Purification and Characterization of a Multienzyme Complex in the Tryptophan Pathway of Neurospora crassa*

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SUMMARY

A multienzyme complex which carries out the anthranilate synthetase, N-(S'-phosphoribosyl)anthranilate isomerase and indole-3-glycerol phosphate synthetase reactions in the tryptophan pathway of Neurospora crassa has been purified and characterized. The complex, as prepared here, is a single homogeneous protein molecule having a sedimentation coefficient of 10.3 S and a molecular weight of 240,000. Sedimentation equilibrium studies in 6 M guanidine HCl and in 0.1% sodium dodecyl sulfate reveal that the complex consists of six 40,000 molecular weight subunits. Amino acid analysis and titration with p-chloromercuribenzoate show that the complex contains 20 sulfhydryl groups and no disulfide bridges. Titration of 12 of the sulfhydryl groups or more with p-chloromercuribenzoate leads to the quantitative dissociation of the complex into 4.4 S and 7.4 S fragments. Based on the sedimentation values, the 4.4 S fragment is a dimer and the 7.4 S fragment is a tetramer of the 40,000 molecular weight subunits. The 4.4 S fragment is itself inactive but in the presence of dithiothreitol a 7 S form of anthranilate synthetase develops as the only enzymic activity. The 7.4 S fragment on the other hand, is fully active in indole-3-glycerol phosphate synthetase and N-(S'-phosphoribosyl)anthranilate isomerase. Electrophoresis in 8 M urea shows that the 4.4 S and 7.4 S fragments consist of single but distinct polypeptides, termed here the A and the I subunits, respectively. An A1I model for the complex is discussed.

* The abbreviations used are: PR-anthranilate, N-(S'-phosphoribosyl)anthranilate; InGP, indole-3-glycerol 5-phosphate; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; CMB, p-chloromercuribenzoate.

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ments. The $x$ and $y$ coordinates of the refractive index gradients were measured with a Scherr-Turner Optical Comparator. The protein was equilibrated in the solvent to be used in centrifugation by filtration through Sephadex G-25 (fine). The elution buffer was used in the solvent chambers for sedimentation equilibrium and synthetic boundary experiments.

Three methods were used in the determination of molecular weights by sedimentation equilibrium. In the midpoint or short column (0.7 mm) method (9) molecular weights were calculated according to the formula:

$$ M = \frac{1}{RT} \left( \frac{dc}{dr} \right) \omega \left( \frac{1 - \bar{v}}{\bar{v}} \right) $$

where $r$ is the radial distance, $c$ is the concentration, $\omega$ is the angular velocity, $\bar{v}$ is the partial specific volume, $p$ is the density, $R$ is the gas constant, $T$ is the absolute temperature, and $M$ is the molecular weight. The initial concentration ($C_0$) was determined by integrating the schlieren peak obtained in a synthetic boundary run and $(dc/dr)$, $\bar{v}$ was determined after equilibrium was achieved by measuring the height of the schlieren refractive index gradient at the midpoint ($r$). In the meniscus depletion or high speed, long column (3 mm) method (10), molecular weights were calculated according to the formula:

$$ M = \frac{d \ln F}{d(r^2)} \frac{2RT}{(1 - \bar{v})\omega^2} $$

$F$ was determined by measuring the displacement of the Rayleigh interference fringes from the value at the meniscus. A solvent blank was examined at the same rotor speed to account for extraneous fringe displacement. Molecular weights were determined from least squares interpretation of the slope obtained by plotting $\ln F$ against $r^2$. In the third method (11) relatively slow speeds and long columns (3 mm) were used. Molecular weights were calculated according to the formula:

$$ M = \frac{d(\ln h - \ln r)}{d(r^2)} \frac{2RT}{(1 - \bar{v})\omega^2} $$

The values of $h$ at increasing values of $r$ were determined by measuring the height of the schlieren refractive index gradient relative to the solvent blank. Molecular weights were determined from a least squares interpretation of the slope obtained by plotting $\ln h - \ln r$ against $r^2$. A bar angle of 70° was used in each case. In the latter two methods data were analyzed and plots were made with the aid of Portran IV programs (MOLWTS and MOLWTR, respectively) prepared by D. W. Hopkins (University of California, San Diego).

**Sucrose Gradient Analyses (12)**—Linear gradients from 5 to 20% sucrose were made to a final volume of 4.4 ml. All gradients contained 0.05 M potassium phosphate (pH 7.0), 0.1 mM EDTA, and 0.1 mM GSH. Centrifugation was for 12 hours at 37,000 rpm with an SW 39 rotor. Ten-drop fractions were collected, providing a total of 25 fractions.

**Amino Acid Analysis**—The sample was dialyzed exhaustively against 0.1 M ammonium bicarbonate buffer (pH 8), lyophilized, and dried at 100° under vacuum against P2O5 for 16 hours in an Albedehalken apparatus. Dry weight measurements were made and the extinction of the protein at 280 nm in 0.01 M potassium phosphate buffer, pH 7, was recorded. A known quantity of the dried protein was hydrolyzed under vacuum with constant boiling HCl for 21 and 48 hours at 105°. Analyzes were performed on the short and long column of the Beckman-Spinco amino acid analyzer, model 120R (13). Cysteic acid was measured on samples oxidized with performic acid (14) and tryptophan was measured spectrophotometrically (15).

