Biosynthesis of Tetrahydrofolate
STEREOCHEMISTRY OF DIHYDRONEOPTERIN ALDOLASE*

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Victoria Illarionova†, Wolfgang Eisenreich§, Markus Fischer, Christoph Hausmann‡, Werner Römisch, Gerald Richter†, and Adelbert Bacher
From the Lehrstuhl für Organische Chemie und Biochemie, Technische Universität München, Lichtenbergstrasse 4, D-85747 Garching, Germany

7,8-Dihydronicotinamide aldolase catalyzes the formation of the tetrahydrofolate precursor, 6-hydroxymethyl-7,8-dihydropterin, and is a potential target for antimicrobial and anti-parasitic chemotherapy. The last step of the enzyme-catalyzed reaction is believed to involve the protonation of an enol type intermediate. In order to study the stereochemical course of that reaction step, [1',2',3',6,7,13C6]dihydronicotinamide was treated with aldolase in deuterated buffer. The resulting, partially deuterated [6a,6,7,13C4]6-hydroxymethyl-7,8-dihydropterin was converted to partially deuterated [6-\(\text{H}\)]7,8-dihydropteroate by a sequence of three enzyme-catalyzed reactions followed by treatment with \([1^3C]\)formaldehyde. The product was analyzed by multidimensional NMR spectroscopy. The data show that the carbinol group of enzymatically formed 6-hydroxymethyl-dihydronicotinamide contained \(^2\text{H}\) predominantly in the pro-\(S\) position.

Tetrahydrofolate and its derivatives are essential coenzymes of one-carbon metabolism. Although plants and many microorganisms obtain folate coenzymes by \textit{de novo} synthesis, vertebrates are absolutely dependent on nutritional sources (1). Insufficient supply of the vitamin is conducive to anemia in adults and to neural tube malformation in human embryos (2).

The biosynthesis of tetrahydrofolate has been studied in some detail (for review see Ref. 3). The first committed step catalyzed by GTP cyclohydrolase I converts GTP into dihydropteroate synthase and dihydrofolate reductase. This paper describes studies on the mechanism of dihydronicotinamide aldolase.

**EXPERIMENTAL PROCEDURES**

Materials—7,8-Dihydro-v-neopterin, 6-hydroxymethyl-7,8-dihydropterin hydrochloride, pteroic acid, and 7,8-dihydropteronic acid were purchased from Schircks Laboratories, Jona, Switzerland. \([U-1^3\text{C}]\)Glucose was obtained from Cambridge Isotope Laboratories, Miamisburg, OH. \([1',2',3',4',5',13\text{C}6]\)GTP was prepared as described (11). All other reagents used were of the highest purity available. A NucleoScan C18 HPLC column (4 \(\times\) 250 mm) was from Schambach, Bad Homb, Germany. Superdex 75, Superdex 200, Q-Sepharose Fast Flow, and DEAE-Sepharose Fast Flow were purchased from Amersham Biosciences. Nanosep 10K Omega Ultrafilter and Omega membrane 10K were purchased from Pall-Gelman (Dreieich, Germany).

**Enzymes**—Alkaline phosphatase was from Roche Molecular Biochemicals. Recombinant GTP cyclohydrolase I and dihydronicotinamide aldolase of \textit{Escherichia coli} were prepared as described (11, 12).

**Construction of Expression Plasmids**—The \textit{folK} gene and \textit{folP} genes of \textit{Haemophilus influenzae} were amplified by PCR using the plasmids GHHIC06 and GHHIF04 (Table I) as templates. The oligonucleotides HKsense and HKanti (Table II) served as primers for amplification of \textit{folK}, and the oligonucleotides HSsense and HPasanti served as primers for amplification of \textit{folP}. The resulting DNA fragments served again as templates for PCR with the primer \textit{Ec}eORI for both reactions and the oligonucleotides HKanti and HPansi for amplification of \textit{folK} and \textit{folP}, respectively. After restriction with \textit{EcoRI} and \textit{BamHI}, the amplificates were ligated into the vector pcMO113 affording the plasmids pHPPK and pDHPS comprising \textit{folK} and \textit{folP} gene, respectively. The expression plasmids were transformed into \textit{E. coli} M15[Rep4]. Kanamycin (20 mg/liter) and ampicillin (50 mg/liter) were added for the maintenance of the plasmids in the host strain.

