THE SIMILAR PROPERTIES OF TRYPTOPHAN SYNTHETASE AND A MUTATIONALLY ALTERED ENZYME IN *NEUROSPORA CRASSA**

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SUMMARY

A method has been developed for the stabilization and further purification of tryptophan synthetase from *N. crassa*. The sedimentation constant, diffusion coefficient and approximate molecular weight of the enzyme from strain C-84 have been determined. A marked similarity in physical properties was found between the enzyme and an antigenically related protein in one tryptophan-requiring mutant. These properties include behavior during purification, elution pattern during DEAE cellulose column chromatography, and sedimentation velocity constants. The results support the hypothesis that the antigenically related proteins in the td mutants are in fact genetically altered tryptophan synthetase molecules.

INTRODUCTION

Certain mutant strains of *Neurospora crassa* (td mutants), which require tryptophan for growth and which lack the enzymic ability to synthesize L-tryptophan from L-serine and indole, contain proteins (CRM) which are antigenically very similar to wild-type Tsase[1-3]. Since these mutations represent distinct genetic alterations[4-6], a comparison of the properties and ultimately the structure of the normal enzyme and the CRM proteins offers one approach to understanding the types of changes in a protein molecule which can occur as a consequence of mutations within a single genetic locus.

Studies with Tsase from *N. crassa* have been severely handicapped in the past by the lability of the enzyme, limiting the degree of purification which could be attained. This is a report of the stabilization of Tsase and the subsequent purification of the enzyme and a CRM from one td mutant. The proteins have been compared in several of their physical properties and the results strongly support the contention that the CRM proteins represent genetically altered tryptophan synthetase molecules.

Abbreviations: Tsase, tryptophan synthetase; TCA, trichloroacetic acid; PALP, pyridoxal phosphate; EDTA, ethylenediaminetetraacetate; DEAE, diethylaminoethylcellulose; GSH, reduced glutathione.

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MATERIALS AND METHODS

Preparation of crude extracts

Tryptophan synthetase was obtained from *N. crassa*, strain C-84 (see ref. 7), a histidine mutant, grown on minimal medium[8] supplemented with L-histidine (53 mg/l). The enzyme from C-84 resembles Tsase obtained from several wild-type strains (74A, 5297, and 5256A) in those properties which have been examined, viz. stability and antigenic activity.

CRM was obtained from *N. crassa*, strain td_2^9, a tryptophan-requiring mutant grown on minimal medium supplemented with DL-tryptophan (150 mg/l). Both strains were grown in 10 l carboys with vigorous aeration for 50 to 80 h, harvested by filtration through cheesecloth, washed with distilled water, promptly frozen, and lyophilized. Dried mycelia were powdered with a mortar and pestle and the crude extracts were prepared by stirring the powder into cold potassium phosphate buffer (0.1 *M*, pH 7.8). After standing for 20 min at 2–5° the slurry was centrifuged at 15,000 × *g* for 20 min and the supernatant solution, containing the enzyme, was decanted.

Tryptophan synthetase assay

Tryptophan synthetase activity was measured by following indole disappearance colorimetrically, using the *p*-dimethylaminobenzaldehyde reaction[10]. Tryptophan formation by purified enzyme preparations was verified by chromatography in a butanol–acetic acid–water system using a Precision Chromatofuge. One enzyme unit (TSU) is defined as that amount of enzyme which will catalyze the conversion of 0.1 μmole of indole to tryptophan during a 60-min incubation period at 37°. The reaction mixtures contained 0.1 *M* K_2HPO_4, pH 7.8, indole (0.5 μmole), DL-serine (80 μmoles), and pyridoxal phosphate (0.96 μmoles), with ethylenediaminetetraacetate (EDTA, 2 μmoles) added to protect the enzyme.

CRM assay

CRM activity was measured using rabbit anti-CRM serum[3]. A highly specific anti-CRM was prepared by injecting rabbits with a fractionated preparation of td_2-CRM which had been further purified by precipitation of non-CRM proteins with rabbit anti-td_1 serum[3] (strain td_1 contains neither Tsase nor CRM and is similar to mutant td_2 at loci other than the td locus[10]). Agar diffusion data indicate that the protein remaining after precipitation of td_2-CRM preparations with anti-td_1 is capable of inducing the formation of antisera which contain anti-CRM as the major component[11]. Samples containing CRM in 0.1 *M* potassium phosphate buffer, pH 7.8 were pre-incubated with antiserum for 15 min at 0°. The quantity of uncombined anti-CRM remaining after pre-incubation was determined by the Tsase-inhibition reaction[3]. One unit of CRM is defined as the amount of CRM which will combine with that quantity of anti-CRM necessary to inhibit one tryptophan synthetase unit. It has been previously shown that the reaction of anti-CRM with CRM and Tsase is complete and irreversible under the conditions of the assay[3]. All CRM values are based on quantitative absorption curves such as the one shown in Fig. 1.

