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5-(*p*-Aminophenyl)-1,2,3,4-Tetrahydroxypentane, a Structural Component of the Modified Folate in *Sulfolobus solfataricus*

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The partial characterization of the modified folate present in *Sulfolobus solfataricus* has been carried out. Separation of ethanol-water extracts of these cells on a DEAE-Sephadex column led to the isolation of a small amount of intact oxidized cofactor, which, when subjected to reductive cleavage with Zn-HCl, produced 6-methylpterin. This indicated that the modified folate in these cells contained a nonmethylated pterin linked, via a methylene group at the C-6 position of the pterin, to an arylamine, as is found in folate. Oxidative cleavage of intact reduced cofactor produced pterin and a single arylamine. The azo dye derivative of this arylamine was prepared and purified by chromatography on a Bio-Gel P-6 column. The resulting purified compound was shown to be readily hydrolyzed in dilute acid to the azo dye derivative of 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane, which was, in turn, readily cleaved to 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane by Zn-HCl reduction. The stereochemistry of the resulting 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane was shown to be *ribo*, the same as that of the 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane moiety found in methanopterin. The complete arylamine side chain of the modified folate thus contains 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane attached, via an acid-labile bond, to a currently unidentified substituent. The modified folate present in *S. solfataricus* thus contains structural features common to both folates and methanopterin.

Recent work on the analyses of the different types of modified folates present in thermophilic bacteria has indicated that at least three members of the thermophilic archaebacteria, *Pyrococcus furiosus*, *Thermococcus celer*, and *Sulfolobus solfataricus*, contain a different modified folate as their C_1 carrier (16). For *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 (Fig. 1), the first modified folate to be identified and characterized (10, 11). The major difference, however, between these modified folates and any of the presently identified modified folates was found to be the chemical structures of the arylamine side chains (6, 7, 16).

In this paper, we describe our work directed at the chemical characterization of the modified folate present in *S. solfataricus*. Our results showed that most of the modified H_4folate present in these cells is oxidatively cleaved to pterin and an arylamine (Fig. 2, reaction a) during aerobic extraction of the cells. Reductive cleavage of the small amount of intact oxidized cofactor present in the cell extracts to 6-methylpterin (Fig. 2, reaction c) showed that this modified folate contained a nonmethylated pterin linked, through a methylene group at the C-6 position of pterin, to an arylamine. The results also showed that the acid-resistant arylamine fragment of this compound was 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane, which had the same structure and *ribo* stereochemistry as the 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane moiety found in methanopterin.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. Cells of *S. solfataricus* 98-3 and *S. solfataricus* ATCC 35091 were used in this work. *S. solfataricus* 98-3 was originally isolated and characterized in 1970 by Thomas Brock and was supplied to us by Thomas A. Langworthy, University of South Dakota, Vermillion, S.D. *S. solfataricus* 98-3 was grown at 75°C for 3 to 4 days in batch culture in 20-liter bottles as previously described (14). Typically, 16 g (wet weight) of cells was isolated per bottle. These cells were frozen at −20°C for periods of up to 4 years before use.

S. solfataricus ATCC 35091 was grown in either Fernbach flasks or a chemostat with the same defined salts medium containing yeast extract as was used for *S. solfataricus* 98-3 (14). In a typical growth, six 2.8-liter Fernbach flasks, each containing 2 liters of medium, were shaken at 150 rpm and in a New Brunswick Scientific Co. model G25 Incubator Shaker at 70°C. By using a 10% inoculum (vol/vol) of culture from a previous cell growth, typically 12 g of cells (wet weight) could be harvested every 3 to 4 days. However, because of the requirement to produce much larger amounts of harvested cells, this method of cell production was replaced by growing *S. solfataricus* ATCC 35091 in a chemostat. The chemostat consisted of a 9.5-liter bottle containing 9 liters of the defined salts medium with yeast extract which was maintained at 75°C and stirred by the addition of filtered air (100 to 200 ml/min) through a fritted glass cylinder (12 by 250 mm) held at the bottom of the bottle. Fresh medium was pumped into the bottle at the same rate at which the medium containing the grown cells was removed; by using a pumping speed of 15 ml/min, a constant absorbance (A_{540}) for the culture of 1.0 could be obtained. (This A_{540} was the same as that obtained with cultures grown in the Fernbach flasks.) The culture that was removed from the chemostat (20 liters/24 h) was collected at room temperature and

