JOURNAL OF BACTERIOLOGY, May 1997, p. 3374–3377 Vol. 179, No. 10
0021-9193/97/$04.00+0

Purine Biosynthesis in the Domain *Archaea* without Folates or Modified Folates

ROBERT H. WHITE*

Department of Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061-0308

Received 19 December 1996/Accepted 17 March 1997

The established pathway for the last two steps in purine biosynthesis, the conversion of 5-aminoimidazole-4-carboxamide ribonucleotide (ZMP) to IMP, is known to utilize 10-formyl-tetrahydrofolate as the required C_1 donor cofactor. The biosynthetic conversion of ZMP to IMP in three members of the domain *Archaea*, *Methanobacterium thermoautotrophicum* ΔH, *M. thermoautotrophicum* Marburg, and *Sulfolobus solfataricus*, however, has been demonstrated to occur with only formate and ATP serving as cofactors. Thus, in these archaea, which use methanopterin (MPT) or another modified folate in place of folate as the C_1 carrier coenzyme, neither folate nor a modified folate serves as a cofactor for this biosynthetic transformation. It is concluded that archaea, which function with modified folates such as MPT, are able to carry out purine biosynthesis without the involvement of folates or modified folates.

The evolution of a group of organisms which carry out their metabolism with a modified coenzyme is an unprecedented occurrence but an event that nonetheless has occurred in most of the archaea. This event is the replacement of folate as the C_1 carrier coenzyme with a series of different modified folates, examples of which include methanopterin (MPT), sarcinapterin, taliopterin, and thermopterin in the methanogens (14, 15, 17); modified folates with poly-β(1→4)-linked *N*-acetylglucosamine side chains in the thermophilic, sulfur-dependent archaea (20, 21); and sulfopterin in *Sulfolobus solfataricus* (19, 24). As a rule, the exchange of C_1 derivatives of tetrahydro-MPT (H_4MPT) and H_4folates as cofactors for enzymes derived from archaea (which use modified folates) with enzymes derived from bacteria and eukarya (which use folate) is not possible (5, 6, 9, 10). (The one exception to this rule is the N^5-methyl H_4MPT:coenzyme M methyltransferase, which can use either N^5-methyl-H_4MPT or N^5-methyl-H_4folate as the methyl donor [1].) Thus, in adapting to changes in the structure of a functioning coenzyme, the archaea must have evolved the ability to perform the biosynthesis of the modified folate (for details on the biosynthesis of MPT, see reference 22), and the enzymatic reaction(s) using the modified folate as a coenzyme must have evolved such that either the coenzyme is no longer required or the enzymatic step(s) in the biosynthetic pathway can proceed using the modified folate. I now report that in archaea, the expected substitution of a 10-formyl-H_4-modified folate, such as 10-formyl-H_4MPT for the 10-formyl-H_4folate, in the next-to-last step of purine biosynthesis does not occur, but instead a pathway which is not dependent on folates or modified folates functions. In archaea, 5-aminoimidazole-4-carboxamide ribonucleotide (ZMP) is found to be readily converted to IMP by cell extracts in the presence of only ATP and formate (Fig. 1).

For the experiments described in Table 1, anaerobically prepared cell extracts or anerobically prepared Sephadex G-25-purified cell extracts of *Methanobacterium thermoautotrophicum* ΔH (18) were incubated at 60°C, under an argon atmosphere, with ZMP and the substrates as indicated. Experiments 1 to 3 were performed with cell extracts of *M. thermoautotrophicum* ΔH prepared in 50 mM TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid]–10 mM $MgCl_2$–2 mM mercaptoethanol (pH 7.5) buffer as previously described (18). Experiments 4 and 5 were performed with the protein-containing fraction of the cell extract purified by Sephadex G-25 chromatography. All experiments used 100 μl of the cell extract or Sephadex G-25-purified cell extract and contained 4.3 mM ZMP and the indicated concentration(s) of substrate(s). The substrates were added as concentrated anaerobic solutions in the same buffer. The amount of hypoxanthine reported is based on the ratios of the hypoxanthine peaks to the adenine peaks in the gas chromatography-mass spectrometry data and the amount of adenine added as ATP to the samples.

