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In Vivo Regulation of Intermediate Reactions in the Pathway of Tryptophan Biosynthesis in *Neurospora crassa*

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The in vivo regulation of intermediate reactions in the pathway of tryptophan synthesis in *Neurospora crassa* was examined in a double mutant (*tr-2*, *tr-3*) which lacks the functions of the first and last enzymes in the pathway from chorismic acid to tryptophan. The double mutant can convert anthranilic acid to indole and indole-3-glycerol, and the production of these indolyl compounds by germinated conidia was used to estimate the activity of the intermediate enzymes in the pathway. Indole-synthesizing activity was maximal in germinated conidia obtained from cultures in which the levels of L-tryptophan were growth-limiting; the formation of this activity was markedly repressed when the levels of L-tryptophan exceeded those required for maximal growth. D-, 5-methyl-DL-, and 6-methyl-DL-tryptophan were less effective than L-tryptophan, and 4-methyl-DL-tryptophan, tryptamine, and indole-3-acetic acid were ineffective in repressing the formation of indole-synthesizing activity; anthranilic acid stimulated the formation of indole-synthesizing activity. Preformed indole-synthesizing activity was strongly and specifically inhibited by low levels of L-tryptophan; several related compounds were ineffective as inhibitors. These results suggest that, in addition to repression, an end product feedback inhibition mechanism is operative on an intermediate enzyme(s) in tryptophan biosynthesis. The relation of these results to other in vivo and in vitro studies and to general aspects of the regulation of tryptophan biosynthesis in *N. crassa* are discussed.

The unique aspects of the biosynthesis of tryptophan in *Neurospora crassa* can be summarized by the reaction scheme shown in Fig. 1. Previous studies (6, 7, 8) on the regulation of this metabolic pathway showed that tryptophan could repress the formation of activities associated with the *tr-2* and *tr-3* loci, and possibly those of the *tr-4* and *tr-1* loci as well. These studies also indicated that the preformed activity of the first reaction, associated with the *tr-2* locus, could be inhibited by tryptophan. However, quantitative differences in the effectiveness of tryptophan and its analogues as repressors or inhibitors were observed, depending on the segment of the biosynthetic sequence examined. Such observations suggested that the intermediate steps in the biosynthesis of tryptophan differed from the first and last steps in their sensitivity to repression. Also, these studies suggested that the preformed activity of one of the intermediate reactions, as well as the first reaction, could be inhibited by tryptophan.

This paper describes an analysis of the regulation of the intermediate reactions in the biosynthesis of tryptophan, in an in vivo system. By using a double tryptophan auxotroph (*tr-2*, *tr-3*) of *N. crassa*, it has been possible to circumscribe the metabolic activities which convert anthranilic acid to indole and indole-3-glycerol. These studies indicate that these activities are subject to regulation by tryptophan, both by repression of the formation of these activities and by end product inhibition of preformed activity.

MATERIALS AND METHODS

Organism. *N. crassa* RC-5-1 (*tr-2*, *tr-3*) was obtained from the ascospore progeny of a cross between *tr-2A* and *tr-3* (td_5)*a* strains (provided, respectively, by D. R. Stadler and S. R. Suskind). The *tr-2* strain requires anthranilic acid, indole, or tryptophan for growth; it lacks a functional anthranilate synthetase (which catalyzes the formation of anthranilic acid from chorismic acid). The *tr-3* strain can grow only on tryptophan, and it accumulates indole and indole-3-glycerol in cultures; this strain produces a defective

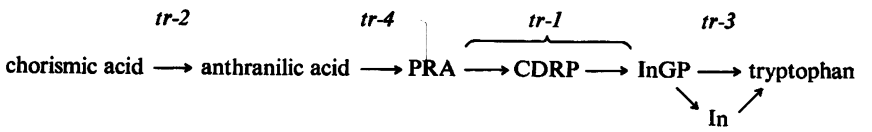

FIG. 1. *Reactions involved in the biosynthesis of tryptophan in Neurospora crassa. Abbreviations: PRA, N-(5'-phosphoribosyl) anthranilic acid; CDRP, 1-(o-carboxyphenylamino)-1-deoxyribulose-5-phosphate; InGP, indole-3-glycerol phosphate; In, indole. The tr notations above the arrows indicate the genetic loci determining the corresponding reactions.*

tryptophan synthetase which can convert indole-3-glycerol phosphate to indole but which cannot convert either of these compounds to tryptophan.

