CHAPTER III

PARTIAL PURIFICATION AND PROPERTIES

OF GTP CYCLOHYDROLASE FROM B. ASHYII.
It was established that conversion of adenine, hypoxanthine or xanthine to riboflavin must pass through guanine (1-5). The question, whether the precursor is guanine, guanine riboside or guanine ribotide has been answered recently.

Mehta, Mattoo and Modi (6) have shown that U-14C GMP was taken up intact by E.ashbyii and was incorporated into riboflavin as well as in blue fluorescent compound (BFC), and the radioactivity from this BFC was incorporated into riboflavin by the cell free extract of E.ashbyii.

A considerable body of evidence shows that the biosynthesis of all pteridines and related compounds studied so far begins at the level of GTP. This was shown for folic acid (7), neopterin (8), biopterine (9) toxoflavin (10) and pyrolopyrimidine nucleosides (11). The initial step in all cases consists of the removal of C-8 from the imidazole ring of GTP.

An enzyme from E.coli that catalyzes formation of formic acid from C-8 of GTP and dihydronopterin triphosphate has been reported (7) and purified (12) by Brown and associates. The enzyme was termed as GTP cyclohydrolase and the reaction it catalyzes is the first step in a series of reactions in biosynthesis of pterin part of folic acid. It was suggested that the biosynthesis of riboflavin may follow the same general path (13,14).
Recently Poor and Brown reported in *E. coli*, the presence of a similar activity which also catalyzed removal of C-8 of GTP as formic acid. However, instead of dihydro-neopterin, a pyrimidine derivative and pyrophosphate were produced (15). The authors implicated its role in riboflavin biosynthesis in *E. coli* and named it GTP cyclohydrolase II to distinguish it from the first enzyme, GTP cyclohydrolase I. Almost simultaneously, Mailander and Bacher suggested GTP as the precursor of flavinogenesis while working with a mutant of *S. typhimurium* (16).

Apart from the work of a number of investigators on riboflavin synthetase, the last enzyme of the pathway (17), nothing was known about the early enzymes involved in the biosynthesis of riboflavin until Brown and colleagues reported GTP cyclohydrolase II, a deaminase and a reductase in *E. coli* (15,18). Much of the work in elucidation of riboflavin biogenetic pathway has been carried out in weak overproducers like bacteria (13,15,19,20) and yeasts (21,22).

As in case of other organisms, except the last step, the other intermediates and the enzymes catalyzing their formation are unknown in a strong overproducer like *E. ashbyii*. Though Mitsuda *et al* (23), using resting cell system, indicated that the nucleotide precursor of riboflavin in *E. ashbyii* is GTP, a more convincing enzymatic evidence is not available.
The present studies with flavinogenic *E. ashbyii* support GTP as nucleotide precursor for flavinogenesis and report the presence of GTP cyclohydrolase with some properties of the partially purified preparation.

The correlation between the appearance of riboflavin and GTP cyclohydrolase with respect to growth is shown in Fig. 1. It is evident from the figure, that riboflavin synthesis starts relatively late in the growth stage and GTP cyclohydrolase formation precedes riboflavin synthesis. The enzyme showed exponential rise up to the third day and the peak was attained on the fourth day. Further incubation showed marginal decrease in the level.

The partial purification of this enzyme was carried out and some of its properties were studied because of the following four reasons, (i) GTP has been strongly suggested as a precursor of riboflavin, (ii) GTP cyclohydrolase has been postulated to catalyse first step in a series of reactions leading to riboflavin formation by chanelling cellular pool of GTP, (iii) GTP is a key compound in cell metabolism that can act as a precursor for nucleic acid synthesis and a variety of products (iv) and (iv) to establish role of GTP cyclohydrolase in flavinogenesis in *E. ashbyii*.

All operations of purification were carried out at 0-4°C. A four day old culture of *E. ashbyii* was used to isolate the enzyme. To the cell free extract prepared, as
Figure-1: Kinetics of flavin formation (□) GTP cyclohydrolase activity (△) and growth (○) of _E. ashbyii_.

