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Structures of the Modified Folates in the Extremely Thermophilic Archaebacterium *Thermococcus litoralis*

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The chemical structures of the two modified folates present in *Thermococcus litoralis* were established. These compounds, each containing a core structure of 1-[4-[[1-(2-amino-7-methyl-4-oxo-6-pteridinyl)-ethyl]amino]phenyl]-1-deoxy-[1-α-D-ribofuranosyl]-ribitol, were characterized. The five position of the ribose in this core structure was β-linked to the C-1 of a poly-β(1→4)*N*-acetylglucosamine having a chain length of four or five *N*-acetylglucosamine residues. Thus, these compounds are *N*-acetylglucosamine homologs of the modified folates found in *Pyrococcus furiosus*.

Recent work on the analysis of the modified folates present in thermophilic archaea has shown that species from both genera of the order *Thermococcales* (i.e., *Pyrococcus* and *Thermococcus*) contain different modified folates (5). Structural characterization of the modified folates in *Pyrococcus furiosus* show them to consist of the three similar structures shown in Fig. 1 (6). In this paper the structural characterization of the modified folates present in *Thermococcus litoralis* is described. These structures were found to be *N*-acetylglucosamine (GluNAc) homologs of the modified folates found in *P. furiosus*, supporting the close taxonomic association of these two different species as established by 16S rRNA sequence data (8).

The structural characterization of these compounds was accomplished by procedures analogous to those used for the characterization of the modified folates present in *P. furiosus* (6). These procedures consisted of the identification and structural characterization of the pterin and arylamine products resulting from the oxidative cleavage of the intact reduced modified folate present in the bacterial cells. Reductive cleavage of the oxidized intact cofactor to 6-ethyl-7-methylpterin resulted in the establishment of the C-9 methylation in the modified folate (4).

Frozen cells of *T. litoralis* NS-C (DSM 5473) were kindly supplied by Michael W. W. Adams, Department of Biochemistry and Center for Metalloenzyme Studies at the University of Georgia, Athens. One to five grams (wet weight) of these cells was extracted with 50% ethanol, and the resulting cell extracts, after oxidative cleavage (2), were separated by DEAE-Sephadex column chromatography as previously described (6). The arylamines in the separated DEAE column fractions were determined by a modification (8) of the Bratton and Marshall assay procedure (1) and showed one peak eluting at a position where noncharged molecules eluted from the column. This was the same position at which the arylamines resulting from the oxidative cleavage of the modified folates present in *P. furiosus* eluted. Pterins present in the column fractions were detected by fluorescence spectrometry (excitation maximum, 350 nm; emission maximum, 450 nm); one major pterin peak was detected and identified as 7-methylpterin (5).

The fractions eluted from the DEAE-Sephadex column containing the arylamines were combined and concentrated to 1 ml by evaporation with a stream of N_2. The resulting arylamines were converted into their azo dye derivatives and purified on a Bio-Gel P-6 column eluted with 50 mM HCl as previously described (6, 7). Two poorly resolved purple peaks, both eluting slightly earlier than any of the azo dye derivatives of the arylamines derived from *P. furiosus*, were observed. Fractions containing the respective peaks were combined, lyophilized, and redissolved in 100 mM citrate buffer (pH 4.0) for further analysis. Thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC) analyses of the isolated azo dye derivatives of the arylamines contained in these peaks were performed as previously described (7). The analytical results showed that the arylamine azo dye derivative contained in each of these column peaks was more polar than any of the arylamine azo dye derivatives derived from the modified folates present in *P. furiosus*. The azo dye derivative contained in the first peak of the Bio-Gel P-6 column had an R_f of 0.017 (TLC) and a retention time of 26.21 min (HPLC). The azo dye derivative contained in the second peak had an R_f of 0.033 (TLC) and a retention time of 26.49 min (HPLC). This is to be compared with an R_f of 0.038 (TLC) and a retention time of 26.80 min (HPLC) for the 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane-ribose-$(GluNAc)_3$ [*p*APT-ribose-$(GluNAc)_3$] derivative isolated from *P. furiosus* (7).

