Tryptophan Biosynthesis in *Coprinus lagopus*: A Genetic Analysis of Mutants

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SUMMARY

Thirty-one tryptophan-requiring mutants of *Coprinus lagopus* have been assigned by genetic and complementation analyses to four loci designated *trp-1*, *trp-2*, *trp-3* and *trp-4*. The *trp-1* and *trp-3* loci were located in group III and *trp-2* in group G of the linkage map. The *trp-4* locus showed no linkage to the other *trp* loci or to markers in three additional linkage groups tested. From auxanographic tests and a study of accumulated biosynthetic intermediates, the enzymes controlled by each locus have been provisionally assigned. The *trp-2* and *trp-3* loci both appear necessary for anthranilate synthetase activity since mutants accumulated no intermediates. Only the *trp-3* mutant could utilize anthranilic acid, therefore the *trp-2* locus must also be involved in a subsequent step in the pathway. The *trp-4* mutants utilized indole and accumulated anthranilic acid, and hence this locus is involved in the conversion of anthranilic acid to indoleglycerol phosphate. The *trp-1* mutants utilized only tryptophan and accumulated indoleglycerol phosphate and anthranilic acid. They are therefore blocked in the final steps of the pathway catalysed by tryptophan synthetase.

INTRODUCTION

Studies of tryptophan biosynthesis in diverse micro-organisms and higher plants have revealed a common basic reaction sequence catalysed by a wide variety of enzyme structures (Fig. 1; see also Crawford, 1975). In bacteria, the number of genetic loci involved in specifying the necessary enzymes ranges from seven in *Bacillus subtilis* to five in *Escherichia coli* (Crawford, 1975). In fungi, there are five in *Saccharomyces cerevisiae* (Bonner, DeMoss & Mills, 1965) and four in *Neurospora crassa* (DeMoss, Jackson & Chalmers, 1967), *Aspergillus nidulans* (Roberts, 1967) and *Coprinus radiatus* (Henke, Guerdoux & Hutter, 1973). In bacteria the loci are located in various types of clusters, generally forming operons, whereas, in fungi they are not clustered.

This report describes for the first time tryptophan-requiring mutants of *Coprinus lagopus*. The work is primarily a genetic study designed to provide a foundation for subsequent studies on the action of analogues of tryptophan. An indication as to the enzymic steps affected in the mutants is derived from auxanographic tests and by identification of accumulated intermediates. This allows some comparison of the enzymes in *C. lagopus* with those in other fungi.

Fig. 1. Outline of the tryptophan biosynthetic pathway. PRA, *N*-(5'-phosphoribosyl)-anthranilic acid; CDRP, 1-(*o*-carboxylphenylamino)-1-deoxyribulose 5-phosphate; InGP, indoleglycerol phosphate. Enzyme activities: 1, anthranilate synthetase; 2, phosphoribosyl transferase; 3, PRA isomerase; 4, InGP synthetase; 5a + 5b, tryptophan synthetase.

METHODS

Strains. The wild-type strains TC4 A_2B_3 , JT7 A_6B_6 , JT15 A_5B_5 and JT16 A_6B_6 were derived from the original stocks isolated from the wild by Professor D. Lewis. The following strains carrying markers representing five of the known linkage groups were used: group I, NG184 *ade-8* A_6B_6 and ZBR17 *met-5* A_6B_6 ; group III, PR2301 *met-1* A_2B_3 ; group IV, ZBR23 *met-9* A_5B_5 ; group V, ZBR9 *met-8* A_5B_5 ; group G, C692 *ade-3*, A_3B_1 .

Tryptophan mutants. Most mutants were derived from strain TC4 following irradiation of oidia with ultraviolet light. Two mutations, 35 and 36, were induced in JT15. To satisfy the genetic restrictions on dikaryon formation, the prerequisite for complementation tests and for fruiting, a few of the original mutations were introduced into a new mating type by out-crossing to JT7 or JT16.

Media. Glucose minimal medium was that used by Lewis (1961) with the addition of 0.25 g $MgSO_4$ /l (Casseltan & Casseltan, 1966). Filter-sterilized growth supplements were added after autoclaving to give the following concentrations: adenine sulphate, L-methionine, L-tryptophan and anthranilic acid, 0.5 mM, indole, 0.1 mM (higher indole concentrations are growth inhibitory). Liquid medium contained 10 g ammonium acetate/l as carbon source in place of glucose, because the latter may react with the anthranilic acid accumulated by mutants (Doy & Gibson, 1959).