**Purification Procedure**

**Growth of Organism**—*N. crassa*, til 48 R (17), was grown and harvested as described by DeMoss (4). An average yield of lyophilized mycelium grown at 30° under forced aeration in 10 20-liter Nalgene carboys was 300 to 350 g. In later experiments the mold was grown in a New Brunswick Fernmazzell Fermentor. Fifteen liters of a 48-hour culture were used as inocula for 80 liters of medium. Incubation was at 30° with an air flow rate of 4 cubic feet per min and an agitation rate of 250 rpm. The mycelium was harvested 6 hours after the disappearance of indole from the medium (usually within 30 hours after incubation). An average yield of lyophilized mycelium was 700 g.

**Preparation of Extracts**—Crude extracts were prepared according to the procedure of Wegman and DeMoss (5). Fractionation of crude extracts with protamine sulfate and ammonium sulfate was carried out as described previously (4).

**Gel Filtration (Sephadex G-25)**—The 40 to 50% ammonium sulfate cut originating from 600 g of lyophilized mycelium was placed on a column, 4 × 90 cm, of coarse grade Sephadex G-25 and eluted at 4° with 0.05 M potassium phosphate buffer, pH 7.0. DTT, 0.1 mM, and EDTA, 0.1 mM, were also included in the elution buffer and, unless otherwise indicated, all other buffers used in the purification procedure included these reagents at the above concentrations. The excluded fraction (dark brown in color) was collected, warmed to 20° to 25° to induce precipitation of heat-labile contaminants, and then returned to the cold. The sample was maintained at 0° to 4° throughout the remainder of the procedure. The precipitate which formed at 20° to 25° was removed by centrifugation.

**DEAE-cellulose Chromatography I**—The entire sample from the previous step was fractionated on a column, 4 × 80 cm, of DEAE-cellulose, Brown Paper Company, Batch 1340. The DEAE-cellulose was prepared by suspending 300 g of dry DEAE-cellulose in 2 liters of 0.1 M NaOH. After 80 min at room temperature the DEAE-cellulose was collected by filtration and re-suspended in 2 liters of water for 15 min. The DEAE-cellulose was washed five more times with water, suspended in 0.01 M KH2PO4 for 1 hour, filtered, resuspended in 0.01 M potassium phosphate buffer pH 6.5 (minus DTT and EDTA), and allowed to equilibrate overnight at 4°. Filtration and equilibration in 0.01 M potassium phosphate buffer were continued until the pH was established at 6.5. Just prior to packing the column, the DEAE-cellulose was filtered and resuspended in 3 liters of 0.1 M potassium phosphate buffer containing 0.1 mM DTT and 0.1 mM EDTA. The column was packed by gravity with constant stirring of the above suspension. Elution of the column was accomplished with a 6000-ml linear gradient of 0.01 M to 0.15 M potassium phosphate, pH 6.5. The flow rate was 150 ml per hour with a Buchler Micropump and fractions were collected every 8 min. Enzyme activity eluted in Fractions 260 to 300.

**Gel Filtration (Sephadex G-75)**—The entire sample from the previous step was concentrated with ammonium sulfate (60% saturation) and then desalted and further fractionated with a

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* D. W. Hopkins and W. L. Butler, unpublished experiments.
column, 4 × 50 cm, of Sephadex G-75. The column was eluted
with 0.01 M potassium phosphate buffer, pH 6.5, at about 200
ml per hour and 8.5-ml fractions were collected. Enzymic ac-
tivity eluted in Fractions 25 to 32.

DEAE-cellulose Chromatography II—A second chromato-
graphing separation was accomplished with a column, 2 × 70 cm,
of DEAE-cellulose, prepared as described under "DEAE-cellulose
Chromatography I," and eluted with a 1500-ml linear
gradient of 0.01 to 0.15 M potassium phosphate, pH 6.5. The
column was eluted at 50 ml per hour and fractions were collected
every 15 min. Enzymic activity was found in Fractions 100 to
120. The fractions were pooled, concentrated with ammonium
sulfate, and dissolved in 0.05 M potassium phosphate, pH 7.0.

Gel Filtration (Sephadex G-200)—The concentrated sample
was eluted from a column, 2 × 70 cm, of Sephadex G-200 with
0.01 M potassium phosphate, pH 7.0. Three-milliliter fractions
were collected with a flow rate of 18 ml per hour. Enzymic ac-
tivity was located in Fractions 24 to 36.

