Articles

Structures of the Modified Folates in the Thermophilic Archaebacteria *Pyrococcus furiosus*†

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ABSTRACT: The structures of the modified folates present in *Pyrococcus furiosus* have been determined. This was accomplished largely by the characterization of the arylamines resulting from the air oxidative cleavage of the reduced modified folates present in these cells, using both chemical and enzymatic methods. Cell extracts separated on DEAE-Sephadex columns showed one major peak containing the arylamines derived from the modified folates. These arylamines were not retained on the DEAE-Sephadex columns, indicating that they contained no net negative charge. Purification of the azo dye derivatives of these arylamines on a Bio-Gel P-6 column showed the presence of three different compounds (compounds **1**, **2**, and **3**) in an average amount of 4.1, 7.6, and 22 nmol/g dry weight of cells, respectively. Each of these compounds readily underwent mild acid hydrolysis (0.1 M HCl, 110 °C, 1 min) to produce the azo dye derivative of 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane (*p*APT). The structure and stereochemistry (*ribo*) of the *p*APT was the same as the *p*APT present in methanopterin. In addition, compounds **1**, **2**, and **3** were each shown to contain 1 mol equiv of ribose and 1, 2, and 3 mol equiv of *N*-acetylglucosamine (gluNAc), respectively, and were designated as the azo dye derivatives of *p*APT-ribose-gluNAc, *p*APT-ribose-$(gluNAc)_2$, and *p*APT-ribose-$(gluNAc)_3$. Each of these compounds was readily cleaved to the azo dye derivative of *p*APT-ribose by the enzymatic action of β-*N*-acetylglucosaminidase, indicating that all the gluNAc residues were β-linked. The linkage between the ribose and the *p*APT in the *p*APT-ribose was assigned as $\alpha(1\rightarrow5)$ because of its identity with *p*APT-ribose isolated from methaniline (1-(4-aminophenyl)-1-deoxy-5-*O*-[5-*O*-[(1,3-dicarboxypropoxy)hydroxyphosphinyl]-α-D-ribofuranosyl]ribitol) after enzymatic cleavage with the phosphodiesterase I from *Crotalus atrox* venom. *p*APT-ribose-$(gluNAc)_3$ and *p*APT-ribose-$(gluNAc)_2$ were found to be readily cleaved by chitinase to *p*APT-ribose-gluNAc and *p*APT-ribose, whereas *p*APT-ribose-gluNAc was not cleaved by chitinase. These observations indicated that all linkages between gluNAc residues were $\beta(1\rightarrow4)$ and that the oxidized modified folates present in *P. furiosus* consist of three 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 linked to 1, 2, and 3 gluNAc. In each of these compounds the first gluNAc of the chain is β-linked to the 5-position of the ribose. Thus, the modified folates in *P. furiosus* are in the same family of modified folates as is methanopterin. For a coenzyme, the structures of these modified folates are very useful in that they contain no charged groups.

Folic acid, one of the central coenzymes found in biological systems, and its polyglutamyl derivatives (Figure 1) function as C_1 carriers in over twenty different biochemical reactions (Blakley & Benkovic, 1985). The core of the folate coenzyme structure was established in 1946 (Pfiffner et al., 1946; Angier et al., 1947); no modifications of this original structure were found until 1980 when the structurally-modified folate methanopterin (Figure 1) was identified (Van Beelen et al., 1984a,b). Methanopterin was isolated and characterized because of its involvement as a C_1 carrier in the reduction of CO_2 to CH_4 in methanogenic archaebacteria, where it functions in many ways analogous to folate (Rouviere & Wolfe, 1988). Subsequent work has indicated that methanopterin appears to completely replace folate in the methanogens which lack the normal folate coenzymes (Leigh, 1983; Worrell & Nagle, 1988). This substitution of methanopterin for folic acid in a given organism has been verified at the enzyme level by the direct measurement of specificity of several enzymatic reactions involved in C_1 metabolism. Enzymes involved in C_1 metabolism isolated from the methanogens use methanopterin instead of folate as a coenzyme, whereas the opposite is true for enzymes isolated from eubacteria. Thus, tetrahydromethanopterin, not tetrahydrofolic acid, functions as the C_1 carrier for serine hydroxymethyltransferase (Hoyt et al., 1986), 5,-10-methylenetetrahydromethanopterin dehydrogenase (Hartzell et al., 1985), and 5,10-methenyltetrahydromethanopterin cyclohydrolase (Donnelly et al., 1985; DiMarco et al., 1986). This observation of folate replacement by methanopterin in the C_1 metabolism of cells, presumably also extends to the replacement of folate by sarcinapterin, tatiopterin, and thermopterin, three recently described structural analogs of methanopterin (Figure 1) that have been found in some methanogens (Raemakers-Franken et al., 1989, 1991). These substitutions of structurally-modified coenzymes for any given coenzyme are unprecedented, since the structures of coenzymes utilized in specific biochemical reactions have all been found to be the same regardless of their origin. The discovery of

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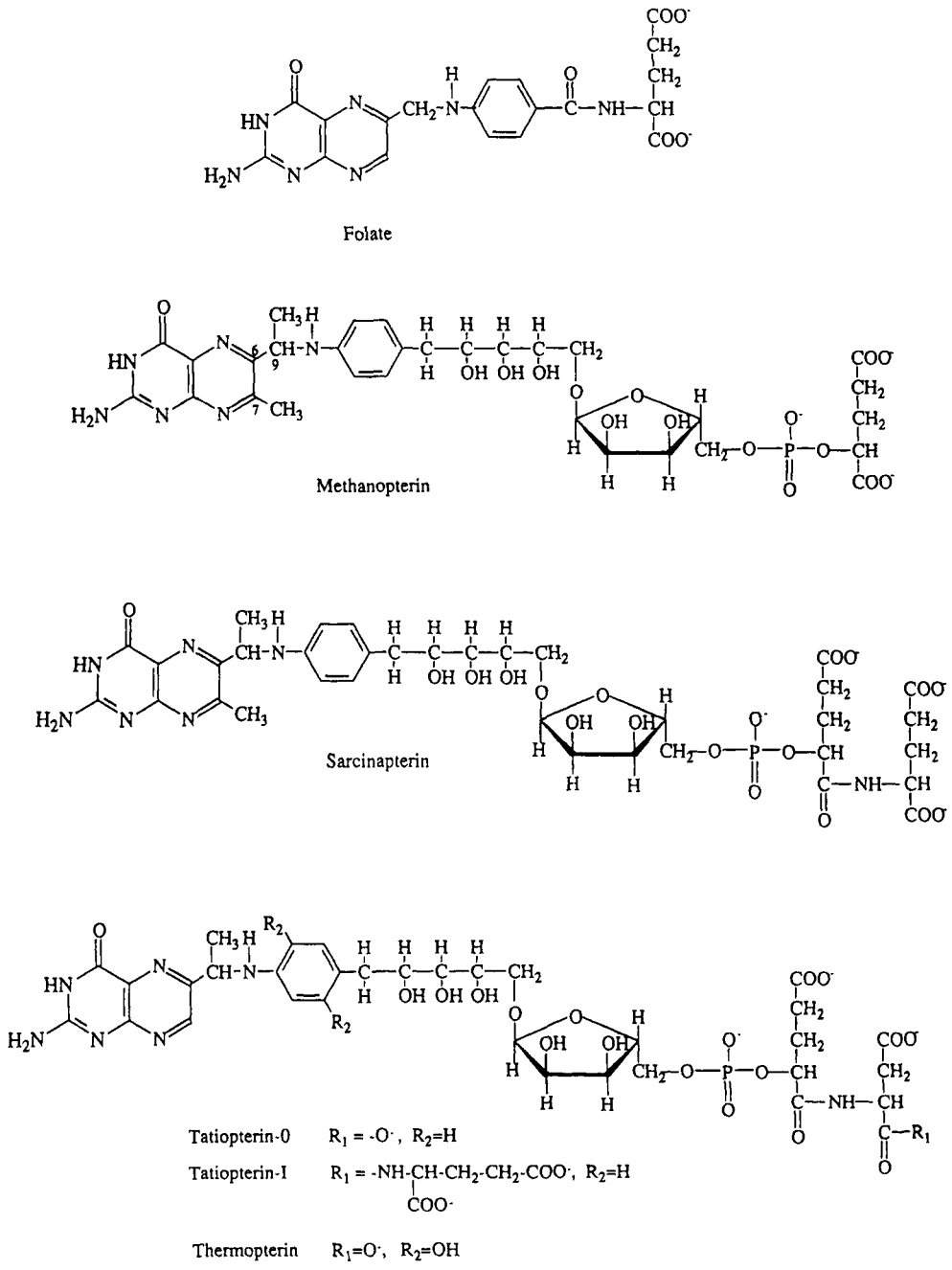