Culture. General techniques for using *Coprinus* have been described by Lewis (1961) and Anderson (1971). All mycelial cultures were grown at 37 °C. For fruiting a lower temperature (28 °C) and light were required. Cultures grown in liquid medium were initiated from oidia (2.5×10^5 oidia/ml final concentration) inoculated into 200 ml of medium in 1 l Erlenmeyer flasks and placed on a rotary shaker (140 rev./min; throw, 12 mm).

Accumulation of biosynthetic intermediates. Liquid cultures were grown for 5 days in medium supplemented with tryptophan. The mycelia were collected by centrifugation (1000 g for 15 mins), washed with water (200 ml), collected and resuspended in 100 ml of medium without tryptophan. After a further 2 days' incubation the cultures were each shaken with toluene (0.3 ml), left for an hour, and the toluene evaporated. The media were separated from the mycelia by filtration (Whatman No. 1). Dry weights of all mycelia were similar.

Identification of intermediates. Anthranilic acid and 1-(*o*-carboxylphenylamino)-1-deoxyribulose 5-phosphate (CDRP) were identified by a procedure based on that of Doy & Gibson (1959). Fifty ml of acidified medium (pH 4) were extracted with 50 ml ethyl acetate which was then dried over anhydrous Na_2SO_4 and reduced under vacuum to 2 ml, taking care not to evaporate to dryness. A 0.1 ml portion of the concentrate was subjected to paper chromatography, using the solvent methanol-*n*-butanol-benzene-water (2:1:1:1, by vol) and

Whatman No. 1 paper. Anthranilic acid was identified as a spot, strongly fluorescent under u.v. light, with the same R_f as pure anthranilic acid. CDRP should appear as a slower-moving fluorescent spot. Indoleglycerol phosphate (InGP) was assayed in the medium using the ferric chloride reagent and the periodate oxidation methods (Yanofsky, 1956). Indole was tested for with the indole reagent (Yanofsky, 1955).

RESULTS

Complementation analysis

Thirty-one tryptophan-requiring mutants were studied. Only six of these were recovered in strains with a new, compatible, mating type because of a technical difficulty encountered in the early work. Basidiospores carrying a *trp*⁻ mutation germinated poorly, making it difficult to recover *trp*⁻ progeny. However, the few mutants recovered through crosses were sufficient to allow a limited complementation analysis in dikaryons. On this basis the mutants could be separated into four well-defined groups (Table 1). Taken together with the genetical data presented below, it was concluded that there are four genes involved and these were designated *trp-1*, *trp-2*, *trp-3* and *trp-4*. Interallelic complementation was detected between the *trp-1* mutants, 14 and 7.

Genetic analysis

In crosses between *trp*⁺ and *trp*⁻ strains, germination of basidiospores on medium supplemented with tryptophan was generally no more than 50 % and most of the progeny carried the *trp*⁺ allele. The phenomenon was neither allele nor locus specific, and was unaffected by tryptophan concentration. By plating spores at low density it was found that *trp*⁻ spores germinated very slowly. Once growth was established, *trp*⁻ colonies grew at similar rates to *trp*⁺ colonies. This suggests that basidiospores have a poorly developed system for uptake or utilization of exogenous tryptophan. Interestingly, medium supplemented with indole supported normal germination of basidiospores of those *trp*⁻ mutants which could utilize indole as an alternative growth supplement to tryptophan. Uptake of exogenous indole is therefore independent of tryptophan uptake.

Mutants, representative of the four *trp*⁻ complementation groups identified, were crossed to strains carrying markers in each of five of the eight known linkage groups of *Coprinus lagopus* (Moore, 1967; Moore & Stewart, 1971). Although it was possible to recover all the progeny genotypes in crosses involving those *trp*⁻ mutants which utilized indole this was not always attempted, and in some crosses only the *trp*⁺ progeny were analysed (Table 2). Two of the loci, *trp-1* and *trp-3*, showed close linkage to the *met-1* locus in linkage group III, while *trp-2* was located in linkage group G some 15 units from *ade-3*. No linkage was detected between *trp-4* and either the other three *trp* loci or the linkage group markers tested.

To confirm that *trp-1* and *trp-3* are not identical and to determine their relative order with respect to *met-1*, the following three point cross was made:

$$trp-1\ met-1^+ trp-3^+ \times trp-1^+ met-1\ trp-3.$$

Recombinant progeny of genotype *trp-1*⁺ *met-1*⁺ were selected by plating basidiospores from the cross on medium supplemented with anthranilic acid. *trp-3* mutants can utilize this supplement and hence the alleles at this locus remain unselected.

