Characterization of Human S Protein, an Inhibitor of the Membrane Attack Complex of Complement. Demonstration of a Free Reactive Thiol Group†

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ABSTRACT: S protein, an inhibitor to the membrane attack complex of complement, was purified from human plasma. The procedure involved barium citrate adsorption and fractionation by poly(ethylene glycol) 4000 precipitation, followed by chromatography on DEAE-Sephacel, Blue Sepharose, Sephacryl S-200, and finally anti-albumin–Sepharose. Reduced glutathione was added throughout to inhibit spontaneous formation of disulfide-linked S-protein dimers. The recovery was 7%, resulting in approximately 10 mg of pure S protein from 1 L of starting plasma. S protein is a single-chain molecule; sedimentation equilibrium ultracentrifugation yielded a molecular weight of 83 000; the $s^0_{20,w}$ value was estimated to be 4.0 S. The purified protein contained a free, reactive thiol group causing spontaneous formation of disulfide-linked S-protein dimers. Alkylated and nonalkylated S proteins were equally active in inhibiting C9 polymerization, catalyzed by the C5b–8 complex. In parallel with the inhibition of C9 polymerization, nonalkylated S protein catalyzed the formation of disulfide-linked C9 dimers, presumably through disulfide interchanges.

The membrane attack sequence is the common cytolytic pathway of the classical and alternative pathways of complement and involves five plasma proteins, C5–C9 (Muller-Eberhard, 1980). Assembly of the membrane attack complex (MAC)[1] is initiated by the proteolytic modification of C5 to C5b by the classical or alternative pathway C5 convertase (Muller-Eberhard & Schreiber, 1980; Reid & Porter, 1981). C5b remains associated with the C5 convertase of either pathway and successively reacts with C6, C7, and C8 to form the C5b–8 complex which then catalyzes polymerization of C9, resulting in the formation of a membrane-penetrating protein tubule with cytolytic activity (Tschopp & Podack, 1981; Tschopp et al., 1982; Bhakdi & Tranum-Jensen, 1981; Ramm et al., 1982). Although the five individual proteins (C5–C9) are hydrophilic, their assembly product (the MAC) is amphiphilic. The first hydrophilic–amphiphilic transition occurs upon binding of C7 to C5b–6 (Podack et al., 1978b; Podack & Muller-Eberhard, 1978). The functional correlate of this reaction is the hydrophobic insertion of C5b–7 into the target membrane.

S protein is a plasma protein functioning as a regulator of the membrane attack complex (Podack et al., 1978a; Podack & Muller-Eberhard, 1979, 1980b; Bhakdi & Tranum-Jensen, 1982). It binds to the newly exposed hydrophobic region of the C5b–7 complex, thereby inhibiting membrane insertion and subsequent membrane attack. S protein renders the forming SC5b–7 complex soluble. SC5b–7 reacts with C8 and C9; however, C9 polymerization does not occur in SC5b–9 (Podack et al., 1984). S protein may also have a regulatory role in the coagulation system. It interacts with thrombin and decreases the rate of inactivation by antithrombin III (AT III). S protein remains bound to the resulting thrombin–AT III complex, and the trimolecular complex can be demonstrated in serum (Podack & Muller-Eberhard, 1979).

Studies of S proteins have been hampered by difficulties in purifying the protein. In 1979, Podack and Muller-Eberhard reported the successful isolation of S protein from plasma, with a yield of 0.5% (Podack & Muller-Eberhard, 1979). The authors pointed out that the low recovery was due to a high tendency of the S protein to aggregate and to adsorb to various surfaces. We now report an improved purification procedure resulting in approximately 15 times higher yield. With this new procedure, the larger quantities of S protein available permitted a further biochemical and functional characterization of the protein.

Experimental Procedures

DEAE-Sephacel, Blue Sepharose, and Sephacryl S-200 were from Pharmacia Fine Chemicals. Iodo[1-^{14}C]acetamide was purchased from Amersham Corp. Bio-rad protein assay and molecular weight standard proteins were from Bio-Rad. Phenylmethanesulfonyl fluoride (PMFS), *p*-nitrophenyl *p*-guanidinobenzoate (NPGB), reduced glutathione, benzamidine hydrochloride, poly(ethyleneglycol) 4000, 2,2′dithiodipyridine, and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Ellmans' reagent) were from Sigma Chemical Corp. Freshly frozen citrated plasma was purchased from the San Diego Blood Bank.

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[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate; PEG, poly(ethylene glycol); MAC, membrane attack complex; AT III, antithrombin III; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; PTH, phenylthiohydantoin; HPLC, high-pressure liquid chromatography; EDTA, ethylenediaminetetraacetic acid; kDa, kilodalton; PAGE, polyacrylamide gel electrophoresis; CPD, citrate–phosphate–dextrose. The nomenclature for complement proteins is that recommended by the World Health Organization (1968, 1981).

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The complement proteins [C5b–6 (Podack & Muller-Eberhard, 1980), C7 (Podack et al., 1979), C8 (Kolb & Muller-Eberhard, 1976), and C9 (Biesecker & Muller-Eberhard, 1980)] were purified as described.

