Tryptophan Synthetase in *Euglena gracilis* Strain G

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The five enzyme activities in the synthesis of L-tryptophan have been obtained in extracts of *Euglena gracilis*. One of these, tryptophan synthetase, has been studied in detail. The general catalytic properties of tryptophan synthetase, including the range of reactions catalyzed and its substrate and cofactor affinities, are similar to those reported for other organisms. The *Euglena* enzyme has two properties never previously observed for tryptophan synthetase. First, the rate of catalysis of the conversion of indole-glycerol phosphate to L-tryptophan remained at its maximal value and was unaffected by the ionic environment up to 0.3 M KCl. In contrast, the conversion of indole to tryptophan showed a sharp maximum at 0.08 M KCl. Second, the enzyme is a component of a complex that includes every enzyme in the pathway committed to tryptophan biosynthesis with the exception of anthranilate synthetase, the regulatory enzyme.

*Euglena gracilis* is a photosynthetic single-celled flagellate with morphological resemblances to the algal flagellates and to the protozoa. There is considerable morphological diversity among the euglenoids and they are difficult to classify. While they share chlorophyll a and b with plants and green algae (4), they also share certain biochemical characteristics otherwise restricted to the higher fungi. As an example, Vogel (27), studying the lysine biosynthetic pathway in organisms ranging from bacteria to plants, found that only the euglenoids and the higher fungi use the L-α-aminoacidic acid pathway for lysine biosynthesis. In contrast, bacteria, algae, and plants use the meso-α, ε-diaminopimelic acid pathway.

Another biochemical marker useful for taxonomic studies is the terminal enzyme in L-tryptophan biosynthesis, tryptophan synthetase [EC 4.2.1.20]. L-serine hydrolyase (adding indole). This enzyme has been studied in a wide range of organisms, most intensively in bacteria (3, 30) and the fungus *Neurospora crassa* (12). In these organisms the enzyme catalyzes three reactions:

\[
\text{indole} + \text{L-serine} \rightarrow \text{L-tryptophan} \quad (1)
\]

\[
\text{indole-3-glycerol phosphate (InGP)} + \text{L-serine} \rightarrow \text{L-tryptophan} + \text{D-glyceraldehyde-3-phosphate} \quad (2)
\]

\[
\text{InGP} \rightleftharpoons \text{indole} + \text{D-glyceraldehyde-3-phosphate} \quad (3)
\]

In *Escherichia coli* the enzyme has the formula α₂β₂. The α subunit alone catalyzes reaction 3 at one-hundredth the rate of the tetramer, whereas the isolated β₂ subunit catalyzes reaction 1 at one-thirtieth the normal rate (6). For the physiological reaction (reaction 2) the complex is required.

In contrast to the bacteria, the *N. crassa* enzyme is not dissociable into active subunits (20). This also appears to be true of the ascomycete *Aspergillus nidulans* (16) and the yeast *Saccharomyces cerevisiae* (18). In blue-green and green algae (23) and in the higher plant *Nicotiana tabacum* (8) the data suggest that the enzyme is of the bacterial type.

Given this information we examined the enzyme in *Euglena gracilis* in the hope that this would provide additional information on the taxonomic status of this organism. The evidence obtained shows that the enzyme in *Euglena* has certain properties different from those heretofore described in other organisms.

**MATERIALS AND METHODS**

*Growth and maintenance of E. gracilis strain G.* A culture of the organism, obtained from Francis Haxo, was grown and maintained in Hutton’s liquid medium at pH 3.5 (14). For mass cultivation, 15 liters of medium in glass carboys was inoculated with 300 ml of culture (2 × 10⁴ cells/ml) and grown under continuous aeration at room temperature. Constant illumination (800 foot-candles at surface) was pro-
vided by a bank of fluorescent lamps rated at 40 watts. After 4 days, cells were harvested in a Sharples centrifuge, washed in distilled water, lyophilized, and stored at -60 °C. Under these conditions enzyme activity could be extracted without loss after several months of storage. The extracts contained negligible amounts of chlorophyll and other pigments.

