JOURNAL OF BACTERIOLOGY, Jan. 1997, p. 563–566 Vol. 179, No. 2
0021-9193/97/$04.00+0

Occurrence and Biosynthesis of 5-Aminoimidazole-4-Carboxamide Ribonucleotide and *N*-(β-D-Ribofuranosyl)Formamide 5′-Phosphate in *Methanobacterium thermoautotrophicum* ΔH

ROBERT H. WHITE*

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

Received 6 September 1996/Accepted 6 November 1996

5-Aminoimidazole-4-carboxamide ribonucleotide (ZMP) and *N*-(β-D-ribofuranosyl)formamide 5′-phosphate (FAR-P) have been identified as products of the metabolism of ATP and 5-phospho-α-D-ribosyl diphosphate by *Methanobacterium thermoautotrophicum* ΔH, a member of the domain *Archaea*. Evidence indicates that the first three steps in the pathway to the formation of these compounds are the same as the first three steps of histidine biosynthesis and lead to the generation of pro-phosphoribosyl formimino-5-aminoimidazole-4-carboxamide ribonucleotide (5′-proFAR). The 5′-proFAR then undergoes hydrolysis to ZMP and FAR-P. The reaction was detected by an unexpected high concentration of ZMP in cell extracts of *M. thermoautotrophicum* ΔH.

5-Aminoimidazole-4-carboxamide ribonucleotide (ZMP) is an intermediate in the biosynthesis of purines (15) and is also formed during the biosynthesis of histidine (14). The following is a report of a new biosynthetic pathway for the formation of ZMP that has been uncovered in *Methanobacterium thermoautotrophicum* ΔH, a member of the domain *Archaea*. The first three steps of this pathway appear to be the same as the first three steps in the biosynthesis of histidine (14) and begin with the condensation of ATP with 5-phospho-α-D-ribosyl diphosphate (PRPP) to form N^1-(5′-phosphoribosyl)-ATP (PR-ATP) (left side of Fig. 1). The hydrolytic removal of pyrophosphate from the PR-ATP, followed by the hydrolysis of the purine ring, leads to the formation of pro-phosphoribosyl formimino-5-aminoimidazole-4-carboxamide ribonucleotide (5′-pro-FAR). In the normal histidine biosynthetic pathway, the resulting 5′-proFAR would undergo an Amadori rearrangement to phosphoribulosyl formimino-5-aminoimidazole-4-carboxamide ribonucleotide (PRFAR), which would eventually lead to histidine (top right side of Fig. 1). In *M. thermoautotrophicum* ΔH, however, most of the resulting 5′-proFAR undergoes hydrolysis to ZMP and *N*-(5-*O*-phosphono-β-D-ribofuranosyl) formamide (FAR-P), a compound not previously observed as a biochemical product. Thus far, this biosynthetic pathway has been detected only in *M. thermoautotrophicum* ΔH and to a lesser extent in *M. thermoautotrophicum* Marburg, and it has not been detected in *Methanosarcina thermophila*, *Methanococcus voltae*, or *Escherichia coli.*

Cell extracts prepared from *M. thermoautotrophicum* ΔH produced by growth heterotropically with H_2 and CO_2 in the presence of yeast extract and Trypticase were found to contain 0.91 mM ZMP. In contrast, no ZMP was detected in cell extracts of *M. thermoautotrophicum* ΔH grown autotropically on H_2 and CO_2 (Table 1). Incubation of cell extracts which contained ZMP at 50°C for 1 h under argon, resulted in the complete loss of any detectable amount of ZMP (<7 μM). Mild acid hydrolysis (50 mM HCl, 100°C, 5 min) of cell extracts obtained from cells grown under either condition did not increase the amount of ZMP found. This indicates that the ZMP was not present in the extracts bound as 5′-proFAR, which is known to readily undergo complete hydrolysis under these conditions (1).

Despite the observed difference in the amounts of ZMP detected when the cells were grown in the presence or absence of yeast extract and Trypticase, cell extracts from either group of cells readily formed about the same amount of ZMP when incubated with ATP and PRPP (experiments 5 and 8, Table 1). Cell extracts of *M. thermoautotrophicum* Marburg were found to produce a small amount of ZMP, whereas *Methanosarcina thermophila* and *E. coli* were found not to contain detectable levels of ZMP or to produce detectable levels of ZMP when the extracts were incubated with PRPP and ATP. ZMP was not detected in cells of *Pyrococcus furiosus* or *Sulfolobus sulfataricus* by the procedures described in this paper.