Quantitation of the amounts of these compounds present in the cell extracts, based on the amount of each azo dye derivative isolated from the Bio-Gel P-6 column, showed that the first and second peaks were present in amounts corresponding to 34 and 46 nmol/g (dry weight) of cells, respectively. These values are comparable to the amount of modified folates in *P. furiosus* (33.7 nmol/g [dry weight] of cells). These values are about 10 times higher than the amount of folate in eubacteria and 100 times less that the amount of methanopterin present in the methanogens (5).

The arylamine azo dye derivatives of the modified folates in *P. furiosus* and methaniline, the arylamine derived from methanopterin, as well as the arylamine azo dye derivatives of the modified folates isolated from *T. litoralis*, all readily underwent rapid and complete acid hydrolysis (0.1 M HCl, 100°C, 1 min) to produce the azo dye derivative of *p*APT. The identity of the *p*APT was confirmed by TLC and HPLC comparisons with the known compound (3, 7).

Treatment of either of the isolated azo dye derivatives with β-*N*-acetylglucosaminidase led to the rapid production

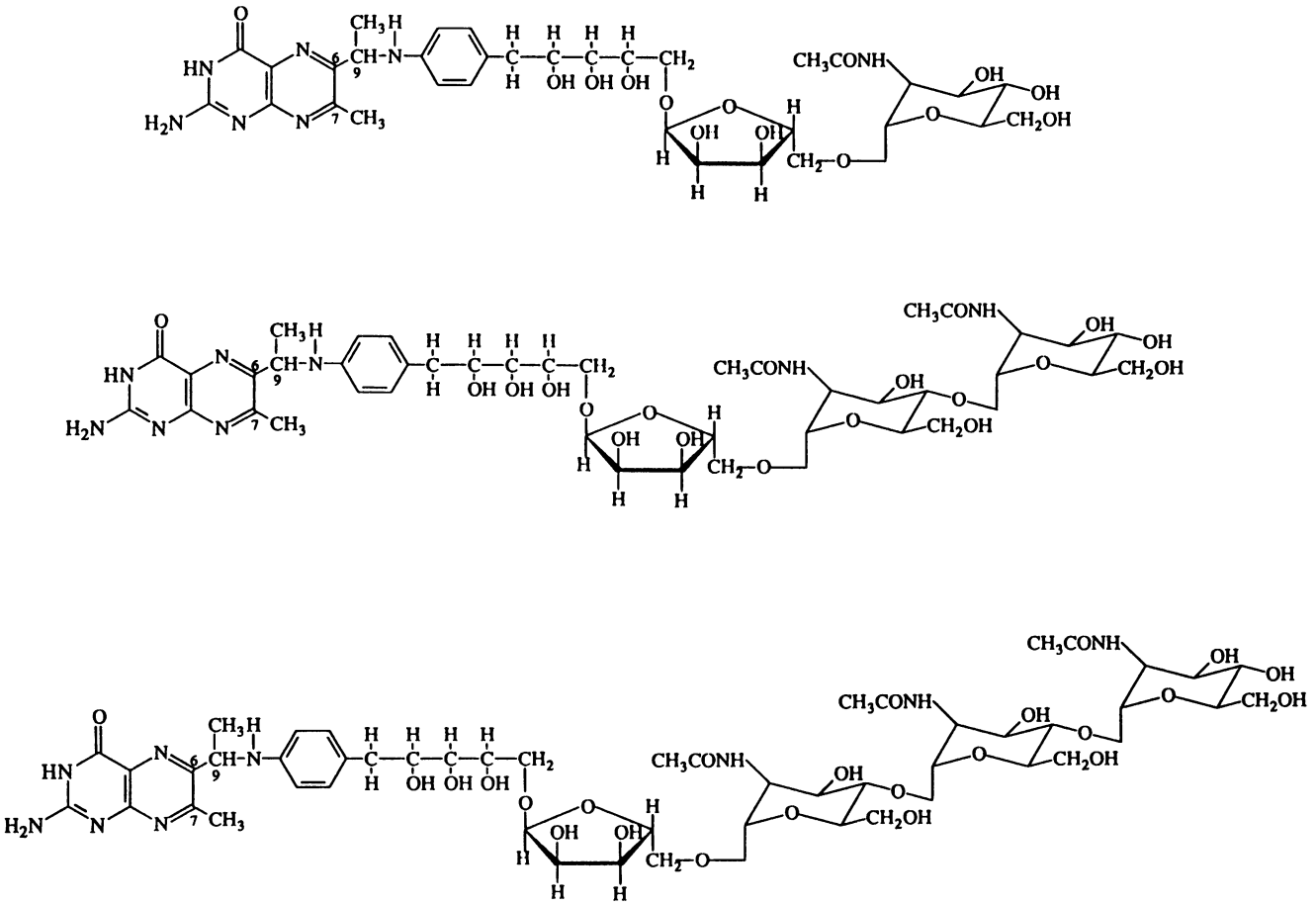

FIG. 1. Chemical structures of the modified folates present in *P. furiosus*. The compounds present in *T. litoralis* are homologs of these same compounds with poly-GluNAc side chains containing four or five GluNAc residues.

of a new compound which had the same R_f (TLC) and HPLC retention time as the azo dye derivative of *p*APT-ribose-GluNAc isolated from *P. furiosus*. This compound, in turn, was then slowly hydrolyzed to *p*APT-ribose as the enzymatic incubation progressed. By using a lower initial enzyme concentration, it was possible to observe, with HPLC, all of the poly-GluNAc intermediates as they were generated by the enzymatic hydrolysis of the substrate. This partial enzymatic hydrolysis of a sample also allowed for the unambiguous assignment of the number of GluNAc residues in the molecule from the HPLC chromatographic data (Fig. 2), which shows the clear separation of each of the intermediates.

