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Reaction Mechanism of GTP Cyclohydrolase I: Single Turnover Experiments Using a Kinetically Competent Reaction Intermediate

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Elsevier Science Ltd.GTP cyclohydrolase I catalyses the transformation of GTP into dihydroneopterin 3′-triphosphate, which is the first committed precursor of tetrahydrofolate and tetrahydrobiopterin. The kinetically competent reaction intermediate, 2-amino-5-formylamino-6-ribosylamino-4(3*H*)-pyrimidinone, was used as substrate for single turnover experiments monitored by multiwavelength photometry. The early reaction phase is characterized by the rapid appearance of an optical transient with an absorption maximum centred at 320. This species is likely to represent a Schiff base intermediate at the initial stage of the Amadori rearrangement of the carbohydrate side-chain. Deconvolution of the optical spectra suggested four linearly independent processes. A fifth reaction step was attributed to photodecomposition of the enzyme product. Pre-steady state experiments were also performed with the H179A mutant which can catalyse a reversible conversion of GTP to 2-amino-5-formylamino-6-ribosylamino-4(3*H*)-pyrimidinone but is unable to form the final product, dihydroneopterin triphosphate. Optical spectroscopy failed to detect any intermediate in the reversible reaction sequence catalysed by the mutant protein. The data obtained with the wild-type and mutant protein in conjunction with earlier quenched flow studies show that the enzyme-catalysed opening of the imidazole ring of GTP and the hydrolytic release of formate from the resulting formamide type intermediate are both rapid reactions by comparison with the subsequent rearrangement of the carbohydrate side-chain which precedes the formation of the dihydropyrazine ring of dihydroneopterin triphosphate.

Keywords: GTP; GTP cyclohydrolase I; tetrahydrobiopterin biosynthesis; dihydroneopterin triphosphate; pteridine

Introduction

GTP cyclohydrolase I catalyses a ring expansion conducive to the formation of the pteridine derivative, dihydroneopterin triphosphate (compound **10**, Figure 1), from GTP.[1,2] The enzyme product is the first committed precursor in the biosynthetic pathway of the vitamin, tetrahydrofolate, which is produced by plants and many microorganisms.[3] Dihydroneopterin triphosphate also serves as the first committed precursor in the biosynthetic pathway of tetrahydrobiopterin, which is an endogenous metabolite of vertebrates where it serves as an essential cofactor for the biosynthesis of nitric oxide and of catecholamine-type neurotransmitters.[4,5]

GTP cyclohydrolase I of microbial and vertebrate origin share considerable sequence similarity.[6] The catalytic domain of the human protein features 37 % identical amino acid residues by comparison with the protein from *Escherichia coli*. Both proteins are d_5 symmetric toroid-shaped decamers[7,8] where each of the ten active sites is located at the interface of three adjacent subunits. An essential zinc ion is bound to conserved cysteine and histidine residues at each active site.[8]

The multistep reaction catalysed by GTP cyclohydrolase I is mechanistically complex. Kinetic studies showed that the opening of the imidazole ring of GTP under formation of compound **4**

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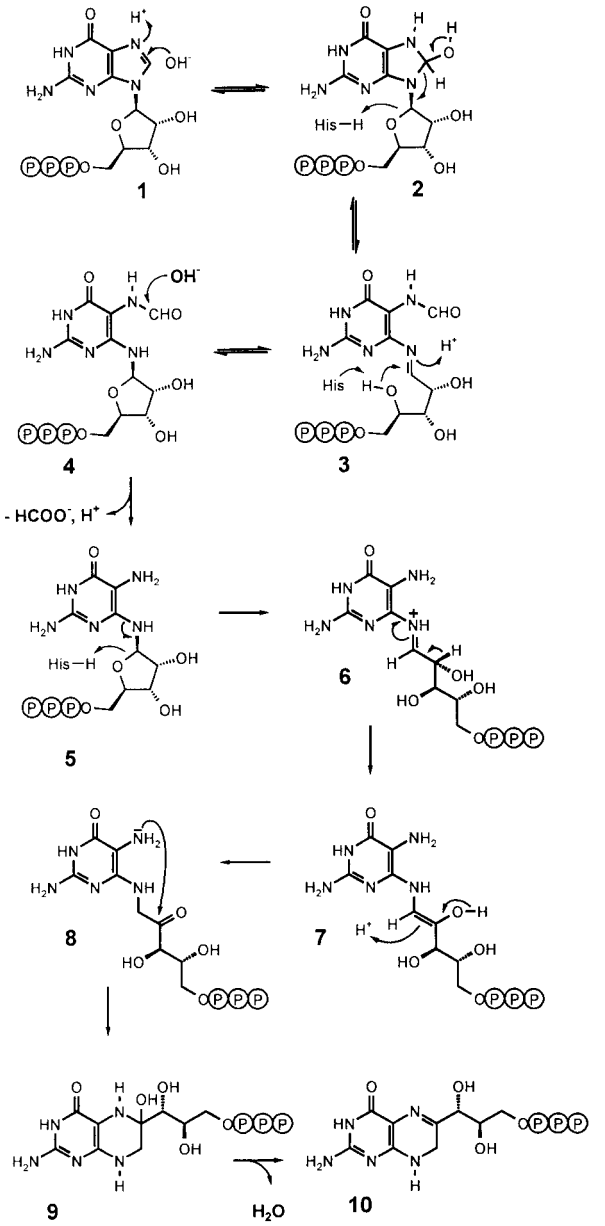

Figure 1. Hypothetical reaction mechanism of GTP cyclohydrolase I.[9]

is rapid as compared to the subsequent carbohydrate rearrangement and ring closure.[9] In order to facilitate the kinetic analysis of the complex reaction sequence, we decided to utilize the kinetically competent reaction intermediate, compound **4** (Figure 1), as substrate for pre-steady state experiments.

