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Biosynthesis of the 7-Methylated Pterin of Methanopterin

ROBERT H. WHITE

Department of Biochemistry and Nutrition, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

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The incorporation of [^{15}N]glycine and [U-*methyl*-^{2}H]methionine into methanopterin by growing cells of a methanogenic bacterium was measured to establish the biosynthetic route to the methylated pterin in this structure. The tetrahydromethanopterin produced by the cells was oxidatively cleaved to produce 7-methylpterin, and the amount of label incorporated into this pterin was measured by gas chromatography-mass spectrometry of the ditrimethylsilyl derivative of this compound. Approximately 27% of the 7-methylpterin and the guanine present in the cell was derived from the fed [^{15}N]glycine. [U-*methyl*-^{2}H]methionine was incorporated with the initial retention of all three deuteriums. These results are consistent with the biosynthesis of the pterin of methanopterin originating from GTP and its 7-methyl group arising from the methyl group of methionine.

Methanopterin is a recently characterized cofactor (4, 16, 17) which, along with several other cofactors or coenzymes, functions in the conversion of CO_2 to methane (5). Based on both its structural similarity to folic acid and on chemical evidence which shows that methanopterin can serve as a single carbon carrier at the oxidation level of methenyl, methylene, and methyl (4, 6, 7, 18), there is little doubt that this cofactor functions in cells in a manner analogous to folic acid. The marked similarity between the structures of folic acid and methanopterin (Fig. 1) would also indicate that these molecules are biosynthesized by similar reactions. Folic acid is known to be biosynthesized by the condensation of 6-hydroxymethyl-7,8-H_2-pterin pyrophosphate with either *p*-aminobenzoic acid or *p*-aminobenzoylglutamate to produce the corresponding 7,8-dihydropteroates (13, 15, 20). The required substrates for the reaction, 6-hydroxymethyl-7,8-H_2-pterin pyrophosphate and *p*-aminobenzoic acid, arise from GTP (2) and chorismic acid (21), respectively. Methanopterin could be produced by a similar reaction in which a dimethylated 6-hydroxymethyl-7,8-H_2-pterin pyrophosphate is condensed with an arylamine which has been modified so as to generate the required structure of methanopterin. The required dimethylated pterin would be derived from GTP, and the required arylamine would be derived from *p*-aminobenzoic acid (24). Several of these ideas on the biosynthesis of methanopterin have been supported by the demonstration that the 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane moiety of methanopterin is derived from *p*-aminobenzoic acid (24) and that 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane conjugated to ribose, phosphate, and α-hydroxyglutaric acid has been found in methanogenic bacteria (23), where it could serve as a biosynthetic precursor to methanopterin.

I now report evidence which indicates that the pterin ring of methanopterin most likely arises from guanosine in the same manner as the pterin of folic acid and that the 7-methyl group of this pterin arises from the methyl group of methionine.

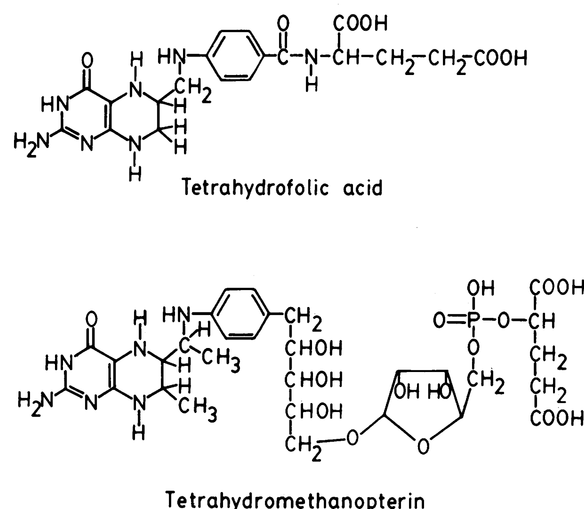

FIG. 1. Structures of folic acid and methanopterin as their tetrahydro derivatives.

MATERIALS AND METHODS

Growth of microorganism. Rumen isolate 10-16B, as described by Lovely et al. (9), was used for the work described herein. The organism was grown in 2-liter bottles pressurized to 30 lb/in^2 with H_2-CO_2 (80:20) on 500 ml of the minimal salts medium of Lovely et al. (9). Acetate (62.5 mM), either unlabeled or labeled (98 atom% ^{2}H) with three deuteriums in the methyl group, and [^{15}N]glycine (99 atom% ^{15}N) or [U-*methyl*-^{2}H]methionine (98 atom% ^{2}H) were added to this medium at the concentrations indicated in the tables. Strain 10-16B was selected for this work because it grows rapidly on the defined medium and readily takes up glycine and methionine and incorporates them into cellular material (data not shown).

