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Altered Protein Formation as a Result of Suppression in *Neurospora Crassa*

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The mutant td201 of *Neurospora crassa* is mutated in the *trp-3* locus and forms an altered tryptophan synthetase. A suppressor mutation, *su2-6*, in this mutant, unlinked to the *trp-3* locus, results in the production of wild-type tryptophan synthetase activity, which accounts for the alleviation of the tryptophan or indole requirement. This enzyme activity is associated with a protein physically dissimilar to the wild-type enzyme. A second altered protein, a serologically cross-reacting material, is also formed in the suppressed mutant, in addition to the altered enzyme normally formed by the td201 mutant. Normal growth, equivalent to that of wild type, is not restored in the suppressed mutant even with tryptophan supplementation. The relationship of the data to possible mechanisms of suppression is discussed.

Tryptophan synthetase (TSase) catalyzes three reactions in vitro:

$$\text{indoleglycerol phosphate} + \text{L-serine} \xrightarrow[\text{phosphate}]{\text{pyridoxal}} \text{L-tryptophan} + \text{glyceraldehyde-3-phosphate} \quad (1)$$

$$\text{indole} + \text{L-serine} \xrightarrow[\text{phosphate}]{\text{pyridoxal}} \text{L-tryptophan} \quad (2)$$

$$\text{indoleglycerol phosphate} \rightleftharpoons \text{indole} + \text{glyceraldehyde-3-phosphate} \quad (3)$$

Reaction 1 is the terminal reaction in tryptophan biosynthesis in vivo, and is used as the indicator of wild-type enzyme activity. A tryptophan mutant of *Neurospora crassa*, td201, mutated at the *trp-3* locus, has been shown to form an altered protein related enzymatically and serologically to the enzyme TSase (15). Specific suppressor gene mutations in this strain result in the alleviation of the tryptophan requirement; this has been shown to be due to the formation of wild-type TSase in several suppressed strains of this mutant (24-26). Recent evidence indicates that missense suppression can occur due to altered transfer ribonucleic acid (tRNA) molecules, which lead to mistakes in the translation process; e.g., two amino acids are specified by the same codon (5, 10). In the strains studied, the proper amino acid substitution has led to synthesis of some wild-type enzyme.

In the present study, the proteins related enzymatically or serologically to TSase which are formed in the suppressed mutant, td201su2-6, have been compared to both the wild-type and mutant proteins. The suppressor mutation in this strain is nonallelic with those previously studied (17). It is shown that the enzymatically active enzyme found in the suppressed mutant is physically dissimilar to the wild-type enzyme.

MATERIALS AND METHODS

Organisms and culture conditions. N. crassa strain 74A was the source of wild-type enzyme. The mutant used in this study, td201, has several distinctive properties which make it quite useful for this work (15). It requires either indole or tryptophan for growth, and it accumulates indole-glycerol and anthranilic acid. The altered tryptophan synthetase of td201 catalyzes only reaction 2, and its specific activity is usually three times higher than that of the wild type. This altered enzyme acts as serologically cross-reacting material (CRM) when assayed by reaction 1.

The *N. crassa* cultures were grown in medium N (19) plus 2% sucrose with vigorous aeration for 60 hr at 35 C; for mutant growth, DL-tryptophan, 100 μg/ml, was added to the medium. The mycelium was filtered through cheese cloth, washed with cold distilled water, squeezed dry, and lyophilized. The filtrate was tested for indole by adding Ehrlich's reagent to a toluene extract (22). Indole-glycerol was qualitatively assayed by the use of ferric chloride reagent (23). Anthranilic acid, if present, gave a bright yellow color when 0.05 ml of Ehrlich's reagent was added to 1 ml of culture filtrate.

Escherichia coli strain T3, a mutant blocked at an early step in tryptophan biosynthesis, was the source of tryptophanase, used in the assay of reaction 1, and tryptophan synthetase used for indoleglycerol-3-phosphate (InGP) production. For the former, it was grown in medium E (20) plus 0.2% glucose supplemented with DL-tryptophan, 100 μg/ml; for the latter, anthranilic acid, 2 μg/ml, was substituted for

tryptophan. Tryptophanase was prepared according to the procedure of Burns and DeMoss (3).

