*THE ENZYMATIC ACTIVITY ASSOCIATED WITH THE PROTEIN IMMUNOLOGICALLY RELATED TO TRYPTOPHAN SYNTHETASE**

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It has been well established in a variety of organisms that gene mutations affect the formation of specific enzymes. In view of the complexity of the gene in terms of numbers of distinct mutational sites,[1] it might be expected that different changes within any one gene would result in different alterations of a specific enzyme or protein. Some enzyme alterations have, in fact, been associated with gene changes,[2–5] and, in one case,[6] a single amino acid substitution has been shown to distinguish the mutant from the normal form of a protein. These instances strengthen the view that the amino acid sequence of each protein may reflect the arrangement of the subunits of its determining gene.

In *Neurospora*, certain mutants defective in the ability to form the enzyme tryptophan synthetase (TSase) are distinguishable from one another on the basis of analyses of the following types: growth in the absence of tryptophan, accumulation of intermediates, reversion frequency, response or lack of response to specific suppressor genes, enzyme activity measurements, and immunological comparisons.[7–11] Since these strains represent mutations of the same gene and are distinguishable from one another, it may be assumed that in some cases the dissimilarities detected result from different alterations of TSase. In this regard the immunological findings are of particular interest because they demonstrate that certain of these mutants form a protein (designated CRM) which may be an inactive or incomplete form of TSase.[10] This protein is immunologically similar to TSase and is absent from at least one mutant which lacks TSase.[10,11] Whether the CRM's formed by distinguishable mutant types differ from one another has not been determined, but differences might be expected if the CRM's represent altered forms of TSase.

Studies with the analogous system in *Escherichia coli* have shown that mutants lacking TSase fall into the same two groups, viz., those which form a cross-reacting protein (CRM) and those which do not.[12] In *E. coli*, mutants in the latter group have two additional characteristics which distinguish them from strains which form CRM; they accumulate indoleglycerol instead of indole,[12, 13] and they lack an enzyme required for the conversion of indoleglycerol phosphate to indole.[14] The absence from these mutants of both CRM and an enzyme involved in the synthesis of indole suggested a second interpretation of the nature of CRM, viz., that it is an enzyme catalyzing an earlier step in tryptophan synthesis.[12] This report is concerned with a study of this possibility.

MATERIALS AND METHODS

The characteristics of the various mutant strains of *E. coli* employed in this

study are summarized in Table 1, in which the probable pathway of tryptophan synthesis in this organism is also indicated. With the exception of the stocks designated "B" (derived from the B strain of *E. coli*), the mutants listed were obtained from the K-12 strain of *E. coli*. Mutant T-3 was considered equivalent to the wild type for the purposes of this study. It forms large amounts of TSase and the other enzymes involved in tryptophan synthesis when grown on low levels of anthranilic acid.[14]

TABLE 1

CHARACTERISTICS OF THE STRAINS EMPLOYED

STRAIN	GROWTH ON*				ACCUMULATION	ENZYME ACTIVITY ABSENT†
	0	0 + A	0 + In	0 + T		
Wild-type K-12	+	+	+	+	...	...
T-3	−	+	+	+	?	?
T-4, T-8	−	+	+	+	InG	A
T-41, T-84	−	−	−	+	InG	B, TSase
$td_{2,3,4,5}$, B-82	−	−	−	+	In	TSase
B/IT	−	−	−	+	?	A, B, TSase‡
B/IT-N	−	−	−	+	InG	A, B, TSase

→ anthranilic acid → indoleglycerol phosphate → indole → tryptophan

* 0 = unsupplemented minimal medium; A = anthranilic acid; In = indole; T = tryptophan; InG = indoleglycerol.
† A and B are enzyme components required for the conversion of indoleglycerol phosphate to indole.
‡ Also lacks the enzymes involved in the conversion of anthranilic acid to indoleglycerol phosphate.