Preparative Electrophoresis—Final purification was accom-
plished by electrophoresis through acrylamide gel with a Buchler
preparative electrophoresis apparatus (PolyPrep). The large
core spacer gel and small pore resolving gel were prepared accord-
ing to the method of Davis (7). The buffer used in the elec-
rophoresis upper chamber was 0.01 M Tris-base, 0.0525 M glycine,
0.01 M GSH, 0.1 mM EDTA, and 0.1 mM DTT. The lower
chamber buffer was 0.1 M Tris-base and 0.05 M ICI. The elution
buffer was the same as the lower buffer but also contained 0.1
mM EDTA and 0.1 mM DTT. The membrane chamber buffer
was 0.5 M Tris-ICl, pH 9.0. The resolving gel was 90 ml and
the spacer gel was made to equal the volume of the sample.
Preparations were at room temperature, after which the appar-
atus was taken to a cold room, attached to a circulating water
bath, and left to equilibrate to 4° for 2 hours. The sample was
adjusted to 5% in sucrose just prior to being siphoned onto
the surface of the spacer gel. Electrophoresis was at 50 ma (200
to 400 volts). Fractions were collected every 30 min with a flow
rate of 15 ml per hour by positive pressure from a Beckman
Acou-Flow Pump. Enzyme activity eluted in Fractions 43-47
The fractions were pooled, concentrated by pressure dialysis,
and stored in liquid nitrogen.

RESULTS

The location of the three enzymic activities in the various
steps of the purification procedure was routinely monitored by
assaying for either anthranilate synthetase or InGP synthetase.
On occasions when all three activities were measured they were
always found in the same fractions after DEAE-cellulose chro-
natography, gel filtration, and preparative electrophoresis. As
can be seen in Fig. 1, after preparative electrophoresis through
polyacrylamide gel, the three peaks of enzymic activity coincided
with each other and with a single protein peak. In addition, as

![Figure 1. Preparative electrophoresis. Elution profile of the
three enzymic activities and the protein from the final step in the
purification procedure. Preparation of the acrylamide electropho-
resis apparatus and the sample was as described under "Purifi-
cation Procedure." Electrophoresis was toward the anode with
a constant current of 50 ma. Fractions were collected every 30
min at a flow rate of 15 ml per hour. Enzymic activities are in
arbitrary units. Protein is represented by absorption at 280 mμ.
The first large peak of absorbing material is a nonproteinaceous
contaminant present in the electrophoresis reagents. PR α-
PR-anthranilate.

### TABLE I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Total protein</th>
<th>Anthranilate synthetase</th>
<th>Recovery</th>
<th>Specific activity</th>
<th>Anthranilate synthetase: PR-anthranilate isomerase ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>2100 ml</td>
<td>(200,000)a</td>
<td>(10,000)</td>
<td>100</td>
<td>0.50</td>
<td>1:1.12</td>
</tr>
<tr>
<td>Protamine and (NH₄)₂SO₄</td>
<td>218 ml</td>
<td>19,800</td>
<td>9,900</td>
<td>100</td>
<td>0.50</td>
<td>1:1.0:12</td>
</tr>
<tr>
<td>Sephadex G-25</td>
<td>380 ml</td>
<td>14,400</td>
<td>9,500</td>
<td>90</td>
<td>0.66</td>
<td>1:0.91:10</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>700 ml</td>
<td>1,680</td>
<td>8,150</td>
<td>82</td>
<td>4.9</td>
<td>1:0.85:10</td>
</tr>
<tr>
<td>Protamine by (NH₄)₂SO₄</td>
<td>30 ml</td>
<td>1,560</td>
<td>6,830</td>
<td>69</td>
<td>4.4</td>
<td>1:0.8:12</td>
</tr>
<tr>
<td>Sephadex G-75</td>
<td>68 ml</td>
<td>415</td>
<td>5,880</td>
<td>59</td>
<td>14.2</td>
<td>1:0.9:13</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>144 ml</td>
<td>141</td>
<td>4,200</td>
<td>43</td>
<td>20.6</td>
<td>1:0.92:10</td>
</tr>
<tr>
<td>Concentration by (NH₄)₂SO₄</td>
<td>6 ml</td>
<td>133</td>
<td>3,820</td>
<td>39</td>
<td>28.7</td>
<td>1:0.91:12</td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td>94 ml</td>
<td>70</td>
<td>3,440</td>
<td>35</td>
<td>49.2</td>
<td>1:1.1:12</td>
</tr>
<tr>
<td>PolyPrep</td>
<td>29 ml</td>
<td>19.6</td>
<td>1,890</td>
<td>18.5</td>
<td>93.4</td>
<td>1:1.1:12</td>
</tr>
</tbody>
</table>

a Values in parentheses are approximate.

b Specific activity by dry weight: 82 units per mg.
summarized in Table I, the ratio of the three activities remained essentially constant throughout the 2000-fold purification.

