Enzymic characterization of *Bacillus subtilis* GTP cyclohydrolase I

Evidence for a chemical dephosphorylation of dihydroneopterin triphosphate

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GTP cyclohydrolase I catalyses the first committing step in the biosynthesis of the pterin moiety of folic acid: conversion of GTP to dihydroneopterin triphosphate. GTP cyclohydrolase I of *Bacillus subtilis* was purified to homogeneity and shown to have a homo-octameric structure. The enzyme had an apparent K_m for GTP of 4 μM and, in the absence of cations, a $V_{max.}$ of 80 nmol/min per mg of protein. K^+ ions moderately increased its $V_{max.}$, whereas UTP and Ca^{2+} and Mg^{2+} ions drastically increased its K_m for GTP. Dihydrofolate and other products of the folate and tetrahydrobiopterin pathways did not inhibit GTP cyclohydrolase I. In addition to their effect on the enzyme activity, Ca^{2+} and Mg^{2+} ions catalysed the chemical dephosphorylation of dihydroneopterin triphosphate to non-cyclic dihydroneopterin monophosphate, the substrate for the phosphomonoesterase reaction in folate biosynthesis. This dephosphorylation was specific and did not require the action of a phosphatase. We suggest a physiological role for Ca^{2+} ions and UTP in regulation of folate biosynthesis at the levels of GTP cyclohydrolase I and dephosphorylation of dihydroneopterin triphosphate.

INTRODUCTION

GTP is used as a substrate for various biosynthetic pathways, such as the synthesis of RNA, riboflavin and folic acid or tetrahydrobiopterin (for reviews, see [1,2]). The mechanisms controlling the flow of GTP into each of these pathways are largely unknown. GTP cyclohydrolase I is one of the enzymes that uses GTP as a substrate. It catalyses the conversion of GTP to dihydroneopterin triphosphate and formic acid, and thus performs the first committing step in the biosynthesis of the pteridine moiety of folic acid in micro-organisms and of tetrahydrobiopterin in higher animals [3,4]. Tetrahydrofolate is a coenzyme for a variety of one-carbon-transfer reactions [5]. Tetrahydrobiopterin functions as a cofactor for various amino acid hydroxylases, the *O*-alkylglycerolipid cleavage enzyme [3,4] and nitric oxide synthases [6–8]. A mechanism of action for GTP cyclohydrolase I is known, which involves at least two discrete enzyme-bound intermediates [9]. GTP cyclohydrolases I of several different organisms have been described (for review, see [10]), and the enzyme has been purified to apparent homogeneity from *Escherichia coli* [11,12], *Drosophila melanogaster* [13] and human, mouse and rat liver [14–16].

GTP cyclohydrolase I as the first committing step in the biosynthesis of folic acid and tetrahydrobiopterin is a logical target for regulation of the biosynthesis of these compounds. GTP cyclohydrolase I is indeed the rate-limiting enzyme in the biosynthesis of tetrahydrobiopterin. The enzyme activity is regulated at the transcriptional [17–21] and substrate [15,22] levels. In addition, GTP cyclohydrolase I of rat liver is subject to feed-back inhibition by tetrahydrobiopterin [23,24]. This feed-back inhibition is mediated by a p35 protein [25]. L-Phenylalanine, the substrate of the tetrahydrobiopterin-dependent phenylalanine hydroxylase, stimulates the enzyme activity. This effect is also mediated by the p35 protein [25]. Less is known about the regulation of microbial GTP cyclohydrolases I. The purified GTP cyclohydrolase I of *E. coli* is weakly inhibited by tetrahydrobiopterin [12]. The relevance of this finding is, however, questionable, because *E. coli* does not produce this compound. The *E. coli* enzyme may be subject to feed-back regulation by dihydrofolate, as GTP cyclohydrolase I has been specifically purified using a dihydrofolate–Sepharose affinity column [26].

The next step in folate biosynthesis is the dephosphorylation of dihydroneopterin triphosphate to dihydroneopterin monophosphate. The latter compound is the substrate for a phosphomonoesterase reaction in which dihydroneopterin is produced. Relatively little is known about the removal of the pyrophosphate group from dihydroneopterin triphosphate. A pyrophosphohydrolase activity has been purified from *E. coli* [27]. It has a molecular mass of ~ 17 kDa and requires Mg^{2+} ions for activity. This finding was later questioned by the original author [1], and the pyrophosphohydrolase activity was assigned to a non-specific enzyme.

Here, we describe the purification and subunit structure of GTP cyclohydrolase I from *Bacillus subtilis*. The kinetic and regulatory properties of the enzyme were studied. In addition, we studied the next step in folate biosynthesis, the dephosphorylation of dihydroneopterin triphosphate to dihydroneopterin monophosphate. We show that this conversion can be efficiently catalysed *in vitro* by divalent cations. In summary, our data suggest functions for divalent cations and UTP in regulation of folate biosynthesis in *B. subtilis*.

MATERIALS AND METHODS

Chemicals

Derivatives of neopterin, biopterin and folate were obtained from Dr. B. Schircks Laboratories. GTP (lithium salt) and periodate-oxidized GTP were obtained from Sigma Chemical Co. The gel-filtration resins Ultrogel AcA 34 and AcA 54 were from IBF Sepracor; Partisil-10 SAX anion-exchange radial pack was purchased from Millipore.

Bacterial strains and plasmids

Plasmid pSI45 containing the wild-type *mtr* operon was kindly provided by Dr. P. Gollnick [28]. Plasmid pSPOCy6, which contains the *mtr* operon under control of the $spoI_{26}$ promoter,

Abbreviations used: b, base; PEI, polyethyleneimine; TRAP, tryptophan RNA-binding attenuation protein.