**Enzyme extraction.** All operations were carried out at 0 to 5 °C. The buffer used, unless otherwise stated, was 0.08 M potassium phosphate (pH 7.6) containing 10⁻² M dl-serine, 10⁻² M sodium ethylenediaminetetraacetate (EDTA), 10⁻⁴ M 2-mercaptoethanol, and 10 mM pyridoxal phosphate (PLP) per ml. Centrifugations were at 39,000 × g for 15 min. To approximately 10 g of lyophilized cells, 150 ml of buffer was added and the mixture was stirred for 10 min. After centrifugation, 10 ml of protamine sulfate (1.5%, w/v) was added slowly to 100 ml of the crude extract. After stirring for 15 min the precipitate was removed by centrifugation. To each 100 ml of extract, 14.5 g of powdered (NH₄)₂SO₄ was slowly added and stirred for 15 min. After removal of the precipitate, an additional 9.1 g of (NH₄)₂SO₄ was added. The resulting precipitate was dissolved in 8 ml of buffer and desalted by overnight dialysis against buffer. Two volumes of enzyme solution were then added to one volume of calcium phosphate gel A grade (CalBiochem lot 010211), previously equilibrated with buffer, and mixed for 10 min, and the gel was removed by centrifugation.

**Sucrose density gradients.** Velocity sedimentation analysis was performed by the method of Martin and Ames (19). The enzyme extract (0.5 ml) was layered on a linear 5 to 20% sucrose density gradient made up with 0.1 M potassium phosphate (pH 7.8) containing 10⁻³ M 2-mercaptoethanol, and centrifuged for 12 hr at 4 °C in a SW41 rotor at 39,000 rev/min in a Beckman model L2-65 centrifuge. Beef liver catalase, E. coli a subunit of tryptophan synthetase, pig heart malate dehydrogenase (MDH), and lactate dehydrogenase (LDH) were used as markers for molecular weight determinations.

**Gel filtration.** Sephadex G-200 was handled as suggested by the Sephadex products manual. Columns (2.2 by 44.5 cm) were equilibrated with buffer, with or without PLP depending on the form of the enzyme applied to the column. Proteins were eluted from the column with the equilibration buffer at a flow rate of 20 ml per hr.

**Formation of apoenzyme.** A precursor was prepared by overnight dialysis of the enzyme solution against 100 volumes, with two changes, of buffer without PLP and with a 10-fold increase in dl-serine.

**Enzyme assays.** The effect of ionic strength on the catalytic activity of tryptophan synthetase from N. crassa was examined in collaboration with Peter Matchett in his laboratories. For the experiment, the enzyme was prepared by extraction from lyophilized mycelia and partially purified by protamine sulfate and ammonium sulfate fractionation to a specific activity of 0.15 μmoles per mg per min.

Reaction 1 of tryptophan synthetase (indole + l-serine → l-tryptophan) was measured by a modification of the method of Crawford and Yanofsky (5).

The enzyme mixture contained potassium phosphate buffer (pH 7.8) instead of tris(hydroxymethyl)aminomethane (Tris), and NaCl was omitted. Both Tris and NaCl were found to be inhibitory to the Euglena enzyme. In the salt experiments with KCl, 0.01 M buffer was used. Reactions 2 and 3 were measured as previously described (26). The enzyme unit employed was the appearance of 1 μmole of substrate per min. Specific activity was defined as enzyme units per milligram of protein. Anthranilate synthetase (10), phosphoribosyl (PR) transferase (16), phosphoribosyl anthranilate (PRA) isomerase (16), and InGP synthetase (28) were assayed as previously described. MDH was measured by the method of Ochoa (21). LDH was measured by following the oxidation of reduced nicotinamide adenine dinucleotide (22), and catalase was measured spectrophotometrically (1).

Protein was assayed by the method of Lowry et al. (17) with bovine serum albumin as a standard.

**Chemicals.** InGP was the generous gift of I. P. Crawford, 1-(O-carboxyphenylamino)-1-deoxyribose-5-phosphate was prepared by the method of Smith and Yanofsky (23); indole and PLP were purchased from CalBiochem and used without further purification.

**Purification of enzyme activity.** In Table 1 are recorded the data obtained in a typical enzyme purification, in this case ammonium sulfate precipitation followed directly by affinity chromatography. The characteristics of the enzymes reported in this paper were invariant over a 200-fold purification range.

