Structure of Sulfohalopterin 2 from *Halobacterium marismortui*†

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ABSTRACT: Sulfohalopterin 2 (SHP-2) isolated from *Halobacterium marismortui* is shown to be a negatively charged pterin containing sulfate esters and carboxylates on the side chain. Proton magnetic resonance and carbon magnetic resonance studies prove that the pterin is bound at its 6-position to the terminal methylene of a 7-deoxyheptonic acid. Periodate cleavage of SHP-2 yields threonic acid 3-sulfate and a dimeric pterin containing an ether linkage between the two pterin side chains. It is concluded that SHP-2 is a novel symmetrical dimeric pterin containing an ether bond between the 6-positions of the 7-deoxyheptonic side chains.

Recent work on the distribution of pterins in the nonmethanogenic archaebacteria has uncovered several previously undescribed 6-substituted pterins (X. Lin and R. H. White, unpublished experiments, 1987). In the halobacteria, two new pterins, designated sulfohalopterin 2 (SHP-2)[1] and phosphohalopterin 1 (PHP-1), were discovered. These pterins, along with methanopterin (Van Beelen et al., 1984) and sarcinapterin (Van Beelen et al., 1984), which were isolated from the methanogenic archaebacteria, illustrate the unexpected diversity in structure and distribution of the pterins found in the archaebacteria. In this paper, we report on the isolation and structural characterization of SHP-2 (Figure 1), which was isolated from *Halobacterium marismortui*.

Materials and Methods

UV–visible spectra were recorded on a Varian Cary 219 double-beam spectrophotometer. Fluorescence spectra were recorded on a Perkin-Elmer 650-40 fluorescence spectrophotometer. NMR spectra were obtained on an IBM System WP-270 SY. Infrared spectra were run on a Perkin-Elmer 700 infrared spectrophotometer. Mass spectra and GC–MS were obtained with a VG 70-70HF mass spectrometer. A Varian 2400 gas chromatograph was used for the GC analyses. CD spectra were recorded on a Jobin Yvon Auto. Dichrograph Mark-5.

Halobacterium marismortui was supplied by Barbara J. Javor (Scripps Institution of Oceanography, LaJolla, CA). Carrier-free [^{35}S]sulfuric acid was supplied by ICN Biomedicals, Inc. Biopterin and D-(+)-neopterin were supplied by Fluka Chemical Corp.

Growth of H. marismortui and Isolation of SHP-2. H. marismortui cells were grown at 39 °C for 5 days with shaking and constant illumination in 2.8-L Fernbach flasks containing 1.1 L of the complex medium described by Javor (1984). The cells were isolated by centrifugation for 30 min at 17000*g*, and 700 g wet weight of cells was collected from 40 L of the growth medium (most of the wet weight of the cell pellet is salt). The resulting cell pellet was mixed with 3 L of 70% ethanol and kept in the dark at room temperature for 12 h to complete the oxidation of the reduced pterin. The mixture was centrifuged, the clear, 70% ethanol fraction was separated, and the insoluble material was reextracted (10 times) with 3 L of 70% ethanol. The combined 70% ethanol fractions were evaporated, dissolved in ~500 mL of distilled water, and mixed with 500 mL of chloroform. The well-shaken water and chloroform mixture was centrifuged for 30 min at 25000*g*, and the resulting clear, yellow water layer, which contains a high concentration of salt, was removed and diluted to 20 L with distilled water to reach a final sodium chloride concentration of 0.15 M as measured by conductivity. The water solution was then passed through a 3.5 × 23 cm column of QAE-Sephadex (40–120 μm) that was preequilibrated with 0.1 M NH_4HCO_3. The column was further washed with 2 L of 0.2 M NH_4HCO_3, and the anionic compounds were eluted with a linear gradient of NH_4HCO_3 from 0.2 to 2 M. Fractions (7 mL) were collected from the eluent (800 mL), and SHP-2 was detected by its absorbance at 350 nm. Those fractions containing the major SHP-2 peak, between 1.5 and 2.0 M NH_4CO_3, were combined, lyophilized, dissolved in approximately 3 L of distilled water, and applied to another QAE-Sephadex column (1.5 × 27 cm) that was preequilibrated with 0.1 M NH_4HCO_3. A 0.2–2 M NH_4HCO_3 linear gradient was applied, and the fractions containing SHP-2 were collected and lyophilized. The resulting lyophilized material was dissolved in the smallest possible volume of distilled water (5–10 mL) and applied to a 3.5 × 37 cm column of Bio-Gel P-2 (200–400 mesh) from Bio-Rad Laboratories that was preequilibrated with distilled water. Distilled water was used as the eluting solvent, and SHP-2 fractions were quantitated by its absorbance at 342 nm ($\epsilon = 6.3 \times 10^3$ M^{-1} cm^{-1}). The combined SHP-2 fractions were evaporated, dissolved in a minimal amount of water, and purified by paper chromatography developed with 1-butanol–AcOH–water (12:3:5 v/v) on Whatman No. 3 paper (23 × 55 cm). The major blue fluorescent band with the lowest R_f was cut off, eluted from the paper with water, dried, dissolved in a minimal amount of water, and reapplied to the above Bio-Gel P-2 column, which was eluted with distilled water. The fractions containing a total of 39 μmol of SHP-2 were combined, evaporated, and dissolved in D_2O for NMR and subsequent analyses.

Analysis of Organic Sulfate and Phosphate in SHP-2. Three samples of SHP-2 (24 nmol in each sample) were hydrolyzed with 1 M HCl at 100 °C for 2 h to release the bound sulfate for assay. As a control, three unhydrolyzed samples of SHP-2 were analyzed along with the hydrolyzed samples.