Isolation of 7-methylpterin. The tetrahydromethanopterin present in the cells, which were harvested at the end of log-phase growth by centrifugation, was oxidatively cleaved during extraction of the cells in the presence of air to produce the 7-methylpterin. The extracted 7-methylpterin was then purified by preparative thin-layer chromatography as previously described (23).

Isolation of guanine. A portion of the cell pellet (50 mg) which remained after extraction of the 7-methylpterin was placed in 1 ml of 7.5 M $HClO_4$ and heated at 100°C for 1 h. At the end of this period, 1 ml of 7.5 M KOH was added, and the resulting $KClO_4$ and precipitated carbon particles were

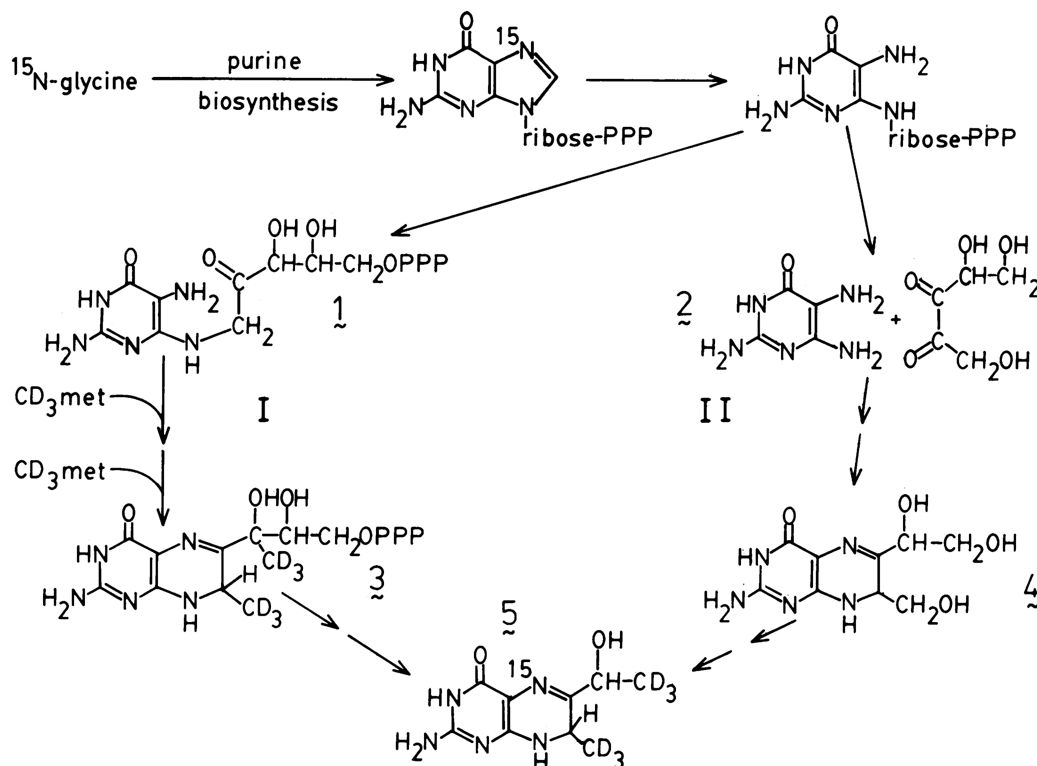

FIG. 2. Proposed routes for the biosynthesis of the methylated pterin present in methanopterin. Compound 5 would serve as a precursor for the biosynthesis of tetrahydromethanopterin.

removed by centrifugation for 3 min in an Eppendorf model 5412 centrifuge. The guanine in the resulting solution was purified by elution from a 0.6 by 6-cm column of Dowex 50-8X H^+ with 4 M HCl as described by Wall (19).

