Biosynthesis of Riboflavin

SINGLE TURNOVER KINETIC ANALYSIS OF GTP CYCLOHYDROLASE II*

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GTP cyclohydrolase II catalyzes the conversion of GTP into a mixture of 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5'-phosphate (Compound 2), formate, and pyrophosphate. Moreover, GMP was recently shown to be formed as a minor product. The major product (Compound 2) serves as the first committed intermediate in the biosynthesis of the vitamin, riboflavin. Numerous pathogenic microorganisms are absolutely dependent on endogenous synthesis of riboflavin. The enzymes of this pathway are therefore potential drug targets, and mechanistic studies appear relevant for development of bactericidal inhibitors. Pre-steady state quenched flow analysis of GTP cyclohydrolase II shows the rate-determining step to be located at the beginning of the reaction sequence catalyzed by the enzyme. Thus, GTP is consumed at a rate constant of 0.064 s⁻¹, and the reaction product, Compound 2, is formed at an apparent rate constant of 0.062 s⁻¹. Stopped flow experiments monitored by multiwavelength photometry are well in line with these data. 2-Amino-5-formylamino-6-ribosylamino-4(3H)-pyrimidinone triphosphate can serve as substrate for GTP cyclohydrolase II but does not fulfill the criteria for a kinetically competent intermediate. A hypothetical reaction mechanism involves the slow formation of a phosphoguanosyl derivative of the enzyme under release of pyrophosphate. The covalently bound phosphoguanosyl moiety is proposed to undergo rapid hydrolytic release of formate from the imidazole ring and/or hydrolytic cleavage of the phosphodiester bond.

GTP cyclohydrolase II catalyzes the first committed step in the biosynthesis of the vitamin, riboflavin. The enzyme was discovered in Escherichia coli by Foor and Brown (1) who noted the apparently simultaneous formation of three products from GTP, namely formate, pyrophosphate, and an organic compound that was assigned as 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5'-phosphate (Compound 2) on basis of indirect evidence (Fig. 1).

GTP cyclohydrolase II constitutes a potential antibiotics target for the following reasons: (i) numerous pathogenic bacteria, in particular Gram-negative organisms, are absolutely dependent on endogenous synthesis of the vitamin, because they are unable to absorb the vitamin from the environment because of the absence of an appropriate uptake system; (ii) GTP cyclohydrolase II is absent in animals that are dependent on exogenous supply of vitamin.

The exploration of novel antibiotic targets is urgent in light of the fast development of microbial resistance against all antibiotic agents that are currently used. Studies on the reaction mechanism of riboflavin biosynthetic enzymes from bacteria could benefit the development of bactericidal inhibitors. It should also be noted that the enzymes of the riboflavin pathway are potential herbicide targets.

The mechanistical details of GTP cyclohydrolase II are incompletely understood. Blau and co-workers (2) reported the formation of two different heterocyclic products but did not determine their structures. The unknown second product was recently identified as the α-anomer of Compound 2, which is formed by spontaneous isomerization of the enzyme product and constitutes an in vitro artifact (3). On the other hand, GMP was recently identified as a genuine enzyme product that is formed by GTP cyclohydrolase II at a rate of about 10% as compared with Compound 2 (3).

The hydrolytic opening of the imidazole ring of GTP catalyzed by GTP cyclohydrolase II has notable parallels in the reaction catalyzed by GTP cyclohydrolase I, which converts GTP to dihydronopterin 3'-triphosphate, the first committed intermediate in the biosynthetic pathways of tetrahydrobiopterin (4–6) and tetrahydrofolate (Fig. 2) (7). GTP cyclohydrolase I was shown to hydrolyze the C-8—N-9 bond of GTP affording the formamide derivative, Compound 4, which is then converted to the 5-aminopyrimidine derivative, Compound 5, by hydrolytic release of formate (8). Compound 5 subsequently undergoes an Amadori reaction followed by ring closure affording the pteridine chromophore of dihydronopterin 3'-triphosphate (Compound 6). Despite the mechanistic similarity, GTP cyclohydrolases I and II have no detectable sequence similarity.