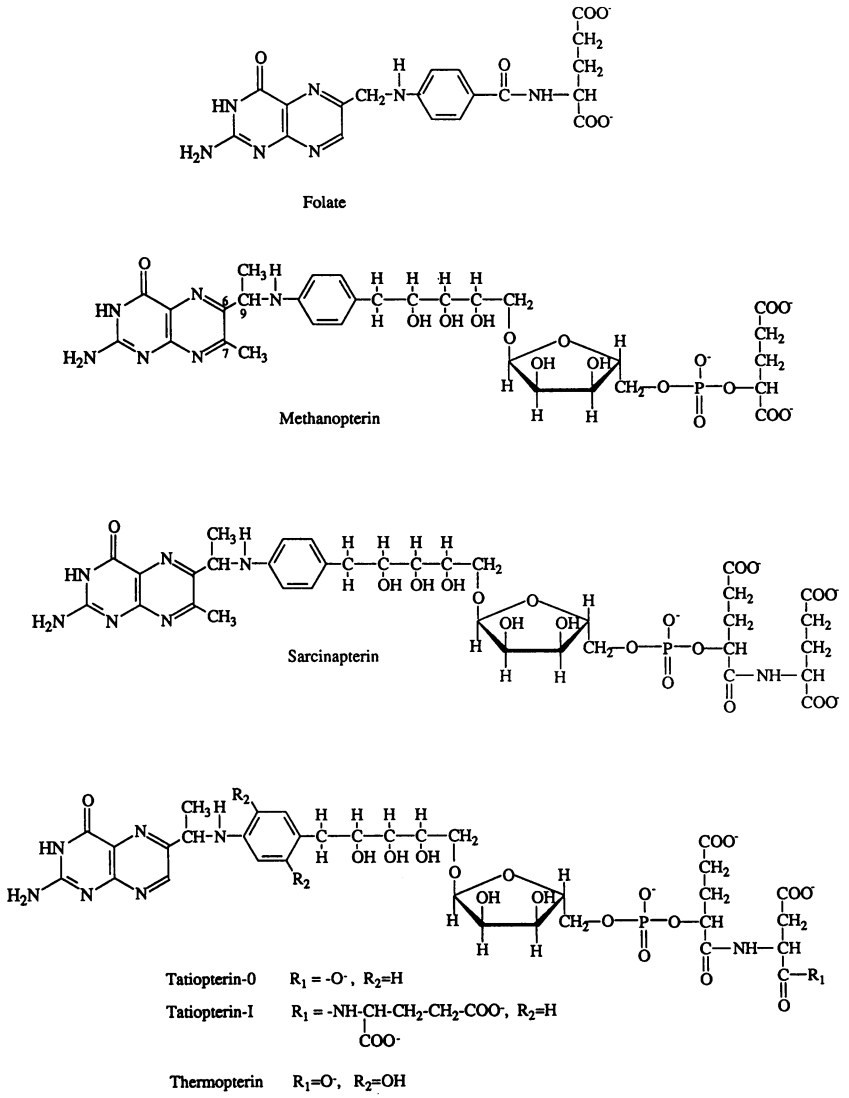

FIG. 1. Chemical structures of folate and the presently characterized modified folates.

stored at 3°C daily. Every 3 to 4 days, these collected and stored cultures were concentrated by membrane filtration at room temperature, and the wet cells were isolated by centrifugation (14). The production of cells by the chemostat procedure generated about 30 to 35 g (wet weight) of cells per day, a 10-fold increase in the amount of cells produced by growth in the Fernbach flasks.

Preparation and separation of cell extracts. Frozen cells (5 g) of *S. solfataricus* 98-3 were thawed and extracted with 50% ethanol, and the resulting extracts were oxidatively cleaved, concentrated, and separated on a DEAE-Sephadex column by using an ammonium bicarbonate gradient as previously described (16).