Determination of Physical Parameters of S Protein. The sedimentation coefficient of S protein was determined in a Beckman Model E analytical ultracentrifuge equipped with a photoelectric scanner. Three S-protein concentrations (0.41, 0.20, and 0.10 mg/mL in 50 mM Tris-HCl and 0.15 M NaCl (pH 7.4) were analyzed. The rotor velocity was 47 660 rpm, and the temperature was 20 °C. The sedimentation values were extrapolated to zero protein concentration to obtain $s^0_{20,w}$. The molecular weight of S protein was determined by sedimentation equilibrium ultracentrifugation in the same instrument. The initial protein concentration was adjusted to give an adsorbance at 280 nm of 0.4. The solvent was 50 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl. Human serum albumin (molecular weight 66 200) was used in control runs to ascertain the validity of the results. The centrifuge was run at 20 °C at a speed of 13 410 rpm. An assumed partial specific volume of 0.72 mL/g was used in the molecular weight calculation. The extinction coefficient at 280 nm of S protein was calculated by amino acid analysis as follows: The absorbance at 280 nm of an S-protein solution, in 0.1 M NH_4HCO_3, pH 7.5, was measured. Aliquots (100 and 150 μL) were drawn in triplicate into hydrolysis tubes containing a known amount of L-norleucine. The tubes were evaporated to dryness and then subjected to hydrolysis (24 h) in vacuo in 6 N HCl. The amount of S protein in each tube was determined by amino acid analysis (Asp and Glu were used). A molecular weight of 80 000 was used in the calculations.

Amino Acid Composition. Protein samples were hydrolyzed for 24 and 120 h at 110 °C in 6 N HCl under vacuum and analyzed on a Beckman Model 121 M amino acid analyzer (Moore & Stein, 1963). Half-cystine and methionine were determined after performic acid oxidation (Moore, 1963) or after reduction and carboxymethylation with iodoacetic acid.

Amino-Terminal Sequence Analysis. Automated Edman degradation (Edmann & Begg, 1967) was performed with a Beckman Model 890D sequenator using a 0.1 M Quadrol program. Phenylthioydantoin (PTH) derivatives of amino acids were identified by HPLC as described previously (Meuth et al., 1983).

Electrophoretic and Immunochemical Techniques. Agarose gel electrophoresis was performed at pH 8.6 as described (Jeppsson, 1979), using a 15 mM Tris and 40 mM glycine buffer containing 4 mM EDTA. Crossed immunoelectrophoresis (Ganrot, 1972), electroimmunoassay (Laurell, 1972), and fused rocket immunoelectrophoresis (Svendson, 1973) were run at pH 8.6 in 38 mM Tris, 0.1 M glycine, and 10 mM EDTA, pH 8.6. SDS–polyacrylamide slab gel electrophoresis was performed as described (Podack & Tschopp, 1982), using 7.5–12.5% or 2.5–10% gradient gels. The buffer system of Laemmli (1970) was used. The molecular weight was determined by comparison with those of standard proteins (molecular weights in parenthesis): myosin (200 000), β-galactosidase (116 250), phosphorylase *b* (92 500), bovine serum albumin (66 200), and ovalbumin (45 000).

Determination of SH Content. The SH content of S protein was determined spectrophotometrically with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) as described by Janatova et al. (1968). The S-protein concentrations used were 0.41 and 0.58 mg/mL. The SH content was also determined by radioalkylation. A solution of iodo[1-^{14}C]acetamide with a specific activity of 1 μCi/mol was prepared by adding 0.5 mL of 0.1 M iodoacetamide to an ampule containing 50 μCi of dry iodo[^{14}C]acetamide. Forty microliters of this solution was added to 200 μL of S protein (1.4 mg/mL) in 0.2 M Tris-HCl, pH 8.2, containing 2 mM EDTA. After 2-h incubation at 37 °C in the dark, the bound radioactivity was determined after dialysis and the absorbance at 280 nm measured. The alkylated protein was also analyzed by SDS–polyacrylamide gel electrophoresis. The stained gel was sliced and the amount of radioactivity associated with each slice measured. The described procedure was also used to quantify the SH content in denatured S protein by using 0.1 M Tris-HCl and 6 M guanidine hydrochloride, pH 8.0, containing 5 mM EDTA.

Effect of S Protein on C9 Polymerization. The polymerization of C9 induced by C5b–8 in the fluid phase was followed by SDS–polyacrylamide gel electrophoresis essentially as previously described (Podack et al., 1982). ^{125}I-Labeled S protein (100, 250, and 500 μg/mL) was incubated with C5b–6 (150 μg/mL), C8 (75 μg/mL), and ^{131}I-labeled C9 (225 μg/mL) in 50 mL of Tris-HCl and 0.15 M NaCl, pH 7.4, at 37 °C. The reaction was started by the addition of C7 (50 μg/mL). After 30 min, 80 μL of the reaction mixtures was mixed with SDS (2% final solution) and analyzed by SDS–polyacrylamide slab gel electrophoresis. The effect of S protein with a blocked SH group (alkylated under nondenaturing conditions as described above) was studied in a parallel experiment.

