JOURNAL OF BACTERIOLOGY, June, 1965 Vol. 89, No. 6

Increased Activity of Tryptophan Biosynthetic Enzymes in Histidine Mutants of *Neurospora crassa*

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Received for publication 12 January 1965

ABSTRACT

CARSIOTIS, M. (University of Cincinnati College of Medicine, Cincinnati, Ohio), AND ANN M. LACY. Increased activity of tryptophan biosynthetic enzymes in histidine mutants of *Neurospora crassa*. J. Bacteriol. **89**:1472–1477. 1965.—The activities of tryptophan synthetase and indoleglycerol phosphate synthetase, the last two enzymes in the tryptophan biosynthetic pathway, are elevated twofold or more in all histidine mutants of *Neurospora crassa*. The elevation occurs only when the mutants are grown in a histidine-limited medium. Several possible mechanisms for this elevation were disproved experimentally. The cause of the elevated enzyme activities remains unknown.

Genetic, biochemical, and immunological studies have contributed to an understanding of the relationship between tryptophan synthetase of *Neurospora crassa* and its structural gene, the *td* locus (Yanofsky, 1960; Bonner, Suyama, and DeMoss, 1960). The tryptophan synthetase of wild-type strains catalyzes three reactions: the formation of tryptophan from indole-3-glycerol phosphate (InGP) and serine, the conversion of indole and serine to tryptophan, and the formation of indole from InGP (DeMoss, 1962).

An understanding of the regulation of tryptophan synthetase formation in *N. crassa* is still limited, and can be summarized as follows: (i) the wild-type enzyme is maximally repressed in growing cultures of wild-type strains (Lester, 1961; Matchett and DeMoss, 1962); (ii) a threefold increase of tryptophan synthetase activity occurs when strain 10575 (an auxotroph blocked early in tryptophan biosynthesis) is grown in media containing low levels of tryptophan (Lester, 1961); (iii) conditions which derepress fivefold the synthesis of a mutationally altered form of tryptophan synthetase in strain *td* 201 [catalyzes only indole + serine → tryptophan (Rachmeler and Yanofsky, 1961)] fail to derepress synthesis of the enzyme in wild-type strains (Matchett and DeMoss, 1962); (iv) the hist-1 mutant C84 produces two- to threefold more tryptophan synthetase activity than does the parental wild-type strain (Hogness and Mitchell, 1954).

Further investigation of this last finding seemed warranted. The genetic crossing data of Hogness and Mitchell (1954) indicated that the increased tryptophan synthetase activity segregated with the C84 gene, but the data were not sufficient to indicate whether this characteristic was inherent in the C84 mutant gene itself or was caused by a closely linked gene. The elimination of the latter possibility by enzyme assays of single spore isolates seemed endless. Consequently, enzyme assays were carried out on a number of hist-1 mutants isolated from two different wild-type strains: Em a and 74A.

The hist-1 mutant strains used were chosen because they differed from each other with regard to induced reversion rate, complementation pattern, and genetic map location within the hist-1 locus (M. E. Case, *personal communication;* Catcheside, 1960). To determine whether the raised level of tryptophan synthetase was limited to hist-1 mutants or characteristic of histidine mutants generally, the enzyme level was measured in isolates from each of the nine known histidine mutant classes in *N. crassa* (Webber and Case, 1960; Fink, 1964). [Although genetic data indicate seven histidine loci in *N. crassa*, the hist-3 mutants can be divided into three classes. One class (3D) accumulates histidinol, whereas the other two classes (3A, 3B) do not (Fink, 1964).] It was also of interest to know whether the activity of other tryptophan biosynthetic enzymes was increased. For this reason,

the activity of InGP synthetase (the enzyme preceding tryptophan synthetase) was also measured. Finally, when it was found that both tryptophan biosynthetic enzyme activities were elevated in all histidine mutants, the mechanism and specificity of the increased enzyme levels were studied.

MATERIALS AND METHODS

Cultures. Seventeen histidine mutants and the parental wild-type strain, Em a, were obtained from D. G. Catcheside (1960). An additional 15 histidine mutants and their parental wild-type strain 74A were obtained from M. E. Case (Webber and Case, 1960).