Fresh, nonfrozen *S. solfataricus* cells (ATCC 35091) (60 to 100 g [wet weight] of cells), grown either in Fernbach flasks or in the chemostat, were suspended in 300 ml of water and sonicated in two equal portions at 20 to 40°C with four 2-min 150-W pulses by using a Sonifier Cell Disruptor fitted with a macrotip. The combined, sonicated extracts were adjusted to pH 4.0 with 1 M HCl and centrifuged at approximately 40,000 × *g* for 2 h. The clear brown supernatant was decanted and saved; the cell pellets were resuspended in 200 ml of water and were resonicated and centrifuged as described above. The combined supernatants (400 ml) were then readjusted to pH 4.0 (1 M NaOH) and magnetically stirred overnight in a 1-liter beaker to complete the oxidative cleavage of the modified folate. Bacterial growth in the stirred supernatant was inhibited by the addition of a few milliliters of methylene chloride. The resulting solution was then adjusted to pH 8.5 by the addition of 1 M NaOH and applied to a DEAE-Sephadex column (1.5 by 25 cm). The column was eluted with 400 ml of a linear gradient of sodium chloride (0 to 2 M) in 0.04 M phosphate buffer (pH 5.8) and collected in 6-ml-volume fractions.

Analysis of the intact oxidized cofactor by using reductive cleavage. The position of elution of the intact cofactor in the DEAE-Sephadex fractions was determined by establishing which fractions produced 6-methylpterin upon Zn-HCl reduction. The procedure was patterned after the one developed for the cleavage of folic acid (4). An aliquot of each fraction (0.5 ml) was mixed with 0.5 ml of 1 M HCl and 0.1 ml of a suspension of Zn dust (0.5 g in 1 ml of 0.5% gelatin),

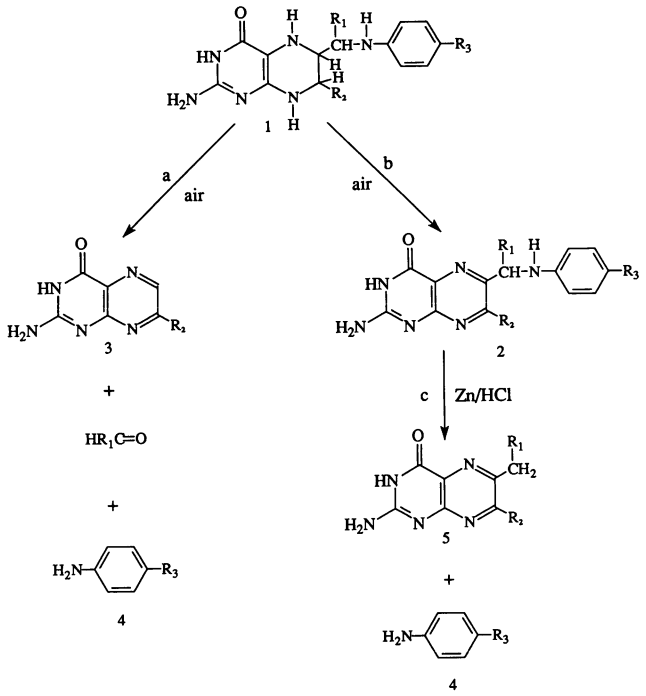

FIG. 2. Chemical transformations of reduced folates and modified folates. Reactions: a, one of several known pathways for the oxidative cleavage of reduced folate or modified folates; b, oxidation of reduced folate or modified folates without cleavage; c, reductive cleavage of oxidized folate or modified folates. When $R_1 = R_2 = H$ and $R_3 = CO(glu)_X$, the structure is that of folate and its polyglutamates.

magnetically stirred for 8 min, and centrifuged at 1,000 × *g* for 5 min to remove the insoluble material. The resulting pellet was washed with 0.5 ml of 0.5 M HCl and recentrifuged, and both supernatants were combined; 1 M NaOH was added until the first appearance of a $Zn(OH)_2$ precipitate. After adjustment of the pH of the suspension to 4.5 by the addition of acetic acid (2 drops), the $Zn(OH)_2$ precipitate redissolved, and the resulting clear solution was applied to a C_{18} Sep-Pak cartridge (Waters Associates, Milford, Mass.). After this column was washed with water (2 ml) to remove the salts, the pterins were eluted with methanol (2 ml). The residue resulting from evaporation of the methanol with a stream of nitrogen was assayed by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) as described below.

