D-erythro-Neopterin Biosynthesis in the Methanogenic Archaea

Methanococcus thermophila and Methanobacterium thermoautotrophicum ΔH

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The steps in the biosynthetic transformation of GTP to 7,8-dihydro-o-erythro-neopterin (H₉neopterin), the precursor to the modified folates found in the methanogenic archaea, has been elucidated for the first time in two members of the domain Archaea. In Methanococcus thermophila and Methanobacterium thermoautotrophicum ΔH, it has been demonstrated that H₉neopterin 2'-3'-cyclic phosphate is an intermediate in this conversion. In addition, the formation of the pterin ring of the H₂neopterin 2'-3'-cyclic phosphate is catalyzed by a single enzyme, as is known to occur with GTP cyclohydrolase I in the Eucarya and Bacteria, but rather by two or more enzymes. A 2,4,5-triamo-4-(3H)-pyrimidinone-containing molecule, most likely 2,5-diamo-6-ribosylamino-4(3H)-pyrimidinone 5'-triphosphate, has been identified as an intermediate in the formation of the H₂neopterin 2'-3'-cyclic phosphate. Synthetic H₂neopterin 2'-3'-cyclic phosphate was found to be readily hydrolyzed by cell extracts of M. thermophila via the H₂neopterin 3'-phosphate to H₉neopterin, a known precursor to the pterin portion of methanopterin.

GTP cyclohydrolase I is recognized as the enzyme that catalyzes the first committed step in the biosynthesis of the pterin ring of folic acid, biopertin, and sepiapterin (2). In this reaction, the C-8 carbon of the GTP is removed as formate and the resulting enzyme-bound intermediate is converted to 7,8-dihydro-o-erythro-neopterin (H₉neopterin) 3'-triphosphate (12). In the case of E. coli, the required four steps of this transformation are carried out by the folE gene product (6). Genes encoding highly conserved sequences homologous to the sequence of this enzyme have been identified from a wide range of different Eucarya and Bacteria (12), and in a few cases, the proteins have been cloned, sequenced, overexpressed, and shown to catalyze the expected reaction (8, 25). Early labeling experiments using archaea indicated that GTP cyclohydrolase I also catalyzed the first committed step in the biosynthesis of the pterin portion of the modified folate, methanopterin (9, 20). Recent results from the analysis of the archaeal genome of Methanococcus jannaschii, however, show no genes with sequences identifiable as GTP cyclohydrolase I (3). This brought into question the nature of the enzyme(s) responsible for the generation of the pterin portion of the modified folates, such as methanopterin, which occur in the archaea (23). In this report, we demonstrate the absence of an Escherichia coli-like GTP cyclohydrolase I in the archaeal Methanococcus thermophila and Methanobacterium thermoautotrophicum ΔH and show that in these archaea, GTP is transformed via a multistep process into H₂neopterin 2'-3'-cyclic phosphate. The H₂neopterin 2'-3'-cyclic phosphate is then hydrolyzed to H₉neopterin via H₂neopterin 3'-phosphate. The H₂neopterin is then converted to d-7,8-dihydro-6-hydroxymethylpterin, a known precursor to the pterin portion of methanopterin and sarcinopterin (22).

MATERIALS AND METHODS

Materials. Chromatographically purified E. coli alkaline phosphatase (type III, E. coli; catalog no. P 4252) and GTP were obtained from Sigma Chemical Co.

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RESULTS AND DISCUSSION

The first approach to determining the metabolism of GTP into pterin-containing compound(s) was to isolate cell extracts of the methanogens with GTP and to look for the production of fluorescent, pterin-containing products. Incubation of a cell extract of *M. thermophila* with GTP, followed by treatment of the incubation mixture with alkaline phosphate and separation of the reaction mixture on a Sephadex G-25 superfine column (1 by 46 cm), gave the elution profile shown in Fig. 1. (A control incubation without the addition of GTP produced only sarcinapterin [peak A] and 7-methylpterin [peak D], both of which resulted from the oxidation of the tetrahydrosoarcinapterin present in the cell extract [19].) Five major fluorescent peaks, designated A, B, C, D, and E, were observed. The major pterin components in peaks A, B, C, and D were established to be sarcinapterin, *d-*erythro-neopterin, 2′,3′-cyclic-phosphate, *d-*erythro-neopterin, and 7-methylpterin, respectively, based on comparison of the pterin in each peak with known pterin samples, using the following rationale. Each of the four peaks eluted from the Sephadex G-25 column at the same position as the respective known compound. The fluorescence excitation and emission spectra (excitation *λ*<sub>max</sub> = 360 nm; emission *λ*<sub>max</sub> = 445 nm) as well as the absorbance spectra of each peak were the same as those of the known compound. Finally, the TLC *R*<sub>p</sub> of the fluorescent compounds recovered from each peak were the same as those of the known pterins, using the two different TLC solvent systems. In addition to these methods of identification, the sarcinapterin contained in peak A was confirmed by its milic acid cleavage to the same pterin-containing fragment as derived from the acid cleavage of methanopterin (23). The *d-*erythro-neopterin 2′,3′-cyclic-phosphate contained in peak B was confirmed by its acid and base cleavage to either *d-*erythro-neopterin 3′-phosphate or *d-*erythro-neopterin 2′-phosphate followed by alkaline phosphatase cleavage to *d-*erythro-neopterin. Both the synthetic and biosynthetic *d-*erythro-neopterin 2′,3′-cyclic-phosphate were found to be resistant to cleavage by alkaline phosphatase, as had been previously reported for this compound (10).