The \textit{folK} gene of \textit{E. coli} was amplified by PCR using chromosomal DNA as template and the oligonucleotides EC-FolA-NdeI-sense and EC-FolA-BamHI-anti (Table II) as primers. The amplificate was cleaved with \textit{NdeI} and \textit{BamHI} and was then ligated into the expression vector pET-5a that had been digested with the same enzymes. The resulting expression plasmid designated pDHFR was transformed into \textit{E. coli} BL21(DE3)[pLysS]. Chloramphenicol (20 mg/liter) and ampicillin (50 mg/liter) were added for the maintenance of the plasmids in the host strains.

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† On leave of absence from the Institute for Biophysics, Krasnoyarsk 660036, Russia.

‡ To whom correspondence may be addressed: Lehrstuhl für Organische Chemie und Biochemie, Technische Universität München, Lichtenbergstr. 4, D-85747 Garching, Germany.

¶ To whom correspondence may be addressed: Lehrstuhl für Organische Chemie und Biochemie, Technische Universität München, Lichtenbergstr. 4, D-85747 Garching, Germany. Tel.: 49-89-289-13363; Fax: 49-89-289-13363; E-mail: gerald.richter@ch.tum.de.

§ To whom correspondence may be addressed: Lehrstuhl für Organische Chemie und Biochemie, Technische Universität München, Lichtenbergstr. 4, D-85747 Garching, Germany. Tel.: 49-89-289-13043; Fax: 49-89-289-13363; E-mail: wolfgang.eisenreich@ch.tum.de.

¶ To whom correspondence may be addressed: Lehrstuhl für Organische Chemie und Biochemie, Technische Universität München, Lichtenbergstr. 4, D-85747 Garching, Germany. Tel.: 49-89-289-13043; Fax: 49-89-289-13363; E-mail: wolfgang.eisenreich@ch.tum.de.

† Present address: Bogenstr. 2, D-40724 Hilden, Germany.

To whom correspondence may be addressed: Lehrstuhl für Organische Chemie und Biochemie, Technische Universität München, Lichtenbergstr. 4, D-85747 Garching, Germany. Tel.: 49-89-289-13363; Fax: 49-89-289-13363; E-mail: gerald.richter@ch.tum.de.

The abbreviations used are: HPLC, high pressure liquid chromatography; IPTG, isopropyl-1-thio-\(\beta\)-galactopyranoside.
mm, and incubation was continued for a period of 4 h. Cells were harvested by centrifugation, washed with 0.9% sodium chloride, and stored at −20°C.

Frozen cell mass (5.5 g) was suspended in 40 ml of 20 mM potassium phosphate, pH 7.0, containing 100 mM potassium chloride and 1 mM EDTA. The suspension was ultrasonically centrifuged. The supernatant was applied to a DEAE-Sepharose FF column (2 × 15 cm) that had been equilibrated with 20 mM potassium phosphate, pH 7.0, containing 100 mM potassium chloride and 1 mM EDTA. The column was developed with a linear gradient of 0–0.5 M potassium chloride (flow rate, 1 ml min⁻¹; total volume, 400 ml). Dihydrofolate reductase was monitored photometrically using an absorbance coefficient of 31,100 M⁻¹ cm⁻¹ (280 nm) (17). The specific activity was 17 mol mg⁻¹ h⁻¹. The enzyme was stored at −70°C in 60 mM potassium phosphate, pH 7.6.

