Samples were removed at 1, 2, and 4 h and assayed for PAL. Gilbert et al. (8) reported that maximal functional PAL mRNA levels are reached in 60 min after a shift to a medium containing phenylalanine as carbon and nitrogen source. Therefore, one would expect to see at least a transient buildup of PAL before repression starts. We did not observe, however, any change in PAL levels in this experiment. The second possibility was tested by checking strain FP1 for growth on a few of the intermediates in the phenylalanine degradative pathway, namely, benzoate, *p*-hydroxybenzoate, and 3,4-dihydroxybenzoate. The PFP-sensitive parental strain grew on all three compounds as carbon sources, whereas the mutant FP1 was unable to grow or grew very poorly on each of these intermediates. These results suggest that strain FP1 contains a mutation in a regulatory gene that controls the phenylalanine degradative pathway.

Derivatives of FP1 able to grow on phenylalanine as a carbon source. Mutant FP1 was plated onto a yeast nitrogen base medium containing 0.2% phenylalanine and incubated at 30°C until colonies appeared. Five mutants, designated FP1a to FP1e, were picked, purified, and examined to determine the biochemical basis for their growth on phenylalanine. The results shown in Table 4 illustrate that all five mutants regained an inducible PAL, but the specific activities of PAL varied by a factor of 3. Strain FP1a was examined more closely. This mutant was more sensitive to PFP than its parent FP1, but considerably more resistant than the prototrophic strain (Fig. 2). As expected by its resistance to PFP, FP1a did cross-feed a phenylalanine auxotroph of *B. subtilis*. In addition, FP1a regained the capacity to grow on benzoate, *p*-hydroxybenzoate, and

TABLE 4. PAL activity in five derivatives of strain FP1

Strain designation	Sp act[a]	
	Fructose	Phenylalanine
FP1a	8.5	116
FP1b	5.3	85
FP1c	6.0	83
FP1d	8.4	113
FP1e	8.8	40

[a] Cells were grown in yeast nitrogen base medium containing 0.2% fructose or phenylalanine. Cultures were harvested at early stationary phase. Specific activity is expressed as nanomoles of cinnamate formed per minute per milligram of protein.

3,4-dihydroxybenzoate. Interestingly, when FP1a was grown with *p*-hydroxybenzoate as carbon source, PAL was two- to threefold greater than that found in the wild-type strain growing in the same medium. In the parental strain PAL was not induced and was equivalent to the activity found in cells grown in a fructose-$(NH_4)_2SO_4$ medium.

Since FP1a was phenotypically and biochemically distinct from the PFP-sensitive wild-type strain, we can assume that FP1a contains two mutations, that is, the original one in FP1 plus a suppressor mutation that allows for growth on phenylalanine. If these two mutations were in the PAL structural gene, one would expect to see some kinetic differences between the PAL from FP1a and that from the wild-type strain. However, we did not see any differences in these two enzymes.

DISCUSSION

Four major conclusions emerged from this study. First, the analog PFP is a very potent inhibitor of growth of *R. glutinis*. Resistance to this compound occurs by loss of feedback control of DAHP synthase (7) or by a loss of PAL inducibility. In both cases, the consequence is an accumulation of phenylalanine that can be detected in cross-feeding tests with a phenylalanine auxotroph of *B. subtilis*. Thus, increasing the pool of phenylalanine ameliorates the inhibitory effects of PFP. We have found that PAL will deaminate PFP (M. J. Fiske, unpublished data) and we know that cinnamate is also growth inhibitory (10; Fiske, unpublished data). It is not unreasonable to propose that PFP is converted by PAL to *p*-fluorocinnamate, which is, in fact, the inhibitory compound. This hypothesis is supported by the following observations. (i) *R. glutinis* contains three isozymes of DAHP synthase, only one of which is inhibited by phenylalanine or PFP (7). One would not expect inhibition of the phenylalanine-sensitive isozyme to inhibit growth since two isozymes remain active, and no other steps in the aromatic pathway appear to be inhibited by phenylalanine or PFP. (ii) A loss of PAL leads to PFP resistance, suggesting that conversion of PFP is essential for inhibition. It is also interesting to note that these mutants cross-feed a Phe^- mutant so that PAL may play a role in regulating the pools of phenylalanine.