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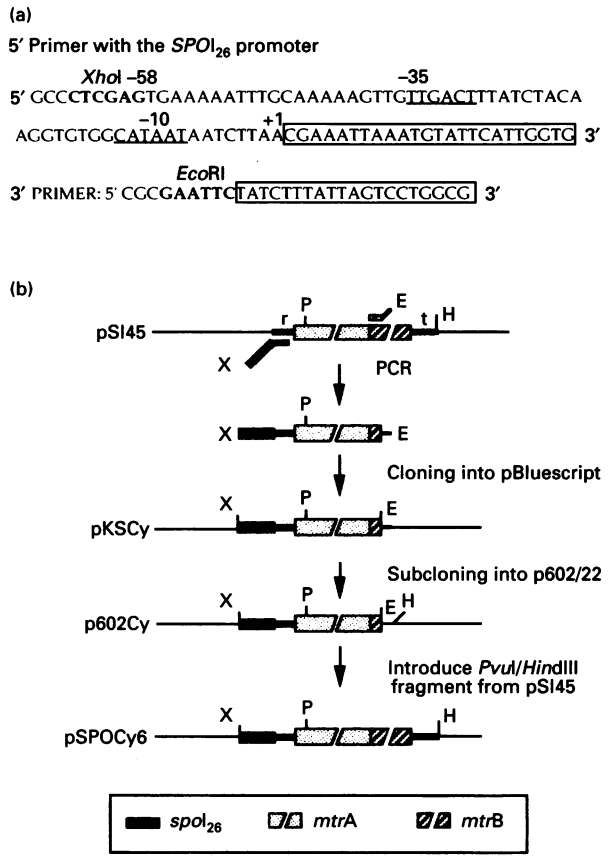

Figure 1 Construction of plasmid pSPOCy6 containing the *mtr* operon under control of the strong $spoI_{26}$ promoter

(**a**) Oligonucleotides used for PCR amplification of *mtr*A, the gene encoding GTP cyclohydrolase I. Bases −58 to +1 represent the $spoI_{26}$ promoter. Underlined are the −35 and −10 sequences of the promoter. Boxes identify sequences complementary to the *mtr*A sequence. The restriction sites are shown in bold characters. (**b**) Construction of plasmid pSPOCy6. Construction was as described in the Materials and methods section. r, Ribosomal binding site; t, transcriptional terminator; E, *Eco*RI; H, *Hin*dIII; P, *Pvu*I; X, *Xho*I.

was constructed as follows. A 91 base (b) oligonucleotide was synthesized (Figure 1a), which contains an *Xho*I site followed by 58 b of $spoI_{26}$ promoter [29] and a 24 b sequence complementary to the leader sequence of the *mtr* operon. PCR amplification [30] was performed using plasmid pSI45, the 91 bp oligonucleotide and a 29 bp oligonucleotide containing the 3′ end of the coding sequence for *mtr*A followed by an *Eco*RI site (Figure 1a). The first five cycles were performed with an annealing temperature of 50 °C, and were followed by 25 cycles with 65 °C as annealing temperature.

The isolated PCR fragment and vector pBluescript KS (Stratagene Inc.) were digested with *Xho*I and *Eco*RI, ligated and transformed into the *E. coli* strain SCS-1 (Stratagene Inc.). Plasmid pKSCy contained the PCR fragment (Figure 1b). All positive clones harboured variants of the plasmid with very low copy numbers, suggesting that the presence of plasmid pKSCy is deleterious to *E. coli*. The *Xho*I–*Eco*RI fragment of pKSCy was transferred to the *B. subtilis* shuttle vector p602/22 [31]. The DNA sequence of the *Xho*I–*Pvu*I part of the resulting plasmid p602Cy was checked using the dideoxy DNA sequence analysis procedure. Plasmid p602Cy was then digested with *Pvu*I (present within the coding sequence of *mtr*A) and *Hin*dIII (located in p602/22 at the 3′ end of the *mtr*A sequence). The vector-

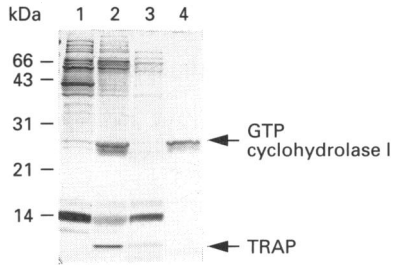

Figure 2 Overexpression and purification of GTP cyclohydrolase I

GTP cyclohydrolase I was overexpressed in *B. subtilis* 1012 using plasmid pSPOCy6 (Figure 1b). The resulting strain SPCy was used for purification of GTP cyclohydrolase I. Fractions obtained during purification were analysed on a 4–16% (w/v) SDS/polyacrylamide gel. Lane 1, lysate of wild-type 1012 cells; lane 2, lysate of cells containing plasmid pSPOCy6; lane 3, flow-through of the GTP-affinity column; lane 4, combined fractions with GTP cyclohydrolase I activity obtained by elution of the GTP-affinity column. The positions of GTP cyclohydrolase I (encoded by *mtr*A) and TRAP (encoded by *mtr*B) are indicated. The strong band at 14 kDa is lysozyme used for cell lysis.

containing part was then ligated with a *Pvu*I–*Hin*dIII fragment of pSI45 containing part of the *mtr*A gene, the *mtr*B gene and the transcription terminator of the *mtr*AB operon.