Conversion of S-Protein Monomer to Dimer. S protein (0.4 mg/mL) in Tris-buffered saline, pH 7.4, was incubated with 2,2′-dithiodipyridine (50 μM final concentration) at 37 °C for 60 min. After dialysis in the same buffer, the sample was analyzed in the analytical ultracentrifuge for comparison to monomeric S protein. S-protein dimers were reconverted to monomers with 1 mM dithiothreitol (1 h, 37 °C) and subsequent dialysis.

Radiolabeling. S protein was labeled with ^{125}I and C9 with ^{131}I by using the iodogen method (Fraker & Speck, 1978).

Purification of S Protein. Chromatographies and all manipulations of the samples were performed in the cold room (4–6 °C). All centrifugations were done at 4 °C.

Five units (0.95 L) of freshly frozen human citrated (CPD-adenine; Travenol Laboratories) plasma were thawed at 37 °C and then cooled in an ice bath. Reduced glutathione (1 mM) and the following protease inhibitors were added: benzamidine hydrochloride (10 mM), PMSF (1 mM), and NPGB (0.5 mM). Barium citrate adsorption was performed essentially according to Moore et al. (1965). Eighty milliliters of 1 M $BaCl_2$ was added dropwise per liter of plasma. After the mixture was stirred for H 1 h, the barium citrate was removed by centrifugation at 6000*g* for 15 min. To the supernatant plasma (1 L) was added 220 mL of 50% PEG 4000 in 30 mM Tris-HCl, pH 7.4, 0.1 M NaCl, and 1 mM EDTA, giving a final PEG concentration of approximately 9%. The plasma was stirred for 1 h and then centrifuged at 6000*g* for 15 min. The precipitate was discarded, andd 315 mL of the 50% PEG 4000 solution (see above) was added to the supernatant plasma (1.12 L), resulting in a final PEG concentration of 20%.

The plasma was stirred for another h, and the PEG precipitate was collected by centrifugation at 6000*g* for 15 min. The 9–20% PEG precipitate was dissolved in 20 mM sodium phosphate and 2 mM EDTA, pH 7.0, containing 2 mM benzamidine hydrochloride, 1 mM glutathione, andl mM PMSF and applied to a column (2.9 × 40 cm) with DEAE-Sephacel in the same buffer (Figure 1). After the column was washed with approximately 600 mL of equilibration

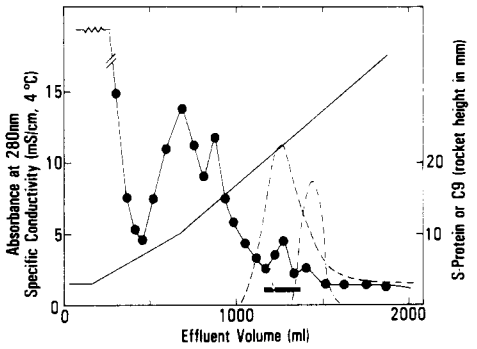

FIGURE 1: DEAE-Sephacel chromatography of 9–20% PEG 4000 fraction. The column (2.9 × 40 cm) was equilibrated in 10 mM sodium phosphate and 2 mM EDTA, pH 7.0, containing 2 mM benzamidine hydrochloride, 1 mM glutathione, and 1 mM PMSF. After application of the sample, the column was washed with approximately 600 mL of equilibration buffer containing 25 mM NaCl. The adsorbed proteins were eluted with a gradient of NaCl (0.025–0.3 M), 1 L in each vessel. The flow rate was 70 mL/h, and 12-min fractions were collected. (●) Absorbance at 280 nm; (—) specific conductivity; (--) S protein, height of fused rockets; (-·-) C9, height of fused rockets. The black bar indicates the S-protein pool.

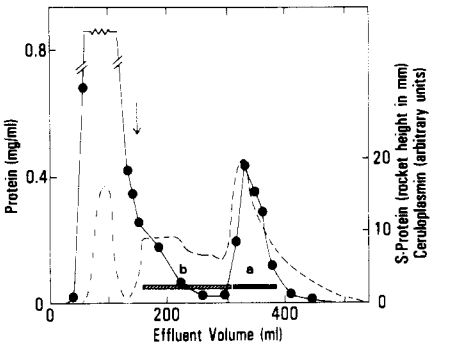

FIGURE 2: Blue Sepharose chromatography. The column (2.5 × 25 cm) was equilibrated in 50 mM Tris-HCl, 0.15 M NaCl, and 2 mM EDTA, pH 7.4, containing 2 mM benzamidine hydrochloride and 1 mM reduced glutathione. After application of the sample, the column was washed with approximately 120 mL of starting buffer. The arrow indicates the elution with 1 M NaCl in equilibration buffer. The flow rate was 110 mL/h, and 4-min fractions were collected. (●) Protein measured according to Bradford (1976); (-···-) ceruloplasmin estimated by the absorbance at 660 nm; (--) S protein, height of fused rockets. The two horizontal bars represent pools a and b.