That the observed synthesis of ZMP in *M. thermoautotrophicum* ΔH was connected with histidine biosynthesis was confirmed by the observation of a 92% decrease in the amount of ZMP produced when the cell extracts were incubated with ATP and PRPP in the presence of 10 mM histidine (compare experiments 5 and 6, Table 1). The inhibition of the first step of histidine biosynthesis by histidine is well-known (7), with as little as 0.1 mM histidine causing complete inhibition of the reaction (1). If the ZMP was produced by the normal histidine biosynthetic pathway, then incubation of cell extracts of *M. thermoautotrophicum* ΔH with ATP, PRPP, and glutamine would be expected to produce imidazoleglycerol phosphate (IGP) and/or other imidazole-containing intermediates in the histidine biosynthetic pathway (11). However, no detectable levels of imidazole derivatives (<0.1 nmol/mg of protein) were found when such an incubation was conducted and assayed by the Pauly method (8). This observation demonstrated that the major route for the metabolism of 5′-proFAR was not its expected conversion via PRFAR, which leads to the latter steps in the histidine biosynthetic pathway. The most likely explanation for ZMP formation would then be the hydrolysis of 5′-proFAR to ZMP and FAR-P. This was confirmed by the identification of FAR-P as the other product formed during the incubation of cell extracts of *M. thermoautotrophicum* ΔH with ATP and PRPP.

ZMP was detected during experimentation to establish the pathway for the biosynthesis of 4-(β-D-ribofuranosyl)aminobenzene 5′-phosphate (β-RFA-P), an intermediate in the biosynthesis of methanopterin in different members of the domain

* Phone: (540) 231-6605. Fax: (540) 231-9070.

