

Biosynthesis of Pteridines

NMR STUDIES ON THE REACTION MECHANISMS OF GTP CYCLOHYDROLASE I, PYRUVOYL-TETRAHYDROPTERIN SYNTHASE, AND SEPIAPTERIN REDUCTASE*

(Received for publication, May 14, 1998, and in revised form, August 10, 1998)

Andreas Bracher, Wolfgang Eisenreich, Nicholas Schramek, Harald Ritz, Eva Götze, Anja Herrmann, Markus Güthlich, and Adelbert Bacher‡

From the Institut für Organische Chemie und Biochemie, Technische Universität München, Lichtenbergstrasse 4, D-85747 Garching, Federal Republic of Germany

GTP cyclohydrolase I catalyzes a ring expansion affording dihydroneopterin triphosphate from GTP. [1',2',3',4',5'-¹³C₅,2'-²H₁]GTP was prepared enzymatically from [U-¹³C₆]glucose for use as enzyme substrate. Multinuclear NMR experiments showed that the reaction catalyzed by GTP cyclohydrolase I involves the release of a proton from C-2' of GTP that is exchanged with the bulk solvent. Subsequently, a proton is reintroduced stereospecifically from the bulk solvent. This is in line with an Amadori rearrangement mechanism. The proton introduced from solvent occupies the pro-7R position in the enzyme product. The data also confirm that the reaction catalyzed by pyruvoyltetrahydropterin synthase results in the incorporation of solvent protons into positions C-6 and C-3' of the enzyme product. On the other hand, the reaction catalyzed by sepiapterin reductase does not involve any detectable incorporation of solvent protons into tetrahydrobiopterin.

Pteridines serve as cofactors for a variety of enzyme-catalyzed reactions. Specifically, tetrahydrofolate (in bacteria and eukaryotic organisms) and tetrahydromethanopterin (in archaea) mediate the transfer of one-carbon fragments, tetrahydrobiopterin (BH₄)¹ is implicated in the hydroxylation of aromatic amino acids and the formation of nitric oxide in animals (1, 2), and molybdopterin is required as cofactor by a variety of redox enzymes, *e.g.* xanthine dehydrogenase. The metabolic roles of these cofactors have been reviewed repeatedly (3–6).

The formation of pterins by ring expansion of guanosine, including an Amadori rearrangement of the ribose moiety, was first suggested by Weygand *et al.* (7) on basis of *in vivo* studies using ¹⁴C-labeled precursors. Subsequent studies by Brown and Burg (8) and by Shiota *et al.* (9) showed that the first committed step in the biosynthesis of tetrahydrofolate and BH₄ is catalyzed by the enzyme GTP cyclohydrolase I. More specifically, C-8 of GTP (Fig. 1, compound 1) is released as formate, carbon atoms 1' and 2' of the ribose moiety are utilized for the formation of the dihydropyrazine ring, and carbon atoms 3'–5' of GTP afford the position 6 side chain of dihydroneopterin

triphosphate (NH₂TP) (Fig. 1, compound 2) (for a review, see Ref. 3).

The product of GTP cyclohydrolase I, NH₂TP, is converted to BH₄ (compound 4) by the consecutive action of pyruvoyltetrahydropterin synthase (PPH₄ synthase) and sepiapterin reductase (10–12). PPH₄ synthase catalyzes the elimination of triphosphate from NH₂TP as well as a series of tautomerization reactions that are conducive to the formation of a tetrahydropterin from the dihydropterin substrate. Both carbonyl groups of the resulting pyruvoyltetrahydropterin (PPH₄, compound 3) are subsequently reduced by the action of sepiapterin reductase.

The three-dimensional structures of GTP cyclohydrolase I from *Escherichia coli* (13, 14), PPH₄ synthase from rat (15, 16), and sepiapterin reductase (17) from mouse have been determined by x-ray crystallography. The folding patterns of GTP cyclohydrolase I and PPH₄ synthase are surprisingly similar in the absence of significant sequence similarity (13).

A hypothetical mechanism for GTP cyclohydrolase I proposed by Brown and Burg (8), Wolf and Brown (18), and Shiota *et al.* (9, 19) implicates the hydrolytic opening of the imidazole ring of GTP followed by an Amadori rearrangement of the ribose moiety. Whereas the details of this complex reaction sequence are incompletely understood, it is immediately obvious that the reaction formally requires the removal of hydrogen from C-2' of GTP followed by the reintroduction of hydrogen, which becomes ultimately located at C-7 of the product. The experiments reported in this paper were designed in order to determine the details of the hydrogen transfer process and the stereochemical course of the reaction.

EXPERIMENTAL PROCEDURES

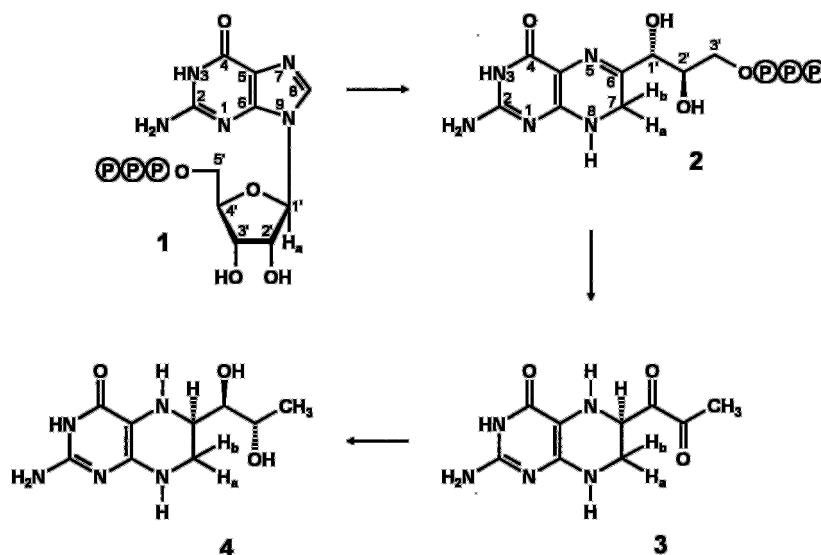
Materials—[U-¹³C₆]Glucose (99% enrichment) and D₂O (99.9% enrichment) were purchased from Isotec (Miamisburg, OH) and Euriso-Top (Gif-sur-Yvette, France), respectively. D-Neopterin, L-sepiapterin, L-biopterin, and (6R)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride were obtained from Schircks Laboratories (Jona, Switzerland). Alkaline phosphatase and lysozyme were from Boehringer Mannheim. Other enzymes were purchased from Sigma. All other reagents were of the highest available purity.

Preparation of [U-¹³C₅]Ribulose 5-Phosphate—The reaction mixture contained 100 mM Tris hydrochloride, pH 8.0, 300 mM KCl, 60 mM MgCl₂, 55.5 mM [U-¹³C₆]glucose, 1 mM DTT, 66 mM phosphoenol pyruvate, 5.5 mM ATP, 225 mM ammonium acetate, 225 mM ketoglutarate, 5.5 mM NADP⁺, 5 units of hexokinase (EC 2.7.1.1), 4.3 units of pyruvate kinase (EC 2.7.1.40), 1.7 units of 6-phosphogluconate dehydrogenase (EC 1.1.1.44), 5 units of glucose-6-phosphate dehydrogenase (EC 1.1.1.49), and 5 units of L-glutamate dehydrogenase (EC 1.4.3.11) in a total volume of 2 ml. The reaction mixture was incubated at 37 °C and was monitored by ¹³C NMR spectroscopy at intervals. The reaction was terminated when the ¹³C signals of 6-phosphogluconate had disappeared. The solution was lyophilized, and the residue was dissolved in 2 ml of D₂O. The compounds involved were characterized by the following ¹³C NMR parameters (ppm values; determined in H₂O/D₂O solution

* This work was supported by grants from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 49-89-289-13360; Fax: 49-89-289-13363; E-mail: Bacher@oc3gra.org.chemie.tu-muenchen.de.

¹ The abbreviations used are: BH₄, (6R)-5,6,7,8-tetrahydro-L-biopterin; DTT, dithiothreitol; NH₂TP, 7,8-dihydroneopterin triphosphate; PPH₄, 6-pyruvoyl-5,6,7,8-tetrahydropterin.

FIG. 1. Biosynthesis of BH₄.

at pH 8.0 at 90.55 MHz). [U-¹³C₆]6-Phosphogluconate, 65.49 (d, broad, $J = 40.0$ Hz (5), C-6), 74.62 (dd, $J = 53.8$ Hz (1), $J = 39.2$ Hz (3), C-2), 75.5–78.1 (m, C-3, C-4, C-5), 179.21 (d, $J = 54.5$ Hz (2), C-1); [U-¹³C₅]ribose 5-phosphate, 64.05 (d, broad, $J = 42.2$ Hz (4), C-5), 66.89 (dd, $J = 41.4$ Hz (2), $J = 14.5$ Hz (3), C-1), 72.21 (td, $J = 41.4$ Hz (3, 5), $J = 7.7$ Hz (³¹P), C-4), 75.4 (td, $J = 41.8$ Hz (2, 4), $J = 14.2$ Hz (1), C-3), 213.1 (t, $J = 42.2$ Hz (3, 4), C-2).