buffer, also containing 25 mM NaCl, the adsorbed proteins were eluted with a linear gradient of NaCl (0.025–0.3 M), 1 L in each vessel. The positions of S protein and C9 were determined by fused rocket immunoelectrophoresis and ceruloplasmin by its absorbance at 600 nm. The fractions containing S protein were pooled as indicated in Figure 1 and concentrated with solid $(NH_4)_2SO_4$ at 70% saturation. After the mixture was stirred for 1 h, the precipitate was collected by centrifugation at 10000*g* for 20 min and dissolved in 50 mM Tris-HCl and 0.15 M NaCl, pH 7.4, containing 2 mM benzamidine hydrochloride and 1 mM reduced glutathione. The sample was dialyzed against the same buffer and layered on top of a column (2.5 × 25 cm) containing Blue Sepharose (Figure 2), equilibrated in the same buffer. After the sample had entered the gel, the column was washed with approximately 120 mL (one column volume) of equilibration buffer. The adsorbed proteins were then eluted with 1 M NaCl in starting buffer. The protein concentration of each fraction was quantified according to Bradford (1976). The positions of S protein, C9, and ceruloplasmin were determined as described above. The fractions were pooled as indicated in Figure 2. The two S-protein pools were then concentrated by ultrafiltration, using a YM 10 membrane, and applied to a gel

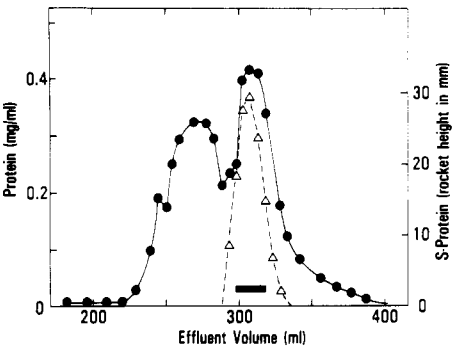

FIGURE 3: Gel filtration chromatography on Sephacryl S-200. The column (2.8 × 105 cm) was equilibrated in the same buffer as that given in the legend to Figure 2. The figure represents the chromatogram obtained with pool a from the Blue Sepharose chromatography. The flow rate was 30 mL/h, and 10-min fractions were collected. (●) Protein measured according to Bradford (1976); (△) S protein, height of rockets; note in this case it is not a fused rocket. The S-protein pool is indicated by the black bar.

filtration column (2.8 × 105 cm) with Sephacryl S-200 equilibrated in a buffer having the same composition as that used in the Blue Sepharose chromatography (Figure 3). Fractions were monitored for S protein by using an electroimmunoassay and pooled as indicated by the bar in Figure 3. Contaminating albumin was removed by passing the material over a column (1.4 × 12 cm) with anti-albumin–Sepharose in 50 mM Tris-HCl, pH 7.4, and 0.15 M NaCl, pH 7.4, containing 2 mM EDTA, 2 mM benzamidine hydrochloride, and 1 mM reduced glutathione. The purified S protein was concentrated to a concentration of 2–3 mg/mL by ultrafiltration using a YM 20 filter and stored at −70 °C.

RESULTS

Comments on the Purification Procedure. The major problem hampering a successful purification of S protein has been the S-protein heterogeneity as suggested from the trailing of S protein over a large part of the chromatogram (Podack & Muller-Eberhard, 1979). This is partly due to a tendency of S protein to form noncovalent aggregates. In some earlier preparations, we have also observed that S protein forms disulfide-linked dimers during its purification, suggesting the presence of a reactive thiol in the protein. The isolation procedure described here was designed so as to minimize these problems. The initial steps were rapid and avoided dialysis and concentration by ultrafiltration. To inhibit proteolysis, several protease inhibitors were added throughout the purification. Reduced glutathione was included to inhibit the formation of disulfide-linked dimers. The purification procedure is summarized in Table I.

The barium citrate adsorption was included to separate S protein from prothrombin, which otherwise contaminated the final product in trace amounts. The advantage of the fractionated PEG precipitation was that the after the proteins dissolved, they could be directly applied to DEAE-Sephacel chromatography within 1 day.

The DEAE-Sephacel chromatography resulted in a 50-fold purification. S protein eluted as a distinct, relatively symmetrical peak (Figure 1). The small tendency to trail was much more pronounced when the starting material was outdated plasma and when protease inhibitors and glutathione were not added throughout. The positions of ceruloplasmin and C9 were used as a reference. Under optimal conditions, S protein coincided with ceruloplasmin and preceded C9. S protein eluting after C9 usually was aggregated as judged by gel filtration chromatography (not shown). Disulfide-linked

Table I: Purification of S Protein

fraction	volume (mL)	total protein	S protein[a] (mg)	x-fold purification	% recovery
plasma	950	57000	142		100
barium citrate, supernate	1000	53000	116	0.88	81.7
9–20% PEG 4000 ppt	470	38000	76	0.8	53.5
DEAE-Sephacel	215	305	29	38.2	20.4
Blue Sepharose					
pool a	74	28.5	9.7	137	11.6
pool b	147	16.4	6.8	167	
Sephacryl S-200					
pool a	23.5	8.5	6.2	292	7.4
pool b	23.5	6.6	4.3	261	
anti-albumin–Sepharose					
pool a	26.5	7.6	5.5	289	6.5
pool b	27	5.0	3.6	288	

[a] Determined by electroimmunoassay.