Initial studies indicated that a reducing environment was required to stabilize the enzymes. However, it was only in later experiments that DTT was found to be the most effective reagent for this purpose and GSH was used initially as the reducing agent of choice. Immediately after the final purification step, preparations stabilized with GSH exhibited a single band on analytical acrylamide gels (Fig. 2A) and a single peak in the analytical ultracentrifuge (Fig. 3A) with an $s_{20,w}$ of 10.3 (Fig. 4). However, storage of these preparations in liquid nitrogen for several weeks or at room temperature overnight resulted in the selective loss of anthranilate synthetase (InGP synthetase and PR-anthranilate isomerase remained fully active) and in the dissociation of the 10.3 S protein. Two additional bands of greater mobility were found on analytical gels (Fig. 2B) and sedimentation velocity experiments revealed two additional peaks at 7.4 S and 4.4 S (Fig. 3C). Sucrose gradient analysis verified that the bands

![Image of analytical acrylamide gels]

**Fig. 2.** Analytical acrylamide gels of three different preparations of the purified protein: A, in the presence of 0.01 mM GSH, a few hours after the final step in the purification procedure (as shown in Fig. 1 except that in this case 0.01 mM DTT was substituted for 0.1 mM DTT in the elution buffer); B, in the presence of 0.01 mM GSH, after several weeks storage in liquid nitrogen; C, as A except in the presence of 0.1 mM CMB for 15 min at room temperature (GSH was removed by filtration through Sephadex G-25, fine). The protein concentration was 0.5 mg per ml. The sample volume was 0.1 ml. Electrophoresis was at 3 ma per gel (0.6 mm x 70 mm) at pH 8.3 for 90 min (top, -; bottom, +). Migration was toward the anode. Other conditions were as described under "Experimental Procedure."

Fig. 3. Schlieren sedimentation patterns of four different preparations of the purified protein: A, in the presence of 0.01 mM GSH, a few hours after the final step in the purification procedure (cf. Fig. 2A); B, in the presence of 1 mM CMB for 1 hour at room temperature and 4 to 5 hours at 4°C (cf. Fig. 2C; the protein concentration was 5 mg per ml); C, in the presence of 0.01 mM GSH, after several weeks storage in liquid nitrogen (cf. Fig. 2B); D, in the presence of SDS (treated for 6 hours at 25°C in 1% SDS, filtered through Sephadex G-25, fine, with buffer containing 0.01 mM GSH and no SDS). Except as modified above, the buffer used in each case was the electrophoresis elution buffer described under "Purification Procedure." A double sector cell was used in A and D. Standard cells and a 1° wedge were used in B and C. The rotor speed was 60,000 rpm in each case and the temperature was 20°C. Protein concentrations were: A, 0.4%; B, 0.5%; C, 0.5%; D, 0.8%.

![Image of schlieren sedimentation patterns]

**Fig. 4.** The $s_{20, w}$ of the purified native protein in 0.01 M potassium phosphate, pH 7.0, with 0.1 mM DTT and 0.1 mM EDTA.

seen in analytical acrylamide gels corresponded to the centrifuge peaks. Analytical gels were prepared on each fraction from a sucrose gradient of 2 mg of a partially dissociated sample (cf. Fig. 2B). In this way it was shown that the sedimentation values of the proteins represented in the three bands shown in Fig. 2B were, beginning from the top of the gel, 10 S, 7 S, and 4 S, respectively.

These results indicated that the 10.3 S protein dissociated upon storage in the presence of GSH into two nonidentical fragments, one of which, or both, retained the InGP synthetase and PR-anthranilate isomerase activities. DTT prevented this dissociation and greatly reduced the loss of anthranilate synthetase activity during storage.

Initial attempts to determine the molecular weight of the 10.3 S protein by sedimentation equilibrium analyses were unsuccessful because of the instability of the protein in the presence of GSH. With DTT, the protein remained intact and appeared homogeneous even after 48-hour centrifugation at 20°C. Equilibrium was routinely achieved within 4 hours for 0.7-mm columns and within 24 hours for 3-mm columns. Plots of $\ln h - \ln r$ (11)
or in fringe displacement (10) against $r^2$ showed that the 10.3 S protein was homogeneous at concentrations as low as 0.18 mg per ml and as high as 4 mg per ml (Fig. 5, A and B). A partial specific volume of 0.728 was calculated (18) from the amino acid analyses presented in Table III (see Footnotes a, b, c, and d). The protein concentrations are: A, 4 mg per ml; B, 0.18 mg per ml; C, 3 mg per ml; D, 4 mg per ml.

**TABLE II**

**Amino acid composition of 10.3 S protein**

Conditions are described in the text. Analyses were performed on duplicate samples. Values are given as the number of residues per molecule of 240,000 molecular weight. The per cent by weight recovery of the 10.3 S protein as amino acids was 94%.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>24 hrs</th>
<th>48 hrs</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>residues/molecule</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>94</td>
<td>118</td>
<td>106</td>
</tr>
<tr>
<td>Histidine</td>
<td>39</td>
<td>48</td>
<td>44</td>
</tr>
<tr>
<td>Arginine</td>
<td>95</td>
<td>121</td>
<td>108</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>154</td>
<td>190</td>
<td>187</td>
</tr>
<tr>
<td>Threonine</td>
<td>103</td>
<td>111</td>
<td>107</td>
</tr>
<tr>
<td>Serine</td>
<td>157</td>
<td>157</td>
<td>157</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>221</td>
<td>230</td>
<td>226</td>
</tr>
<tr>
<td>Proline</td>
<td>111</td>
<td>112</td>
<td>112</td>
</tr>
<tr>
<td>Glycine</td>
<td>155</td>
<td>162</td>
<td>158</td>
</tr>
<tr>
<td>Alanine</td>
<td>103</td>
<td>105</td>
<td>104</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>20</td>
<td>—</td>
<td>20</td>
</tr>
<tr>
<td>Valine</td>
<td>97</td>
<td>128</td>
<td>112</td>
</tr>
<tr>
<td>Methionine</td>
<td>26</td>
<td>28</td>
<td>27</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>66</td>
<td>91</td>
<td>78</td>
</tr>
<tr>
<td>Leucine</td>
<td>190</td>
<td>206</td>
<td>193</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>45</td>
<td>33</td>
<td>39</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>66</td>
<td>75</td>
<td>70</td>
</tr>
<tr>
<td>Tryptophan$^a$</td>
<td>—</td>
<td>—</td>
<td>20</td>
</tr>
</tbody>
</table>