InGP was prepared from an incubation mixture containing 500 μmoles of indole, 5 mmoles of fructose-1,6-diphosphate, 0.75 mmole of aldolase (five times crystallized, Worthington Biochemical Corp., Freehold, N.J.), 50 mM KPO_4 buffer (*p*H 7.8), and 5,000 units of tryptophan synthetase, in a total volume of 500 ml. The mixture was incubated at 37 C for 60 min, and the InGP was isolated as described by Yanofsky (23).

Quantitative growth assays were performed in minimal medium N or minimal medium supplemented with L-tryptophan, 100 μg/ml, or indole, 10 μg/ml (16).

Crosses were performed on synthetic medium (21) according to the procedure of Ryan (16).

Protein purification. The proteins were partially purified according to the procedure of Mohler and Suskind (14). The extracts obtained after step III, the first ammonium sulfate step, and subsequent dialysis, were used in these studies.

Protein was determined by the method of Lowry et al (13).

Enzyme assays. The indole to tryptophan and InGP to indole reactions were assayed according to Yanofsky (22). The latter was measured in the forward reaction only, by the appearance of indole from InGP. The InGP to tryptophan reaction was measured by use of the tryptophanase method of DeMoss (7).

A unit of enzyme activity equals 1 μmole of substrate utilized or product formed in 60 min; specific activity equals units divided by milligrams of protein.

Immunological assays. Antiserum was prepared to the various proteins by injecting rabbits with increasing doses of partially purified antigen (from 2 to 10 mg) intravenously four times a week for 3 weeks. After an additional 14 days, a subsequent injection of 20 mg of antigen protein diluted 1:1 in incomplete Freund's adjuvant was given intramuscularly. After 7 to 10 days, and periodically thereafter, the rabbits were bled from the central ear vein by use of a hypodermic syringe. The clot formed from overnight incubation of the blood at 0 to 4 C was removed by centrifugation at 2,000 rev/min for 10 min. The antibody precipitated by 50% ammonium sulfate saturation was collected from the serum, resuspended in a small volume of 0.1 M KPO_4 buffer (*p*H 7.8), and dialyzed for 16 hr against this buffer in the cold.

CRM assays were performed as described previously (15, 18). A unit of CRM is the amount of mutant protein necessary to protect 1 unit of wild-type enzyme activity from neutralization by antiserum.

RESULTS

Isolation of suppressors and genetic analysis. Conidia from td201 were ultraviolet-irradiated and plated on minimal medium. Slow-growing colonies were selected and crossed to wild type to determine the presence of suppressor mutations. One suppressed mutant, designated td201-su2-6, was selected for further study. This mutant is the SuR-6 strain of St. Lawrence et al. (17). The suppressor mutation was determined to be nonallelic with su1 and su3 of Yourno and Suskind (17, 24–26). The four progeny from a single ascus in a cross of td201su2-6 × wild type were isolated and used in the subsequent analyses (17).

The *su2* suppressor appears to be fairly specific in so far as its action on the *td* locus is concerned; crosses to other td mutants, td1, td2, td3, td24, and td71, failed to yield any tryptophan-independent growth.

Growth studies. Two important points to note from the growth studies (Table 1) are that the suppressed mutant does not reach wild-type growth even with tryptophan supplementation, and that the suppressed wild type is also slightly inhibited in its growth as compared with wild type. These findings suggest that the suppressor affects the cell deleteriously, in addition to causing suppression of the tryptophan requirement. The suppressed mutant still accumulates indoleglycerol and anthranilic acid in its culture filtrate, indicating that an enzymatic defect still exists in the tryptophan pathway (Table 1).

Enzyme studies. The three reactions of tryptophan synthetase are catalyzed in a fairly constant ratio, during initial stages of purification. A disparity in the ratio of reactions 1 to 2 from that normally observed in partially purified wild-type extracts is used as one indication of the presence of an altered protein in the presence of wild-type enzyme (Table 2). A ratio well below the normal 0.5 indicates either the presence of td201-type mutant enzyme in the presence of wild-type enzyme or an altered enzyme which catalyzes the two reactions in an unusual ratio. The suppressed mutant is found to contain a small amount of wild-type activity, InGP → tryptophan; however,

TABLE 1. *Growth and accumulation products of progeny of td201su2-6 × wild type cross*