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[1] Abbreviations: PMR, proton magnetic resonance; CMR, carbon magnetic resonance; SHP-2, sulfohalopterin 2; HP-2, halopterin 2; SHP-OR, sulfohalopterin 2 oxidized with periodate and reduced by borohydride; TMS, trimethylsilyl; GC, gas chromatography; GC–MS, gas chromatography–mass spectrometry.

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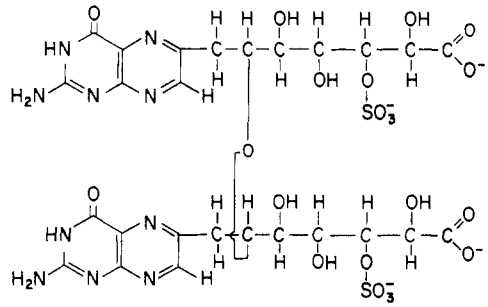

FIGURE 1: Proposed structure for SHP-2.

In addition, three blanks with the same amount of 1 M HCl as contained in the samples were subjected to the same "hydrolysis" procedures and were assayed in the same way as the samples. A water solution of Na_2SO_4 (4 mM) was used to generate the standard curve. The sulfate assay method used, which is based on the reaction of barium rhodizonate with sulfate, is basically the same as that described by Terho and Hartiala (1971).

The phosphate assay used was that of Martin et al. (1971). The stock solution is a mixture of 1 part 10% ascorbic acid and 6 parts molybdate mix composed of 0.42% ammonium molybdate in 1 N H_2SO_4. The samples and the standard phosphate solutions and the samples (prepared by the acid hydrolysis of SHP-2 in 1 M HCl at 100 °C for 12 h and then dried under nitrogen) were dissolved in 0.3 mL of 0.5 M HCl. Stock solution (0.7 mL) was added to the standards and the samples, and these were mixed well and heated at 45 °C for 20 min before the absorbance was read at 820 nm.

Periodate Oxidation of HP-2. Samples (~50 nmol) of HP-2 and neopterin were each dissolved in 2 mL of 0.01 M HCl, and 0.5 mL of 0.01 M HIO_4 was added to each. After the samples stood at room temperature for ~15 min, 0.5 mL of 0.1 M KOH was added to give a final pH of 10.5, and a UV–visible spectrum was taken immediately of each in order to determine the maximum absorbance. For the controls, HP-2 and neopterin were each dissolved in 2.5 mL of 0.01 M HCl, and 0.5 mL of 0.1 M KOH was added before each spectrum was taken.

Periodate Oxidation of SHP-2 and Reduction of the Resulting Aldehyde. SHP-2 (1.3 μmol) was dissolved in 0.1 mL of water, and the resulting solution was mixed with 0.2 mL of 65 mM $NaIO_4$ (13 μmol) in water. The mixture was kept in the dark for ~2 h, and the resulting pterin aldehyde precipitate was dissolved by adding 0.1 mL of 2 M NH_4OH. After the addition of 0.1 mL of 1 mM $NaBH_4$ in 2 M NH_4OH, the reaction mixture was kept in the dark for ~1 h. The reaction mixture was then evaporated under a stream of nitrogen, and the extra $NaBH_4$ was destroyed by addition of 1 M HCl. A few drops of methanol were added to the acidic solution, and the mixture was evaporated under nitrogen. The resulting material was dissolved in water and passed through a small Dowex 50W-8X H^+ (0.7 × 3.0 cm) column. The column was washed with water, and the pterin was eluted with 2 M NH_4OH into the top of a 0.7 × 4.5 cm column of Dowex 1-X8-200 OH^-. The Dowex 1 column was washed with water and 0.01 M HCl, and the pterin was eluted with 0.1 M HCl. This pterin, which will be called SHP-OR, is ready to be derivatized by the TMS reagent for mass spectral analysis. For mass spectrometric analysis, about 0.1 μmol of the purified SHP-OR was dried in a 0.2-mL bottle, and then 20 μL of pyridine–hexamethyldisilazane–chlorotrimethylsilane (9:3:1 v/v) was added. The resulting sample was heated at 110 °C for ~15 min, and then a 2-μL sample was evaporated directly into a MS probe for mass spectrometric analysis.

Periodate Oxidation and Sodium Borotritide Reduction of the Resulting Aldehydes. Experimental procedures were basically the same as those described above except that $NaBT_4$ was used instead of $NaBH_4$. SHP-2 (0.6 μmol) was reacted with 3.0 μmol of $NaIO_4$ in water, and the reaction mixture was kept in the dark at room temperature for 3 h. After the pterin precipitate was dissolved by addition of 2 M NH_4OH, ~3 μCi of $NaBT_4$ was added to the reaction mixture. The mixture was kept in a hood overnight, and then ~6 μmol of $NaBH_4$ was added to make sure that the reduction was completed. The above reaction mixture was evaporated under nitrogen to remove excess ammonia, ~0.5 mL of 50% acetic acid was carefully added, and the solution was evaporated again. The residue was redissolved in 0.5 mL of an equal mixture of 1 M HCl and methanol and then evaporated with heating under nitrogen. The evaporation of the acidic methanol was repeated once more in order to ensure the complete removal of boric acid. The total counts per minute of the resulting "hot" sample was 3.7×10^7.