Second, a spontaneous mutagenic event results in resistance to PFP, a loss of PAL inducibility, and the inability to use benzoate, *p*-hydroxybenzoate, or 3,4-dihydroxybenzoate as carbon sources. It is highly probable that this was a single mutation event and that such a mutation would result in the loss of a function rather than a gain in function. These observations are most consistent with an alteration in a common, positive regulatory gene that affects expression of the entire catabolic pathway.
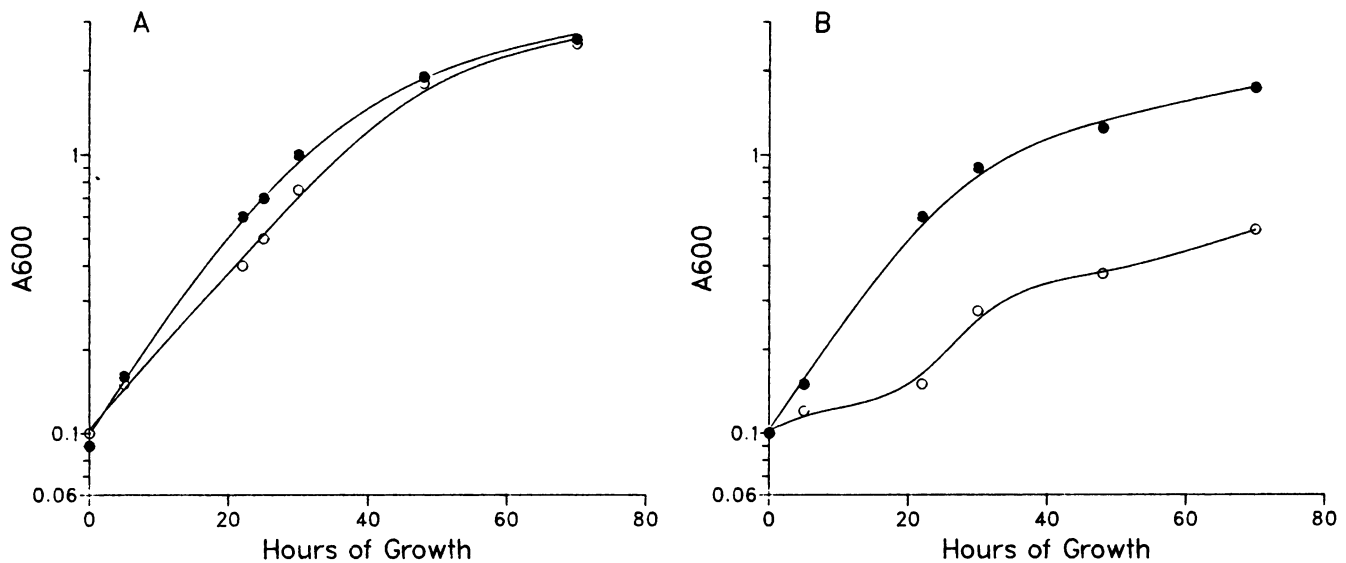
FIG. 2. Effect of PFP on growth of *R. glutinis* FP1 and FP1a. *R. glutinis* strains were grown in yeast nitrogen base medium containing 0.2% fructose with no PFP (●) and 20 μg of PFP ml⁻¹ (O). Results obtained with FP1 are shown in (A); those obtained with FP1a are given in (B). A600, Absorbance at 600 nm.

Third, strain FP1a must contain two mutations since it does not resemble the prototroph. The PAL level appears to be increased twofold and *p*-hydroxybenzoate apparently induces PAL in FP1a but not in the wild-type strain. Such an observation again would be consistent with the common regulatory product proposed above.

Finally, at low concentrations of carbohydrate PAL activity is expressed. Most of the work of Gilbert et al. (8, 9) was done in media containing 2% glucose so one would not expect to see this "carbon controlled" PAL activity. However, we have demonstrated that PAL is expressed in the absence of exogenous phenylalanine as the concentration of utilizable carbohydrate disappears. It is reasonable to assume that, under this physiological stress, phenylalanine is generated intracellularly by either the release of compartmentalized metabolite pools of phenylalanine or the action of a protease (13). Since previous work (8, 9) suggests that there is one PAL, we propose that this PAL gene is regulated in a positive fashion by phenylalanine that is exogenously supplied or endogenously generated.

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