Preparation of [1',2',3',4',5']-¹³C₅,2'-²H₁]GTP—The reaction mixture contained 100 mM Tris 2-(*N*-morpholino)ethanesulfonic acid, pH 7.4, 300 mM KCl, 50 mM MgCl₂, 10 mM DTT, 2 mM ATP, 10 mM phosphoenolpyruvate, 20 units of phosphoriboisomerase (EC 5.3.1.6), 0.12 unit of phosphoribosyl pyrophosphate synthetase from *E. coli* (EC 2.7.6.1), 0.12 unit of hypoxanthine guanine phosphoribosyltransferase from yeast (EC 2.4.2.8), 2 units of nucleoside-monophosphate kinase from bovine liver (EC 2.7.4.4), 2 units of guanylate kinase from bovine brain (EC 2.7.4.8), and 8 units of pyruvate kinase (EC 2.7.1.40) in 4 ml of D₂O. The mixture was incubated at 37 °C. At intervals, aliquots of 100 mM guanine in 0.5 M NaOD (total volume, 740 μl) and of [U-¹³C₅]ribose 5-phosphate (see above) (total volume, 1200 μl) in D₂O were added. Phosphoenolpyruvate was replenished, and the pH was adjusted by addition of concentrated HCl. The reaction was monitored by ion pair high pressure liquid chromatography using a column of Nucleosil RP18 (5 μm, Macherey & Nagel, Düren, Germany; 4 × 250 mm). The eluent contained 1% triethylammonium phosphate, pH 7.0, and 0.8% isopropanol (20). The effluent was monitored by a Hewlett-Packard HP1504A diode array detector. The reaction was terminated after 17 h. The pH of the solution was adjusted to 4.0 by the addition of concentrated HCl. Precipitate was removed by centrifugation. The supernatant was placed on a column of DEAE-cellulose DE 52 (20 × 145 mm) that had been equilibrated with 30 mM LiCl, pH 3.5. The column was developed with a linear gradient of 30–195 mM LiCl at a temperature of 4 °C. The effluent was monitored photometrically at 280 nm. Fractions containing GTP were combined. The pH was adjusted to 6.5 by the addition of ammonium bicarbonate. The solution was lyophilized. The residue was extracted with dry methanol. The lithium salt of GTP was collected by centrifugation. The residue was dried under reduced pressure. The yield of GTP was 12.7 mg (23 μmol, 33% based on glucose) as estimated photometrically ($\epsilon_{252\text{ nm}}^{\text{cm}} = 13,700 \text{ M}^{-1}$) (21).

Construction of an Expression Plasmid for PPH₄ Synthase—The open reading frame specifying human PPH₄ synthase was amplified by PCR using ATCC clone 79818 (22) as a template. The oligonucleotides PPH4-1F (5'-GAGGAGAAATTAACATGAGCACGGAAGGTGGTGGCCGTCG-3') and PPH4S-2Pst (5'-ATTATTCTGCAGTGCTAACCCCAATAGCTATTCTCC-3') were used as primers. The resulting DNA fragment was reamplified using the oligonucleotides UniEcoRI (5'-CAATTGAATTCATTAAAGAGGAGAAATTAACATG-3') and PPH4-2Pst as primers. After restriction with *Eco*RI and *Pst*I, the resulting PCR fragment was ligated into the vector pMAL-c2 (New England Biolabs, Schwalbach, Germany) yielding the plasmid pMAL-PPH4S, which specifies a fusion protein comprising the maltose-binding protein of *E. coli* and human PPH₄ synthase. The plasmid was transformed into *E. coli* strain XL-1 blue, yielding the recombinant strain *E. coli* XL-1 blue pMAL-PPH4S.

Construction of an Expression Plasmid for Sepiapterin Reductase—Plasmid pNCO-SR (17) was digested with *Eco*RI and *Hind*III. The resulting 0.8-kilobase pair fragment was ligated into the vector pMAL-c2, which had been prepared with the same enzymes. The resulting plasmid pMAL-SR specifies a fusion protein comprising maltose-binding protein of *E. coli* and murine sepiapterin reductase. The plasmid was transformed into *E. coli* strain XL-1 blue by electroporation yielding the recombinant strain *E. coli* XL-1 blue pMAL-SR.

Purification of Recombinant GTP Cyclohydrolase I of *E. coli*—The recombinant *E. coli* strain M15 (pREP4, pECHI) (14) was grown aerobically as described in LB medium containing 150 mg of ampicillin and 22 mg of kanamycin per liter. At an A_{600} of 0.7, isopropylthiogalactoside was added to a concentration of 1 mM. Cells were harvested by centrifugation after 18 h of incubation. Wet cell mass (8 g) was suspended in 100 ml of a solution containing 20 mM Tris hydrochloride, pH 7.5, 10 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 14 mg of lysozyme. The suspension was incubated at 37 °C for 40 min and was then subjected to ultrasonic treatment and centrifuged. The supernatant was applied to a column of DEAE-cellulose (DE 52, Whatman, 40 × 160 mm) that had been equilibrated with 20 mM Tris hydrochloride, pH 7.5. The column was washed with 300 ml of 20 mM Tris hydrochloride, pH 7.5, and was subsequently developed with a gradient of 0–0.25 M NaCl in 20 mM Tris hydrochloride, pH 7.5. Fractions were combined. Alkaline phosphatase (0.14 mg/ml) was added, and the solution was incubated for 2 h at 37 °C in order to hydrolyze phosphorylated pteridines. The solution was loaded on a column of DEAE-cellulose (20 × 100 mm) that was developed as described above. Fractions were collected and concentrated by ultrafiltration. The protein showed a single band in SDS-polyacrylamide gel electrophoresis. The specific activity was 96 nmol min⁻¹ mg⁻¹. Fractions were combined and concentrated. The concentration of GTP cyclohydrolase I was monitored photometrically using an absorbance coefficient of $\epsilon_{280\text{ nm}}^{\text{cm}} = 0.33 \text{ ml mg}^{-1}$. The enzyme was stored at 4 °C in 10 mM potassium phosphate, pH 7.5, containing 1 mM DTT.

Purification of Fusion Proteins—The recombinant fusion proteins consisting of maltose-binding protein and PPH₄ synthase or sepiapterin reductase were purified by the following procedure. The respective *E. coli* strains were grown in LB medium containing ampicillin (170 mg per liter). At an A_{600} of 0.6, isopropylthiogalactoside was added to a concentration of 1 mM. After 5 h, the cells were harvested by centrifugation. Cells were homogenized by ultrasonic treatment in 50 mM Tris hydrochloride, pH 8.0. The suspension was centrifuged. The crude extract was applied to an amylose resin column (New England Biolabs; 20 × 30 mm). The column was washed with 50 mM Tris hydrochloride, pH 8.0, and was then developed with 50 mM Tris hydrochloride, pH 8.0, containing 100 mM maltose.

Enzyme Assays—Assay mixtures for the determination of GTP cyclohydrolase I activity contained 100 mM Tris hydrochloride, pH 8.5, 100 mM KCl, 2.5 mM EDTA, 0.2 mM GTP, and protein. The samples were incubated at 37 °C, and absorbance at 330 nm was recorded. The concentration of NH₂TP was estimated using an absorption coefficient of $\epsilon_{330\text{ nm}}^{\text{cm}} = 6300 \text{ M}^{-1}$ (23).