SHP-2 (~2 μmol) was used to prepare an unlabeled, carrier sample that was combined with the tritiated sample and then directly applied to paper for purification by paper electrophoresis. After acid hydrolysis, the sample was dried, dissolved in 0.5 mL of water, and passed through a small Dowex 50W-8X H^+ (0.7 × 1.0 cm) column. The resulting elution was dried, and the lactone, formed by cyclization during the Dowex column chromatography, was derivatized by addition of 50 μL of BSTFA [bis(trimethylsilyl)trifluoroacetamide] and heating at 60 °C for 1 h (Izumi et al., 1979). Two microliters of sample was injected into the gas chromatograph; fractions were collected at the unignited flame ionization tip by condensation in a glass tube, eluted from the tube with methanol, and counted. Greater than 90% of the counts injected into the GC column were recovered in the collected fractions. The column used was a 0.3 × 183 cm glass column containing 10% SP-2100 on Chromosorb Q programmed from 100 °C at 10 deg/min. Gas chromatography–mass spectrometry was done on the sample under identical chromatographic conditions in order to obtain the mass spectra of the radioactive peaks.

Preparation of Threonic and Erythronic Acids. Erythronic acid was prepared by oxidizing erythrose with iodine in base solution according to Schaffer and Isbell (1963). The threonic acid was prepared by two methods. The first was to epimerize erythronate to threonate in base according to the method of Hamilton and Smith (1954). The second and preferred method was to oxidize ascorbic acid to threonic acid with H_2O_2 in base according to the method of Isbell and Frush (1979). The TMS derivatives of the lactones of the tetraonic acids were prepared as described above.

Catalytic Reduction of SHP-2 and Reoxidation by Air. The procedures used are basically those of Fukushima and Nixon (1980), Fukushima et al. (1978), and Kaufman (1967) for the reduction of pterins.

SHP-2 (0.15 μmol) was put into a small bottle (1 × 3 cm) fitted with a rubber septum. Platinum(IV) oxide (1 mg) was added, and the bottle was flushed with hydrogen. Forty microliters of 1 M HCl was then added, and the bottle was pressurized with 24 psi of H_2 and kept at room temperature with constant stirring for 2 h. The resulting tetrahydropterin (as confirmed by its UV–visible spectrum) was separated from the platinum by filtration and equally distributed into two small bottles. The samples in both bottles were evaporated under nitrogen, and then 10 μL of 0.1% I_2 in 0.1 N NaOH was added to one bottle and 10 μL of 0.1% I_2 in 0.1 N HCl

was added to the other bottle. The bottles were kept in the dark at room temperature for ~30 min, then the reaction product (oxidized pterin) was directly applied to the TLC plate for analysis. As a control, standard neopterin was reduced and reoxidized in exactly the same manner.

Isolation of ^{35}S-Labeled SHP-2. In order to get the highest specific activity of the radioactive sulfate with a given amount of $^{35}SO_4^{2-}$, it was important to use a minimal amount of "cold" sulfate in the growth medium so that the cells would grow normally and the yield of SHP-2 would not decrease. In order to determine this minimal sulfate concentration, the influence of the sulfate content in the growth medium on the growth of *H. marismortui* and on the yield of SHP-2 was studied. The normal complex medium (CM) described by Javor (1984) contains 81 mM $MgSO_4$. A sulfate-free medium was made which was essentially the same as the CM medium except that $MgCl_2$ was used instead of $MgSO_4$. Ten milliliters of *H. marismortui* cells, which were grown for 2 days in the CM medium, was transferred to 90 mL of sulfate–free medium to make the final sulfate concentration 8.1 mM. After the cells were grown under normal conditions, 10 mL of the cells that were grown for 2 days on the 8.1 mM sulfate medium was transferred to the sulfate-free medium to make the final concentration of sulfate 0.81 mM. The growth curves of the cells that were grown in the CM media that contained 81, 8.1, and 0.81 mM sulfate were compared, as were the yields of SHP-2 in these growth media.

For the radioactive experiment, 10 mL of *H. marismortui* cells, which were grown in the CM medium (81 mM sulfate) for 2 days, was added to 90 mL of sulfate-free CM medium in a 250-mL Erlenmeyer flask to bring the sulfate concentration to 8.1 mM. Approximately 2 mCi of H_2SO_4 (carrier free) from ICN Biomedicals, Inc., was then added to this medium, and the cells were grown for 5 days before being centrifuged and extracted as described above for the isolation of SHP-2. The ^{35}S-labeled SHP-2 was purified first by paper chromatography, then by paper electrophoresis (2 times), and finally by Bio-Gel P-2 column chromatography.

Results and Discussion

Optical Spectroscopy of SHP-2. The UV–visible spectra of SHP-2 showed λ_{max} at 255 and 362 nm at pH 11.0, at 273 and 342 nm at pH 4.8, and at 250 (shoulder) and 320 nm at pH 2.0. These spectra were the same as those recorded for neopterin under the same conditions.

The fluorescence excitation and emission spectra of SHP-2 (excitation maximum 366 nm and emission maximum 445 nm in water; excitation maximum 373 nm and emission maximum 454 nm in ~0.01 N NaOH) were also the same as those of neopterin. These spectra formed the basis for the identification of the chromophore of SHP-2 as a pterin.

Circular Dichroism Spectrum of SHP-2. If a chiral group is bound to the 6-position of the pterin, as it is in biopterin and neopterin, then this asymmetry may be reflected in the CD spectrum of the pterin. This was demonstrated in the CD spectra of biopterin and neopterin (Figure 2). The spectra are mirror images of each other because they have opposite stereochemistry at the 1′-carbon. The figure also shows that SHP-2 has essentially no CD-active bands, which supports the idea that SHP-2 has no asymmetric center at the 1′-carbon. These data support the proposed structure of SHP-2 (Figure 1) with a 1′-methylene carbon that is bound to the C-6 position of the pterin.

Periodate Cleavage of SHP-2. Electrophoresis has shown that the pterin which results from the periodate cleavage of SHP-2 is not charged. This pterin is also produced by the periodate cleavage of HP-2, and it was shown to be clearly different from the pterin product that was derived by periodate cleavage of neopterin (data not shown) by TLC and mass spectrometric analysis of the TMS derivative.