Tryptophan Synthetase Assay.—TSase activity (the coupling of indole and serine to form tryptophan) was determined as described previously,[15] with the following minor changes: (1) tris buffer, pH 7.8, was substituted for phosphate buffer, and (2) 0.03 ml. of a saturated solution of sodium chloride was added per milliliter of incubation mixture. Under these conditions the activity of the TSase of *E. coli* is approximately double that which is observed in the presence of phosphate buffer alone. Immunological tests indicate that this increase is not accompanied by an increase in TSase antigen. One unit of TSase activity is defined as the amount of enzyme which will convert 0.1 μmole of indole to tryptophan in 20 minutes at 37° C. in an incubation mixture containing tris buffer, sodium chloride, pyridoxal phosphate, indole, and serine. Specific activity is expressed as units of TSase per milligram protein. Protein was determined by the method of Lowry *et al.*[16]

Component B Assay.—As will be shown subsequently, two enzyme components are required for the conversion of indoleglycerol phosphate to indole. One of these has been arbitrarily designated component A, and the second, component B. Either activity can be determined in the presence of an excess of the other. The incubation mixture employed in assaying component B activity contained 0.15 μmoles of indoleglycerol phosphate; 0.1 ml. of 0.5 *M* phosphate buffer at pH 7.0; 0.05 ml. of 5×10^{-2} *M* NH_2OH; and a suitable excess of partially purified component A in a final volume of 0.5 ml. Reaction mixtures were incubated for 20 minutes at 37°, 0.1 ml. of 1 *N* sodium hydroxide was then added to stop the reaction, and the indole formed was subsequently extracted with toluene and determined colorimetrically.[15] One unit of component B activity is defined as the amount of enzyme which will form 0.1 μmole of indole from indoleglycerol phosphate in 20 minutes at 37° under the foregoing conditions. Specific activity is expressed as units of B per milligram protein.

Preparation of Antisera.—Antibodies were produced in rabbits in response to the injection of partially purified preparations of TSase or CRM.[12] The antisera were heated for 30 minutes at 56°, fractionated with ammonium sulfate, and then dialyzed before use. In some cases, antisera were repeatedly treated with portions of extracts of K-12 mutants (T-84 and T-41) lacking both CRM and TSase to remove antibodies to other *coli* antigens. The absorbed antisera were then heated, fractionated with ammonium sulfate, and dialyzed before use.

Neutralization of TSase.—Antibodies to TSase or to CRM are capable of completely neutralizing TSase activity.[12] Over a considerable portion of the neutralization curve (see Fig. 1), however, some of the enzyme is precipitated without being neutralized (from 0.08 to 0.2 ml. antiserum). It is also evident from Figure 1 that the amount of enzyme activity remaining in the supernatant solution decreases linearly as increasing amounts of antiserum are added. As previously reported,[12] 15 minutes is sufficient for an antiserum to exert its maximal inhibitory effect.

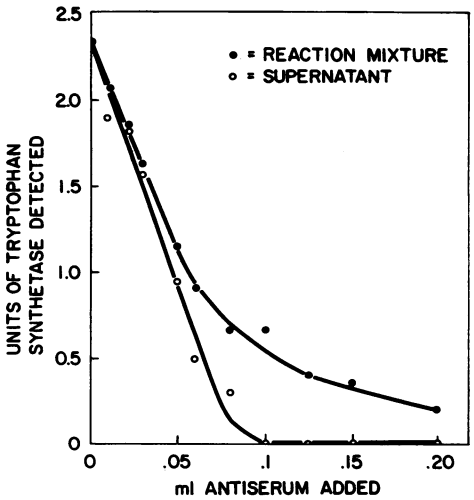


FIG. 1.—Neutralization and precipitation of TSase. Mixtures of TSase and antiserum (a 1–20 dilution) were incubated for 18 hours at 4° C. Aliquots of the reaction mixture and the supernatant solution obtained after centrifugation were then assayed for TSase activity.

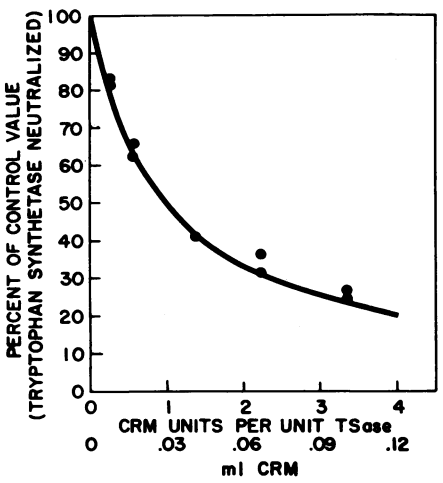


FIG. 2.—Titration of the CRM in a td extract. Values given are in percentages of the amount of TSase neutralized in control tubes. The solid line is the theoretical curve, and the points plotted were obtained experimentally with a 1–9 dilution of an extract of strain td_4.