At the end of the incubation, the sample was cooled to room temperature and simultaneously exposed to air with the addition of 50 μl of an aerobic solution of 1 M HCl. All subsequent steps were performed under aerobic conditions. The sample was mixed and centrifuged to remove the precipitated proteins. The pellet was extracted with 100 μl of water and centrifuged, and the supernatant was combined with the original supernatant; 1 ml of water was added, and the solution was adjusted to pH 7 to 8 by the addition of 1 M NaOH. The IMP and AMP present in the sample were then purified by a procedure that was specifically developed for the purification of the cellular mononucleotides from this resulting solution. A small DEAE-Sephadex column (5 × 12 mm) was washed with 4 ml of 2 M NH_4HCO_3 and 4 ml of water before the sample was applied. The column was then washed with 2 ml of water and 2 ml of 0.1 M NH_4HCO_3 in sequence, and the IMP as well as other cellular nucleotides were eluted with 2 ml of 0.4 M NH_4HCO_3. Evaporation of the solvent from the 2-ml 0.4 M NH_4HCO_3 fraction with a stream of nitrogen gas gave a sample containing the cellular nucleotides, including the biosynthesized IMP. Hypoxanthine and adenine were quantitatively released from the purine mononucleotides present in the evaporated sample by hydrolysis for 10 min at 100°C with 100 μl of 1 M HCl. Under these hydrolysis conditions, both AMP and IMP were found to quantitatively release their purines as measured by thin-layer chromatography analysis of the hydrolysis of known samples.

After evaporation of the acid with a stream of nitrogen gas at 100°C, the sample was redissolved in 0.05 M HCl (100 μl) and applied to a Dowex 50W-8X H^+ column (2 × 4 mm), equilibrated in water, which absorbed the hypoxanthine and

* Corresponding author. Phone: (540) 231-6605. Fax: (540) 231-9070. E-mail: rhwhite@vt.edu.