The resulting plasmid pSPOCy6 contains the entire *mtr*AB operon under control of the $spoI_{26}$ promoter. *B. subtilis* strain 1012 was transformed with undigested plasmid DNA. This leads to a Campbell-type integration of the plasmid pSPOCy6 into the endogenous *mtr* locus, resulting in duplication of the *mtr*AB operon, one copy having the $spoI_{26}$ promoter. Proper integration of the plasmid into the genome in strain SPCy was confirmed using PCR analysis.

Purification and characterization of *B. subtilis* GTP cyclohydrolase I

Frozen SPCy cells (25 g) were resuspended in 2 ml of lysis buffer/g of cell wet weight and were incubated for 1 h at 37 °C and 1 h on ice. The lysis buffer contained 50 mM potassium phosphate buffer, pH 6.5, 2.5 mM EDTA, 2 mM phenylmethanesulphonyl fluoride and 3×10^4 units/ml lysozyme (Sigma), 50 units/ml DNAase (Boehringer) and 4 units/ml RNAase (Boehringer). The supernatant obtained after centrifugation for 1 h at 4000 *g* was dialysed at 4 °C against 50 mM potassium phosphate buffer, pH 7.5. The protein (60 mg) was passed through an Ultrogel AcA 34 gel-filtration column (1.8 cm × 96 cm). GTP cyclohydrolase I was eluted at a flow rate of 0.2 ml/min using 50 mM Tris/HCl, pH 7.5, containing 0.3 M KCl. Fractions containing enzyme activity were combined and applied to a GTP-affinity column. GTP- and UTP-affinity gels were freshly made by coupling nucleotides via their sugar moiety to a hydrazide agarose gel (Affi-gel Hz, Bio-Rad). The coupling was done essentially as described by Robberson and Davidson [32]. The column contained ~ 10 μmol of bound nucleotide/ml of settled gel. Protein (10 mg) containing 130 m-units of GTP cyclohydrolase I was applied with a flow rate of 0.3 ml/min to a 1 cm × 7 cm column with a bed volume of 5 ml. The column was washed with 15 ml of 50 mM Tris/HCl, pH 7.5, 0.3 M NaCl followed by 15 ml of Tris/HCl, pH 7.5. Bound proteins were eluted with 10 ml of 50 mM Tris/HCl, pH 7.5, containing 0.25 mg/ml GTP. Fractions with GTP cyclohydrolase I activity were combined and passed through Ultrogel AcA 54 to remove GTP. The purity of the GTP cyclohydrolase I was analysed by silver straining of SDS-polyacrylamide gels (Figure 2) and by

protein sequence analysis. One nmol of GTP cyclohydrolase I was used for N-terminal sequence analysis. The detection limit for individual amino acids in each step of the protein sequence analysis was ~ 1 pmol (results not shown). Using 1 nmol of purified enzyme, this procedure thus allows detection of single contaminating proteins each present at levels as low as 0.1 % of total protein, provided that their N-termini are not blocked.

The molecular mass of the native purified enzyme was determined using gel filtration on a Bio-Sil SEC 250 h.p.l.c. column (Bio-Rad). GTP cyclohydrolase I (0.7 μg) was applied at room temperature to a 7.5 mm × 300 mm column equilibrated with 50 mM potassium phosphate buffer, pH 7, containing 100 mM KCl. The flow rate used was 1 ml/min. The column was eluted with the same buffer. Reference proteins for calibration were from Bio-Rad.

M.s. was used as described by Smith et al. [33] to determine the exact molecular mass of the GTP cyclohydrolase I subunit. The mass spectrometric analyses were performed in positive ion mode by flow injection of samples on an API III mass spectrometer (SCIENX, Thornhill, Canada), equipped with an ionspray interface.

Enzyme assays

GTP cyclohydrolase I activity was determined at 37 °C in 100 mM Tris/HCl, pH 8, 100 mM KCl using either total cell extracts, dialysed cell extracts or the purified GTP cyclohydrolase I. Cell extracts were made as described above. Extracts were dialysed for 12 h at 5 °C against 50 mM potassium phosphate buffer, pH 7.5. A standard reaction of 0.2 ml contained up to 500 ng of purified enzyme and 1 μM–2 mM GTP. Other compounds were added at the concentrations indicated. Experiments with reduced compounds were carried out in the same buffer containing 20 mM 2-mercaptoethanol. The reduced state of the compounds was confirmed by spectral analysis. After the indicated incubation period, the reaction was stopped by the addition of SDS to a final concentration of 0.5 % (w/v). Precipitates were removed by centrifugation for 3 min in a microfuge and the supernatants were frozen on solid CO_2. The products of the enzyme reactions were analysed by h.p.l.c. Samples (10–50 μl) were applied to a Partisil-10 SAX column (6 mm × 100 mm). This column allows separation of the various phosphorylated forms of dihydroneopterin. The column was developed at a flow rate of 1.2 ml/min with 250 mM ammonium dihydrophosphate, 50 mM sodium pyrophosphate, pH 5.9, containing 250 mM KCl. The neopterin derivatives were detected fluorometrically (excitation wavelength 365 nm, emission wavelength 446 nm), and the substrate consumption was monitored by u.v. adsorption at 255 nm. Where indicated, samples were applied to a Nucleosil 10 RP 18 (6 mm × 100 mm). This column allows detection of dihydroneopterin phosphates, but does not allow separation of differently phosphorylated forms of dihydroneopterin. Elution was performed in 7 % (v/v) methanol, 30 mM formic acid at a flow rate of 2 ml/min. One unit of GTP cyclohydrolase I catalyses the formation of 1 μmol of dihydroneopterin triphosphate/min at 37 °C. The dihydroneopterin triphosphate concentrations were calculated using a standard curve.

T.l.c.