Neutralization of Component B.—Antisera to TSase or to CRM, whether absorbed or not, are capable of precipitating component B. However, only a fraction of the B activity which is precipitated by antibody is neutralized.[17] In determinations of antibody–component B interactions, therefore, precipitates were removed by centrifugation (following 40-hour incubations), and only supernatant solutions were assayed for component B activity.

Immunological Assay of CRM.—Two methods were used for estimating the amount of CRM in various mutant extracts and preparations. In the first method, unknown amounts of CRM were preincubated with an excess of antibody, and, after a suitable period, the amount of antibody remaining unneutralized was determined.[12] In the second or "competition" method, aliquots of CRM solutions

were thoroughly mixed with a known amount of TSase prior to the addition of antibody. Antibody was then added, and the reduction in neutralization of TSase activity resulting from the presence of CRM was determined. In this assay procedure, which gave more reproducible results than the first method, one unit of CRM was defined as the amount of material which was antigenically equivalent to (i.e., would combine with as much antibody as) one unit of TSase. The theoretical curve based on this assumption is shown in Figure 2, as well as the experimental points obtained in a typical titration of the CRM in an extract of mutant td_4. Assays by the second method were reproducible to within 10–20 per cent, and this procedure was accordingly used in instances where a quantitative titration of CRM was desired. CRM specific activity is expressed in units of CRM per milligram protein.

RESULTS

In initial studies on formation of indole from indoleglycerol phosphate by mutant extracts it became apparent that at least two enzymes are required for this conversion. This is illustrated by the data in Table 2. Extracts of neither strain T-84 nor strain T-8 alone were capable of converting indoleglycerol phosphate to indole. A mixture of these two extracts, however, rapidly carried out this conversion. We have arbitrarily designated the active T-84 and T-8 components as A and B, respectively. It can also be seen from Table 2 that extracts of wild-type strains contain two distinct components, both of which are essential for the conversion of indoleglycerol phosphate to indole. One of these components, A (determined by mixing experiments with T-84 and T-8 extracts), remains in the supernatant solution when extracts are treated with acetic acid until precipitation just occurs. The second component, B, is more stable than A to heating at 60°.

TABLE 2

SEPARABILITY OF COMPONENTS A AND B

	EXTRACT	μG INDOLE FORMED*
1.	T-84 alone (A)	0
2.	T-8 alone (B)	0
3.	1 plus 2	12.1
4.	Acid supernatant (wild type)	0
5.	Heat supernatant (wild type)	1.2
6.	4 plus 5	8.8

* Under standard conditions employed in assaying component B.

The present study is concerned with the possibility that CRM is a participant in the conversion of indoleglycerol phosphate to indole. If CRM is involved in this conversion, *it must be component B and not component A, since some mutants which lack CRM—T-41* and T-84—*form component A.*

The B and CRM Content of Mutant Extracts.—To study the relationship between B and CRM, extracts of five mutants containing CRM but lacking TSase were assayed for component B and CRM levels. The results of these analyses are presented in Table 3. It can be seen that, with the exception of strain td_3, the B/CRM ratios observed were fairly constant. Approximately the same ratio was found in extracts of cells grown in the presence of high levels of tryptophan;

under these conditions the formation of both component B and CRM is inhibited. Strain td_3 consistently exhibits somewhat higher B/CRM ratios than the other td mutants. This observation and other data which will be presented elsewhere indicate that in extracts of mutant td_3 there is slightly more component B activity per unit of CRM than in other mutant extracts.

TABLE 3

B AND CRM CONTENT OF VARIOUS MUTANT EXTRACTS

EXTRACT	CRM SPECIFIC ACTIVITY	B SPECIFIC ACTIVITY	B/CRM × 100
td_2	71.5	1.7	2.4
td_2	87.5	2.15	2.5
td_2 (high tryp.)*	1.6	0.04	2.5
td_3	72	2.6	3.6
td_3	41.3	1.35	3.3
td_4	14	0.33	2.3
td	42	0.93	2.2
td_5	14	0.34	2.4
B-82	82	2.3	2.8

* Grown on a medium containing 200 μg L-tryptophan/ml.

