NONALLELIC SUPPRESSORS OF A NEUROSPORA TRYPTOPHAN SYNTHETASE MUTANT

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WILD-TYPE tryptophan synthetase (TSase) of *Neurospora crassa* catalyzes the following reactions (YANOFSKY 1960; GARRICK, ELBERFELD, and SUSKIND 1964):

(1) indole 3-glycerolphosphate + L-serine → L-tryptophan + D-glyceraldehyde 3-phosphate + H_2O
(2) indole + L-serine → L-tryptophan + H_2O
(3f) indole 3-glycerolphosphate → indole + D-glyceraldehyde 3-phosphate
(3r) indole + D-glyceraldehyde 3-phosphate → indole 3-glycerolphosphate + "compound x"

TSase mutant td_{201} forms elevated levels of a mutationally altered enzyme (CRM) which retains only reaction (2) activity, and the mutant requires either indole or L-tryptophan for growth.

It has been reported previously that seven independently isolated td_{201} specific suppressors, hereafter designated $su_1(201)$ suppressors, are all allelic or closely linked and map in the right arm of linkage group VII (YOURNO and SUSKIND 1963 1964a). The suppressors restore a low level of wild-type-like TSase activity to the mutant while causing no gross deleterious effects on generalized cell function. The restored activity was associated with a protein which was wild-type-like by several enzymic and physical criteria (YOURNO and SUSKIND 1964b). Among four successfully characterized new suppressed mutant isolates, three were found to have the heretofore observed slow-growth characteristics on minimal medium and to contain suppressors mapping in the $su_1(201)$ region of linkage group VIIR. The remaining suppressed mutant however, $su_3(201)$-*1*, grew much more slowly in the absence of tryptophan or indole and was not linked to the others. These phenotypically different suppressors have been compared with respect to site-specificity, location, general effects on cell growth, and properties of the functional TSase produced.

$su_1(201)$ and $su_3(201)$ will be symbolized by the abbreviated symbols su_1 and su_3 throughout the paper. su^+ will indicate the wild-type allele of the suppressor gene which is *inactive*, and *su* the mutant allele which is *active* in suppression.

MATERIALS AND METHODS

Isolation of prototrophic strains from td_{201}, growth studies, genetic analyses, and enzymological

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characterizations were performed as previously described (YOURNO and SUSKIND 1964a,b). Briefly, these were as follows:

Prototrophic strains were derived from td_{201} by ultraviolet irradiation of conidial suspensions and plating of these on minimal sorbose agar. Slow-growing strains, isolated after one week of incubation at 30°C, were crossed with a wild-type stock to obtain homocaryotic slow-growing progeny.

Growth rates were compared by measuring the dry weight of mycelial pads grown in still culture (RYAN 1950).

All crosses were performed on Westergaard medium slants at 25°C as described by RYAN (1950). td_{201} suppressors were tested for linkage to group VIII markers by crosses of td_{201} su_1 or td_{201} su_3 strains to a group VII marker stock, *arg-10, sk*. The segregation of the suppressors relative to the group VII markers was scored among td_{201} progeny by appropriate nutritional tests, as were the analyses for recombination between su_1 and su_3. The specificity of su_1 and su_3 genes was examined by crosses of the wild-type carrying su_1 or su_3 to a number of *td* mutants, and slow growth on minimal medium was employed as the initial criterion for cross-suppression.

Cultures were grown in 200 ml batches on a shaker at 30°C or in 18 l carboys with vigorous aeration. Crude extracts of TSase were prepared from lyophilized mycelia. R_{25-31} preparations of TSase (MOHLER and SUSKIND 1960), about 100-fold purified, were obtained by ammonium sulfate fractionation of 100 gram lots of lyophilized mycelia.