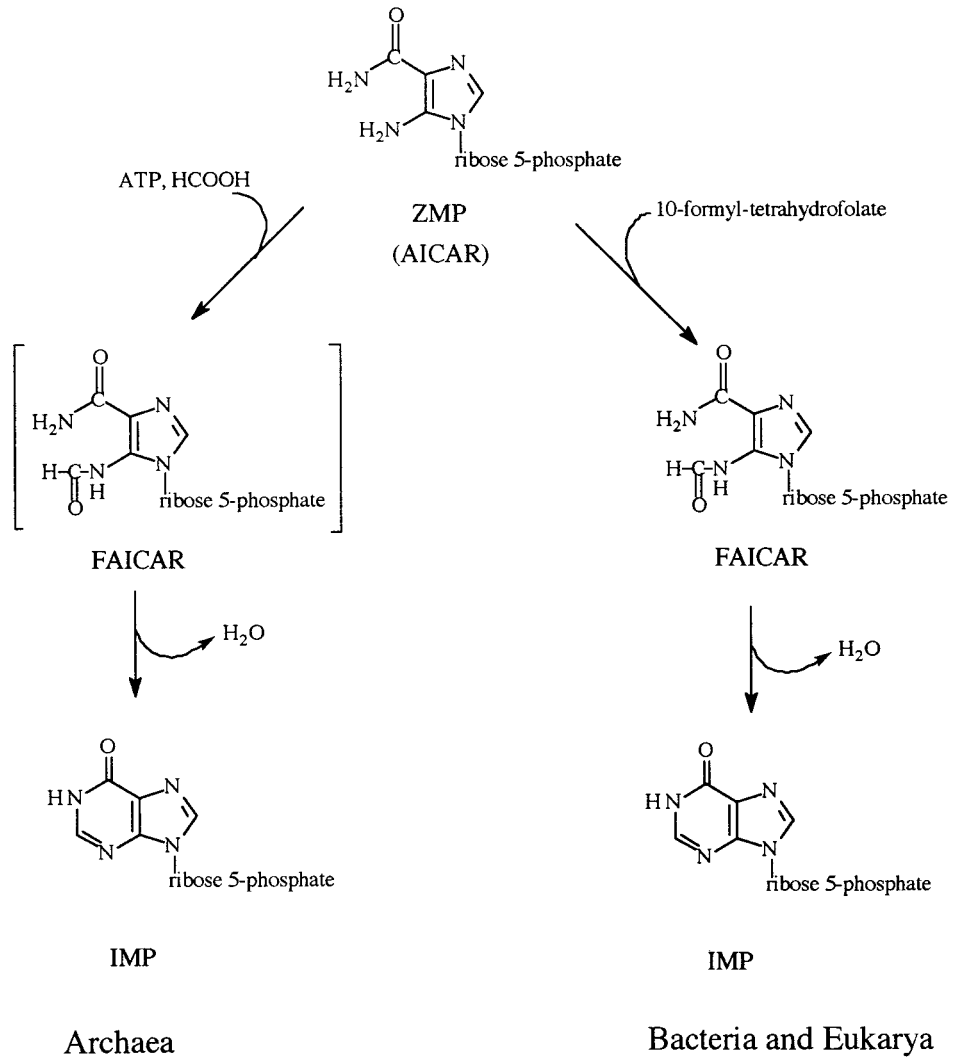

FIG. 1. The different pathways for the formation of IMP in archaea, bacteria, and eukarya. The intermediacy of 5′-phosphoribosyl-4-carboxamide-5-formamidoaminoimidazole (FAICAR) in the archaeal pathway has not been established.

adenine. After the column was washed with 0.05 M HCl (0.5 ml) and then water (0.5 ml), the hypoxanthine and adenine were eluted with 0.5 ml of 3 M aqueous ammonia. The sample, evaporated to dryness with a stream of nitrogen gas, was then converted into the ditrimethylsilyl derivative by reacting with 10 μl of *N*,*O*-bis(trimethylsilyl)acetamide for 10 min at 100°C. Gas chromatography-mass spectrometry of the resulting derivatized sample was performed at 70 eV on a VG-7070EHF mass spectrometer equipped with a high-performance capillary column (0.32 mm × 30 m; HP-5) programmed from 70 to 260

TABLE 1. Incorporation of ^{13}C-labeled precursors into IMP by *M. thermoautotrophicum* ΔH

Expt. no.[a]	Substrate (concn[s] [mM])	Hypoxanthine detected (mM)	Portion of IMP derived from labeled precursor (%)
1	$^2H_2$-formaldehyde (4.0)	0.017	<1
2	[^{13}C]formaldehyde (8.5), 2-Bromoethanesulfonic acid (1.3)	0.037	28
3	[^{13}C]formate (8.5), ATP (8.5)	1.7	79
4	[^{13}C]formate (8.5), ATP (8.5)	4.1	89
5	[^{13}C]formate (8.5)	<0.0002	—[b]

[a] Experiments 1 to 3 were performed with 100 μl of cell extract (15 to 20 mg of protein per ml) of *M. thermoautotrophicum* ΔH prepared in 50 mM TES–10 mM $MgCl_2$–2 mM mercaptoethanol (pH 7.5) buffer. Experiments 4 and 5 were performed with the protein-containing fraction of the cell extract purified by Sephadex G-25 chromatography. For all experiments, the mixtures used contained 4.3 mM ZMP and the indicated substrate concentration(s). The mixtures were incubated for 2 h at 60°C under an argon atmosphere.

[b] Since no hypoxanthine was detected, the portion derived from the [^{13}C]formate could not be measured.

at 10°C per min. Under these conditions, the ditrimethylsilyl derivatives of hypoxanthine and adenine eluted at 16.97 and 17.86 min, respectively.