**Assay of 6-Hydroxymethyl-7,8-dihydropterin Pyrophosphokinase**—Assay mixtures containing 50 mM Tris hydrochloride, pH 8.0, 5 mM MgCl₂, 5 mM mercaptoethanol, 10 mM ATP, 100 μM 6-hydroxymethyl-7,8-dihydropterin hydrochloride, and protein in a total volume of 100 μl were incubated at 37°C (12, 18). The reaction was terminated by the addition of 50 μl of 1 M hydrochloric acid containing 1% (w/v) iodine and 2% (w/v) potassium iodide. 6-Hydroxymethyl-7,8-dihydropterin diphosphate was determined by reversed phase HPLC using a column of ODS silica with a flow rate of 1 ml min⁻¹. The effluent was monitored fluorometrically (excitation, 350 nm; emission, 450 nm). The retention time of 6-hydroxymethyl-7,8-dihydropterin diphosphate was 3.2 min.

**Assay of Dihydrofolate Reductase Activity**—Assay mixtures containing 60 mM potassium phosphate, pH 7.6, 5, 5 mM MgCl₂, 10 mM ATP, 100 μM 6-hydroxymethyl-7,8-dihydropterin hydrochloride, 10 mM 4-aminobenzoate, 10 μM 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase, and protein in a total volume of 0.3 ml were incubated at 37°C. Aliquots (100 μl) were analyzed by HPLC using a reversed phase column of Nucleosil C18 (4 × 250 mm) and an eluent containing 5 mM tetra-n-butylammonium phosphate, pH 6.8, 25 mM sodium chloride, 0.5 mM dithioerythritol, and 10% acetonitrile. The flow rate was 1 ml min⁻¹. The effluent was monitored photometrically using a TIDAS diode array photometer (J & M Analytische Mess und Regeltechnik GmbH, Aalen, Germany). The retention volume of 7,8-dihydropteridic acid was 9 ml.

**Assay of Dihydrofolate Reductase Activity**—Assay mixtures containing 60 mM potassium phosphate, pH 7.8, 5 mM mercaptoethanol, 0.25 mM NADPH, 0.12 mM 7,8-dihydropterate, and protein (19) were incubated at 37°C under an atmosphere of nitrogen. The conversion of dihydropterate to tetrahydropterate was analyzed by HPLC.

**Ion Pair HPLC Analysis of GTP, 7,8-Dihydropterin, and 7,8-Dihydropteridic Acid**—Experiments were performed using a column of Nucleosil C18 (4 × 250 mm) that was developed with a mixture of isopropyl alcohol, triethylamine, 85% phosphoric acid, water (3:10:9:984, v/v) (20). The flow rate was 1.5 ml min⁻¹. The effluent was monitored spectrophotometrically using a TIDAS diode array photometer (retention times: GTP, 14 min; 7,8-dihydropteridic acid, 3.5 min; and 7,8-dihydropteridic acid, 8.9 min).

**Ion Pair HPLC Analysis of Pteroate Derivatives**—Experiments were performed using a column of Nucleosil C18 (4 × 250 mm) and an eluent containing 5 mM tetra-n-butylammonium phosphate, pH 6.8, 25 mM sodium chloride, 0.5 mM dithioerythritol, and 10% acetonitrile (21). The flow rate was 1 ml min⁻¹. The effluent was monitored spectrophotometrically using a TIDAS diode array photometer (retention volumes: 7,8-dihydropteridic acid, 9 ml; tetrahydropteridic acid, 6.7 ml; and 5,10-methylenetetrahydropteridic acid, 11.5 ml).

**Synthesis of [11-13C1]6-(RS)-5,10-Methylenetetrahydropteroate**—To a reaction mixture containing 60 mM potassium phosphate, pH 7.6, and 1 mM dihydropteridic acid in a total volume of 1 ml, sodium borohydride was added to a final concentration of 10 mM under a stream of nitrogen (22). After 20 min, the excess of sodium borohydride was destroyed by the addition of 20 μl of 17 M acetic acid, and the solution was adjusted to pH 7.1 with 10 N NaOH. Mercaptoethanol was added to a final concentration of 2 mM, and [13C]formaldehyde was added to a concentration of 3 mM. The formation of 6-6-[13C]5,10-methylenetetrahydropterate was monitored using reversed phase HPLC and NMR spectroscopy.