The ratio of TSase enzyme to antigen was determined with crude extracts according to the method of SUSKIND (1957). For all other measurement of TSase activity, R_{25-31} preparations were employed. The energy of activation of reaction 1 was determined with preparations diluted to 5 to 7 units of reaction 1 per ml in Solution A (see heat inactivation, below) containing 1 mg BSA per ml. Reaction 1 activity was assayed at 17°C, 28°C, and 37°C, and the activation energy calculated from the Arrhenius plot of the log of the initial velocity *vs* 1/T. The K_m for indole 3-glycerolphosphate (InGP) in reaction 3f was determined with preparations diluted in pH 6.2 buffer to approximately 1 unit of reaction 3f per ml. The reaction mixture contained 0.1 ml enzyme, 260 μmoles pH 6.2 phosphate buffer, 0.2 μmoles pyridoxal phosphate, 1 mg BSA, 2×10^{-3} M EDTA, and 0.04 to 0.80 μmoles InGP in a final volume of 1 ml. In order to insure a linear rate, the reaction was stopped before reaching 10% completion. To measure the pH optimum of reaction 3f, enzyme was diluted in 0.1 M pH 6.2 buffer to approximately 1 unit of reaction 3f activity per ml. The reaction mixture consisted of 0.1 ml enzyme 250 μmoles phosphate buffer at pH indicated, 1 mg BSA, 0.2 μmoles InGP, 0.2 μmoles pyridoxal phosphate and 6×10^{-3} M EDTA in a final volume of 0.9 ml. Heat inactivation of TSase was measured with preparations diluted to 2 mg protein per ml in Solution A, X, or Z. Solution A contained 10^{-3} M EDTA, 0.12 μmoles pyridoxal phosphate and 100 μmoles phosphate buffer, pH 7.8 ml. Solutions Z and X were identical to Solution A except that Solution Z contained only 0.014 μmoles pyridoxal phosphate per ml while Solution X contained 0.5 μmoles pyridoxal phosphate and 10 μmoles DL-serine per ml. 0.5 ml aliquots were preheated at 37°C for 3 to 5 minutes, then placed in a water bath at the appropriate temperature and swirled for the first 30 seconds. At specified times the tubes were plunged in ice water and swirled for 30 seconds, then stored in an ice bath until assayed. The relative $T_{\frac{1}{2}}$ in the presence of pyridoxal phosphate and DL-serine was obtained from the ratio of the $T_{\frac{1}{2}}$ in Solution X to that in Solution Z.

All enzyme assays were performed as previously described (YOURNO and SUSKIND 1964a).

RESULTS AND DISCUSSION

General comparison su_3 *and* su_1 *suppressed mutants:* Suppressed mutant td_{201} su_{3-1}, which appeared considerably later than the $td_{201}su_1$ types on minimal sorbose agar isolation plates, exhibits a greater growth lag and slower rate on minimal agar slants. This property was not completely eliminated by the addition of tryptophan to the minimal medium, as seen in quantitative growth experiments

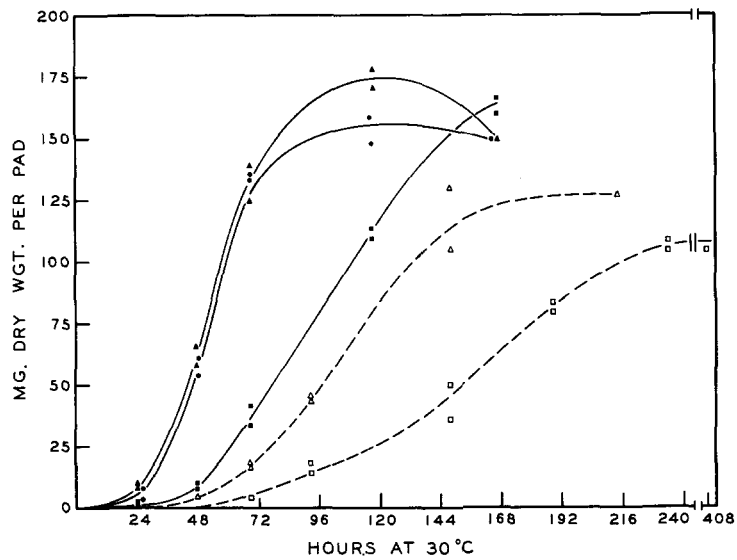

FIGURE 1.—Comparison of still-culture growth of td_{201}, $td_{201}su_{1-20}$, and $td_{201}su_{3-1}$ on various media. ●, td_{201} on minimal medium + 150 μg DL-tryptophan per ml; ▲, $td_{201}su_{1-20}$ on minimal medium + 150 μg DL-tryptophan per ml; ■, $td_{201}su_{3-1}$ on minimal medium + 150 μg DL-tryptophan per ml; △, $td_{201}su_{1-20}$ on minimal medium; □, $td_{201}su_{3-1}$ on minimal medium. Viable conidial inocula per culture flask were: td_{201}, 1.7×10^5; $td_{201}su_{1-20}$, 3.9×10^5; $td_{201}su_{3-1}$, 3.9×10^5.