Strain RC-5-1 was obtained from an ascus which also contained tryptophan-independent ascospores, indicating that strain RC-5-1 represented the *tr-2, tr-3* ditype. This was further demonstrated by the observations that the tryptophan requirement of strain RC-5-1 could not be replaced by anthranilic acid or indole, no indolyl or anthranil compounds accumulated in the growth medium, but, if anthranilic acid as well as tryptophan was added to the growth medium, then indole and indole-3-glycerol were produced. These observations indicated that strain RC-5-1 could not carry out the first and last reactions in the biosynthesis of tryptophan, but could accomplish the intermediate reactions attributable to the *tr-4* and *tr-1* loci.

Estimation of the activity of the intermediate reactions in tryptophan biosynthesis. The production of indole and of indole-3-glycerol by germinated conidia suspended in buffer containing glucose and anthranilic acid was used as a measure of the activity of the portion of the tryptophan pathway still present in strain RC-5-1. The preparation of germinated conidia and of samples taken for assay has been described elsewhere (6, 7). Indole synthesis was carried out in 125-ml Erlenmeyer flasks containing 15 to 25 ml of reaction mixture, with cell concentrations of 1.5 to 5.0 mg (dry weight) per ml. The flasks were incubated at 30 C with agitation. Samples of the mixture were taken at 0, 1.5, and 3.0 hr and assayed for indole (13) and indole-3-glycerol (14). Since the rate of production of indole was nearly linear with time, and also directly proportional to cell concentration, the estimates of indole in the 1.5- and 3.0-hr samples were averaged; indole-3-glycerol was measured only in 3.0-hr samples, since the assay system for this compound was less sensitive than that for indole. The specific synthesizing activity of the system is designated as nmoles of indole or indole-3-glycerol produced per mg of cells (dry weight) per 3.0 hr. (All specific activities were based on the dry weight of cells at 0 hr.) In most of the experiments, only indole was measured, since, as will be shown, there is a close, direct correspondence between the production of indole and indole-3-glycerol.

RESULTS

Conditions for indole synthesis by strain RC-5-1. Several variations in the composition of the medium used for evaluating indole-synthesizing activity by germinated conidia were examined. The presence of anthranilic acid was crucial for the synthesis of indole; the additional presence of glucose afforded a modest increase in indole synthesis (Table 1). No significant differences in indole synthesis were observed whether the suspending medium was a buffer solution or a nutrient salts solution. Other experiments indicated that the optimal concentration of anthranilic acid for indole synthesis was between 0.2 and 0.4 μmole/ml, that glucose was at least as effective as sucrose, D-ribose, D-xylose, or L-arabinose in stimulating indole synthesis, and that a *p*H of 5.8 was more favorable than a *p*H of 7.0 or higher. Such experiments provided the rationale for the composition of the basal reaction mixture used to evaluate the indole-synthesizing activity of germinated conidia: 0.02 M NaH_2PO_4 in 0.05 M NaCl (*p*H 5.8), containing 85 μmoles of glucose and 0.3 μmole of anthranilic acid per ml.

Effect of L-tryptophan on the formation of indole-synthesizing activity. Table 2 shows the influence of the concentration of tryptophan in the germination medium on growth and the indole-synthesizing activity of germinated conidia. A maximal yield of germinated conidia was obtained with a concentration of 0.12 to 0.16 μmole of L-tryptophan per ml. Maximal indole-synthesizing activity was observed in the cells obtained from cultures in which the level of L-tryptophan afforded somewhat less then maximal growth. As the level of tryptophan approached and exceeded a concentration optimal for growth, a marked diminution of indole-synthesizing activity was observed. Indole-3-glycerol-synthesizing activity was consistently about twice that of indole, indicating that the ability to convert indole-3-glycerol phosphate (InGP) to indole was not differentially influenced by tryptophan. The external levels of tryptophan did not greatly influence the intracellular levels of tryptophan in germinated conidia, which suggests that the observed differences in indole-synthesizing activities were not a consequence of inhibition of the indole-synthesizing system by intracellular tryptophan. The relationship of indole-synthesizing activity to the concentration of tryptophan in the RC-5-1 culture

TABLE 1. *Influence of the composition of the reaction mixture on indole synthesis by germinated conidia*[a]

Base	Additions[b]	Indole synthesis[c]
Buffer[d]	None	0.9
	Glucose	1.3
	Anthranilic acid	10.0
	Anthranilic acid + glucose	13.7
0.5× Fries salts (2)	None	2.4
	Glucose	3.6
	Anthranilic acid	9.5
	Anthranilic acid + glucose	13.5

[a] The germination medium contained 0.10 μmole of L-tryptophan per ml.