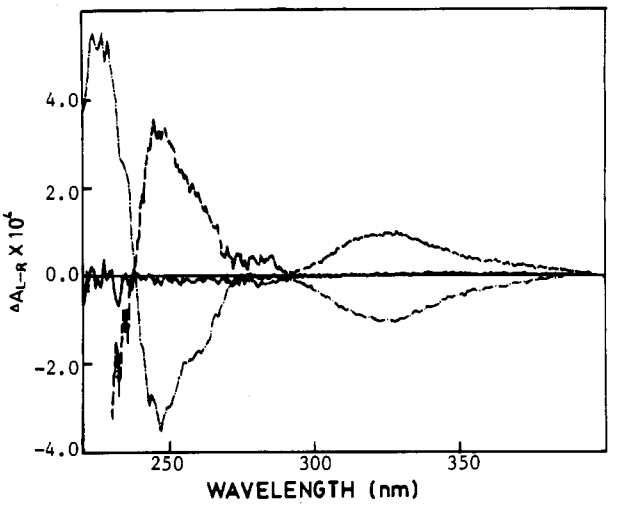

FIGURE 2: CD spectrum of SHP-2. SHP-2 (—), biopterin (– –), and neopterin (–·–) were each dissolved in water at a concentration of ~0.1 M for the determination of their circular dichroism spectra.

In order to test for functionality at the 1′-carbon, SHP-2 was cleaved with periodate, and a UV–visible spectrum was recorded before and after the reaction. [If SHP-2 had any structural features similar to those of neopterin, then the aldehyde produced by periodate cleavage would conjugate with the aromatic system of the pterin ring, and the UV–visible spectrum would change.] As a control, neopterin was subjected to the same reaction procedures. The results show that, after reaction with periodate, there is a 7-nm red shift for neopterin because of the conjugation of the resulting aldehyde with the aromatic ring of the pterin. No shift was observed for HP-2, which means that the aldehyde produced by the periodate cleavage of HP-2 is not conjugated with the pterin. This is the expected outcome if the 1′-carbon of HP-2 is a methylene, as discussed below, and if the HP-2 is a dimeric pterin as shown in Figure 1.

Oxidation of Tetrahydro-SHP-2 in Acid or Base. It is well-known that tetrahydroneopterin and tetrahydrobiopterin are degraded to pterin when oxidized in basic solutions (Fukushima & Nixon, 1980). If SHP-2 has a methylene group as suspected, then it may not undergo this type of oxidative cleavage. This was checked by reducing the SHP-2 to tetrahydro-SHP-2 and then reoxidizing it back to SHP-2 with iodine in either 0.1 M HCl or 0.1 M NaOH. The results of the reduction and reoxidation of SHP-2 and neopterin clearly show that tetrahydroneopterin is cleaved to pterin when oxidized in base but that tetrahydro-SHP-2 is not cleaved under the same conditions. This means that SHP-2 and neopterin have some fundamental difference in their side-chain structures.

Acid Hydrolysis of SHP-2. A negatively charged side chain was recognized for SHP-2 from its paper electrophoresis data and from its retention on a QAE-Sephadex column. The time-dependent removal of this charge by acid hydrolysis of SHP-2, as monitored by paper electrophoresis of the fluorescent pterin products, is complex. Two major intermediates, intermediate 1 and intermediate 2, and two final hydrolysis products, HP-2-I (charged) and HP-2-II (noncharged), have been identified (Figure 5).

The easy removal of a negative charge from a compound by acid hydrolysis is characteristic either of sulfate esters,

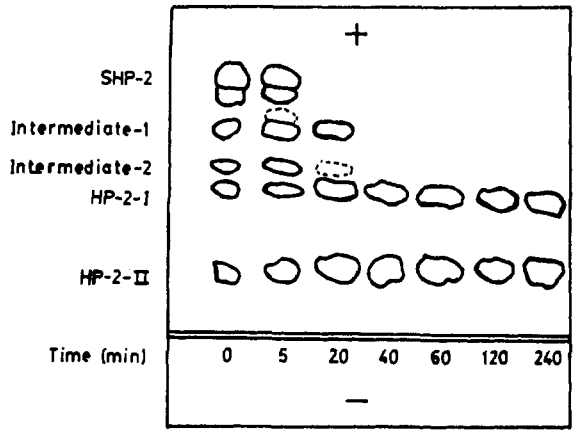

FIGURE 3: Paper electrophoresis of the time-dependent acid hydrolysis of SHP-2. Paper electrophoresis was done as described in the text. The hydrolysis was done in 1 M HCl at 100 °C. Samples were hydrolyzed for the times indicated in the figure and then evaporated in vacuo. The dried samples were redissolved in water and applied to paper. The recorded spots are the fluorescent spots observed by exposing the paper to UV light.

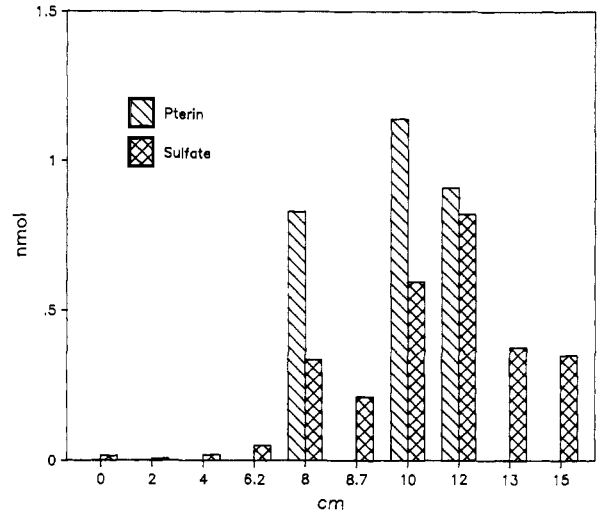

FIGURE 4: Paper electrophoresis of the partially hydrolyzed ^{35}S-labeled SHP-2. ^{35}S-Labeled SHP-2 was hydrolyzed in 1 M HCl for 5 min and separated by paper electrophoresis as described in Figure 3. The last bar in the figure represents the cutting from 15 to 17 cm, and the width of the cutting is 2.0 cm. The 12-cm bar corresponds to the R_f of SHP-2, the 10-cm bar corresponds to the R_f of intermediate 1, and the 8-cm bar corresponds to the R_f of intermediate 2. The amount of sulfate was obtained from the specific activity of the sulfate in the medium and the number of counts in each area of the paper. Pterin was measured by fluorescence after elution from the paper.