$^a$ Half-cystine was measured as cysteic acid in a sample oxidized with performic acid (14).

$^b$ Tryptophan was determined spectrophotometrically (15).

analyzes presented in Table II and was used in the estimation of molecular weights. Molecular weights were determined from the slopes of the $\ln h - \ln r$ and in fringe displacement plots and by the midpoint method of Yphantis (10). A relatively high concentration of supporting electrolyte was needed to eliminate concentration dependence. The molecular weights determined by the three methods were in good agreement and indicated a molecular weight for the 10.3 S protein of approximately 240,000 (Table III).

Information concerning the subunit structure of the 10.3 S protein was obtained by sedimentation equilibrium analyses in 6 M guanidine HCl and in 0.1% SDS. As shown in Fig. 5, C and D, in both guanidine HCl and in SDS, plots of $\ln h - \ln r$ against $r^2$ were completely linear, indicating that the protein was homogeneous in these reagents and that the polypeptide chains of the 10.3 S protein were equal in size. The molecular weights obtained from the slopes of the $\ln h - \ln r$ plots are summarized in Table III. An uncorrected partial specific volume of 0.728 was used for these determinations. The partial specific volume of proteins in guanidine HCl has been found to be the same as, or only slightly less than, the partial specific volume in dilute salt solution (20, 21). The partial specific volume and the molecular size of a protein in SDS depend upon the amount of SDS bound

### TABLE III

<table>
<thead>
<tr>
<th>Solvent system and (analytical technique)</th>
<th>protein concentration in guanidine HCl, and in SDS</th>
<th>Average ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer$^a$</td>
<td>r2</td>
<td>238,000 ± 5,000</td>
</tr>
<tr>
<td>Phosphate buffer$^b$</td>
<td>(C, dc/dr at mid-point)</td>
<td>236,000 ± 4,000</td>
</tr>
<tr>
<td>Phosphate buffer$^c$</td>
<td>(ln fringe versus $r^2$)</td>
<td>226,000 ± 23,000</td>
</tr>
<tr>
<td>6 M guanidine HCl$^d$</td>
<td>(ln h - ln r versus $r^2$)</td>
<td>43,000 ± 6,000</td>
</tr>
</tbody>
</table>

$^a$ Potassium phosphate, 0.05 M, pH 7; NaCl, 0.1 M; EDTA, 0.1 mM; DTT, 1.0 mM; SDS, 0.1%.

$^b$ Tryptophan was determined spectrophotometrically (15).

$^c$ Methionine was measured as cysteic acid in a sample oxidized with performic acid (14).

$^d$ Tryptophan was determined spectrophotometrically (15).
to the protein. However, even if as much as 0.1 g of SDS was bound per g of protein, the assumption that no SDS was bound to the protein would result in a molecular weight that was only 4% greater than the actual value (22). A molecular weight of approximately 40,000 was therefore indicated for the polypeptide chains of the 10.3 S protein (mol wt 240,000). The fact that the polypeptide chains of the 10.3 S protein were equal in size was also shown by sedimentation velocity experiments. After treatment with SDS the protein sedimented as a single peak at 2.0 S (Fig. 3D).

The requirement for reducing agents for the stabilization of the 10.3 S protein suggested that the observed spontaneous dissociation involved sulfhydryl groups. Treatment of the protein with a 40-fold molar excess of CMB or o-iodoacetamide for 15 min at 4°C resulted in the quantitative conversion of the 10.3 S protein to the two, more mobile, electrophoretic components and in the total loss of anthranilate synthetase (Fig. 2C). On the other hand, the protein was surprisingly stable in iodoacetamide. No dissociation or enzyme inactivation was observed after 15-min exposure at room temperature to a 40-fold molar excess of iodoacetamide, pH 7.0. A more detailed analysis of the effect of CMB is shown in Fig. 6. Titration of the sulfhydryl groups of the protein by the method of Boyer (23) showed that modification of even an average of one sulfhydryl group resulted in the loss of some anthranilate synthetase activity. Thereafter inactivation proceeded in a nearly linear manner until complete inactivation was observed after 15 min at 25°C. The CMB-treated sample (1 ml) was made dense with sucrose ions. EDTA (1.0 mM) was included in the electrophoresis buffer.