**Preparation of [1,2′,3′,6′,7′,8′-13C6]7,8-Dihydropteridic Acid**—All enzymes used were dialyzed against 60 mM potassium phosphate, pH 7.6. For experiments to be performed in D₂O, enzyme solutions were lyophilized and subsequently dissolved in D₂O. Reactions were performed in a sealed vial under an atmosphere of nitrogen. Reagents were injected through a rubber septum.
Stereochemistry of Dihydroneopterin Aldolase

TABLE I
Strains and plasmids used in this study

| Strain or plasmid | Genotype or relevant characteristic | Ref. |
|------------------|-----------------------------------|------|
| E. coli strains  |                                   |      |
| XL1-Blue         | recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lacY1 proAB lacI215 ΔlacZΔM15 Tn109 | 13   |
| M15[rep4]        | lac ara gal mtl recA1 araD139 (F'ompT gal dcm hsdR15 (rK-mK) (DE3) [pLysS,Cm]) | Novagen |
| Bl21(DE3)[pLYS8] |                                   | 14   |
| Plasmids         |                                   |      |
| pNCO113          | High copy number plasmid vector    |      |
| pET-5a           | High copy number plasmid vector    |      |
| GHHIC06          | pUC18 plasmid containing the folK gene of H. influenzae |      |
| GHHDF84          | pUC18 plasmid containing the folP gene of H. influenzae |      |
| pHPPK            | pNCO113 plasmid containing the folK gene of H. influenzae |      |
| pHPSFS           | pNCO113 plasmid containing the folP gene of E. coli |      |
| pHFR             | pET-5a plasmid containing the folE gene of E. coli |      |

TABLE II
Oligonucleotides used for PCR amplification

| Restriction sites are shown in boldface type. |
|-----------------------------------------------|
| EC-FolA-Ndel-sense 5’ataataaatcatgctgattggegc3’ |  |
| EC-FolA-BamHI-anti 5’tattcatggactctagctgactc-3’ |  |
| HKKense 5’ggagaataaatcaggctatgatt-3’ |  |
| HKanti-BamHI 5’catggactctagcggattaatttcatg-3’ |  |
| HPsense 5’ggagaataaatcaggctatgatt-3’ |  |
| HPanti-BamHI 5’tggtaggtatctagcggattaatt-3’ |  |
| kEcoRI 5’acacaggacttaaagggagtaacctg-3’ |  |

A mixture containing 60 mM potassium phosphate, pH 7.6, 10 mM [1,2,3,4,5-13C5]GTP, and 20 mg of GTP cyclohydrodase I of E. coli in a total volume of 1 ml was incubated at 37 °C for 5 h. A solution (163 μl) made up from 100 μl of 60 mM potassium phosphate, pH 7.6, 10 μl of 100 mM magnesium chloride, 1 μl of 100 mM zinc chloride, and 50 μl of alkaline phosphatase solution (50 units) was added, and incubation at 37 °C was continued for 2 h. The solution was passed through an ultrafiltrator (Nanosep 30K Omega) by centrifugation at 20,000 g. The ultrafiltrate was collected under a stream of nitrogen.