which have a half-life of less than 1 h in 0.25 N HCl at 100 °C (Rees, 1963), or, less likely, of phosphate esters, which have a half-life of 30 min in 1 M HCl at 100 °C (Robbins & Lipmann, 1956). The results of the phosphate assay showed that less than 2 nmol of phosphate was present in 45 nmol of SHP-2. This clearly ruled out the possibility of phosphate esters in SHP-2 being responsible for the negative charge. With the sulfate assay method, an average value of 23 ± 3 nmol of sulfate was found from three samples containing 24 nmol of SHP-2. This result clearly indicates that one sulfate ester was present for each pterin in the SHP-2.

Incorporation of [^{35}S]Sulfate into SHP-2 and Its Acid Hydrolysis. Acid hydrolysis of SHP-2 by 1 M HCl at 100 °C for 1 h produced two pterins, one negatively charged and the other neutral (Figure 3). The charged HP-2 (HP-2-I) could not be further hydrolyzed to uncharged HP-2 (HP-2-II) in acid with longer heating time, but the uncharged HP-2 was changed to charged HP-2 when heated in base or maintained for long periods of time in water (data not shown). This means that if HP-2-I is an acid, then HP-2-II is a lactone of that acid and the charge on HP-2-I results from a carboxylate such as $R-(CHOH)_x-CO_2^-$. The presence of a carboxylate group was proved by IR spectroscopy of SHP-2 in a KBr pellet, which showed absorbance bands at 1400 and 1700 cm^{-1} for the asymmetric and symmetric carboxylate anion stretch, respectively.

The SHP-2 that was isolated from *H. marismortui* cells grown with [^{35}S]sulfate was labeled with ^{35}S and purified as described for SHP-2. The SHP-2-containing fractions collected from the Bio-Gel P-2 column were combined and subjected to acid hydrolysis in 1 M HCl at 100 °C for 1 h. Paper electrophoresis of this hydrolyzed sample showed a complete separation of the radioactive sulfate from the pterin, proving that the sulfate was bound as an ester.

Data on the partial hydrolysis of ^{35}S-labeled SHP-2 are shown in Figure 4. The figure shows that at least two intermediates which contain both ^{35}S and pterin arise during the short-term hydrolysis of SHP-2 to HP-2 (5 min). The figure also shows approximately one sulfate per pterin for intact SHP-2 (12-cm bar), and more interestingly, it shows that the two intermediates (10- and 8-cm bars) each contain only half a sulfate per pterin. This is best explained by the idea that SHP-2 is a dimer, that there are two sulfates in the pterin dimer (one sulfate per pterin), and that if the dimer lost one

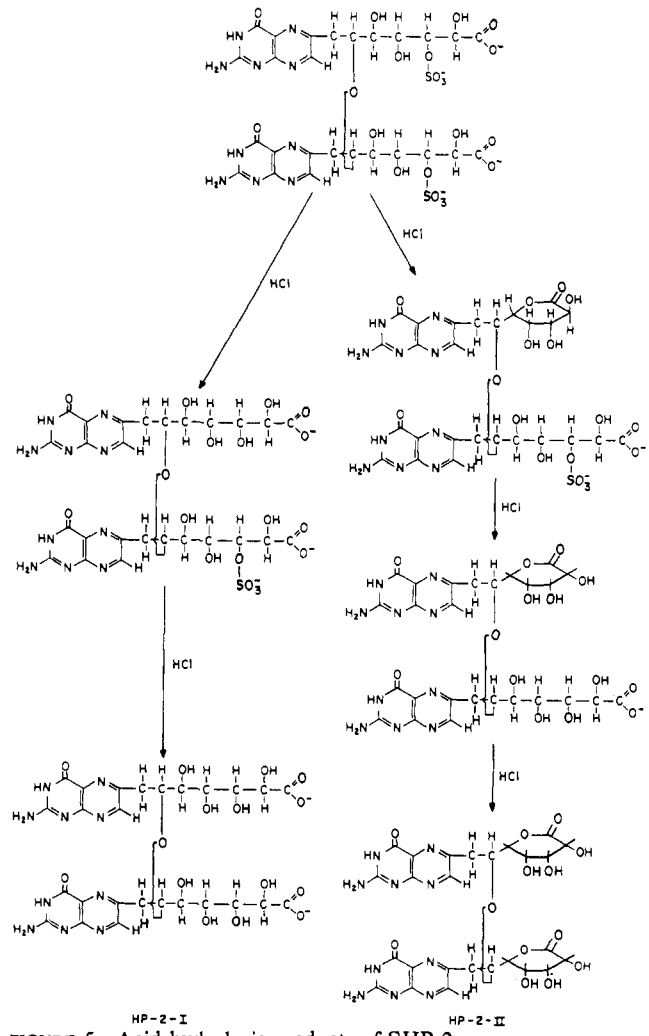

FIGURE 5: Acid hydrolysis products of SHP-2.

of its sulfates, there would be one sulfate per dimer or half a sulfate per pterin (Figure 5).