**RESULTS**

Crude extracts of E. gracilis were found to contain low levels of tryptophan synthetase activity. The specific activity determined for reaction 1 was 7.8 × 10⁻⁴ μmoles per min per mg. By treatment of the crude extracts with protamine sulfate, precipitation of the activity between 25 and 40% ammonium sulfate, and subsequent treatment with calcium phosphate gel, about 60% of the original activity was recovered with an eight- to tenfold increase in specific activity. This partially purified preparation catalyzed all three reactions previously described. Furthermore, the ratio of reaction 1 to 2 remained constant at 1.7 during purification when reaction 1 was measured at the optimum salt concentration. The specific activity of reaction 3 in the partially purified preparation was 10⁻² μmoles per min per mg.

**Some properties of the enzyme.** Figure 1A illustrates the effect of salt on the activity of Euglena tryptophan synthetase. The rate of reaction 1 increased sharply to a maximum at 0.05 M KCl while the rate of reaction 2 remained constant between 0.01 and 0.3 M KCl. This type of effect was unusual; in the case of the N. crassa enzyme (Fig. 1B) both rates re-
Table 1. Purification of tryptophan synthetase and InGP synthetase from Euglena gracilis

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Tryptophan synthetase (1)</th>
<th>InGP synthetase (2)</th>
<th>Rate of specific activity (1) to (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total units*</td>
<td>Specific activity</td>
<td>Total units</td>
</tr>
<tr>
<td>Crude</td>
<td>860</td>
<td>0.49</td>
<td>360</td>
</tr>
<tr>
<td>Pellet 25-40% (NH₄)₂SO₄ fraction</td>
<td>550</td>
<td>2.9</td>
<td>230</td>
</tr>
<tr>
<td>Affinity chromatography†</td>
<td>95</td>
<td>10.0</td>
<td>11</td>
</tr>
</tbody>
</table>

*All anthranilate synthetase activity in the crude extract was recovered in 25-40% (NH₄)₂SO₄ supernatant fraction. PR transferase (not shown) copurified with the two enzyme activities shown above.

†Tryptophan synthetase was measured in the reaction indole + L-serine → L-tryptophan.

†The sample layered onto the affinity column was taken from the 25 to 40% (NH₄)₂SO₄ pellet. Activity in peak fraction as measured in the conversion of indole plus L-serine to L-tryptophan.

Fig. 1. Effect of K⁺ concentration on reactions 1 and 2. The enzyme was prepared from (NH₄)₂SO₄ precipitate and desalted with Sephadex G-25 equilibrated with 0.01 M potassium phosphate (pH 7.8). (A) Enzyme from Euglena; (B) enzyme from N. crassa obtained as described in Materials and Methods. Symbols: reaction 1, ●; reaction 2, ○.

Sponded identically to increasing KCl with a maximum at 0.1 M KCl. A third type of salt effect has been reported for the enzyme from the plant Nicotiana tabacum (8) and appears to be true of reaction 1 in bacteria (24). In N. tabacum, the rate of reaction 1 showed a hyperbolic increase with added salt whereas it was the rate of reaction 2 that showed a maximum.

The Kₘ values obtained for the enzyme substrates and the cofactor are summarized in Table 2. The data show the similarity between N. crassa and E. gracilis as compared with E. coli. An unexpected inhibition of enzyme activity that occurred with increasing concentrations of PLP will be discussed later.

Over the relatively broad pH range of 6.2 to 8.2, at the salt concentration optimum for reaction 1, the rates of reactions 1 and 2 remained constant. At a pH below 6.2 the enzyme was precipitated.