Assay mixtures for the determination of PPH₄ synthase contained 50 mM Tris hydrochloride, pH 8.0, 1 mM MgCl₂, 1 mM DTT, 1 mM NADPH,

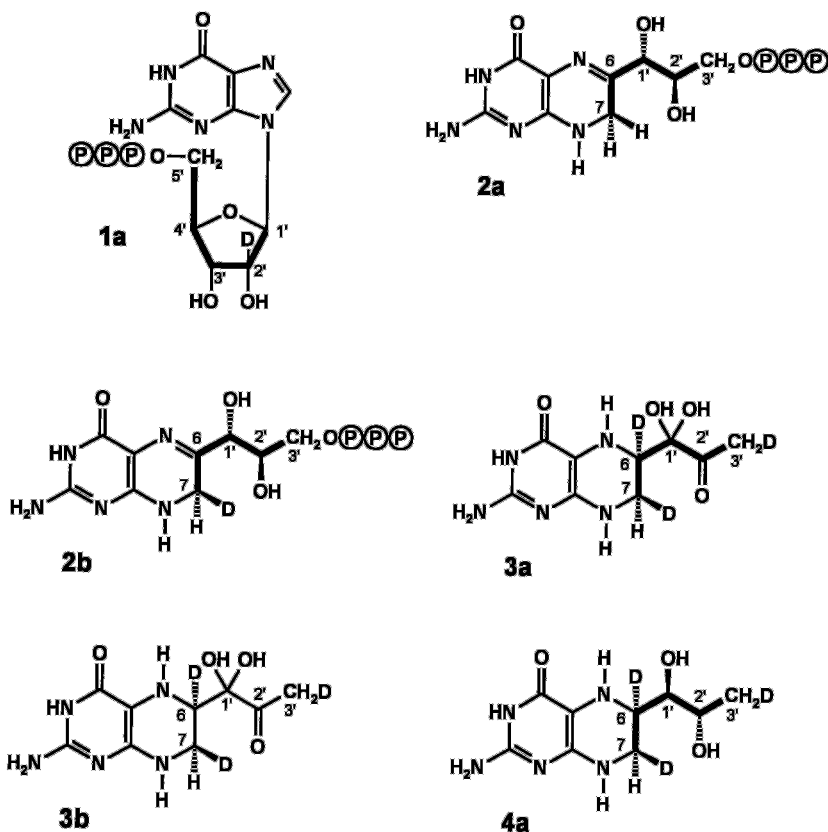


FIG. 2. Isotopomers of the BH_4 pathway metabolites discussed under "Results." Contiguous ^{13}C labeling is indicated by boldface lines.

an excess of sepiapterin reductase (2 mg per ml), and protein in a total volume of 300 μl . The solutions were incubated at 37 $^\circ\text{C}$. The reaction was terminated by the addition of 50 μl of 1 M hydrochloric acid containing 1% iodine and 2% potassium iodide. Biopterin was determined by reversed phase high pressure liquid chromatography using a column of Nucleosil 10-C18 (Macherey & Nagel; 4×250 mm). The eluent contained 3% methanol, 2% acetonitrile, 0.4% phosphoric acid, and water in a total volume of 1 liter. The flow rate was 1 ml min^{-1} . The effluent was monitored fluorometrically (excitation, 350 nm; emission, 450 nm). The retention time of biopterin was 8 min (24).

Assay mixtures for the determination of sepiapterin reductase activity contained 50 mM Tris hydrochloride, pH 8.0, 100 μM NADPH, 40 μM sepiapterin, and protein in a total volume of 300 μl . The reaction mixture was incubated at 37 $^\circ\text{C}$. The reaction was terminated as described above. Biopterin was determined by high pressure liquid chromatography (see above). One unit of each of the enzymes under study catalyzes the formation of 1 nmol of product/min at 37 $^\circ\text{C}$.

Enzymatic Formation of Isotope-labeled Pteridines—For experiments to be performed in D_2O , all reaction components were lyophilized repeatedly and subsequently dissolved in 99.9% D_2O . Enzyme reactions were performed as follows. The reaction mixtures were subjected to NMR analysis without further processing.

7,8-Dihydroneopterin Triphosphate—The reaction mixture contained 10 mM potassium phosphate, pH 7.5, 100 mM KCl, 1 mM DTT, 2.5 mM GTP, and 70 units of GTP cyclohydrolase I in a volume of 500 μl . Reactions were performed at 37 $^\circ\text{C}$ for 4 h in sealed vials under an atmosphere of nitrogen.

Pyruvoyl-5,6,7,8-tetrahydropterin—To a solution of NH_2TP prepared as described above, a solution (100 μl) containing 60 mM MgCl_2 , 10 mM potassium phosphate, pH 7.5, 1 mM DTT, and 27 units of PPH_4 synthase (fusion protein) was added anaerobically by injection through the rubber septum. The reaction mixture was incubated for 1 h at 37 $^\circ\text{C}$.

(6R)-5,6,7,8-Tetrahydro-L-biopterin—To a solution of NH_2TP obtained as described above, a solution (100 μl) containing 10 mM potassium phosphate, pH 7.5, 60 mM MgCl_2 , 2 mM DTT, 31 mM NADPH, 76 mM 6-phosphogluconate, 125 milliunits of 6-phosphogluconate dehydrogenase, 36 units of sepiapterin reductase (fusion protein), and 27 units of PPH_4 synthase (fusion protein) were added anaerobically by injection through the rubber septum. The solution was incubated at 37 $^\circ\text{C}$ for 90 min.

NMR Spectroscopy—NMR measurements were performed at 27 $^\circ\text{C}$ using a Bruker DRX 500 spectrometer operating at 500.13 and 125.7

MHz for ^1H and ^{13}C NMR experiments, respectively. The spectrometer was equipped with a lock-switch unit for ^2H -decoupling experiments using the lock channel. D_2O (100 μl) was added anaerobically to reaction mixtures containing H_2O as solvent. The NMR tubes were flushed with argon for 10 min. The solution was injected into the NMR tube, and the tube was sealed with an Omni-Fit sample tube valve (Wilmad, Buena, NY).

One-dimensional ^1H and ^{13}C NMR experiments and two-dimensional double-quantum-filtered COSY, NOESY, HMQC, and HMQC-TOCSY experiments were performed with standard Bruker software (XWIN-NMR). ^{13}C NMR spectra were obtained using a dual $^{13}\text{C}/^1\text{H}$ probehead, and proton-detection experiments were obtained using a $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ inverse triple resonance probehead. Simultaneous ^1H and ^2H decoupling of ^{13}C was achieved with a WALTZ16 pulse sequence during relaxation (^1H) and acquisition ($^1\text{H}, ^2\text{H}$). ^2H decoupling was via the lock channel using a lock-switch unit (Bruker). Prior to Fourier transformation, the free induction decay was multiplied with a Gaussian function. NMR spectra were simulated using the NMR-SIM software package from Bruker.

RESULTS

Experiments were designed to study the transfer of deuterium in the enzyme reaction. At the outset of the experiments, we assumed tentatively that the putative Amadori rearrangement might proceed by reincorporation of a proton released from C-2' into the enzyme product. Based on this working hypothesis (which turned out to be incorrect), we decided to conduct the experiments with GTP labeled with deuterium in the 2' position. In order to increase the selectivity and sensitivity of ^{13}C NMR observations, it also appeared advantageous to label the ribose side chain of substrate GTP with ^{13}C . Consequently, $[1', 2', 3', 4', 5', 6', 7', 8', 9', 10']\text{-}^{13}\text{C}_9\text{GTP}$ (Fig. 2, compound 1a) was prepared from $[\text{U-}^{13}\text{C}_6]\text{glucose}$ as described under "Experimental Procedures." Briefly, $[\text{U-}^{13}\text{C}_6]\text{glucose}$ was converted to $[\text{U-}^{13}\text{C}_5]\text{ribulose 5-phosphate}$ in a one-pot reaction using hexokinase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase. NADPH generated by the reaction sequence was dehydrogenated using glutamate dehydrogen-

TABLE I
NMR data of metabolites shown in Figs. 1 and 2
An aqueous buffer of 10 mM potassium phosphate, pH 7.5, was used as solvent for NMR spectroscopy

Compound position	Chemical shift ^a			Coupling constants			Isotope shift			Correlation experiments		
	¹ H	¹³ C		¹ J _{CD}	¹ J _{HH}		² J _{HD}	¹ H(² H)	¹³ C(² H)	DQF-COSY	HMQC-TOCSY	NOESY
1a	1'	5.82 (s) ^b										
	2'	— ^b	86.70 (d)	42.8 (2')								
	3'	4.48 (m) ^b	73.21 (dd)	43.3 (1'), 37.9 (3')								
	4'	4.23 (m) ^b	70.21 (t)	36.7 (2', 4')	ND							
	5'	4.14, 4.09 (m) ^b	83.89 (dt) ^c 65.10 (dd) ^d 154.17 (dd)	40.2 (3', 5') 42.7 (4') 55.2 (1'), 39.3 (7)	ND							
2a	6		41.33 (dd)	39.1 (6), 7.8 (1')								
	1'	4.17 (d)	72.79 (ddd)	55.0 (6), 42.0 (2'), 7.6 (7)								
	2'	3.90 (m)	70.85 (dt) ^e	42.4 (1', 3')	8.1 (2')							
	3'a	4.04 (dd)	66.72 (d)	45.1 (2')	ND							
	3'b	4.06 (dd)			3.0 (2'), 6.0 (3'b) 5.0 (2'), 6.5 (3'a)							
3a	6	3.32 (dd)	53.76 (ddd)	41.5 (1'), 35.8 (7), 3.4 (2')								
	7 ^f	3.45 (dd)	39.54 (d)	35.8 (6)	7.5 (7 _R), 3.3 (7 _S)							
	7 _R ^f	3.22 (dd)	96.30 (dt)	47.1 (6, 2'), 11.3 (3')	12.3 (7 _R), 3.1 (6)							
	1'	—	210.50 (ddd)	49.0 (1'), 41.3 (3'), 3.4 (6)	12.3 (7 _S), 7.5 (6)							
	2'	—										
4a	3'	2.31 (s)	24.62 (dd)	41.6 (2'), 11.5 (1')	ND							
	6	3.05 (dt)	50.68 (dd)	41.8 (1'), 36.0 (7)	7.0 (7 _R 1'), 3.3 (7 _S)							
	7 _S ^f	3.38 (dd)	40.88 (d)	36.8 (6)	12.5 (7 _R), 3.3 (6)							
7 _R ^f	1'	3.25 (dd)	73.2 (t)		12.5 (7 _S), 6.6 (6)							
	1'	3.36 (dd)			7.5, 6.0 (6, 2')							
	2'	3.81 (qui) ^g	68.56 (t)	40.6 (1', 3')	6.4 (1', 3')							
3'	1.13 (d)	17.14 (d)		38.9 (2')	6.4 (2')							

^a Chemical shifts were referenced to external 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt. ¹H data refer to natural abundance compounds except where indicated. Coupling patterns of ¹³C NMR signals observed in (¹H,²H) decoupled spectra are indicated in parentheses.