Chromatography on polyethyleneimine (PEI)–cellulose was carried out by the ascending method in a 1.5 M KH_2PO_4buffer, pH 3.4 [34]. [α-^{32}P]GTP and [γ-^{32}P]GTP were used as substrate for the *in vitro* enzymic reaction. After reaction for 10 min at 37 °C, samples were loaded on the t.l.c. plates. After development of the chromatogram, radioactive spots were detected using a phosphorimager.

RESULTS

Overexpression, purification and subunit composition of GTP cyclohydrolase I

The endogenous promoter of the *mtr* operon of *B. subtilis* was replaced by the strong *spo*I$_{26}$ promoter (Figure 1). This resulted in a massive accumulation in *B. subtilis* of both GTP cyclohydrolase I and tryptophan RNA-binding attenuation protein (TRAP) (Figure 2; compare lanes 1 and 2). The steady-state level of GTP cyclohydrolase I in clone SPCy was increased 800–850-fold, as shown by quantitative immunoblotting analysis. This value corresponded well with the 1000-fold increase measured for GTP cyclohydrolase I enzyme activity in the dialysed cell extracts (results not shown). Thus the specific activity of the overproduced GTP cyclohydrolase I in clone SPCy is comparable with that of the GTP cyclohydrolase I present in wild-type 1012 cells. GTP cyclohydrolase I was purified to homogeneity (Figure 2, lane 4) from the overproducing clone SPCy. The specific activity of GTP cyclohydrolase I was increased 24.8-fold by this purification procedure (Table 1) or 24800-fold compared with the wild-type strain 1012.

The purified GTP cyclohydrolase I was essentially free of contaminating proteins, as shown by silver staining of SDS-polyacrylamide gels (Figure 2, lane 4). Based on the protein sequence and m.s. data shown below, the smear seen just below the protein band (Figure 2, lane 4) is likely also to represent GTP cyclohydrolase I. The N-terminal sequence of the purified GTP cyclohydrolase I was Met-Lys-Glu-Val-Asn (results not shown), which is identical to the sequence predicted from DNA sequence analysis [28]. In each sequencing step only a single amino acid was seen. Based on the detection limit for amino acids in the sequence analysis, no additional proteins are present at levels > 0.1 % each. The predicted molecular mass of the GTP cyclohydrolase I (21220 Da; [28]) was confirmed by m.s. (21221 Da; results not shown). H.p.l.c. gel filtration was used to determine the molecular mass of the native GTP cyclohydrolase I. The purified enzyme had an apparent molecular mass of 180 kDa (results not shown), suggesting that the native enzyme is a homo-octameric protein.

Reaction kinetics of GTP cyclohydrolase I

Kinetic properties of purified GTP cyclohydrolase I are shown in Table 2. The enzyme follows Michaelis–Menten kinetics. The hyperbolic relationship between initial velocity and GTP concentration indicates that no positive co-operativity between substrate molecules occurs. The $V_{max.}$, but not the K_m, of the enzyme was affected by the potassium chloride concentration. For all later assays, 100 mM potassium chloride was used, because this reflects a physiologically relevant concentration. GTP cyclohydrolase I activity was similar in Tris/HCl or phosphate buffers. The K_m for GTP at 100 mM potassium chloride was 4 μM.

Addition of Mg^{2+}or Ca^{2+} Ions increases the K_m of purified GTP cyclohydrolase I:

The addition of $MgCl_2$ (Figure 3) or $CaCl_2$ (results not shown) resulted in an increase of the K_m of GTP cyclohydrolase I for GTP (Table 2 and Figure 3a). At 5 mM $MgCl_2$, the K_m of the GTP cyclohydrolase I for GTP was increased to 300 μM whereas the $V_{max.}$ of the enzyme was not affected (Table 2 and Figure 3a).

Table 1 Purification of GTP cyclohydrolase I from *B. subtilis* strain SPCy

Strain 1012 was transformed with plasmid pSPOCy6 (Figure 1). The resulting transformant SPCy has a 1000-fold increased level of GTP cyclohydrolase I activity and was used to purify GTP cyclohydrolase I. Cell lysates of SPCy were dialysed before enzyme activity measurement. One unit of GTP cyclohydrolase I catalyses the formation of 1 μmol of dihydroneopterin triphosphate per min at 37 °C.

Protein sample	Total activity (m-units)	Specific activity (m-units/mg)	Relative specific activity	Recovery (%)
Cell lysate from SPCy	420	7	1	100
Eluate from Ultrogel AcA 34	350	25	3.6	80
Eluate from GTP affinity	290	173	24.8	69
Eluate from Ultrogel AcA 54	290	173	24.8	69

Table 2 Kinetic parameters of purified GTP cyclohydrolase I

Kinetic analysis of GTP cyclohydrolase I was performed. The K_m and $V_{max.}$ values were determined in the absence and presence of various effectors. The data were analysed using double reciprocal Lineweaver–Burk plots. Enzyme assays were performed as described in the Materials and methods section. Standard deviations were ~ 3%. A Nucleosil 10 RP18 column was used to determine the amount of dihydroneopterin phosphates produced.

	Kinetic parameters	
Effector concentration	K_m (μM)	$V_{max.}$ (nmol/min per mg)
100 mM KCl	4	173
50 mM KCl	4	130
0 mM KCl	4	80
100 mM KCl + 500 μM UTP	120	173
100 mM KCl + 5 mM $MgCl_2$	300	173

As a result, the $V_{max.}$ of GTP cyclohydrolase I was already reached at GTP concentrations $< 200\ \mu$M in the absence of $MgCl_2$, but only at 2 mM GTP in the presence of 5 mM $MgCl_2$. Figure 3(b) shows the effect of increasing $MgCl_2$ concentration on the formation of dihydroneopterin phosphates using 500 μM GTP as substrate. Maximal inhibition (80%) was found with 12 mM $MgCl_2$ (Figure 3b) or $CaCl_2$ (results not shown).