FIGURE 1: Chemical structures of folate and the presently characterized modified folates.

these modified folates in the methanogenic archaebacteria prompted an investigation to determine if other archaebacteria contained other uniquely-modified folates.

To date, my investigation has revealed that three members of the thermophilic archaebacteria, *Pyrococcus furiosus*, *Thermococcus celer*, and *Sulfolobus solfataricus*, each contained a new and uniquely-modified folate (White, 1991). In the case of *P. furiosus* and *T. celer*, these modifications consist, in part, of the methylation of the C-7 and C-9 carbons of the pterin as is found in methanopterin (Figure 1). The modified folate present in *S. solfataricus*, however, was found to contain the normal C-7 and C-9 nonmethylated pterin as is found in folate (White, 1991) and the *p*APT as is found in methanopterin (Zhou & White, 1992). The major difference, however, among each of these modified folates and any of the presently known modified folates was found to be the chemical structure of the arylamine side chain (White, 1991).

In this paper, the first step toward exploring the biochemistry of these modified folates was taken by characterizing the structures of the modified folates in *P. furiosus*, an extreme thermophilic bacteria with a growth temperature optimum of 100 °C (Fiala & Stetter, 1986), using both chemical and enzymatic methods. This was accomplished by the characterization of the arylamine resulting from the air oxidative cleavage of the reduced modified folates present in these cells. This information along with earlier work establishing that the modified folate in *P. furiosus* contains a 7,9-dimethylated pterin, as is found in methanopterin (White, 1991), was used to determine the complete structure.

MATERIALS AND METHODS

Materials. [1-^{2}H]-D-Ribose was obtained from Cambridge Isotope Laboratories, Woburn, MA. *N,N'*-Diacetylchitobiose, *N,N',N''*-triacetylchitotriose, chitinase from *Streptomyces griseus* (200–600 units/g of solid), and *β-N*-acetylglucosaminidase from *Aspergillus niger* (30 units/mg of protein) were all obtained from Sigma Chemical Co. Phosphodiesterase I from *Crotalus atrox* venom (46 units/mg of solid)

was obtained from Pharmacia, Molecular Biology Division, Piscataway, NJ.

Synthesis of 1-(p-Aminobenzyl)-2,3-dihydroxytetrahydrofuran. [*N*,*N*-Bis(trimethylsilyl)-*p*-anilinyl]lithium was coupled with 2,5-anhydro-3,4-*O*-isopropylidene-D-ribose (Defaye & Reyners, 1968) to form the isopropylidene derivative of 1-(*p*-aminophenyl)-1-(2,3-dihydroxytetrahydrofuranyl)-methanol, which was subsequently reduced with $LiAlH_4$ and hydrolyzed to 1-(*p*-aminobenzyl)-2,3-dihydroxytetrahydrofuran by the same series of reactions used for the preparation of the different stereochemical isomers of 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane (White, 1986).

Bacterial Strains and Growth Conditions. Frozen cells of *P. furiosus* (DSM 3638) were supplied by Dr. Michael W. W. Adams, Department of Biochemistry and Center for Metalloenzyme Studies at the University of Georgia, Athens, GA. These cells were grown using maltose as a carbon source as previously described (Bryant & Adams, 1989).

Extraction and Initial Purification of the Arylamines. During the course of this work, cells of *P. furiosus* were extracted by two different procedures. In early experiments, 5–10 g wet weight of cells was extracted with 50% ethanol, and the resulting cell extracts were separated by DEAE-Sephadex chromatography as previously described (White, 1991). The arylamines in the separated column fractions were determined using a modification (Zhou & White, 1992) of the Bratton and Marshall assay procedure (Bratton & Marshall, 1939). A typical chromatographic elution is shown in Figure 2. After it was established that the arylamines were retained on Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA) and subsequently eluted with methanol, the isolation procedure was modified in the following manner. Frozen cells of *P. furiosus* (~20 g) were suspended in 100 mL of water, heated at 100 °C for 30 min, and centrifuged at 15000*g* for 20 min. After removal of the clear, dark brown liquid, the resulting pellet was again extracted with 75 mL of water using this same procedure. The combined extracts were stirred for 4 h at room temperature in a 1-L beaker, the pH was adjusted from ~4.9 to 5.8 with 1 M NaOH, and the resulting solution was concentrated in vacuo to a volume which was approximately the same as that of the original cell mass. The resulting thick brown liquid was passed through Sep-Pak C_{18} cartridges (5 mL per cartridge), and each cartridge was then washed with water (2 mL) and eluted with 66% methanol (3 mL).

Preparation and Purification of the Azo Dye Derivative of the Arylamines on a Bio-Gel P-6 Column. The arylamines contained in the above methanol elutions from the Sep-Pak C_{18} cartridges were recovered by evaporation of the solvent with a stream of nitrogen gas, placed in 500 μL of water, and converted into azo dye derivatives as previously described (Zhou & White, 1992). After 1–2 h the purplish reaction mixture was applied to a Bio-Gel P-6 column (1.5 × 26 cm) previously equilibrated with 50 mM HCl, and the azo dye derivatives were eluted with 50 mM HCl. Three purple bands eluting maximally as peaks centered at 66, 108, and 126 mL were observed. Fractions containing these individual bands were combined and lyophilized. After being redissolved in 1 mL of 50 mM HCl, each sample was then rechromatographed on the original column using the same procedure. Samples purified in this manner were chromatographically pure as determined by thin-layer chromatography (TLC) (visual inspection of the purple spots) and HPLC (A_{556}). Each of the azo dye derivatives had an absorbance maximum at 556 nm in 50 mM HCl. These samples were used for the enzymatic cleavage reactions discussed below. Samples used for chemical analyses were further purified using gravity flow on a reverse-phase C_{18} column (7 × 300 mm), eluted with a linear gradient generated from 50 mL of aqueous 1% trifluoroacetic acid and 50 mL of 1% trifluoroacetic acid in 50% acetonitrile. For the quantitation of the azo dyes of *p*APT and its derivatives, a molar extinction coefficient of 4.8×10^4 cm^{-1} M^{-1} was used.