Protein assay

Specific activities of Tsase and CRM were calculated as tryptophan synthetase

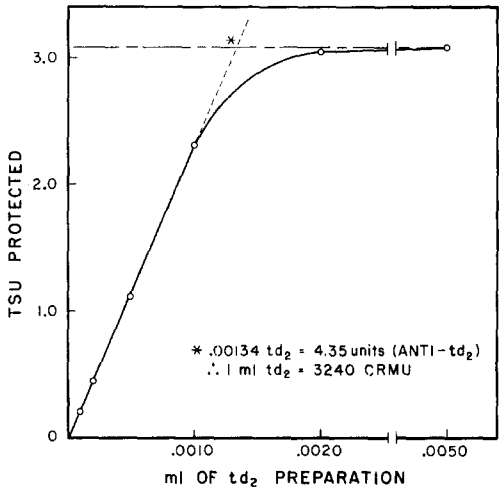

Fig. 1. CRM assay. CRM diluted with 0.1 *M* K-PO_4, pH 7.8, containing BSA 1.0 mg/ml was added to tubes containing 4.35 units of anti-td_2 in 0.15 ml. The mixture was incubated for 5 min at 37° and recooled to 3°. 5.50 units of crude C-84 Tsase in 0.10 ml was added with sufficient buffer to bring tubes to 0.90 ml. After 15 min, the substrate–coenzyme mixture was added in 0.75 ml, and residual enzymic activity assayed. Control enzyme–antibody tubes contained 1.85 TSU, indicating that they were in the non linear portion of the enzyme–antibody curve. This accounts for the difference between maximum TSU Protected (3.08) and Anti-td_2 added (4.35). CRM activity per/ml was calculated by extrapolating the linear portion of the curve to 3.08 TSU, the value at which all anti-CRM was absorbed.

units (TSU) and CRM units (CRMU)/mg protein. Protein was measured colorimetrically by the method of LOWRY *et al.*[12], spectrophotometrically by the ratio of absorption at 260 mμ and 280 mμ[13], or turbidometrically by the absorption at 600 mμ after the addition of TCA to a final concentration of 3% (see ref. 14).

Stability assays

The stability of Tsase at 2–5° was studied by storing aliquots of a given preparation in stoppered test tubes. At intervals duplicate tubes were removed and the reaction mixture for the Tsase assay was added directly to these tubes. In other experiments 1-ml aliquots of Tsase were dialyzed in the cold. Individual sacs were removed at intervals and duplicate 0.15-ml samples were assayed for residual enzymic activity.

Heat stability was measured by immersing test tubes containing aliquots of Tsase or CRM in a water bath at the desired temperature. The Tsase reaction mixture was added directly to the tubes after they were cooled to 0°.

Purification of Tsase and CRM

A method of purification was developed for Tsase and applied without modification to td_2-CRM. All operations were performed in a cold room, refrigerated centrifuge or ice bath at temperatures below 3°. Low temperature is essential for the preservation of maximal activity during the first two steps of purification. All solutions used in purification as well as in assays were made up in glass-distilled water. Low conductivity water was used since it is known that Tsase is inhibited by certain trace metals[10].

During a typical purification 768 ml of crude extract was obtained from 50 g of powdered mycelia extracted in 800 ml of phosphate buffer. *Step I*: The pH was adjusted from 7.4 to 6.9 with 17 ml of 1 *N* acetic acid, and 93 ml (14 ml /100 ml extract) of a 1.5 % protamine sulfate solution was added rapidly drop by drop with stirring over a 15-min period. After standing for 15 min the samples were centrifuged at 15,000 × *g* for 20 min.