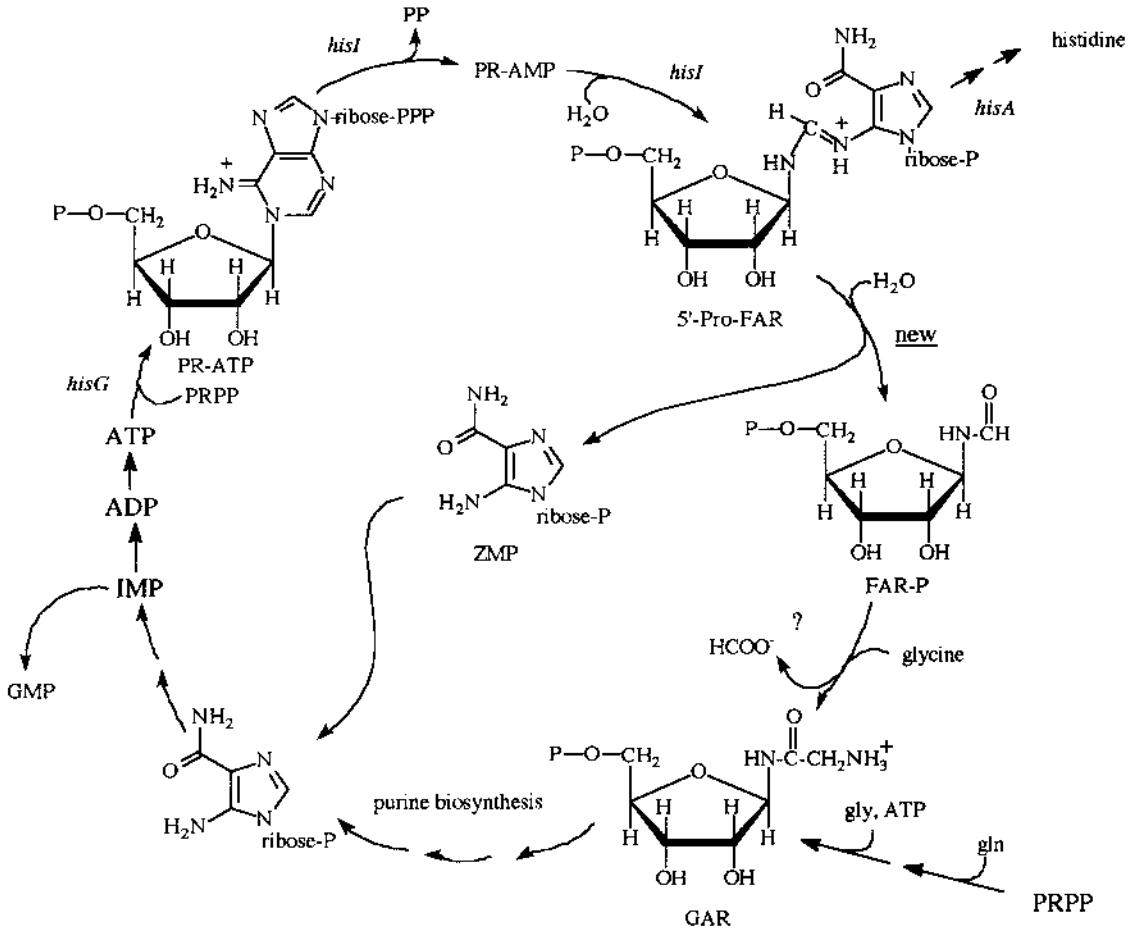

FIG. 1. A possible purine shunt biosynthetic pathway involving the histidine (top portion) and the purine (bottom portion) biosynthetic pathways which are coupled via ZMP and FAR-P.

Archaea (13). In these experiments, cell extracts of *M. thermoautotrophicum* ΔH were incubated under argon in the presence of PRPP and 4-aminobenzoic acid (*p*AB) and assayed for the generation of arylamines. The assay procedure involved the formation of an azo dye derivative of the arylamines, followed by their separation on a Bio-Gel P-4 column as previously described (12). When this procedure was done with cell extracts of *M. thermoautotrophicum* ΔH grown in the presence of yeast extract and Trypticase, two large, unknown, colored peaks were observed in the column fractions, the concentrations of which increased when the cell extract was incubated with PRPP and *p*AB or with PRPP alone. The first peak, purple, eluted with a relative retention volume of 1.04, and the second peak, pink, eluted with a relative retention volume of 1.78. [The retention volume of 1-(4-aminophenyl)-1-deoxy-D-ribitol is set at 1.00 (12).]

These compounds were identified by the following observations. The compound(s) responsible for generating these peaks had a low molecular weight (eluted in the stationary phase volume of a G-25 Sephadex column) and was only slightly retarded when chromatographed on a C_{18} reverse-phase column with 0.1 M citrate (pH 3.6) as the eluting solvent. Based on the 562-nm absorbance of the fractions eluted from the Bio-Gel P-4 column, the ratio of the first peak to the second peak was about 2 to 1. Hydrolysis of each peak with *E. coli* alkaline phosphatase (12) showed that only the retention volume of the first peak was altered, indicating that this peak contained a monophosphate ester. The relative retention volume of this dephosphorylated compound on the Bio-Gel P-4 column was 1.33. The azo dye in each of the isolated peaks was reduced to its original arylamine with Zn dust in 1 M HCl (12), followed by reformation of the azo dye derivative and rechromatography on a Bio-Gel P-4 column. This experiment showed that the compound contained in peak 1 now produced the same two original peaks 1 and 2 in the same 2-to-1 ratio as seen in the initial separation, whereas the compound in peak 2 gave only the original peak 2. Mild acid hydrolysis (1 M HCl, 100°C, 5 min) of the compound recovered from peak 1 gave peak 2. The azo dye contained in peak 1 remained at the origin upon analysis of the sample by silica gel thin-layer chromatography (TLC) with the solvent system acetonitrile-water-formic acid (88%) (40:10:5; vol/vol/vol) and then moved to an R_f of 0.24 after treatment of the sample with *E. coli* alkaline phosphatase. Both the phosphorylated and nonphosphorylated azo dye compounds showed an absorbance maximum at 554 nm in 50 mM HCl. The azo dye contained in peak 2 had an R_f of 0.33 in the same TLC solvent system and an absorbance maximum at 524 nm in 50 mM HCl.

These data were consistent with the compound being a nucleotide monophosphate containing a diazotizable nitrogen-containing base and an *N*-ribosidic linkage which is relatively acid labile. The only currently known, naturally occurring bi-

TABLE 1. ZMP occurrence and formation in cell extracts of *M. thermoautotrophicum* ΔH

Expt[a]	Source of cell extract	Substrate(s)	Amt of ZMP[b]	
			mM	nmol/mg of protein
1	Normal[c]	None[d]	0.91	28
2	Normal	None	<0.007	<0.8
3	Purified[e]	None[d]	<0.007	<0.8
4	Normal	PRPP	0.25	17
5	Normal	PRPP, ATP	4.0	270
6	Normal	PRPP, ATP, His	0.3	20
7	Auto[f]	None	<0.007	<0.8
8	Auto	PRPP + ATP	2.1	78