TLC Analysis of Azo Dye Derivatives of the Arylamines. The azo dye derivatives of the different arylamines were identified by TLC analysis on silica gel 60 F-254 TLC plates (E. Merck, Darmstadt, West Germany) using acetonitrile–water–formic acid (88%) (40:10:5 v/v/v) as the developing solvent. With this solvent, the following azo dye derivatives had the indicated R_f's: *p*-aminophenylacetic acid, 0.67; *p*-aminobenzoic acid, 0.65; 1-(*p*-aminobenzyl)-2,3-dihydroxytetrahydrofuran, 0.62; *p*APT, 0.57; *p*APT-ribose, 0.41; *p*APT-ribose-gluNAc, 0.17; *p*APT-ribose-$(gluNAc)_2$, 0.10; and *p*APT-ribose-$(gluNAc)_3$, 0.038. The purple color of the azo dyes was most intense immediately after evaporation of the solvent from the TLC plate, and it completely faded from the plate after a few hours. This fading of the TLC spots was prevented by placing the TLC plates in a chamber containing HCl vapors.

HPLC Analysis of the Azo Dye Derivatives. The azo dye derivatives of the arylamines were assayed by reverse-phase chromatography using two different HPLC systems and columns. One system consisted of a Du Pont Instrument 8800 series gradient liquid chromatographic system fitted with a Whatman Partisil PX5 10/25 ODS column. The azo dyes were eluted with a linear gradient generated from 50% methanol containing 1% trifluoroacetic acid and methanol containing 1% trifluoroacetic acid, over a 20-min period at a flow rate of 1 mL/min. Under these conditions, the azo dye derivatives of methaniline, *p*APT-ribose, and *p*APT eluted at 5.16, 5.78, and 6.01 min, respectively. The other system consisted of a Shimadzu liquid chromatography system (Model LC-6A) mounted with an Axxiom octadecylsilane column (5 μm, 4.6 × 250 mm) and equipped with a Shimadzu SPD 6AV UV–visible spectrometer detector. The azo dyes were eluted using a gradient generated with 0.1% aqueous trifluoroacetic acid as solvent A and 0.075% trifluoroacetic acid in acetonitrile as solvent B. The gradient was linear from 3.5% B to 45% B over a 40-min period, and the flow rate was 1 mL/min. Elution was monitored by the absorbance at 556 nm. Under these conditions, the azo dye derivatives of *p*APT-ribose-$(gluNAc)_3$, *p*APT-ribose-$(gluNAc)_2$, *p*APT-ribose-gluNAc, *p*APT-ribose, and *p*APT eluted at 26.74, 27.46, 28.08, 29.44, and 30.60 min, respectively.

Recovery of the Arylamines from their Azo Dye Derivatives and Subsequent Gas Chromatography–Mass Spectrometry (GC–MS) Analysis. The azo dye derivative of either 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane or 1-(*p*-aminobenzyl)-2,3-dihydroxytetrahydrofuran was reduced with Zn/HCl and the resulting arylamine was isolated from the reduction mixture as previously described (Zhou & White, 1992). The arylamine was then converted into its trifluoroacetyl derivative and assayed by GC–MS as previously described (White, 1986). Since both of these arylamines eluted close to the *n*-butyl trifluoroacetyl derivative of glutamic acid, this glutamic acid derivative was used as a internal standard in order to accurately establish relative GC retention times.

GC–MS Identification and Quantitation of the Sugars Present in the Azo Dye Derivatives of the Arylamines. Ribose and glucosamine present in the individually purified arylamines were identified and quantiated by GC–MS analysis of their

methyl glycoside trifluoroacetyl derivatives. These sugars were identified by direct comparison of their gas chromatographic retention times and mass spectra to those of known sugars. These assignments were further strengthened by comparing the GC patterns of the α- and β-pyranoside and furanoside derivatives formed from each of these sugars with that formed from known sugars. The quantitation of the ribose was achieved by organic isotope dilution analysis using [1-^{2}H]-ribose as an internal standard (Caproli, 1972). The glucosamine was quantitated by comparing the ratios of the intensities of specific ions in the [1-^{2}H]ribose derivative to the intensities of specific ions of the glucosamine derivative, with the same ratios observed in known samples containing a known ratio of [1-^{2}H]ribose to glucosamine.

Thus, to solutions of the individually purified azo dye derivatives of the arylamines was added an equivalent amount of [1-^{2}H]ribose. The amount of [1-^{2}H]ribose used was typically from 10 to 40 nmol and was calculated from the A_{556} and volumes of the individual solutions used. The resulting solutions were then made 1 M in HCl by the addition of 6 M HCl, heated under nitrogen for 2 h at 100 °C, and evaporated to dryness with a stream of nitrogen. The sugars present in the resulting residue were converted into the methyl glycosides by heating at 70 °C for 5 h in 500 μL of 3 M HCl in methanol. After evaporation of the solvent from the samples, the resulting methyl glycosides were reacted overnight at room temperature with 200 μL of a equal mixture of trifluoroacetic anhydride and methylene chloride. Upon evaporation of the solvents, the resulting residue was dissolved in methylene chloride for GC–MS analysis. The sugar derivatives were separated on a HP-5 column (25 m × 0.32 mm × 0.17 μm film thickness) programmed from 60 °C to 200 °C at a constant rate of 10 °C/min.

Ions in the mass spectrum of the [1-^{2}H]ribose derivative which were suitable for quantitation of the ribose (i.e., those ions retaining all of the C-1 deuterium) were the $M^+ - CH_3OH$ ion at m/z 422, the $M^+ - CH_3OH - H_2O$ ion at m/z 404, the $M^+ - CF_3COO$ ion at m/z 340, and the m/z 194 ion. The analytical results obtained from all of these ions were similar. These ions, along with the m/z 176 and m/z 404 ions of methyl β-glycoside of the glucosamine, were used for the analysis of the glucosamine.

β-N-Acetylglucosaminidase Cleavage of the Azo Dye Derivatives of pAPT-ribose-(gluNAc)$_x$. Lyophilized samples of the individual azo dye derivatives (10–20 nmol) purified on the Bio-Gel P-6 column were dissolved in 1–2 mL of 100 mM citrate buffer (pH 4.0). To these samples was added β-*N*-acetylglucosaminidase (0.04–0.4 unit) dissolved in the same citrate buffer. (Samples of the enzyme were prepared by filtering the ammonium sulfate suspension of the enzyme and dissolving the collected protein in the citrate buffer.) At timed intervals, aliquots of the enzymatically-digested samples were assayed by TLC and HPLC to identify the products.