Analysis of pterins. Pterin-containing compounds in the DEAE-Sephadex-separated fractions were detected by measuring either fluorescence intensity (excitation, 350 nm; emission, 450 nm) or A_{342} or both. TLC of the pterin-containing compounds was accomplished on precoated silica gel TLC plates (E. Merck AG, Darmstadt, Germany) with two solvent systems, acetonitrile-water-formic acid (88%) (80:20:10, vol/vol/vol) and butanol-acetic acid-water (12:3:5, vol/vol/vol) as previously described (15). The pterins were also analyzed and quantified by HPLC with fluorescence detection by using known compounds for the identification and quantification of the unknowns. A Perkin-Elmer Series 400 HPLC system interfaced to a Perkin-Elmer LS-3 Fluorescence Spectrophotometer was used. The system was equipped with two Perkin-Elmer Picosphere C_{18}-cartridge columns in series, and the samples were eluted with a linear gradient (0 to 100%) of methanol in water. Pterin, 7-methylpterin, 6-methylpterin, 6,7-dimethylpterin, and 6-ethyl-7-methylpterin were readily resolved by using this HPLC system.

Analysis of arylamines by the BM assay. Arylamines in the separated DEAE fractions were determined by means of the Bratton and Marshall (BM) assay (1). To an aliquot (0.5 ml) of each fraction was added, at room temperature, 0.1 ml of 6 M HCl followed by 0.1 ml of 1.5% aqueous $NaNO_2$. After 2 min, 0.1 ml of 7.5% aqueous ammonium sulfamate was added with mixing to destroy the excess nitrous acid. After an additional 2 min, 0.5 ml of 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride was added, and the solution was left for at least 1 h at room temperature to complete the conversion of the diazonium derivative to the purple azo dye. Quantification was achieved by comparing the A_{556} of the azo dye derivative to that of the azo dye derivative produced under identical conditions with known amounts of *p*-aminophenylacetic acid. The azo dye product of *p*-aminophenylacetic acid was found to have a molar extinction coefficient of 4.8×10^4 cm^{-1} M^{-1}.

Preparation and purification of the azo dye derivative of the arylamine. Fractions from the DEAE column peak which were positive by the BM test were combined and concentrated until the point of salt precipitation. The solution was then reacted with the BM assay reagents in proportion to the volume of the solution used. After 1 to 2 h, the resulting solution containing the purple azo dye derivative was applied to a Bio-Gel P-6 column (1.5 by 26 cm) that had been washed and equilibrated with 0.05 M HCl. The azo dye derivative of the intact arylamine was eluted from the column with 0.05 M HCl as a sharp purple band whose center of elution was at 171 ml. Combined fractions containing this purple band were concentrated under vacuum to 1 to 2 ml and hydrolyzed at 100°C for 5 min. TLC analysis with the acetonitrile solvent system showed that this process led to the complete hydrolysis of the arylamine. (The intact azo dye derivative had an R_f of 0.00, and the hydrolysis product had an R_f of 0.64. The azo dye derivatives became visible as purple bands on the TLC plate only after evaporation of the eluting solvent.) The hydrolysis product, rechromatographed on the same Bio-Gel P-6 column, eluted as a single purple peak whose center of elution was at 211 ml. The combined peak fractions were evaporated under vacuum, dissolved in 0.1 M DCl, and analyzed by ^1H nuclear magnetic resonance (NMR).

NMR of the azo dye derivative. The ^1H NMR spectrum of the azo dye derivative was obtained at 399.952 MHz on a Varian Unity 400 instrument. A two-dimensional ^1H NMR spectrum was also obtained on the same instrument by means of double-filtered correlated spectroscopy.

HPLC analysis of the azo dye derivatives. The azo dye derivative of the arylamine was assayed by reverse-phase chromatography on a Shimadzu liquid chromatography system (model LC-6A) mounted with an Axxiom octadecylsilane column (5 μm, 4.6 by 250 mm) and equipped with a Shimadzu SPD 6AV UV-visible spectrometer detector. The azo dyes were eluted by using a trifluoroacetic acid-H_2O-CH_3CN gradient with 0.1% trifluoroacetic acid in water as solvent A and 0.075% trifluoroacetic acid in acetonitrile as solvent B. The gradient was linear from 3.5 to 45% B over a 40-min period, and the flow rate was 1 ml/min. Elution was monitored by the A_{556}.

Recovery of 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane from its azo dye derivative and GC-MS. Approximately 1 mg of Zn dust was added, with stirring, to a solution (1 ml)

of the azo dye derivative of 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane in 0.1 M HCl. After 2 min, the remaining Zn dust was removed by filtration, and the solution was evaporated to dryness with a stream of nitrogen gas. The resulting white solid was then dissolved in water and passed through a very small column of Dowex 50W-X8 H^+ (3 by 4 mm). After the Dowex column was washed with water, the 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane was eluted with 6 M ammonia (1 ml). After evaporation of the solvent (N_2 stream), the 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane was converted to its pentatrifluoroacetyl derivative and assayed by gas chromatography-mass spectrometry (GC-MS) as previously described (3).