Step II: A supernatant solution of 790 ml was obtained and the pH was adjusted from 6.9 to 5.7 with 1 *N* acetic acid. Saturated ammonium sulfate solution (3°) was added dropwise over a 40-min period to give 30 % saturation. Small amounts of a 10 % (v/v) ammonium hydroxide solution were added to maintain the pH at 5.60 ± 0.05 during the ammonium sulfate addition. The final mixture was stirred gently for 25 min and centrifuged at 15,000 × *g* for 20 min. The supernatant solution was discarded and the precipitate taken up to a volume of 50 ml in a mixture containing 0.1 *M* potassium phosphate buffer (K-PO_4) pH 7.8, $1 \cdot 10^{-4}$ *M* PALP and $1 \cdot 10^{-2}$ *M* EDTA.

Step III: To this slightly turbid solution were added 8 ml of 0.2 *M* DL-serine, 15 ml of $4 \cdot 10^{-3}$ *M* PALP, 15 ml of 0.1 *M* EDTA, 40 ml of 0.5 *M* K-PO_4 buffer pH 7.0, and 22 ml of 0.1 *M* K-PO_4, pH 7.8. The resulting 150 ml were brought to 54° over a 15-min period and then maintained at 54° for 5 min. After cooling in an ice bath, the heavy precipitate was centrifuged for 20 min at 15,000 × *g*. The resulting 142 ml of clear supernatant solution was first dialyzed for 5 h at 3° against 1500 ml of glass-distilled water containing $1 \cdot 10^{-5}$ *M* PALP and $1 \cdot 10^{-3}$ *M* EDTA, followed by a 10-h dialysis against 1500 ml of 0.01 *M* K-PO_4, pH 7.8 containing the same concentrations of PALP and EDTA.

Step IV: To the dialyzed material were added 5 ml of $4 \cdot 10^{-3}$ *M* PALP and 3 ml of 0.1 *M* EDTA. The pH was adjusted to 5.8 with 1 *N* acetic acid. Saturated ammonium sulfate was added dropwise to give 25 % saturation. (pH was maintained at 5.8 with NH_4OH as in *step II*). The slightly turbid mixture was stirred gently for 15 min and centrifuged at 15,000 × *g* for 20 min. The precipitate was discarded. The pH was adjusted to 5.0 with 1 *N* acetic acid and saturated ammonium sulfate was added to give 31 % saturation. After gentle stirring for 5 min, the mixture was centrifuged for 20 min at 15,000 × *g* and the precipitate taken up in 0.1 *M* K-PO_4, pH 7.8 containing $1 \cdot 10^{-4}$ *M* PALP and $1 \cdot 10^{-2}$ *M* EDTA. The sample was dialyzed for successive 6- and 12-h periods against 0.01 *M* K-PO_4, pH 7.8 containing $1 \cdot 10^{-5}$ *M* PALP and $1 \cdot 10^{-3}$ *M* EDTA.

Step V: Details of the preparative fractionation on a DEAE cellulose column are similar to those given below for analytical chromatography. However, for preparative work, a 1.8 · 10 cm column was used and elution was effected rapidly within a 4 to 5 h period under a pressure of approximately 6 pounds/in^2. A non-linear gradient was obtained by adding 100 ml of mixture "2" to a constant volume mixing chamber initially containing 80 ml of mixture "1" (see below).

Analytical chromatography

Commercial DEAE cellulose was repeatedly washed with 0.3 *M* KOH and distilled water, followed by 0.3 *M* KH_2PO_4 and distilled water. After adjusting the pH to 7.8, with 0.3 *M* KOH it was washed with several liters of 0.01 *M* K-PO_4 pH 7.8 containing $1 \cdot 10^{-3}$ *M* EDTA and suspended in this buffer 2 % (w/v). Columns

(0.8 × 10 cm) were filled with the suspension, allowed to settle under gravity, packed briefly under a pressure of 3 pounds/in^2 and decanted. The final column was washed with 20 to 40 ml of mixture "1" (0.01 M K_2HPO_4, pH 7.8 with $1 \cdot 10^{-5}$ M PALP and $1 \cdot 10^{-3}$ M EDTA). The samples of Tsase or CRM which had been dialyzed against mixture "1" were put on the column under a pressure of 2 pounds/in^2. A linear gradient was developed by adding 60 ml of mixture "2" (0.5 M K_2HPO_4, pH 7.8, with $1 \cdot 10^{-4}$ M PALP and $1 \cdot 10^{-2}$ M EDTA) to a mixing chamber containing an equal volume of mixture "1" in such a way that the volumes of the two chambers remained approximately equal throughout. Elution took place over 12 to 15 h at a rate of 8–12 ml/h.