(Figure 1). While these results may indicate a dissimilarity in the mechanism of action of the suppressors in the two mutants, the differential response to tryptophan more likely reflects the nonisogenicity of the different strains. On complete medium the growth rates of the su_1 and su_3 suppressed mutants are identical.

Random spore analysis of crosses of $td_{201}su_{1-2}$ and $td_{201}su_{3-1}$ by td^+ strains carrying markers in linkage group VII in both instances yielded unsuppressed td_{201} progeny in frequencies expected for the segregation of suppressor genes unlinked or distantly linkage to the *td* locus (Table 1).

The design of our isolation experiments undoubtedly selects mainly for $td_{201}su$ strains having the more rapid growth rate characteristic of the $td_{201}su_1$ types, since we routinely isolate strains for study within a week following ultraviolet irradiation and incubation of td_{201} conidia on minimal sorbose agar. After about one week the sorbose isolation plates become overgrown by secondary faster-growing tryptophan-independent colonies, which tend to obscure incipient ultra-slow growing colonies. It appears significant that all of the more rapidly growing suppressed strains we have so far isolated carry a suppressor gene of the su_1 type. ST. LAWRENCE, NAISH, and BURR (1965) have studied two nonallelic groups of td_{201} suppressors, and it seems that one of these is identical with our su_1 group. The other group, the su_2 suppressors, is nonallelic with both the su_1 and su_3 groups.

Genetic studies: Recombination of su_{3-1} *with linkage group VII markers:* Three of the four newly isolated *su* mutations, su_{1-17}, su_{1-20}, and su_{1-25}, mapped in the

TABLE 1

Recombination analysis of td_{201} su_{1-2} *and* td_{201} su_{3-1} *with an* arg-10,sk *(linkage group VIIR) marker strain*

Progeny genotype	Cross	
	arg-10 sk × $td_{201}su_{1-2}$	*arg-10 sk* × $td_{201}su_{3-1}$
td su arg+ *sk*+	21	5
td su+ *arg sk*	15	13
td su arg+ *sk*	6	0
td su+ *arg sk*+	4	1
td su arg sk+	0	0
td su+ *arg*+ *sk*	0	1
td su+ *arg*+ *sk*+	2	14
td su arg sk	2	12
**td*+ *arg*+ *sk*+	22	20
**td*+ *arg sk*	19	15
**td*+ *arg*+ *sk*	2	3
**td*+ *arg sk*+	2	3
Percent recombination *su–arg-10*	8.0	59
Percent recombination *su–sk*	28.0	59
Percent recombination *arg-10–sk*	14.7	9.2
Percent germination	99	90

* *su* and *su*+ cannot be scored in *td*+ progeny.
Number of random isolates is given in the body of the Table.

su_1 region on VII. This was determined by crossing the $td_{201}su_1$ strains by an *arg-10,sk* (VIIR) marker stock previously utilized (YOURNO and SUSKIND 1964a). su_{3-1}, however, showed free recombination with these same markers.

Recombination of su_{3-1} *and* su_1: su_{3-1} was shown to be nonallelic to su_1 suppressors by recovery of unsuppressed td_{201} progeny in crosses of $td_{201}su_{3-1}$ by $td_{201}su_{1-2}$ and by $td_{201}su_{1-4}$. su_1 and su_3 segregate independently.

Site-specificity of su_{3-1}: The site-specificity of su_{3-1} with respect to various CRM-negative and CRM-positive tryptophan synthetase point mutants, namely td_1, td_6, td_{71}, and td_{141}, was investigated by crosses of these to a td^+su_{3-1} strain. No slow-growing recombinants (cross-suppressed mutants) were isolated (48 progeny tested from each cross) with the exception of the td_{201} control cross, where suppressed mutants appeared at close to the expected 25% frequency. The 1:1 segregation of wild-type (*td*+ *su*+, *td*+ *su*) and mutant (*td su*+, *td su*) progeny indicates a site-specificity of su_{3-1} similar to that previously shown for the su_1 suppressors (YOURNO and SUSKIND 1964a).