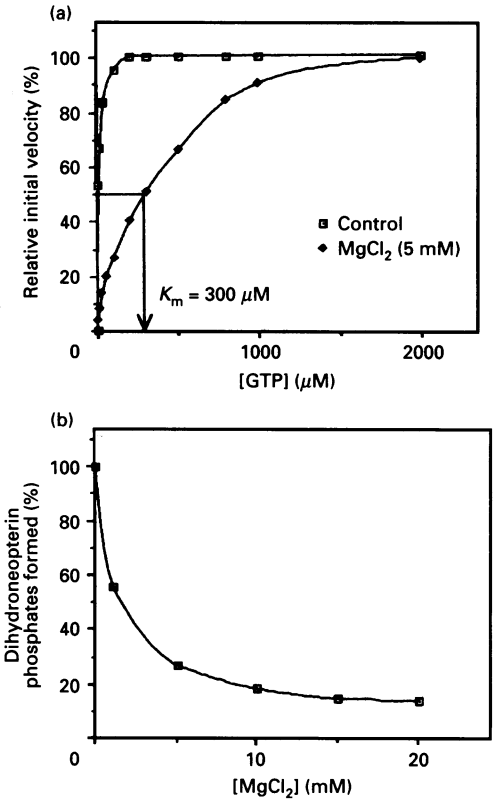

Figure 3 $MgCl_2$ inhibits the GTP cyclohydrolase I-dependent formation of dihydroneopterin phophates

(**a**) Effect of $MgCl_2$ on the initial velocity of the GTP cyclohydrolase I reaction. Enzyme incubations were performed using 0.2 ml assays containing 1 m-unit of GTP cyclohydrolase I and 100 mM KCl. To calculate the initial velocities four different reaction times (5, 10, 15 and 20 min) were used. Control, enzyme assay without divalent cations. 100% represents the $V_{max.}$ of 173 munits/mg of protein. (**b**) The amounts of dihydroneopterin phosphates formed during 10 min enzyme reactions each containing 500 μM GTP, 100 mM KCl and the indicated amount of $MgCl_2$ was measured. $MgCl_2$ was added to the reaction buffer without pre-incubation with the enzyme. 100% represents 1.5 nmol of dihydroneopterin phosphates formed in an enzyme assay without divalent cations.

Addition of Mg^{2+} or Ca^{2+} ions *in vitro* leads to accumulation of dihydroneopterin monophosphate in an enzyme-independent manner

When the GTP cyclohydrolase I enzyme assay was performed in the absence of $MgCl_2$ or $CaCl_2$, dihydroneopterin triphosphate accumulated (Figure 4a, top panel). An additional product was detected when the enzyme assay with purified GTP cyclohydrolase I was performed in the presence of Mg^{2+} or Ca^{2+} ions (Figure 4a, middle and bottom panels). With $CaCl_2$ concentrations > 10 mM, this new product represented the main product obtained after reaction for 5 min (Figure 4a, bottom panel). The retention time of the new product was identical to that of a dihydroneopterin monophosphate standard. The formation of dihydroneopterin monophosphate was not dependent on the presence of the GTP cyclohydrolase I enzyme. [α-^{32}P]- and [γ-^{32}P]-labelled dihydroneopterin triphosphate were purified by h.p.l.c. When 12 mM $CaCl_2$ was added to the labelled, purified dihydroneopterin triphosphates, radioactivity was as expected only observed in the new products when α-labelled dihydroneopterin triphosphate was used as substrate (Figure 4b, middle panel). The minor peak between dihydroneopterin triphosphate and dihydroneopterin monophosphate could represent dihydroneopterin diphosphate.

Similar results were also obtained when t.l.c. was used instead of h.p.l.c. to detect the products formed (Figure 4c). The observed conversion was specific for dihydroneopterin triphosphate: no

