REGULATION OF TYROSINE BIOSYNTHESIS BY PHENYLALANINE IN ANTHRAMYCIN-PRODUCING *STREPTOMYCES REFUINEUS*

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(Received for publication February 26, 1980)

The regulation of tyrosine production in the anthramycin-producing organism *Streptomyces refuineus* var. *thermotolerans* has been studied with wild-type and tyrosine auxotrophic organisms. Growth of the auxotroph on minimal medium plus phenylalanine suggested that phenylalanine may increase the supply of tyrosine. In incubation with whole cells, tyrosine levels increased in response to added phenylalanine. However, no radiolabeled tyrosine was detected after incubation with ^{14}C-phenylalanine. Thus, no phenylalanine hydroxylase is present. Phenylalanine was found to feedback inhibit prephenate dehydratase, resulting in an increase in NAD-dependent prephenate dehydrogenase activity, thus channeling prephenic acid toward tyrosine.

Anthramycin is a pyrollo (1,4)benzodiazepine antitumor antibiotic produced by *Streptomyces refuineus* var. *thermotolerans* (NRRL 3143)[1]. Hurley and coworkers have found that anthramycin is derived from tryptophan, tyrosine, and methionine[2]. As part of a study of the control mechanisms regulating anthramycin production, we are examining the control of biosynthesis of the aromatic amino acid precursors. Tyrosine auxotrophic mutants were created and one such mutant was found to grow on phenylalanine-supplemented minimal medium, although not as well as on tyrosine-supplemented medium. This observation suggested that phenylalanine was capable of increasing tyrosine levels to a concentration which would support growth. This communication presents the outcome of a study of two possible mechanisms for the observed increase, *i.e.*, (1) direct conversion of phenylalanine to tyrosine, and (2) indirect stimulation of tyrosine biosynthesis *via* the prephenate - *p*-hydroxyphenylpyruvate pathway.

Materials and Methods

Materials

All the amino acids used in this study, as well as NAD, barium prephenate, pyridoxal phosphate *p*-hydroxyphenylpyruvate, and N-methyl-N′-nitro-N-nitrosoguanidine (NTG) were obtained from Sigma Chemical Co. NMR was used to rule out significant contamination of phenylalanine with tyrosine. All amino acids were the L-stereoisomers unless otherwise specified. Media constituents were obtained from Difco and Baltimore Biological Laboratory. (U-^{14}C)Tyrosine and (U-^{14}C)phenylalanine were obtained from ICN Chemical and Radioisotope Division. Recrystallized 1-nitroso-2-naphthol was kindly supplied by Dr. N. Zenker. All chemicals used were analytical reagent grade or the highest purity available.

Growth of the organism

Streptomyces refuineus var. *thermotolerans* NRRL 3143 was maintained by growth on slants of nutrient agar at 46°C and storage at 4°C. Spore suspensions of the organism were prepared by washing the surface of an agar slant with 8 ml of sterile distilled water and filtering through sterile Whatman No. 1 filter paper. The usual spore concentration was 1.6×10^7 CFU/ml.

Mutation

For mutation the spore suspensions was treated with 2.3 mg/ml NTG in nutrient broth made with pH 6.4, 0.05 M phosphate buffer. The treatment was done on a rotatory shaker at 250 r.p.m. for 12 hours at 28°C. This procedure has been used previously to create streptomycete mutants blocked in antibiotic pathways[3]. A variety of standard mutational procedures [*e.g.* irradiation with 254 nm ultraviolet light[4], short-term NTG treatment at basic pH[5], irradiation with 364 nm ultraviolet light in the presence of 8-methoxypsoralen[6]] were also tried, but only the 12-hour NTG treatment yielded a substantial rate of auxotroph production.

Survivors of the mutagenic treatment were plated on complete medium (CM) (CM=nutrient agar) without dilution and were incubated for 48 hours at 46°C. Spore suspensions of mutants were made from these plates and diluted $1/10^4$ to yield 100 to 150 colonies per plate on CM plates. When the colonies were well sporulated, the cultures were replicated with sterile velvet first to plates of minimal medium (MM) and then to CM. The MM consisted of $NaNO_3$, 0.2 g; K_2HPO_4, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; KCl, 0.05 g; $FeSO_4 \cdot 7H_2O$, 0.001 g; dextrose, 1.0 g; trace element solution[7], 0.1 ml; distilled water to 100 ml; agar, 2.0 g. The colonies which did not grow on MM but grew on CM were picked from the CM plates and were further plated on MM and MM plus various amino acids to identify specific nutritional requirements.