[b] Glucose was added at a concentration of 85 μmoles/ml; anthranilic acid, at 0.3 μmole/ml.

[c] Expressed as specific activity.

[d] The buffer consisted of 0.02 M NaH_2PO_4 in 0.05 M NaCl, adjusted to *p*H 5.8 with HCl.

TABLE 2. *Effect of the concentration of L-tryptophan in the germination medium on the formation of indole-synthesizing activity*

L-Tryptophan concn	Germinated conidia	Intracellular tryptophan[a]	Indole synthesis[b]	Indole-3-glycerol synthesis[b]	Indole-3-glycerol to indole ratio
μmoles/ml	*mg/ml*				
0.04	1.1	0.7	11.0	26	2.4
0.08	1.8	0.8	10.2	22	2.2
0.12	2.5	0.9	8.1	16	2.0
0.16	2.7	1.0	3.2	7	2.2
0.20	2.7	1.0	1.9	—[c]	
0.24	2.8	1.3	2.0	—	

[a] Intracellular tryptophan, expressed as micromoles per gram (dry weight), was estimated from the indole formed after incubation of hot-water extracts of germinated conidia with a partially purified preparation of tryptophanase from *E. coli* (4).

[b] Expressed as specific activity.

[c] Insufficient indole-3-glycerol for measurement.

medium is similar to that observed previously (7) with a strain of *N. crassa* carrying only a mutation of the *tr-3* locus. The present results indicate that L-tryptophan can repress the formation of one or more of the intermediate activities in the biosynthesis of tryptophan.

Specificity of repression by L-tryptophan. The indole-synthesizing activities of germinated conidia obtained from cultures containing compounds related to tryptophan were examined. D-Tryptophan, 5-methyltryptophan, 6-methyltryptophan, and indole repressed the formation of indole-synthesizing activity, but not as effectively as did L-tryptophan; other indolyl compounds were ineffective as repressors (Table 3). Generally, this pattern of specificity is qualitatively similar to that observed with the *tr*-3 strain (7). However, there are some quantitative differences in that 6-methyltryptophan appears to be a less effective repressor in strain RC-5-1 (*tr-2, tr-3*) than in the *tr-3* strain, and anthranilic acid appears to stimulate markedly the formation of indole-synthesizing activity in the double mutant.

Effect of anthranilic acid on the formation of indole and indole-3-glycerol-synthesizing activities. The stimulatory effect of anthranilic acid noted above was investigated further to determine (i) the dependence of this stimulatory effect on the concentration of anthranilic acid in the germination medium, (ii) the possibility of the stimulatory effect resulting from a change in the relative amounts of indole and indole-3-glycerol produced, and (iii) whether anthranil compounds which could be converted to indole were present in the cells. The stimulatory effect of anthranilic acid was concentration-dependent with maximal indole-synthesizing activity being formed at a concentration of 0.10 μmole of anthranilic acid per ml of germination medium (Table 4). The ability of cells grown in the presence of various levels of anthranilic acid to subsequently synthesize indole was closely paralleled by their ability to produce indole-3-glycerol. Thus, the stimulatory effect of anthranilic acid is not merely a reflection of a change in the relative amounts of indole and indole-3-glycerol synthesized by germinated conidia.

TABLE 3. *Effect of L-tryptophan and related compounds on the formation of indole-synthesizing activity*

Addition to germination medium[a]	Concn	Indole synthesis (specific activity)
	μmoles/ml	
None		10.8
L-Tryptophan	0.5	2.2
D-Tryptophan	0.5	5.7
4-Methyl-DL-tryptophan	1.0	10.1
5-Methyl-DL-tryptophan	1.0	7.2
6-Methyl-DL-tryptophan	1.0	6.8
Indole	0.5	7.0
Indole-3-acetic acid	0.5	10.3
Tryptamine	0.5	10.3
Anthranilic acid	0.5	20.2

[a] The germination medium contained 0.10 μmole of L-tryptophan per ml.