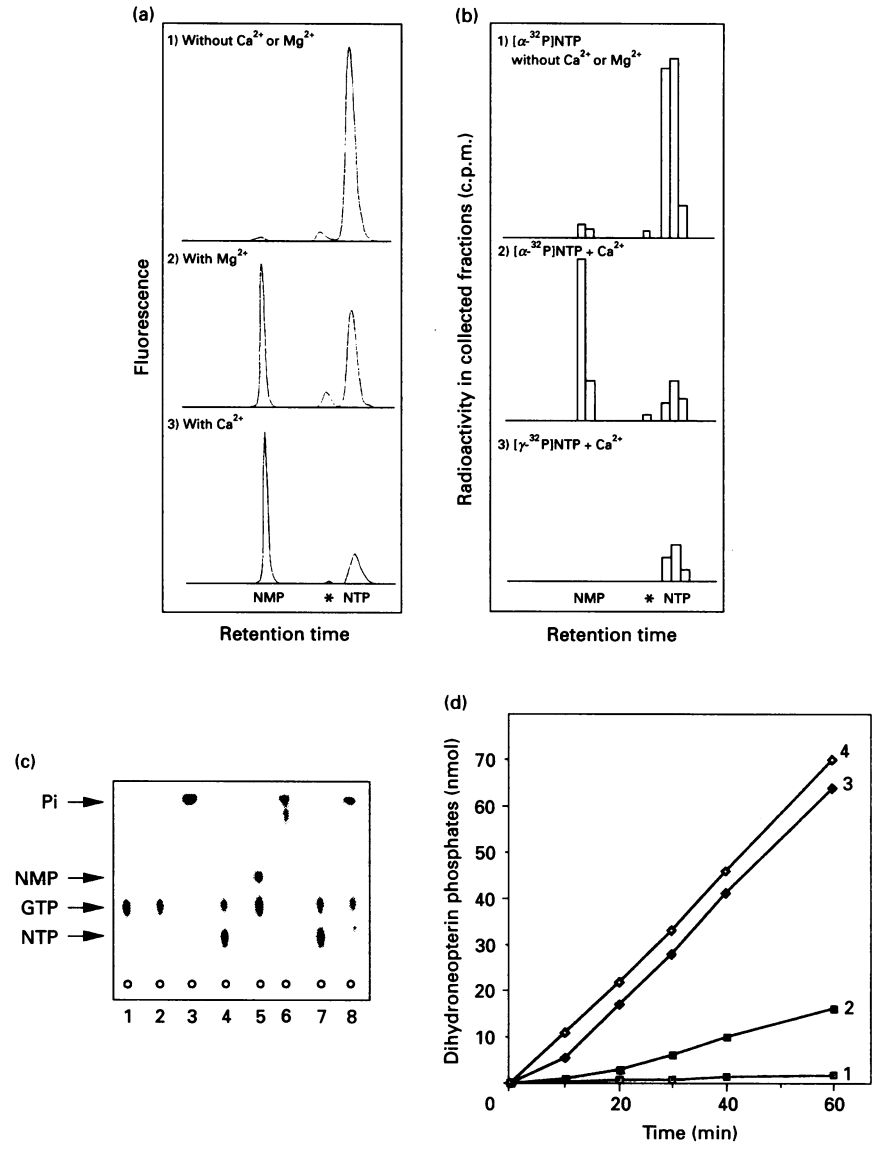

Figure 4 Accumulation of dihydroneopterin monophosphate in the presence of magnesium or calcium ions

(**a**) GTP cyclohydrolase I assays were performed for 5 min in the presence of 100 mM KCl using the purified enzyme. The products of the reaction were analysed by h.p.l.c. using a Partisil-10 SAX column and fluorescence measurement. Enzyme reactions were performed in the absence of divalent ions (top panel) or in the presence of 10 mM $MgCl_2$ (middle panel) or $CaCl_2$ (bottom panel). The mobilities of dihydroneopterin triphosphate (NTP, retention time 8 min) and dihydroneopterin monophosphate (NMP, retention time 3.2 min) were determined using reference compounds. The asterisk indicates an uncharacterized compound which may represent dihydroneopterin diphosphate (retention time 6.4 min). (**b**) [α-^{32}P]- and [γ-^{32}P]GTP were used as substrates in the GTP cyclohydrolase I reaction to prepare [α-^{32}P]- and [γ-^{32}P]-labelled dihydroneopterin triphosphate respectively. Radiolabelled dihydroneopterin triphosphates were then purified by h.p.l.c. The purified [α^{32}P]-dihydroneopterin triphosphate (top and middle panels) or [γ^{32}P]-dihydroneopterin triphosphate (bottom panel) was then incubated in the absence of divalent cations (top panel), or for 10 min in the presence of 12 mM $CaCl_2$ (middle and bottom panels). (**c**) Analysis of radiolabelled reaction products by t.l.c. Lanes 1 and 2, [γ-^{32}P]GTP incubated without (lane 1) and with 12 mM $CaCl_2$ (lane 2). Lane 3, [γ-^{32}P]GTP incubated with alkaline phosphatase. Lanes 4–8, GTP cyclohydrolase I enzyme reactions. Enzyme (1 m-unit) and 500 μM substrate in 0.2 ml of buffer containing 100 mM KCl) were incubated for 10 min with: lane 4, [α-^{32}P]GTP; lane 5, [α-^{32}P]GTP + 12 mM $CaCl_2$; lane 6, same as for lane 5 but followed by an additional incubation for 10 min with 10 units of alkaline phosphatase; lane 7, [γ-^{32}P]GTP; or lane 8, [γ-^{32}P]GTP + 12 mM $CaCl_2$. The products formed were analysed by PEI–cellulose t.l.c. Assignment of radioactive spots was based on comparison with the h.p.l.c. data. NTP, dihydroneopterin triphosphate; NMP, dihydroneopterin monophosphate. (**d**) Kinetics of formation of dihydroneopterin monophosphate in the absence and presence of divalent cations. Enzyme reactions (1 ml) contained 1 m-unit of GTP cyclohydrolase I, 2 mM GTP and 100 mM KCl. Samples (50 μl) were taken after the indicated times to analyse by h.p.l.c. the amounts of dihydronepterin phosphates formed. 1, dihydroneopterin monophosphate formed without addition of divalent cations; 2 and 3, dihydroneopterin monophosphate formed in reactions containing 5 mM $MgCl_2$ or $CaCl_2$ respectively. The total amounts of dihydroneopterin phosphates formed (mono-, di- and triphosphates) in all three cases were identical [for a reaction lacking divalent cations, see (4)] (results not shown for reactions with Mg^{2+} or Ca^{2+} ions). The concentration of GTP was suffficiently high to allow the reactions to proceed at $V_{max.}$.

conversion of GTP (Figure 4c, lane 2) or UTP (not shown) was seen upon addition of divalent cations. In addition, the dihydroneopterin monophosphate formed (Figure 4c, lane 5) can be dephosphorylated when incubated with alkaline phosphatase (Figure 4c, lane 6). This excludes the possibility that the dihydroneopterin monophosphate formed as product of the chemical dephosphorylation reaction is a cyclic compound.