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described in Chapter II, crystalline ammonium sulfate was gradually added with constant slow stirring until 30 percent saturation was attained (176 gm/litre). The mixture was stirred for additional 3 hrs and centrifuged at 10,000 X g for 10 minutes. The precipitates were discarded after testing for activity and, to the resultant supernatant, more ammonium sulfate was added with care, to obtain 60 percent saturation (198 gm/litre). The mixture was left overnight at 0-4°C after dissolving ammonium sulfate. The mixture was centrifuged at 15,000 X g for 15 min. The supernatant was discarded after checking the activity. The precipitates obtained were dissolved in 0.01 M tris-HCl buffer (pH 7.5) and dialysed against the same buffer. The dialysed enzyme was passed through a sephadex G-200 column (1.5 x 30 cm) previously equilibrated with 0.01 M tris-HCl buffer, pH 7.5.

The purification summary of GTP cyclohydrolase is described in Table-I. About 74 fold purification was achieved. The fractions showing maximum activity (fractions 7,8 and 9) were pooled and used for further studies. A typical elution profile of GTP cyclohydrolase on sephadex G-200 column is shown in Fig.2.

The molecular weight (M.W.) of the enzyme was estimated at 250,000 (Fig.3) by gel filtration on sephadex G-200 column previously equilibrated with standard proteins, viz. catalase (230,000), bovine serum albumin (dimer=137,000 and monomer=68,000) and lysozyme (14,400). GTP cyclohydrolase from
Table 1: Purification summary of GTP cyclohydrolase of E. ashbyii.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total Protein (mg)</th>
<th>Total Units* (mg)</th>
<th>Total Volume (ml)</th>
<th>Protein mg/ml</th>
<th>Specific Activity Units/mg Protein</th>
<th>Fold purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>311.50</td>
<td>319.59</td>
<td>25.0</td>
<td>12.46</td>
<td>1.026</td>
<td>1.00</td>
</tr>
<tr>
<td>Ammonium sulfate ppt. (30-60%)</td>
<td>101.36</td>
<td>230.08</td>
<td>7.0</td>
<td>14.48</td>
<td>2.270</td>
<td>2.21</td>
</tr>
<tr>
<td>Sephadex G-200 filtration (fraction 8)</td>
<td>1.30</td>
<td>98.35</td>
<td>5.0</td>
<td>0.260</td>
<td>75.65</td>
<td>73.73</td>
</tr>
</tbody>
</table>

* A unit of enzyme is defined as the amount of enzyme that catalyzes formation of 1 μmole of formic acid per 60 min. at 30°C.
Figure-2: Gel filtration of GTP cyclohydrolase on sephadex G-200 column (1.5 X 30 cm). Ammonium sulfate precipitates were loaded on the column and the effluent fractions (5.0 ml) were collected at a flow rate of 20 ml/hr. Key: Protein (0) and GTP cyclohydrolase activity (●).
Figure-3: Molecular weight determination by gel-sieving of GTP cyclohydrolase from *E. ashbyii*.
different sources have been reported to have their molecular weights ranging between 44,000 to 650,000. GTP cyclohydrolase II, implicated to play a role in flavinogenesis in \textit{E. coli}, is reported to have M.W. of 44,000 (15). Other GTP cyclohydrolases known to catalyze first step in folic acid biosynthesis, have M.W. of 170,000, 210,000, 500,000 and 650,000 from \textit{S. indica} (24), \textit{E. coli} (12), \textit{Streptomyces rimosus} (11) and \textit{Comamonas sp.} (25) respectively. Since the M.W. of GTP cyclohydrolase I from \textit{E. coli} is relatively high (250,000), the suggestion was made (7) that the purified GTP cyclohydrolase might be an aggregate containing two or more subunits and that only one of these might possess the catalytic activity for the removal of formate from GTP. Whether \textit{E. ashbyii} GTP cyclohydrolase is made up of a single protein or more subunits is not known.

Treatment of the enzyme with 6 M urea or 1 percent SDS (sodium dodecyl sulfate) in 0.1 M tris-HCl buffer (pH 7.2) for 60 min. at 30°C resulted in complete loss of activity. The treated enzyme after dialysis against cold buffer for 5-6 hrs. did not show any activity under our experimental conditions.

In our studies to confirm that \textit{E. ashbyii} GTP cyclohydrolase participates in biogenesis of riboflavin, the UV absorption spectra of the enzymatic product was analysed at different time intervals upto 60 min. A time dependent increase in absorbance at 290 nm (Fig.4) indicated that the
Figure-4 : UV absorption spectra of a reaction mixture at different time. A reaction mixture containing (in μmoles) tris-HCl buffer (pH 8.0), 100; GTP, 2.5; Mg^{2+}, 2.0 and appropriate enzyme concentration in a total volume of 2.0 ml was incubated at 30°C and the spectrum was taken at the times indicated in min. The same mixture without GTP was used as the blank.
reaction catalyzed by E. ashbyii GTP cyclohydrolase yielded a product that has been proposed as an intermediate in flavinogenesis. This observation suggests the formation of a pyrimidine product participating in riboflavin biogenesis.