Growth and assay procedures. All strains were grown in 125-ml Erlenmeyer flasks containing 50 ml of Vogel's (1964) medium and 2% sucrose. A supplement of 3.4×10^{-4} M L-histidine was generally added to this medium, except where indicated in the appropriate tables and figure. In most cases, dry conidia from a slant were used for inoculation, but whenever appropriate a conidial suspension was employed. The flasks were incubated at 30 C on a rotary shaker (180 rev/min), generally for 72 hr. The mycelia were recovered by filtration, washed twice with distilled water, lyophilized, weighed, and ground to a powder which was stored at −20 C until assayed. [Grinding in a mortar and pestle often proved difficult. Therefore, the lyophilized mycelia were placed in a test tube (18 by 150 mm) with two stainless-steel spatulas. The tube was held firmly at the lip and agitated on a mechanical agitator (Vortex Junior Mixer). This procedure rapidly produced a finely ground powder suitable for extraction (D. B. Fankhauser, *unpublished data*).]

Crude extracts of the powder were prepared in the cold by the method of Mohler and Suskind (1960), with the use of 0.1 M potassium phosphate (*p*H 7.8) containing 10^{-3} M ethylenediaminetetraacetate when assaying tryptophan synthetase and InGP synthetase. Tris(hydroxymethyl)aminomethane buffer (0.02 M, *p*H 7.2) was used when extracts were prepared for assaying inorganic pyrophosphatase. Tryptophan synthetase activity in the extracts was determined by measuring the rate of indole disappearance in the reaction, indole + serine → tryptophan (Yanofsky, 1955). One tryptophan synthetase unit catalyzes the disappearance of 0.1 μmole of indole in 1 hr at 37 C. InGP synthetase was assayed by measuring the formation of InGP from 1-(*o*-carboxyphenylamino) - 1 - deoxyribulose 5 - phosphate (CDRP; Wegman and DeMoss, *in press*). The CDRP was synthesized by the method of Smith and Yanofsky (1960). One InGP synthetase unit catalyzes the appearance of 0.1 μmole of InGP in 15 min at 37 C; InGP was assayed chemically (Yanofsky, 1956). Protein concentration was measured by a modification of the biuret method (Zamenhof, 1957). Specific activity is expressed as units of the respective enzymatic activity per milligram of protein. Inorganic pyrophosphatase was assayed essentially by the method of Heppel (1955).

The formation of tryptophan from InGP and serine was measured by the method of DeMoss (1962). Chorismic acid was synthesized by use of an extract of *N. crassa* (DeMoss, 1965), and its conversion to anthranilic acid was measured fluorimetrically (DeMoss, 1965). A highly purified fraction of tryptophan synthetase (designated R-2) was prepared according to Carsiotis and Suskind (1964). A 40 to 50% ammonium sulfate fraction which contains anthranilate synthetase was prepared by the procedure of DeMoss (1965).

RESULTS

Tryptophan synthetase, InGP synthetase, and inorganic pyrophosphatase levels. All 15 hist-1 strains tested exhibited more tryptophan synthetase and InGP synthetase activity than did their parental wild-type strain (Table 1). Similar data were obtained for the other classes of histidine mutants tested (Table 2). The specific activity of

TABLE 1. *Enzymatic activities of hist-1 mutants and parental wild-type strains*

Expt no.	Strain	Mycelial dry wt/flask	Specific activity	
			Tryptophan synthetase	InGP* synthetase
		mg		
1	K646a	139	4.8	0.59
	K744a	83	4.4	0.50
	K651a	78	3.6	0.34
	K626a	123	4.3	0.52
	K85a	196	5.3	0.61
	K90a	181	5.8	0.72
	Em a†	273	0.95	0.15
	Em a‡	309	1.1	0.23
2	A-5	148	4.3	0.46
	A-36	85	4.4	0.48
	A-11	99	4.4	0.51
	74A†	256	1.3	0.22
	74A‡	279	1.2	0.22
3	A-10	115	5.2	0.34
	A-30	79	4.6	0.32
	A-40	99	4.8	0.31
	A-52	80	4.4	0.31
	A-61	69	4.8	0.30
	Y-155-M302	200	3.6	0.34
	74A†	227	1.4	0.13
	74A‡	251	1.3	0.13

* Indoleglycerol phosphate.

† Medium supplemented with 3.4×10^{-4} M L-histidine.

‡ No histidine in medium.