Phosphodiesterase I Treatment of Methaniline. Methaniline (10–20 nmol) obtained from bacterial cells (rumen isolate 10-16B) (Lovley et al., 1984; White, 1985b) by extraction and chromatography of the extracts on DEAE-Sephadex as previously described (White, 1990) was dissolved in 200 μL of 0.1 M glycine buffer (pH 8.8). To this solution was added ~1 unit of phosphodiesterase I from *Crotalus atrox*. The sample was then incubated for 12 h at 37 °C and the resulting *pAPT*-ribose converted into the Bratton and Marshall azo dye derivative as previously described (Zhou & White, 1992). The reaction mixture was separated on a small column of Bio-Gel P-6 and eluted with 50 mM HCl to recover the azo dye derivative of *p*APT-ribose. This sample was then used as a TLC and HPLC reference sample.

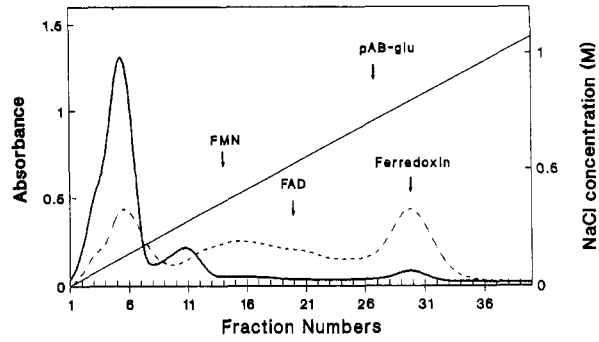

FIGURE 2: Elution profile of a cell extract of *P. furiosus* separated on DEAE-Sephadex using a NaCl gradient. The dashed line is the absorbance of the fractions at 342 nm. The solid line is the absorbance at 566 nm resulting from the Bratton and Marshall assay. The straight solid line is the concentration of NaCl in the eluted fractions. The compounds present in peaks 11 and 30 that were responsible for the color development in the Bratton and Marshall assay were isolated and identified as tryptophan and indole-3-acetic acid, respectively.

Chitinase Cleavage of the Azo Dye Derivatives of pAPT-ribose-(gluNAc)$_x$. Lyophilized samples of the individual azo dye derivatives (10–20 nmol) purified on the Bio-Gel P-6 column were dissolved in 1–2 mL of 50 mM phosphate buffer (pH 6.0). The samples were then concentrated by evaporation with a stream of nitrogen gas to 200 μL. To 30 μL of these samples was added 2 μL of a solution of chitinase containing about 0.02 unit of activity. The resulting solutions were then incubated at room temperature and the products were assayed by TLC at 5-, 15-, 30-, and 60-min timed intervals.

Periodate Cleavage. The azo dye derivative of *p*APT-ribose-gluNAc (9 nmol) obtained by chitinase cleavage of the azo dye derivative of *p*APT-ribose-(gluNAc)$_3$ was dissolved in 50 μL of water, and 40 μL of 500 mM sodium periodate was added. After 1 h at 25 °C, 10 μL of ethylene glycol was added, followed after 30 min by the addition of 9 nmol of [1-^{2}H]ribose. The sample was then hydrolyzed and analyzed for ribose by GC–MS as described above.

Generation and Analysis by Reductive Cleavage of the Intact Oxidized Modified Folate. Frozen cells of *P. furiosus* (2.5 g) were suspended in 7 mL of 0.05 M phosphate buffer (pH 5.8) containing 0.58 mmol of potassium ferricyanide, and the suspension was adjusted to pH ~7, heated at 100 °C for 15 min, and centrifuged at 15000*g* for 20 min. The clear supernate was passed through a Sep-Pak C_{18} cartridge, which, after being washed with water, was eluted with methanol. After evaporation of the methanol, the intact ferricyanide-oxidized modified folates present in the samples were reduced with Zn/HCl and the resulting pterins were isolated as previously described (Zhou & White, 1992). The pterins in these samples were then further purified by chromatography on a Sephadex G-25 column (1.0 × 26 cm) using water as the eluting solvent (Fukushima & Nixon, 1980). Fractions corresponding to the elution positions of the expected pterins, that is, 6-methylpterin, 6,7-dimethylpterin, and 6-ethyl-7-methylpterin, were each combined and analyzed by both TLC and HPLC to establish the identity of the pterin (White, 1991).

RESULTS

Extraction and Initial Purification of the Arylamines. Separation of ethanol–water extracts of *P. furiosus* by DEAE-Sephadex chromatography followed by subsequent analyses of the fractions gave the results shown in Figure 2. Three peaks containing Bratton and Marshall positive compounds

were detected. The azo dye derivatives of the Bratton and Marshall positive compounds present in the first and major peak eluted in the same positions from the Bio-Gel P-6 column as the azo dye derivatives prepared from the arylamines isolated from cell extracts using Sep-Pak C_{18} cartridges. In each case, three purple bands eluting maximally at fractions 14, 18, and 22 containing, respectively, 22, 7.6, and 4.1 nmol of azo dye/g dry weight of extracted cells were observed. Their identities were confirmed by their cochromatography using both TLC and HPLC. [The compounds present in peaks 11 and 30 that were responsible for the color development in the Bratton and Marshall assay were isolated and identified as tryptophan and indole-3-acetic acid, respectively. The generation of a Bratton and Marshall color reaction from these compounds was not expected, but, on the basis of their structures, they could not be the arylamines derived from the C_1 carrier.] That the Bratton and Marshall assay generated by peak 30 did not result in part from the ferredoxin contained in this peak was confirmed by the lack of the formation of color when a pure sample of ferredoxin was assayed.)

Chromatographic Characteristics of the Native Arylamines. The Bratton and Marshall positive compounds present in the major peak shown in Figure 2 were not retained on a Dowex 50W-X8 H^+ column or an SP-Sephadex column at neutral pH. The compounds were, however, retained on an SP-Sephadex column equilibrated with 50 mM formic acid and were subsequently eluted as a broad band by a NaCl gradient. At neutral pH the arylamines were found to be retained on a Sep-Pak C_{18} cartridge and be eluted with methanol. Preparative TLC of these arylamines using the acetonitrile solvent system showed that the major Bratton and Marshall assayable compound had an R_f of ~0.2. HPLC separation of these arylamines on a Axxiom octadecylsilane column using 0.1% trifluoroacetic acid as the eluting solvent, however, showed an area of Bratton and Marshall positive material eluting just after the solvent peak.

Hydrolysis Products of the Native Arylamines. Strong acid hydrolysis (6 M HCl, 6 h, 100 °C) of the purified arylamines obtained from the major peak of the DEAE-Sephadex column gave one major peak with an R_f, on TLC, between 0.53 and 0.6. The synthetic sample of the 1-(*p*-aminobenzyl)-2,3-dihydroxytetrahydrofuran displayed the same TLC chromatographic characteristics. Final confirmation that the compound isolated from the acid hydrolysis was 1-(*p*-aminobenzyl)-2,3-dihydroxytetrahydrofuran was established by the identical GC–MS data for the pentatrifluoroacetyl derivatives of both the unknown and known compounds.

Hydrolysis Products of the Azo Dye Derivatives of the Arylamines. Each of the isolated azo dye derivatives readily underwent mild acid hydrolysis (100 mM HCl, 100 °C, 1 min) to produce the azo dye derivative of *p*APT. The identity of this compound was confirmed by its cochromatography on TLC and HPLC with the azo dye derivative of 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane isolated from methanopterin. The stereochemistry of the polyol side chain of the *p*APT was found to be *ribo* by gas chromatography of the trifluoroacetyl derivative as previously described (White, 1992).