The rate-determining step of GTP cyclohydrolase I occurs relatively late in the reaction catalyzed by GTP cyclohydrolase I (9). In contrast, this paper shows that the rate-determining step of GTP cyclohydrolase II is located at the start of the reaction sequence.

EXPERIMENTAL PROCEDURES

Materials—2-Amino-5-formylamino-6-ribosylamino-4(3H)-pyrimidinone 5'-triphosphate (Compound 4) was prepared as described earlier (8). Recombinant GTP cyclohydrolase II of E. coli was prepared as described (10).

Single Turnover Experiments—Stopped flow experiments were performed with an SFM4/QS apparatus (Bio-Logic, Claix, France) equipped with a linear array of three mixers and four independent syringes. For stopped flow measurements, the content of a 1.5-mm light path quartz cuvette behind the last mixer was monitored with a Tidas diode array spectrophotometer (200–610 nm) equipped with a 15-watt deuterium lamp as light source (J&M Analytische Mess- und Regeltechnik, Aalen, Germany). The reaction buffer contained 50 mM Tris hydrochloride, pH 8.5, and 5 mM MgCl₂. GTP cyclohydrolase II was used at a
concentration of 6.3 mg ml\(^{-1}\) (equivalent to 290 \(\mu\)M enzyme subunits), GTP was used at a concentration of 210 \(\mu\)M, and an equilibrium mixture of Compound 4 isomers (8) was used at a concentration of 440 \(\mu\)M. The enzyme solution was mixed with substrate solution at 30 °C at a ratio of 1:1 with a total flow rate of 4 ml s\(^{-1}\). At that flow rate, the calculated dead time is 7.6 ms. During the reaction, spectra integrated over 48 ms were recorded at intervals of 100 ms.

Quenched flow experiments were performed with a SFM4/QS apparatus (Bio-Logic, Clai, France) equipped with a linear array of mixers, four independent syringes, and a computer-controlled valve. The apparatus was thermostated at 30 °C. A delay loop of 230-\(\mu\)l nominal volume was used. Syringe 1 contained a reaction buffer consisting of 50 mM Tris hydrochloride, pH 8.5, and 5 mM MgCl\(_2\). Syringe 2 contained 210 \(\mu\)M GTP in reaction buffer. Syringe 3 contained 6.3 mg ml\(^{-1}\) GTP-cyclohydrolase II (equivalent to 290 \(\mu\)M active sites) in 50 mM Tris hydrochloride, pH 8.5, and 5 mM MgCl\(_2\). At the start of each experiment, the delay loop was washed with reaction buffer from syringe 2 and with enzyme solution from syringe 3. The solutions were mixed at a ratio of 1:1 (v/v) and at a flow rate of 4 ml s\(^{-1}\). After an appropriate delay, the reaction mixture in the delay loop was quenched by mixing with 0.2 M trichloroacetic acid from syringe 4 at a ratio of 1:1 (v/v) and a flow rate of 4 ml s\(^{-1}\). The effluent was collected. Samples were centrifuged to remove protein and were frozen at −70 °C until further analysis.

**HPLC\(^{1}\)** Analysis—Aliquots of 100 \(\mu\)l were injected into a reversed phase column (4.6 \(\times\) 250 mm, Hypersil RP18, 5 \(\mu\)M, Schambeck, Bad Honnef, Germany). The column was developed with isopropanol/trifluoroacetic acid (8:1), pH 2.02 (MicroMath Inc., Salt Lake City, UT).

**Global Analysis of Data from Stopped Flow Experiments**—Prior to numerical analysis, the data were corrected for absorbance of buffer and enzyme by subtraction of a blank data set obtained without addition of substrate. Data reduction and stronger weighting of early spectra were achieved by extracting 300 spectra on a pseudo-logarithmic time base from the difference data sets. These data sets were then analyzed using the program SPECFIT/32, Spectrum Software Associates, Marlborough, MA.