Fractionation and Comparison of the Stability of B and CRM.—As a further test of the association of component B activity with CRM, fractionation experiments were performed. The results of a typical fractionation are summarized in Table 4. Here and in several similar experiments, all the fractions obtained (representing about an 80-fold spread in B-specific activity) showed approximately the same B/CRM ratio, suggesting that these two activities were inseparable under the various conditions employed.

TABLE 4

FRACTIONATION OF COMPONENT B AND CRM*

FRACTION	CRM/ML	B/ML	B SPECIFIC ACTIVITY	B/CRM ×100
Starting material	728	21	0.53	2.9
25% amm. sulfate	1,534	45	1.6	2.9
45% amm. sulfate	300	9	0.23	3
25%, 53°, 2 min.	1,391	43.5	2.2	3.1
20% amm. sulfate	2,200	75	10	3.4
40% amm. sulfate	770	22.5	2.4	2.9
20% calcium phosphate gel supernatant	1,100	34.5	16	3.1

* The starting material was treated with protamine sulfate, and the supernatant solution obtained after centrifugation was adjusted to pH 6.0. Solid ammonium sulfate was added to 25 per cent of saturation and the precipitate which formed was collected by centrifugation. The supernatant solution was fractionated further at 45 per cent of saturation. The 25 per cent fraction was heated at 53° for 2 minutes, and the precipitate was discarded. The supernatant solution was fractionated with ammonium sulfate at 20 per cent and 40 per cent of saturation. The 20 per cent fraction was purified further by calcium phosphate gel treatment.

Sensitivity of B and CRM to heat and alkaline pH was tested and both B and CRM were found to be affected to the same extent (see Table 5). Similarly, determinations on the supernatant solutions obtained following precipitation of extracts with dilute acetic acid indicate that, although only small amounts of B activity remain, corresponding levels of CRM are present. Partially purified preparations of component A (from an extract containing both components) have only *trace* amounts of component B but contain a *corresponding* amount of CRM. Thus these tests,too, suggest an association of B activity with CRM.

TABLE 5

INACTIVATION OF B AND CRM

TREATMENT	PER CENT DECREASE IN B	PER CENT DECREASE IN CRM
Alkali at pH 11 for 5 min.	88	89
Heat at 60° for 10 min.	53	60
Heat at 52° for 17 min.	72	68
Acid supernatant	90	87

Neutralization of the TSase in Extracts with High Levels of B.—If component B is actually CRM, it would be expected that the TSase in extracts of mutants which have a B/TSase ratio higher than that observed in extracts of wild type strains would be relatively more difficult to neutralize with antiserum. That is, if, in addition to TSase, a mutant extract contains an excess of CRM, a given amount of antiserum should be less effective in inhibiting its TSase than in inhibiting the TSase in a wild-type extract. The results presented in Table 6 show that mutants which lack component A (T-4 and T-8) do exhibit a B/TSase ratio which is higher than that observed in wild-type extracts. The fifth column of this table shows that a given amount of antiserum is much less effective in inhibiting the TSase of T-4 and T-8 extracts than in inhibiting the TSase activity of the wild-type preparation. Using the B/CRM ratio characteristic of mutants td_2, td_4, and td_5 and the B/TSase ratio characteristic of wild-type strains, it is possible to calculate the reduction in neutralization expected with T-4 and T-8 extracts (assuming that the excess B is equivalent to a corresponding amount of CRM). Comparison of the values in the last two columns indicates that the experimental values compare favorably with those calculated on this basis.

TABLE 6

NEUTRALIZATION OF TSASE OF STRAINS LACKING COMPONENT A

EXTRACT	SPECIFIC ACTIVITY TSase	SPECIFIC ACTIVITY B	B/TSASE × 100	TSASE UNITS Per Cent of Control	NEUTRALIZED Per Cent Expected*
Wild type K-12 (control)	6.3	0.17	2.7†	100	
T-4	1.1	0.12	10.9	26	24
T-8	3.5	0.5	14.3	15	18

* Based on theoretical curve in Fig. 2.