[a] A typical incubation used either 40 μl of a cell extract obtained by sonication of the cells under anaerobic conditions or 40 μl of the protein fraction generated by the separation of the cell extract on Sephadex G-25 under anerobic conditions. To these cell extracts were added anaerobic solutions of ATP (2 μl of 0.2 M) and PRPP (5 μl of 0.1 M). The samples were incubated under argon at 60°C for 1 h and then cooled, and 0.4 ml of water was added; the azo dyes were formed and separated on Bio-Gel P-4 columns as previously described (12). When required, anaerobic solutions (0.1 M) of L-histidine, ATP, and/or PRPP were added at the start of the incubations. The final concentrations of the substrates in the incubation mixture were 8.5 mM ATP, 10.6 mM PRPP, and 10 mM histidine.

[b] The amount of ZMP was determined from the measured absorbance at 562 nm using $\varepsilon = 2.25 \times 10^4\ M^{-1}\ cm^{-1}$.

[c] Cells used to obtain the "normal" cell extract were grown on H_2 and CO_2 in the presence of yeast extract and Trypticase (2).

[d] The cell extract was not incubated for 1 h at 60°C.

[e] Sephadex G-25-purified protein fraction obtained from normal cell extract.

[f] Cells used to obtain the Auto cell extract were grown autotropically on CO_2 and H_2 (2).

ological compound with this characteristic is 5-aminoimidazole-4-carboxamide ribonucleotide (ZMP). Analysis of a known sample of ZMP (Sigma) showed that it produced chemical, chromatographic, and spectroscopic data (Table 2) identical to those generated from the unknown compound obtained from cell extracts of *M. thermoautotrophicum* ΔH, thus confirming the unknown compound as ZMP.

For the identification of FAR-P, incubations were performed as described in Table 1, and at the completion of the incubation, the FAR-P produced in the reaction was isolated by diluting the sample with water (0.4 ml) and passing the reaction mixture through a small DEAE-Sephadex column (3 by 5 mm) which retained the FAR-P. After the column was washed with water, the FAR-P was eluted with 2 ml of 2 M ammonium bicarbonate. After evaporation of the solvent with a stream of nitrogen gas the sample was dissolved in 50 μl of 0.1 M glycine buffer (pH 10.4) treated with *E. coli* alkaline phosphatase and the *N*-(β-D-ribofuranosyl)formamide (FAR), isolated by elution of the sample from a Dowex 50 H^+ column (3 by 5 mm) and then a Dowex-1 acetate column (3 by 5 mm). Reaction of the dried residue with 20 μl of a mixture of chlorotrimethylsilane, hexamethyldisilane, and pyridine (1:3:9; vol/vol/vol) afforded the tritrimethylsilyl derivative, which was assayed by gas chromatography-mass spectrometry on a DB-5 column (30 m by 0.32 mm; J&W Scientific Co.) programmed from 75 to 280°C at 10°C/min. The sample eluted as a single peak showing an M^+ ion at *m/z* 393 and an $M^+ - 15$ ion at *m/z* 378. Reference samples of FAR and FAR-P, prepared as described by Czarnik and Leonard (4), showed the same characteristics as the biologically produced compound.

TABLE 2. Chromatographic and spectroscopic characteristics of azo dye derivatives

Compound	Retention vol[a]	TLC R_f[b]	λ max (nm)[c]
1-(4-Aminophenyl)-1-deoxy-D-ribitol	1.0	0.61	557
β-RFA-P	0.79	0.10	558
ZMP	1.04	0.00	554
5-Amino-4-imidazole carboxamide riboside	1.33	0.24	554
5-Amino-4-imidazole carboxamide	1.78	0.33	524

[a] The retention volumes are related to the retention volume of 1-(4-aminophenyl)-1-deoxy-D-ribitol, which is set at 1.00.

[b] TLC on silica gel with the solvent system (acetonitrile-water-formic acid [88%] [40:10:5, vol/vol/vol]).

[c] Samples were dissolved in 50 mM HCl.