Sedimentation velocity

Sedimentation velocities of Tsase and CRM were determined either by measuring the decrease in enzymic or antigenic activity in the separation cell of TISELIUS[15] or by conventional optical technics, employing a Spinco Model E Ultracentrifuge. Calculations of sedimentation velocity in the separation cell were made assuming stable sedimentation with a well defined boundary[16]. This assumption was confirmed by the experimental results. All sedimentations were made in 0.1 M K-PO_4 pH 7.8 with $1 \cdot 10^{-4}$ M PALP and $1 \cdot 10^{-3}$ M EDTA.

Diffusion coefficient

The diffusion coefficient for Tsase was measured by the diaphragm-cell method of NORTHROP AND ANSON[18,19]. Two cells (volumes 4.42 and 5.19 ml) were run simultaneously. They were calibrated immediately prior to this experiment with 1 N HCl assuming a diffusion coefficient of 1.54 cm^2/day at 4° (see ref. 20). Partially purified Tsase (1016 TSU/ml and 5.7 mg protein/ml) in 0.1 M K_2HPO_4 buffer, pH 7.8 containing $1 \cdot 10^{-4}$ M PALP, $1 \cdot 10^{-3}$ M EDTA and $1 \cdot 10^{-4}$ M serine was dialyzed twice against the same buffer-stabilizer solution. An identical buffer-stabilizer solution was used for the external bath (20 ml) into which Tsase diffused. Bovine serum albumin (BSA-100 μg/ml) was added to the bath to protect the purified enzyme against inactivation during dilution. The effect of BSA can be seen in Table I. Tsase assays were performed in quadruplicate or sextuplicate.

In order to confirm the stability of Tsase during the diffusion process (83 h)

TABLE I

LOSS OF ACTIVITY ON DILUTION IN ABSENCE OF BOVINE SERUM ALBUMIN

The table gives the apparent activity (TSU/ml) of a partially purified preparation of C-84 Tsase (specific activity 280 TSU/mg protein) when various buffer mixtures were used to dilute the sample in preparation for assay. The three diluents shown are: (a) 0.1 M K-PO_4, pH 7.8, alone, (b) K-PO_4 with $1 \cdot 10^{-4}$ M PALP and $1 \cdot 10^{-2}$ M EDTA, and (c) K-PO_4 with BSA 1.0 mg/ml. The assays themselves were carried out in the presence of PALP, EDTA and BSA. Other experiments showed that washing of glassware with 5 N nitric acid did not prevent loss of activity. Gelatin but not protamine sulfate or heparin will prevent loss. After initial loss during dilution, the activity remained stable in K-PO_4, EDTA, and PALP for 170 h at 3°.

	KPO_4	*TSU in KPO_4, PALP, EDTA*	*KPO_4, BSA*
Dilution 1:10	680	680	800
Dilution 1:100	470	470	800

and to average out errors due to fluctuations in the temperature of the Tsase assay (approx. 6 %/ degree at 37°), each diffusate sample was assayed 3 times at daily intervals. Although slight variations were observed in each set of three assays, there was no evidence for enzymic inactivation. Therefore, each set of three values was combined and the average value used. All of the initial enzymic activity was quantitatively recovered from the baths and the cells at the end of the experiment.

RESULTS

Stabilization of Tsase

YANOFSKY[10] reported that partial stabilization of Tsase at 5° could be achieved by the addition of GSH and PALP. More than 90 % of the activity of crude enzyme preparations is preserved at 3° for 160 h by adding PALP, DL-serine and EDTA. Tsase from four different strains of *N. crassa* (5256A, 5297a, 74A and C-84) were found to behave similarly in this respect. Virtually complete stabilization of the partially purified Tsase from C-84 is found under the same conditions for 215 h. PALP, EDTA and serine each increase the stability of Tsase in crude extracts when used separately, but the rate of inactivation of crude enzyme and the degree of protection afforded by each compound alone is somewhat different for the four strains (Table II).

TABLE II

A COMPARISON OF THE STABILITIES OF TSASE FROM SEVERAL STRAINS OF *N. crassa*

Crude enzyme was diluted with 0.1 *M* K-PO_4, pH 7.8 to approx. 10 TSU/ml and 0.40-ml aliquots placed in tubes with additives and K-PO_4 to make 0.55 ml. The tubes were stored covered with Saran wrap at 3°. Duplicate tubes were assayed at 0, 4, 18, 72 and 140 h. The values listed in the table are the percent of 0 h control activity remaining at 100 h as read from the plotted curves.