RESULTS AND DISCUSSION

Assuming that the reduced modified folate present in *S. solfataricus* behaves chemically in a manner analogous to folate and methanopterin, one can envision three approaches that could be used to establish its chemical structure. These include the isolation and structural characterization of the intact reduced cofactor (Fig. 2, structure 1), the isolation and structural characterization of the intact oxidized cofactor (Fig. 2, structure 2), and the isolation and structural characterization of the oxidative cleavage fragments of the cofactor (Fig. 2, structures 3 and 4). 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 for both H_4folate (3, 8) and H_4methanopterin (5, 12).

After careful consideration of the feasibility and compatibility of these different approaches, it was decided that both the second and third approaches should be used in order to determine the structure of the modified folate present in *S. solfataricus*. One reason for this decision was that a single separation method (i.e., chromatography on DEAE-Sephadex) could be used to isolate not only the products resulting from the oxidative cleavage of the modified folate, but also the intact oxidized modified folate, should any of it be present.

Since ethanol-water mixtures were used for the extraction of the intact oxidized modified folate, methanopterin (10, 11), and tatiopterin and its derivatives from methanogens (6, 7), this was considered to be an extraction procedure that could yield some of the intact oxidized modified folate from *S. solfataricus*. Thus, cells of *S. solfataricus* 98-3 were extracted with 50% ethanol, and the extract was separated on a DEAE-Sephadex column with an ammonium bicarbonate gradient. The resulting fractions were then assayed for the presence of the intact oxidized cofactor, pterins, and arylamine. The elution profile obtained is shown in Fig. 3.

The fractions containing the oxidized intact cofactor were identified by measuring the production of 6-methylpterin by Zn-HCl cleavage of individual DEAE-Sephadex fractions (Fig. 2, reaction c). A single peak eluting around fraction 27 (Fig. 3) produced 6-methylpterin, indicating that this was the position of elution of the intact oxidized cofactor. The fact that the 6-methylpterin was derived from a modified folate was supported by the observations that a wide range of different non-folate-type pterin-containing compounds (e.g., neopterin, pterin, and biopterin) failed to produce 6-methylpterin during Zn-HCl reduction and that folate or any of its polyglutamate derivatives did not elute from the column with the gradient used. The fact that only 6-methylpterin, and not 6,7-dimethylpterin or 6-ethyl-7-methylpterin, was produced by the Zn-HCl reduction demonstrated that this modified

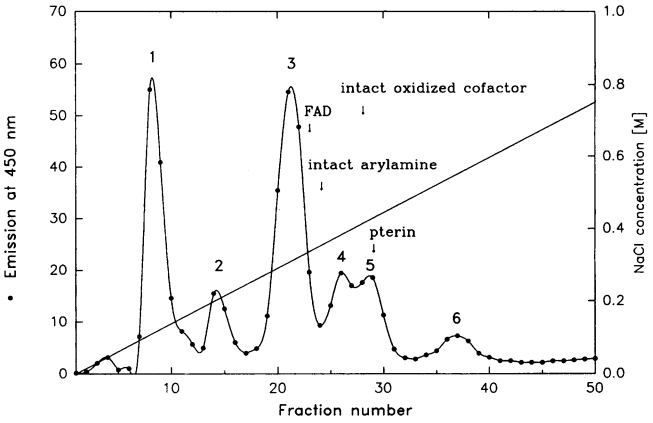

FIG. 3. Elution profile resulting from the separation of a 50% ethanol extract of *S. solfataricus* 98-3 on a DEAE-Sephadex column. Fractions were analyzed for the presence of pterins by measuring the fluorescence intensity at 450 nm, with excitation at 350 nm. Arylamines in the fractions were determined by means of the BM assay (3). Peak 1 was tentatively identified as sulfopterin (*erythro*-neopterin-3'-D-2-deoxy-2-aminoglucopyranoside) (14) by TLC analysis in two separate solvent systems and by its position of elution from the DEAE-Sephadex column. Peak 3 was also found to have exactly the same R_f on TLC as sulfopterin in the two different solvent systems, and at present its structure is unknown. No information on the structures of peaks 2 and 6 is presently available. Peaks 4 and 5 were identified as neopterin and pterin, respectively, on the basis of comparison with the known compound by using TLC, HPLC, fluorescence spectroscopy, and GC-MS of the trimethylsilyl derivatives.

cofactor was not methylated at either C-7 or C-9, as is found in methanopterin (11) or tatiopterin and its derivatives (Fig. 1) (6, 7). The detection of 6-methylpterin also demonstrated that the pterin was linked at its C-6 position through a methylene group to an arylamine, as is found in folate.