TABLE 2. *Enzymatic activities of hist-2 through hist-7 mutants and parental wild-type strains*

Expt no.	Strain	Hist allele	Mycelial dry wt/flask	Specific activity	
				Trypto-phan syn-thetase	InGP[a] syn-thetase
			mg		
1	K53a	3D	136	3.1	0.51
	K937a	4	168	5.6	0.51
	K52a	5	97	6.2	0.55
	K694a	6	153	3.6	0.60
	K277a	7	96	3.0	0.46
	Em a[b]	—	330	1.5	0.23
	Em a[c]	—	367	1.7	0.23
2	K74a	2	159	2.8	0.45
	K26a	3D	196	3.4	0.48
	C141a[d]	4	113	5.9	0.32
	K54a	5	147	9.2	0.55
	K692a	6	99	4.5	0.37
	K620a	7	188	5.5	0.55
	Em a[b]	—	260	—	0.18
	Em a[c]	—	315	1.2	0.20
3	Y152-M43	2	149	3.7	0.71
	Y155-M261	3D	113	2.7	0.39
	Y152-M111	3A,B,D	124	3.7	0.59
	Y152-M108	5	115	4.5	0.41
	Y152-M105	6	112	2.9	0.52
	Y152-M31	7	160	3.7	0.64
	74A[b]	—	256	1.3	0.22
	74A[c]	—	279	1.2	0.22

[a] Indoleglycerol phosphate.
[b] Medium supplemented with 3.4×10^{-4} M L-histidine.
[c] No histidine in medium.
[d] An isolate of backcrosses to Emerson stocks of the original C141 obtained from B. Ames.

inorganic pyrophosphatase was about the same in Em a and hist-1 mutant K85a, indicating that the elevation of the two tryptophan biosynthetic enzymes was not due to a generalized increase in protein synthesis.

Of the 32 mutant strains listed in Tables 1 and 2, 27 consistently exhibited elevated enzyme levels. The remaining five mutant strains showed some decrease in enzyme levels after repeated transfers of the stock cultures.

Effect of growth conditions. Since Hogness and Mitchell (1954) used minimal medium supplemented only with 3.2×10^{-4} M L-histidine, the effect of varying amounts of histidine on the enzyme levels was tested. The hist-1 mutant K85a was used, because it had consistently exhibited elevated tryptophan synthetase and InGP synthetase. The results (Fig. 1) clearly show that both enzymes were elevated only at the lower concentrations of histidine, and that these concentrations were growth-limiting. The wild-type strain showed some decrease of enzyme levels when supplemented with histidine. The finding that elevation of enzyme levels depends upon growth-limiting amounts of histidine raised the possibility that limited growth *per se* caused the elevations. This hypothesis was discounted by the failure of wild-type strain Em a to exhibit elevated enzyme levels when its growth was limited by decreasing the sucrose content of the medium (Table 3). Similarly, an adenine mutant failed to show any elevation when grown on limited amounts of adenine.

Effect of histidine intermediates. Ames and Garry (1959) reported that a limiting amount of histidine causes the accumulation of histidine intermediates. Such intermediates might be present within the extracts being assayed (Ames and Mitchell, 1955), and could possibly activate tryptophan synthetase and InGP synthetase during the enzymatic assay. However, upon mixing extracts of three different classes of hist- mutants with an extract of the wild-type strain,

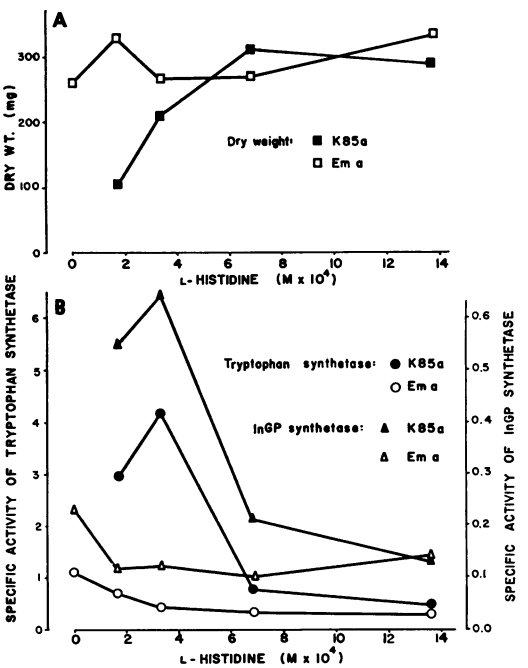

FIG. 1. *Effect of histidine concentration on growth, tryptophan synthetase, and indoleglycerol phosphate (InGP) synthetase activity of hist-1 mutant K85a and wild-type strain Em a. (A) Dry weight (average of duplicate flasks). (B) Specific activity of tryptophan synthetase and InGP synthetase.*

the calculated and observed activities agreed closely (Table 4). This result also discounts the possibility that the lower specific activities of the enzymes in wild-type strain Em a are due to an inhibitor. Another possible effect of histidine intermediates is that they may act in vivo as false feedback inhibitors (Moyed and Friedman, 1959) of some step in tryptophan biosynthesis. This possibility was negated by the fact that the presence of histidine intermediates had little or no effect on the three tryptophan biosynthetic reactions studied (Table 5).