GC–MS Identification and Quantitation of the Sugars Present in the Azo Dye Derivatives of the Arylamines. Using isotope dilution analysis GC–MS with [1-^{2}H]ribose as an internal standard, it was shown that each of the azo dye derivatives isolated from the Bio-Gel P-6 column released 1 mol of ribose upon acid hydrolysis. In addition, from the ratios of the ribose derivative peak to the gluNAc derivative peak, it was possible to establish that peaks 1, 2, and 3 contained 1, 2, and 3 glucosamines, respectively.

Enzymatic Cleavages of the Azo Dye Derivatives. Each of the isolated azo dye derivatives were readily cleaved to the azo dye derivative of *p*APT-ribose by the action of β-*N*-acetylglucosaminidase. The $\beta(1\rightarrow4)$ linkage of the gluNAc was confirmed by the hydrolysis of the samples with chitinase, an enzyme specific for $\beta(1\rightarrow4)$-linked gluNAc. An authentic sample of *p*APT-ribose was obtained by the cleavage of methanopterin with phosphodiesterase I.

Periodate Cleavage. The ribose present in each of the azo dye derivatives was found to be rapidly and quantitatively destroyed by incubation of the sample with periodate.

Generation and Analysis by Reductive Cleavage of the Intact Oxidized Modified Folate. By using ferricyanide to oxidize the intact reduced cofactor, during its extraction from the cells, it was possible to isolate a small amount of the intact oxidized cofactor. The pterin in this intact oxidized cofactor was determined to be methylated at C-7 and C-9 by its reductive cleavage with Zn/HCl to 6-ethyl-7-methylpterin.

DISCUSSION

Assuming that the reduced modified folates present in *P. furiosus* behave chemically in a manner analogous to H_4-folate and H_4-methanopterin, one can envision three approaches that could be used to establish their chemical structures. These include the isolation and structural characterization of (a) the intact reduced cofactors, (b) the intact oxidized cofactors, and (c) the air oxidative cleavage fragments of the cofactors. This last method requires that the original structure be deduced from the structures of the products resulting from the oxidative cleavage of the reduced cofactor, a process studied in the case of both H_4-folate (Reed & Archer, 1980) and H_4-methanopterin (Keltjens et al., 1984; White, 1985b).

Because of experimental difficulties required for the isolation and purification of the oxygen-sensitive reduced modified folates, as well as a demonstrated very low recovery of the intact air oxidized cofactors from the thermophilic bacteria (Zhou & White, 1992), the structure of the modified folate in *P. furiosus* was determined by the structural characterization of the air oxidative cleavage products (White, 1985b). This approach has been successfully applied to the partial structural characterization of the modified folate in *S. solfataricus* (Zhou & White, 1992). Work on the characterization of the pterin portion of the modified folate in *P. furiosus* had already established the presence of a dimethylated pterin like that found in methanopterin (White, 1991). Thus, the complete structure could be established by the isolation and structural characterization of the arylamines which result from the air oxidative cleavage of the reduced cofactors.

Each work on the separation of the air oxidative cleavage products of the 6-substituted tetrahydropterins in the cell extracts of *P. furiosus* consisted of eluting the products from a DEAE-Sephadex column with an ammonium bicarbonate gradient (White, 1991). This particular separation system was chosen because it gave a good separation of methaniline, the arylamine derived from methanopterin, from 7-methylpterin. Analysis of the fractions from these DEAE-Sephadex columns for the presence of arylamines, by the Bratton and Marshall assay, showed the presence of one major peak consisting of compounds not retained by the DEAE column, followed by four minor peaks. None of these peaks eluted at positions of any of the known arylamines (e.g., methaniline,

p-aminobenzoylglutamate, etc.) resulting from the oxidative cleavage of any of the known folates or modified folates. One problem with these early column separations was that none of the different *p*-aminobenzoylpolyglutamates [*p*AB(glu)$_x$] was eluted from the column with the ammonium bicarbonate concentrations used. However, by eluting the DEAE-Sephadex columns with a sodium chloride gradient at pH 5.8, the different *p*AB(glu)$_x$ could be separated and detected. Analysis of these sodium chloride-eluted fractions for the presence of arylamines using the Bratton and Marshall assay procedure showed three peaks (Figure 2), none of which corresponded to the elution position of any of the arylamines resulting from the air oxidative cleavage of H_4-folate or any of the known modified H_4-folates. These results indicated that the early eluting peak from both the ammonium bicarbonate- and sodium chloride-eluted DEAE-Sephadex columns contained the arylamine from *P. furiosus* and that this arylamine had a structure different from that present in any of the previously-described modified folates.

Calculation of the amount of Bratton and Marshall positive material present in this early eluting peak (Figure 2) showed it to contain 20 nmol/g wet cell weight. This amount of Bratton and Marshall positive material was 5–10 times more than that in any of the other Bratton–Marshall positive peaks and was close to the amount of 7-methylpterin (8 nmol/g dry cell weight) previously reported to occur in cell extracts of *P. furiosus* (White, 1991). [The observation that the amount of pterin in the cell extracts was much less than the amount of arylamine is consistent with the low yields of pterin produced during the air oxidation of H_4-folate (Reed & Archer, 1980).] The amount of the arylamines assayed in this peak as well as the amount of 7-methylpterin present in cell extracts was also found to decrease when cells of *P. furiosus* were extracted in the presence of 0.1 M mercaptoethanol, a molecule known to prevent the oxidative cleavage of H_4-folate (Zakrzewski, 1966). These results further supported the idea that the Bratton and Marshall positive material present in the peak centered at fraction 5 was the arylamine(s) produced during the oxidative cleavage of the modified folate in these cells.

As a first step in determining the structure of these arylamines, information on the number of charges on these molecules was obtained. Two experimental observations indicated that the arylamines contained no net charge. First, the elution position of the Bratton and Marshall positive compounds in the peak centered at fraction 5 of the DEAE-Sephadex column was the same as the elution position of noncharged molecules from this column. Second, the Bratton and Marshall positive compounds present in this fraction were not retained on a Dowex 50W-X8 H^+ column or an SP-Sephadex column at neutral pH. Separation of the compounds present in fraction 5 on a SP-Sephadex column in 50 mM formic acid eluted with a salt gradient, however, showed a broad peak eluting at about 0.12 M NaCl, indicating that at the acidic pH of this column (ca. 2.5) the arylamines were cationic. One explanation for the origin of this positive charge would be the protonation of the arylamine nitrogen. At neutral pH the arylamines were found to be retained on a Sep-Pak C_{18} cartridge and eluted with methanol. Preparative TLC purification of these arylamines using the acetonitrile solvent system showed that the major Bratton and Marshall assayable compound had an R_f of ~0.2. HPLC separation of the compound on a Axxiom octadecylsilane column using 0.1% trifluoroacetic acid as the eluting solvent, however, showed an area of Bratton and Marshall positive material eluting just after the solvent peak. This change in the elution positive with the change in pH of the column can be attributed to the protonation of the arylamine when applied to the acidified C_{18} HPLC column, which greatly reduced the arylamine's affinity for this column.