Penicillin selection for auxotrophs was performed with 3,000 units/ml potassium penicillin G using procedures outlined by KIRKPATRICK and GODFREY[8]. The procedure resulted in a 10-fold enrichment in amino acid auxotrophs.

Determination of the effect of phenylalanine *in vivo*

To confirm the postulated effect of phenylalanine on tyrosine production, tyrosine auxotrophic organisms were grown on complex medium 2 (consisting of cornstarch, 1.0 g; peptonized milk, 2.0 g; yeast extract, 0.3 g; in distilled water to 100 ml, adjusted to pH 7.0), were transferred to MM containing 0.3 mM phenylalanine, and were incubated with shaking for 16 hours. Cells were collected, washed, and resuspended in 0.05 M, pH 7.0 potassium phosphate buffer. Baffled flasks containing 5 ml of cell suspension (A_{540nm}=15.0), 0.48 μmol or 2.4 μmol L-phenylalanine, and 0.05 M, pH 7.0 phosphate buffer in a total volume of 10 ml were incubated for 3 hours and 6 hours at 46°C. Reaction mixtures with buffer in place of phenylalanine and with dead cells were used as controls. When the reaction period ended the mixtures were subjected to sonic oscillation and centrifuged, one ml of 30% trichloroacetic acid was added to the supernatant, and the mixture was recentrifuged. Tyrosine concentration in the supernatant was determined using the fluorometric procedure of WAALKES and UDENFRIEND which measures the fluorescence of a nitrosonaphthol derivative of tyrosine[9].

Enzyme assays

To assay for direct conversion of phenylalanine to tyrosine, a cell suspension was prepared as above and incubated with 0.48 μmol phenylalanine and 6.4 μCi (U-^{14}C)phenylalanine (specific activity: 60 mCi/mmol). Buffer in place of cell suspension was used in the control. At the end of the reaction period the mixture was treated by sonic oscillation and deproteinized as before. L-Tyrosine, 0.1 mg/ml, was added as carrier. The solutions were chromatographed on Whatman 3M paper with *n*-butanol - glacial acetic acid - water (12: 3: 5) with a reference spot of tyrosine (Rf tyrosine=0.49, Rf phenylalanine =0.70). The area corresponding to tyrosine was cut out and eluted with 40 ml of 95% ethanol. The solvent was removed by vacuum distillation at 30°C and the eluted material was cocrystallized with cold tyrosine using the procedure described by COX and KING[10]. The product of each recrystallization step was dried and weighed, and 2 mg of crystals were dissolved and counted in a liquid scintillation counter (Model 6880, Searle Analytic, Inc.). Recrystallization was repeated six times.

The assay for *p*-hydroxyphenylpyruvate aminotransferase (EC 2.6.1.15) was modified from that of KUPCHIK and KNOX[11]. The cells of wild-type and mutant *S. refuineus* were grown separately in complex medium-2 and then in MM for 16 hours, harvested by filtration, resuspended in 0.02 M, pH 7.8 potassium phosphate buffer, subjected to sonic oscillation for 4 minutes at 5~10°C at setting 3.5 on a Branson sonifier, and centrifuged at 20,000×*g* for 20 minutes. The supernatant was dialyzed for 2×1 hour against 100-fold excess phosphate buffer. The activity of the crude cell extract was measured

by monitoring the loss of *p*-hydroxyphenylpyruvate-borate complex at 300 nm on a Beckman DB-2 spectrophotometer. The reaction mixture contained 0.5 ml pH 8.5, 0.75 M borate buffer, 60 μmol L-glutamine, 0.4 μmol *p*-hydroxyphenylpyruvate, 0.003 μmol pyridoxal phosphate, 0.2 ml crude cell extract, and phosphate buffer to a total volume of 1.5 ml. Other amino group donors tried included L-alanine and L-aspartic acid. The optical blank contained phosphate buffer in place of *p*-hydroxyphenylpyruvate. Boiled enzyme was used as a control. Protein was estimated by the Bio-Rad protein assay[12].