Results

The H179A mutant of GTP cyclohydrolase I can catalyse the hydrolytic release of formate from GTP affording the reaction intermediate, 2-amino-5-formylamino-6β-ribosylamino-4(3*H*)-pyrimidinone (compound **4a**, Figure 2) but is unable to catalyse the subsequent reaction steps.[10] The primary product of the mutant enzyme, compound **4a**, is subject to rapid isomerisation affording the α-glycoside **4b**.[10]

GTP was removed from the equilibrium mixture obtained by treatment of GTP with recombinant H179A mutant GTP cyclohydrolase I by anion exchange chromatography. The resulting mixture of the β and α isomers (**4a** and **4b**) was used for pre-steady-state stopped-flow kinetic experiments monitored by multiwavelength photometry.

Only the β-glycoside **4a** can serve as substrate for GTP cyclohydrolase I. Since the spontaneous anomerisation of the compound **4** type compounds is slow by comparison with the time frame of the stopped-flow experiments, compound **4b** is not consumed to a significant extent in single turnover experiments. It was therefore appropriate to correct the optical spectra for the absorption contribution of this ultraviolet-absorbing material whose concentration did not change significantly during the reaction progress.

A set of corrected optical spectra from a stopped-flow experiment with wild-type GTP cyclohydrolase I of *E. coli* and the isomer mixture

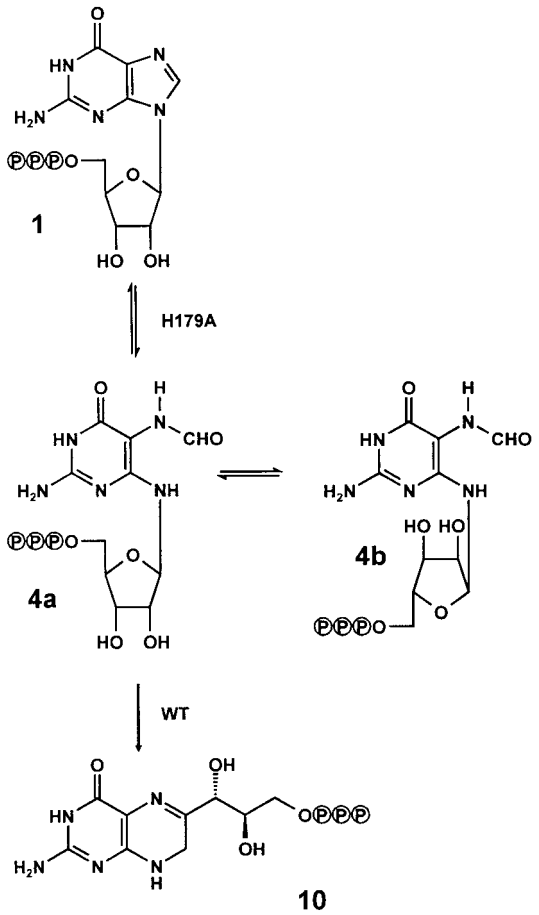

Figure 2. Enzyme-catalysed and spontaneous reactions involving the reaction intermediates, compound **4**.

as substrate is shown in Figure 3(a). Data from a similar stopped-flow experiment performed with GTP as substrate are shown for comparison in Figure 3(c).[11]

The landscapes in Figure 3(a) and (c) are similar with the exception of the initial reaction period. Selected spectra from the early and the late phases of both experiments are displayed in Figures 4 and 5.

Compounds **4a** and **4b** are characterized by absorption maxima at 273 nm (Figure 6). In the stopped-flow experiment shown in Figure 3(a), we note a rapid decrease of the absorption at 275 nm in conjunction with the emergence of an absorption band centred at about 320 nm. Due to the rapid depletion of compound **4**, the first spectrum acquired after 0.1 second already shows substantial absorption above 300 nm indicative of the optical transient C (see below) (Figure 4(c)).

In the experiment with GTP as substrate (Figure 3(c)) a closely similar transient with long wavelength absorbance above 300 nm appears significantly later. Hence, the measurement at 0.1 second shows a virtually unperturbed GTP spectrum. The long wavelength optical transition is clearly noted after 0.7 second (Figure 4(a)). At that time, the 320 nm transient is already approaching its maximum value in the experiment using compound **4** as substrate (Figure 4(c)).

The dynamic evolution of the transient characterized by the 320 nm maximum with GTP and compound **4** is best compared in Figure 7. The absorption at 320 nm reached its maximum value at 2.5 seconds in the experiment with 2-amino-5-formylamino-6-ribosylamino-4(3*H*)-pyrimidinone 5′-triphosphate (Figure 7(b)) and at four seconds in the experiment with GTP (Figure 7(a)). In both experiments, the absorption at 320 nm subsequently passes through a shallow minimum around 19 seconds. Coincidentally, optical spectra with a characteristic absorption maximum at 285 nm are observed in both experiments (Figure 5). It should also be noted that the transient species absorbing at 320 nm disappears in the course of the reaction progress.