**RESULTS**

Single turnover quenched flow experiments were performed by mixing recombinant GTP cyclohydrolase II of *E. coli* with substoichiometric amounts of GTP (0.72 equivalents per enzyme subunit) at 30 °C. Reaction mixtures were quenched with trifluoroacetic acid. The substrate, GTP, the major product, Compound 2, and GMP formed were similar to the partitioning observed in earlier steady state kinetic experiments (−9,600 \(\text{cm}^{-1}\)).

The abbreviations used are: HPLC, high pressure liquid chromatography.

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The observed reaction sequence is represented by Eq. 1 as follows:

$$\begin{align*}
\text{GTP} & \xrightarrow{k_1} \text{Compound 2} \\
\text{GMP} & \xrightarrow{k_2} \text{Compound 1}
\end{align*}$$

(Eq. 1)

and can be described by the following set of equations.

$$
\begin{align*}
\frac{d[\text{GTP}]}{dt} &= -k_1 \cdot [\text{GTP}] - k_2 \cdot [\text{GTP}] \\
\frac{d[\text{Compound 2}]}{dt} &= k_1 \cdot [\text{GTP}] \\
\frac{d[\text{GMP}]}{dt} &= k_1 \cdot [\text{GTP}]
\end{align*}
$$

(Eq. 2, Eq. 3, Eq. 4)

The program SCIENTIST 2.0.2 was used to fit these equations to the experimental data in Fig. 3. The best fit was obtained with the rate constants summarized in Table I. The lines in Fig. 3 were calculated with that data set.

The production of Compound 2 requires the hydrolytic cleavage of 3 bonds (specifically, the N-7–C-8, and N-9–C-8 bonds in the imidazole ring and the α-β phosphoanhydride bond). Because the consumption of the substrate is essentially equivalent to the formation of products throughout the reaction sequence, the rate-determining step must be the first hydrolytic bond cleavage in the reaction sequence. Otherwise, a sigmoidal curve should have been observed for the formation of product.

In an extended search for reaction intermediates, we performed stopped flow experiments monitored by multiwavelength photometry. These experiments were designed on basis of the absorbance properties of the reactants, GTP and the minor product, GMP, are virtually indistinguishable by ultraviolet spectrometry, because both compounds have the same chromophore absorbance at 254 nm and shoulders around 273 nm (Fig. 7). The major product, Compound 2, has an absorption maximum at 293 nm. The hypothetical formamide type intermediate, Compound 3, which had been expected to be formed on the basis of earlier experiments with GTP cyclohydrolase I (8), can be assumed to have the same ultraviolet absorbance as Compound 4, which has been reported earlier (Fig. 2) (8).

The experimental conditions were similar to those used in the quenched flow experiments. The enzyme and the substrate, GTP, were mixed at a substoichiometric ratio of 0.7. Ultraviolet spectra were acquired at intervals of 100 ms. The spectra were superimposed in Fig. 6. The absorbance at 254 nm (i.e. at the λ_max value of GTP) is characterized by a relatively rapid increase followed by a steady decrease (Fig. 7, A and B). On the other hand, the absorbance at 295 nm (i.e. at the λ_max of the enzyme product, Compound 2) shows a steady increase. Notably, this trace does not appear to contain a sigmoidal component.

The absorbance at 279 nm remained virtually constant over a period of 280 s (Fig. 7, A and B). This is equivalent to the presence of an apparent isosbestic point at that wavelength (Fig. 6A). The formation, in substantial amounts, of a formamide-type intermediate similar or identical to the intermediate 4 in the reaction sequence catalyzed by GTP cyclohydrolase I would have been expected to afford a temporary increase of absorbance at 279 nm, which was not observed.