The level of 6-methylpterin produced by the reductive cleavage of the intact oxidized cofactor was low, approximately 0.1 nmol/g (wet weight) of cells, indicating that the level of the intact oxidized cofactor recovered from the cell extracts was also low. This result indicated that it would be very difficult to isolate enough of the intact oxidized cofactor for its characterization. A similar analysis of a 50% ethanol extract of *S. solfataricus* ATCC 35091 grown in the chemostat produced an identical DEAE-Sephadex elution profile, as measured by pterin fluorescence, but none of these fractions produced any methylated pterins after Zn-HCl reduction. This suggested that in extracts of these cells, the intact modified folate was completely destroyed by oxidative cleavage.

Analysis of these DEAE-column fractions for pterins showed the presence of at least six different compounds that had excitation and emission spectra characteristic of pterins (Fig. 3). Only the fifth peak, which was identified as pterin on the basis of comparisons with the known compound by using TLC, HPLC, fluorescence spectroscopy, and GC-MS of the trimethylsilyl derivatives, could have arisen from the oxidative cleavage of the original reduced modified folate. As 7-methylpterin, a product of the oxidative cleavage of C-7 methylated modified folates (16), was found to elute at exactly the same fraction position from the DEAE-Sephadex column as pterin, its absence in peak 5 again confirmed that *S. solfataricus* 98-3 contained no C-7 methylated modified folates (16). Therefore, the above information confirms that

the C_1 cofactor in *S. solfataricus* 98-3 contains a nonmethylated pterin linked to an arylamine, as is found in folate, and that most of this cofactor was oxidatively cleaved either before, during, or after the cell extract was obtained.

The DEAE-Sephadex fractions of the 50% ethanol extracts of *S. solfataricus* 98-3 were assayed for the occurrence of aromatic amines by using the BM assay (1, 2). One weakly BM-positive peak was detected eluting maximally at fraction 24, which was slightly after where flavin adenine dinucleotide eluted. The absorbance maximum of the BM azo dye product of this material was at 569 nm, compared with 556 nm for the azo dye product of *p*-aminobenzoylpolyglutamate and 567 nm for the azo dye derivative of *p*-aminophenylacetic acid. These results confirmed that the arylamine present in *S. solfataricus* 98-3 was not a *p*-aminobenzoylpolyglutamate. Quantitation of the amount of arylamine material present in the cell extracts by using the BM assay showed that this DEAE column-eluted peak corresponded to about 1.4 nmol of arylamine per g (dry weight) of cells.

At this stage, the characterization of the C_1 carrier was completely dependent upon the chemical characterization of the structure of the arylamine fragment. The ability to recover the arylamine from cells, however, was dependent upon a large number of factors. These factors included the strains and species of *S. solfataricus* used, the carbon sources used for the growth of the cells (e.g., yeast extract or tryptone), the means used to grow the cells (e.g., shake flasks, batch culture, or chemostat), and the length of time between cell isolation from the medium and extraction. The yield of arylamine also depended on the method used to extract the cells (e.g., boiling, sonication, or ethanol-water extraction), as well as the pH of the extracting solution, during both the extraction steps and subsequent oxidation steps. After more than a year of work, the best yields of the arylamine were obtained from chemostat-grown cells of *S. solfataricus* ATCC 35091 and by extracting the freshly harvested cells (less than 3 days old), using the sonication procedure described in Materials and Methods. (The lowest yields of the arylamine were obtained from cells extracted with boiling water and from cells grown on tryptone as the carbon source.) By using the procedure that generated the best yield of arylamine, a clear, intense BM peak was observed in the DEAE-Sephadex fractions eluted with the NaCl gradient. As was found also in the ethanol extracts, this arylamine peak eluted slightly after the flavin adenine dinucleotide that was also present in the extract. From the BM assay of this peak, it was determined that 16.2 nmol of arylamine could be typically extracted from 1 g (dry weight) of cells. This yield of product was 11.6 times greater than that obtained from the ethanol extracts of *S. solfataricus* 98-3.