TABLE 3. *Effect of sucrose concentration on enzyme activities in wild-type strain Em a*

Sucrose	Mycelial dry wt*	Specific activity	
		Tryptophan synthetase	InGP† synthetase
%	mg		
0.25	34	0.82	0.19
0.5	83	0.73	0.18
1.0	127	0.97	0.20
2.0	221	0.74	0.14

* Average of duplicate flasks.
† Indoleglycerol phosphate.

TABLE 4. *Additive effect of mixing crude extracts from wild-type and histidine mutant strains*[a]

Expt no.	Strain	TSU[b]/ sample	TSU[b] found upon mixing samples		InGP-SU[c]/ sample	InGP-SU[c] found upon mixing samples	
			Calculated	Observed		Calculated	Observed
1	K74a[d]	1.93	2.63	2.26	0.38	0.58	0.56
	Em a	0.70			0.20		
2	K626a[e]	1.28	2.06	2.01	0.50	0.98	1.1
	Em a	0.78			0.48		
3	K53a[f]	3.16	3.86	3.40	0.71	0.91	0.82
	Em a	0.70			0.20		

[a] Crude extracts prepared as described in Materials and Methods.
[b] Units of tryptophan synthetase activity.
[c] Units of indoleglycerol phosphate synthetase activity.
[d] K74a is a hist-2 mutant and therefore blocked in the first step in histidine biosynthesis.
[e] K626a is a hist-1 mutant and therefore blocked in an intermediate step in histidine biosynthesis.
[f] K53a is a hist-3D mutant and therefore blocked in the final step in histidine biosynthesis.

TABLE 5. *Effect of histidine intermediates on three tryptophan biosynthetic enzymes*

Expt no.	Enzymatic reaction[a]	Histidine intermediate	Relative rate
			%
1[b]	Chorismic acid → anthranilic acid	None	100
		L-Histidinol, 10^{-3} M[c]	85
2	CDRP[d] → InGP[e]	None	100
		L-Histidinol, 10^{-3} M[c]	104
3	InGP[e] + serine → tryptophan	None	100
		Imidazoleglycerol phosphate, 10^{-3} M[c]	90

[a] Prepared and assayed as described in Materials and Methods. The enzyme fractions used were as follows: experiment 1, a 40 to 50% ammonium sulfate fraction of *Neurospora crassa;* experiment 2, crude extract; experiment 3, an R-2 fraction of tryptophan synthetase.
[b] This experiment was performed in the laboratory of I. P. Crawford. We are grateful to him and T. Baker for their hospitality and advice.
[c] These histidine intermediates were generously provided by J. C. Loper.
[d] 1-(*o*-Carboxyphenylamino)-1-deoxyribulose 5-phosphate.
[e] Indoleglycerol phosphate.

DISCUSSION

The observation that both tryptophan synthetase and InGP synthetase activity in all histidine mutants of *N. crassa* are higher than in wild-type strains is a new finding. Before discussing possible mechanisms of the increased enzyme activities, it is pertinent to compare these results with those of others. First, it was noted that about the same three- to fivefold increase of tryptophan synthetase observed in strain 10575 (Lester, 1961) and strain *td* 201 (Matchett and DeMoss, 1962) occurs in the histidine mutants. Second, the ratio tryptophan synthetase-InGP synthetase is not constant in the various histidine mutants. Thus, if the phenomenon of increased tryptophan enzymes in histidine mutants is due to derepression, it is apparently not coordinate (Ames and Garry, 1959). Ito (*personal communication*) found coordinate repression of all tryptophan biosynthetic enzymes in *Escherichia coli*, whereas Lester (1963), working with *N. crassa*, failed to find coordinate repression in tryptophan biosynthesis. Gross (1964), however, reported coordinate repression of leucine biosynthetic enzymes in *N. crassa*.