If anthranilic acid in the germination medium gave rise to anthranil precursors of indole which accumulated within the cell, it might be expected that the dependence of the indole-synthesizing system on anthranillic acid would be modified. That is, indole should be produced by anthranilate-grown cells in the absence of anthranilic acid. Table 5 indicates that this was not the case; as previously shown (Table 1), the presence of anthranilic acid was still crucial for the synthesis of indole, and, again, indole synthesis was enhanced by glucose. These experiments suggest that the presence of anthranilic acid in the culture medium stimulates the formation of indole-synthesizing activity; some possible explanations for this effect of anthranilic acid will be discussed later.

Effect of L-tryptophan on preformed indole-synthesizing activity. Germinated conidia were grown on a level of tryptophan which was optimal for the formation of indole-synthesizing activity, and the expression of this activity in the presence of various levels of tryptophan was examined.

TABLE 4. *Effect of the concentration of anthranilic acid in the germination medium on the formation of indole- and indole-3-glycerol-synthesizing activities*[a]

Anthranilic acid concn	Indole synthesis (specific activity)	Indole-3-glycerol synthesis (specific activity)	Indole-3-glycerol to indole ratio
μmoles/ml			
0	10	24	2.4
0.05	12	25	2.1
0.10	18	35	1.9
0.25	17	35	2.1
0.50	17	37	2.2
0.80	19	40	2.1

[a] The germination medium contained 0.10 μmole of L-tryptophan per ml.

TABLE 5. *Dependence on anthranilic acid and glucose for indole synthesis by germinated conidia cultured in the presence of anthranilic acid*[a]

Additions to reaction mixture[b]	Indole synthesis (specific activity)
None	1.3
Glucose	1.4
Anthranilic acid	12.8
Anthranilic acid + glucose	17.5

[a] The germination medium contained 0.10 μmole of L-tryptophan per ml and 0.5 μmole of anthranilic acid per ml.

[b] Glucose was added at a level of 85 μmoles/ml; anthranilic acid, at 0.3 μmole/ml.

L-Tryptophan, at low concentrations, was capable of almost completely inhibiting preformed indole-synthesizing activity (Table 6). The relative effectiveness of tryptophan observed here is similar to that observed previously (7) with the *tr-3* strain. It is pertinent to note that, in contrast to the *tr-3* strain, the double mutant lacks the first enzymatic activity in the sequence of reactions which is unique to the biosynthesis of tryptophan. Consequently, it might be assumed that L-tryptophan can act as an end product inhibitor of another one of the intermediate reactions in the biosynthesis of tryptophan.

Specificity of the inhibition of preformed activity by L-tryptophan. Of the compounds examined, only L-tryptophan markedly inhibited preformed indole-synthesizing activity in strain RC-5-1 (Table 7). This is in marked contrast to the results of a similar experiment (7) performed with the *tr-3* strain, wherein 4-methyl-, 5-methyl-, and

TABLE 6. *Effect of the concentration of L-tryptophan on preformed indole-synthesizing activity*[a]

L-Tryptophan concn	Indole synthesis (specific activity)	Indole-3-glycerol synthesis (specific activity)	Indole-3-glycerol to indole ratio
μmoles/ml			
0	12.9	24	1.9
0.015	7.0	15	2.1
0.03	4.5	10	2.2
0.06	3.8	8	2.1
0.10	2.4	—[b]	
0.20	1.7	—	

[a] The germination medium contained 0.10 μmole of L-tryptophan per ml.

[b] Insufficient indole-3-glycerol for measurement.

TABLE 7. *Effect of L-tryptophan and related compounds on preformed indole-synthesizing activity*[a]

Addition to reaction mixture	Concn	Indole synthesis (specific activity)
	μmoles/ml	
None		11.1
L-Tryptophan	0.5	2.0
D-Tryptophan	0.5	11.1
4-Methyl-DL-tryptophan	1.0	9.2
5-Methyl-DL-tryptophan	1.0	10.9
6-Methyl-DL-tryptophan	1.0	10.1
Indole-3-acetic acid	0.5	12.4
Tryptamine	0.5	11.1
Anthranilic acid	0.5	10.2

[a] The germination medium contained 0.10 μmole of L-tryptophan per ml.