^b Data from (¹³C)-decoupled spectrum of 1a.

^c ³J_{CP}, 7.4 Hz.

^d ²J_{CP}, 5.1 Hz.

^e ³J_{CP}, 8.3 Hz.

^f 7_S, pro-7S; 7_R, pro-7R.

^g Pseudo-quintet.

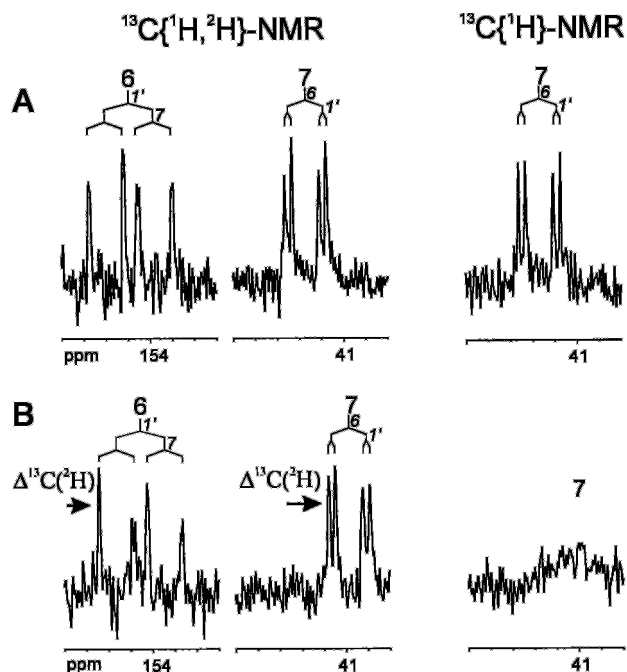


FIG. 3. ^{13}C NMR signals of NH_2TP prepared from $[1',2',3',4',5'\text{-}^{13}\text{C}_5, 2'\text{-}^2\text{H}_1]\text{GTP}$ in H_2O (A) and D_2O (B). Left, $\{^1\text{H}, ^2\text{H}\}$ -decoupled; right, $\{^1\text{H}\}$ -decoupled.

ase, with α -ketoglutarate serving as hydride acceptor (25). $[\text{U}\text{-}^{13}\text{C}_5]\text{Ribulose phosphate}$ was converted to GTP in a one-pot reaction performed in D_2O as solvent. The reaction mixtures contained all enzymes required for generation of GTP. Pentose phosphate isomerase was added in a large excess (26). The phosphate donor was phosphoenolpyruvate. Under these experimental conditions, deuterium from solvent was incorporated into the 2' position of the ribose moiety with an enrichment above 90%. The labeling pattern of the multiply labeled GTP sample was confirmed by multinuclear NMR experiments (Table I). The presence of ^2H at the 2' position was confirmed by the large upfield ^{13}C shift of C-2' and the smaller upfield shifts of C-1' and C-3'. All carbon atoms of the ribose moiety showed the expected $^{13}\text{C}\text{-}^{13}\text{C}$ coupling patterns (data not shown).

The labeled GTP was converted to NH_2TP by the catalytic action of GTP cyclohydrolase I in H_2O or D_2O solution. In the experiment using H_2O as solvent, the ^{13}C signal of C-7 of NH_2TP appears as a doublet of doublets due to $^{13}\text{C}\text{-}^{13}\text{C}$ coupling via one bond (to C-6) and via two bonds (to C-1') (Fig. 3A). This signal pattern was not affected by ^2H decoupling. It follows that the enzyme product had the structure 2a (Fig. 2).

In a similar experiment with D_2O as solvent, the $\{^1\text{H}\}$ -decoupled ^{13}C signal of C-7 appeared as a broad hump (Fig. 3B). Simultaneous $\{^1\text{H}, ^2\text{H}\}$ -decoupling revealed a doublet pattern for C-7 that differed from that in Fig. 3A by an upfield shift of 0.30 ppm. The signal of C-6 was also offset to high field by 0.06 ppm. The difference between the spectra obtained in the two experiments indicates that, contrary to our initial hypothesis, the reaction in water resulted in the virtually complete loss of the deuterium bound at C-2' of the GTP substrate, whereas the product obtained in D_2O as solvent contained deuterium in the position 7 methylene group of NH_2TP . In summary, the structure of the enzyme product obtained in D_2O is described by the structure 2b (Fig. 2).

It follows that the formation of NH_2TP (compound 2) involves the efficacious exchange of a proton released from C-2' of substrate GTP with solvent water. For the analysis of the stereochemical course of the H addition step, it was therefore in

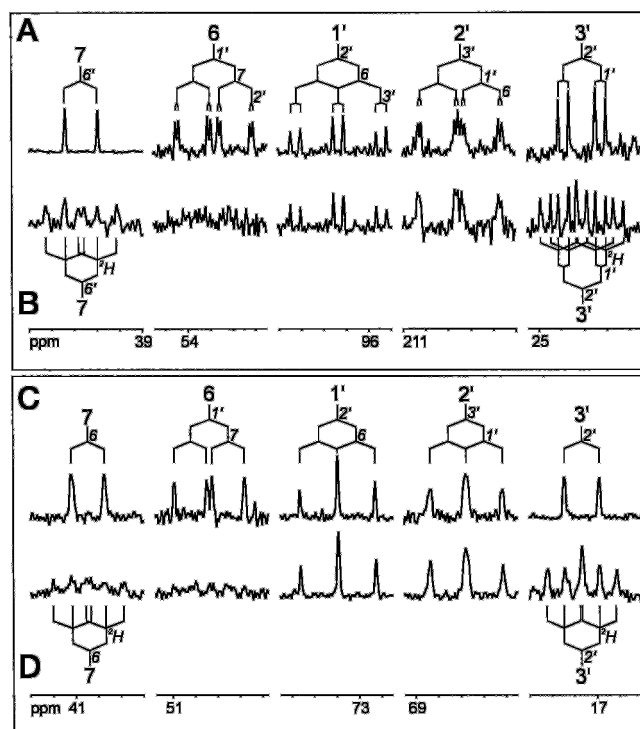


FIG. 4. ^{13}C NMR signals of PPH_4 (top) and BH_4 (bottom) obtained from $[1',2',3',4',5'\text{-}^{13}\text{C}_5, 2'\text{-}^2\text{H}_1]\text{GTP}$ by enzyme catalysis in D_2O as solvent. A and C, $\{^1\text{H}, ^2\text{H}\}$ -decoupling; B and D, $\{^1\text{H}\}$ -decoupling.

order to conduct experiments in D_2O , irrespective of the presence or absence of deuterium in the 2' position of substrate GTP.

The chemical shifts of the diastereotopic H-atoms at C-7 of NH_2TP (compound 2) are degenerate, because the effect of the chirality in C-1' and C-2' does not extend far enough to cause any significant chiral induction at the C-7 methylene group (data not shown). In order to determine the configuration of the CHD group in position 7 of the pteridine obtained in D_2O as solvent, it was therefore necessary to generate a more closely adjacent chiral center. This was achieved by enzymatic conversion of NH_2TP to pyruvoyltetrahydropterin (PPH_4 , compound 3) or BH_4 (compound 4).