TLC analysis of portions of the individual fractions making up peak D showed that the beginning of this peak eluted at the same elution position as a known sample of 6-hydroxymethylpterin and was 6-hydroxymethylpterin, a known metabolite of *M. thermophila* incubated in the methane fermenter with GTP. Chromatographic and chemical analyses of the fluorescent peaks, which were about 10 times less than those observed with *M. thermophila*, showed that they were identical to those observed for *M. thermophila* except that peak A was identified as methanopterin. Because of the low production of the desired products in cell extracts of *M. thermophila*.
moautotrophicum ΔH, the remaining experiments were conducted with cell extracts of *M. thermophila*.

Based on the observed fluorescence intensities of the *M. thermophila*-derived peaks, 10 nmol of D-neopterin and 2.7 nmol of D-neopterin 2′:3′-cyclic phosphate were recovered from the incubation mixture after cleavage of the phosphate monoesters with alkaline phosphatase. Based on the CD spectra (positive CD band at 247 nm) and the TLC Rf of the recovered neopterin peak, the neopterin isomer was identified as D-erythro-neopterin (14, 24). Treatment of the pterin contained in peak B with base followed by phosphatase cleavage led to the isolation of 2.1 nmol of D-erythro-neopterin, indicating that peak B was originally D-erythro-neopterin 2′:3′-cyclic phosphate. An identical incubation and Sephadex column separation of an incubation mixture without the phosphatase treatment gave only 2.5 nmol of neopterin, indicating that about 7.7 nmol of neopterin 3′-phosphate was generated during the incubation. Phosphatase treatment of pterin(s) contained in peak B, which eluted at the same position as neopterin-P3, produced no neopterin, indicating that no neopterin-P3 was present in the incubated sample. Incubation of H2-neopterin-P3 and neopterin-P3 under conditions even harsher than those used in the incubation (2 h at 70°C in 0.2 M Tris hydrochloride buffer [pH 8.5]) failed to produce any detectable neopterin cyclic phosphate.

Further proof of the involvement of H2-neopterin 2′:3′-cyclic phosphate in the pathway leading to H2-neopterin was confirmed by the observation that the cyclic phosphate was rapidly hydrolyzed first to H2-neopterin-3′-phosphate and then finally to neopterin by cell extracts of *M. thermophila*. These observations, along with previously published results showing that D-erythro-neopterin was readily converted into 6-hydroxymethylpterin by cell extracts of *M. thermophila* (22), indicate that H2-neopterin 2′:3′-cyclic phosphate is very likely involved in the sequence of reactions leading to the pterin portion of methanopterin and sarcinapterin.

The involvement of H2-neopterin 2′:3′-cyclic phosphate in the pathway to neopterin is in contrast to the established pathway of pterin formation where neopterin-P3, generated by the action of GTP cyclohydrolase I on GTP, is an intermediate. Attempts to isolate GTP cyclohydrolase I from *M. thermophila* and *M. moautotrophicum* ΔH by the method of Yim and Brown (27) gave negative results; no enzyme producing a fluorescent neopterin-containing product could be found. These observations, along with the absence of a protein with a sequence homologous to that of GTP cyclohydrolase I in the genome of the archaeon *Methanococcus jannaschii* (3), make one suspect that the routes for the formation of neopterin may be different in the archaea. Since the conversion of GTP to H2-neopterin-P3 requires at least four chemical steps, then it is possible that more than one enzyme is required for the formation of H2-neopterin 2′:3′-cyclic phosphate in the archaea. To test this idea, the proteins present in an extract of *M. thermophila* were separated on an FPLC Mono Q column into 12 fractions, and each fraction was assayed for its ability to produce pterin-containing compound(s) when incubated with GTP. This was established by iodine oxidation of the reaction product followed by its separation on a Sephadex G-25 column and identification of the pterins by fluorescence and TLC as described above. We found no single Mono Q fraction that produced a pterin-containing compound upon incubation with GTP, whereas the combined Mono Q fractions 5 to 10, eluting from 0.4 to 1 M KCl, readily produce H2-neopterin 2′:3′-cyclic phosphate, indicating that more than one enzyme or an enzyme and a coenzyme were required for the transformation. Since the product of this incubation was H2-neopterin 2′:3′-cyclic phosphate, this finding indicates that the enzyme(s) re-
FIG. 2. Proposed pathways for the formation of d-H2-neopterin 2'-3' cyclic phosphate, riboflavin, and F420 in *M. thermophila*. It is proposed that the first step in the reaction is catalyzed by new enzyme termed GTP cyclohydrolase III.
quired for the further metabolism of H2neopterin 2′:3′-cyclic phosphate were not contained in Mono Q fractions 5 to 10.