Enzymological comparison of td_{201} su_{1-2} *and* td_{201} su_{3-1} *tryptophan synthetase:* td_{201} produces 5 to 10-fold the normal level of mutationally altered TSase (RACHMELER and YANOFSKY 1961). We have observed that this derepressed formation of defective enzyme occurs in older tryptophan-supplemented cultures of td_{201} and is correlated with the depletion of external (supplied in medium) and intracellar tryptophan. Similar derepression in tryptophan-supplemented cultures is also found with TSase mutants td_{141} (reaction 2 positive), td_2 and td_{71} (reaction

TABLE 2

Analysis of recombination between su_1 *and* su_3

Cross	Number of spores of phenotypes		Percent germination
	T+	T-	
$td_{201}su_{3-1} \times td_{201}su_{1-2}$	62	23	86
$td_{201}su_{3-1} \times td_{201}su_{1-4}$	41	19	63
Pooled	103	42	. .
Expected if independent	109	36	. .

Number of random isolates is given in the body of the table.
Nutritional phenotypes of spore progeny: T+, significant growth in 5 days or less without tryptophan at 30°C; T-, no growth without tryptophan.

3 positive), and td_{37}R, td_{48}R, and td_{132}R, which contain CRM with very weak reaction 2 activity. In each case mutant enzymic and CRM specific activities (only CRM specific activity in the case of the latter three mutants) peak at about 5 to 10-fold those of wild type and this occurs only when external and intracellular tryptophan is depleted or reaches a low threshold value. The wild type, whether grown on minimal medium or tryptophan supplemented minimal medium, maintains a high concentration of intracellular tryptophan, a plausible explanation for the low levels of TSase observed throughout the culture period (YOURNO, JUHALA, and SUSKIND, unpublished).

Crude extracts of $td_{201}su_{3-1}$, (like those of su_1 suppressed mutants), while containing considerable quantities of accumulated indoleglycerol and retaining the high reaction-2 specific activity characteristic of td_{201} mutant tryptophan synthetase, exhibit about 10% of wild-type levels of reaction-1 activity. Although the low levels of reaction 1 activity of suppressed mutants are somewhat difficult to quantitate precisely in crude extract because of the high background of indoleglycerol, the ratio of reaction-2 to reaction-1 enzymic activity appears to be 50 to 100% higher in the su_1 suppressed mutants than it is in $td_{201}su_{3-1}$. This relation is maintained in partially purified R_{25-31} preparations where reaction 1 measurements are much more exact. Reaction 3f is not detectable in crude extracts of either type of suppressed mutant but is measurable in R_{25-31} preparations.

The recovered wild-type-like enzymic activities of R_{25-31} preparations of $td_{201}su_{1-2}$ and $td_{201}su_{3-1}$ TSase were similar to each other and to that of wild-type enzyme. As can be seen in Table 3, both types of suppressed mutant TSase, wild-type TSase, and a reconstituted mixture of td_{201} CRM and wild-type TSase showed very similar ratios of reaction 1 to reaction 3f activity, K_m for indoleglycerol-3-phosphate in reaction 3f, pH optimum of reaction 3f and energy of activation of reaction 1.

Furthermore, the heat lability ($T_{1/2}$) of reaction 1 activity of R_{25-31} TSase preparations of both suppressed mutants grossly resembles the wild type in Solution A and is stabilized to the same extent by pyridoxal-phosphate and DL-serine. The possible significance of the slightly higher $T_{1/2}$ for reaction 1 of $td_{201}su_1$ in Solution A has been discussed (YOURNO and SUSKIND 1964b). Reaction 2 activity of each suppressed mutant shows the much greater heat stability

TABLE 3

Comparison of properties of TSase or CRM from su_{1-2} *and* su_{3-1} *strains*

Strain	Ratio of activities, reaction 1	2	3f	Enzyme/antigen ratio, reaction 2	Activation energy reaction 1†	K_mInGP, reaction 3f (molar)	pH optimum, reaction 3f
$td^+su^+_{1-2}$*	39	100	0.8	1.0 ± 0.1	14,200	3.1×10^{-4}	6.2
td_{201}	0	100	0	0.7 ± 0.5	.....	.....	..
$td_{201}su_{1-2}$	0.8	100	0.012	0.7 ± 0.5	13,800	3.1×10^{-4}	6.2
$td_{201}su_{3-1}$	0.5	100	0.008	0.7 ± .05	13,500	2.6×10^{-4}	6.2
reconstitute ($td_{201} + td^+su^+_{1-2}$)	1.2	100	0.020	.....	13,600	3.0×10^{-4}	6.2