The optical spectrum showed a slow increase of absorbance in the long wavelength range of about 340 nm (Fig. 7, A and B). The formation of that component continues progressively even after the conversion of GTP to product is virtually complete. The process conducive to the long wavelength absorption can therefore not be part of the enzyme reaction. In fact, a similar formation of components with long wavelength absorption had been noted earlier in studies with GTP cyclohydrolase I where it could be conclusively attributed to photo-oxidation of reaction components caused by the intense ultraviolet irradiation required for high-speed optical monitoring of the reaction process (13).

Numerical deconvolution of the data was performed using the program package SPECFIT/32. The analysis revealed an optical transient with a reconstructed spectrum closely similar to that of GTP. Moreover, the numerical deconvolution afforded spectra with clear similarity to these of GTP/GMP, the enzyme product, Compound 2, and the photoproduc. The reaction can be described by the following scheme.

$$
\begin{align*}
\text{GTP} & \xrightarrow{k_1} \text{Compound 2} \\
\text{GMP} & \xrightarrow{k_2} \text{photoproduc}
\end{align*}
$$

(Eq. 5)

By combining the concentrations of Compound 2 and the artifactual photoproduc resulting from its decomposition, this scheme can be simplified as follows.

$$
\begin{align*}
\text{GTP} & \xrightarrow{k_3} \text{GTP} \\
\text{GMP} & \xrightarrow{k_2} \text{photoproduc}
\end{align*}
$$

(Eq. 6)

Table I

| Rate constant (s⁻¹) | 
|-------------------|
|--------------------|
| 0.052 ± 0.002 | 
| 0.0023 ± 0.00091 |
It is important to note that the rapid appearance of the optical transient with a $\lambda_{\text{max}}$ of 248 nm appears not to be associated with a modification of the covalent structure of the substrate, GTP. More specifically, the transient reaches a maximum at 2 s, but the acid quench of the reaction mixture at that time showed hardly any decrease of GTP (see above). Hence, the optical transient in Fig. 8A is suggested to represent a conformational modulation of substrate and/or enzyme.

The rate constants for the optically observed processes are summarized in Table II. Values of 0.052 and 0.062 s$^{-1}$ were obtained for the rate constants of the formation of enzyme-product by quenched flow and stopped flow analysis in close agreement.

Throughout the reaction sequence, the sum of the GMP, Compound 2, and the artifactual photoproducts rather accurately reflected the amount of GTP consumed. In agreement

Fig. 5. Optical spectra from single turnover stopped flow experiments with GTP (A) and Compound 4 (B) as substrate.

Fig. 6. Selected spectra from the single turnover experiments shown in Fig. 5.

Fig. 7. Absorbance changes observed during single turnover stopped flow experiments with GTP cyclohydrolase II and GTP, respectively, Compound 4. Symbols represents experimental data from Fig. 4. Solid lines represent the data obtained from numerical simulation using the kinetic constants in Table II.
with the quenched flow data, we found no evidence for formation of an intermediate between GTP and the product, Compound 2, in appreciable concentrations. Most notably, we obtained no evidence for the formation of a formamide-type intermediate identical or similar to the intermediate 4 in the reaction catalyzed by GTP cyclohydrolase I.

This failure prompted additional stopped flow experiments using Compound 4 as substrate (see Fig. 5B, Fig. 6B, Fig. 7, C and D, and Fig. 8, C and D). This non-natural substrate is characterized by an absorbance maximum at 272 nm (Fig. 6). At this wavelength, we note a monotonous decrease of absorbance in Fig. 7, C and D. This transient was characterized by a slight shift of absorption properties as compared with Compound 4 and is reminiscent of the optical transient found with GTP as substrate, thus indicating that the formamide 4 is less rapidly converted to product than the natural substrate, GTP.

Numerical deconvolution disclosed a transient species whose optical spectrum was distinct from, but closely similar to, that of the substrate (Fig. 8C). This transient was characterized by a slight shift of absorption properties as compared with Compound 4 and is reminiscent of the optical transient found with GTP as substrate (see A in Fig. 8A). There was no evidence for the formation of GTP or GMP (i.e. by ring closure). The formation of a photoproduct caused by the intense ultraviolet exposure was similar to that observed in the earlier experiment with GTP as substrate. The reaction sequence can be described by the following scheme.

\[
\begin{align*}
\text{Compound 4} & \xrightarrow{k_3} \text{Compound 4'} & \xrightarrow{k_4} \text{Compound 2} & \xrightarrow{k_5} \text{product} \\
\end{align*}
\]

This scheme can be simplified as follows.

\[
\begin{align*}
\text{Compound 4} & \xrightarrow{k_3} \text{Compound 4'} & \xrightarrow{k_4} \text{Compound 2} & \xrightarrow{k_5} \text{photoproduct} \\
\end{align*}
\]

The numerical deconvolution of the absorbance data in Fig. 5 shows that the pyrimidine product, Compound 2, is produced less rapidly from Compound 4 than from GTP. Thus, Compound 4 does not fulfill the criteria for a kinetically competent intermediate.

**DISCUSSION**

The GTP cyclohydrolases catalyzing the initial steps in the biosynthesis of folate and unconjugated pteridines (type I enzymes) and of riboflavin (type II enzymes) are slow catalysts with turnover numbers in the range of 1 to 5 per min and per subunit. The slow enzymes are likely to be rate-limiting for the biosynthesis of the heterocyclic cofactors that are only required in small amounts by comparison with bulk metabolites such as amino acids and nucleic acid precursors. Earlier studies on pre-steady state kinetics of GTP cyclohydrolase I identified the Amadori rearrangement of the carbohydrate side chain as the rate-determining step.

The data in the present study suggest that the rate-limiting reaction in the reaction catalyzed by GTP cyclohydrolase II is the release of pyrophosphate under formation of a covalently protein-bound intermediate that can undergo three rapid hydrolytic reactions resulting in the opening of the imidazole ring, the release of formamide from the resulting formamide type intermediate, and the cleavage of the phosphoester or phosphoamide bond affording the enzyme product, Compound 2. More specifically, the covalently bound intermediate could be GMP linked to an amino acid side chain via a phosphoester or phosphoamide bond.

This hypothetical scenario can explain the formation of the second enzyme product, GMP, if the hydrolysis of the covalent linkage of the intermediate to the enzyme is prior to the release of formate. The observed partitioning factor for the formation of GMP and Compound 2 of ~10 would then suggest that the
The formation of a covalent linkage between enzyme and substrate is supported by the failure of GMP to serve as substrate for GTP cyclohydrolase II, which had already been noted by Foor and Brown (1). It is also in line with the finding that $^{18}$O from solvent is incorporated into Compound 2 but not into pyrophosphate as shown recently (3). Nevertheless, it must be emphasized that the involvement of a covalently enzyme-bound intermediate is as yet hypothetical. In light of the data reported in this paper, it is also obvious that the concentration of the enzyme-bound species would always be low, because the rate-determining step is the formation and not the decay of the enzyme-bound species.

In any case, we must conclude that the initial section of the reaction catalyzed by GTP cyclohydrolase II proceeds in a strictly ordered fashion in which the release of pyrophosphate must precede the hydrolysis of the imidazole ring. In contrast, the second part of the reaction is subject to partitioning, which results in the formation of GMP as a minor product. The failure of the enzyme to convert Compound 4 into GTP by ring closure is also well in line with the hypothetical mechanism in Fig. 9.

If the release of pyrophosphate is an obligatory first reaction step for any substrate to enter the reaction cascade, the enzyme-catalyzed transformation of Compound 4 to GTP would be impossible despite the favorable equilibrium constant for that reversible reaction. It should be noted in this context that Compound 4 is reversibly converted to GTP by the H179A mutant of GTP cyclohydrolase I. On the other hand, wild type GTP cyclohydrolase I does not detectably catalyze that reaction, probably because the transformation of the 5-formamide-type intermediate, Compound 4, to the 5-amino-type intermediate, Compound 5, is a relatively rapid reaction.

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