The spectra obtained at 20 seconds and later are similar in the experiments with GTP or compound **4**. In Figure 5(a) and (b), the final reaction stage is characterized by the progressive increase of absorbance in the 320 to 380 nm range, indicating the formation of dihydroneopterin triphosphate (compound **10**) as well as an artifactual photo-oxidation product which has been discussed earlier.[11]

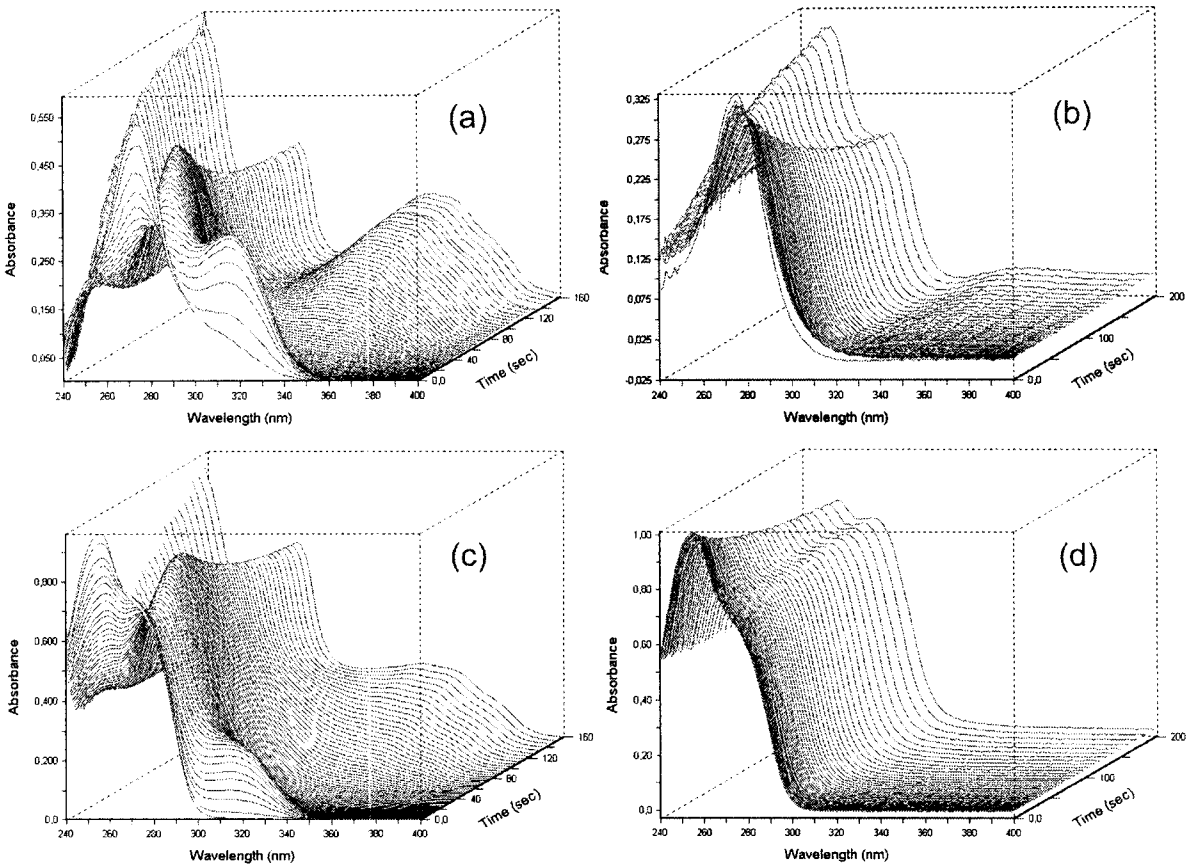

Figure 3. Optical spectra from single turnover stopped-flow-experiments. (a) Wild-type GTP cyclohydrolase I, compound **4** as substrate; (b) H179A mutant, compound **4** as substrate; (c) wild-type GTP cyclohydrolase I, GTP as substrate; (d) H179A mutant, GTP as substrate.