Strain	Wild-type growth (%) on indicated medium		Accumulation[a]		
	Minimal	Minimal + tryptophan	Indoleglycerol	Indole	Anthranilic acid
td201	0	100	+	−	+
td201su2-6	15	80	+	−	+
Wild type (WT)	100	100	−	−	−
WTsu2-6	90	90	−	−	−

[a] Accumulations after growth in minimal medium except for td201 which was grown in minimal medium plus tryptophan; + = presence; − = absence.

the indole → tryptophan activity is far in excess of what would be expected if only wild-type protein were present. This can be accounted for, as indicated, either by the presence of td201 altered protein in the suppressed mutant, *in addition* to a "wild-type-like" protein, or by the presence of an altered wild-type protein that catalyzes the two reactions in an unusual ratio. The amount of InGP → indole activity found is what would normally be expected from the InGP → tryptophan activity present. The possibility that the mutant strain or the suppressed mutant strain produced an inhibitor of wild-type enzyme was tested by assaying mixtures of extracts of wild type with the two mutant strains. The presence of enzyme-inhibiting material would explain the low wild-type activity observed in the suppressed mutant, but the data indicate that no appreciable amount of enzyme-inhibiting material was present in any of the extracts.

TABLE 2. *Enzyme activities of partially purified extracts*

Strain	Activity (units/mg of protein)[a]			
	InGP → Trp (1)	In → Trp (2)	InGP → In (3)	Ratio (1/2)
Wild type (WT)..	0.36	0.72	0.063	0.5
WTsu2-6.....	1.14	2.42	0.15	0.47
td201.....	0.0	7.9	0.0	0
td201su2-6.....	0.044	4.3	0.004	0.01

[a] A specific activity of 250 for reaction 2 has been reported for the pure enzyme (9). InGP = indoleglycerol-3-phosphate; Trp = tryptophan; In = indole.

Enzyme stability studies. The thermostability of the enzyme activities was compared. Extracts of the four strains in solutions containing approximately equal concentrations of protein were heated at 60 C, and samples were assayed for residual activity in reactions 1 and 2 after varying periods of time (Fig. 1). The InGP → tryptophan activity