As can be seen from the data presented in Table 1 (experiment 1), only a small amount of IMP, as assayed by the amount of hypoxanthine recovered, was detected in cell extracts incubated with ZMP and $^2H_2$-formaldehyde and processed as described above. The hypoxanthine recovered was found to contain no detectable deuterium. If H_4MPT were involved in the formation of IMP, then one would have expected to find some labeled hypoxanthine. This follows from the fact that formaldehyde is known to readily react chemically with H_4MPT to form methylene-H_4MPT (7), which is, in turn, enzymatically oxidized to methenyl-H_4MPT (16). The methenyl-H_4MPT can then be converted to 5-formyl-H_4MPT by an enzymatic route (2, 6) or to 10-formyl-H_4MPT by chemical hydrolysis (2, 6). If these reactions lead to the production of a labeled formyl-H_4MPT which could be involved in IMP biosynthesis, then there are several possible explanations to account for the lack of incorporation of the deuterated formaldehyde into IMP: (i) the methylene-H_4MPT was all reduced to methane, (ii) the methenyl-H_4MPT exchanged its deuteriums with water protons (25), (iii) the C-2 deuterium of the IMP and/or hypoxanthine exchanged with water protons during sample workup and hydrolysis, and/or (iv) a formyl-H_4MPT was not involved in the reaction. To surmount the first three of these problems, the experiment was repeated with [^{13}C]formaldehyde (91.2 atom% ^{13}C) in the presence of 2-bromoethanesulfonate, a potent inhibitor of methanogenesis, which prevents the reduction of formaldehyde to methane (8). In this experiment, experiment 2 (Table 1), more IMP production was measured, with 28% of the molecules being derived from the labeled formaldehyde. The increased amount of IMP can be accounted for by the increased concentration of formaldehyde in the incubation.

Assuming that the labeled precursor was only incorporated at the formate oxidation state, this result indicates that some oxidation of the formaldehyde to formate occurred and that the formate was subsequently incorporated into IMP. Again however, the amount of product formed and the extent of incorporation of ^{13}C were small. Although it is possible that formyl-H_4MPT served as a donor in this experiment, it is also possible that chemical hydrolysis of the formyl-H_4MPT to formate had occurred and that the resulting free formate along with the cellular ATP was responsible for the formation of the IMP. To check out the latter possibility, a cell extract was incubated with [^{13}C]formate and ATP. This led to the production of 1.7 mM IMP, of which 79% was derived from the labeled formate (experiment 3 [Table 1]). This result strongly suggests that only ATP and formate and not a derivative of H_4MPT were involved in this step of purine biosynthesis.

Since H_4MPT was present in the cell extract used in these experiments, it is still possible, however, that the ATP and formate formylated the H_4MPT to formyl-H_4MPT, which in turn serves as the formyl donor for the formylation of ZMP. This idea was discounted by the data presented in experiment 4 (Table 1), which show that an anaerobically prepared Sephadex G-25-purified cell extract, found to be free of H_4MPT, also readily produced IMP from ZMP, formate, and ATP. That the reaction was dependent on ATP was confirmed by the results of experiment 5 (Table 1), which showed no detectable production of IMP in the absence of ATP.

Experiments identical to those described above for *M. thermoautotrophicum* ΔH but with anaerobically prepared extracts of *M. thermoautotrophicum* Marburg and aerobically prepared cell extracts of *S. solfataricus* gave very similar results. Incubation of 100 μl of cell extract of *M. thermoautotrophicum* Marburg for 2 h at 60°C in the presence of 8.5 mM [^{13}C]formate and 8.5 mM ATP produced 2.9 mM IMP with a 20.5 atom% excess of ^{13}C. Incubation of 100 μl of cell extract of *S. solfataricus* for 2 h at 60°C in the presence of 8.5 mM [^{13}C]formate and 8.5 mM ATP produced 0.18 mM IMP with a 20.8 atom% excess of ^{13}C.