The optimum conditions for the enzyme reaction were studied. The effect of pH on the activity of enzyme was investigated over a wide pH range. The optimum pH for maximal activity of E. ashbyii GTP cyclohydrolase was found to be 8.0 (Fig. 5-A) and was 8.5 for E. coli enzyme (15). The optimum temperature for the enzyme was 30°C (Fig. 5-B) and that for E. coli GTP cyclohydrolase II, was 42°C (15). A shift in temperature on either side of 30°C considerably reduced the activity. High enzyme protein concentration inhibited enzyme action (Fig. 5-C). The enzyme activity as a function of time was found to be linear up to 60 min. (Fig. 5-D).

The effect of divalent cations on the activity of GTP cyclohydrolase is listed in Table-2. Among all the cations tested Mg$^{2+}$ was the most effective stimulator followed by Mn$^{2+}$, Co$^{2+}$, Zn$^{2+}$ and Ba$^{2+}$ inhibited enzyme activity. Mg$^{2+}$ at 1.0 mM concentration in cell free extract stimulated activity by approximately 2.5 fold. EDTA (2.0 mM) addition prior to incubation in assay system containing Mg$^{2+}$ reduced the activity to half the control value.
Figure-5: pH profile (A), enzyme activity as a function of temperature (B), initial reaction velocity as a function of enzyme protein concentration (C) and time (D) of *E. ashbyii* GTP cyclohydrolase.
Table-2: Effect of divalent cations on *E. ashbyii*
GTP cyclohydrolase.

<table>
<thead>
<tr>
<th>Metal ions*</th>
<th>Enzyme activity (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None, Control</td>
<td>0.441</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>1.124</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>0.763</td>
</tr>
<tr>
<td>Fe$^{2+}$</td>
<td>0.404</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>0.462</td>
</tr>
<tr>
<td>Cu$^{2+}$</td>
<td>0.233</td>
</tr>
<tr>
<td>Co$^{2+}$</td>
<td>0.111</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>0.104</td>
</tr>
<tr>
<td>Ba$^{2+}$</td>
<td>0.088</td>
</tr>
<tr>
<td>Mg$^{2+}$ + EDTA **</td>
<td>0.201</td>
</tr>
</tbody>
</table>

* All the cations were added at 1.0 mM concentration.

** EDTA was added at 2.0 mM concentration.
The heat inactivation kinetics of GTP cyclohydrolase at 40°C and 50°C are plotted in Fig.-6. The activity decreased rapidly at both the temperatures. The half life at 40°C and 50°C were 6.5 and 3.5 minutes respectively, suggesting heat labile nature of the enzyme. The heat sensitivity of the enzyme is in agreement with the strict temperature dependence of flavinogenesis in E.ashbyii (26,27).

The enzyme was tested for its substrate specificity with various nucleotides. No noticeable activity was found with GDP, GMP and guanosine. But unlike E.coli GTP cyclohydrolase, this enzyme exhibited about 15 percent activity with guanine. ATP, AMP (adenosine monophosphate), XTP and XMP did not serve as substrates (Table-3), indicating high specificity of enzyme for GTP. As a result of this observation, the substrate saturation kinetics for GTP cyclohydrolase with varying concentrations of GTP and guanine were studied. The curves hyperbolic (inset-A, Fig.-7). The Lineweaver-Burk plot was linear (Fig.-7). Km values for GTP and guanine were 5.88 x 10^{-4}M and 4 x 10^{-3}M respectively. This Km value for GTP is much higher than that reported for E.coli GTP cyclohydrolase II (15). The substrate dependence (inset-A) and Lineweaver-Burk plot of GTP cyclohydrolase in the presence of different concentrations of guanine are reported in Fig.8.

At all concentrations tested, guanine inhibited the enzyme activity in a competitive manner with the gradual increase in Km. At 12 mM guanine, Km for GTP was increased by about
Figure-6: Heat stability of GTP cyclohydrolase from *E. ashbyii*. 
Table 3: Substrate specificity of GTP cyclohydrolase from *E. ashbyii*.