The kinetics of formation of dihydroneopterin monophosphate in the presence of $MgCl_2$ were slower than with $CaCl_2$ (Figure 4d, curves 2 and 3). Thus Mg^2 and Ca^{2+} ions catalyse an enzyme-

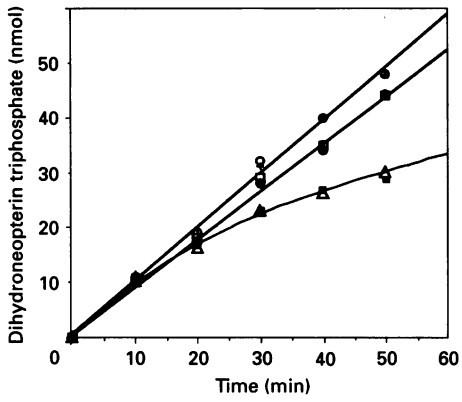

Figure 5 GTP cyclohydrolase I of *B. subtilis* is not inhibited by dihydrofolate

Enzyme assays were performed in the presence of 100 mM KCl using 1 m-unit of enzyme and 1 mM GTP in a final volume of 1 ml. Accumulation of dihydroneopterin triphosphate was monitored by h.p.l.c. using a Partisil-10 SAX column and fluorescence. Enzyme assays were performed in the absence (○, ●, △) or presence (+, □, ■) of 1 mM dihydrofolate using the pure enzyme (+, ○), a crude dialysed lysate from strain 1012 (□, ●) or a non-dialysed lysate (■, △). Cell extracts were diluted 10-fold in the enzyme assay and the KCl concentration was adjusted to 100 mM.

independent and specific dephosphorylation of dihydroneopterin triphosphate to non-cyclic dihydroneopterin monophosphate.

UTP competitively inhibits GTP cyclohydrolase I

GTP cyclohydrolase I may bind GTP and UTP at distinct binding sites. GTP cyclohydrolase I bound to a UTP-affinity matrix can be eluted with UTP, but not with GTP. Conversely, GTP cyclohydrolase I bound to the GTP-affinity matrix can be eluted with GTP, but not with UTP (results not shown). Addition of 500 μM UTP, however, resulted in an increased K_m of the purified GTP cyclohydrolase I for GTP from 4 to 120 μM (Table 2). Thus UTP interferes with the binding of GTP to the GTP cyclohydrolase I, suggesting that changes in the intracellular concentrations of UTP can affect GTP cyclohydrolase I activity and folic acid biosynthesis.

Dihydrofolate and other products of the folate or biopterin biosynthetic pathways do not affect the kinetic properties of GTP cyclohydrolase I

The initial velocities of purified GTP cyclohydrolase I and of GTP cyclohydrolase I in dialysed and non-dialysed crude cell extracts of wild-type *B. subtilis* cells are similar (Figure 5). This suggested that no strong inhibitor of GTP cyclohydrolase I is present in cell extracts before the enzyme reaction. In addition, the GTP cyclohydrolase I reaction is linear for at least 2 h using purified enzyme or dialysed cell extracts, but only for 10–20 min if undialysed cell extracts are used (Figure 5, and results not shown). Thus, no strongly inhibiting compounds are produced during the first 2 h in enzyme reactions containing the purified GTP cyclohydrolase I or dialysed crude cell extracts. This suggests that at least dihydroneopterin triphosphate does not inhibit GTP cyclohydrolase I.

The *E. coli* GTP cyclohydrolase I binds strongly to dihydrofolate, but not folate, allowing specific purification of the enzyme from cell extracts [26]. GTP cyclohydrolase I of *B. subtilis*, however, is not inhibited by dihydrofolate. Addition of 1 mM folate (results not shown) or dihydrofolate (Figure 5; compare curves ○ with +, ● with □ and △ with ■; to purified GTP cyclohydrolase I or to crude dialysed or undialysed extracts of wild-type *B. subtilis* cells did not affect the activity of GTP cyclohydrolase I. None of the other compounds tested had any effect on the activity of purified GTP cyclohydrolase I (results not shown). The compounds used were: dihydroneopterin monophosphate (1 mM), dihydroneopterin (1 mM), tetrahydrofolate (1 mM), folylpolyglutamates with two, five and eight glutamate residues (500 μM each), formyltetrahydrofolate (500 μM), methenyltetrahydrofolate (500 μM), biopterin (1 mM), dihydrobiopterin (1 mM), tetrahydrobiopterin (500 μM), *S*-adenosyl methionine (500 μM), methionine (0.2%, w/v), tryptophan (0.1%, w/v), glycine (0.2%, w/v), ATP (500 μM) and CTP (500 μM). Dihydroneopterin, tetrahydrofolate, tryptophan and methionine also had no effect on the GTP cyclohydrolase I activity in a dialysed cell extract from the wild-type strain 1012.

DISCUSSION

GTP cyclohydrolase I catalyses the first committing step in the multi-enzyme biosynthetic pathways leading to folates in micro-organisms, or tetrahydrobiopterin in animals. In animals, GTP cyclohydrolase I is the rate-limiting step in the biosynthesis of tetrahydrobiopterin (see the Introduction section). Is GTP cyclohydrolase I the key regulatory step in folate biosynthesis in micro-organisms such as *B. subtilis*? As a first step towards answering this question, we over-expressed the active enzyme in *B. subtilis*, purified it to homogeneity and studied its catalytic properties.