† It may be noted that this ratio is exactly the same as the B/CRM ratios obtained with extracts of mutants td_2, td_4, and td_5. This finding indicates that, per unit of antigenic material, there is as much B activity in mutants as in the wild-type strain.

In a further study, neutralization of the TSase in a T-4 extract was compared with neutralization of a mixture containing T-3 TSase and td_2 B in amounts which reproduced exactly the B/TSase ratio of the T4 extract. The results of this experiment (Fig. 3) show that the T-4 TSase and the TSase in the T-3-td_2 mixture were both inhibited to the same extent by the antiserum employed.

Comparison of the Neutralization of B and CRM.—In other experiments, the precipitation of component B and CRM by absorbed antisera was compared. The results of two such experiments are presented in Figure 4. Both CRM and component B were affected to approximately the same extent by increasing amounts of antiserum, and both were completely removed at the same point. In similar tests, antiserum to td_2-CRM (absorbed) also precipitated both CRM and component B to the same extent. When excesses of antibody were incubated (2

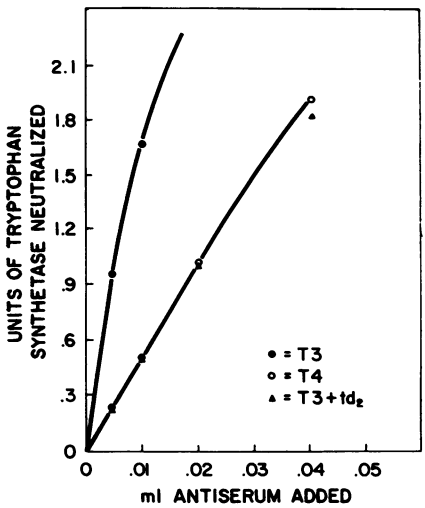


FIG. 3.—Comparison of the neutralization of the TSase in a T-4 extract and in a mixture containing T-3 TSase and td_2 B. The mixture contained T-3 TSase and td_2 B in amounts which reproduced exactly the B/TSase ratio of the T-4 extract. The neutralization curve obtained with T-3 TSase alone is included for comparison.

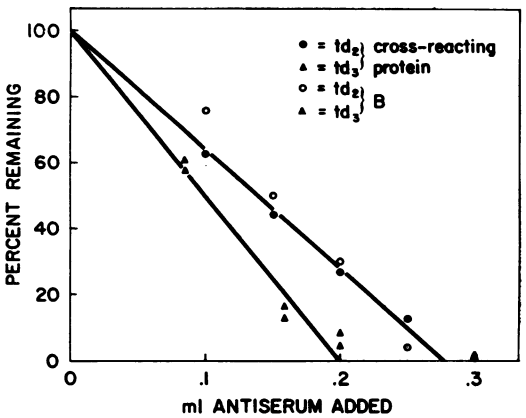


FIG. 4.—Comparison of the neutralization of component B and CRM in td extracts. Varying amounts of absorbed TSase-antiserum were incubated with constant amounts of td_2 or td_3 extracts in the presence of glutathione and pyridoxal phosphate. After 2 days at 4° C. the reaction mixtures were centrifuged and the supernatant solutions assayed for remaining activity.

days at 4°) with component B and CRM, the antibody remaining in the supernatant solution following centrifugation also precipitated component B and CRM to approximately the same extent. Although both CRM and component B were generally precipitated to the same degree by the various antisera employed, a significant difference was often observed in the region of high antigen excess. In this region, somewhat more B activity was detected in the supernatant solution than was expected on the basis of the amount of CRM which was removed. This may be due to the often observed partial solubilization of an antigen-antibody precipitate by excess antigen.

DISCUSSION

Biochemical studies with *E. coli*[14, 18] have implicated the following reactions as steps in the biosynthesis of tryptophan:

$$\text{Indole-3-glycerol phosphate} \xrightarrow[\text{component A + component B}]{} \text{indole} \xrightarrow[\text{TSase}]{\text{+ serine}} \text{tryptophan}$$

The data presented in this paper demonstrate a striking similarity in the behavior of component B, an enzyme required for the conversion of indoleglycerol phosphate to indole, and CRM, a protein immunologically related to TSase. On the basis of the similarities observed, it appears likely that component B activity and CRM represent, respectively, the enzymatic and immunological properties of the same protein.