<table>
<thead>
<tr>
<th>Substrate*</th>
<th>Activity Units/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guanosine-5'-triphosphate (GTP)</td>
<td>0.955</td>
</tr>
<tr>
<td>Guanosine-5'-diphosphate (GDP)</td>
<td>-</td>
</tr>
<tr>
<td>Guanosine-5'-monophosphate (GMP)</td>
<td>0.052</td>
</tr>
<tr>
<td>Guanosine</td>
<td>-</td>
</tr>
<tr>
<td>Guanine</td>
<td>0.139</td>
</tr>
<tr>
<td>Adenosine-5'-triphosphate (ATP)</td>
<td>-</td>
</tr>
<tr>
<td>Adenosine-5'-monophosphate (AMP)</td>
<td>-</td>
</tr>
<tr>
<td>Xanthosine-5'-triphosphate (XTP)</td>
<td>-</td>
</tr>
<tr>
<td>Xanthosine-5'-monophosphate (XMP)</td>
<td>-</td>
</tr>
</tbody>
</table>

* Substrates were used at a concentration of 1.25 mM.

(-) means no activity.
Figure 7A: Substrate saturation curves with respect to GTP (▲) and guanine (●) for GTP cyclohydrolase.

B: The Lineweaver-Burk plot of data from A.
Figure-8, A: Substrate saturation curves of the initial reaction velocity versus GTP concentrations at several fixed concentrations of guanine. The following concentrations of guanine were used: 0.0 (▲), 0.4 mM (○), 4.0 mM (□) and 12.0 mM (●).

B: The Lineweaver-Burk plot of data from A.

C: The replot of slope versus inhibitor concentrations.
6 fold, indicating lowered affinity of GTP for the enzyme. The secondary plot of guanine concentrations versus slope of the double reciprocal plot (inset-c, fig.-8) gave $K_i$ for guanine 2.5 mM. GTP cyclohydrolase from other sources have been reported to be inhibited competitively by GDP (24) or ATP, dGTP, guanosine-5'-tetraphosphate and XTP (12,15).

As an essential step towards the understanding of the regulatory mechanism of flavinogenesis, the product inhibition studies were carried out. The inhibition by pyrophosphate and formic acid at several fixed concentrations against varying concentrations of GTP were non-competitive (Figures 9 and 10). HTP, when tested at several fixed concentrations against varying concentrations of GTP, exhibited competitive inhibition pattern with a change in $K_m$ for GTP (Fig.11). The difference in the degree of inhibition is evidenced by the difference in $K_i$ values, for the three end products, obtained from secondary plots (Insets Figs.9-11). HTP and pyrophosphate with $K_i$ values 2.1 mM and 3.6 mM respectively seemed to be much more effective inhibitors than formate with higher $K_i$ (11.4 mM). The effect of these reaction end products in various combinations (at a concentration equivalent to their $K_i$ values) was studied as, under in vivo condition, they may act in a coordinated fashion (Table-4). Pyrophosphate and HTP together showed maximum (75 percent) inhibition among the
Figure-9, A: Substrate saturation curves of the initial reaction velocity versus GTP concentrations at several fixed concentrations of pyrophosphate. The following concentrations of pyrophosphate were used: 0.0 mM (▲), 0.5 mM (○), 5.0 mM (□) and 10 mM (●).

B: The Lineweaver-Burk plot of data from A.

C: The replot of slope versus inhibitor concentrations.
Figure-10, A: Substrate saturation curves of the initial reaction velocity versus GTP concentrations in the presence of 0.0 mM (▲), 0.5 mM (○), 5.0 mM (□) and 10.0 mM (●) formate concentrations.

B: The Lineweaver-Burk plot of data from A.

C: The replot of slope versus inhibitor concentrations.
Figure 11, A: Substrate saturation curves of the initial reaction velocity versus GHDP concentrations in the presence of 0.0 mM (▲), 0.5 mM (●), 2.5 mM (□) and 10.0 mM (●) HTP concentrations.

B: The Lineweaver-Burk plot of data from A.

C: The replot of slope versus inhibitor concentrations.
Table 4: Effect of reaction end products in combinations on GTP cyclohydrolase.

<table>
<thead>
<tr>
<th>Additions*</th>
<th>Residual activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None, Control</td>
<td>100</td>
</tr>
<tr>
<td>Pyrophosphate + HTP**</td>
<td>24.12</td>
</tr>
<tr>
<td>Pyrophosphate + Formate</td>
<td>40.33</td>
</tr>
<tr>
<td>HTP + Formate</td>
<td>38.82</td>
</tr>
<tr>
<td>Pyrophosphate + HTP + Formate</td>
<td>10.20</td>
</tr>
</tbody>
</table>

* The concentration of the endproduct was equivalent to its Ki value.