Purified GTP cyclohydrolase I of *B. subtilis* has a $V_{max.}$ of 173 nmol/min per mg of protein and a K_m for GTP of 4 μM in cases where 100 mM KCl is present and divalent cations are absent. In the absence of KCl, the $V_{max.}$ was decreased ~ 2-fold. Variations in the intracellular concentrations of K^+ ions could affect the activity of the GTP cyclohydrolase I. The effect is, however, too small to be of real relevance for the regulation of folate biosynthesis under physiological conditions. Addition of Mg^{2+} or Ca^{2+} ions to purified GTP cyclohydrolase I resulted in a drastic increase of the K_m from 4 μM in the absence of divalent cations to 300 μM in the presence of 5 mM $MgCl_2$. A similar inhibition was also described for the purified GTP cyclohydrolases I of *Serratia indicata* [35], *Drosophila melanogaster* [13] and *E. coli* [36]. This effect could ultimately be caused by a different affinity of the MgGTP or CaGTP salts for the enzyme compared with free GTP. In contrast with our data, Schoedon et al. [12] demonstrated stimulation of the *E. coli* enzyme by Mg^{2+} ions. Such ions, however, did not affect the activity of purified GTP cyclohydrolase I from *Comamonas* [37], although addition of a stimulatory protein plus Mg^{2+} ions increased the enzyme activity. The reason for these different and in part opposite effects of divalent cations on GTP cyclohydrolases I from different species are not yet clear.

Intracellular concentrations of Mg^{2+} ions fluctuate within the millimolar range. Thus, although the purified *B. subtilis* GTP cyclohydrolase I exhibits 90% of the maximal velocity of formation of dihydroneopterin triphosphate at GTP concentrations as low as 30 μM and in the absence of divalent cations, under physiologically relevant conditions considerably higher GTP concentrations will be needed to reach the $V_{max.}$ of the enzyme. Where the concentration of intracellular GTP available to the enzyme is less than a few hundred micromolar, GTP will thus be rate-limiting for dihydroneopterin triphosphate formation and folate production. GTP cyclohydrolase I itself may only become limiting *in vivo* where the intracellular concentration of GTP available to the enzyme lies within the millimolar range.

Millimolar concentrations of Ca^{2+} ions also inhibit formation of dihydroneopterin triphosphate *in vitro*. The cytoplasmic concentration of free calcium in *B. subtilis* is tightly regulated within the micromolar range, but can vary drastically during different growth phases. Calmodulin-like proteins have been described for *B. subtilis* [38]. Ca^{2+} concentrations of up to 9 mM can be reached in spores of *Bacillus megaterium* [39]. Sporulation and proteolysis in *B. subtilis* [40] and possibly initiation of major cell-cycle events [41] are strongly dependent on the Ca^{2+} concentration. Thus, if the effect of Ca^{2+} ions on the activity of GTP cyclohydrolase I has any physiological relevance for the regulation of folate biosynthesis, then such an effect could eventually be coupled to cell-cycle control and especially to sporulation.

Mg^{2+} and Ca^{2+} ions also catalysed the non-enzymic conversion *in vitro* of dihydroneopterin triphosphate to non-cyclic dihydroneopterin monophosphate, but not dephosphorylation of GTP or UTP. Schoedon et al. [12] described the appearance of an unknown new product upon addition of Ca^{2+} to an enzyme reaction with the purified GTP cyclohydrolase I of *E. coli*. Their unknown product may also represent dihydroneopterin monophosphate. The existence of a dihydroneopterin triphosphate pyrophosphatase has been described [27,42]. More recently, the evidence for the existence of a specific phosphatase has been questioned [1]. In our experiments, the kinetics of production of dihydroneopterin monophosphate in the presence of 5 mM $MgCl_2$ were similar with crude extracts of *B. subtilis* and purified GTP cyclohydrolase I (results not shown). This suggested that no specific phosphatase is present in the cell extracts used and that the conversion of dihydroneopterin triphosphate to dihydroneopterin monophosphate *in vivo* may also be determined by the concentration of divalent cations. If so, then chemical conversion of dihydroneopterin triphosphate to dihydroneopterin monophosphate may be relevant for regulation of folate biosynthesis. Our results thus suggest, but do not prove, that divalent cations could also reduce the need *in vivo* for an enzyme-catalysed conversion dephosphorylation of dihydroneopterin triphosphate. A similar situation was found in the riboflavin biosynthetic pathway. Mailander and Bacher [43] have suggested that the product of GTP cyclohydrolase II could be dephosphorylated through a non-specific phosphatase activity or through a chemical reaction not involving the action of an enzyme.

High concentrations of UTP (500 μM) inhibited GTP cyclohydrolase I. Thus folate production could be inhibited under conditions in which cells accumulate UTP, e.g. in non-growing cells. No feed-back inhibition of the purified GTP cyclohydrolase I of *B. subtilis* was seen by any of the intermediates or products of folate or tetrahydrobiopterin synthesis we tested. Dihydrofolate, which was described to strongly bind to the GTP cyclohydrolase I of *E. coli* [26], did affect neither the activity of purified *B. subtilis* GTP cyclohydrolase I nor enzyme activity in dialysed or undialysed extracts of wild-type cells. Tetrahydrobiopterin, which was reported to weakly inhibit the purified GTP cyclohydrolase I of *E. coli* [12] and to inhibit rat liver GTP cyclohydrolase I, had no effect on the purified *B. subtilis* enzyme. The feed-back inhibition of the rat enzyme by tetrahydrobiopterin and its stimulation by phenylalanine are mediated by a p35 protein [25]. The existence of a stimulatory protein for GTP cyclohydrolase I activity was also previously shown for *Comamonas* sp. [37]. We currently have no evidence for the involvement of another protein in the regulation of GTP cyclohydrolase I activity in *B. subtilis*. More work will, however, be needed to clarify this point.

In summary, our data suggest that divalent cations and UTP may be important factors in the regulation of folate biosynthesis in *B. subtilis*.

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