Attempts to define the subunit structure. Tryptophan synthetase in bacteria, algae, and N. tabacum can be separated into two components by differential precipitation with (NH₄)₂SO₄ or by subjecting the apo-form of the enzyme to either Sephadex G-150 chromatography or to density gradient centrifugation. By none of the above methods were we able to show subunit structure for the Euglena enzyme. Figure 2 shows the sedimentation pattern of a preparation of apoenzyme which contained 1% of the original activity but was fully restorable upon addition of 10⁻⁴ M PLP. Only one peak of activity for reactions 1 and 2 was detected, and the peak contained 80% of the (restorable) activity layered on to the gradient. The same result was obtained with Sephadex.
TABLE 2. Summary of $K_m$ values for tryptophan synthetase from Euglena gracilis as compared with Escherichia coli and Neospora crassa

<table>
<thead>
<tr>
<th>Reference</th>
<th>Substrate</th>
<th>$K_m$ (mm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>E. coli</td>
</tr>
<tr>
<td>9, 13</td>
<td>Indole</td>
<td>0.165</td>
</tr>
<tr>
<td>9</td>
<td>InGP</td>
<td>0.15</td>
</tr>
<tr>
<td>11, 13</td>
<td>L-Serine</td>
<td>24.6</td>
</tr>
<tr>
<td>11, 13</td>
<td>Pyridoxal phosphate</td>
<td>1.1 $\mu$M</td>
</tr>
</tbody>
</table>

*The numbers in parentheses after $K_m$ values indicate the reaction measured for the determination. Note difference in units for pyridoxal phosphate.

Fig. 2. Velocity sedimentation pattern of E. gracilis tryptophan synthetase. A 0.5-ml amount of apoenzyme was layered onto a linear 5 to 20% sucrose density gradient in 0.08 M potassium phosphate (pH 7.8) containing $10^{-3}$ M 2-mercaptoethanol, and the gradient was centrifuged for 12 hr at 4°C at 39,000 rev/min.

G-200; enzyme activity for reactions 1 and 2, at the normal ratio, was found in a single peak when the enzyme activity of the fractions was restored with PLP (Fig. 3). Four trials with Sephadex G-200 (29) and three trials with density gradient centrifugation gave estimated molecular weights for the enzyme of 229,000 ± 18,000 and 240,000 ± 16,000. The average of all seven trials was 234,000 ± 18,000.

Evidence for an enzyme aggregate. The highest molecular weight previously reported for tryptophan synthetase was 150,000. The value of 234,000, coupled with the difficulty in resolving the Euglena enzyme into components, led us to examine the possibility of an enzyme complex. Tryptophan biosynthesis, in all organisms studied, requires four additional enzyme activities. These were found in Euglena extracts. Two of them, InGP synthetase and PRA isomerase, purified with tryptophan synthetase through the calcium phosphate step and gave coincident profiles on Sephadex G-200 (Fig. 4). In the peak fractions, with reaction 1 at a specific activity of $5.4 \times 10^{-2}$ umoles per min per mg (100-fold-purified), the ratio of each of the activities to reaction 1 was identical with the ratio determined in the crude extract. PR transferase also purified with tryptophan synthetase through the calcium phosphate step and, as will be shown later, gave an identical elution pattern with all three activities on affinity chromatography. The remaining pathway activity, anthranilate synthetase, was found in the 40% $(NH_4)_2SO_4$ supernatant solution.

The copurification of the four enzyme activities could be merely a coincidence, but that it was not was suggested by the following evidence. First, concentrations of PLP that inhibited reactions 1 and 2 of Euglena tryptophan synthetase (and which did not inhibit equivalent enzyme activities from E. coli and N. crassa) also inhibited the reaction catalyzed by InGP synthetase, a reaction which does not require PLP. Second, V. Rocha of our laboratory has shown that the $B_2$ subunit of E. coli tryptophan synthetase is retained by indoleacetic acid-substituted agarose and is specifically eluted by aqueous indole. The Euglena enzyme was retained on this column (Fig. 5) and, upon washing with an aqueous indole solution ($5 \times 10^{-2}$ M), was eluted coincident with the other three enzyme activities. The material obtained from the column was 200-fold-purified and the ratios of the enzyme activities to each other were identical to those determined in the crude extracts.