Forty-two *trp-1*⁺ *met-1*⁺ recombinants were recovered from 1.7×10^4 viable spores plated, giving an estimated recombination frequency between these two loci of 0.5 % (0.3 %, Table 2). Of these recombinants, 36 were *trp-3*⁺ and six *trp-3*. Together with the observed recombination frequency between *met-1* and *trp-3* of 6.2 % (Table 2), these results indicate

Table 1. Complementation analysis of tryptophan-requiring mutants

		<i>trp-1</i>		<i>trp-2</i>	<i>trp-3</i>	<i>trp-4</i>	
		7	8	11	29	1	30
<i>trp-4</i>	1	+	+	+	+	—	—
	30	+	+	+	+	—	—
	33	+	+	+	+	—	
<i>trp-3</i>	29	+	+	+	—		
<i>trp-2</i>	11	+	+	—			
	12	+	+	—			
	18	+	+	—			
	35	+	+	—			
	36	+	+	—			
<i>trp-1</i>	7	—	—				
	8	—	—				
	9	—	—				
	10	—	—				
	14	+	—				
	16	—	—				
	17	—	—				
	20	—	—				
	21	—	—				
	22	—	—				
	23	—	—				
	24	—	—				
	25	—	—				
	27	—	—				
	28	—	—				
	31	—	—				
	32	—	—				
	34	—	—				

+, Complementation; —, no complementation.

Table 2. Number of progeny of each genotype recovered from two point crosses between mutants of the four *trp* loci and markers on linkage groups III and G

Linkage group	Marker	<i>trp</i> locus	Number of progeny of genotype:				Percentage recombination ± S.E.
			<i>trp</i> ⁺ <i>met</i> [—]	<i>trp</i> [—] <i>met</i> ⁺	<i>trp</i> ⁺ <i>met</i> ⁺	<i>trp</i> [—] <i>met</i> [—]	
III	<i>met-1</i>	<i>trp-1</i>	777	—*	2	—*	0.3 ± 0.3
			71	70	1	0	
		<i>trp-2</i>	75	—*	93	—*	55†
		<i>trp-3</i>	188	188	15	10	6.2 ± 0.9
		<i>trp-4</i>	22	38	25	26	46†
G	<i>ade-3</i>		<i>trp</i> ⁺ <i>ade</i> [—]	<i>trp</i> [—] <i>ade</i> ⁺	<i>trp</i> ⁺ <i>ade</i> ⁺	<i>trp</i> [—] <i>ade</i> [—]	
		<i>trp-2</i>	152	153	25	30	15.1 ± 0.8
		<i>trp-4</i>	49	—*	41	—*	45.6†

* No *trp*[—] progeny expected because the medium was not supplemented with tryptophan or indole.

† Not significantly different from 50 %.

Table 3. *Growth of tryptophan-requiring mutants on minimal medium and supplemented minimal medium*

Mutants	Growth supplement to minimal medium			
	None	Anthranilic acid	Indole	Tryptophan
Wild type	+	+	+	+
<i>trp-1</i>	—	—	—	+
<i>trp-2</i>	—	—	+	+
<i>trp-3</i>	—	+	+	+
<i>trp-4</i>	—	—	+	+

+, Normal growth; —, no growth. Growth was scored after 48 h incubation.

Table 4. *Accumulation of biosynthetic intermediates by tryptophan-requiring mutants*

Established mycelia were incubated for 2 days in medium without tryptophan, treated with toluene, the mycelium filtered off and the medium tested for tryptophan biosynthetic intermediates as described under Methods. The amount of anthranilic acid was estimated from the size and intensity of fluorescence of chromatographic spots.

Locus	Strain	Anthranilic* acid	Indoleglycerol† phosphate
Wild type	TC4	+	—
<i>trp-1</i>	7	+++	+
	8	+++	+
	14	+++	+
<i>trp-2</i>	11	+	—
	12	++	—
<i>trp-3</i>	29	+	—
<i>trp-4</i>	30	++++	—

* +, ++, +++, +, Decreasing quantities of anthranilic acid.

† +, Easily detectable accumulation; —, no detectable accumulation.

the order of these three loci as: *trp-1 met-1 trp-3*. Thus it is confirmed that *trp-1* and *trp-3* are not identical.