PPH_4 was obtained from $[1',2',3',4',5'\text{-}^{13}\text{C}_5, 2'\text{-}^2\text{H}_1]\text{GTP}$ in D_2O as solvent by the consecutive action of GTP cyclohydrolase and PPH_4 synthase in D_2O as solvent. ^{13}C NMR signals of the product are shown in Fig. 4. In the $\{^1\text{H}, ^2\text{H}\}$ -decoupled spectrum (Fig. 4A), all carbon atoms appear as multiplets due to $^{13}\text{C}\text{-}^{13}\text{C}$ coupling. On basis of chemical shift and coupling arguments, all signals can be unequivocally assigned. Notably, the signal of C-1' appears at 96.3 ppm, thus suggesting that the carbonyl group is hydrated. On the other hand, C-2' resonates at 210.5 ppm and is apparently not hydrated to an appreciable degree. In the absence of ^2H -decoupling (Fig. 4B), the signal of C-7 appears as a doublet of triplets indicating $^{13}\text{C}\text{-}^2\text{H}$ coupling of 22 Hz. Simultaneous $\{^1\text{H}, ^2\text{H}\}$ -decoupling simplifies this multiplet to a doublet (Fig. 4A). These findings again confirm the presence of exactly one deuterium atom at C-7.

The 3' methyl carbon appears as a quartet in the $\{^1\text{H}, ^2\text{H}\}$ -decoupled spectrum and as a pattern of 10 lines in the $\{^1\text{H}\}$ -decoupled spectrum. Closer analysis shows that the latter pattern is a quartet of triplets arising by ^2H coupling. It follows that the methyl group contains exactly one deuterium atom. The signals of the 1' and 2' carbon atoms are not affected by $\{^2\text{H}\}$ decoupling, as they do not carry directly bound deuterium. The signal of C-6 is only resolved by simultaneous $\{^1\text{H}, ^2\text{H}\}$ -

decoupling, indicating the presence of a directly bound deuterium atom. In summary, the molecular species can be described by structure 3a (Fig. 2).

As a consequence of CH acidity, the protons at C-3' of PPH₄ (compound 3) are subjected to further hydrogen exchange with solvent at prolonged (10 h at 30 °C) incubation. Samples that were kept for extended time periods after completion of the enzyme reaction showed spectroscopic evidence of a molecular species carrying two deuterium atoms at the 3' methyl group (0.23 ppm upfield shifted, but otherwise unchanged doublet of doublets in a ¹H/²H-decoupled ¹³C NMR spectrum obtained after 10 h at 30 °C; data not shown).

In a subsequent experiment, BH₄ (compound 4) was obtained from [1',2',3',4',5'-¹³C₅,2'-²H₁]GTP in D₂O as solvent by the consecutive action of GTP cyclohydrolase I, PPH₄ synthase, and sepiapterin reductase (Fig. 4, C and D). ¹H/²H-Decoupling (Fig. 4C) did not affect the signals of C-1' and C-2', thus indicating that these carbon atoms did not carry directly bound deuterium. On the other hand, it was obvious from comparison of the ¹H-decoupled spectrum (Fig. 4D) with the ¹H/²H-decoupled spectrum (Fig. 4C) that each of the carbon atoms 6, 7, and 3' carried one directly bound deuterium atom. The biopterin species obtained in this experiment is represented by structure 4a (Fig. 2).

Based on these results, we can now address the configuration of the C-7 methylene groups in the isotopomers 3b and 4a. Two different strategies were used for the analysis, (i) one-dimensional ¹H NMR analysis of PPH₄ obtained from unlabeled GTP (*i.e.* Fig. 2, compound 3b), and (ii) ¹H/¹³C correlation spectroscopy with BH₄ obtained from [1',2',3',4',5'-¹³C₅,2'-²H₁]GTP (*i.e.* Fig. 2, compound 4a).

¹H NMR signals of PPH₄ formed from unlabeled GTP in H₂O as solvent by the consecutive action of GTP cyclohydrolase and PPH₄ synthase are shown in Fig. 5C. Signals could be assigned by analysis of the coupling pattern in the one-dimensional spectrum and by two-dimensional DQF-COSY (not shown) and NOESY experiments (see Fig. 7A). The prochiral C-7 protons of PPH₄ devoid of isotope labels afford two double doublets centered at approximately 3.22 and 3.45 ppm (Fig. 5C). The double doublet at 3.22 ppm is contaminated by three lines (marked by *asterisks*), which were contributed by an unidentified component of the reaction mixture. The hydrogen at C-6 appears as a double doublet arising by coupling to both C-7 protons. A simulation of the nonlinear three-spin coupling pattern involving the geminal protons at C-7 and the proton at C-6 of compound 3 is shown in Fig. 5D.

The ²H-decoupled ¹H NMR spectrum of PPH₄ obtained in D₂O from unlabeled GTP (*i.e.* isotopomer 3b) is shown in Fig. 5A. The downfield C-7 proton appears as a singlet at 3.43 ppm. The signals of the proton at C-6 and the upfield geminal proton at C-7 are both absent, thus indicating the incorporation of ²H into the respective positions (the signals marked by *asterisks* in Fig. 5A represent the unknown contaminant mentioned above). The upfield shift of the singlet at 3.43 ppm relative to the double doublet pattern in Fig. 5C is caused by the α and β shift contributions of the deuterium atoms present at C-6 and C-7 of PPH₄ in isotopomer 3b (see Fig. 4, A and B, for analysis of ²H substitution). The data indicate that deuterium incorporated from solvent D₂O by GTP cyclohydrolase I is specifically contributed to the position 7 methylene proton resonating at higher field (3.22 ppm) in the enzyme product, PPH₄. As shown below, this signifies that deuterium is incorporated into the pro-7R position.

Without ²H-decoupling, the ¹H signal of the 3' methyl group appears as a triplet with 2.2 Hz spacing in Fig. 5B. ²H-Decoupling converts this triplet to a singlet with an up-