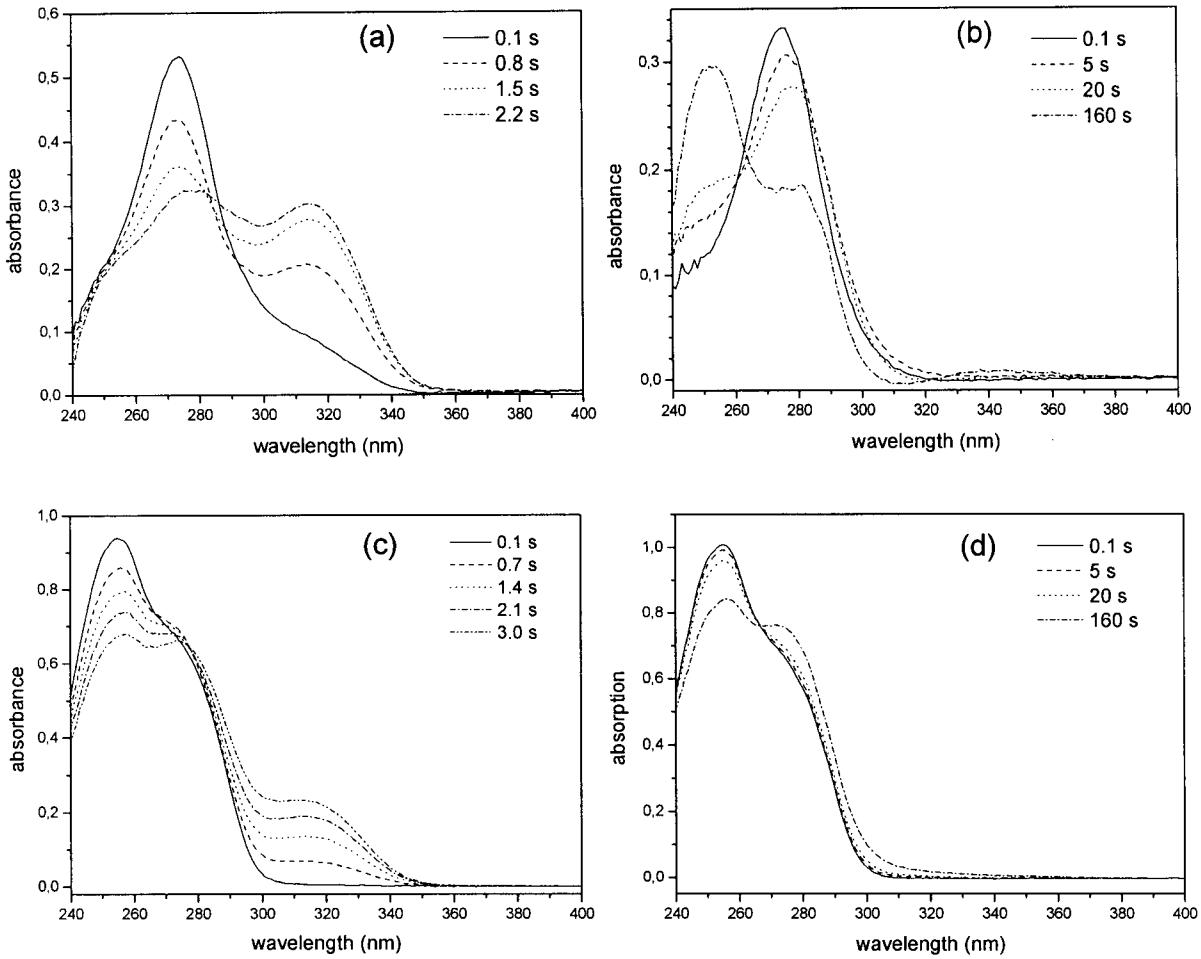

Figure 4. Selected ultraviolet spectra from single turnover reaction sequence of GTP cyclohydrolase I (early reaction phase). See the legend to Figure 3 for details.

Earlier experiments using the H179A mutant had shown that the transformation of GTP into the formamide derivative, compound **4**, is a reversible reaction with an equilibrium constant around 0.1. The predominant species at equilibrium was found to be GTP. Based on these findings and our earlier stopped-flow and quenched-flow experiments,[9,11] we had expected that wild-type GTP cyclohydrolase would catalyse a reverse reaction conducive to the formation of GTP from proffered compound **4** at an appreciable rate, in addition to the formation of the final product, dihydroneopterin triphosphate (compound **10**).

This question can be addressed by inspection of the absorbance at 254 nm, the absorption maximum of GTP, in the stopped-flow experiments in Figure 7(a) and (b). With GTP as substrate, the absorbance at 254 nm decreased to about 40 % of its initial value over a period of 30 seconds where it reaches a minimum. This behaviour is well in line with the consumption of proffered GTP as observed by both stopped-flow and quenched-flow analysis.[9,11]

An absorption minimum of the formamide derivative, compound **4**, at 250 nm coincides almost exactly with the absorption maximum of GTP at 254 nm. Moreover, compounds **5** and **7-10** (Figure 1) are also characterized by low absorbency at the maximum of GTP at 254 nm (cf. Figure 5 and the discussion of absorbance properties in Ref. [11]). The expected formation of GTP from compound **4** would be expected to increase the absorbance of the reaction mixture at 254 nm. Contrary to that expectation, we observe a slight absorbance decrease affording to a shallow minimum at about ten seconds in Figure 7(b).

The formation of GTP in appreciable amounts from compound **4** appears unlikely in light of these findings. The unexpected result requires that the hydrolysis of compound **4** to form formate is rapid by comparison with the reverse reaction conducive to the formation of GTP by condensation of the 5-formyl group with the glycosidated position 5 amino group.

In a previous study, we had analysed the data in Figure 3(c) (GTP as substrate) using a model that emphasized the reversibility of the transformation of GTP and compound **4**.[11] With that model, it was necessary to introduce the experimentally determined absorbance spectra into the numerical

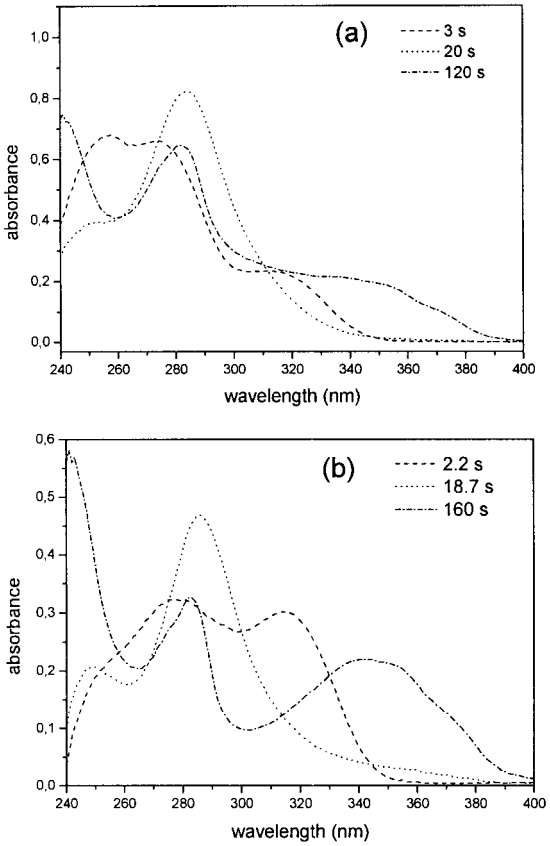

Figure 5. Selected ultraviolet spectra from single turnover reaction sequence with wild-type GTP cyclohydrolase I and GTP (a), resp. compound **4** (b) as substrate.