Auxanographic tests

All mutants were tested for ability to utilize anthranilic acid or indole as alternative growth supplements to tryptophan. With one exception, mutants assigned to each locus gave the growth responses recorded in Table 3. The exception was a *trp-1* mutant (14) which utilized indole as well as tryptophan. These results allow the following conclusions: (i) *trp-1* mutants are blocked in the final steps of the pathway which, in other organisms, are catalysed by tryptophan synthetase (reactions 5a and 5b, Fig. 1); (ii) *trp-2* and *trp-4* mutants are blocked in the reactions between anthranilic acid and indole (reactions 2, 3, 4 or 5a, Fig. 1); and (iii) the *trp-3* mutant is blocked before anthranilic acid in the reaction which, in other organisms, is catalysed by anthranilate synthetase (reaction 1, Fig. 1).

Accumulation of intermediates

Representative mutants were tested for the accumulation of tryptophan biosynthetic intermediates (Table 4). All tests for CDRP and indole were negative. The results support the conclusions drawn from the auxanographic tests and allow the additional conclusion that *trp-2* mutants are also blocked, at least partially, in anthranilic acid formation. A block

only in the conversion of anthranilic acid to indole, as indicated by auxanographic data, would cause strong accumulation of any anthranilic acid produced, but such accumulation was barely detectable. The small amounts of anthranilic acid produced by the *trp-3*, *trp-2* and wild-type strains (Table 4) probably resulted from tryptophan catabolism. This occurs in *N. crassa* (Matchett & DeMoss, 1963) and probably also in *C. radiatus* (Guerdoux, 1967).

DISCUSSION

Analysis of 31 tryptophan-requiring mutants has shown that at least four different genes (*trp-1*, *trp-2*, *trp-3* and *trp-4*) are necessary for tryptophan biosynthesis in *C. lagopus*. All the enzyme activities of tryptophan biosynthesis (Fig. 1) except tryptophan synthetase have been detected in *C. lagopus* (Hutter & DeMoss, 1967). The enzymes controlled by each genetic locus are inferred in the present study from auxanographic tests and accumulated intermediates.

The *trp-3* locus is assumed to control anthranilate synthetase since the *trp-3* mutant was blocked in anthranilic acid formation. Further support for this comes from the observation that the accumulation of anthranilic acid caused by the *trp-4* mutation is prevented in the double mutant *trp-3 trp-4*. The *trp-3* mutant showed no requirement for phenylalanine or tyrosine, which are also synthesised from chorismic acid (Fig. 1). In all organisms studied, anthranilate synthetase is the only enzyme preceding anthranilic acid and specific to tryptophan biosynthesis.

Similar evidence indicates that the *trp-2* locus also controls anthranilate synthetase. In addition, it controls an activity necessary for the conversion of anthranilic acid to indole. Hutter & DeMoss (1967) presented evidence for an enzyme aggregate in *C. lagopus* which carries anthranilate synthetase, *N*-(5'-phosphoribosyl)-anthranilic acid (PRA) isomerase and InGP synthetase activities (reactions 1, 3 and 4, Fig. 1) similar to a well characterized aggregate in *N. crassa*. It is probable that the *trp-2* locus of *C. lagopus* is similar to the locus in *N. crassa* that codes for a polypeptide which carries PRA isomerase and InGP synthetase activities, and which must aggregate with the product of a second locus to form normal anthranilate synthetase activity (Arroyo-Begovich & DeMoss, 1973), the equivalent of the latter locus in *C. lagopus* being *trp-3*. In *C. radiatus*, anthranilate synthetase activity appears to be less dependent on the integrity of the whole aggregate (Henke *et al.*, 1973).

The *trp-4* locus is necessary for the conversion of anthranilic acid to InGP and may well control phosphoribosyl transferase, which was not detectably aggregated with other enzymes of the pathway in *C. lagopus* and *N. crassa* (Hutter & DeMoss, 1967). The very strong accumulation of anthranilic acid by the *trp-4* mutant is consistent with this.

The *trp-1* locus controls tryptophan synthetase because all but one of the *trp-1* mutants were blocked in the last reaction of the pathway (5b). The accumulation of InGP and not indole by the *trp-1* strains tested indicates that they were also blocked in reaction 5a, also catalysed by tryptophan synthetase. The one *trp-1* mutant (14) which could utilize indole appears blocked in only reaction 5a. Mutants of this type have been described in *N. crassa* (Ahmad, Choudhury & Islam, 1969) and *Saccharomyces cerevisiae* (Manney *et al.*, 1969). It appears that, as in the above fungi, both tryptophan synthetase activities in *C. lagopus* are coded in the same genetic region.

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