S-protein dimers, occasionally observed when glutathione was not included during the preparation, also eluted in this region.

Blue Sepharose chromatography was found to separate ceruloplasmin and C9 from S protein (Figure 2). This was difficult to achieve by other chromatographic techniques. The adsorbed S protein was eluted in one step with 1 M NaCl since gradients resulted in broad peaks dispersing the protein in inconveniently large volumes. As seen in Figure 2, the S protein was eluted already in the wash even when the NaCl concentration in the washing buffer was lowered to 0.1 M. However, S protein always separated from ceruloplasmin which passed directly through the column. C9 was recovered in the later part of the breakthrough peak. The two pools (a and b) from the Blue Sepharose were concentrated by ultrafiltration and applied separately to gel filtration chromatography on Sephacryl S-200 from which they eluted as identical protein peaks of equal purity (shown for pool a in Figure 3). S protein, after gel filtration, was recovered in the second protein peak together with albumin which was subsequently removed by an anti-albumin–Sepharose. The purified S protein was stable at −70 °C.

The S-protein concentration in human plasma was estimated by electroimmunoassay to be approximately 140 mg/L. S protein was purified 290-fold with recovery of approximately 7%, yielding 10 mg from 1 L of starting material.

Electrophoretic Characterization of Purified S Protein. To address the question as to whether the previously observed two-chain structure of S protein (Podack & Muller-Eberhard, 1979) was due to cleavage during purification S protein was prepared with and without added PMSF (I and II, respectively) and compared. Outdated plasma was used as starting material for the preparation performed in the absence of PMSF (preparation II). Each preparation yielded two pools (a and b) after Blue Sepharose chromatography and gel filtration. On SDS–polyacrylamide gel electrophoresis (Figure 4), S protein of preparations I + II appeared as doublet bands under reducing (M_r 80000 and 63000) and nonreducing conditions. However, preparation I (PMSF present) contained more of the 80-kDa protein than preparation II. In addition, pools a of both preparations contained more of the 80-kDa species than pools b. Upon reduction, the proportion of the 63-kDa protein increased in all pools. The small peptide chain (M_r 17000) liberated after reduction was seen only after the gel was heavily overloaded (Podack & Muller-Eberhard, 1979). In contrast to the heterogeneity of S protein observed on SDS–PAGE, all preparations showed a single band on agarose gels (Figure 5A) which reacted with monospecific anti-S-protein antiserum (Figure 5B). A minor slower migrating band, detected in pools Ib and IIb, has not been further characterized. The results suggest proteolytic cleavage of S protein during the purification procedure, which was more pronounced in the absence of PMSF.

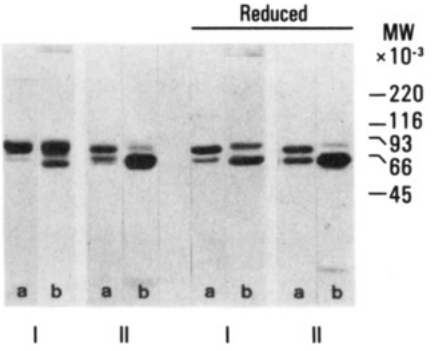

FIGURE 4: SDS–polyacrylamide gradient (7.5–12.5%) slab gel electrophoresis of purified S-protein pools. S protein (pools a and b) from two different preparations (I and II) was analyzed before (left lanes) and after (right lanes) reduction of disulfide bridges with 5% β-mercaptoethanol at 100 °C for 2 min. Ten to twenty micrograms was applied per lane. The positions of molecular weight standard proteins are indicated to the right.

Table II: Amino Acid Composition of S Protein

amino acid[a]	prepn pool Ia	prepn pool IIa
Lys	36.6	31.5
His	14.0	11.7
Arg	50.0	43.6
Cys	20.1[e]	12.4[f]
Asp	70.0	61.5
Thr[b]	28.0	29.6
Ser[b]	51.1	47.2
Glu	89.5	78.8
Pro	52.2	44.5
Gly	57.0	50.8
Ala	42.7	41.1
Val[c]	37.6	31.5
Met	7.7	8.0
Ile[c]	20.7	19.3
Leu	41.0	40.9
Tyr	30.8	24.5
Phe	34.6	29.2
Trp[d]	n.d.[g]	9.1

[a] Residues per molecule of protein calculated with an assumed molecular weight of 70000 for the apoprotein. [b] Extrapolated to zero-time hydrolysis. [c] 120-h hydrolysis value. [d] Determined according to Hugli & Moore (1972). [e] Determined as cysteic acid after performic acid oxidation. [f] Determined as carboxymethylcysteine. [g] Not determined.