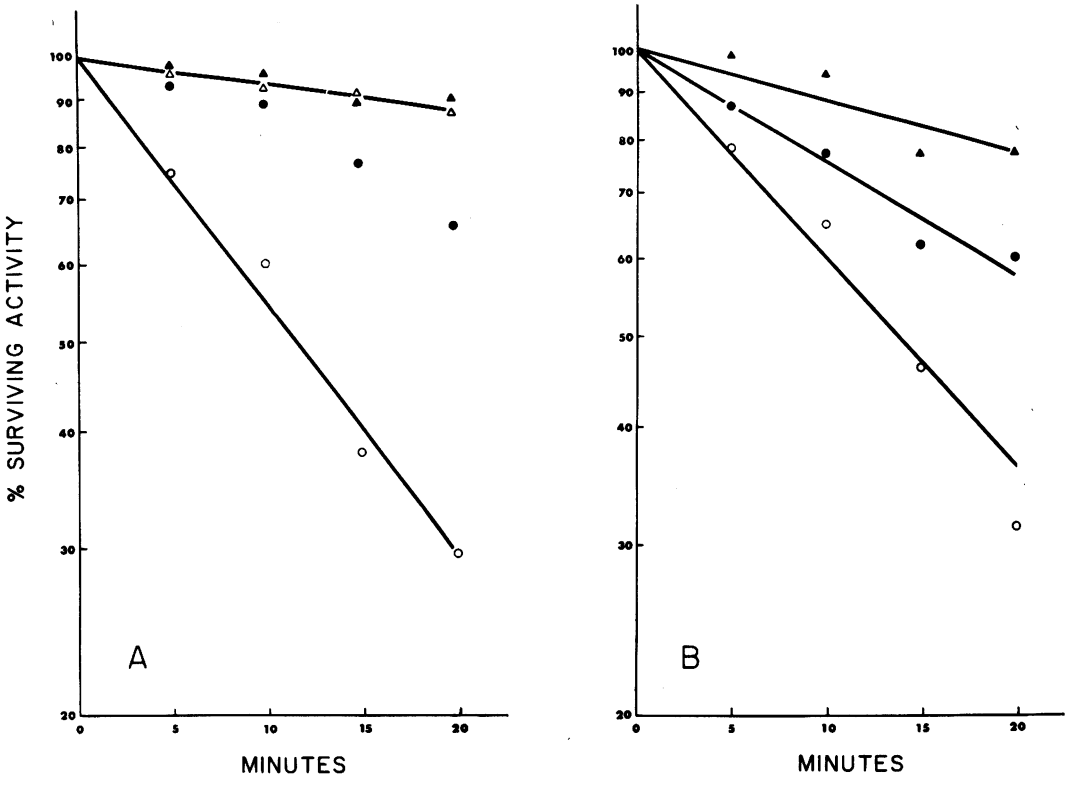

FIG. 1. *Stability of extracts of various strains to heating at 60 C. At various times, samples were assayed from tubes containing the extracts, 1.0 mg of bovine serum albumin per ml, 0.1 M KPO_4 buffer (pH 7.8), 10^{-4} M pyridoxal phosphate, and 10^{-2} M ethylenediaminetetraacetate. In A, samples were assayed at various times by use of reaction 2; in B, samples were assayed by use of reaction 1. Symbols: ○, wild type; ●, WTsu2-6; △, td201 ▲, td201su2-6. The final protein concentration of the extracts per milliliter in the final heating mixture varied from 0.2 mg for the wild-type extract to 0.02 mg for the td201 extract.*

was appreciably more thermostable in the suppressed mutant than in the wild type, suggesting that this activity in the suppressed strain is associated with an enzyme physically dissimilar to wild-type enzyme. The indole → tryptophan activity in td201su2-6 was also appreciably more thermostable than that of the wild type; it was, in fact, similar in thermostability to the td201 protein. The two enzyme activities in wild type su2-6 were also more thermostable than that found in the wild type, although less so than those found in either of the mutant strains. The possibility that the thermolability of the wild-type extract was due to the production of an enzyme inhibitor during heating was tested by assaying mixtures of heated and unheated extracts and by assaying various heated mixtures. The data in Table 3 indicate that the observed inhibitions do not account for the results of stability studies.

When compared in their stability to changes in *p*H and storage at 4 C (Table 4), it was found that the enzyme activity in extracts of the suppressed mutant strain again differed markedly from the wild-type enzyme. This further supports the contention that the wild-type enzyme activity in the suppressed strain is associated with a protein physically different from the wild-type enzyme.

Immunological studies. Previous work has shown that the enzyme of strain td201 is as enzymatically active in reaction 2 as wild-type enzyme, per unit of antigen. This was determined by showing that a given anti-TSase serum has an equal potency to inactivate the enzyme activity in extracts of both (15). Anti-TSase serum was less effective in neutralizing the reaction 2 activity of the suppressed mutant than the same activity in either wild-type or td201 extracts (Table 5). This decrease in sensitivity to neutralization by antiserum indicates the presence of a protein in td201su2-6 that is active antigenically but not enzymatically; e.g., it differs from the enzymatically active altered protein of td201 or the wild-type enzyme. This CRM represents either an enzymatically inactive protein formed in the suppressed mutant as a result of the suppressor activity or an enzymatically active protein possessing a lower indole → tryptophan activity per unit of antigen

TABLE 3. *Effects of heating on the enzyme activity of mixtures of extracts*

Extracts	Percentage of expected reaction 2 activity[a]
td201 heated[b] + wild type unheated	86
td201su2-6 heated + wild type unheated	84
Wild type heated + wild type unheated	80
Extracts heated together for 20 min	
Wild type + td201	100
Wild type + td201su2-6	100
Wild type + WTsu2-6	80

[a] The percentage of expected activity is the activity observed in the mixture multiplied by 100 divided by the sum of the activities of the individual extracts treated as indicated. The heating was carried out under conditions indicated in Fig. 1.

[b] The extracts were heated separately for 20 min at 60 C, then mixed with unheated extracts and assayed.