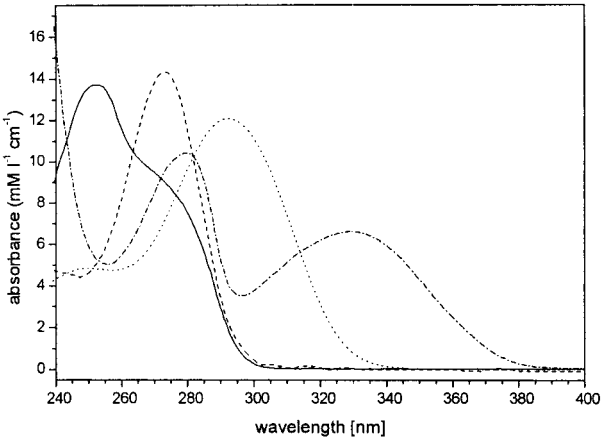

Figure 6. Ultraviolet spectra of compounds occurring in the reaction trajectory of GTP cyclohydrolase I.[11] (—) GTP (compound **1**); (- - -) 2-amino-5-formylamino-6-ribosylamino-4(3*H*)-pyrimidinone 5′-triphosphate (compound **4**); (· · · · ·) 2,5-diamino-6-ribitylamino-4(3*H*)-pyrimidinone 5′-monophosphate (compound **5**-monophosphate); (-·-·-) dihydroneopterin (the triphosphate isomer compound **10**).

deconvolution procedure. A re-evaluation of these earlier data in light of our failure to observe the expected formation of GTP from compound **4** is given below.

For a detailed analysis, the data set in Figure 3(a) was subjected to singular value decomposition using the program SPECFIT/32 (Spectrum Software Associates, Marlborough, MA). The analysis indicated five linearly independent optical processes significantly above the noise floor. These data were numerically fitted to a kinetic model indicating four consecutive enzyme catalysed reaction steps followed by a fifth reaction step accounting for the artifactual photodecomposition of dihydroneopterin triphosphate which has been described in detail earlier:[11]

$$B \xrightarrow{k_2} C \xrightarrow{k_3} D \xrightarrow{k_4} E \xrightarrow{k_5} F \xrightarrow{k_6} G \qquad (1)$$

The deconvoluted spectra of the molecular species B to F are shown in Figure 8(c). In contrast to our earlier approach the artifactual photoproduct is not shown explicitly in the concentration *versus* time profile and has instead been added to the concentration of the terminal enzyme product.

The quality of the fit can be estimated from the comparison between the experimental absorbance data (symbols in Figure 7(b)) and the sum of the fractional absorption contributions of components B to G (lines in Figure 7(b)) according to the kinetic model using the parameters shown in Table 1.

The data of an experiment using GTP as substrate are again shown for comparison (Figure 8(a) and (b); data from Ref.[11]). Notably, six linearly independent processes had to be assigned in order to deconvolute these data, i.e. one more than in the experiment with compound **4**. In contrast to the data analysis in an earlier publication, experimental spectra were not introduced into the deconvolution procedure, and the conversion of GTP to

Table 1. Rate constants of the GTP cyclohydrolase I reaction

	Rate constant (s^{-1}) GTP as substrate	Rate constant (s^{-1}) compound **4** as substrate
GTP → Compound **4**	0.16 ± 0.001	-
k_2	1.5 ± 0.05	0.76 ± 0.008
k_3	0.50 ± 0.007	0.19 ± 0.002
k_4	0.019 ± 0.0008	0.035 ± 0.003
k_5	0.023 ± 0.0002	0.043 ± 0.005
k_6	0.0071 ± 0.000	0.0050 ± 0.00002

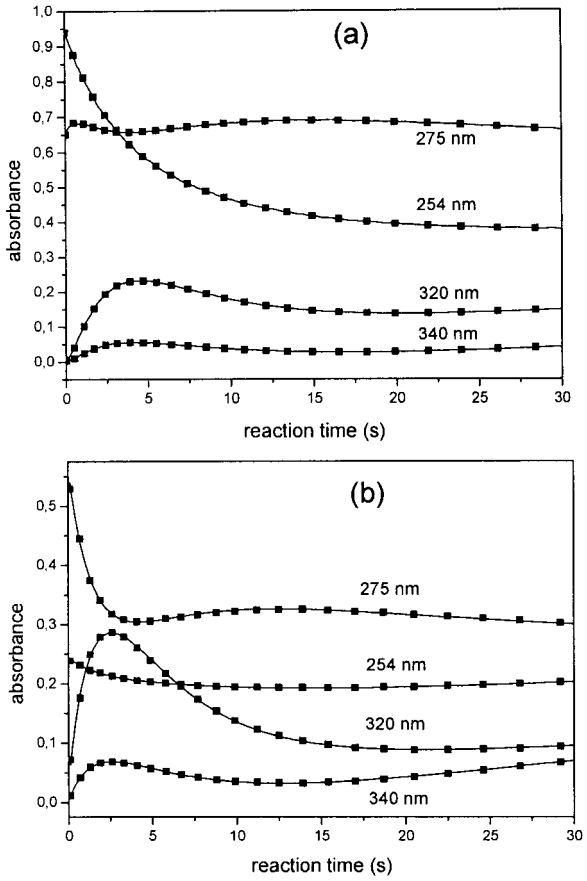

Figure 7. Absorbance changes obtained from single turnover stopped-flow experiment with wild-type GTP cyclohydrolase I and GTP (a), resp. compound **4** (b). Symbols represents the original data, lines represents the data obtained from numerical simulation using the kinetic constants in Table 1.

compound **4** was modelled as an irreversible process.