Prephenate dehydrogenase activity could not be detected using the standard method of monitoring the reduction of NAD[13]. A subsequent experiment demonstrated rapid oxidation of NADH by the crude cell extract, thus explaining the inability to detect NADH formation. An alternative assay was developed which permitted the simultaneous measurement of prephenate dehydratase (EC 4.2.1.51, yielding phenylpyruvate) and prephenate dehydrogenase (EC 1.3.1.12, yielding *p*-hydroxyphenylpyruvate) as follows: Phenylpyruvate in NaOH develops a maximum absorbance at 318 nm within 5 minutes and this absorbance disappears completely within 50 hours as the product is oxidized to benzaldehyde (λ_{max}=250 nm)[14]. On the other hand, *p*-hydroxyphenylpyruvate in NaOH is oxidized to *p*-hydroxybenzaldehyde in 30 minutes and the maximum absorbance of this compound at 331 nm is stable for at least 50 hours[15]. This difference in oxidation rates makes it possible to detect the product of the prephenate dehydratase reaction at 318 nm after 5 minutes and the product of the prephenate dehydrogenase reaction at 331 nm after 50 hours.

The reaction mixture contained 0.6 μmol barium prephenate, 0.5 μmol NAD, 25 μmol Tris buffer, pH 8.4, 0.7 ml crude cell extract, and buffer to a total volume of 1.2 ml. At the end of a reaction period 1.2 ml 2 N NaOH was added and the absorbances read at the appropriate times as described. Controls included the reaction mixture with boiled enzyme, without NAD, and without prephenate. The activity was also determined using NADP in place of NAD, and with the addition of 0.5 μmol phenylalanine.

Various concentrations of *p*-hydroxyphenylpyruvate were added to the reaction mixture with boiled enzyme and the absorbance read at 331 nm. The absorbance difference between the boiled control and the boiled control plus *p*-hydroxyphenylpyruvate was linear with the concentration of *p*-hydroxyphenylpyruvate. The molar absorptivity was calculated as $\varepsilon=1.4\times10^{4}$ M^{-1} cm^{-1}.

Results and Discussion

The mutant used in this study grew on MM containing tyrosine but not on MM alone, thus confirming that it is an auxotroph with a single nutritional deficiency. The auxotroph was stable, having <1% revertants, and did not have the appearance of a "leaky" mutant, *i.e.*, did not yield any background growth of small colonies on tyrosine deficient medium. The organism failed to grow when *p*-hydroxyphenylpyruvate was added to the MM plates, suggesting that the lesion was in the *p*-hydroxyphenylpyruvate aminotransferase enzyme. However, the specific activity of this enzyme in cell-free extracts from the auxotroph was approximately the same as that from the wild-type organism (0.0014 μmol/min/mg protein for the wild-type; 0.0018 μmol/min/mg protein for the auxotroph). In both cases the enzyme was specific for glutamine; neither aspartate nor alanine was utilized. The boiled control had no activity. Failure of *p*-hydroxyphenylpyruvate to support growth may be due to lack of uptake of this precursor.

The addition of 50 μg/ml L-phenylalanine to the MM plates supported the growth of the auxotroph although the colonies were smaller and fewer in number than when the medium contained tyrosine. That phenylalanine was either indirectly or directly increasing the tyrosine concentration was confirmed by incubating whole cells with phenylalanine. The cells were grown under starvation conditions with phenylalanine added to induce any inducible enzymes (phenylalanine hydroxylase in *Pseudomonas*

is an inducible enzyme[16]) and then transferred to buffer containing various concentrations of phenylalanine and incubated another 3~6 hours. Tyrosine concentration in the sonicated cell extracts increased in response to added phenylalanine, as can be seen in Table 1.

Table 1. Tyrosine production in whole cells in response to phenylalanine.