TABLE 5. *Serologically cross-reacting material (CRM) formation*[a]

Extract	Indole → tryptophan (units/mg of protein)	
	Enzyme	CRM
Wild type	0.72	0.0
WTsu2-6	2.7	0.3
td201	8.4	0.0
td201su2-6	4.5	3.0

[a] The units of CRM are determined from the percentage of enzyme activity neutralized by a given quantity of antiserum as compared with its effectiveness against wild type, e.g., the anti-TSase serum is only 90% effective in neutralizing the reaction 2 activity in extracts of WTsu2-6; thus, there is 10% CRM in this extract.

TABLE 4. *Relative stabilities of enzyme activity of wild-type and suppressed strains*

Treatment	Inactivation (%)					
	Reaction 1 (indoleglycerol-3-phosphate → tryptophan)			Reaction 2 (indole → trypytophan)		
	Wild type	td201su2-6	WTsu2-6	Wild type	td201su2-6	WTsu2-6
Treated with 1 N acetic acid (*p*H 4.5) and neutralized	15	75	45	37	100	25
Stored for 18 hr at 4 C	50	25	..	90	18	0

than the wild-type enzyme. The small amount of CRM found in WTsu2-6 suggests that an altered protein is also formed in this strain.

DISCUSSION

The data presented establish that the physiologically important terminal reaction in tryptophan biosynthesis in *N. crassa*, the conversion of InGP to tryptophan, is restored in td201 as a result of a suppressor mutation. This suppressor mutation is also apparently responsible for the inhibition of growth noted in the suppressed strain. Although the inhibition can be attributed, in part, to the small amount of wild-type enzyme activity present in these strains, the failure of tryptophan to restore normal growth is indicative that the alleviation of the tryptophan requirement of the cells by the formation of active TSase is not the only effect of the suppressor mutation.

The fact that the mutated *td* locus is unlinked to the suppressor gene (17) supports the hypothesis that the suppressor activity occurs through the cytoplasm, probably in the translation apparatus of the cell. It has been proposed that "mistakes" in translation could result from altered tRNA molecules or amino acyl synthetases (1, 2) or mutationally altered ribosomes (6). Recently, studies with extracts from two suppressed TSase A-protein mutants of *E. coli* have implicated altered tRNA molecules in the mechanism of suppression (5, 10). In each case, the suppressor action resulted in a substitution of one amino acid for another during the translation process, e.g., arg → gly in one mutant and cys → gly in the second. In vitro studies showed that the extracts of each suppressed mutant were able to incorporate glycine into trichloroacetic acid-insoluble material by use of templates which normally code for arginine and cysteine, respectively. It is implicit in the mechanisms proposed that the suppressor action is specific for a given codon. The substitutions which occurred resulted in wild-type enzymes.

In this study, the wild-type activity in the suppressed mutant, td201su2-6, is associated with a protein physically dissimilar to the wild-type enzyme. It seems likely, therefore, that the activity in the td201 mutant was restored either by a second-site amino acid change in the td201 altered protein or by substitution of an amino acid at the primary site of change that is normally not present at that site in the wild-type protein. Such amino acid changes have been shown to be able to restore enzyme activity in an altered protein without restoring the normal amino acid sequence (11, 12). Not all of these types of substitutions might be expected to restore activity to an altered enzyme; in fact, it is likely that in many cases it might result in a further altered protein, e.g., the altered td proteins in the suppressed mutant, or in an inactivation of normal enzyme, e.g., the CRM in the suppressed wild type. Inactivation of many normal enzymes in a cell is probably the reason for the growth inhibition, observed in the suppressed strains, that cannot be overcome by tryptophan supplementation.

Previous studies have shown that two groups of nonallelic suppressor mutations, Su_1 and Su_3, are effective in restoring wild-type enzyme activity in td201 (24–26). In each case, no differences could be observed between the enzyme formed in the suppressed mutant and the wild-type enzyme. Growth inhibition in minimal media was observed with Su_3 strains, but, although tryptophan could not overcome this inhibition completely, the suppressed mutants grew well on a nutritionally complete medium. The suppressor used in the present study has been shown to be nonallelic with those previously studied, although linked to Su_1 (17; St. Lawrence, *unpublished data*). It is, therefore, apparent that several possibilities exist by which suppression can correct the missense substitution in the td201 altered protein. It is likely that we are dealing with mutations which may be affecting similar or different components of the translation process, e.g., ribosomes, aminoacyl synthetases, or tRNA molecules. At present, we have not distinguished among these possibilities in this system.

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