Physical Parameters. The molecular weight of S protein was determined by sedimentation equilibrium ultracentrifugation to be 83000 (two determinations, 81700 and 84200). The $s^0_{20,w}$ value was estimated to be 4.0 S which is lower than the reported value of 4.7 S determined by sucrose density

gradient centrifugation (Podack & Muller-Eberhard, 1971a). The extinction coefficient (ϵ) at 280 nm was 1.38 cm^2 mg^{-1}.

Amino Acid Composition and Amino-Terminal Sequence Analysis. The amino acid compositions of S-protein pool Ia and pool IIb are given in Table II. Although appearing quite different on SDS–polyacrylamide gel electrophoresis, the two S proteins had similar amino acid compositions. Reduced and carboxymethylated S protein, Ia and IIb, was subjected to NH_2-terminal sequence analysis. Two sequences were observed, but the ratios of observed amino acids differed in the two preparations. Approximately 11 mol of S-protein pool Ia gave the following result (nmol in parentheses): step 1, Asp (4.4) and Ala (2.1); step 2, Gln (3.2) and X; step 3, Cys (2.7) and Trp (0.6). Approximately 18 nmol of S-protein pool Ib yielded the following: step 1, Asp (7.7) and Ala (16); step 2, Gln (7.2) and X; step 3, Cys (4.6) and Trp (4.1). The heterogeneity in the NH_2-terminal region was probably caused by proteolysis during purification. Undegraded S protein presumably has Asp in the NH_2-terminal position, whereas Ala occupies the NH_2-terminus after proteolysis.

Demonstration of Free SH Groups in S Protein. The number of free SH groups in S protein (pool IIa) was estimated spectroscopically by using DTNB at 0.78 and 0.88 mol/mol (measured at S-protein concentrations of 0.41 and 0.58 mg/mL, respectively). Bovine serum albumin, used at three different concentrations (0.56, 0.8, and 1.3 mg/mL), gave values of 0.27, 0.24, and 0.35 SH group per molecule, respectively. Estimated by radioalkylation under denaturing conditions, the number of free SH groups in S protein pool Ia and pool IIb was 1.20 and 1.28, respectively. On SDS–polyacrylamide gel electrophoresis, the radiolabel was associated with the dominating protein band, i.e., with the 80-kDa band in pool Ia and the 63-kDa band in pool IIb (not shown). Under nondenaturing conditions, S protein (pool IIa) incorporated 1.9 mol of [^{14}C]carboxamidomethyl groups per mole of protein, and the radiolabel was equally distributed in the 80- and 63-kDa bands on SDS–polyacrylamide gel electrophoresis (not shown).

Interconversion of S-Protein Monomers and Dimers by Sulfhydryl Reagents. The free sulfhydryl group of S protein can give rise to disulfide-linked S-protein dimers during purification. This reaction was studied with purified S-protein using the analytical ultracentrifuge. The sedimentation coefficient of monomeric S protein was 4.0 S (Figure 6A), whereas purified disulfide-linked S-protein dimer had an approximate sedimentation coefficient of 8 S (Figure 6C). Incubation of the dimer with reducing agent changed its sedimentation rate to that of the monomer (Figure 6D), whereas monomeric S protein was converted to a faster sedimenting species after incubation with the sulfhydryl reagent 2,2′-dithiodipyridine (Figure 6B). The interconversion of monomeric and dimeric forms could also be demonstrated on SDS–polyacrylamide gel electrophoresis (not shown).

Inhibition of C9 Polymerization. It was shown recently that S protein inhibits C9 polymerization in the SC5b–9 complex (Podack et al., 1984). Because Yamamoto et al. (1982) and Ware & Kolb (1981) have demonstrated that disulfide-linked C9 polymers and dimers, respectively, are formed during MAC assembly, it was of interest to determine the role of the free SH group in S protein in this reaction. C9 polymerization, induced by the C5b–8 complex, was monitored by SDS–polyacrylamide gel electrophoresis. Alkylated (Figure 7B) and nonalkylated (Figure 7A) S protein added to the reaction mixture resulted in an equal inhibition of C9 polymerization as measured by the decrease of the (1.1–1.3) × 10^6 molecular

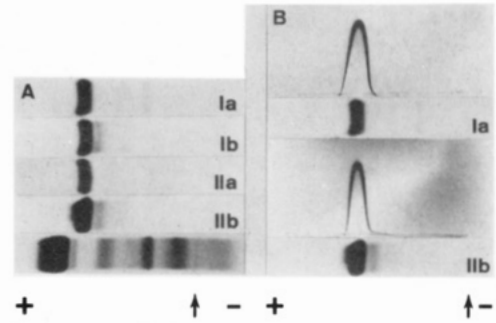

FIGURE 5: Agarose gel electrophoresis and crossed immunoelectrophoresis of purified S protein. Pools a and b from preparations I and II were analyzed by agarose gel electrophoresis (A). A reference plasma is shown. Pools Ia and IIb were also analyzed by crossed immunoelectrophoresis (B) with an antiserum against S protein. Arrows indicate application of samples.