The arylamine was determined to be an alkyl-substituted aniline, as found in methaniline, rather than a *p*-aminobenzoic acid-containing compound as found in the folates, on the basis of the 556-nm absorbance maximum of its azo dye product in 50 mM HCl. This is the same absorbance maximum as that observed for the azo dye derivative of *p*APT and is close to the 557.5-nm absorbance maximum measured for the azo dye derivative of *p*-aminophenylacetic acid but is quite different from the absorbance maximum of 550.7 nm for the azo dye derivative of *N*-(*p*-aminobenzoyl)-L-glutamic acid. All of these observations are consistent with the arylamines found in *P. furiosus* consisting of an alkylaniline attached to noncharged molecules.

The arylamines isolated by DEAE-Sephadex, SP-Sephadex, and Sep-Pak C_{18} chromatography were found to elute in the same position as salts from a G-25 Sephadex column, indicating the molecules had molecular weights less than 1000. Acid hydrolysis (6 M HCl, 6 h, 100 °C) of the material purified using the above chromatographic steps, followed by preparative TLC purification of the resulting products, showed only one area of the TLC plate, between R_f 0.53 and R_f 0.60, that contained compounds giving a Bratton and Marshall color reaction. Unlike the native arylamines, which were not retained by a Dowex 50W-X8 H^+ column, the arylamine recovered from acid hydrolysis was readily retained by a Dowex 50W-X8 H^+ column and eluted with aqueous ammonia. GC–MS of the trifluoroacetyl derivative of this material showed only one GC peak containing a m/z 202 fragment ion ($[CF_3CONHC_6H_4CH_2]^+$), which is characteristic of alkylanilines. The highest mass in the 70-eV spectrum of this peak was at m/z 497, indicating that the trifluoroacetyl derivative of the molecule had a molecular weight of 497. This was confirmed by the chemical ionization mass spectrum of the molecule which showed an MH^+ ion at m/z 498. This molecular weight corresponds to a molecule in which a ditrifluoroacetyltetrahydrofuranyl moiety is added to the m/z 202 fragment ion. Thus, this molecule corresponds to the trifluoroacetyl derivative of 1-(*p*-aminobenzyl)-2,3-dihydroxytetrahydrofuran, a cyclic ether that could be derived from 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane (*p*APT), a compound already characterized as a component of the methanopterins (VanBeelen, 1984a,b; White, 1985a,b) and the modified folate in *S. solfataricus* (Zhou & White, 1992). The identity of the isolated compound as 1-(*p*-aminobenzyl)-2,3-dihydroxytetrahydrofuran was confirmed by comparing it to a known synthetic sample using TLC, GC–MS of their trifluoroacetyl derivatives, and Bio-Gel P-6 column chromatography. Since the synthetic sample, with the *ribo* stereochemistry, had the same GC retention time as the unknown sample, and since it is known that the different stereoisomers of *p*APT can be separated by the GC method used (White, 1986), then it is most likely that the furan isolated from the cells also had the *ribo* stereochemistry. This assignment of the *ribo* stereochemistry to the 1-(*p*-aminobenzyl)-2,3-dihydroxytetrahydrofuran isolated from the cells thus indicated that the unknown furan was likely derived from the naturally-occuring *ribo* isomer of *p*APT.

The above results indicated that the arylamine of *P. furiosus* contained 1-(*p*-aminobenzyl)-2,3-dihydroxytetrahydrofuran. However, because there were a large number and amount of impurities detected in the sample during GC–MS analysis of

1-(*p*-aminobenzyl)-2,3-dihydroxytetrahydrofuran, it was clear that a better procedure for the purification of the intact arylamine must be found before further structural characterization could be attempted. To this end the purification and analysis of the azo dye derivatives of the intact arylamines was undertaken.

Experiments designed to determine the best methods to prepare the maximum yield of the azo dyes of the arylamines indicated that this was best accomplished after the arylamines were first separated from crude cell extracts using the Sep-Pak C_{18} cartridges. Separation of the azo dye derivatives of the arylamines which were purified in this manner, on the Bio-Gel P-6 column, showed the presence of three purple bands eluting maximally at fractions 14, 18, and 21 which contained 22, 7.6, and 4.1 nmol of azo dye/g dry weight of extracted cells, respectively. [The azo dye derivatives of the arylamines contained in each of these peaks will be referred to as samples 3, 2, and 1, respectively.] In addition, a minor peak eluting at fraction 28 was sometimes observed. A small amount of this peak was always observed when any of samples 3, 2, or 1 were rechromatographed on the Bio-Gel P-6 column. This continuous generation of the compound eluting at fraction 28 was shown to be the result of partial acid hydrolysis that occurred during the concentration and rechromatography of the sample. The rapid rate of this hydrolysis (~30-s half-life in 100 mM HCl at 100 °C) was found to be the same as that observed for the acid hydrolysis of the azo dye derivative of methaniline or the azo dye derivative of the arylamine present in *S. solfataricus* (Zhou & White, 1992) and suggested the presence of an α-linked ribose in all of these compounds. In addition, since no intermediates were ever detected during the acid hydrolysis of these molecules, the α-linked ribose must be the most acid-labile bond in the molecules.

Analysis of the azo dye present in fraction 28 by TLC and chromatography on the Bio-Gel P-6 column showed it to be identical to the azo dye derivative of *p*APT derived from methanopterin. To confirm that this compound had the *ribo* stereochemistry, a portion of sample 3 was subjected to mild acid hydrolysis, and the resulting azo dye isolated by Bio-Gel P-6 chromatography was reduced with Zn/HCl (Zhou & White, 1992). The resulting *p*APT was shown, using GC–MS of the trifluoroacetyl derivative (White, 1986), to have the *ribo* stereochemistry, the same as the *p*APT found in methanopterin and the modified folate in *S. solfataricus* (Zhou & White, 1992).

These findings indicated that the 1-(*p*-aminophenyl)-2,3-dihydroxytetrahydrofuran isolated and characterized above was in fact an artifact resulting from the cyclization of the *p*APT present in these samples. The specific step at which this cyclization occurred was most likely at the acid hydrolysis step. This was confirmed by the quantitative conversion of a sample of *p*APT into 1-(*p*-aminophenyl)-2,3-dihydroxytetrahydrofuran by heating it in 6 M HCl at 100 °C for 1 h.

The presence of ribose in each of the three samples was confirmed by GC–MS analysis of the ribose as the methyl trifluoroacetyl derivative released by acid hydrolysis (1 M HCl, 100 °C, 4 h). Quantitation of the ribose using an internal standard of [1-^{2}H]ribose, established the presence of 1 ± 0.1 mol equiv of ribose in each of the samples. The GC–MS data also established that samples 1, 2, and 3 contained 1, 2, and 3 mol equiv of *N*-acetylglucosamine (gluNAc), respectively. [Since it was the methyl glycoside pentatrifluoroacetyl derivative of glucosamine that was assayed by GC–MS, deacetylation of the gluNAc must have occurred. This deacetylation would have occurred during the hydrolytic release of the sugars and/or during their subsequent derivatization (Chambers & Clamp, 1971). Additional evidence that the glucosamine in the original samples was in some way derivatized at the nitrogen also comes from the early observation that the original samples were not cationic.]