Buffer mixture	*Strain*			
	5256A	*5297a*	*74A*	*C-84*
(1) 0.1 *M* K-PO_4, pH 7.8	32 %	40 %	—	13 %
(2) (1) + 0.01 *M* EDTA	50	42	37	58
(3) (1) + 4×10^{-4} *M* PALP	70	87	76	74
(4) (1) + 0.02 *M* DL-serine	49	55	27	47
(5) (3) + (4)	98	91	88	92
(6) (2) + (3)	91	87	85	95
(7) (2) + (3) + (4)	99	92	92	95

At temperatures above 50° neither PALP nor EDTA significantly alter the rate of inactivation of partially purified Tsase. L-serine or D-serine is required in addition to PALP to protect against inactivation at 55–56° for 30 to 40 min. Partially purified td_2-CRM is also stabilized at 55° by PALP and serine. Alanine and glycine have some stabilizing effect on the enzyme, while phenylalanine, cysteine, threonine, aspartic acid, and tyrosine are of little or no value. Potassium propionate and acetate decrease heat stability as does pyruvate even in the presence of pyridoxamine phosphate.

Pyridoxal phosphate is specifically required to stabilize the enzyme both at low temperatures and above 50°. Pyridoxine, pyridoxal, pyridoxamine and pyridoxamine phosphate failed to protect. They have also been shown to be ineffective as coenzymes for Tsase activity[10].

Although phosphate buffer has been used exclusively in the work reported here, the stability of Tsase in glycylglycine, veronal, potassium maleate and Tris-HCl (0.1 *M*, pH 7.8) was examined. Enzymic activity is lost in Tris-HCl (50% in 17 h during dialysis) and Tris itself inhibits the enzymic assay. Tris may compete with the enzyme for PALP, since Tris-PALP mixtures develop an intense yellow color with a spectrum similar to PALP-serine in the 350 to 450 mμ range. The activity in glycylglycine is also somewhat unstable (25% loss in 160 h). In veronal or maleate the enzyme was stable for 160 h but the assay itself is slightly inhibited by these buffers.

Purification of Tsase and CRM

Results of the purification of Tsase and CRM are shown in Table III and Fig. 2. It is evident that the method developed for Tsase is effective for purifying CRM. Step III is possible because PALP and DL-serine stabilize partially purified Tsase at 55° while considerable denaturation of other proteins occurs. Heat treatment of

TABLE III

PURIFICATION OF C-84 TRYPTOPHAN SYNTHETASE AND CRM FROM MUTANT td_2

	C-84 Tsase			*td_2-CRM*		
	Total activity	*Specific activity**	*% recovery*	*Total activity*	*Specific activity**	*% recovery*
Crude enzyme	38,000 TSU	3.2	100	40,300 CRMU	4.5	(100)%
Step I						
Protamine sulphate	36,000	8.1	95	44,000	7.5	100%
Step II						
Ammonium sulphate pH 5.6	23,000	53	61	33,000	53	75
Step III						
Heat	20,700	190	55	32,200	230	73
Step IV						
Ammonium sulphate pH 5.0	17,000	430	45	23,000	360	52
DEAE column**	9,750	880	26	13,000	630	30

* Specific activity: units of Tsase or CRM/mg of protein as measured by absorption at 260 mμ and 280 mμ.

** These figures apply to a pool of the three fractions from each column which contained the highest specific activity. Considerably higher % recovery was obtained from the total of all column fractions: viz., 13,000 TSU for the Tsase column and 17,000 CRMU for the CRM column. Furthermore, the peak specific activity for each column is higher than this average.

crude extracts has not proved to be a satisfactory purification step. The conditions for the two ammonium sulfate fractionations are based on data derived from the precipitation of Tsase at different pHs. (Fig. 3). Although maximum total activity is precipitated in Step II at pH 5.9, the specific activity of the enzyme recovered by pH 5.6 precipitation is higher.

Some purification can also be achieved by absorption on alumina gel at pH 5.8 and elution at higher pH and ionic strength. Purification by absorption and elution from Ca-PO_4 gel has been reported[10].

No gradients other than ionic strength have been employed for the DEAE column purification step.