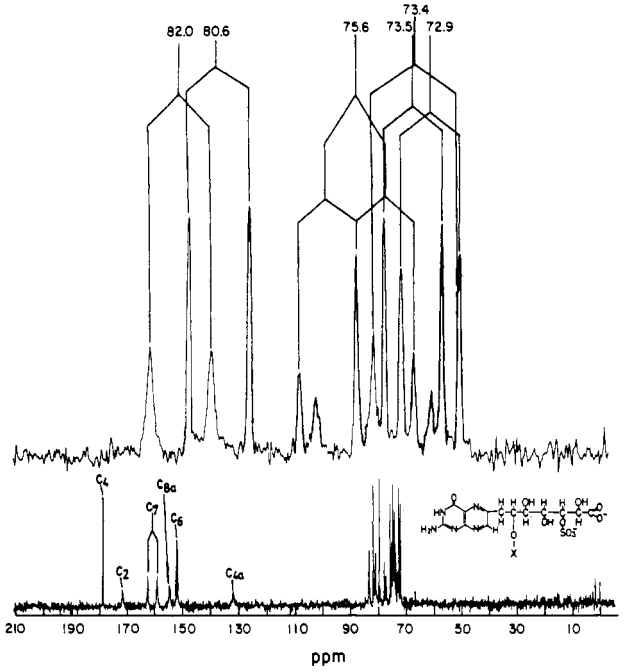

FIGURE 6: The 67.9-MHz Fourier transform CMR spectrum of SHP-2. SHP-2 (39 μmol) was dissolved in ~0.5 mL of ~1 M deuteriated ammonium hydroxide in deuteriated water in a 5-mm NMR tube. The spectrum was recorded at room temperature with sodium [2,2,3,3-$^2H_4$]-3-(trimethylsilyl)propionate (TSP) as the internal standard. The X in the formula represents an undefined group.

CMR of SHP-2. Figure 6 shows the CMR spectrum of SHP-2 that has been dissolved in dilute ammonia. The spectrum shows the expected resonances for a 6-substituted pterin on the basis of the assignments of carbon resonances in known pterins (Ewers et al., 1973, 1974a,b; Geerts et al., 1976; Schircks et al., 1976; Tobias et al., 1985). The substitution of the pterin at the 6-position is confirmed by the large splitting observed for the C-7 pterin carbon at 160 ppm due to the ^{13}C-7–^1H-7 coupling. In addition to the pterin carbon resonances, five hydroxymethenyl carbon resonances and one methylene carbon resonance were also observed. The hydroxymethenyl resonances were all observed as doublets, and the single methylene resonance was observed as a triplet. This indicates that the methenyl carbons have one attached proton and that the methylene carbon has two protons.

The chemical shift assignments for the side-chain carbons of SHP-2 as polyol carbons were according to Koerner et al. (1973), O'Connor et al. (1979), and Bock and Thøgersen (1982). The methylene resonances were assigned on the basis of the NMR of a synthetic sample of 2′,3′,4′-(trihydroxybutyl)pterin (X. Lin and R. H. White, unpublished results).

Figure 7 shows the proton-decoupled CMR spectrum of SHP-2. A comparison of the polyol carbon resonances of the proton-coupled (Figure 6) and proton-decoupled (Figure 7) ^{13}C NMR spectra of SHP-2 is as follows: the 82.0, 80.6, and 72.9 ppm peaks are the same; the 75.6 ppm peak in Figure 6 corresponds to the 75.5 ppm peak in Figure 7 (methylene carbon); the two peaks, 73.5 and 73.4 ppm, in Figure 6 have fused into one peak (73.5 ppm) in Figure 7. Actually, it is not uncommon for two separate carbons in a polyol spectrum to display one peak in CMR spectra (Koerner et al., 1973; O'Connor et al., 1979; Bock & Thøgersen, 1982).

By using the technique of insensitive nucleus enhancement through polarization transfer (INEPT), it is possible to distinguish between carbons that contain one or three protons from those that contain two protons. Carbons with one or three protons are found to produce a positive signal, whereas

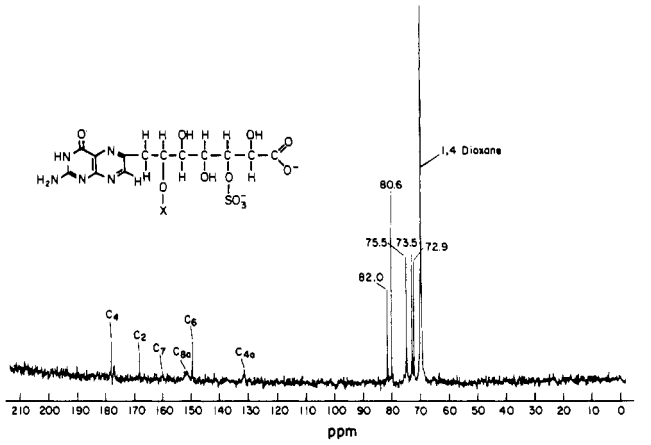

FIGURE 7: The 67.9-MHz Fourier transform proton-decoupled ^{13}C NMR spectrum of SHP-2. SHP-2 (19 μmol) was dissolved in ~0.5 mL of 1 M deuteriated ammonium hydroxide in deuteriated water in a 5-mm NMR tube. The spectrum was recorded at room temperature with 1,4-dioxane as the internal standard, and the chemical shift was then corrected to TSP.