Thus, fresh cells of *S. solfataricus* ATCC 35091 from the chemostat were extracted by sonication, and the cell extract was separated on a DEAE-Sephadex column eluted with an NaCl gradient. The BM-positive fractions were concentrated until NaCl precipitation, the arylamine contained therein was converted to the azo dye derivative, and the entire mixture was separated on a Bio-Gel P-6 column with 0.05 M HCl as the eluting solvent. (This chromatographic separation of the azo dye derivatives was based on the special affinity of the azo dyes for these polyacrylamide resins and has been used previously by others for the purification of folate-derived arylamines [2, 9].) This resulted in the isolation of a single purple peak which was shown to consist of a single azo dye by HPLC analysis when monitored at 556 nm. Monitoring the HPLC separation at 220 nm, however, showed that the material was quite impure. This P-6 column-purified azo dye derivative of the intact arylamine (Fig. 4, compound 1) was found to be insoluble at neutral and basic pHs and thus could be further purified only under acidic conditions. This pH insolubility of the azo dye greatly restricted the possible routes for its further purification. An even more severe problem, however, was the discovery that the purified azo dye derivative of the intact arylamine (Fig. 4, compound 1) was found to readily cleave to a second purple compound (Fig. 4, compound 2) when stored either at 3°C or at room temperature in acidic solutions. This second purple compound was readily resolved from the parent purple compound by chromatography on a Bio-Gel P-6 column, where it eluted at 211 ml compared with 171 ml for the intact compound.

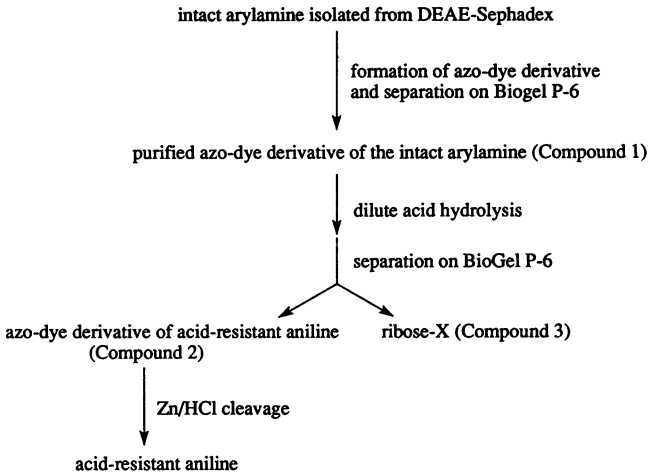

FIG. 4. Steps in the purification and analysis of azo dye derivatives of the arylamines produced from the modified folate.

When the intact arylamine molecule was hydrolyzed with 6 M HCl (6 h at 100°C) and converted into the azo dye derivative, the resulting azo dye was the same compound (Fig. 4, compound 2) as that derived by the mild acid hydrolysis of the intact azo dye derivative (Fig. 4, compound 1) as determined by chromatography on Bio-Gel P-6. This result indicated that the aniline portion of the molecule contained no acid-labile groups and that this aniline is attached to a group, via an acid-labile bond, to form the intact arylamine side chain of the modified folate.

The azo dye derivative of the acid-released aniline was purified on a Bio-Gel P-6 column and identified from its ^1H HMR spectrum. The chemical shifts and intensities of the observed resonances (Fig. 5 and Table 1) are consistent with the compound being the azo dye derivative of 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane. This was confirmed by comparison with the ^1H NMR spectrum of an authentic sample of the azo dye derivative of 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane prepared from a sample of methanopterin (10). The identity of these samples was also confirmed with TLC and HPLC by comparison of the azo dye derivatives of the compounds isolated from the *S. solfataricus* cell extracts and methanopterin.