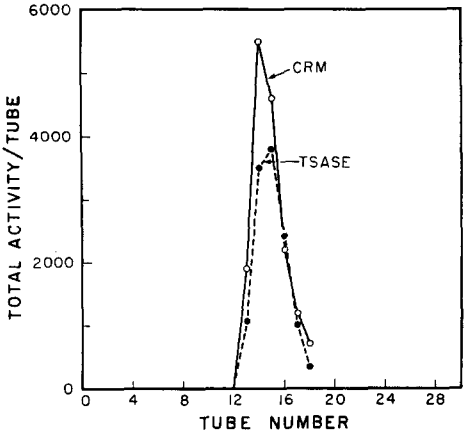

Fig. 2. Elution patterns for preparative DEAE cellulose columns. The figure shows the remarkable similarity between the elution patterns for Tsase and CRM during the purification of each on separate preparative DEAE columns. Only tubes 12 to 18 were assayed in detail. All tubes were given a preliminary screening which would have detected 100 units per tube and no evidence was obtained for a second peak of activity. See text for details of method.

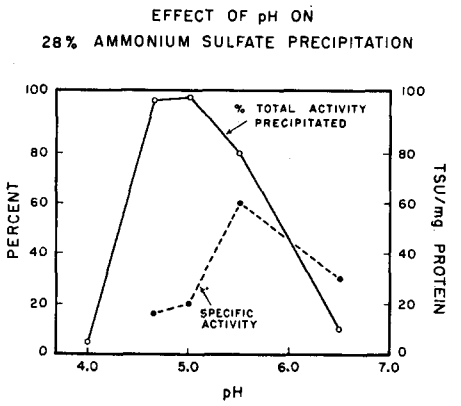

Fig. 3. Precipitation of C-84 Tsase with ammonium sulfate. Crude C-84 which had been treated with protamine sulfate (step II) was divided into 25-ml aliquots containing 1000 TSU. The pH was adjusted with 1 *N* acetic acid at 3°. Saturated ammonium sulfate was added dropwise to a final saturation of 28% while maintaining the pH within 0.05 units variation. The precipitate was centrifuged at 15,000 $\times g$ for 20 min and resuspended in 0.1 *M* $K\text{-}PO_4$, pH 7.8 with $1 \cdot 10^{-4}$ *M* PALP and $1 \cdot 10^{-2}$ *M* EDTA. Protein assays were done by the method of LOWRY[12] at dilutions where the PALP did not give a significant color.

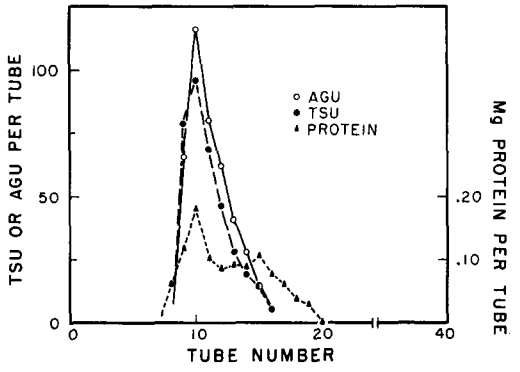

Fig. 4. Elution patterns for a mixture of Tsase and CRM from analytical DEAE cellulose column. A mixture containing 810 enzymic units (TSU) and 700 CRMU (total = 1180 Antigenic units* (AGU)) and 4.80 mg protein in 8.0 ml was chromatographed by methods detailed in the text. In tubes 8 through 20, 425 TSU, 427 AGU, and 1.06 mg protein were recovered, with less than 1.0 TSU or AGU or 0.02 mg protein in any of the other tubes. Tube volumes were 3.95 ± 0.05 ml. Tubes 39 and 40 were eluates from washing the column with mixture "2" undiluted, in an effort to remove any residual protein.

* The enzyme–antigen activity ratio greater than 1.00 which was found in tube 9 (E/A 1.21) may reflect the high ratio (E/A 1.69) of the purified Tsase preparation used in the original mixture. Such high ratios have been seen frequently and may simply indicate a lower enzyme–antigen ratio in the Tsase preparation used to standardize the anti-CRM serum.