The information above established the structures of the arylamines resulting from the oxidative cleavage of the reduced modified folates present in *T. litoralis* but gave no information as to the extent of methylation of the pterin. The detection of 7-methylpterin in the cell extracts supported the idea that the pterin in the intact modified folate was methylated at C-7. This observation, however, did not establish whether the C-9 was also methylated as is found in methanopterin. The presence of the C-9 methyl group in the structure was established by isolating the intact oxidized cofactor and demonstrating that it underwent reductive cleavage to 6-ethyl-7-methylpterin. This was accomplished by the reductive cleavage of the compounds present in the individual fractions from the DEAE-Sephadex column and determination of which one produced 6-ethyl-7-methylpterin. One peak, eluting at the position at which noncharged molecules eluted, was detected. Since this is the expected position of elution of the intact oxidized cofactor, it is assumed that the 6-ethyl-7-methylpterin arose from the modified folate present in these cells. Since 6-ethyl-7-methylpterin arises only from a 7,9-dimethylated modified folate (4), this result proves that the modified folate present in *T. litoralis* is methylated at both C-7 and C-9, as is methanopterin. (The identity of the 6-ethyl-7-methylpterin was confirmed by comparing it with samples of 6-ethyl-7-methylpterin produced both synthetically from the condensation of 6-hydroxy-2,3,5-triaminopyrimidine with 2,3-pentanedione and from the reductive cleavage of methanopterin by procedures previously described [4].)

From a comparison of the structures of the modified folates in *T. litoralis* with those of the presently characterized modified folates, it is clear that these structures are more closely related to methanopterin than to folate. In addition, the present data indicate that the modified folates in *Thermococcus celer* (6; unpublished results) are identical to the modified folates found in *T. litoralis*, indicating that all presently known members of the order *Thermococcales* contain GluNAc homologs of the modified folates found in *P. furiosus*.