Strain	$T_{1/2}$ (seconds and inactivation order at 60°C), for reaction 1	2	3f	Relative $T_{1/2}$ in presence of pyridoxal phosphate and DL-serine at 58°C, reaction 1	2
$td^+su^+_{1-2}$*	60,1°	52,1°	63,1°	4.6×	4.6×
td_{201}	....	1200, > 1°	....	..	1.1
$td_{201}su_{1-2}$	140, > 1°	1320, > 1°	130, > 1°	5.6	1.2
$td_{201}su_{3-1}$	125, > 1°	1200, > 1°	125, > 1°	4.1	1.0
reconstitute ($td_{201} + td^+su^+_{1-2}$)	56,1°	1200, > 1°	56,1°	4.6	1.2

* No significant differences in these values were observed for td^+su_{1-2}, $td^+su^+_{3-1}$, and td^+su_{3-1} enzymes.

† Calories per mole InGP.

R_{25-31} preparations of TSase or CRM were used in all determinations except for the enzyme/antigen ratio, where freshly prepared crude extracts were tested.

characteristic of td_{201} reaction 2, and is likewise not significantly protected by pyridoxal phosphate and DL-serine.

It appears therefore that nonallelic suppressor genes specific for the same single-site TSase missense mutant can affect the synthesis of very similar wild-type or wild-type-like proteins. An alterative has also been demonstrated. RACHMELER and ST. LAWRENCE (1964) have found that the su_2 suppressor gene restores a tryptophan synthetase activity with properties more similar to the mutant than to the wild-type protein. WEIGERT and GAREN (1965) have recently demonstrated a relationship between nonallelic suppressors of an *E. coli* alkaline phosphatase nonsense mutant and amino acid substitutions in the protein.

Effect of su_{3-1} *on wild-type:* Like td^+su_{1-2} TSase, td^+su_{3-1} enzyme was indistinguishable from that of a td^+su^+ control with respect to several enzymological and physical criteria (Table 3), indicating that neither td_{201} suppressor gene had a detectable effect on the wild-type enzyme.

On either minimal or complete medium (YOURNO and SUSKIND 1964b), all td^+su_1 strains exhibited a slight growth depression relative to $td^+su^+_1$ controls. td^+su_{3-1} showed no growth inhibition on either medium.

On the basis of the similar enzymatic properties of both types of suppressed td_{201} mutants, the site-specificity of the suppressor genes and the lack of gross effects of both suppressors on the wild type, it is likely that despite their non-

allelism these suppressors of missense mutant td_{201} function by a similar mechanism. These observations can be readily fitted into the mistake mechanism hypothesis of suppressor action, whether at the level of the adaptor system (YANOFSKY and ST. LAWRENCE 1959; YANOFSKY, HELINSKI, and MALING 1961; CAPECCHI and GUSSIN 1965; BRODY and YANOFSKY 1965), or at the level of the ribosome (GORINI and KATAJA 1964; DAVIES, GILBERT, and GORINI 1964). It would seem on the basis of suppressor nonallelism, however, that different components of the adaptor system or ribosome are affected in each case.

The one difference noted between $td_{201}su_{3-1}$ and $td_{201}su_1$ tryptophan synthetase was the lower ratio of wild-type to mutant enzyme activity in the $td_{201}su_{3-1}$ suppressed mutant (ca. 60% that of the $td_{201}su_{1-2}$ strain). The lower ratio in $td_{201}su_{3-1}$ strains could reflect a less efficient mechanism of amino acid substitution or the substitution by a less functional amino acid. This may correlate with the significantly lower growth rate of the $td_{201}su_{3-1}$ suppressed mutant on minimal medium, where the tryptophan synthetase level in growth-limiting. STADLER and YANOFSKY (1959), however, examined a series of suppressed TSase A-protein mutants of *E. coli* and found no correlation of minimal medium growth rates with levels of active enzyme.

SUMMARY

Two site-specific nonallelic suppressors of tryptophan synthetase mutant td_{201}—su_1 on linkage group VII, and su_3 which segregates independently—affect the formation of low levels of tryptophan synthetase that is similar to wild-type enzyme. No other gross effects are noted in either td_{201} or the wild-type strain. The lower TSase specific activity of $td_{201}su_{3-1}$ relative to $td_{201}su_1$ may be correlated with the slower growth rate of the former on unsupplemented medium.

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