Analytical chromatography

Because of the striking similarity of the individual elution patterns of CRM and Tsase on preparative DEAE columns, it was of interest to determine the behavior of a mixture of the two proteins. Three experiments with different batches of material gave similar results. The data from one experiment are shown in Fig. 4. It is clear that enzymic activity (TSU) and antigenic activity (AGU), which measures the ability of both Tsase and CRM to combine with anti-CRM, present a very similar elution pattern. If they were identical, however, the ratio of AGU to TSU should be the same for all eluate fractions and this was not observed. The early samples have a higher TSU/AGU ratio than would be expected from identical elution patterns. This could be explained if Tsase was eluted off the column earlier than CRM, although other explanations are possible.

In many preparative columns with Tsase or CRM the recovery of activity has always been less than 90 % (usually 70–80 %). The cross-mixing of inactive fractions from the column has failed to demonstrate any separation of materials which can be reconstituted to yield active enzyme. In addition, if two components were being separated by the column procedure, one might expect to find an increase in activity when mixing early and late eluate samples. No increase has been found (Table IV).

TABLE IV

RESULTS OF MIXTURES OF COLUMN ELUATE FRACTIONS

Assays contained 0.10 ml from each of the two tubes in a given mixture. Assays performed in duplicate. For details, see text.

	TSU		*AGU*	
	Observed	*Calculated*	*Observed*	*Calculated*
Tubes 8 + 11	21.0	20.9	21.4	22.3
Tubes 9 + 12	31.8	31.7	33.5	32.2

Sedimentation velocity studies

Sedimentation velocity studies on Tsase by the separation-cell technic gave an S_{20} for Tsase of approx. 6.4. The value for CRM by the separation-cell method was higher, approx. 6.9. These data are shown in Fig. 5.

An S_{20} of 6.0 was obtained for the major peak in a purified preparation of Tsase when optical methods were employed. The relative area of this peak in different preparations appeared proportional to the specific activity of the enzyme. The less well-defined, smaller maximum has an S_{20} of approximately 1.8. The S_{20} for CRM based on optical measurements was 6.7. In order to compare the sedimentation velocities of CRM and Tsase under identical conditions, the sedimentation of a mixture of approximately equal amounts of purified CRM and Tsase was examined optically. The sedimentation pattern of the mixture was quite similar to that of Tsase alone and the major peak had an S_{20} of about 5.9. There is a slight widening of this peak, which is compatible with a difference of about 10 % (5–15 %) in the sedimentation velocities of CRM and TSase. This is in agreement with the difference observed when Tsase and CRM were compared separately. The sedimentation patterns for Tsase, CRM and the Tsase-CRM mixture are shown in Fig. 6.

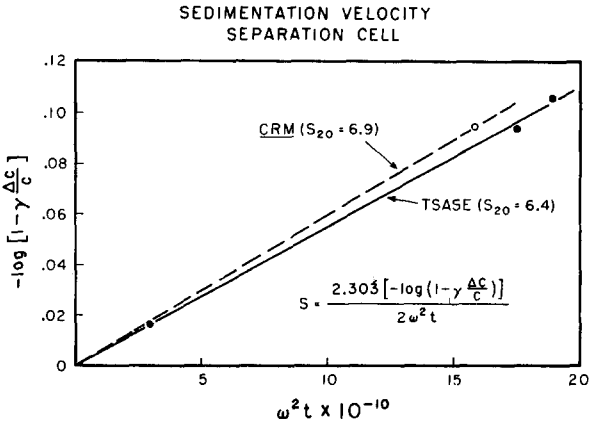

Fig. 5. This figure plots data corrected to 20°. The viscosity of the solvent has been assumed to be that of pure water. For details of method see text[16].

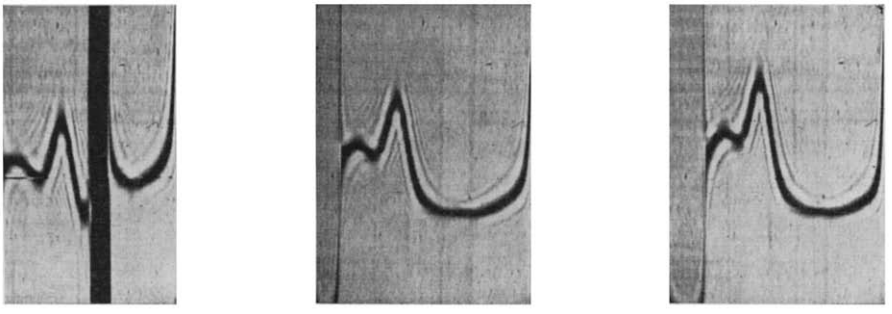

Fig. 6. Optical patterns of sedimentation velocity studies. A. CRM, 2600 CRMU and 3.6 mg protein/ml. Separation cell. 118 min, 42,040 rev./min., 4°, diaphragm 25°. B. Tsase, 4600 TSU and 6.6 mg protein/ml; 116 min, 42,040 rev./min, 6°, diaphragm 25°. C. Mixed Tsase and CRM-equal volumes of A and B mixed; 116 min, 42,040 rev./min., 8°, diaphragm 25°.