The numerical deconvolution shown in Figure 8 faithfully reflects the course of events that has been described qualitatively in the previous section. The proffered substrate, compound **4** (β anomer) decays rapidly. The decay process can be modelled as a first order reaction. The 50 % value for compound **4** is reached after 1.0 second and the 10 % value is reached after 3.2 seconds. By comparison, the time required for 50 % consumption of proffered GTP in the experiment shown in Figure 7(a) is 4.2 seconds. Thus compound **4** is consumed more rapidly than GTP and fulfills the formal criteria for a kinetically competent intermediate.

The optical transient C (green line in Figure 8) reaches a maximum value after 2.4 seconds in the experiment with compound **4** as substrate (Figure 8(c)) and substantially later, at four seconds, in the experiment with GTP as substrate (Figure 8(a)). The maximum level of transient C is higher in the experiment with compound **4** as substrate as compared to the experiment with GTP. The results of the numerical deconvolution are well in line with the preliminary conclusions from the raw data (see above).

Earlier, we had identified transient D (blue line in Figure 8) as the dominant species at 19 seconds in experiments with GTP as substrate (Figure 8(b)). A closely similar transient was found in the experiment with compound **4** as substrate where it reaches a maximum value at an earlier time, around 14 seconds (Figure 8(d)).

Transients E and F have similar shapes and time-courses in the experiments with compound **4** with GTP as substrate. Since the conversion of GTP to compound **4** is quite rapid by comparison with the overall reaction, the close similarity of the late reaction stages in experiment with 2 different substrates is well in line with the *a priori* expectation.

As described earlier, transient F resembles the spectrum of the enzyme product, dihydroneopterin triphosphate (compound **10**). The relatively weak transient E cannot be unequivocally attributed to one of the proposed reaction intermediates in Figure 1. An association of transient E with the hydrated pteridine structure, compound **9**, is conceivable but tentative.

As reported earlier, the H179A mutant of GTP cyclohydrolase I can catalyse the release of formate from GTP but is unable to convert the resulting compound **4** any further.[10] We had also shown earlier that the reaction catalysed by the mutant enzyme is reversible. In light of our failure to detect the retrograde formation of GTP from compound **4** by the wild-type enzyme (see above), it was in order to perform additional kinetic studies with the H179A mutant protein. It should be noted that the reaction velocity of the mutant enzyme is substantially lower as compared to the wild-type enzyme.

Stopped flow experiments using H179A mutant and compound **4** with GTP are shown in Figure 3(b) and (d). The landscapes are relatively featureless by comparison with the data discussed above (Figure 3(a) and (c)). An isosbestic point appears likely to be present in the experiment with GTP as substrate shown in Figure 4(b). Moreover, isosbestic points appear to be present at least in the early phase of the similar experiment using the compound **4** stereoisomers mixture in Figure 4(d).

The most important finding in these experiments is the absence of a transient with a significant absorbance at wavelength above 300 nm; a slight absorbance increase at long wavelengths occurring in the raw data after prolonged incubation in the optical chamber can be attributed to photodecomposition rather than enzyme catalysis.

In line with this preliminary description, the numerical deconvolutions shown in Figure 9(a) and (b) shows only two optical species contributing at a significant level. They are closely similar to the spectra of GTP with compound **4**.