Finally, attempts to dissociate the enzyme activities by ion exchange chromatography have been unsuccessful. In these studies diethylaminoethyl cellulose columns (2 by 24 cm) were equilibrated with buffer, and the enzyme was eluted with a 0.08 to 0.4 M potassium phosphate buffer (pH 7.6) gradient. Under these experimental conditions all four enzyme activities co-eluted and were not resolvable.
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FIG. 3. Gel filtration of the apo-form of tryptophan synthetase on Sephadex G-200. A 2.5-ml amount of apo-enzyme was applied to a Sephadex G-200 column, and the enzyme was eluted in 3-ml fractions with 0.08 M potassium phosphate (pH 7.8) containing $10^{-3}$ M D,L-serine, $10^{-3}$ M 2-mercaptoethanol, and $10^{-3}$ M EDTA. Symbols: absorbancy at 280 nm, $x$; reaction 1, ●; reaction 2 of tryptophan synthetase, ○.

FIG. 4. Distribution of enzymes involved in L-tryptophan biosynthesis following gel filtration. The apo- or holo-form of the enzyme (2.5 ml) was applied to a Sephadex G-200 column and eluted as described in Materials and Methods. Tryptophan synthetase activity was measured by reaction 1.

DISCUSSION

The enzymes committed to L-tryptophan biosynthesis in *E. gracilis* strain G were examined and found to be unique in several respects. The inability to separate tryptophan synthetase into active subunits by the usual methods [(NH$_4$)$_2$SO$_4$ fractionation, gel filtration, or velocity sedimentation], together with the $K_m$ values measured for the enzyme substrates, initially suggested that the *Euglena* enzyme more closely resembled the fungal enzyme. However, that the *Euglena* enzyme is distinct from the *N. crassa* enzyme was shown by the effect of salt on reactions 1 and 2. Moreover, the finding that tryptophan synthetase from *Euglena* appears to be in an aggregate with PR transferase, PRA isomerase, and InGP synthetase differentiates the enzyme from its analogue in the fungi (15). The inability to separate tryptophan synthetase from the other enzymes by the above methods in addition to ion exchange and affinity chromatography prevents us from making any statement about the subunit structure of the enzyme. Nevertheless, this is the first reported case in which tryptophan synthetase is asso-
Fig. 5. Distribution of enzymes involved in L-tryptophan biosynthesis following affinity chromatography. A 2.5-ml amount of enzyme was applied to an indoleacetic acid-substituted agarose column and washed with 0.02 M potassium phosphate (pH 7.0) containing 10^{-4} M PLP and 10^{-3} M 2-mercaptoethanol. Tryptophan synthetase (O) was eluted from the column with an aqueous solution of indole (5 \times 10^{-3} M). Symbols: InGP synthetase, ●; PRA isomerase, Δ; PR-transferase, ©; absorbance at 280 nm, ×.

associated with any of the enzymes in the terminal pathway for tryptophan biosynthesis. To our knowledge it is the first difference of a general nature that has been reported between the higher fungi and Euglena with respect to amino acid biosynthesis.

Hütter and DeMoss (15) reported three aggregate patterns characteristic of the ascomycetes and basidiomycetes. In these patterns, at most three of the five terminal enzymes were aggregated; in all, anthranilate synthetase was a component. In Euglena, in no case were we able to detect anthranilate synthetase with the enzyme complex. The evidence offered here, coupled with the data of Berlyn, Ahmed, and Giles (2), indicate that, in Euglena, nine of the twelve enzymes in the entire pathway of L-tryptophan biosynthesis starting with D-erythrose-4-PO_4 and phosphoenolpyruvate are in two aggregates. These two aggregates offer the prospect that in vivo there is a "pathway particle" consisting of the aggregates conjoined, perhaps loosely so, by chorismate synthetase and the two regulatory enzymes, anthranilate synthetase and 3-deoxy-D-arabino-heptulosonic acid-7-PO_4 synthetase. A system such as this would provide the structural framework for the biochemical efficiency suggested in the channeling of substrates and intermediates (27).

Studies directed at trying to elucidate the aggregate nature of the complex are presently hampered by the low enzyme yields available in wild-type strains of E. gracilis. We are currently trying to obtain auxotrophic mutants of Euglena and, if possible, to obtain partial or incomplete aggregates. Such studies will allow us to learn more about the genes and enzymes involved in tryptophan biosynthesis in a flagellated photosynthetic organism which shares certain features with the higher fungi.

ACKNOWLEDGMENTS

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LITERATURE CITED