The basic outline of the 10 steps in purine biosynthesis was established in the late 1950s by Buchanan and coworkers, using pigeon and chicken livers, and represents the established pathway for purine biosynthesis in most organisms (23). In the originally proposed pathway, the 3rd step, catalyzed by glycinamide ribonucleotide (GAR) transformylase, and the 10th step, catalyzed by 5′-phosphoribosyl-4-carboxamide-5-aminoimidazole (AICAR) transformylase, involved the incorporation of a C_1 unit of formate from N^{10}-formyl-H_4folate. Later experiments with *Escherichia coli*, however, demonstrated that a non-folate-dependent GAR transformylase (GAR transformylase T) was also present in *E. coli* (13). This enzyme catalyzes the production of β-formyl-GAR from formate, ATP, and β-GAR and is not homologous to the N^{10}-formyl-H_4folate-utilizing enzyme which is now referred to as GAR transformylase N (12). It is the GAR transformylase T that appears to function in the methanogens, since an open reading frame for only GAR transformylase T is found in the genome of Methanococcus jannaschii (3). The other reaction requiring formyl-N^{10}-formyl-H_4folate is in the conversion of ZMP to IMP and, as shown in this paper, is carried out with only ATP and formate; neither N^{10}-formyl-H_4folate nor N^{10}-formyl-H_4MPT is required. This may explain the absence of a gene for AICAR transformylase in *Methanococcus jannaschii* (3) and the differences observed in the labeling patterns at the C-2 position of purines produced in the different methanogens labeled with different carbon sources (4). Thus, it appears that *M. thermoautotrophicum* ΔH either has retained a more primitive route for the conversion of ZMP to IMP that is independent of folates or modified folates or has developed another route to generate IMP that avoids having to replace N^{10}-formyl-H_4folate with N^{10}-formyl-H_4MPT. The use of the reaction described here by the methanogens also solves the problem of the route for the formation of N^{10}-formyl-H_4MPT, the expected formyl donor for the reaction, which is currently not known to be biologically produced by the methanogens (11).

I thank Kim Harich for running the mass spectra and Constance D. Anderson for editing the manuscript.

This work was supported by grant DMB-871-18216 from the National Science Foundation.

REFERENCES

1. **Becher, B., V. Müller, and G. Gottschalk.** 1992. N^5-methyl-tetrahydromethanopterin:coenzyme M methyltransferase of *Methanosarcina* strain Göl is an Na^+-translocating membrane protein. J. Bacteriol. **174:**7656–7660.
2. **Brietung, J., R. A. Schmitz, K. O. Stetter, and R. K. Thauer.** 1991. N^5,N^{10}-methenyltetrahydromethanopterin cyclohydrolase from the extreme thermophile *Methanopyrus kandleri*: increase of catalytic efficiency (K_{cat}/K_M) and thermostability in the presence of salt. Arch. Microbiol. **156:**517–524.
3. **Bult, C. J., et al.** 1996. Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii*. Science **273:**1058–1072.
4. **Choquet, C. G., J. C. Richards, C. B. Patel, and G. D. Sprott.** 1994. Purine and pyrimidine biosynthesis in methanogenic bacteria. Arch. Microbiol. **161:** 471–480.
5. **DiMarco, A. A., M. I. Donnelly, and R. S. Wolfe.** 1986. Purification and properties of the 5,10-methenyltetrahydromethanopterin cyclohydrolase from *Methanobacterium thermoautotrophicum*. J. Bacteriol. **168:**1372–1377.
6. **Donnelly, M. I., J. C. Escalante-Semerena, K. L. Rinehart, Jr., and R. S. Wolfe.** 1985. Methenyl-tetrahydromethanopterin cyclohydrolase in cell extracts of *Methanobacterium*. Arch. Biochem. Biophys. **242:**430–439.
7. **Escalante-Semerena, J. C., K. L. Rinehart, Jr., and R. S. Wolfe.** 1984. Tetrahydromethanopterin, a carbon carrier in methanogenesis. J. Biol. Chem. **259:**9447–9455.