The biochemical and physiological mechanisms considered were the following. The possibility that an activator exists in the extracts of histidine mutants or an inhibitor in the extracts of wild-type strains was disproven. Since no increase of enzyme activities in a wild-type strain occurred when various amounts of histidine were added to the medium, a direct effect of histidine seems improbable. Although the increases are only noted when growth of the mutants is limited by the amount of histidine, neither a wild-type nor an adenine mutant strain formed more tryptophan synthetase or InGP synthetase when its growth was limited. The fact that the specific activity of inorganic pyrophosphatase is the same in a histidine mutant and its parental wild-type strain indicates that a generalized increase of enzyme synthesis is not the cause of the elevated enzyme activities.

Having discounted these possibilities, we sought an explanation within the framework of known control mechanisms. This seemed reasonable because of the elevation of activities of two enzymes in the tryptophan pathway and because their synthesis could be depressed to wild-type levels by excess histidine. Tryptophan biosynthesis in *N. crassa* can be schematized as follows (DeMoss and Wegman, 1964; Wegman and DeMoss, 1964): chorismic acid $\xrightarrow{\text{I}}$ anthranilic acid $\xrightarrow{\text{II}}$ *N*-(5′-phosphoribosyl) anthranilate $\xrightarrow{\text{IIIa}}$ [CDRP] $\xrightarrow{\text{IIIb}}$ InGP $\xrightarrow{\text{IV}}$ tryptophan. This pathway and the histidine pathway share no common intermediates (Ames and Hartman, 1963). However, the fact that elevation of tryptophan synthetase and InGP synthetase depended upon growth of the histidine mutants in a histidine-limited medium, which is known to cause the accumulation of histidine intermediates (Ames and Garry, 1959), prompted the following hypothesis. If an accumulated histidine intermediate caused false feedback inhibition (Moyed and Friedman, 1959) of any step in tryptophan biosynthesis, the result would be decreased tryptophan formation. This would cause derepression of tryptophan synthetase and InGP synthetase, since tryptophan represses the formation of tryptophan synthetase (Lester, 1961; Matchett and DeMoss, 1962) and other tryptophan biosynthetic enzymes (Matchett and DeMoss, 1963; Lester, 1963). We found, however, that reactions I, IIIb, and IV in tryptophan biosynthesis are hardly affected by a histidine intermediate(s).

Obviously, this hypothesis could be discarded if one knew which hist locus corresponds to the first histidine biosynthetic enzyme, because such a mutant accumulates no intermediate. Hence, the hypothesized false feedback inhibition in the tryptophan pathway could not occur. At the time these studies were begun, it was not certain whether the first histidine enzyme is controlled by the hist-2 or hist-3 locus (Webber and Case, 1960); furthermore, the latter gene was known to be complex (Webber, 1960). Therefore, we did not know whether any of the hist-2 or hist-3 mutants in our possession included a mutant of the locus for the first histidine enzyme. Recently, the hist-2 locus has been shown to control the first histidine enzyme (Ahmed, Case, and Giles, 1964; Fink, 1964), nullifying this hypothesis.

Although the elevation and depression of enzyme levels in the histidine mutants resemble derepression and repression, respectively, we have failed to repress the elevation by the addition of either tryptophan or indole to the growth medium (*unpublished data*). Whether these results are due to the existence of two metabolically active pools of tryptophan in *N. crassa* (Matchett and DeMoss, 1964) must be determined.

Several other amino acid mutants have been tested for their levels of tryptophan synthetase and InGP synthetase (*unpublished data*). Although some of these contain significantly higher levels of both enzymes than does either 74A or Em a, these levels are almost unaffected by the amount of amino acid added to the medium. Furthermore, these amino acid mutants are not derived from 74A or Em a, so comparison with 74A and Em a is unwarranted. We are currently working with the histidine mutants and also with a variety of auxotrophic strains (amino acid, vitamin, purine) to determine the specificity and mechanism of the observed elevation of tryptophan biosynthetic enzymes.

ACKNOWLEDGMENTS

We wish to thank Mervyn Klein, holder of a research training fellowship supported by the Public Health Service General Research Support Grant, and David Fankhauser for able technical assistance.

This investigation was supported by National Science Foundation grants GB-1651 (M.C.) and G-14059-GB-5 (A.M.L.), and by an Institutional Grant (IN-79) from the American Cancer Society (M.C.).

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