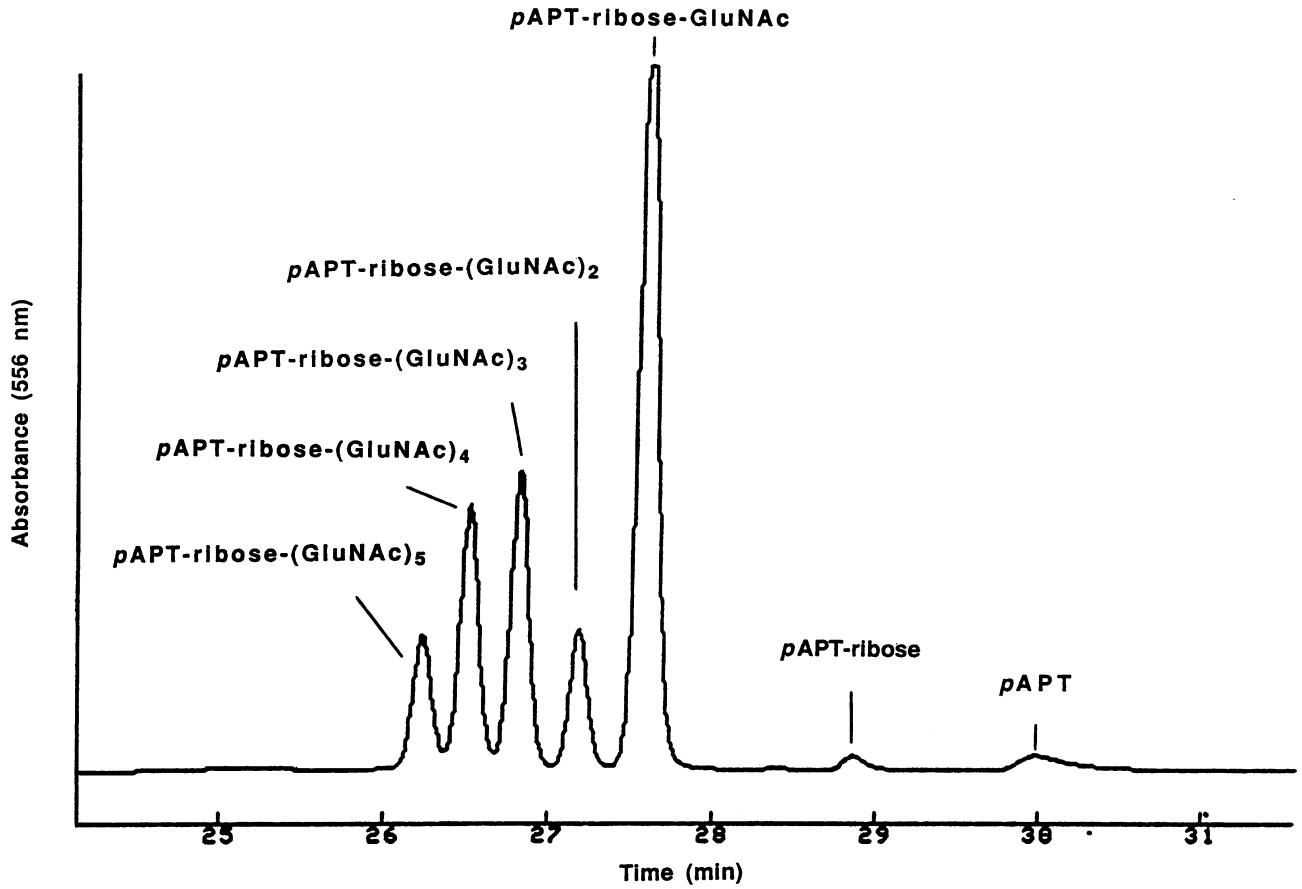

FIG. 2. HPLC elution of a sample of *p*APT-ribose-$(GluNAc)_5$ that has been partially digested with β-*N*-acetylglucosaminidase. The number of GluNAc residues in the original molecule can be readily determined by counting the number of intermediate peaks observed.

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