Preparation of [[6,7,9,11-13C]8]-[R]-5,10-Methylene-tetrahydropterin—To a solution of [1,2,3,4,5-13C5]7,8-dihydroneopterin (800 μl), a mixture made up of 40 μl of 250 mM ATP, 50 μl of 100 mM magnesium chloride, 100 μl of 100 mM 4-aminobenzoate, 4 μl of 1.4 mM mercaptoethanol, 40 μl of 0.25 mM dihydropteroate aldolase, 20 μl of 1 mM 6-hydroxymethyl-7,8-dihydroneopterin pyrophosphokinase, and 16 μl of 0.15 mM dihydropteroate synthase was added, and the resulting mixture was incubated for 4 h at 37 °C. A solution (152 μl) made up of 10 mg of NADPH, 2 μl of 1.4 mM mercaptoethanol, and 150 μl of 0.25 mM dihydrofolate reductase was added, and the mixture was incubated for 1 h at 37 °C. (15C)Formaldehyde was added at a molar ratio of 2:1 based on the amount of tetrahydropterin acid present in the reaction mixture (23). The mixture was kept at room temperature and was then analyzed by NMR spectroscopy without further treatment.

NMR Spectroscopy—NMR measurements were performed at 27 °C using a Bruker DRX 500 spectrometer operating at 500.13 and 125.7 MHz for 1H and 13C NMR experiments, respectively. The spectrometer was equipped with a lock-switch unit for 2H-decoupling experiments using the lock channel.

One-dimensional 1H and 13C NMR experiments and two-dimensional COSY, NOESY, ROESY, and HMOC-TOCSY experiments were performed with standard Bruker software (XWIN-NMR 3.0). 13C spectra were obtained using a dual 13C/1H probe head, and proton detection experiments were performed using a 1H/13C/15N inverse triple resonance probe head. The mixing time was 500 ms for NOESY, 300 ms for ROESY, and 60 ms for TOCSY transfer.

RESULTS
Dihydroneopterin aldolase had been proposed to cleave 7,8-dihydro-D-neopterin (2, Fig. 2) by a retroaldol-type mechanism, although no experimental evidence has been reported (24). The hypothetical reaction sequence requires the protonation of carbon 6c of the postulated intermediate (8). The proton for that reaction step could be supplied by an acidic group of the protein, by the solvent, or by internal return of a proton abstracted from the substrate in an earlier reaction step. In order to determine the stereochemical features of the protonation, we decided to study the incorporation of deuterium from solvent D2O into the heterocyclic reaction product by multinuclear NMR spectroscopy.

For the purpose of NMR analysis, we designed a reaction sequence in order to convert the enzyme product, 6-hydroxymethyl-7,8-dihydroneopterin (3), into 5,10-methylene-tetrahydropterate (11, Fig. 3). In that tricyclic compound, carbon atom 9 which is equivalent to the 6a carbinal group of 6-hydroxymethyl-7,8-dihydroneopterin (3) is embedded into a five-membered, relatively rigid ring system, which was expected to enable the stereospecific assignment of the diastereotopic protons of the position 7 and 9 methylene groups of 11 by NMR spectroscopy using carbon 6 as a chiral reference center.

The reaction steps designated A, C, D, and E in Fig. 1 are catalyzed by enzymes of the tetrahydrofolate pathway and all were documented in the literature (4, 6, 25–30). The details of removal of the triphosphate motif under physiological conditions in the biosynthetic pathway (Fig. 1, reaction B) are still not known with certainty; however, alkaline phosphatase could be used for the dephosphorylation of 1.

The enzymatic reduction of dihydropterate (5) affording 6-(S)-tetra-hydropterate (6-S-10) (Fig. 3) has not been described to the best of our knowledge, but we could show that the non-physiological reaction can be catalyzed at an appreciable rate by dihydrofolate reductase of E. coli. The conversion of 6-(S)-tetrahydropterate into 6-(R)-5,10-methylene-tetrahydropterate (6-R-11) could be performed by treatment with formaldehyde following a suggestion of R. Matthews.2

It should be noted that the experimental strategy required the recombinant expression of the folK, folP, and folA genes in order to prepare 6-hydroxymethyl-dihydroneopterin pyrophosphokinase, dihydropterate synthase, and dihydrofolate reductase as described under “Experimental Procedures.”