8. **Gunsalus, R. P., J. A. Romesser, and R. S. Wolfe.** 1978. Preparation of coenzyme M analogues and their activity in the methyl coenzyme M reductase system of *Methanobacterium thermoautotrophicum*. Biochemistry **17:** 2374–2377.
9. **Hartzell, P. L., G. Zvilius, J. C. Escalante-Semerena, and M. I. Donnelly.** 1985. Coenzyme F_{420} dependence of the methylene-tetrahydromethanopterin dehydrogenase of *Methanobacterium thermoautotrophicum*. Biochem. Biophys. Res. Comm. **133:**884–890.
10. **Holt, J. C., A. Oren, J. C. Escalante-Semerena, and R. S. Wolfe.** 1986. Tetrahydromethanopterin-dependent serine transhydroxymethylase from *Methanobacterium thermoautotrophicum*. Arch. Microbiol. **145:**153–158.
11. **Keltjens, J. T., P. C. Raemakers-Franken, and G. D. Vogels.** 1993. Methanopterin, its structural diversity and functional uniqueness, p. 135–150. *In* J. C. Collins and D. P. Kelly (ed.), Microbial growth or C1 compounds: proceedings of the 7th international symposium. Intercept, Andover, United Kingdom.
12. **Marolewski, A., J. M. Smith, and S. Benkovic.** 1994. Cloning and characterization of a new purine biosynthetic enzyme: a non-folate glycinamide ribonucleotide transformylase from *E. coli*. Biochemistry **33:**2531–2537.
13. **Nygaard, P., and J. M. Smith.** 1983. Evidence for a novel glycinamide ribonucleotide transformylase in *Escherichia coli*. J. Bacteriol. **175:**3591–3597.
14. **Raemakers-Franken, P. C., R. Bogaerts, R. Fokkens, C. van der Drift, and G. D. Vogels.** 1991. Identification of a novel tatiopterin derivative in *Methanogenium tationis*. Eur. J. Biochem. **200:**783–787.
15. **Raemakers-Franken, P. C., C. H. M. van Elderen, C. van der Drift, and G. D. Vogels.** 1991. Identification of a novel tatiopterin derivative in *Methanogenium tationis*. Biofactors **3:**127–130.
16. **Schwörer, B., and R. K. Thauer.** 1991. Activities of formyl-methanofuran dehydrogenase, methylenetetrahydromethanopterin dehydrogenase, methylenetetrahydromethanopterin reductase, and heterodisulfide reductase in methanogenic bacteria. Arch. Microbiol. **155:**459–465.
17. **Van Beelen, P., J. F. A. Labro, J. T. Keltjens, W. J. Geerts, G. D. Vogels, W. H. Laarhoven, W. Guijt, and C. A. G. Haasnoot.** 1984. Derivatives of methanopterin, a coenzyme involved in methanogenesis. Eur. J. Biochem. **139:**359–365.
18. **White, R. H.** 1988. Characterization of the enzymatic conversion of sulfoacetaldehyde and L-cysteine into coenzyme M (2-mercaptoethanesulfonic acid). Biochemistry **27:**7458–7462.
19. **White, R. H.** 1991. Distribution of folates and modified folates in extremely thermophilic bacteria. J. Bacteriol. **173:**1987–1991.
20. **White, R. H.** 1993. Structures of the modified folates in the thermophilic archaebacteria *Pyrococcus furiosus*. Biochemistry **32:**745–753.
21. **White, R. H.** 1993. Structures of the modified folates in the extremely thermophilic archaebacterium *Thermococcus litoralis*. J. Bacteriol. **175:**3661–3663.
22. **White, R. H.** 1996. Biosynthesis of methanopterin. Biochemistry **35:**3447–3456.
23. **Zalkin, H., and P. Nygaard.** 1996. Biosynthesis of purine nucleotides, p. 561–579. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, vol. 1. ASM Press, Washington, D.C.
24. **Zhou, D., and R. H. White.** 1992. 5-(*p*-Aminophenyl)-1,2,3,4-tetrahydroxypentane, a structural component of the modified folate in *Sulfolobus solfataricus*. J. Bacteriol. **174:**4576–4582.
25. **Zirngibl, C., W. Van Dongen, B. Schwörer, R. Van Bünau, M. Richter, A. Klein, and R. K. Thauer.** 1992. H_2-forming methylenetetrahydromethanopterin dehydrogenase, a novel type of hydrogenase without iron-sulfur clusters in methanogenic archaea. Eur. J. Biochem. **208:**511–520.