The above results indicated that each of these samples contained a common structural element of *p*APT-ribose as is found in methanopterin. Analysis of sample 2 for organic phosphate showed it to contain 0.06 mol equiv of phosphate, proving that a ribose-5-P unit as found in methanopterin was not present. The rapid release of ribose from the sample by acid hydrolysis also indicated that the sample did not have a ribose-5-P group which is resistant to acid hydrolysis. The absence of phosphate is also consistent with the lack of negative charge in the molecules.

Considering the structure of methanopterin and the absence of phosphate in these samples suggested that the gluNAc could be directly linked to the ribose. This was confirmed by demonstrating that the single compound produced by the treatment of each of the three peaks with β-*N*-acetylglucosaminidase was the same as that obtained from the enzymatic hydrolysis of methaniline with phosphodiesterase I. The identity of these enzymatically-derived products was established by their cochromatography on TLC, by HPLC analyses using two different columns, and by their mild acid hydrolysis to the azo dye derivative of *p*APT. Establishing the identity of these products also showed that the ribose in the modified folates in *P. furiosus* is α-glucosidically-linked to the C-5 of the *p*APT, because this is the linkage pattern in methaniline.

The cleavage of each of these samples to the common product, *p*APT-ribose, by β-*N*-acetylglucosaminidase also demonstrated not only that the gluNAc was β-linked to the ribose but that each gluNAc was also β-linked to each other, since the β-*N*-acetylglucosaminidase is specific for β-linked gluNAc (Barman, 1969). Thus, the most likely relationship between samples 1, 2, and 3 would be the successive addition of gluNAc to a core structure of *p*APT-ribose. This was demonstrated by following the time course of the enzymatic hydrolysis of samples 1, 2, and 3 with β-*N*-acetylglucosaminidase using HPLC. Sample 3 was converted into sample 2 and then sample 1, which was finally cleaved to the azo dye derivative of *p*APT-ribose. The slowest cleavage step observed was the removal of the final gluNAc. No additional intermediate compounds were observed.

Next to be considered was the position of linkage of the gluNAc to the ribose. Treatment of each of the samples with periodate completely destroyed the ribose as measured by isotope dilution mass spectrometry as described for the analysis of ribose in the samples. Since the ribose was known to be α-linked at C-1, then only if it was also linked at C-5, leaving a free diol at C-2 and C-3, would it have been cleaved by the periodate.

The $\beta(1\rightarrow4)$ linkage of the gluNAc was established by the pattern of cleavage of samples 1, 2, and 3 with chitinase [poly-β-1,4-(2-acetamido-2-deoxy)-D-glucoside glycanohydrolase], which is known to cleave chitin by the removal of gluNAc-$\beta(1\rightarrow4)$gluNAc from the sample (Berger & Reynolds, 1958). Thus, sample 3 [azo dye derivative of *p*APT-ribose-(gluNAc)$_3$] was readily cleaved to sample 1 [azo dye derivative of *p*APT-ribose-gluNAc], which, in turn, was not further cleaved by the enzyme. Sample 2 [azo dye derivative of *p*APT-ribose-(gluNAc)$_2$], on the other hand, was cleaved directly to *p*APT-ribose with no indication of the intermediacy of *p*APT-ribose-gluNAc. These observations are consistent with the removal of an *N*,*N*′-diacetylchitobiose unit, composed of the two

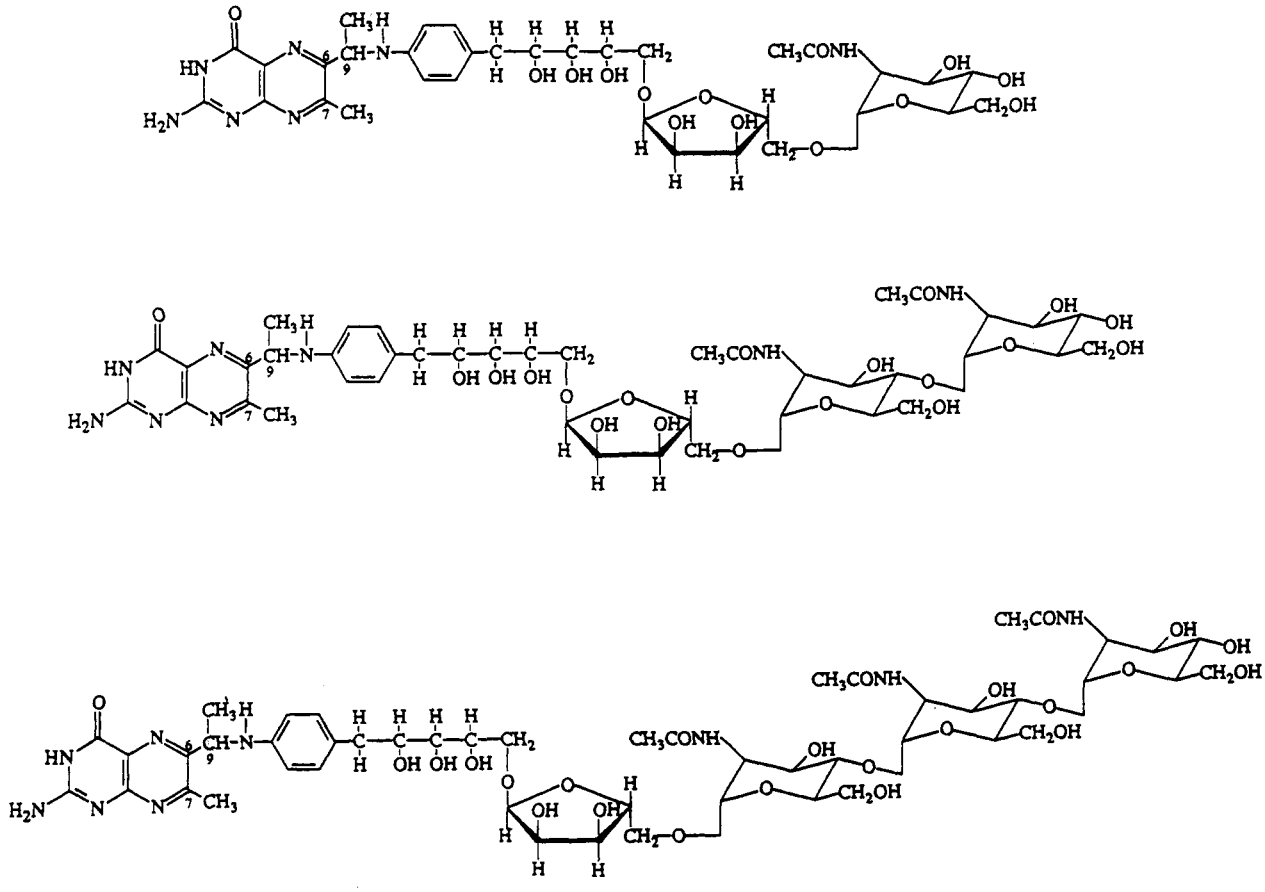

FIGURE 3: Chemical structures of the oxidized form of the modified folates which are present in *P. furiosus*. The structures are different from each other only by the number of *N*-acetylglucosamine residues present.

terminal gluNAc residues, from both samples 2 and 3. These results then confirm that the linkages between the gluNAc are all $\beta(1\rightarrow4)$.