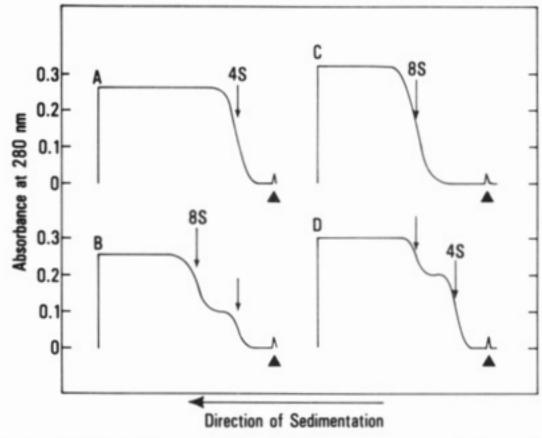

FIGURE 6: Sedimentation of S-protein monomer and dimer in the analytical ultracentrifuge. (A) S protein; (B) S protein after incubation with 2,2′-dithiodipyridine; (C) purified disulfide-linked S-protein dimer; (D) sample in (C) after incubation with 1 mM dithiothreitol at 37 °C for 60 min.

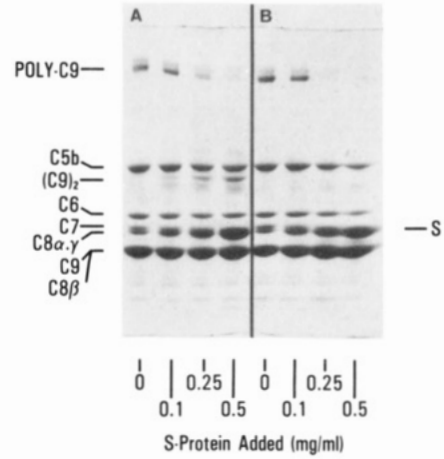

FIGURE 7: Inhibition of C9 polymerization by S protein. C9 polymerization was initiated by the addition of C7 (50 μg/mL) to parallel reaction mixtures containing C5b–6 (150 μg/mL), C8 (75 μg/mL), C9 (225 μg/mL), and the indicated amounts of alkylated or nonalkylated S protein. After 30 min, the reaction was stopped by the addition of SDS (2% final concentration) and analyzed by SDS–polyacrylamide (2.5–10%) gradient slab gel electrophoresis under nonreducing conditions. (A) Nonalkylated S-protein pool Ia; (B) S-protein pool Ia alkylated with iodoacetic acid.

weight band (denoted POLY-C9 in Figure 7). The formation of dimeric C9, $(C9)_2$, was increased with increasing concentrations of S protein containing the free sulfhydryl group. By use of differentially labeled C9 and S protein, no S protein could be detected in the $(C9)_2$ band. Alkylation of S protein

did not affect the ability of S protein to form the SC5b–9 complex as revealed by sucrose density gradient ultracentrifugation (not shown). Furthermore, the S-protein pools Ia and IIb, described above, were equally active in inhibiting C9 polymerization, indicating that proteolytic modification did not affect S protein in this assay.

Discussion

Functional and biochemical characterization of the membrane attack inhibitor protein of plasma, S protein, has been hampered by difficulties in purifying the protein (Podack & Muller-Eberhard, 1979). This has mainly been due to an unusually high tendency of S protein to aggregate and adsorb to various surfaces during its purification. However, once obtained in purified form, the protein is stable and demonstrates no unusual aggregation tendency. This indicates that hydrophobic sites, presumably important for the function as MAC inhibitor, are not exposed in the native molecule. An unknown mechanism exposes these sites during purification, leading to major problems with aggregation and adherence to surfaces. S-protein purification is also complicated by formation of disulfide-linked S-protein dimers and by proteolysis. The procedure we now describe, designed to avoid all these problems, is reproducible and gives pure stable S protein in reasonable yield. The heterogeneous appearance of the purified S protein on SDS–PAGE indicates that despite the abundance of protease inhibitors, proteolysis was not completely inhibited. Furthermore, the data suggest that S protein in vivo is a single-chain 80-kDa molecule.

S protein has a reactive thiol group. Within experimental error, the results are consistent with one SH group per molecule of S protein. The reactivity of the SH group was not important for the inhibitory effect of S protein on C9 polymerization. Interestingly, the reactive thiol group in S protein catalyzed the formation of disulfide-linked C9 dimers through thiol disulfide exchange yet blocked circular C9 polymerization. Low molecular weight compounds containing free thiols, such as glutathione, have also been reported to induce disulfide linking of neighboring C9 molecules in the MAC (Yamamoto et al., 1982), and it was recently suggested that C9 dimerization was directly linked to the polymerization reaction (Yamamoto & Migita, 1983). Our present data indicate that the disulfide-linked dimerization and the polymerization of C9 are distinct processes.