Fig. 7. Microdensitometer traces of analytical gels: A, the 10.3 S protein in 0.1 mM DTT; B, the 10.3 S protein in 0.1 mM CMB for 15 min at 25°C; C, the 10.3 S protein in 0.1 mM CMB for 15 min, followed by 1 mM DTT for 5 min; D, the 10.3 S protein in just enough CMB to titrate fully all of the sulfhydryl groups (cf. the final titration point in Fig. 6). Traces were made as described in Fig. 6. In A, B, and C, the electrophoresis buffer contained 1.0 mM GSH. No reducing agent was used in D. Except as modified above, the samples were prepared as described in Fig. 6.

Fig. 8. Preparative electrophoresis of the CMB-treated 10.3 S protein. Six milligrams of the purified 10.3 S protein were equilibrated with 0.01 M potassium phosphate buffer, pH 7.0 (minus EDTA and reducing agent), by filtration through Sephadex G-25 (fine). Just enough CMB was added to titrate 20 sulfhydryl groups per 240,000 molecular weight protein molecule. After 20-min incubation at 25°C, the CMB-treated sample (1 ml) was made dense with sucrose and siphoned through the electrophoresis buffer onto the surface of an 80-ml resolving gel. Since a spacer gel was not used, the gel was previously run at 30 mA for 4 hours to eliminate persulfate ions. EDTA (1.0 mM) was included in the electrophoresis buffer but no reducing agent was used. In all other respects the procedure was as that described under "Purification Procedure." Enzymic activities were measured in the presence of 1.0 mM DTT. No enzymic activity could be observed in the absence of DTT.

dissociation of the 10.3 S protein. On the other hand, InGP synthetase proved to be fully active even after an average of 14 sulfhydryl groups were titrated and after the 10.3 S protein had dissociated. As can be seen in Fig. 6, InGP synthetase was linearly inactivated by titration of the remaining sulfhydryl groups but the inactivation was not due to the dissociation of the 7.4 S fragment since acrylamide gels of the inactivated sample revealed the 7 S band to be intact. However, prolonged exposure to excess CMB was found to accomplish dissociation of the 7.4 S fragment. The 10.3 S protein was treated with 1.0 mM

Fig. 8. Preparative electrophoresis of the CMB-treated 10.3 S protein. Six milligrams of the purified 10.3 S protein were equilibrated with 0.01 M potassium phosphate buffer, pH 7.0 (minus EDTA and reducing agent), by filtration through Sephadex G-25 (fine). Just enough CMB was added to titrate 20 sulfhydryl groups per 240,000 molecular weight protein molecule. After 20-min incubation at 25°C, the CMB-treated sample (1 ml) was made dense with sucrose and siphoned through the electrophoresis buffer onto the surface of an 80-ml resolving gel. Since a spacer gel was not used, the gel was previously run at 30 mA for 4 hours to eliminate persulfate ions. EDTA (1.0 mM) was included in the electrophoresis buffer but no reducing agent was used. In all other respects the procedure was as that described under "Purification Procedure." Enzymic activities were measured in the presence of 1.0 mM DTT. No enzymic activity could be observed in the absence of DTT.
CMB for 1 hour at room temperature and 4 to 5 hours at 4°C. Sedimentation velocity experiments on such a sample revealed a single peak at 4.2 S with no material visible at 7 S (Fig. 3B). In addition, excess CMB even for short exposure periods was found to accomplish the dissociation of some of the 7.4 S fragment. The microdensitometer trace shown in Fig. 7D indicates that the concentration of 7 S band in the analytical gel is approximately 2-fold greater than the 4 S band. While in Fig. 7, B and C, the 4 S band is in greater concentration. This result can be ascribed to the fact that in the case presented in Fig. 7D just enough CMB was used to titrate all of the sulfhydryl groups of the protein while in Fig. 7, B and C, a 40-fold molar excess of CMB was used. As can be seen in Fig. 7C, the effects of CMB were reversed to some extent by the addition of a 10-fold molar excess of DTT. Approximately 20% of the total protein was restored to the original 10.3 S state. A comparable level of anthranilate synthetase activity was also recovered.