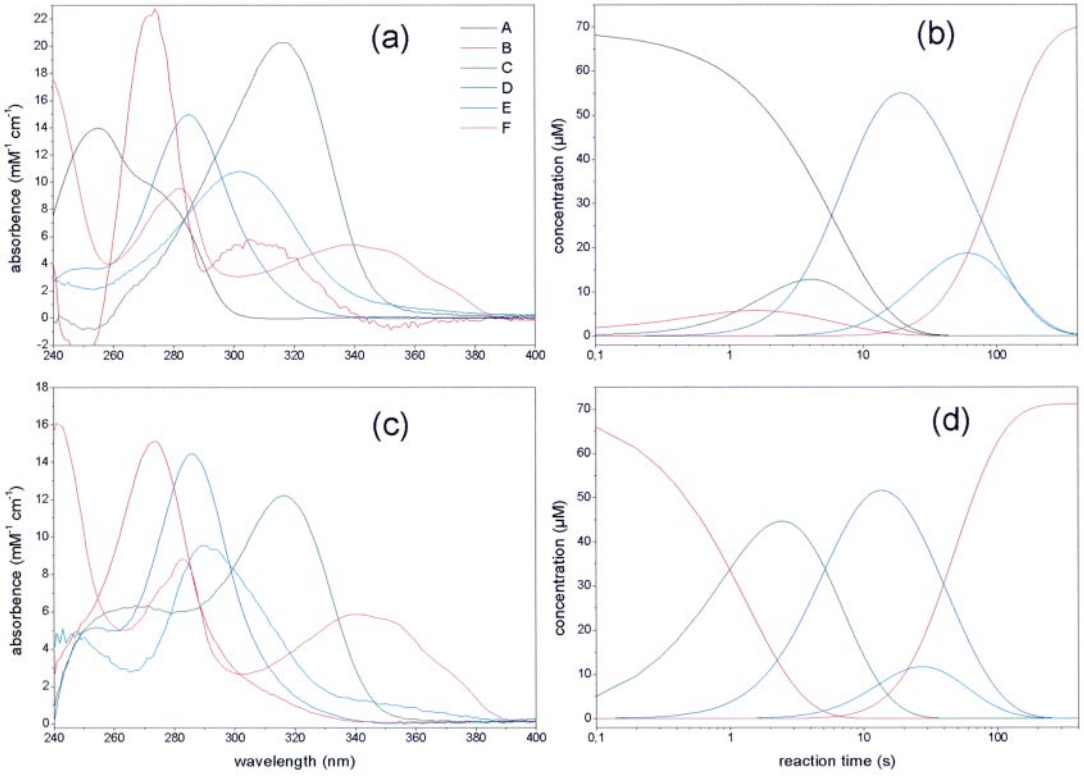

Figure 8. Numerical simulation of stopped-flow data. (a) reconstructed absorbance spectra of transient chromophors using GTP as substrate; (b) concentration of transient species using GTP as substrate; (c) reconstructed absorbance spectra of transient chromophors using compound **4** as substrate; (d) concentration of transient species using compound **4** as substrate.

Discussion

The kinetic analysis of GTP cyclohydrolase I benefits from the characteristic ultraviolet spectra of intermediates and the chemical stability of formate and compound **4** which permits their detection in quenched-flow studies.[9,11] However, it should be noted that neither the optical transients nor the quenched-flow data can be assigned unequivocally to specific reaction intermediates shown in Figure 1. For example, compound **4** detected in quenched-flow analysis could have been formed, at least in part, from the hypothetical intermediates **2** and **3** by the trichloracetic acid quench reaction.

The stopped-flow and quenched-flow data on dihydroneopterin triphosphate formation from GTP cyclohydrolase I reported in earlier papers had shown that the hydrolytic release of formate from GTP is not rate-limiting for the overall reaction. The rate-limiting step was clearly shown to be associated with the rearrangement of the carbohydrate side-chain in the later part of the reaction sequence, although it could not be unequivocally assigned to one specific reaction.[9]

As expected, the data from experiments with wild-type GTP cyclohydrolase I using GTP respectively compound **4** as substrate differ mainly in the early reaction phase, whereas the later reaction phase is similar. All data obtained up to now are consistent with compound **4a** as an obligatory intermediate in the reaction sequence.[10]

A major surprise in the present series of experiments was the failure to detect the formation of GTP from compound **4** by wild-type GTP cyclohydrolase I. Earlier, we had shown that the equilibrium constant for the interconversion of GTP and compound **4** as defined by equation (2) has a value around 0.14 at 30 °C and pH 8.5:[9]

$$\text{GTP} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{compound } \mathbf{4} \quad K = \frac{[\text{Compound } \mathbf{4}]_{\text{eq}}}{[\text{GTP}]_{\text{eq}}} \qquad (2)$$

Based on the kinetic parameters obtained in our earlier stopped-flow study with GTP as substrate, GTP would have been expected to accumulate to a maximum level of 70 μM (as compared to a starting concentration of 250 μM compound **4**) at 1.4 seconds.[11] This expected GTP concentration should have resulted in a positive absorbance increment of 0.14 at 254 nm in Figure 7(b), which is clearly not observed. It should be noted that the calculated concentration of GTP formed from compound **4** is sensitive to the apparent rate constants k_1, k_{-1} and k_2 in equations (1) and (2). In light of the differences in the apparent rate constants for the deformylation of compound **4** in the different experiments using GTP respectively compound **4**

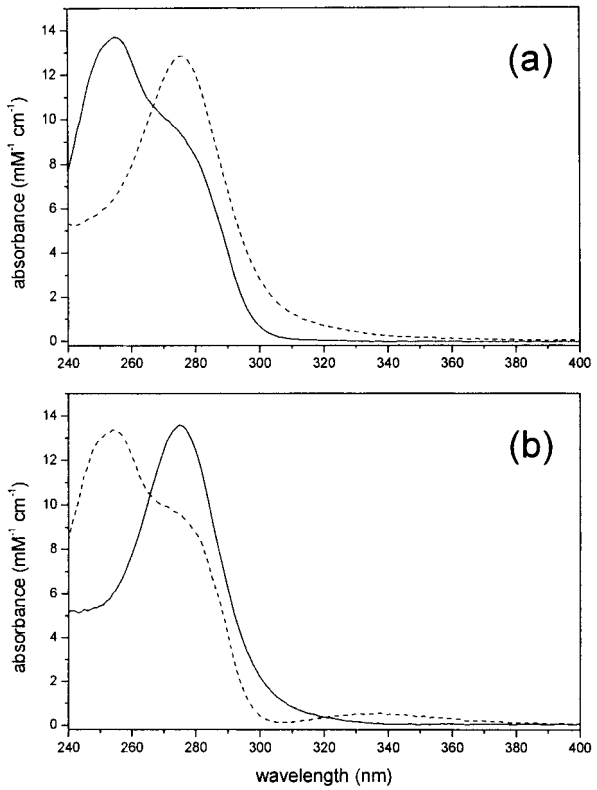

Figure 9. Reconstructed absorption spectra of transient chromophors occurring during single turnover experiments of H179A mutant of GTP cyclohydrolase I with GTP (a), resp. compound **4** (b) as substrate.

as substrate, the rate for the formation of GTP from compound **4** may also be different.