Measurement of label in glycine, methionine, guanine, and 7-methylpterin. Analysis of isotope incorporation into the bound glycine or methionine present in the cells after growth was performed by gas chromatography-mass spectrometry of the *N*-trifluoroacetyl *n*-butyl ester derivatives. The preparation of these derivatives from the bound amino acids released by 6 M HCl hydrolysis of the extracted cell pellet has been previously described (22). Analysis of isotope incorporation into the 7-methylpterin was measured by gas chromatography-mass spectrometry of the ditrimethylsilyl derivative as previously described (23). The label incorporated into the guanine was measured by direct probe mass spectrometry of the trimethylsilyl derivative (8). The label incorporated into these molecules is reported as the moles percent of the molecule or fragment ion that contains the indicated number of enriched atoms and is corrected for the natural abundances of the isotopes as previously described (1).

RESULTS AND DISCUSSION

The two proposed pathways for the biosynthesis of the pterin portion of methanopterin shown in Fig. 2 are patterned after the known biosynthesis of the pterin portion of folic acid (2). Both of these pathways begin with GTP, which would be produced from glycine via the purine biosynthesis pathway. In pathway I, the GTP would be opened by a cyclohydrolase to produce intermediate 1. This compound could then be dimethylated either before or after ring closure to produce dimethyl dihydroneopterin triphosphate 3. Hydrolysis of the triphosphate from this compound, followed by the loss of a C_2 unit, would then generate compound 5. Compound 5 could then be converted into methanopterin by a series of reactions analogous to those known to occur in folic acid biosynthesis; i.e., it is first converted into the pyrophosphate ester with ATP (13, 15, 20) and subsequently condensed with either free 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane or 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane conjugated to ribose, phosphate, and α-hydroxyglutaric acid (11, 15).

Compound 5 could also be produced from GTP by route II. In this route, GTP would be cleaved by the cyclohydrolase reaction, and the ribose would be removed either after or during the cyclohydrolase reaction to form 2,4,5-triamino-6-hydroxypyrimidine, which could be condensed with a pentose to produce compound 4. A possible structure for this sugar would be the diketopentose shown in Fig. 2. Elimination of two water molecules from compound 4, followed by a reduction, would then generate compound 5.

Hybrid pathways, where one of the methyl groups would come from a sugar and the other would come from methionine, are also possible. However, due to analytical constraints, only the origin of the 7-methyl group can be experimentally evaluated at present.

Common to both of these pathways is the idea that GTP serves as an intermediate in the biosynthesis of the methylated pterin. If GTP is a true precursor of the pterin of methanopterin, we would expect glycine, a known precursor of guanine, to be incorporated into both the 7-methylpterin, which is oxidatively derived from methanopterin, and the guanine in the GTP to the same extent. This was confirmed when cells grown with 8 mM [^{15}N]glycine incorporated about the same amount of ^{15}N into the 7-methylpterin as was

incorporated into the guanine in the cells (Table 1). The fact that the total amount of ^{15}N incorporated into these molecules was slightly higher than that observed in the cellular glycine (26.5 atom% ^{15}N) indicated that a small amount of ^{15}N, metabolically derived from the [^{15}N]glycine, was also incorporated into some of the other nitrogen positions of these molecules. This was confirmed by the experimental data (Table 1) in that a small fraction of both the guanine and 7-methylpterin molecules contained more than one ^{15}N.

If route II were operating, we would expect deuterium from a C-1- and C-5-labeled pentose to be incorporated into the 7-methyl group of the pterin. Previous work has shown that the pentoses, as well as the other sugars present in these cells and in other methanogenic bacteria, can be readily labeled with deuterium or ^{13}C at these positions to an extent of >30% by growing the cells with [U-*methyl*-^2H]acetate (R. H. White, Biochemistry, in press) or [2-^{13}C]acetate (3). Examination of the 7-methylpterin isolated from cells grown with [U-*methyl*-^2H]acetate showed that less than 0.5% of them contained deuterium. Thus, route II, which involves the condensation of a sugar with a 2,4,5-triamino-6-hydroxypyrimidine to generate the 7-methylpterin, seems very unlikely.