Cell suspension	Phenylalanine added (mM)	Tyrosine levels (mM)	
		3 hour incubation	6 hour incubation
Whole cells	0	0.033	0.034
	0.48	0.047	0.052
	2.40	0.048	0.076
Dead cells	0.48	0.012	0.009
	2.40	0.010	0.012

Whole cells were used to test for direct hydroxylation of phenylalanine to tyrosine since the previously characterized microbial phenylalanine hydroxylase reaction in *Pseudomonas*[16] required a cofactor, tetrahydropteridine, which was not available to us. Phenylalanine was added to the incubation mixture at the concentration (60 μM) which we had previously shown would yield tyrosine with the greatest percentage conversion, if direct conversion were occurring. (U-^{14}C) Phenylalanine (6.4 μCi) was also added. Following incubation, formation of the cell-free extract, and paper chromatography, the eluant from the portion of the chromatogram corresponding to tyrosine was cocrystallized with cold tyrosine. After six recrystallization steps constant specific activity still had not been achieved. This is strong evidence that radiolabeled tyrosine was not present, especially since a model reaction product mixture with the anticipated ratio of labeled tyrosine to phenylalanine achieved constant specific activity after four recrystallization steps.

Therefore, *Streptomyces refuineus* does not appear to be able to directly hydroxylate phenylalanine to tyrosine. A previous study with streptomycetes, in which labeled tyrosine was determined in cellular protein and in the media after the addition of ^{14}C-phenylalanine, also revealed no evidence of phenylalanine hydroxylase activity[17].

We then turned our attention to effects of phenylalanine which might indirectly result in increased tyrosine production. An ultraviolet assay was used which permitted sequential determination of the products of both the prephenate dehydrogenase and the prephenate dehydratase assays. When the dialyzed crude cell extract was incubated with prephenate but without NAD, only the dehydratase reaction occurred, leading to phenylpyruvate. Table 2 summarizes the results of the assay of prephenate dehydratase in both wild-type and tyrosine auxotrophic organisms. Feedback inhibition by phenylalanine was observed: 90% inhibition by 0.25 mM and 98% by 0.5 mM phenylalanine.

Table 3 summarizes the results obtained when both NAD and prephenate were added to the reaction mixture. L-Phenylalanine (0.75 mM) was added in some cases to inhibit the dehydratase reaction. Phenylalanine increased the formation of *p*-hydroxyphenylpyruvate 4-fold in the wild-type

Table 2. Prephenate dehydratase activity.

Organism	Enzyme preparation	Specific activity (nmol phenylpyruvate/min/mg protein)
Wild-type	Boiled enzyme control	0
	Enzyme	0.810
	+0.25 mM Phenylalanine	0.077
	+0.50 mM Phenylalanine	0.013
Tyrosine auxotroph	Boiled enzyme control	0
	Enzyme	1.150

Table 3. Activity of prephenate dehydrogenase.

Organism	Phenylalanine (mM)	Specific activity (nmol product/min/mg protein)
Wild-type	0	0.023
	0.75	0.097
Tyrosine auxotroph	0	0.008
	0.75	0.047

organism and 4.5-fold in the auxotroph. The phenylalanine-stimulated activity in the auxotroph was greater than the non-stimulated activity in the wild-type extract, suggesting that the amounts formed in the presence of phenylalanine in the auxotroph would be sufficient to support growth. Evidently, the enzyme in the auxotroph was not completely non-functional but simply too inefficient to synthesize growth-supporting amounts of tyrosine.

The mechanism of channeling the prephenate to the tyrosine biosynthetic pathway in the presence of excess phenylalanine has been previously reported in *Pseudomonas aeruginosa*[18], but this is, to our knowledge, the first report of such a control mechanism in a streptomycete. Feedback inhibition of prephenate dehydratase was reported in *Streptomyces* sp. 3022a but no prephenate dehydrogenase activity was detected[19].

One other possible explanation for the observed increase in tyrosine in response to phenylalanine could be induction of an alternative pathway from prephenate to tyrosine *via* pretyrosine (transamination preceding oxidation) as reported in some microorganisms[20]. There is, however, no precedent for the pathway in streptomycetes. Also, our study demonstrates that excess phenylalanine indirectly stimulates one pathway to tyrosine and it seems unlikely that a second alternative pathway would also be dependent upon phenylalanine to function.

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