The important question yet to be answered from this work is why this pathway is present only in these cells. One possibility is that it represents a shunt pathway involved in purine biosynthesis as outlined in Fig. 1. The FAR-P generated by the cleavage of 5′-proFAR could undergo a transpeptidation with glycine to generate 5-phosphoribosylglycinamide (GAR) and formate (lower right side of Fig. 1). The GAR would then be converted via the purine biosynthetic pathway to inosinic acid; in this way, purine biosynthesis could proceed without the established first two steps which involve the reaction of PRPP with glutamine to form 5-phosphoribosylamine (PRA), which is subsequently acylated with glycine in the presence of ATP to produce GAR. A possible advantage to the use of this shunt pathway by these thermophiles would be that purine biosynthesis could proceed without use of the thermally unstable PRA (10) as an intermediate. It has been impossible thus far to confirm the operation of this pathway since it has not been possible to demonstrate the enzymatic conversion of FAR-P to GAR in cell extracts of *M. thermoautotrophicum* ΔH.

ZMP, as its triphosphate derivative (ZTP), has been proposed to function as an alarmone for C_1-folate deficiency in *Salmonella typhimurium* (3), although more recent work has failed to provide evidence to support this idea (9). Although ZTP was not found in *M. thermoautotrophicum* ΔH, it is possible that ZMP is involved in the regulation of C_1-methanopterin metabolism since modified folates such as methanopterin function in place of folates in the methanogens (13). ZMP has also been proposed as a regulator of glycogen biosynthesis (6); however, *M. thermoautotrophicum* ΔH is among the group of methanogens found not to contain glycogen (5).

This work was supported in part by grant no. DMB-871-18216 from the National Science Foundation.

I thank Ralph S. Wolfe and Biswarup Mukhopadayay, Department of Microbiology, University of Illinois, Urbana, for supplying the cells used in this work and Constance D. Anderson for editing the manuscript.

REFERENCES

1. **Ames, B. N., R. G. Martin, and B. J. Garry.** 1961. The first step of histidine biosynthesis. J. Biol. Chem. **236:**2019–2026.
2. **Balch, W. E., G. E. Fox, L. J. Magrum, C. R. Woese, and R. S. Wolfe.** 1979. Methanogens: reevaluation of a unique biological group. Microbiol. Rev. **43:**260–296.
3. **Bochner, B. R., and B. N. Ames.** 1982. ZTP (5-amino-4-imidazole carboxamide riboside): a proposed alarmone for 10-formyltetrahydrofolate deficiency. Cell **29:**929–937.
4. **Czarnik, A. W., and N. J. Leonard.** 1982. Foreshortened nucleotide analogues as potential base-pairing complements for *lin*-bensoadenosine. J. Am. Chem. Soc. **104:**2624–2631.
5. **Konig, H., E. Nusser, and K. O. Stetter.** 1985. Glycogen in *Methanolobus* and *Methanococcus*. FEBS Microbiol. Lett. **28:**265–269.
6. **Leckie, M. P., S. E. Porter, V. L. Tieber, and D. N. Dietzler.** 1981. Regulation of the basal and cyclic AMP-stimulated rates of glygogen synthesis in *Escherichia coli* by an intermediate of purine biosynthesis. J. Bacteriol. **99:**1433–1442.
7. **Martin, R. G.** 1963. The first enzyme in histidine biosynthesis: the nature of feedback inhibition by histidine. J. Biol. Chem. **239:**1848–1855.

8. **Rabinowitz, J. C.** 1963. Intermediates in purine breakdown. Methods Enzymol. **6:**703–713.
9. **Rohlman, C. E., and R. G. Matthews.** 1990. Role of purine biosynthesis intermediates in response to folate stress in *Escherichia coli*. J. Bacteriol. **172:**7200–7210.
10. **Schendel, F. J., Y. S. Cheng, J. D. Otvos, S. Wehrli, and J. Stubbe.** 1988. Characterization and chemical properties of phosphoribosylamine, an unstable intermediate in the de novo purine biosynthesis pathway. Biochemistry **27:**2614–2623.
11. **Smith, D. W. E., and B. N. Ames.** 1964. Intermediates in the early steps of histidine biosynthesis. J. Biol. Chem. **239:**1848–1855.
12. **White, R. H.** 1996. Biosynthesis of methanopterin. Biochemistry **35:**3447–3456.
13. **White, R. H.** 1991. Distribution of folates and modified folates in extremely thermophilic bacteria. J. Bacteriol. **173:**1987–1991.
14. **Winkler, M. E.** 1996. Biosynthesis of histidine, p. 485–505. *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, 2nd ed., vol. 1. American Society for Microbiology, Washington, D.C.
15. **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, 2nd ed., vol. 1. American Society for Microbiology, Washington, D.C.