S protein and serum lipoprotein may both serve a regulatory role in limiting complement-mediated injury to bystander cells, by binding to fluid phase C5b–6 and C5b–7 (Podack et al., 1978a; Podack & Muller-Eberhard, 1979; Lint et al., 1977; Rosenfeld et al., 1983). In a reactive cell lysis system, Podack et al. (1978a) demonstrated that S protein works as a noncompetitive inhibitor with an inhibitory constant of approximately 0.5 μM. The plasma concentration of S protein was now estimated to be 140 mg/L (1.75 μM) which is well above the concentration required for inhibition of reactive lysis by C5b–7. The physiologic importance of S protein in the regulation of the component and coagulation systems still remains to be clarified. The purification procedure we now report will make larger amounts of the protein available which will be helpful for studies of this issue.

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Purification and Properties of the Heat-Released Nucleotide-Modifying Group from the Inactive Iron Protein of Nitrogenase from *Rhodospirillum rubrum*†

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ABSTRACT: Nitrogenase in *Rhodospirillum rubrum* is regulated in vivo by the covalent modification of the Fe protein. This paper reports the isolation, purification, and properties of the modifying group that has been heat released from the Fe protein. The molecule is isolated from the heated mixture by binding to a boronate affinity column. Purification is achieved on an ion-exchange high-performance liquid chromatography column. Structural properties of the molecule have been investigated by using proton and phosphorus NMR, mass spectrometry, enzyme susceptibility, and chromatographic methods. The heat-released modifying group exhibits an unusual signal in the proton NMR spectrum at 1.26 ppm. The molecule also contains a functional group which can be reduced by borohydride. This group is lost on breakdown of the molecule or upon treatment of the molecule with 5′-nucleotidase. The identity of the base and the pentose of modifying group as adenine and ribose, respectively, is confirmed. Ratios of the known components of the modifying group are established.

Nitrogenase activity in the photosynthetic bacterium *Rhodospirillum rubrum* was shown to be inhibited by the addition of ammonia to cell cultures (Gest & Kamen, 1949; Gest et al., 1950; Schick, 1971). This loss of activity has been termed "switch off" by Zumft & Castillo (1978) and has now been demonstrated in several organisms (Bognar et al., 1982; Carithers et al., 1979; Hallenbeck et al., 1982; Jones & Monty, 1979; Ludden et al., 1978; Yoch, 1980; Zumft & Castillo, 1978). In addition to ammonia, glutamine (Neilson & Nordlund, 1975), darkness, oxygen, the oxidizing dye phenazine methosulfate, and the uncoupler CCCP (Kanemoto & Ludden, 1984) have been shown to cause switch off. The biochemical basis for switch off is the modification of the Fe protein by covalently attached modifying group that consists of pentose, phosphate, and adenine (Ludden & Burris, 1978; Gotto & Yoch, 1982) and some previously unknown material (Ludden et al., 1984); when the modifying group is attached, the Fe protein is inactive. At least part of the modifying group is removed by the activating enzyme (AE) which is isolated from the chromatophore membrane fraction of cell extracts (Ludden & Burris, 1976, 1979; Nordlund et al., 1977, Guth & Burris, 1983; Gotto & Yoch, 1982). In addition to removal of the modifyng group (MG)[1] by AE, MG can be removed, and the Fe protein can be activated by heating the protein under relatively mild conditions (Dowling et al., 1982).

The structure of the MG has been elusive. Although the components of the group are similar to AMP, there is no direct evidence that the component is AMP. Glutamine synthetases from some enteric bacteria are known to be inactivated by the attachment of AMP via a phosphodiester bond to a specific tyrosyl residue (Kingdon et al., 1979; Shapiro & Stadtman, 1968). However, the AMP attached to GS is not thermolabile, and it is susceptible to snake venom diesterase; the modifying group on *R. rubrum* Fe protein is thermolabile and is not a substrate for snake venom diesterase. Similarly, ADP-ribose is a known protein modifier (Hayaishi, 1976; Van Ness et al., 1980). ADP-ribose is also susceptible to snake venom diesterase, and the ratio of adenine to ribose to phosphate is 1:2:2 (Hayaishi, 1976). The stoichiometry of modifying group components found on *R. rubrum* Fe protein was reported to be 1:1:1 per protein dimer by us (Ludden et al., 1982), but others are less definitive (Hallenbeck et al., 1982; Gotto & Yoch, 1982). Michalski et al. (1983) have shown that in toluenized *Rhodopseudomonas capsulata* cells, label from [^{14}C]adenine or γ-^{32}P-labeled ATP is incorporated into the MG. Surprisingly, the α-P of ATP is not incorporated into MG as would be expected if ATP or NAD were the donor molecules for AMP or ADP-ribose, respectively. ^{3}H label from both [2-^{3}H]adenine or [8-^{3}H]adenine are incorporated into modifying group in vivo (Nordlund & Ludden, 1983).

In this paper the purification of the heat-released modifying group (ΔMG) is described. The properties of the molecule were investigated, and information about the structure of the

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[1] Abbreviations: MG, modifying group; ΔMG, heat-released modifying group; $H_2\Delta$MG, reduced, heat-released modifying group; DNase, deoxyribonuclease; RNase, ribonuclease; HPLC, high-performance liquid chromatography; EI, electron ionization; CI, chemical ionization.

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