Growth of the cells with [U-*methyl*-^2H]methionine, however, led to the isolation of 7-methylpterin with up to three deuteriums (Table 2). The extent of labeling of the 7-methylpterin was found to increase when the amount of labeled methionine was increased in the medium (Table 2). This increased labeling of the 7-methylpterin reflects the increased utilization of the labeled methionine by the cells, which was confirmed by the increase in the extent of labeling of the cellular methionine (Table 2). However, the moles percent of the molecules found to contain three deuteriums was in each case less than that found to be incorporated into the cells. This can be explained by the exchange of deuterium from the 7-methyl group of methanopterin or the 7-methylpterin during sample work-up. Exchange of the methyl protons from 7-methyl-substituted lumazine derivatives (10, 12) and exchange of the 7-methyl protons of methanopterin during its solution in D_2O (17) have been previously described. This exchange was confirmed by finding 7-methylpterin molecules which contained both one and two deuteriums (Table 2) and by demonstrating that deuterium was incorporated into the methyl group of 7-methylpterin when a sample of this material was extracted in the presence of deuterated water under the conditions used to extract the pterin from the cells.

Thus, it is clear that the 7-methyl group of methanopterin is derived from the methyl group of methionine, with the initial retention of all of its protons. In addition, the observation that the 7-methylpterin and guanine in the cells are labeled to the same extent with [^{15}N]glycine is consistent with GTP serving as a precursor for the biosynthesis of the pterin portion of methanopterin in a manner analogous to its use in the biosynthesis of the pterin portion of folic acid. Since *p*-aminobenzoic acid has been shown to be a precursor for the biosynthesis of the 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane portion of methanopterin, one must conclude that methanopterin represents the first example of a structurally modified folic acid which functions with covalent modifications in both the pterin and *p*-aminobenzoic acid portions of the molecule.

TABLE 1. Incorporation of [^{15}N]glycine into cellular glycine and guanine and methanopterin-derived 7-methylpterin[a]

Compound	No. of ^{15}N per molecule	Distribution of label (%)	Observed normalized ion intensity (%)
Glycine	N_0	73.5	100.0[b]
	N_1	26.5	42.3[b]
Guanine	N_0	71.7	100.0[c]
	N_1	24.5	64.9[c]
	N_2	3.7	30.1[c]
	N_3	0.1	9.9[c]
	N_0	71.8	100.1[d]
	N_1	24.2	33.3[d]
	N_2	3.8	13.9[d]
	N_3	0.2	3.4[d]
7-Methylpterin	N_0	67.6	100.0[e]
	N_1	27.1	69.8[e]
	N_2	4.1	29.3[e]
	N_3	1.1	10.4[e]

[a] [^{15}N]glycine was fed to a growing culture of strain 10-16B at a concentration of 8 mM.

[b] Measured from the M^+-101 ion at *m/z* 127 of the *N*-trifluoroacetyl *n*-butyl ester derivative of the glycine derived from glycine bound in the cell pellet. An unlabeled sample of the glycine had an ion intensity ratio for (*m/z* 128)/(*m/z* 127) of 6.2%.

[c] Measured from the *m/z* 352 (M^+-15) ion of the ditrimethylsilyl derivative of the guanine. The ratios of the ion intensities for an unlabeled sample of this derivative expressed as the percentages of the *m/z* 352 ion were 100, 30.7, 14.5, and 3.1 for the *m/z* 352, 353, 354, and 355 ions, respectively.

[d] Measured from the *m/z* 367 (M^+) ion of the ditrimethylsilyl derivative of the guanine. The ratios of the ion intensities for an unlabeled sample of this derivative expressed as the percentages of the *m/z* 367 ion were 100, 33.2, 13.9, and 3.4 for the *m/z* 367, 368, 369, and 370 ions, respectively.

[e] Measured from the M^+, *m/z* 321 ion of the ditrimethylsilyl derivative.

TABLE 2. Incorporation of [U-*methyl*-^2H]methionine into 7-methylpterin

Concn of labeled methionine fed (mM)	Atom % of cellular methionine with C^2H_3	No. of ^2H per molecule	Distribution of label (%)[a]	Observed normalized ion intensity (%)[b]
0	0.0	H_0	100.0	100.0
		H_1	0.0	28.7
		H_2	0.0	11.1
		H_3	0.0	2.5
		H_4	0.0	0.9
1.3	10.8	H_0	93.6	100.0
		H_1	2.2	31.0
		H_2	1.2	12.8
		H_3	3.0	6.3
		H_4	0.0	2.0
3.4	17.7	H_0	87.2	100.0
		H_1	5.2	35.7
		H_2	3.4	17.0
		H_3	4.2	9.0
		H_4	0.0	2.3

[a] Measured from the M^+, *m/z* 321 ion of the ditrimethylsilyl derivative.

[b] Normalized ion intensity ratios for the *m/z* 321, 322, 323, and 324 ions.

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