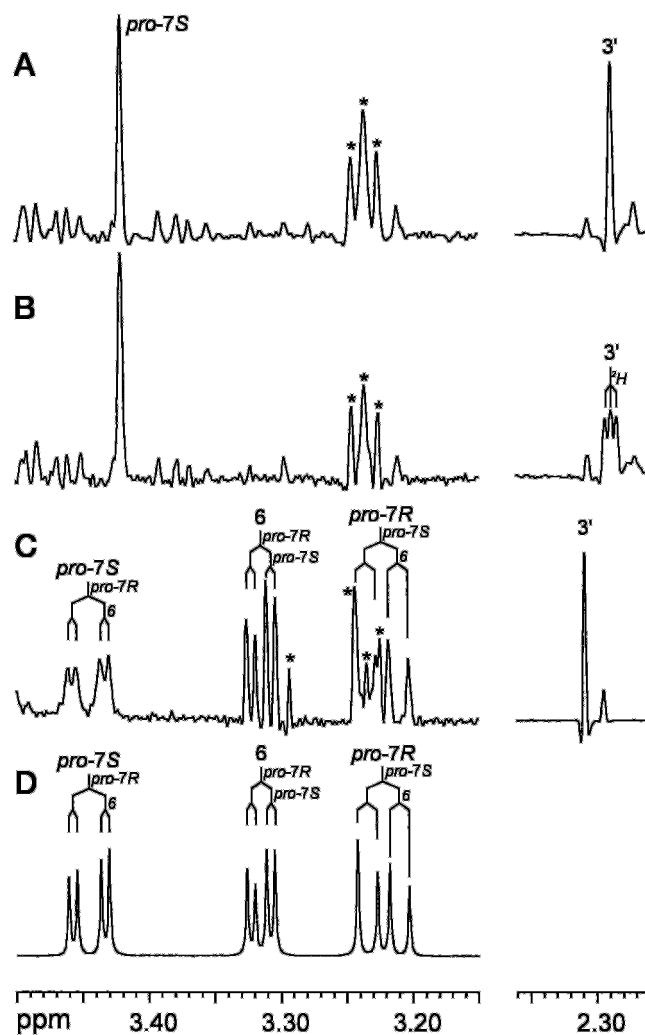


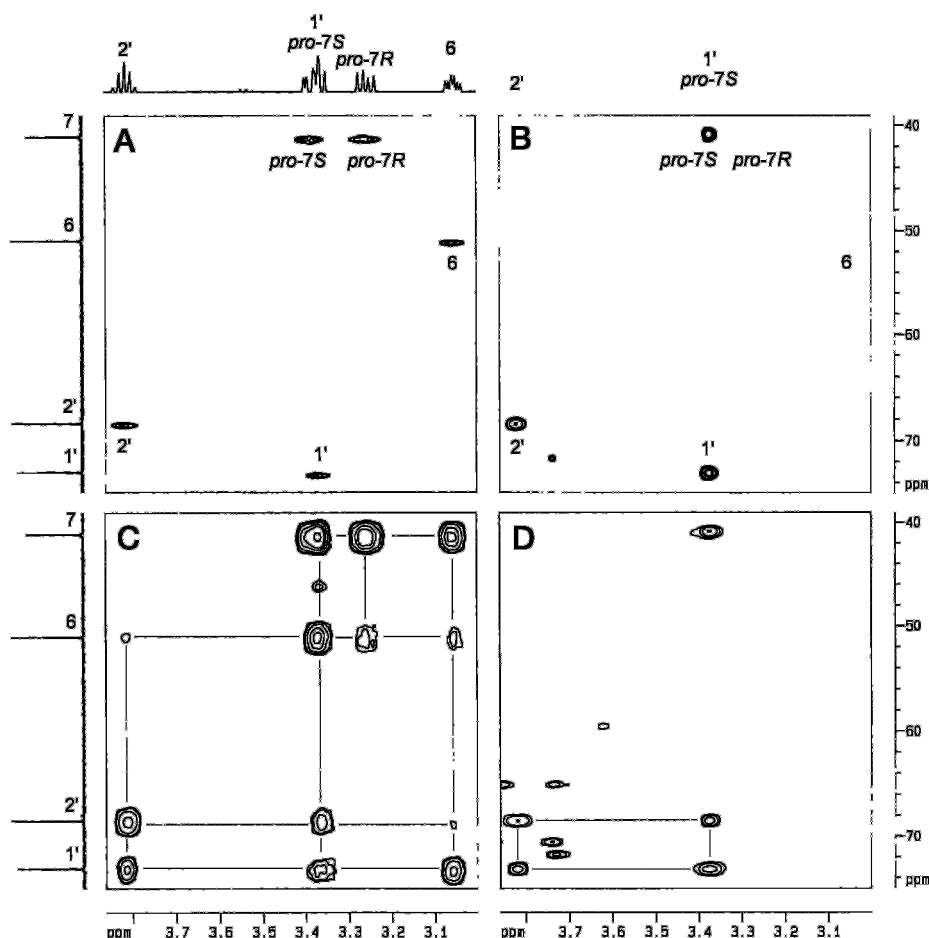
FIG. 5. ¹H NMR-spectra of PPH₄ in D₂O at pH 7.5. A and B, ¹H/²H- and ¹H-NMR-spectra of PPH₄ prepared enzymatically in D₂O; C, unlabeled PPH₄; D, simulated spectrum. Asterisks indicate signals from unidentified components of the reaction mixture.

field shift of 17 ppb as compared with PPH₄ obtained in H₂O as solvent (Fig. 5C). In agreement with the ¹³C observations described above, this indicates the presence of exactly one ²H atom in the methyl group of PPH₄ obtained in D₂O as solvent. However, it should be noted that prolonged incubation in D₂O has been shown to result in additional wash-in of deuterium from solvent (see above).

In a subsequent experiment, a BH₄ sample obtained from [1',2',3',4',5'-¹³C₅,2'-²H₁]GTP in D₂O as solvent (*i.e.* isotopomer 4a) was analyzed by two-dimensional HMQC and HMQC-TOCSY spectroscopy (Fig. 6, B and D). The HMQC spectrum of unlabeled, synthetic BH₄ shown for comparison in Fig. 6A shows correlations of C-1', C-2', C-6, and C-7 with their directly attached protons. In the HMQC-TOCSY spectrum of this sample (Fig. 6C), extended ¹H spin systems connected by ¹H TOCSY transfer are correlated to individual carbon atoms. Thus, C-7 shows correlations to the directly attached diastereotopic H atoms as well as to H-6. C-6 shows strong correlation signals to the protons at C-1' and C-7 and a weak correlation signal extending to the proton at C-2'. C-1' shows correlation to the directly attached proton, the proton at C-6, and the proton on C-2'.

In the corresponding spectra of BH₄ obtained from [1',2',3',4',5'-¹³C₅,2'-²H₁]GTP (Compound 1a) in D₂O solution (*i.e.* the BH₄ isotopomer 4a, Fig. 2), several ¹H/¹³C correlation

FIG. 6. $^1\text{H}/^{13}\text{C}$ Correlation NMR spectra of BH_4 . A and C, unlabeled BH_4 ; B and D, BH_4 obtained enzymatically in D_2O , pH 7.5, from $[1',2',2',4',5',2'-^2\text{H}_1]\text{GTP}$. A and B are expansions of HMQC spectra; C and D are from HMQC-TOCSY spectra.



signals are absent. Specifically, all correlation signals to the upfield C-7 methylene proton and the C-6 proton are absent. This signifies that the upfield position 7 methylene proton and the proton at C-6 have been replaced by deuterium. This finding is well in line with the observations, obtained by a different experimental technique, in the case of PPH_4 (see above). It should be noted that Fig. 6, B and D, shows cross-peaks that do not match either the ^{13}C or ^1H signal positions of BH_4 . The occurrence of these signals is not surprising because the enzymatic reaction mixtures were subjected to NMR analysis without isolation and purification of the chemically labile BH_4 and contain all of the numerous ingredients specified under "Experimental Procedures." It is a specific advantage that these signals are clearly separated from the BH_4 signals in the two-dimensional matrix, in contrast to the signal overlap (Fig. 5, asterisks) occurring in the one-dimensional spectra.

For the interpretation of data in stereochemical terms, the diastereomeric assignment of the geminal C-7 protons remains to be established. Expansion of the relevant portions from NOESY experiments (mixing time, 1 s) with unlabeled PPH_4 and BH_4 are shown in Fig. 7. As expected, the strongest NOE transfer was observed between the proximal geminal protons at C-7. In both spectra in Fig. 7, considerably stronger NOE interactions were found between H-6 and the respective downfield proton at C-7 as compared with the NOE between H-6 and the upfield methylene proton. This indicates that in PPH_4 , as well as in BH_4 , the distances between H-6 and the downfield proton at C-7 are substantially shorter than the distance between H-6 and the upfield proton.

Irrespective of the conformational equilibrium of (6*R*)-5,6,7,8-tetrahydropterins with a side chain in position 6, as a consequence of ring strain, the pro-*S* proton at C-7 will be

closer to H-6 than the pro-*R* proton. This can be easily demonstrated with Newman projections of this model compound (Fig. 8). Therefore, the downfield protons at C-7 of PPH_4 and BH_4 can be assigned as pro-7*S* (natural PPH_4 must be the 6*R* enantiomer, because in both compounds, the proton introduced from solvent by GTP cyclohydrolase I occupies the upfield position).

The average conformations of PPH_4 and BH_4 can be estimated from vicinal coupling constants between the methylene group protons and H-6. In both compounds, small coupling constants of 3.1 and 3.2 Hz, respectively, were found between the protons at positions pro-7*S* and 6, indicating an estimated dihedral angle of about 55° (27). Similar values were found by Armarego *et al.* (28) for BH_4 hydrochloride. The corresponding vicinal coupling constants for the protons at positions pro-7*R* and 6 were 6.6 and 7.5 Hz, respectively. This suggests a conformational equilibrium of pseudo-equatorial and pseudo-axial conformation of the side chain in position 6 (Fig. 8), in agreement with a molecular dynamics simulation for BH_4 (29, 30).

DISCUSSION

Simon *et al.* (31) and Brown and co-workers (8, 18) had proposed an Amadori rearrangement for the remodeling of the ribose moiety by GTP cyclohydrolase, but no direct evidence had been obtained. The experiments reported in this paper show that the removal of hydrogen from C-2' of GTP catalyzed by GTP cyclohydrolase and the introduction of hydrogen conducive to the formation of the position 7 methylene group of NH_2TP occurs by exchange with the bulk solvent as shown by deuterium washout from $[1',2',3',4',5',2'-^2\text{H}_1]\text{GTP}$ occurring in H_2O as solvent, and by deuterium incorporation with unlabeled GTP as substrate (Fig. 9) occurring in D_2O as sol-