In order to check the feasibility of the planned NMR analysis, we prepared racemric 6-(R,S)-5,10-methylene-tetrahydropterate (11) by sodium borohydride reduction of a commercial sample of 7,8-dihydropterate (5) followed by condensation with formaldehyde (Fig. 3). In order to improve the sensitivity and selectivity of product analysis, we used 13C-labeled formaldehyde in the condensation reaction. 1H and 13C NMR sig-

2 R. Matthews, personal communication.
nals of the resulting 6-(R,S)-[11-13C1]-5,10-methylenetetrahydropteroate were assigned by two-dimensional COSY, NOESY, ROESY, and HMQC spectroscopy (Tables III and IV).

The 1H NMR signals for the diastereotopic protons at C-11, C-9, and C-7 of 6-(R,S)-[11-13C1]-5,10-methylenetetrahydropteroate were found to be well separated from each other (Table III and Fig. 4). More specifically, the resonances for H-11 protons and the C-11 carbon atom in 6-(R,S)-[11-13C1]-5,10-methylenetetrahydropteroate were assigned by their enhanced intensities in two-dimensional 1H-13C HMQC experiments. The expected 1H-13C couplings of the H-11 protons with the attached 13C-11 were also observed in the one-dimensional 1H NMR spectra of 6-(R,S)-[11-13C1]-5,10-methylenetetrahydropteroate (cf. Fig. 5A). The doublet of triplets centered at 3.20 ppm showed coupling to the NH-8 proton in the two-dimensional COSY experiment. Moreover, the coupling signature of the well resolved signal in the one-dimensional 1H NMR experiment (Table III and Fig. 4) indicated coupling to NH-8 (coupling constant, 4 Hz). Consequently, the signal at 3.20 ppm was assigned as one of the H-7 protons (H-7e). The signal for the second H-atom (H-7d) attached to C-7 was assigned by HMQC spectroscopy where the signals for H-7e and H-7d were both correlated to the same 13C NMR signal at 38.9 ppm. The signals for H-9 methylene protons could then be assigned via the 1H-13C correlation pattern gleaned from the COSY experiment as well as from 1H-13C correlations in the HMQC experiment (Table III). Stereo-specific assignments were obtained from ROESY and NOESY experiments (Fig. 5 and Table IV).

Thus, negative cross-peaks between resonances for H-9b, H-6, and H-7e (Fig. 5C), respectively H-9a and H-7d (Fig. 5B) indicated syn orientation of H-6, H-9b, and H-7e.

The compounds 10 and 11 are oxygen sensitive; partial degradation of 5,10-methylenetetrahydropteroate (11) was observed after a few hours even at 5 °C. We therefore decided to perform the reaction steps in Fig. 3 as a one-pot reaction with direct NMR analysis. As a substitute for product purification, we made use of 13C labeling (Fig. 6) in order to enhance the sensitivity and selectivity of the NMR analysis. As described below, this approach enabled the acquisition of 13C-filtered 1H NMR data, thus virtually eliminating the 1H signals of unlabeled reagents.