Attempts to confirm the presence of $\beta(1\rightarrow4)$-linked $(gluNAc)_2$ in samples 2 and 3 by their mild acid hydrolysis and subsequent GC–MS analysis of the TMS derivative of the released *N,N'*-diacetylchitobiose (Coduti & Bush, 1977) were never successful. The major reason for the lack of success of this method appeared to be the low recovery of $(gluNAc)_2$ during the acid hydrolysis. A similar low recovery of *N,N'*-diacetylchitobiose was confirmed in the acid hydrolysis *N,N',N''*-triacetylchitobiose. This work did, however, confirm the presence of *N*-acetylglucosamine in samples 2 and 3.

Thus, the above information, together with the previously published data (White, 1991) showing that *P. furiosus* contained C-7 and C-9 dimethylated pterins as is found in methanopterin, indicated that the oxidized form of the modified folates in this organism are as shown in Figure 3. However, because of oxidative reactions, including oxidative cleavage, occurring to the original reduced modified folate, the amount of these structures in cell extracts would be expected to be very low. Indirect evidence for their existence can be obtained by their reductive cleavage with Zn/HCl and identification of the 6-ethyl-7-methylpterin produced (White, 1990). Performing this analysis on both cell extracts as well as individual fractions of cell extracts separated on the DEAE-Sephadex, however, failed to produce any 6-ethyl-7-methylpterin upon reduction. This result indicated that essentially all of the reduced modified folates in these cells were oxidatively cleaved during the preparation of the cell extracts by the methods used. Preparation of cell extracts in the presence of potassium ferricyanide, an oxidant which is known to oxidize a small portion of H_4-folate to folate (Chippel & Schrimgeour, 1970), followed by Zn/HCl reduction of the oxidized modified folates, however, did produce a small amount of 6-ethyl-7-methylpterin. This result then adds support to the idea that the oxidized forms of the modified folates present in *P. furiosus* are as indicated in Figure 3.

Each of these modified folates have a common structural element of *p*APT-ribose which is common to all the currently characterized modified folates (Van Beelen et al., 1984a,b; Raemakers-Franken et al., 1989, 1991). The occurrence of three different structures, each containing a different number of gluNAc, is reminiscent of the pteroylpoly-γ-glutamates and, as with the pteroylpoly-γ-glutamates, may be responsible for channeling C_1 intermediates between the different C_1 enzymatic reactions (McGuire & Coward, 1984). However, unlike any of the other folate or modified folate coenzymes, the side chain of these coenzymes is very unusual in that it contains no charge. Thus, the specificity of the interaction of these modified folates with their enzymes must be controlled by the number of attached gluNAc.

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REFERENCES

Angier, R. B., et al. (1947) *Science 103*, 667–669.
Barman, T. E. (1969) *Enzyme Handbook*, Vol. II, pp 588–589, Springer-Verlag, New York.
Berger, L. R., & Reynolds, D. M. (1958) *Biochim. Biophys. Acta 29*, 522–533.
Blakley, R. L., & Benkovic, S. L., Eds. (1985) *Folates and Pterins, Vol. 1, Chemistry and Biochemistry of Folate*, John Wiley & Sons, New York.

Bratton, A. C., & Marshall, E. K., Jr. (1939) *J. Biol. Chem. 128*, 537–550.
Bryant, F. O., & Adams, M. W. W. (1989) *J. Biol. Chem. 264*, 5070–5079.
Caprioli, R. M. (1972) in *Biochemical Applications of Mass Spectrometry* (Waller, G. R., Ed.) pp 735–776, John Wiley & Sons, New York.
Chambers, R. E., & Clamp, J. R. (1971) *Biochem. J. 125*, 1009–1018.
Chippel, D., & Scrimgeour, K. G. (1970) *Can. J. Biochem. 48*, 999–1009.
Coduti, P. L., & Bush, C. A. (1977) *Anal. Biochem. 78*, 21–38.
Defaye, J., & Reyners, T. (1968) *Bull. Soc. Chim. Biol. 50*, 1625–1635.
DiMarco, A. A., Donnelly, M. I., & Wolfe, R. S. (1986) *J. Bacteriol. 168*, 1372–1377.
Donnelly, M. I., Escalante-Semerena, J. C., Rinehart, K. L., Jr., & Wolfe, R. S. (1985) *Arch. Biochem. Biophys. 242*, 430–439.
Fiala, G., & Stetter, K. O. (1986) *Arch. Microbiol. 145*, 56–61.
Fukushima, T., & Nixon, J. C. (1980) *Methods Enzymol. 66*, 429–437.
Hartzell, P. L., Zvilius, G., Escalante-Semerena, J. C., & Donnelly, M. I. (1985) *Biochem. Biophys. Res. Commun. 133*, 884–890.
Hoyt, J. C., Oren, A., Escalante-Semerena, J. C., & Wolfe, R. S. (1986) *Arch. Microbiol. 145*, 153–158.
Keltjens, J. T., Rozie, H. J., & Vogels, G. D. (1984) *Arch. Biochem. Biophys. 229*, 532–537.
Leigh, J. A. (1983) *Appl. Environ. Microbiol. 45*, 800–803.
Lovley, D. R., Greening, R. C., & Ferry, J. G. (1984) *Appl. Environ. Microbiol. 48*, 81–87.
McGuire, J. J., & Coward, J. K. (1984) in *Folates and Pterins, Vol. 1, Chemistry and Biochemistry of Folate* (Blakley, R. L., & Benkovic, S. L., Eds.) pp 135–190, John Wiley & Sons, New York.
Pfiffner, J. J., Calkins, D. G., Bloom, E. S., & O'Dell, B. L. (1946) *J. Am. Chem. Soc. 68*, 1392.
Raemakers-Franken, P. C., Voncken, F. G. J., Korteland, J., Keltjens, J. T., van der Drift, C., & Vogels, G. D. (1989) *Biofactors 2*, 117–122.
Raemakers-Franken, P. C., Bongaerts, R., Fokkens, R., van der Drift, C., & G. Vogel, G. D. (1991) *Eur. J. Biochem. 200*, 783–787.
Reed, L. S., & Archer, M. C. (1980) *J. Agric. Food Chem. 28*, 801–805.
Rouvière, P. E., & Wolfe, R. S. (1988) *J. Biol. Chem. 263*, 7913–7916.
Van Beelen, P., Stassen, A. P. M., Bosch, J. W. G., Vogels, G. D., Guijt, W., & Haasnoot, C. A. G. (1984a) *Eur. J. Biochem. 138*, 563–571.
Van Beelen, P., Labro, J. F. A., Keltjens, J. T., Geerts, W. J., Vogels, G. D., Laarhoven, W. H., Guijt, W., & Haasnoot, C. A. G. (1984b) *Eur. J. Biochem. 139*, 359–365.
White, R. H. (1985a) *Arch. Microbiol. 143*, 1–5.
White, R. H. (1985b) *J. Bacteriol. 162*, 516–520.
White, R. H. (1986) *J. Am. Chem. Soc. 108*, 6434–6435.
White, R. H. (1990) *Biochemistry 29*, 5397–5404.
White, R. H. (1991) *J. Bacteriol. 173*, 1987–1991.
Worrell, V. E., & Nagle, D. P., Jr. (1988) *J. Bacteriol. 170*, 4420–4423.
Zakrzewski, S. F. (1966) *J. Biol. Chem. 241*, 2957–2961.
Zhou, D., & White, R. H. (1992) *J. Bacteriol. 174*, 4576–4582.