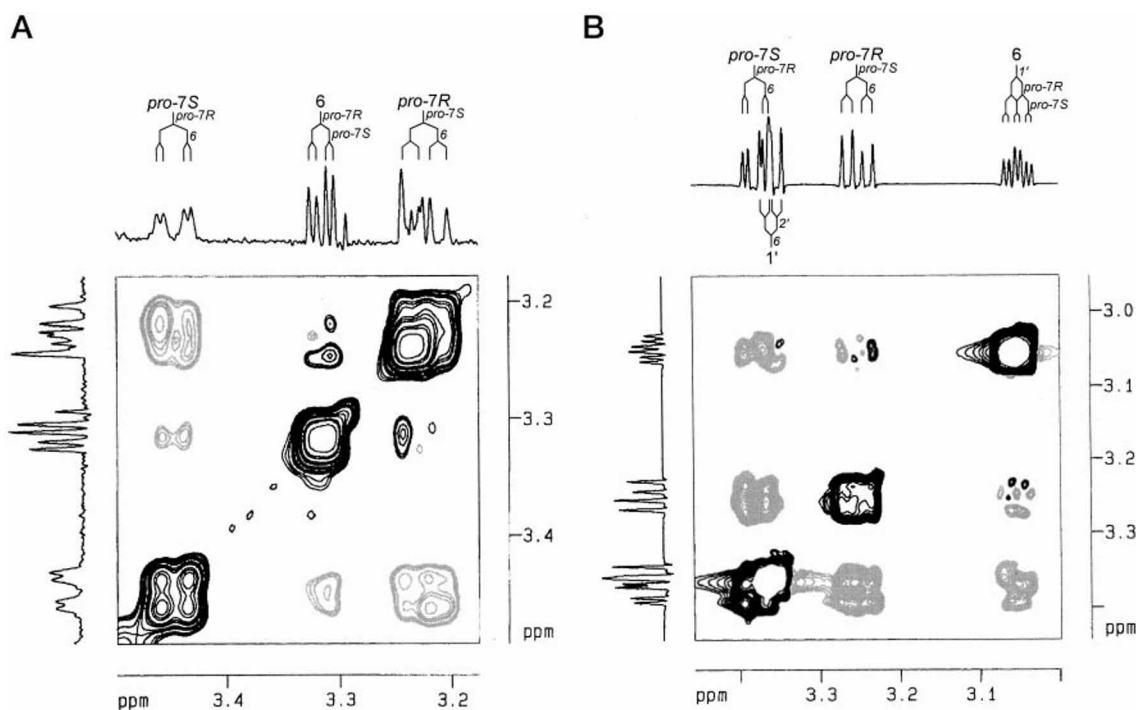
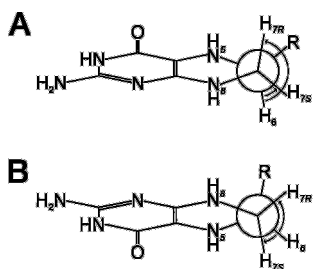
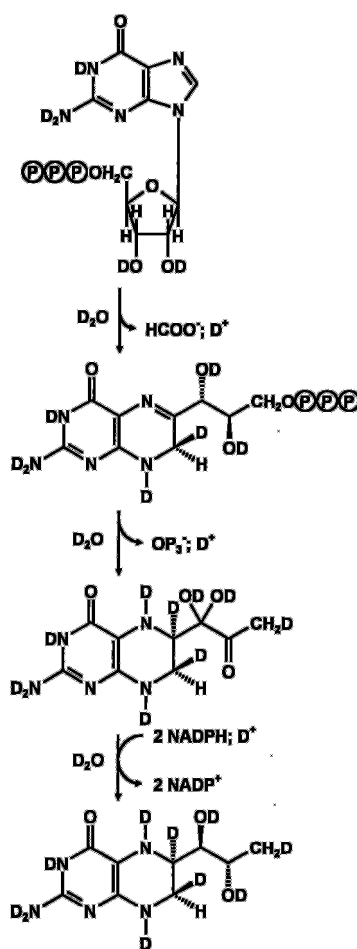
FIG. 7. NOESY spectra of PPH₄ (A) and BH₄ (B). Mixing time, 1 s.

FIG. 8. Conformers of 6-substituted (6R)-5,6,7,8-tetrahydropterins. A, with pseudo-equatorial side chain; B, with pseudo-axial side chain.

vent. It follows that the exchanged species is a proton as opposed to a hydride ion.

Palm and Simon (32, 33) and Simon and Kraus (34) studied the Amadori rearrangement of a glycoside formed from [2-³H]glucose and *p*-toluidine. The tritium label was washed out during the course of the acid-catalyzed reaction. The same experiment with natural abundance glucose and tritiated water yielded Amadori product with tritium in position 1. Our findings are consistent with an Amadori rearrangement of the ribose moiety as an essential part of the GTP cyclohydrolase-catalyzed reaction. Specifically, the hypothetical intermediate 5 obtained by the hydrolytic release of formate from the imidazole ring of GTP could form an iminium ion by opening of the furanose ring (Fig. 10). A proton could then be abstracted from the activated position 2' methine group under formation of an enol-type intermediate. Reprotonation of the enol in the position 1' could then yield the 2'-keto-3',4',5'-trihydroxypentyl-type intermediate 6. Formation of a cyclic imine could occur as the final reaction step. López *et al.* (35) reported a nonenzymatic Amadori rearrangement of a 6-amino-5-ribosylamino-pyrimidine under formation of a pteridine ring system.

The formation of the position 7 methylene group occurs by stereospecific protonation of the carbon atom equivalent to C-1' of the enzyme substrate, GTP. The proton introduced from the solvent occupies the pro-7R position in the enzyme product, NH₂TP (compound 2).

FIG. 9. Stereochemistry of enzyme-catalyzed formation of tetrahydrobiopterin in D₂O as solvent.

As a consequence of the utilization of PPH₄ synthase as an auxiliary enzyme, our data also show that protons are introduced from solvent into positions 6 and 3' of PPH₄ by the action

FIG. 10. Proposed mechanism of the Amadori rearrangement catalyzed by GTP cyclohydrolase I in D_2O buffer.

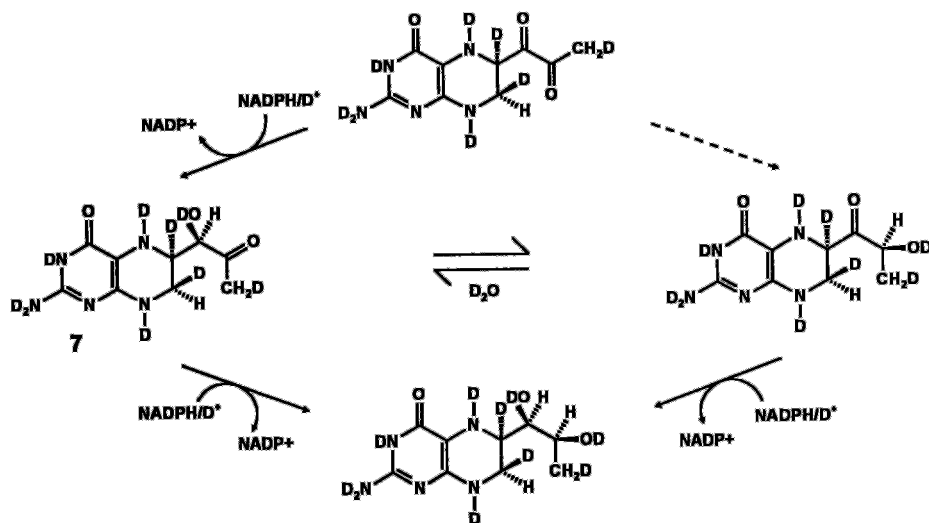
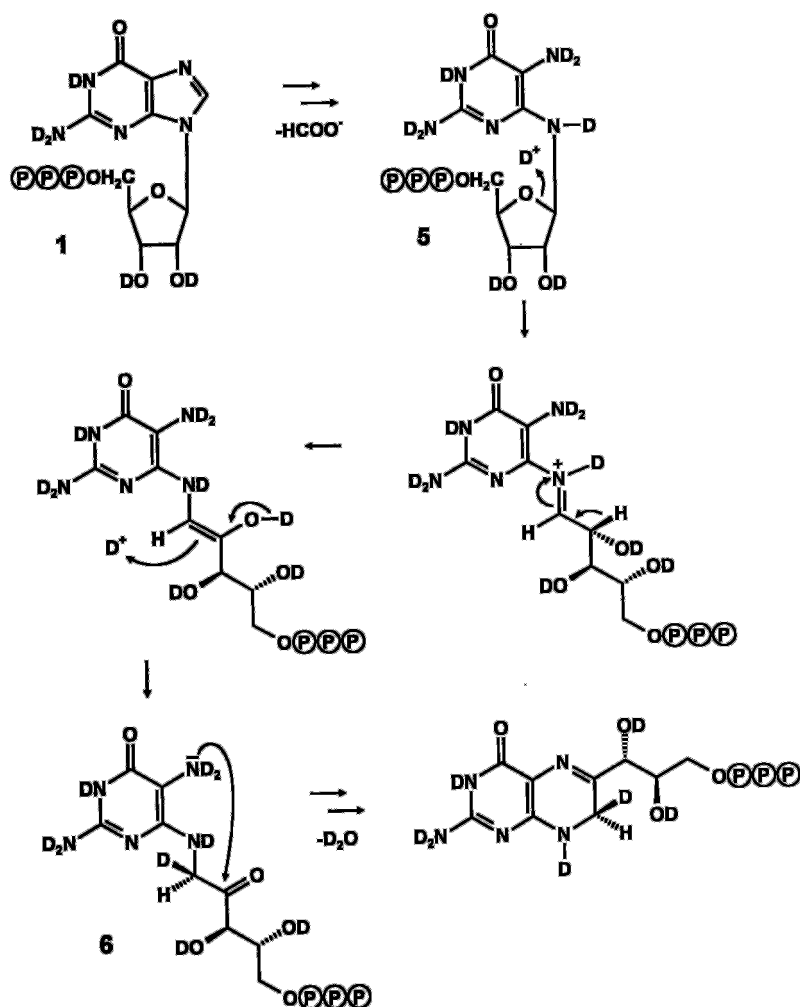


FIG. 11. Hypothetical reactions catalyzed by sepiapterin reductase (17).

of this enzyme. This is well in line with earlier observations. Thus, Ghisla *et al.* (36, 37) had observed 2H incorporation from D_2O in positions C-6 and C-3' of PPH_4 by 1H NMR experiments. On the other hand, Le Van *et al.* (38) found that 3H is not transferred to BH_4 from GTP labeled with 3H in position 3' or 4', thus indicating that the 3' and 4' hydrogen atoms of GTP had been washed out by the enzyme-catalyzed conversion of GTP to BH_4 . Both studies indicated that the hydrogen atoms introduced at C-6 and C-3' had to be introduced from solvent.