On the other hand, the reversible formation of GTP from compound **4** by the H179A mutant has been again confirmed here by the stopped-flow experiments kinetic described in Figure 3(b). We are therefore bound to assume that the release of formate from compound **4** is rapid by comparison with its conversion to GTP. This hypothesis appears to be in conflict with the detection of compound **4** in quenched-flow experiments at an appreciable level.[9] However, as pointed out, it is not possible to unequivocally correlate the experimental observation of compound **4** with one specific reaction intermediate. On the contrary, compound **4** could have been formed from compound **2** and/or **3** under the conditions of the acid quench. It is not possible on the basis of the available data to prove that the transient spectra labelled B in the experiments with GTP and compound **4** as substrates (Figure 8) represent the same molecular species. The hypothetical proposal of an equilibrium between GTP and the GTP hydrate could apply if the optical transient B in the experiment with GTP as substrate represents the GTP hydrate (compound **2**).

In summary, the data suggest that the hydrolytic release of formate from the actual formamide, compound **4**, is rapid by comparison with the rate of formation of compound **4**, and that therefore the steady state concentration of compound **4** in experiments with the wild-type enzyme is low. The hydrolysis of the formamide could be assisted by the zinc ion at the active site in close similarity with the reaction mechanism of the proteases. The zinc would thus have a dual mechanistic function catalysing the addition of water or a hydroxyl ion to C-8 of the purine ring system as well as to the formamide group of the amide type compound **4**.

It is also conceivable that the wild-type enzyme rapidly converts the formamide, compound **4** to a hydrate by the addition of a water molecule. If we assume (i) that the formamide and the hydrate have similar optical spectra and (ii) that the formation of the hydrate proceeds more rapidly than the release of formate, all current findings can be explained satisfactorily.

Our earlier interpretation of stopped-flow experiments with the wild-type enzyme had been based on the hypothesis of a reversible interconversion of GTP and compound **4**.[11] We have now recalculated the data without that assumption; no significant changes occurred in the deconvoluted optical transients. It is not possible to distinguish between a reversible and an irreversible step on the basis of the available data. It should be noted that the equilibrium equation could be simplified by using net rate constant theory[12] leading to a similar equation without equilibrium.

Our earlier studies had also proposed that the optical transient C with a maximum at 320 nm is associated to an intermediate downstream from the formamide type compound **4**. This hypothesis is supported by the finding that the transient C appears more rapidly with compound **4** substrate of the wild-type enzyme as compared to GTP. An even more rigorous proof of the hypothesis is the findings that the reversible interconversion of GTP and compound **4a** does not show a transient with long wavelength absorption. More than that, optical spectroscopy failed to detect any intermediate whatsoever between GTP and compound **4**. This is likely to implicate that the hydrate species, compound **2**, is only populated at a low level. The hypothetical Schiff base, compound **3**, may also go unnoticed due to low population. However, it is also worth considering whether compound **3** should be removed entirely from the reaction scheme. In fact, the hydrated imidazole ring of compound **2** could be opened directly by cleavage of the C-8/N-9 bond accompanied by protonation of N-9 and deprotonation of the hydroxyl group at C-8.

In light of the present findings, there can be hardly any doubt that transient C represents the Schiff base intermediate **6**. Deprotonation of that intermediate affording the enamine type intermediate **7** would then be a slow reaction as compared to all preceding reaction steps. However, the rate-limiting step appears to be downstream from the

enamine; more specifically, the reprotonation of the enamine may limit the overall reaction rate.

Materials and Methods

Protein substrates

Wild-type and H179N mutant GTP cyclohydrolase I of *E. coli* were prepared as described earlier.[13] 2-Amino-5-formylamino-6-ribosylamino-4(3*H*)-pyrimidinone 5′-triphosphate was prepared as described earlier.[10]

Single turnover experiments

Stopped-flow experiments were performed with a SFM4/QS apparatus from Bio-Logic (Claix, France) equipped with a linear array of three mixers and four independent syringes. A 1.5 mm light path quartz cuvette located behind the last mixer was monitored with a Tidas diode array spectrophotometer (200-610 nm) equipped with a 15 W deuterium lamp as light source from J&M Analytische Meß- und Regeltechnik, Aalen, Germany.

The reaction buffer contained 50 mM Tris hydrochloride (pH 8.5), and 100 mM potassium chloride. A solution containing 250 μM 2-amino-5-formylamino-6-ribosylamino-4(3*H*)-pyrimidinone 5′-triphosphate (compound **4**) respectively GTP in reaction buffer was mixed with a 267 μM solution of GTP cyclohydrolase I (6.6 mg ml^{-1}) in reaction buffer at a total flow rate of 4 ml s^{-1} and a temperature of 30 °C. 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.

Data analysis

Prior to data analysis, the absorbance background due to buffer and enzyme was removed by subtraction of a blank data set obtained without addition of substrate. Since only the β anomer of Compound **4** can serve as substrate for GTP cyclohydrolase I, the absorbance contribution of the α anomer was also subtracted.

Data reduction and stronger weighting of early spectra were achieved by extracting 300 spectra on a pseudologarithmic time base from the difference data sets. These data sets were then analysed using the program SPECFIT/32 3.0.12 (Spectrum Software Associates, Marlborough, MA).

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