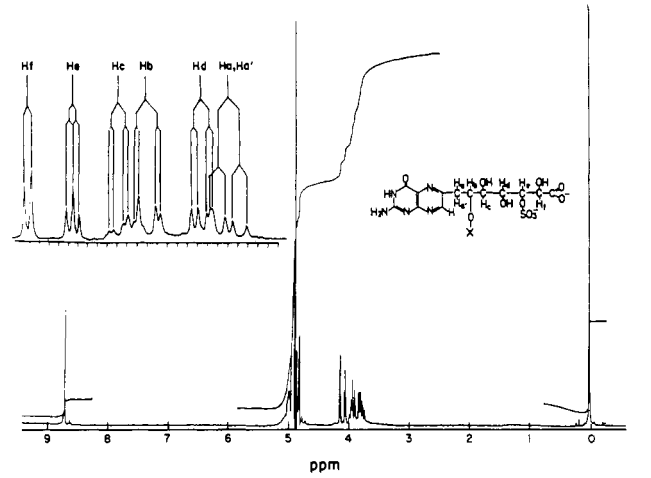

FIGURE 8: The 270-MHz Fourier transform PMR spectrum of HP-2. HP-2 (4 μmol) was dissolved in ~0.5 mL of 1 M deuteriated ammonium hydroxide in deuteriated water in a 5-mm NMR tube. The spectrum was recorded at room temperature with TSP as the internal standard.

those with two protons produce a negative carbon signal. (For the SHP-2 spectrum, no resonances were observed between 5 and 50 ppm, the chemical shift range for methyl groups.) The INEPT spectrum of the proton-decoupled C-13 of SHP-2 shows that the carbon at 75.5 ppm is a methylene carbon and that the other carbons contain only one proton. This is consistent with the proton-coupled ^{13}C NMR of SHP-2 (Figure 6).

The PMR spectra of SHP-2 and HP-2 (Figure 8) are very similar in that each shows a single pterin resonance at ~8.9 ppm and seven additional resonances which occur from 3.5 to 4.2 ppm. Assignments of these resonances are as shown in Figure 8. Proton spin-decoupling experiments with both SHP-2 and HP-2 were in complete agreement with the indicated proton assignments shown in Figure 8 (data not shown).

If the dimer structure for SHP-2 is correct, then periodate oxidation, followed by borohydride reduction, of SHP-2 would result in the dimeric pterin shown in Figure 9. The pterin portion of the products (SHP-OR) can easily be identified by fluorescence and can be purified and converted into the $(TMS)_6$ derivative as described above. The expected TMS derivative of SHP-OR would have an elemental formula of

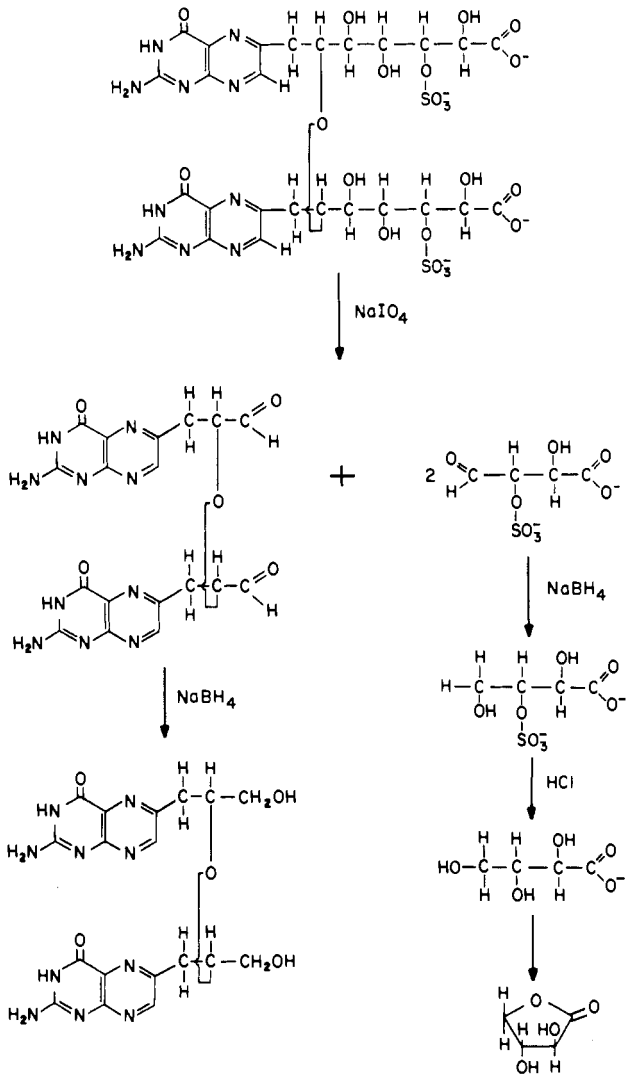

FIGURE 9: Reaction sequence of periodate oxidation and the borohydride reduction of SHP-2.

$C_{36}H_{68}O_5N_{10}Si_6$, which corresponds to a mass of 888. When the mass spectrum of this derivative was recorded, an intense ion was observed at m/z 873 ($M^+ - CH_3$), which is characteristic for TMS derivatives. This information, along with the NMR and periodate cleavage data, confirms that an ether linkage from the 2′-carbons of the pterin side chains must be responsible for the formation of the dimer.

The other product that is derived from the periodate cleavage and sodium borohydride reduction of SHP-2 is the sulfated tetraonic acid shown in Figure 9. In order to isolate this molecule, reactions similar to those shown in Figure 9 were performed except that $NaBT_4$ was used instead of $NaBH_4$ in the reduction step. By replacement of one of the γ-protons of the resulting tetraonic acid with tritium, the tetraonic acid and its derivatives can be detected by radioactivity. Paper electrophoresis of the products derived from the periodate oxidation of SHP-2 and the borotritide reduction of the resulting aldehyde led to the isolation of two anionic, radioactive peaks. The dominant peak is a tetraonic acid 3-sulfate and the other peak is the tetraonic acid that is produced by the hydrolysis of tetraonic acid 3-sulfate during the reaction process. The tetraonic acid 3-sulfate was completely hydrolyzed to tetraonic acid by acid hydrolysis.