Diffusion coefficient

The diffusion coefficient for Tsase at 4° in the two cells was 0.0230 and 0.0211 cm^2/day as calculated from six successive 8-h periods after a constant rate of diffusion had been reached. The difference between the results of the two cells is statistically significant, representing some systematic error. Average values for the diffusion coefficient corrected to 20° are D = 0.0365 cm^2/day or $4.22 \cdot 10^{-7}$ cm^2sec.

Molecular weight of Tsase

Calculated from $S_{20} = 6.4 \cdot 10^{-13}$ sec and D_{20} $4.2 \cdot 10^{-7}$ cm^2/sec obtained on the same preparation, the molecular weight of Tsase is approx. 140,000. The errors from the data themselves place a 95 % confidence limit of ± 12 % on this figure. However, since the choice of a partial molar volume (0.74) for the enzyme was of necessity arbitrary, and since the values for the sedimentation constant obtained by optical and separation-cell methods differ slightly, this molecular weight estimate of Tsase must be considered to have confidence limits of 15–20 %.

DISCUSSION

Current studies on the genetic control of protein formation in microorganisms and higher organisms clearly indicate that certain gene mutations can elicit the formation of structurally aberrant proteins which are, in fact, responsible for the mutant phenotype of the organism.

In the case of many of the tryptophan-requiring mutants of *N. crassa*, the metabolic block, resulting from an inability to form normal tryptophan synthetase, is coupled with the appearance of an inactive, antigenically related protein, CRM. It is known that the CRM from mutant td_2 has retained part of the enzymic activity of Tsase, viz., it will catalyze the conversion of indole glycerol phosphate to indole and triose phosphate, but it is unable to catalyze tryptophan synthesis from indole and serine[21–25]. CRM proteins from other td mutants appear to have their own characteristic enzymic properties, yet they all lack normal catalytic activity[24,26]. Thus it appears that localized, specific damage to a protein can occur as a consequence of particular site mutations within a gene locus.

Evidence has been sought, without success, for inactive *Neurospora* DEAE-protein fractions which can be used to reconstitute an active tryptophan synthetase. RACHMELER AND YANOFSKY[25], and WUST, DEMOSS AND BONNER[32] have also been unable to separate the *Neurospora* Tsase into two or more components. These findings differ significantly from the extensive studies on the tryptophan synthetase of *Escherichia coli*[27–31], in which the enzyme can be separated into two components, A and B, both of which are required for full enzymic activity.

The present degree of purity of *Neurospora* Tsase, based on sedimentation patterns, appears to be about 60 % in preparations having a specific activity of 700. It should be possible to obtain further purification of Tsase and the CRM proteins by combining protein fractionation and immunochemical procedures. A molecular weight of about 140,000 is large for convenient structural analysis, but it may be possible to fragment the protein to a more suitable size by enzymic digestion. In conclusion, the results of these experiments indicate a marked similarity in physical properties between Tsase from strain C-84 and CRM from mutant strain td_2. These include (a) a similar behavior in the purification procedure, (b) similar if not identical DEAE-elution patterns for enzymic and antigenic activities in a mixture of CRM and Tsase, and (c) sedimentation velocity constants which are the same within experimental error (approx. 10 %). These findings, in addition to the antigenic similarity between CRM and Tsase[3], strongly support the view that the td_2-CRM protein represents genetically altered tryptophan synthetase. The close similarity between these two proteins does not imply that the antigenically related proteins in other td mutants of *N. crassa* resemble Tsase to this degree. In fact, in at least one case, the mutation has been of sufficient magnitude to prevent recognition of the protein by any of the presently available means[1,3]. In other td mutants, as mentioned earlier, specific, characteristically modified CRM proteins are formed[24,26]. Consequently, it seems most likely that a variety of mutational changes can occur within the td locus, and that the ultimate configuration and properties of the enzyme tryptophan synthetase reflect these changes.

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