These analytical methods, however, do not allow one to say with certainty that the configuration at the three asymmetric centers of the 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane derived from *S. solfataricus* is the same as that in methanopterin. The correspondence of these asymmetric

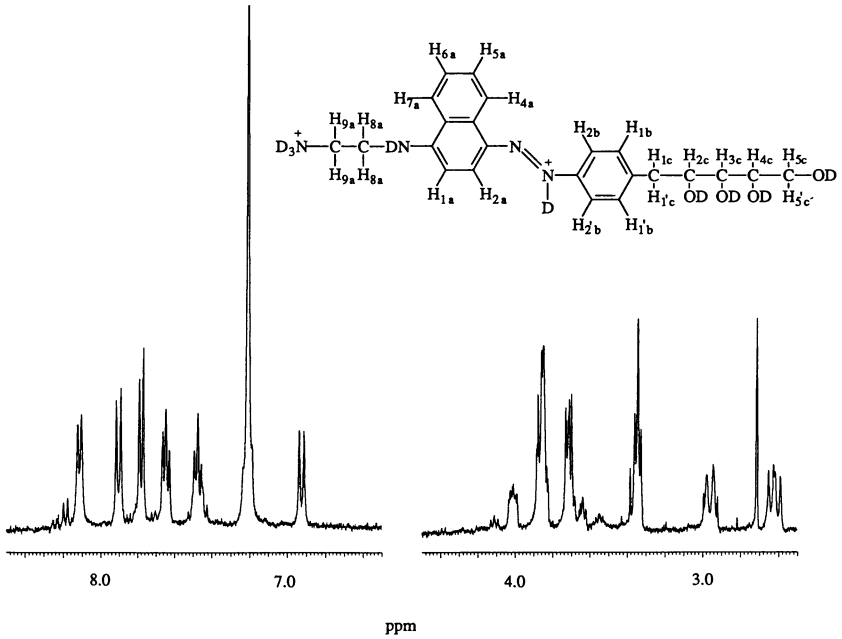

FIG. 5. ^{1}H NMR spectra of the azo dye derivative of the acid-resistant portion of the intact arylamine.

centers was confirmed by GC-MS of the pentatrifluoroacetyl derivative of the 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane under conditions known to resolve each of the four stereoisomers (13). These results then demonstrated that the compound derived from *S. solfataricus* and the compound derived from methanopterin have the same stereochemistry. The present information indicates that the modified folate in *S. solfataricus* ATCC 35091 contains structural features common to both methanopterin and folate. It is similar to folate in having a nonmethylated pterin linked, via a methylene group, to an aniline and similar to methanopterin in having 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane as the arylamine portion of the molecule. Thus, from the point of view of the structure of the modified folate present in *S. solfataricus*, this organism appears to represent an ancestral phenotype of the *Archaea*. This is consistent with its classification into the kingdom *Crenarchaeota* (17). Work is currently in progress to determine the complete structure of this intact arylamine molecule, which is presently known to contain one molecule of ribose, one molecule of phosphate, and no α-hydroxyglutaric acid (16a).

We do not yet have any direct evidence that the modified folate characterized in this paper, in fact, functions as a C_1 carrier in species of *Sulfolobus*. It does, however, contain the correct functional group for a C_1 carrier, and its structure resembles both folates and methanopterin, two established C_1 carriers. In addition, this modified folate occurs in cells at a concentration that is close to that typically found for folates in eubacteria (16). Thus, all of the current evidence indicates that this molecule represents one more example of the growing list of modified folates being characterized in the archaebacteria.

TABLE 1. Proton resonances observed for the azo dye derivative of the acid-resistant portion of the intact arylamine

Proton	Chemical shift (ppm)	Observed coupling pattern,[a] coupling constant (cps), and no. of protons
1c	2.62	dd, (J = 10.4, 13.1), 1H
1′c	2.96	d, (J = 13.4), 1H
8a	3.34	t, (J = 6.7), 2H
9a	3.72	t, (J = 6.4), 2H
5c, 5′c, 3c, 4c	3.87	m, 4H
2c	4.01	m, 1H
1a	6.93	d, (J = 9.7), 1H
1b, 1′b, 2b, 2′b	7.21	s,[b] 4H
6a	7.47	dd, (J = 7.3, 7.9), 1H
5a	7.65	t, (J = ~7.2), 1H
4a	7.78	d, (J = 8.5), 1H
2a	7.91	d, (J = 9.5), 1H
7a	8.12	d, (J = 7.6), 1H

[a] Symbols: s, singlet; d, doublet; t, triplet; dd, doublet of doublets; m, multiplet.

[b] The resonances of these protons can appear as either a singlet or a doublet of doublets, depending on the concentration of DCl in the sample.

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