Radio-GC analysis of the TMS derivative of this acid showed two radioactive peaks. The mass spectrum of the first peak to elute corresponded to the $(TMS)_2$ derivative of the tetraonic acid lactone (M^+, m/z 262), and the spectrum of the second peak corresponded to the $(TMS)_4$ derivative of the acid ($M^+ - 15$, m/z 409) (Petersson, 1970; Thompson et al., 1975). Furthermore, these GC peaks had retention times that corresponded to the $(TMS)_2$ derivative of threonic acid lactone and the $(TMS)_4$ derivative of threonic acid, respectively. Also, since both peaks separated from the corresponding erythronic acid derived compounds, the isomer that was derived from SHP-2 had to be threonic acid. This stereochemistry supports the idea that the side chain of SHP-2 could be biosynthesized by the condensation of the C-1 of galacturonic acid with either 6-methylpterin or 6-(hydroxymethyl)-7,8-dihydropterin pyrophosphate, an important intermediate in folic acid biosynthesis (Jaenicke & Chan, 1960). In the former case, the addition of the anionic methyl of 6-methylpterin to the C-1 of galacturonic acid would generate the required condensation product, which would contain an oxidized pterin. In the latter case, the C-1 of galacturonic acid, made anionic perhaps through the assistance of thiamin pyrophosphate, would displace the pyrophosphate from 6-(hydroxymethyl)-7,8-dihydropterin to generate a dihydropterin-containing condensation product. In either case, reduction of the pterin and formation of the dimer would be required to complete the biosynthesis of reduced SHP-2.

Registry No. SHP-2, 109686-75-1.

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Interaction of Plant Viruses and Viral Coat Proteins with Mixed Model Membranes†

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ABSTRACT: The interaction between model membranes and viruses, empty capsids, and coat protein dimers has been investigated. Spherical plant viruses (cowpea chlorotic mottle virus, brome mosaic virus, and southern bean mosaic virus), a rod-shaped plant virus (tobacco mosaic virus), and well-defined aggregation states of their proteins have been used. Turbidity measurements at 550 nm of neutral and positively and negatively charged small unilamellar vesicles interacting with viral material indicated electrostatic and indirect hydrophobic interactions. Electrostatic interaction resulted in lipid–protein complexes, which precipitate. Indirect hydrophobic interaction produced precipitates which contained lipid but no protein. Virus particles and empty capsids of the spherical viruses reacted with charged vesicles through electrostatic interaction. Coat protein dimers of all plant viruses induced vesicle fusion by interaction of the exposed hydrophobic protein domains with neutral vesicles. Further characterization of the precipitates by ^{31}P nuclear magnetic resonance and electron microscopy indicated that both interactions resulted in formation of multilayer structures. Protein assays after incubation at various salt concentrations showed that protein was never incorporated into the bilayer to form a stable complex held together by direct hydrophobic lipid–protein interactions. From the results, it is concluded that such hydrophobic lipid–coat protein interactions do not occur, although hydrophobic protein domains are able to destabilize membranes and induce fusion.

The initial stages of nonenveloped plant virus infection involve penetration of the virus into the cell and subsequent dissociation of the nucleoprotein particle. Conclusions about the site and mechanism of penetration are still controversial. Various components of the cell are reported to affect the dissociation of plant virus particles: the cell wall (Gaard & De Zoeten, 1979; De Zoeten, 1981), the plasma membrane (Kiho et al., 1976, 1979a,b), lipids (Kiho et al., 1980; Banerjee et al., 1981a,b; Abdel-Salam et al., 1982), protoplast membranes (Watts et al., 1981; Watts & King, 1984; Hull & Maule, 1985), and ribosomes (Wilson, 1984).

At present, several models for initial interactions between virus and cell exist. By analogy with the observation that hydrophobic intersubunit bonds play a role in tobacco mosaic virus (TMV)[1] nucleocapsid assembly, it was proposed that a hydrophobic environment is involved in the uncoating of the viral RNA in vivo (Caspar, 1963). Also, divalent cations have been suggested to regulate assembly and disassembly (Durham et al., 1977). Using this observation, a model including both virus penetration and uncoating was postulated, in which the coat protein subunits become integral membrane proteins stabilized by hydrophobic lipid–protein interactions (Durham, 1978). This model is proven for the filamentous bacteriophage M13, in which the coat protein is assumed to span the cytoplasmic bilayer of *Escherichia coli* during a particular stage of infection (Marvin & Wachtel, 1975). Recently, Wilson (1985) suggested a cotranslational disassembly of destabilized TMV and SBMV as a mechanism for uncoating of viral nucleic acid, and evidence was presented that TMV particles disassemble cotranslationally in vivo (Shaw et al., 1986).

We investigated the nature of the interaction (hydrophobic or electrostatic) between artificial membranes and several plant viruses and their coat proteins to mimic and determine the role of the lipid component of the host plasma membrane in initial

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[1] Abbreviations: TMV, tobacco mosaic virus; CCMV, cowpea chlorotic mottle virus; BMV, brome mosaic virus; SBMV, southern bean mosaic virus; DLPC, dilauroylphosphatidylcholine; DLPA, dilauroylphosphatidic acid; DMPG, dimyristoylphosphatidylglycerol; PALCHOL, palmitoylcholine iodide; CTAB, cetyltrimethylammonium bromide; SUVs, small unilamellar vesicles; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; p*I*, isoelectric point; A_{550nm}, turbidity at 550 nm; EM, electron microscopy; NMR, nuclear magnetic resonance; ppm, parts per million; CSA, chemical shift anisotropy; $\Delta\nu_{1/2}$, line width at half-height; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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