** 6-hydroxy-2,4,5-triaminopyrimidine (HTP).
three pairs of combinations tested. However, 90 percent inhibition of GTP cyclohydrolase was noted when all the three end products were added at their Ki values.

The first evidence of an enzyme catalyzed reaction as the first step in riboflavin synthesis in \textit{E. coli} (weak over producer) was given by Poor and Brown (15). The enzyme, named as GTP cyclohydrolase II (M.W. = 44,000) was purified 2200 fold and used GTP as substrate to form in stoichiometric amounts, formate, pyrophosphate and a purine derivative, at 42°C and at pH 8.5. Since GTP cyclohydrolase II apparently is not concerned with biosynthesis of folic acid, the possible physiological role of this enzyme in the biosynthesis of riboflavin was considered in the light of the observations of Poor and Brown and the previous published work on riboflavin biosynthesis by other investigators.

Several pertinent observations strongly indicate involvement of GTP cyclohydrolase from \textit{E. ashbyii} in flavinogenesis. For example, GTP cyclohydrolase from \textit{E. ashbyii} increased just prior to riboflavin formation and reached the peak value during active flavinogenesis. Secondly, this flavinogenic mold is not known to synthesize folic acid in significant amounts. It is known that a proposed intermediate of riboflavin biosynthetic pathway, 6-hydroxy-2,4,5-triaminopyrimidine (21) gives a peak at 290 nm while the intermediate of folic acid biosynthesis - dihydroneopterine triphosphate (28,29) shows absorption band between 325 nm
to 345 nm. The time dependent increase in absorbance at 290 nm was noted with GTP cyclohydrolase. The formation of pyrophosphate along with formate and HTP further supported the suggestion.

The studies on substrate specificity gave evidence to believe that GTP is the immediate nucleotide precursor in E. ashbyii and that GTP cyclohydrolase catalyzes the first step in riboflavin biosynthesis.

GTP cyclohydrolase from E. ashbyii catalyzed formation of three products using single substrate like GTP cyclohydrolase II from E. coli; but unlike E. coli enzyme the former enzyme is a high molecular weight protein. GTP cyclohydrolases from other organisms have been reported to exist as multiple forms (28,25,11). Since GTP cyclohydrolase I apparently catalyzes four chemical reactions, the suggestion was made earlier (7) that the catalytic unit may be a multi-enzyme aggregate. However, Yim and Brown indicated (12) that the catalytic unit is a single enzyme probably consisting of eight identical polypeptide chains and that this single enzyme catalyzes all of the chemical reactions necessary for the conversion of GTP to dihydroneopterin triphosphate. From the preliminary studies on the effect of dissociating agents on partially purified GTP cyclohydrolase it may be assumed that the treatment resulted in denaturation of the enzyme or irreversible dissociation of GTP cyclohydrolase into inactive monomers.
Meyer and Switzer (30) recently reported that 3 pairs of nucleotide inhibitors which were weak inhibitors when tested singly, exhibited pronounced synergistic inhibition on glutamine phosphoribosyl pyrophosphate amidotransferase. However, the data in Table-4 showed neither synergistic nor additive effect. HTP and pyrophosphate together reduced the activity by 75% and are likely to be important regulators of GTP cyclohydrolase in the cell. Though, the maximum inhibition (90%) is observed in the presence of all the 3 products (at Ki value), this situation may not arise in the cell, as it is unlikely that the concentration of formate, under invivo condition, will reach up to its Ki value (11.4 mM) to regulate flavinogenesis.

In the case of HTP, its affinity for the enzyme (competitive inhibition pattern) might be inhibiting the binding of GTP to GTP cyclohydrolase. Therefore, the lower Ki value of HTP may not be as important as its slow release from the enzyme. This is because HTP formed will be immediately acted upon by the next enzyme in riboflavin biosynthetic pathway, thus preventing its accumulation. On the other hand pyrophosphate formed by GTP cyclohydrolase may accumulate to reach the concentration equivalent to its Ki value.

Thus, GTP cyclohydrolase from E.ashbyii catalyzes first step in a series of reactions leading to riboflavin formation, using GTP as substrate and seems to be feedback inhibited by pyrophosphate - one of the end products - which in turn would regulate flavinogenesis.
References Cited:


