Histidine 179 Mutants of GTP Cyclohydrolase I Catalyze the Formation of 2-Amino-5-formylamino-6-ribofuranosylamino-4(3H)-pyrimidinone Triphosphate*

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GTP cyclohydrolase I catalyzes the conversion of GTP to dihydroneopterin triphosphate. The replacement of histidine 179 by other amino acids affords mutant enzymes that do not catalyze the formation of dihydroneopterin triphosphate. However, some of these mutant proteins catalyze the conversion of GTP to 2-amino-5-formylamino-6-ribofuranosylamino-4(3H)-pyrimidinone 5′-triphosphate as shown by multinuclear NMR analysis. The equilibrium constant for the reversible conversion of GTP to the ring-opened derivative is approximately 0.1. The wild-type enzyme converts the formylamino pyrimidine derivative to dihydroneopterin triphosphate; the rate is similar to that observed with GTP as substrate. The data support the conclusion that the formylamino pyrimidine derivative is an intermediate in the overall reaction catalyzed by GTP cyclohydrolase I.

GTP cyclohydrolase I catalyzes the formation of dihydroneopterin triphosphate from GTP via a mechanistically complex ring expansion. In plants and micro-organisms, the enzyme product serves as the first committed intermediate in the biosynthesis of tetrahydrofolate (1). In animals, the enzyme product is converted to tetrahydrobiopterin, which serves as cofactor for the biosynthesis of catecholamines and of nitric oxide (2–4). Genetic defects of GTP cyclohydrolase I result in severe neurological impairment (5–8).

GTP cyclohydrolase I of Escherichia coli is a 247-kDa homodimer (9, 10). The structure of the protein has been studied by x-ray structure analysis at a resolution of 2.6 Å (11). The torus-shaped protein obeys D2 symmetry. Each of the 10 equivalent active sites is located at the interface of three adjacent subunits.

Brown, Shiota, and their co-workers (12–14) could show the reaction sequence catalyzed by GTP cyclohydrolase I to involve the opening of the imidazole ring of GTP (compound 1, Fig. 1) with release of formate. Carbon atoms 1′ and 2′ of the ribose moiety of GTP are then used to form the dihydropyrazine ring of dihydroneopterin triphosphate (compound 5, Fig. 1). However, the mechanistic details of the highly complex enzyme-catalyzed reactions are incompletely understood.

The catalytic activity of GTP cyclohydrolase is highly sensitive to the replacement of amino acid residues at the active site cavity (11). The amino acid residues Cys110, His112, His113, Glu152, His179, and Cys181 were shown to be indispensable for the formation of dihydroneopterin triphosphate. This paper describes the properties of mutant enzymes with replacement of histidine 179.

EXPERIMENTAL PROCEDURES

Materials—Enzymes were purchased from Boehringer Mannheim (Mannheim, Germany) and Sigma (Deisenhofen, Germany); GTP from Serva (Heidelberg, Germany); [1-14C]glucose was purchased from Iso-tect (Miamisburg, Ohio); [1-13C]formate; phosphoryl chloride, trimethyl phosphate, N,N-dimethylformamide, tri-n-butylamine, and pyrophosphoric acid were purchased from Sigma-Aldrich. All other chemicals were reagent grade.

Enzyme Assays—Assay mixtures contained 100 mM Tris hydrochloride, pH 8.5, 100 mM KCl, 2.5 mM EDTA, 1 mM GTP, and protein in a total volume of 450 μl. The mixtures were incubated at 37 °C, and 100-μl aliquots were retrieved at intervals. The formation of dihydroneopterin triphosphate and 2-amino-5-formylamino-6-ribofuranosylamino-4(3H)-pyrimidinone 5′-phosphate was monitored as follows.

Assay of Dihydronopterin Triphosphate—Aliquots of enzyme assay mixture were mixed with 30 μl of a solution containing 1% isonic and 2% KI in 1 M HCl. After incubation for 30 min at ambient temperature, excess iodine was reduced by addition of 0.11 mM ascorbic acid (10 μl). After adjustment to pH 8.5 by the addition of 1 M Tris-HCl, pH 8.5, a solution (10 μl) containing 1.8 units of alkaline phosphatase, 0.2 μmol of MgCl2, and 0.26 μmol of ZnCl2 was added. The mixture was incubated for 1 h at 37 °C. The reaction was terminated by addition of trichloroacetic acid. Precipitate was removed by centrifugation. Neop-terin was determined by HPLC as described earlier (11).

Assay of 2-Amino-5-formylamino-6-ribofuranosylamino-4(3H)-pyrimidinone 5′-Triphosphate—Aliquots (100 μl) of enzyme assay mixtures were mixed with 100 μl of 1 M HCl. The mixtures were incubated for 5 min at 90 °C. The pH was adjusted to 8.5 by the addition of 1 M NaOH. Butanedione in 1 M Tris-HCl, pH 8.5, was added to a final concentration of 0.5%. The mixtures were incubated at 90 °C for 1 h. 6,7-Dimethylpterin was determined by HPLC as described previously (16).

Preparation of [8-13C]GTP—[8-13C]Guanine was prepared by the route used for [7-15N,8-13C]guanine as described earlier (17), except that unlabeled sodium nitrate was used. The crude material thus ob-
tained was purified by dissolving it in hot 2.5 M HCl and treating with charcoal. Filtration of the hot solution gave a yellow filtrate, which was neutralized to pH 7 with concentrated NH₄OH. Removal of some of the water on a rotary evaporator and cooling the residue overnight at 5 °C gave [8-13C]guanine as a yellowish powdery solid. This was converted (18) into [8-13C]guanidino, which was in turn converted into the required [8-13C]GTP. [8-13C]Guanosine (283 mg; 1 mmol), which had been dried for 24 h over P₂O₅ under high vacuum, was suspended in trimethyl phosphate (5 ml) under an atmosphere of dry nitrogen contained in a flask fitted with a rubber septum and then cooled in an ice bath (19). Freshly distilled phosphoryl chloride was added from a syringe (0.285 ml; 3 mmol) and the mixture stirred at 0 °C. All solid material dissolved within 1 h. HPLC analysis after 3 h (anion exchange column; phosphat gradient 0.02 M KH₂PO₄ to 0.5 M KH₂PO₄) showed only one peak due to product and no peak due to unreacted guanosine. The cold solution was added cold tri-n-butylamine (2 ml; 8.4 mmol), followed immediately by a freshly prepared ice-cold solution of tri-n-butylamine pyrophosphate, prepared from pyrophosphoric acid (1.780 g; 10 mmol) and tri-n-butylamine (4.765 ml; 20 mmol) in dimethylformamide (10 ml) (20). After 1 min, the reaction mixture was quenched with cold water (200 ml) and the pH adjusted to 9–10 with 1 M aqueous potassium hydroxide. The mixture was extracted three times with ether to remove free tributylamine, and all traces of ether were removed from the aqueous layer using a rotary evaporator under vacuum at room temperature, giving a white powder, which was washed with ethanol and stored at 5 °C. Yield was 129 mg. HPLC analysis of the product and no peak due to unreacted guanosine. To the cold solution was added cold tri-n-butylamine (2 ml; 8.4 mmol), followed immediately by a freshly prepared ice-cold solution of tri-n-butylamine pyrophosphate, prepared from pyrophosphoric acid (1.780 g; 10 mmol) and tri-n-butylamine (4.765 ml; 20 mmol) in dimethylformamide (10 ml) (20). After 1 min, the reaction mixture was quenched with cold water (200 ml) and the pH adjusted to 9–10 with 1 M aqueous potassium hydroxide. The mixture was extracted three times with ether to remove free tributylamine, and all traces of ether were removed from the aqueous layer using a rotary evaporator under vacuum at room temperature. The aqueous solution was loaded onto a column of Dowex-1 anion exchange resin in the chloride form (25 × 110 mm). Distilled water (3.5 liters) was first passed through the column, which was then developed with a sodium chloride gradient (0–0.2 M NaCl dissolved in 0.01 M HCl). Monitoring by UV (254 nm) showed three well resolved peaks, the third and largest being the desired GTP, eluted after about 7 liters of the eluting gradient had been passed through. The GTP was separated from the buffer salts by using a toluene-treated charcoal column. The type of charcoal used for this purpose is critical, and some commercial charcoals are not suitable. Riedel de Haën animal charcoal (knochenkohle pulver, catalog number 18008) works satisfactorily. The charcoal column (25 × 85 mm) was prepared in 1 M HCl and then washed with water until the effluent was neutral. It was partially deactivated by washing with a mixture of toluene/0.88 M NH₄OH/isopropanol/water (ratio 1:3:50:46), then with a mixture of EtOH/Water/NH₄OH (2:2:1), and then with distilled water. The column was stored in water and could be reused repeatedly. The GTP-containing fractions from the Dowex column were loaded onto the charcoal column, which was washed with distilled water (500 ml). Elution with 1 liter of a solution of ethanol/water/0.88 M NH₄OH (50:45:5), followed by evaporation of the eluate to dryness in a rotary evaporator under vacuum at room temperature, gave a white powder, which was washed with ethanol and diethyl ether and stored at ~5 °C. Yield was 129 mg. HPLC analysis of this product showed one main peak corresponding to authentic GTP purchased from Sigma. Its UV spectra measured at pH 2, 7, and 13 were identical with the corresponding UV spectra of authentic GTP. The 1H and 31P NMR spectra were measured in buffer solution containing 20 mM Tris-HCl, pH 8.5, 2 mM EDTA, 2 mM dithiothreitol, 100 mM KCl, and 85% D₂O and showed the product to be pure GTP, uncontaminated with either guanosine di- or monophosphate, although containing about 15% inorganic triphosphate. The 1H NMR spectrum showed a single enriched peak at δ 1357.6 (C-8). The 1H spectrum included a doublet at δ 8.03 (J₁,δH1 = 216 Hz; H-8) and a quartet at δ 5.82. The 1H-13C spectrum showed signals at δ -18.35 (t, J = 19.8 Hz; guanosine β-P), -7.51 (d, J = 19.5 Hz; guanosine α-P), and -2.40 (d, J = 21.1 Hz; guanosine γ-P), as well as small signals at δ -17.87 (t, J = 20.9 Hz; P-2 of inorganic triphosphate) and -3.03 (d, J = 20.9 Hz; P-1 and P-3 of inorganic triphosphate). This material was used directly in the biological experiments. 75% phosphoric acid was used as external reference.

Preparation of 1,5-13C-Labeled GTP—[8,1-13C]Guanosine (17).

Preparation of 2-Amino-5-formy lamino-6-ribofur ansanylamino-4(3H)-pyrimidinone 3-Triphosphate—A mixture containing 8.5 mm phosphorus pentoxide, pH 7.0, 7 mM GTP (GDP-free), 85 mM KCl, 2.1 mM EDTA, 4 mM dithiothreitol, and 40 mg of GTP cyclohydrolase I H179A mutant protein in a total volume of 4.7 ml was incubated for 6.5 h at 37 °C. The reaction was terminated by the addition of 1 M LiCl to a final pH of 4.7. Precipitate was removed by centrifugation. Ice-cold, doubly distilled water was added to a total volume of 50 ml. The solution was placed on a column of DEAE-cellulose (DE52, Whatman, Maidstone, United Kingdom; 2 × 14.5 cm) that had been equilibrated with 30 mM LiCl, pH 3.5, at 4 °C. The column was washed with 100 ml of 30 mM LiCl, pH 3.5, and was subsequently developed with a linear gradient of 98–195 mM LiCl (total volume, 400 ml). The effluent was monitored photometrically (280 nm). The yield (4.6 μmol, 13.5% based on GTP) was determined photometrically using an absorbance coefficient of ε₂₅₄ₐₚ₅₃ = 14,300 M⁻¹ cm⁻¹. Fractions were combined, and 0.5 ml of 0.25 mM ammonium bicarbonate was added. The mixture was lyophilized. Dry methanol was added to the residue. The insoluble lithium salt of 2-amino-5-formy lamino-6-ribofur ansanylamino-4(3H)-pyrimidinone 5-triphosphate was collected by centrifugation. LiCl was removed by washing with dry methanol. The residue was stored at ~70 °C.

Preparation of 2,6-Diamino-5-formy lamino-4(3H)-pyrimidinone—

![FIG. 1. Hypothetical reaction mechanism of GTP cyclohydrase I as proposed by Burg and Brown (12).](image)
2,6-Diamino-5-formylamino-4(3H)-pyrimidinone was prepared according to the published synthesis by Pfleiderer (22).

**Site-directed Mutagenesis—**The point mutants of GTP cyclohydrolase I were generated by a polymerase chain reaction strategy (23). All mutated genes were sequenced with the dye terminator method using an ABI Prism377 sequencer (Perkin-Elmer).

**Cell Culture and Preparation of Crude Extracts—**Escherichia coli strain M15[pREP4] harboring the mutated pECHI expression plasmid was grown in LB medium containing 150 mg of ampicillin and 22 mg of kanamycin per liter as described (11). Cells were harvested by centrifugation. Wet cell mass (200 mg) was suspended in 1 ml of 200 mM Tris-HCl, pH 8.0, containing 2.5 mM EDTA, 3 mM sodium azide, 3 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM of lysozyme. The mixture was incubated for 90 min at 37 °C. Cell lysis was completed by ultrasonic treatment. Cell debris was removed by centrifugation.

**Purification of GTP Cyclohydrase I Mutant Protein H179A—**Crude cell extract was dialyzed against 5 liters of buffer A containing 10 mM potassium phosphate, pH 7.0, 2.5 mM EDTA, and 3 mM sodium azide. The dialysate was applied to a column of DEAE-cellulose (DE52, Whatman; 4 × 11 cm) that had been equilibrated at 4 °C in the same buffer. The column was washed with 300 ml of buffer A and developed with a linear gradient of 10–150 mM potassium phosphate, pH 7.0. Fractions containing enzymatic activity (determined as formation of 2-amino-5-formylamino-6-ribosylamino-4(3H)-pyrimidinone) were combined and concentrated by ultracentrifugation. The protein was stored at 4 °C.

**Estimation of Protein Concentration—**GTP cyclohydrase I in crude cell extracts was monitored by radial immunodiffusion on agarose plates containing 60 μl of polyclonal rabbit antiserum against E. coli wild-type GTP cyclohydrase I in 150 μl of 100 mM potassium phosphate, pH 7.0, containing 3 mM sodium azide and 0.56% agarose (DNA-grade). Purified wild-type protein was used as standard (21).

**Total protein was determined by the method of Bradford (24, 25).**

**NMR Experiments—**One-dimensional and two-dimensional 1H and 13C NMR spectra were recorded at 17 °C in D2O using a Bruker DRX500 spectrometer. MLEV17 and GARP sequences were used for decoupling of 1H, 13C, and 15N. Experimental setup and data processing were performed according to standard Bruker software (XWINNMR).

**RESULTS**

The hypothetical reaction mechanism of GTP cyclohydrase I proposed by Brown, Shiota, and their co-workers (12–14) (Fig. 1) implies the formation of at least two *ortho*-diaminopyrimidine-type intermediates. Under appropriate conditions, it appeared possible to convert compounds of this type to highly fluorescent pteridines, thus facilitating their detection with high sensitivity.

Our earlier site-directed mutagenesis study (11) had afforded a number of GTP cyclohydrase I mutants (point mutation of residues Cys110, Cys181, Glu152, His112, His113, and His179), which were unable to form detectable amounts of dihydroleotriphosphate from GTP. These apparently inactive mutants were incubated with GTP, and the reaction mixtures were then boiled with HCl, neutralized, and incubated with butanediol as described under “Experimental Procedures” (Fig. 2). The mixtures were analyzed by fluorescence-monitored HPLC.

A fluorescent compound identified as 6,7-dimethylpterin (compound 6, Fig. 2) was observed in assays with cell extracts of recombinant *E. coli* strains expressing mutant enzymes carrying various amino acids instead of histidine 179, thus suggesting that a diaminopyrimidine-type product had indeed been formed. When the HCl treatment was omitted, little or no 6,7-dimethylpterin was formed in assays with these mutant proteins. This indicates that initially no *ortho*-diaminopyrimidines were present. The rates of product formation detected in these assay mixtures are summarized in Table I. The highest formation rate of the product detected as 6,7-dimethylpterin was observed with the H179S mutant; mutants H179K and H179R did not catalyze any detectable reaction at all. Nevertheless, the specific enzymatic activity for formation of the unknown compound by mutant H179S was only 2.5% as compared with the specific enzymatic activity for the formation of dihydroleotriphosphate by wild-type GTP cyclohydrase I. Mutant H179A was selected for further studies on the basis of preliminary data on 6,7-dimethylpterin formation rate.

**Mutant protein H179A was purified from a recombinant *E. coli* strain. Some properties are summarized in Table II. The formation of the unknown product from GTP by the H179A**

| Enzyme mutation | Formation rate (nmol mg⁻¹ min⁻¹) |
|-----------------|---------------------------------|
| Wild-type       | 2.1                             |
| H179A           | 1.9                             |
| H179D           | 0.64                            |
| H179F           | 0.63                            |
| H179K           | 0.61                            |
| H179N           | 0.61                            |
| H179Q           | 0.61                            |
| H179R           | 0.61                            |
| H179S           | 0.61                            |
| L134S           | 0.61                            |
| Q151A           | 0.61                            |
| Q151E           | 0.61                            |
| S135A           | 0.61                            |
| S135C           | 0.61                            |
| S135H           | 0.61                            |
| S135W           | 0.61                            |

**TABLE II**

**Kinetic properties of wild-type GTP cyclohydrase I and mutant H179A**

All values were determined under standard assay conditions at pH 8.5 and 37 °C. The formation of 5 was monitored photometrically (330 nm) as described (21). The conversion of 2 to GTP and vice versa was monitored photometrically (250 nm) using a Δε of 8.97 × 10⁻³ μM⁻¹ cm⁻¹. Specific activities were determined at substrate saturation. Wild-type GTP cyclohydrase I exhibited weak negative cooperativity. ND, not determined.

| Substrate | Kinetic parameter | Wild-type | H179A |
|-----------|------------------|-----------|-------|
| GTP       | Formed of        | 5         | 2     |
| kₗₑₜ (nmol mg⁻¹ min⁻¹) | 71 ± 4 | 12 ± 0.7 |
| Kₐₛₛ (μM)   | 0.85 ± 0.23 | 8.6 ± 2.1 |
| Hill coefficient | 0.53 | 1 |
| pH optimum | 8 (broad) | 7.2 |
| Formation of | NH₃, TP | GTP |
| kₗₑₜ (nmol mg⁻¹ min⁻¹) | 70 ± 12 | 20 ± 1.5 |
| Kₐₛₛ (μM)   | 0.66 ± 0.50 | 4.7 ± 1.9 |
| Hill coefficient | 0.43 | 1 |
| Formation of | NH₃, TP | GTP |
| Specific activity (nmol mg⁻¹ min⁻¹) | 68 | ND |
mutant is characterized by a value of $K_m = 12 \mu M$ for GTP. For comparison, an apparent $K_m$ value of 0.85 $\mu M$ GTP was observed for the wild-type GTP cyclohydrolase I reaction yielding dihydroneopterin triphosphate.

Whereas replacement of residue H179 affords mutant enzymes converting GTP exclusively to the unknown product, the mutant proteins L134S, Q151A, S135A, and S135C formed both the unknown compound and dihydroneopterin triphosphate from GTP (Table I).

In order to determine the structure of the putative pyrimidine intermediate produced by mutant H179A, we decided to use stable isotope labeled GTP samples as substrates in order to increase the sensitivity and selectivity of NMR detection. [8-$^{13}$C]GTP was synthesized and incubated with H179A mutant protein in phosphate buffer at pH 7.0 in an NMR tube. The sample was kept in the NMR magnet at a temperature of 37 °C, and $^{13}$C NMR spectra were recorded at intervals (Fig. 3). A signal at 165.4 ppm increased at an approximately linear rate, and a signal at 164.9 was found to increase with apparently sigmoidal kinetics. After incubation at 37 °C for 20 h, two small additional signals were observed at 169.9 and 170.1 ppm. These initial data tentatively suggested the formation of four different formamide-type species from the proffered GTP. Formation of significant amounts of $^{13}$C-labeled formate was not observed.

The putative formamidopyrimidine-type compounds could be separated from remaining substrate GTP by ion exchange chromatography. However, they could not be separated from each other. In order to unequivocally assign the structures of the four compounds formed, we prepared [8,1-$^{13}$C,2-$^{13}$C,3-$^{13}$C,4-$^{13}$C,5-$^{13}$C,6-$^{13}$C,7-$^{13}$C,8-$^{13}$C]GTP (Fig. 4a, compound 1a) by enzymatic synthesis. The compound was treated with the H179A mutant protein, and the isolated mixture of four reaction products was analyzed by two-dimensional NMR spectroscopy. Fig. 5 shows a section from a two-dimensional $^1$H-$^{13}$C HMOC-TOCSY spectrum and the corresponding parts from one-dimensional $^1$H-$^{13}$C and $^{13}$C-$^1$H spectra. The one-dimensional $^{13}$C spectrum shows two pairs of doublets whose intensities differ by a factor of about 10. These signals showed correlations to $^1$H doublet signals located in the range of 7.8 and 8.17 ppm. The chemical shift values of both $^{13}$C and $^1$H resonances are in line with the presence of four
TABLE III

NMR data of the compounds discussed under Results

Spectra were measured in D_2O at pH 7.0. ND, not determined.

| Compound | Position | Chemical shift[^a] | Coupling constants | Integrals[^c] | Correlation experiments |
|----------|----------|--------------------|-------------------|--------------|--------------------------|
|          |          | ppm[^b] | 1H[^d] | 13C[^e] | 1H[^f] | 13C[^g] | 1H[^h] | 13C[^i] | 1H[^j] | 13C[^k] | IN | ADEQUATE | HMQC-TOCSY |
| 1a       | 8        | 8.03 (d) | 137.59 (s) | 43.3 (2') | 168 | 5.8 (2') | 10.9 |
|          | 2'       | 4.69 (s) | 73.21 (dd) | 42.7 (1'), 38.0 (3') | ~150 | ND |
|          | 3'       | 4.48 (s) | 70.21 (t) | 38.1 (2', 4') | ~150 | ND |
|          | 4'       | 4.23 (s) | 83.89 (dt) | 40.4 (3', 5') | ~150 | ND |
|          | 5'       | 4.14 (m) | 65.10 (dd) | 42.6 (4') | ~150 | ND |
| 2a (β,α-cis) | -CHO | 8.15 (d) | 165.34 (d) | 45.2 (2') | 165 | 6.0 (2') | 10.5 |
|          | 2'       | 4.19 (t) | 72.61 (dd) | 43.7 (1'), 38.4 (3') | ~150 | 5.7 (1', 3') | 1.2 |
|          | 3'       | 4.36 (t) | 69.4-70.4 (m) | ND | ~150 | 4.2 (2', 4') | ND |
|          | 4'       | 4.01 (m) | 81.75 (dt) | 40.7 (3', 5') | ~150 | ND | 1.2 |
|          | 5'       | 3.88-3.93 (m) | 64.70 (d, broad) | 38.4 (4') | ~150 | ND | ND |
| 2b (α,β-cis) | -CHO | 8.17 (d) | 164.90 (d) | 199.0 | 14.6 | 15.7 | 1.0 |
|          | 1'       | 5.84 (d) | 80.97 (d) | 43.7 (2') | 165 | 6.0 (2') | 1.05 |
|          | 2'       | 4.23 (t) | 69.4-70.4 (m) | ND | ~150 | 4.7 (1', 3') | ND |
|          | 3'       | 4.36 (t) | 69.4-70.4 (m) | ND | ~150 | 4.2 (2', 4') | ND |
|          | 4'       | 4.02 (m) | 80.42 (t, broad) | 40.4 (3', 5') | ~150 | ND | 1.2 |
|          | 5'       | 3.98-4.05 (m) | 64.53 (d, broad) | 39.4 (4') | ~150 | ND | ND |
| 2c (β,α-trans) | -CHO | 7.79 (d) | 170.09 (d) | 197.2 | 14.6 | 13.2 | 0.08 |
|          | 1'       | 5.82 (d) | 86.70 (d) | ND | 4 (2') | ND |
| 2d (α,β-trans) | -CHO | 7.75 (d) | 169.91 (d) | 197.1 | 14.7 | 13.0 | 0.06 |
|          | 1'       | 5.58 (d) | 84.0 (d) | ND | 6 (2') | ND |
| 6a (s-cis) | -CHO | 8.17 (s) | ND | ND | ND | ND | 1.0 |
| 6b (s-trans) | -CHO | 7.80 (s) | ND | ND | ND | ND | 0.12 |

[^a] Chemical shifts were referenced to external TSP.
[^b] 1H data refer to [13C] decoupled spectra.
[^c] 3[^d]J_{CC}: 8.9 Hz.
[^d] 2[^d]J_{CC}: 4.8 Hz.
[^e] 3[^d]J_{CP}: 9.3 Hz.
different formamide-type molecular species. The doublet character of the respective $^{13}$C and $^1H$ signals can be attributed to $^{15}$N coupling as demonstrated by comparison with a $^{15}$N-decoupled spectrum where singlets are observed (not shown). Therefore, all products observed carry the formyl group at the $^{15}$N atom attached to position 5 of the pyrimidine ring.

Chemical shift arguments suggest that the formamide C-N bond has $cis$ configuration in the pair of major products and $trans$ configuration in the pairs of minor products (26, 27). The assignments are well in line with studies on the pyrimidine base aglycon, 2,6-diamino-5-formylamino-4(3H)-pyrimidinone, which was also shown to display formamide $cis/trans$ isomerism. The $^1H$ chemical shifts of the formyl groups in this compound were closely similar to those of the products of GTP cyclohydrolase I mutant H179A (Table III). The ratio of the $cis$ and $trans$ species in the equilibrium mixture of 2,6-diamino-5-formylamino-4(3H)-pyrimidinone were also in close similarity with that in the product mixture generated by the H179A mutant enzyme.

Fig. 6 shows an expansion comprising the carbohydrate signals of a two-dimensional HMQC experiment obtained with the same sample as in Fig. 5. Cross-peaks correlate $^{13}$C atoms with directly bonded $^1H$ atoms. In conjunction with HMQC-TOCSY and $^{13}$C INADEQUATE spectra (not shown), the signals in this spectrum can be assigned unequivocally to an anomeric mixture of N-ribofuranosides for the major product pair (Table III). The ratio of the $cis$ and $trans$ species in the equilibrium mixture of 2,6-diamino-5-formylamino-4(3H)-pyrimidinone were also in close similarity with that in the product mixture generated by the H179A mutant enzyme.

The enzyme reaction catalyzed by the H179A protein can be monitored photometrically. Fig. 7A shows the reaction from GTP to the formylaminopyrimidine product. The UV spectra of the isomeric mixture of 2-amino-5-formylamino-6-ribofuranosylamino-4(3H)-pyrimidinone triphosphates are shown in Fig. 7C for comparison. Fig. 7B shows the reverse reaction using the mixture of four stereoisomers as substrate. The rate of the reverse reaction was approximately two times higher than that of the forward reaction. The reverse reaction slowed down significantly after turnover of about 40% of the formamides present. This is caused by exhaustion of the $\beta$ isomer fraction. The equilibrium constant for the reversible conversion between GTP and the $\beta$ cis isomer is approximately 0.1.

Following on the treatment of GTP with Fenton's reagent (Fe$^{2+}$ and mercaptoethanol in the presence of molecular oxygen), Shiota and co-workers (13, 14) isolated a compound tentatively identified as compound 2 (Fig. 1). Using the published procedure, we isolated a product mixture that had the same UV spectrum and $^1H$ NMR signature as the product mixture obtained from treatment of GTP with the H179A protein (data not shown).

Shiota and co-workers (13, 14) had already shown that the product obtained with Fenton's reagent from GTP could be converted to dihydropyronopterin triphosphate by GTP cyclohydrolase I in cell extracts of Lactobacillus plantarum. We confirmed that the formamide mixture obtained by treatment of
FIG. 7. Ultraviolet spectra recorded during the conversion of GTP with mutant H179A (A) and during the conversion of the isomeric mixture of 2-amino-5-formylamino-6-ribofuranosylamino-4(3H)-pyrimidinone triphosphate (compound 3) with mutant H179A (B) at 30 °C. Reaction mixture A contained 83 μM GTP, 83 μg/ml mutant H179A, 10 mM Tris-HCl, pH 8.5, 10 mM KCl, and 0.25 mM EDTA. Reaction mixture B contained a total amount of 99 μM compounds 2a–2d and 208 μg/ml mutant H179A in the same buffer as above. The insets show difference spectra with the initial spectrum as reference. For comparison, the UV spectra of compounds 2a–2d (equilibrium mixture) and of GTP at pH 7.0 are shown (C).

FIG. 8. Proposed reaction scheme for the formation of the formamide isomeric mixture by enzyme-catalyzed and by nonenzymatic reactions. WT, wild-type enzyme.
GTP with the H179A mutant protein could be converted to dihydroneopterin triphosphate by wild-type *E. coli* GTP cyclohydrolase I. Similarly, we could show that the product obtained by treatment of GTP with Fenton’s reagent according to Shiot-ta’s procedure could be converted to dihydroneopterin triphosphate. The rate of product formation (as determined by the increase of absorbance at 330 nm) was similar to the rate of the reaction with the natural substrate GTP (Table II). NMR experiments with [formyl-\(^{13}\)C]2-amino-5-formylamino-6-ribofuranosylamino-4(3H)-pyrimidinone triphosphate obtained enzymatically from \([8-{\^}\text{\textsuperscript{13}}}\text{C}]\)GTP (data not shown) confirmed that the \(\beta\) cis isomer is preferentially consumed when the mixture is incubated with wild-type enzyme. In summary, the kinetic competency of compound 2a suggests that it is an intermediate in the GTP cyclohydrolase I reaction trajectory.

Although unknown at present, mutants may be discovered that, while unable to utilize GTP as a substrate, are able to convert the product mixture of mutant His\(^{179}\) to dihydroneopterin triphosphate. The mutants C110S, H112S, H113S, and C181S are unable to generate dihydroneopterin triphosphate from GTP.\(^{1}\) None of these mutants, however, led to any detectable dihydroneopterin triphosphate when incubated with the H179A product mixture.

**DISCUSSION**

Aqueous solutions of 2-amino-5-formylamino-6-ribofuranosylamino-4(3H)-pyrimidinone triphosphate obtained by the catalytic action of the H179A mutant of GTP cyclohydrolase I contain a mixture of four stereoisomers 2a–2d. The isomerization reactions between the components probably occur nonenzymatically. Mutarotation of N-glycosides is a proton-catalyzed reaction. From the series of \(^{13}\)C NMR spectra collected during conversion of \([8-{\^}\text{\textsuperscript{13}}}\text{C}]\)GTP with mutant H179A a reaction rate for mutarotation of 0.01 min\(^{-1}\) at pH 7.0 and at a temperature of 37 °C can be estimated. Kene et al. (27) determined a reaction rate of 0.02 s\(^{-1}\) (at 30 °C) for the formamide isomerization of the model compound methyl 4,6-dideoxy-4-formylamino-a-d-mannopyranoside.

The \(\beta\) cis isomer of 2-amino-5-formylamino-6-ribofuranosylamino-4(3H)-pyrimidinone triphosphate appears to be an intermediate in the transformation of GTP to dihydroneopterin triphosphate catalyzed by GTP cyclohydrolase I on the basis of the following arguments. (i) The \(\beta\) cis isomer is the primary product of the GTP cyclohydrolase I mutant H179A, whereas the signal for the \(\alpha\) cis isomer is characterized by sigmoidal kinetics of formation. (The concentration of the trans isomers comprising the minor products of mutant H179A is too small for any interpretation.) (ii) The \(\beta\) cis isomer comprising the major portion of the \(\beta\)-anomeric isomers is preferentially consumed by the H179A mutant during the formation of GTP. (iii) The \(\beta\) cis isomer is preferentially converted to dihydroneopterin triphosphate by wild-type enzyme. (iv) The rate of formation of dihydroneopterin triphosphate (compound 5) from the isomeric mixture by the wild-type enzyme is similar to its rate of formation from GTP. However, we cannot rule out the possibility that the \(\beta\) cis isomer is in equilibrium with an undetected intermediate of the reaction sequence without being itself a proper intermediate. It remains unknown whether formamide cis\(\rightarrow\)trans isomerization plays a mechanistic role in the GTP cyclohydrolase I reaction sequence. These considerations are summarized in Fig. 8.

The conversion of GTP to the formamidopyrimidine nucleotide 2a by H179 mutant proteins is reversible. The equilibrium constant has a value of about 0.1 in aqueous solution at neutral pH. The reversible interconversion of a formamide-type intermediate and GTP reaction is reminiscent of the chemical synthesis of guanine from 2,5,6-triamino-4(3H)-pyrimidinone and formic acid. The latter reaction, however, requires high temperature and the absence of water. The direct formation of a purine nucleoside by ring closure to form the imidazole ring has not been reported to the best of our knowledge.

On the basis of x-ray structure data obtained with the wild-type enzyme, we had proposed earlier that residue H179 is involved in the opening of the imidazole ring of GTP (11). Specifically, it appeared likely that the reaction could be initiated by protonation of N7 of substrate GTP by the amino acid residue. Whereas some mutants with replacement of His\(^{179}\) can still catalyze the ring opening reaction, the maximum velocity observed with the H179S mutant is about 3% as compared with the rate of formation of dihydroneopterin triphosphate by the wild-type. Moreover, preliminary stopped flow experiments suggest that the opening of the imidazole ring by the wild-type enzyme is much faster than the overall reaction. Thus, it appears that the cleavage of the bond between N7 and C8 of GTP is slowed down by more than 1 order of magnitude if His\(^{179}\) is replaced. The slow residual reaction could be enabled by protonation of N7 of the substrate by another amino acid in the neighborhood.

The data also show that His\(^{179}\) is essential for the removal of formate from compound 2 by cleavage of the formamide bond. This would imply that the imidazole moiety of His\(^{179}\) catalyzes two mechanistically important steps at a biosynthetically equivalent nitrogen atom (viz. N-7 of GTP and N-5 of compound 2).

The formamidopyrimidine compound 2 is also observed in enzyme assays conducted with mutants of Gln\(^{151}\), Leu\(^{134}\), and Ser\(^{135}\). Glutamine 151 has been proposed to form a hydrogen bond with His\(^{179}\) in the wild-type enzyme. This could be relevant for optimum positioning of His\(^{179}\) for the hydrolysis of the formamide bond.

We showed recently\(^{1}\) that several mutants of the amino acids Cys\(^{110}\) His\(^{112}\), His\(^{113}\), and Cys\(^{181}\) are able to bind GTP in an apparently normal fashion, but do not catalyze any reaction. In the present paper we have shown further that these same mutants are unable to convert compound 2 to dihydroneopterin triphosphate. It appears, therefore, that their inability to produce dihydroneopterin triphosphate from GTP is not due solely to their inability to catalyze the initial stages of the reaction.

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Reaction Mechanism of GTP Cyclohydrolase I

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