6-methyltryptophan were found to be effective inhibitors of preformed indole-synthesizing activity. Other experiments (8) demonstrated that tryptophan and its methyl analogues also inhibit preformed anthranilate-synthesizing activity, i.e., the first step in the biosynthesis of tryptophan. These disparities in the inhibitory activities by tryptophan analogues in various tryptophan auxotrophs, and the genetic circumvention of the first step in strain RC-5-1, indicate that more than one site of end product inhibition exists in the pathway of tryptophan biosynthesis.

DISCUSSION

The present studies demonstrate that the formation of at least one of the enzymes involved in the intermediate reactions in the biosynthesis of tryptophan is subject to repression under in vivo conditions. Taken together with previous work (6, 8), the present work indicates that the formation of enzymes determined by three of the four genetic loci associated with the biosynthesis of tryptophan can be repressed by tryptophan. Work now in progress indicates that the formation of all of the activities in tryptophan biosynthesis are subject to repression by tryptophan.

The work described reemphasizes previous observations of quantitative differences in the sensitivity of the various reactions in tryptophan biosynthesis to repression by analogues of tryptophan. This might suggest that the regulation of all the activities of tryptophan biosynthesis is not mediated by a common repressor, or, alternatively, that the formation of the various activities is differentially affected by a single repressor depending on the type of tryptophan compound with which it is associated. It might be speculated further that the different sensitivities to repression by tryptophan and its analogues would preclude a coordinate repression or derepression of the formation of the enzymes involved in tryptophan biosynthesis. The plausibility of this speculation is given some support by the fact that each of the genetic loci involved with tryptophan biosynthesis in *N. crassa* is located on a different linkage group (1), rather than being associated as an operon as in *Escherichia coli* (10, 15).

The marked stimulatory effect of anthranilic acid on the formation of indole-synthesizing activity in strain RC-5-1 is not understood, at present. It might be questioned whether anthranilic acid per se or a product of anthranilic acid metabolism, such as indole, InGP,or other anthranil compounds, is responsible for the increase in indole-synthesizing activity. It would be of interest to determine the effect of anthranilic acid on the formation of all of the activities in the biosynthesis of tryptophan. In this respect, it may be noted that anthranilic acid does stimulate the formation of tryptophan synthetase in *E. coli* (9) and in wild-type *N. crassa* (6). In the case of *E. coli*, the increased formation of tryptophan synthetase could be attributed to an inhibition of endogenous tryptophan synthesis, by an inhibition of indole-3-glycerol phosphate synthetase (5), resulting in a derepression of the formation of tryptophan synthetase. Whether a similar inhibition of the same enzyme occurs in *N. crassa* has not been determined. However, a search for anthranilic deoxyribuloside (9) in cells exposed to anthranilic acid did not reveal significant amounts of this compound. Another possibility for the stimulatory effect of anthranilic acid is that it induces a permease which enhances the uptake of anthranilate in the test system.

Many biosynthetic pathways specifically directed towards a particular end product are subject to regulation by an inhibitory action of the end product on the first reaction of the pathway (11). This type of regulation appears to be operative in the biosynthesis of tryptophan in *N. crassa*, since tryptophan inhibits the formation of anthranil compounds in intact cells (8) and the in vitro activity of anthranilate synthetase (3). In the work reported here, the anthranilate synthetase reaction has been circumvented by mutation, and, thus, the inhibitory effect of tryptophan on preformed indole-synthesizing activity must be directed against a later reaction. It has been demonstrated (12) that the conversion of anthranilic acid to InGP is catalyzed by two enzymes, phosphoribosyl (PR) transferase and indole-3-glycerol phosphate synthetase. [PR transferase catalyzes the formation of *N*-(5′-phosphoribosyl) anthranilic acid (PRA) from anthranilic acid and 5-phosphoribosyl-1-pyrophosphate, and indole-3-glycerol phosphate synthetase converts PRA to InGP.] Also, PR transferase activity appears to be inhibited by tryptophan (13). Thus, both in vivo and in vitro studies show that end product inhibition occurs in two of the reactions unique to the biosynthesis of tryptophan. The in vivo studies presented here and elsewhere (8) suggest a differential sensitivity of anthranilate synthetase and PR-transferase to inhibition by methyl analogues of tryptophan. Whether a similar difference in sensitivity occurs in vitro remains to to be determined.

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