The 4 S and 7 S fragments of the 10.3 S protein were then studied in more detail. A purified sample (6 mg) of 10.3 S protein was first treated with just enough CMB to titrate all of the sulfhydryl groups in the protein molecule and was then subjected to preparative electrophoresis (Fig. 8). As anticipated from analytical gels (Fig. 2C), only two peaks of protein were eluted during electrophoresis and no protein was evident beyond Fraction 27, indicating that the 10.3 S protein was completely dissociated. The first peak of absorbing material (280 nm) represents the glycine front and appears to be a nonproteinaceous contaminant of Tris. The leading protein peak contained approximately 1.5 mg of protein and migrated in coincidence with the 4 S band in analytical gels. The trailing peak contained approximately 3 mg of protein and corresponded to the 7 S band. No enzymic activity was observed in either of the protein peaks until DTT was added to the assay mixture. With 1.0 mM DTT in the reaction mixtures, anthranilate synthetase activity was found, but only in the leading peak of 4 S protein, while InGP synthetase activity was only found in the trailing peak of 7 S protein. PR-anthranilate isomerase is not shown but was also only present in the 7 S peak. The appearance of anthranilate in the anthranilate synthetase assay was not linear for the first 2 min of the reaction unless the 4 S protein was previously treated for several minutes with DTT. However, preliminary treatment of the 7 S protein with DTT was not necessary to obtain linear rates in the InGP synthetase assay. Sucrose gradient analyses were then performed on the separated 7.4 S and 4.4 S fragments (isolated in the same way in a second experiment). The InGP synthetase and PR-anthranilate isomerase activities...
The preceding data showed that CMB was capable of dissociating the 10.3 S protein into two distinct fragments, one of which was 4.4 S containing the protein (proteins) necessary to catalyze the anthranilate synthetase reaction and the other of which was 7.4 S containing the protein (proteins) necessary to catalyze the InGP synthetase and PR-anthranilate isomerase reactions. Proof that the 4.4 S and 7.4 S fragments in fact represented distinct proteins was obtained by electrophoresis of the 10.3 S protein and the isolated 4.4 S and 7.4 S fragments in analytical 8 M urea-polyacrylamide gels. As can be seen in Fig. 10A, the 10.3 S protein migrated as two bands in 8 M urea. The 4.4 S fragment migrated in coincidence with the leading band (Fig. 10C) and was free of the slower moving species, while the 7.4 S fragment migrated in coincidence with the trailing band (Fig. 10B) and was only slightly contaminated with the faster moving species.

**DISCUSSION**

A multienzyme complex has been defined as an "order association (not involving peptide linkages) of various enzymes that catalyze successive steps in a reaction sequence" (24). We have shown here that in N. crassa the anthranilate synthetase, PR anthranilate isomerase, and InGP synthetase reactions are catalyzed by a single protein molecule having a sedimentation coefficient of 10.3 S and a molecular weight of 240,000. Although two of the enzymes involved, PR-anthranilate isomerase and InGP synthetase, may be linked together through a peptide bond, and, although the enzyme steps involved are not completely successive, the present enzymic system does at least contain all of the properties outlined in the above definition and can therefore be accurately described as a multienzyme complex.

Fig. 11 provides a summary of the effects of CMB, o-iodosobenzoate, DTT, guanidine HCl, urea, and SDS on the multienzyme complex. Represented are: A1, the proposed hexameric structure of the 10.3 S multienzyme complex (mol wt 240,000); A2, the proposed dimeric structure of the inactive 4.4 S fragment (mol wt 80,000) which is associated with anthranilate synthetase activity; I, the proposed tetrameric structure for the 7.4 S fragment (mol wt 160,000) which catalyzes PR-anthranilate (PRA) isomerase and InGP synthetase. A, the proposed subunit or structural gene product which is required for anthranilate synthetase activity (2.9 S, mol wt 40,000); I, the proposed subunit or structural gene product which contains the active sites for PR-anthranilate isomerase and InGP synthetase activities (2.9 S, mol wt 40,000); X, the unknown structure of the protein which catalyzes the 7 S anthranilate synthetase activity (probably either A or A1).

**FIG. 11. Summary of the effects of CMB (pCMB), o-iodosobenzoate (IOB), DTT, guanidine HCl, urea, and SDS on the multienzyme complex.** Represented are: A1, the proposed hexameric structure of the 10.3 S multienzyme complex (mol wt 240,000); A2, the proposed dimeric structure of the inactive 4.4 S fragment (mol wt 80,000) which is associated with anthranilate synthetase activity; I, the proposed tetrameric structure for the 7.4 S fragment (mol wt 160,000) which catalyzes PR-anthranilate (PRA) isomerase and InGP synthetase. A, the proposed subunit or structural gene product which is required for anthranilate synthetase activity (2.9 S, mol wt 40,000); I, the proposed subunit or structural gene product which contains the active sites for PR-anthranilate isomerase and InGP synthetase activities (2.9 S, mol wt 40,000); X, the unknown structure of the protein which catalyzes the 7 S anthranilate synthetase activity (probably either A or A1).
ase and InGP synthetase activities. However, only a small amount of the active tetrameric form of anthranilate synthetase is ever seen and, assuming that the tetramer has about the same specific activity as the normal complex, less than 1% of the protein can be accounted for by the activity. It is, therefore, not possible to determine from the results presented here whether the anthranilate synthetase tetramer is composed, as might be expected, solely of A subunits. On the contrary, evidence from complementation analyses indicates that the anthranilate synthetase tetramer may in fact contain two inactivite subunits of the protein which catalyze the PR-anthranilate isomerase and InGP synthetase activities. Amidon black staining would not be sensitive enough to detect the presence of the contaminating subunit in our acrylamide gels if only enough of the contaminating subunit is present to account for the small amount of anthranilate synthetase activity which is observed. Contamination of the 4.4 S A2 preparation by the protein contained in the 7.4 S fragment of PR-anthranilate isomerase and InGP synthetase could occur through the dissociation of a small portion of the 7.4 S protein to a 4 S state. Careful titration of the complex with CMB avoids any major dissociation of the 7.4 S fragment but it may not be avoided completely. Attempts to isolate and characterize the tetrameric form of anthranilate synthetase have so far been unsuccessful because of the unstable nature of this species.