The present study also shows that the position 3' methyl group of PPH_4 undergoes slow, spontaneous (*i.e.* nonenzymatic) proton exchange with the solvent. This is not surprising in light of the methylketone structure of PPH_4 .

The position 1' carbonyl group of PPH_4 is hydrated to more than 90%. On the other hand, the position 2' carbonyl group shows no evidence of hydration. Hydration is a common finding in 1,2-diketones, *e.g.* butanedione. However, it is not immediately obvious why the hydration appears to involve exclusively

the 1' carbonyl group of PPH₄.

The experiments also show that the reaction catalyzed by sepiapterin reductase does not involve deuterium incorporation from the solvent into the C-1' and C-2' positions of BH₄ under nonequilibrium conditions. Sepiapterin reductase can catalyze the reduction of both carbonyl groups of PPH₄ (39). On basis of biochemical and crystallographic data (17, 40, 41), it has been proposed that the position 1' carbonyl group is reduced first, and that the enzyme subsequently catalyzes an isomerization via an enediol intermediate conducive to shifting of the carbonyl group from the 2' position in 6-(1'-hydroxy-2'-oxopropyl)tetrahydropterin (Fig. 11, compound 7) to the 1' position in 6-lactoyltetrahydropterin. This rearrangement has been proposed to precede the reduction of the second carbonyl group. If the rearrangement reaction indeed plays a role in the overall reaction mechanism, it must occur without significant hydrogen exchange with the bulk solvent, because no deuterium incorporation from D₂O as solvent has been observed.

Acknowledgments—We thank Professor Helmut Simon for helpful discussions and Angelika Werner for expert help with the preparation of the manuscript.

REFERENCES

1. Tayeh, M. A., and Marletta, M. A. (1989) *J. Biol. Chem.* **264**, 19654–19658
2. Kwon, N. S., Nathan, C. F., and Stuehr, D. J. (1989) *J. Biol. Chem.* **264**, 20496–20501
3. Brown, G. M., and Williamson, J. M. (1987) in *Escherichia coli and Salmonella typhimurium* (Neidhardt, F. C., ed) Vol. 1, pp. 521–538, American Society for Microbiology, Washington, D. C.
4. Keltjens, J. T., and Vogels, G. D. (1988) *BioFactors* **1**, 95–103
5. Nichol, C. A., Smith, G. K., and Duch, D. S. (1985) *Annu. Rev. Biochem.* **54**, 729–764
6. Kisker, C., Schindelin, H., and Rees, D. C. (1997) *Annu. Rev. Biochem.* **66**, 233–267
7. Weygand, F., Simon, H., Dahms, G., Waldschmidt, M., Schliep, H. J., and Wacker, H. (1961) *Angew. Chem.* **73**, 402–407
8. Burg, A. W., and Brown, G. M. (1968) *J. Biol. Chem.* **243**, 2349–2358
9. Shiota, T., Palumbo, M. P., and Tsai, L. (1967) *J. Biol. Chem.* **242**, 1961–1969
10. Switchenko, A. C., and Brown, G. M. (1985) *J. Biol. Chem.* **260**, 2945–2951
11. Smith, G. K., and Nichol, C. A. (1986) *J. Biol. Chem.* **261**, 2725–2737
12. Milstein, S., and Kaufman, S. (1989) *Biochem. Biophys. Res. Commun.* **165**, 854–860
13. Nar, H., Huber, R., Auerbach, G., Fischer, M., Hösl, C., Ritz, H., Bracher, A., Meining, W., Eberhardt, S., and Bacher, A. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 12120–12125
14. Nar, H., Huber, R., Meining, W., Schmid, C., Weinkauff, S., and Bacher, A. (1995) *Structure* **3**, 459–466
15. Nar, H., Huber, R., Heizmann, C. W., Thöny, B., and Bürgisser, D. B. (1994) *EMBO J.* **13**, 1255–1262
16. Bürgisser, D. M., Thöny, B., Redweik, U., Hess, D., Heizmann, C. W., Huber, R., and Nar, H. (1995) *J. Mol. Biol.* **253**, 358–369
17. Auerbach, G., Herrmann, A., Gütlich, M., Fischer, M., Jacob, U., Bacher, A., and Huber, R. (1997) *EMBO J.* **16**, 7219–7230
18. Wolf, W. A., and Brown, G. M. (1969) *Biochim. Biophys. Acta* **192**, 468–478
19. Shiota, T., Baugh, C. M., and Myrick, J. (1969) *Biochim. Biophys. Acta* **192**, 205–210
20. Blau, N., and Niederwieser, A. (1983) *Anal. Biochem.* **128**, 446–452
21. Dawson, R. C. M., Elliot, D. C., Elliot, W. H., and Jones, K. M. (1986) *Data for Biochemical Research*, 3rd Ed., p. 109, Oxford University Press, Oxford, England
22. Thöny, B., Leimbacher, W., Bürgisser, D., and Heizmann, C. W. (1992) *Biochem. Biophys. Res. Commun.* **189**, 1437–1443
23. Pfeleiderer, W. (1985) in *Folates and Pterins* (Blakely, R. L., and Benkovic, S. J., eds.) Vol. 2, pp. 43–114, John Wiley & Sons, New York
24. Kerler, F., Hueltnner, L., Ziegler, I., Katzenmaier, G., and Bacher, A. (1990) *J. Cell. Physiol.* **142**, 268–271
25. Richter, G., Krieger, C., Volk, R., Kis, K., Ritz, H., Götze, E., and Bacher, A. (1997) *Methods. Enzymol.* **280**, 374–382
26. Parkin, D. W., and Schramm, V. L. (1987) *Biochemistry* **26**, 913–920
27. Haasnoot, C. A. G., de Leeuw, F. A. A. M., and Altona, C. (1980) *Tetrahedron* **36**, 2783–2792
28. Armarego, W. L. F., Randles, D., Taguchi, H., and Whittaker, M. J. (1984) *Aust. J. Chem.* **37**, 355–366
29. Estelberger, W., Fuchs, D., Murr, C., Wachter, H., and Reibnegger, G. (1995) *Biochim. Biophys. Acta* **1249**, 23–28
30. Estelberger, W., Mlekusch, W., and Reibnegger, G. (1995) *FEBS Lett.* **357**, 37–40
31. Simon, H., Wacker, H., and Walter, J. (1964) in *Pteridine Chemistry: Proceedings of the Third International Symposium, Stuttgart 1962* (Pfleiderer, W., and Taylor, E. C., eds) pp. 327–339, Pergamon Press, Oxford
32. Palm, D., and Simon, H. (1963) *Z. Naturforsch.* **18b**, 419–420
33. Palm, D., and Simon, H. (1965) *Z. Naturforsch.* **20b**, 32–35
34. Simon, H., and Kraus, A. (1970) *Fortschr. Chem. Forsch.* **14**, 430–471
35. López, M. D., Quijano, M. L., Sánchez, A., Nogueras, M., Low, J. N., and Ferguson, G. (1977) in *Chemistry and Biology of Pteridines and Folates* (Pfleiderer, W., and Rokos, H., eds) pp. 53–56, Blackwell Science, Berlin
36. Ghisla, S., Steinerstauch, P., Hasler, T., Blau, T., and Curtius, H.-C. (1987) in *Unconjugated Pterins and Related Biogenic Amines* (Curtius, H.-C., Blau, N., and Levine, R. A., eds) pp. 67–79, Walter de Gruyter, Berlin
37. Ghisla, S., Kuster, T., Steinerstauch, P., Leimbacher, W., Richter, W. J., Raschdorf, F., Dahinden, R., and Curtius, H.-C. (1990) *Eur. J. Biochem.* **187**, 651–656
38. Le Van, Q., Katzenmaier, G., Schwarzkopf, B., Schmid, C., and Bacher, A. (1988) *Biochem. Biophys. Res. Commun.* **151**, 512–517
39. Katoh, S., and Sueoka, T. (1984) *Biochem. Biophys. Res. Commun.* **118**, 859–866
40. Katoh, S., and Sueoka, T. (1987) *J. Biochem.* **101**, 275–278
41. Katoh, S., and Sueoka, T. (1988) *J. Biochem.* **103**, 286–289