The [1’,2’,3’,6,7,13C6]7,8-dihydro-d-neopterin (2) designed to serve as aldolase substrate was prepared by a complex sequence of enzyme-assisted reactions. Briefly, [1’,2’,3’,4’,5’,13C5]GTP was prepared by enzymatic multistep transformation of [U-13C6]glucose (12) as described earlier (11) and was then converted to [1’,2’,3’,6,7,13C6]7,8-dihydropteroate by treatment with GTP cyclohydrolase I (11). The triphosphate was dephosphorylated by treatment with alkaline phosphatase from calf intestine. Enzymes were removed from the reaction mixture by ultrafiltration, and the resulting solution of [U-13C6]glucose (12) as described earlier (11) and was then converted to [1’,2’,3’,6,7,13C6]7,8-dihydropteroate by treatment with GTP cyclohydrolase I (11). The triphosphate was dephosphorylated by treatment with alkaline phosphatase from calf intestine. Enzymes were removed from the reaction mixture by ultrafiltration, and the resulting solution of [U-13C6]glucose (12) as described earlier (11) and was then converted to [1’,2’,3’,6,7,13C6]7,8-dihydropteroate by treatment with GTP cyclohydrolase I (11). The triphosphate was dephosphorylated by treatment with alkaline phosphatase from calf intestine. Enzymes were removed from the reaction mixture by ultrafiltration, and the resulting solution of [U-13C6]glucose (12) as described earlier (11) and was then converted to [1’,2’,3’,6,7,13C6]7,8-dihydropteroate by treatment with GTP cyclohydrolase I (11). The triphosphate was dephosphorylated by treatment with alkaline phosphatase from calf intestine. Enzymes were removed from the reaction mixture by ultrafiltration, and the resulting solution of [U-13C6]glucose (12) as described earlier (11) and was then converted to [1’,2’,3’,6,7,13C6]7,8-dihydropteroate by treatment with GTP cyclohydrolase I (11). The triphosphate was dephosphorylated by treatment with alkaline phosphatase from calf intestine. Enzymes were removed from the reaction mixture by ultrafiltration, and the resulting solution of [U-13C6]glucose (12) as described earlier (11) and was then converted to [1’,2’,3’,6,7,13C6]7,8-dihydropteroate by treatment with GTP cyclohydrolase I (11). The triphosphate was dephosphorylated by treatment with alkaline phosphatase from calf intestine. Enzymes were removed from the reaction mixture by ultrafiltration, and the resulting solution of [U-13C6]glucose (12) as described earlier (11) and was then converted to [1’,2’,3’,6,7,13C6]7,8-dihydropteroate by treatment with GTP cyclohydrolase I (11). The triphosphate was dephosphorylated by treatment with alkaline phosphatase from calf intestine. Enzymes were removed from the reaction mixture by ultrafiltration, and the resulting solution of [U-13C6]glucose (12) as described earlier (11) and was then converted to [1’,2’,3’,6,7,13C6]7,8-dihydropteroate by treatment with GTP cyclohydrolase I (11). The triphosphate was dephosphorylated by treatment with alkaline phosphatase from calf intestine. Enzymes were removed from the reaction mixture by ultrafiltration, and the resulting solution of [U-13C6]glucose (12) as described earlier (11) and was then converted to [1’,2’,3’,6,7,13C6]7,8-dihydrop...
with 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase and dihydropteroate synthase. The product was then converted to 6-(\(^{\text{73}}\)S)-[6,7,9-\(^{13}\)C\(_3\)]tetrahydropteroate by catalytic action of dihydrofolate reductase. Treatment of the reaction mixture with \([^{13}\text{C}]\)formaldehyde afforded 6-(\(^{\text{86}}\)R)-[6,7,9,11-\(^{13}\)C\(_4\)]5,10-methylenetetrahydropteroate (11). These procedures could all be performed as a one-pot reaction, and the reaction mixture was immediately analyzed by NMR spectroscopy. As described under “Experimental Procedures,” all solutions were made up in D\(_2\)O when required.

The \(^{13}\text{C}\) NMR spectrum of 6-(\(\text{R}\))-6,7,9,11-\(^{13}\text{C}\)_4,5,10-methylenetetrahydropteroate (11) showed intense \(^{13}\text{C}\)-coupled signals at 38.9 ppm (doublet, 35 Hz, C-7), 48.7 ppm (doublet, 36 Hz, C-9), and 52.8 ppm (triplet, 36 Hz, C-6) (see projection in Fig. 7A). The \(^{13}\text{C}\) NMR triplet at 52.8 ppm showed cross-peaks with the aromatic protons of the phenyl ring; hence, the \(^{13}\text{C}\)-enriched carbon atom was assigned as C-6. The \(^{13}\text{C}\)-enriched carbon atom resonating at 67.8 ppm does not show \(^{13}\text{C}\)-coupled cross-peaks and is therefore assigned as the solitary C-11. Hence, the \(^{13}\text{C}\)-enriched C-atom resonating at 38.9 ppm can be assigned as C-7 confirming the assignments obtained with racemic [11-\(^{13}\text{C}\)]5,10-methylenetetrahydropteroate (Table III).