The PR-anthranilate isomerase and InGP synthetase component isolated after polyacrylamide electrophoresis of the CMB-dissociated complex appears to be a tetramer of the 40,000 molecular weight subunits both by sucrose gradient analyses of the enzymic activities and by analyses of the protein in the analytical ultracentrifuge. A sedimentation coefficient of 7.4 S is again consistent with a molecular weight of 160,000. An A4 structure is indicated for this tetramer since it is free of anthranilate synthetase activity (but retains activity for PR-anthranilate isomerase and InGP synthetase at a level comparable to that found in the 10.3 S complex) and since polyacrylamide electrophoresis in 8 M urea reveals the protein to be distinct from the protein in the A2 component. In accord with the above description of the products of SDS, guanidinium HCl, and CMB dissociation, we have assigned an A4 structure to the complex. In additional support of this model, quantitative determination of the fragments produced by careful titration of the complex with CMB provides a 1:2 ratio in the concentration of the A subunit to the I subunit.

Genetic evidence (3) shows that the coding information for PR-anthranilate isomerase and InGP synthetase is within a single gene, which suggests that the active sites for these two enzymic activities exist within a single polypeptide chain. Since anthranilate synthetase is at least in part specified by a single gene, which suggests that the active site for anthranilate synthetase is not on the same polypeptide chain (i.e. the I subunit) we must conclude either that the polypeptide chains involved are electrophoretically and ultracentrifugally indistinguishable or that they cannot be dissociated by 8 M urea. Although both possibilities seem unlikely, a final answer to this question must await the chemical analysis of the I2 fragment.

Titration of the sulphydryl groups of the complex with CMB reveals that anthranilate synthetase is particularly sensitive to this reagent. However, an average of 12 sulphydryl groups must be titrated in order to inactivate anthranilate synthetase fully. The nearly linear relationship between the percentage of sulphydryl groups titrated and the percentage inactivation of anthranilate synthetase indicates an "all or none" reaction similar to the reaction of hemerythin with CMB (25). The fact that inactivation of anthranilate synthetase by titration with CMB parallels dissociation of the complex suggests that loss in anthranilate synthetase activity may be due to dissociation rather than directly due to the oxidation of some enzymically essential sulphydryl group (groups). This view is supported by the fact that both the complex and the anthranilate synthetase activity are stable in the presence of iodoacetamide. Apparently the bulky, hydrophobic nature of CMB is important in the dissociation of the complex which in turn leads to the inactivation of anthranilate synthetase. The fact that the complex dissociates spontaneously in the absence of DTT suggests that oxidation of the sulphydryl groups by molecular oxygen is also capable of bringing about the dissociation, perhaps by the formation of intrapoly-peptide chain disulfide bridges. Similarly, iodoacetobenzene is thought to oxidize sulphydryl groups to disulfide bridges (26) and is effective in dissociating the complex. The formation of intramolecular disulfide bridges or CMB mercaptides may create unfavorable configurational changes which lead to the dissociation of the complex. Apparently no disulfide bridges normally exist in the complex since the three activities are stable in a highly reducing environment (0.01 M DTT) and since the same number of sulphydryl groups are calculated by titration of the native protein with CMB as by amino acid analysis. Dissociation of the complex with CMB does not lead to the loss of InGP synthetase activity which shows that the formation of the multienzyme complex is not necessary for InGP synthetase activity in the same way as it is for anthranilate synthetase. The fact that InGP synthetase is only inactivated after 14 sulphydryls have been titrated shows that the sulphydryl groups important for InGP synthetase activity are less available to titration than those involved with the dissociation of the complex and with the inactivation of anthranilate synthetase.

The physiological significance of the present multienzyme complex is not yet understood. Although genetic and physiological studies (1-5) make it unlikely that the complex is an artifact of cell division, it is possible that the complex as isolated is incomplete. Until now, all of the multienzyme complexes which have been purified (16, 27, 28) catalyze a consecutive series of enzymic reactions. Anthranilate-5-phosphoribosylpyrophosphate phosphoribosyltransferase, the enzyme immediately preceding PR-anthranilate isomerase in the series of enzymic reactions leading to the biosynthesis of tryptophan, is notably absent from the anthranilate synthetase-PR-anthranilate isomerase-InGP synthetase multienzyme complex of N. crassa. It has been suggested that the physiological importance of the multienzyme complex lies in the channeling of biochemical inter-

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mediates (24). Although this concept is an attractive one, no
definitive proof of its validity as a biological principle has been
presented. We must conclude either that the present multien-
yzyme complex has an alternative physiological importance or
that it is only a fragmentary expression of the true organization
which exists in the cell, an organization which includes anthra-
nilate-5-phosphoribosylpyrophosphate phosphoribosyltrans-
ferase.

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