In evaluating this conclusion in terms of the relationship between CRM (B) and TSase, two possibilities are being considered: (*a*) that CRM (B) and TSase are distinct but closely related proteins and (*b*) that one protein normally exhibits

both activities (B and TSase) and that CRM is a mutationally altered form of this protein that retains only component B activity.

The different types of mutants which have been obtained (lacking component B and/or TSase) are of importance in considering the foregoing possibilities. These classes and the class lacking component A are indicated in Table 7, together with the mutants present in each. Although the mutants listed were obtained in several laboratories and from two wild-type strains of *E. coli*, one class of mutants is noticeably absent, the class lacking component B alone. This class is replaced by a category containing strains in which both component B and TSase are absent. All nine of the mutants in this class have been examined in reversion experiments, and all do revert. However, when they do, both activities are invariably regained at the same time. It would appear, therefore, that these strains represent instances in which two enzymatic activities are lost and regained as a result of single mutational events. The fourth category in Table 7 includes mutants in which portions of the genic region controlling tryptophan biosynthesis have been lost by deletions; their behavior is not relevant to present considerations.

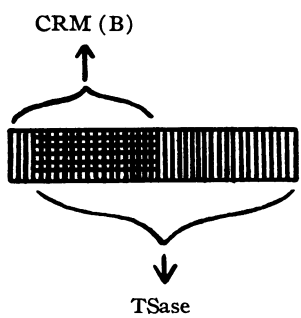
TABLE 7

GROUPING OF MUTANTS LACKING COMPONENTS A, B, OR TSASE

LACK A	LACK B AND TSASE	LACK TSASE	LACK A, B, AND TSASE
T-4, T-8	T-41, T-84	B-82*	B/IT
T-26, T-106	WRT-1, WRT-2	td_2, td_3	B/ITN
B-42,* B-48*	B-81,* B-84*	td_4, td_5	..
B-4*	B-9,† B-10,† B-11†	...	...

* Obtained from Dr. J. Gots.
† Obtained from Dr. P. D. Skaar.

Since one class of mutants (excluding the deletion mutants) lacks both component B and TSase, it appears unlikely that CRM (B) and TSase are different proteins under the control of separate genes. They could, however, be two distinct proteins formed by a complex of two neighboring genes with an overlapping functional region[19] (see diagram). Mutations within the overlap region could affect the formation of both CRM (B) and TSase, thus explaining the origin of one class of mutants, while mutations within the B or TSase-specific regions could affect these activities separately. If the B-specific region were very small in comparison with the rest of the gene complex, mutants lacking only component B would be expected to be very rare. This model has the advantage that it would also explain the tendency of the various genes involved in tryptophan synthesis in *Salmonella typhimurium* and *E. coli* to remain clustered.



The available data do not exclude the possibility, however, that both component B activity and TSase activity are associated with the same protein and that the formation of this protein is controlled by a single gene. Mutations within this gene could presumably affect it so that the ability to form the protein bearing both activities is lost or, when less drastic, could affect only the portion of the gene concerned with the TSase reaction. To

explain the absence of mutants lacking component B alone, it may be assumed that mutations in the genic region which affects B activity result in protein alterations which eliminate both the immunological identity and the TSase activity of the protein. Consistent with this interpretation is the possibility suggested by recent studies with *Neurospora*[20] that the formation of tryptophan from indoleglycerol phosphate (or a compound derived from indoleglycerol phosphate) occurs on one enzyme surface, with enzyme-bound indole generated from indoleglycerol phosphate serving as the true intermediate rather than free indole.

Studies designed to distinguish between the alternative possibilities are now in progress.

SUMMARY

Enzymatic and immunological studies with mutants of *E. coli* have provided evidence suggesting that the cross-reacting protein related to tryptophan synthetase can catalyze a reaction involved in the conversion of indoleglycerol phosphate to indole. The possibility that this reaction and the coupling of indole and serine are normally catalyzed by the same enzyme, tryptophan synthetase, is discussed.

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