**TABLE IV**

| Position | NOE intensity\(^a\) | ROE intensity\(^a\) |
|----------|-------------------|-------------------|
| 9a       | 85 (9b), 9.4 (7d) | 70 (9b), 15 (7d)  |
| 9b       | 85 (9a)           | 70 (9a), 18 (7e), 30 (6) |
| 6        | 30 (9b)           |                   |
| 7d       | 100 (7e), 9.4     | 100 (7e), 15 (9a) |
| 7e       |                   | 100 (7d), 18 (9b) |
| 11f      | 5.5 (7d)          |                   |

\(^a\)Arbitrary units relative to a intensity of 100 for cross-signals between the geminal protons at C-7.
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**Table V**

| Position | $^3$H signal intensity$^a$ | H$_2$O buffer | D$_2$O buffer | $^2$H incorporation$^a$ |
|----------|---------------------------|---------------|---------------|-------------------------|
| $^9$pro-R | 3.16                      | 1.18          | 0.17          | 86                      |
| $^9$pro-S | 3.56                      | 1.06          | 0.36          | 66                      |
| $^9$pro-R | 3.26                      | 1.00          | 1.00          | 0                       |
| $^9$pro-S | 2.84                      | 0.90          | 0.21          | 77                      |
| $^9$pro-R | 3.20                      | 0.94          | 0.95          | 0                       |

$^a$ Intensities of $^3$H/$^13$C correlation signals from HMQC spectra in arbitrary units relative to signal intensities of 1.00 for H-6.

The residual $^3$H in the H-7$_{\text{pro-R}}$ position (23%) of 11 indicates that the solvent had not been completely deuterated. This finding was not unexpected because H$_2$O/D$_2$O exchange had been performed by lyophilization of the multienzyme mixture. The impact of incomplete deuterium of the solvent may have been enhanced by a deuterium isotope effect because release of a proton from bulk water or from an acidic group in exchange with the bulk solvent would occur preferentially by comparison with the release of a deuteron.

Whereas the theoretical reaction mechanism in Fig. 2 suggests the incorporation of precisely one deuterium equivalent at C-9 of 11 by protonation of the hypothetical intermediate 8 (Fig. 2), the data in Table V show a considerable excess of $^3$H. Specifically, 86% deuterium was found in the pro-S position and 66% in the pro-R position at C-9 of 11. This could be due to stereospecific protonation of the enol 8 in the pro-S position in conjunction with wash-in of additional deuterium by spontaneous or enzyme-mediated exchange processes.

The 6α hydrogen atoms of compound 3 have increased CH acidity due to activation by the aminal motif in the dihydropyrazine ring. This activation should also extend to the adjacent ring atom, C-7, but the acidity of the C-6α and C-7 protons may well differ quantitatively, and this could explain the absence of excess deuterium in the position 7 methylene group of compound 11.

The data leave no doubt that the protonation of the intermediate 8 by dihydropterin aldolase occurs preferentially in the pro-S position (Fig. 8). However, due to the excess deuterium at the position 6α carbon of compound 3, the degree of stereospecificity of the protonation of the intermediate 8 cannot be determined unequivocally.

The present data do not answer the question whether the donor for proton transfer to the 6α carbon of the enol 8 is an acidic group of the protein or a solvent water. However, it is clear that the protonation of the enol does not occur by return of the proton that has been abstracted from the substrate. Thus, if the protonation of 8 occurs by an amino acid residue involved in the formation of an anion from the substrate, 2, that residue would have to be water-accessible, and the abstracted proton would have to be exchanged with bulk solvent.

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