Another characteristic of the E. coli GTP cyclohydrolase I is the lack of any detectable intermediate(s) in its enzymatic conversion of GTP to H2neopterin-P3 (27). This is in contrast to our observations where we have tentatively identified 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5′-triphosphate (Fig. 2) as an intermediate in the series of reactions in which H2neopterin 2′:3′-cyclic phosphate is the product. The identification of this intermediate is based on the detection of a molecule, produced in an incubation mixture of a cell extract of M. thermophila, which elutes from a DEAE-Sephadex column in the same position as ATP and GTP and produces 6,7-dimethylpterin when reacted with 2,3-butanedione. These observations are all consistent with 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5′-triphosphate being present in the incubation mixture. The DEAE-Sephadex column separation of the incubation mixture also identified, at about the same concentration, a compound that eluted at the position of AMP and GMP and also produced 6,7-dimethylpterin when heated with 2,3-butanedione. This compound is most likely 2,5-diamino-6-(ribosylamino)-4(3H)-pyrimidinone 5′-phosphate, the first intermediate in the biosynthesis of riboflavin, and arises from the GTP cyclohydrolase II present in the extract (2).

H2neopterin 2′:3′-cyclic phosphate was first thought to be produced by the GTP cyclohydrolase present in Comamonas sp. (4) but was later found to be an artifact of the chromatographic methods employed. This cannot be the reason for the H2neopterin 2′:3′-cyclic phosphate detection here, since none of those analytical methods were used. It should be noted that H2neopterin 2′:3′-cyclic phosphate has been reported several times as a microbiological product (18, 26), which may indicate its involvement in neopterin biosynthesis in organisms other than archaea.

The formation of H2neopterin 2′:3′-cyclic phosphate can be rationalized by the following route. An intramolecular reaction in the 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5′-triphosphate would lead to pyrophosphate and 2,5-diamino-6-(ribitylimino)-4(3H)pyrimidinone 4′:5′-cyclic phosphate as shown in Fig. 2. An aldolase-type rearrangement of the 2,5-diamino-6-(ribitylimino)-4(3H)pyrimidinone 4′:5′-cyclic phosphate followed by cyclization of the resulting product would then produce 7,8-H2neopterin 2′:3′-cyclic phosphate. Hydrolysis of the cyclic phosphate would then produce H2neopterin, a known precursor to the pterin portion of methanopterin, as shown in Fig. 3.

The Methanococcus jannaschii genome has a gene sequence encoding a protein with a sequence homologous to that of GTP cyclohydrolase II (3). GTP cyclohydrolase II of E. coli is responsible for the first step in the biosynthesis riboflavin and converts GTP into 2,5-diamino-6-(ribosylamino)-4(3H)pyrimidinone 5′-phosphate, the first intermediate in the biosynthesis of riboflavin, and arises from the GTP cyclohydrolase II present in the extract (2).

FIG. 3. Steps in the conversion of d-3-H2dihydroneopterin 2′:3′-cyclic phosphate into H2-6-hydroxymethylpterin pyrophosphate.
idinone 5′-triphosphate as shown in Fig. 2. Loss of pyrophosphate from this molecule would then produce 2,5-diamino-6-(ribosylamino)-4(3H)pyrimidinone 5′-phosphate, the established intermediate in riboflavin biosynthesis (1). The presence of 2,5-diamino-6-(ribosylamino)-4(3H)pyrimidinone 5′-phosphate in the archaea is supported by the presence of a riboflavin-specific deaminase in Methanococcus jannaschii (3). We thus propose that the GTP cyclohydrolase in archaea be named GTP cyclohydrolase III, since its product is different from that of GTP cyclohydrolase I or II.

REFERENCES