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Antithrombin III Binding to SC5b-9 Attack Complexes of Human Complement: Dissociation of a Modified Antithrombin III Derivative Subsequent to Complex Formation†

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ABSTRACT: Antithrombin III (ATIII) was firmly bound to fluid-phase complement attack complexes assembled upon complement activation of normal human serum (SC5b-9 complexes). Analysis of gel filtration column profiles of SC5b-9 complex preparations by double immunodiffusion and immunoreplication procedures employing goat anti-human ATIII antibodies clearly indicated the presence of ATIII antigenic determinants in the 1×10^6 molecular weight, SC5b-9 complex containing fractions. Inclusion of ethylenediaminetetraacetic acid in normal human serum prior to the addition of complement activators inhibited SC5b-9 complex formation as well as the appearance of ATIII in gel filtration column fractions containing 1×10^6 molecular weight proteins or protein complexes. Although ATIII was bound firmly to SC5b-9 complexes, as demonstrated by gel filtration column chromatography in the presence of 0.5 M NaCl and 0.1% Triton X-100 and by rocket immunoelectrophoresis, the majority of SC5b-9 complex associated ATIII was dissociated upon incubation with 4 M guanidine hydrochloride or 10% deoxycholate for 16 h at 37% C (the SC5b-9 complex was not dissociated under these conditions). Anti-ATIII immunoreplication procedures further demonstrated that SC5b-9 complex associated ATIII comigrated with C9 as a 70000 molecular weight protein when subjected to sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis under nonreducing conditions. In addition, it was demonstrated that functionally active complement attack complexes assembled on biological membranes (MC5b-9 complexes) were completely devoid of ATIII. Therefore, it appears the protease inhibitor antithrombin III has the potential to react with and thereby modulate complement attack complex assembly and functional activity. The biochemical basis and the biological significance of this interaction are discussed.

Complement (C)[1] is a sequential, multicomponent system of plasma proteins which can be activated by a variety of immunological as well as nonimmunological stimuli (Müller-Eberhard, 1975). C activation can proceed via either the classical (Müller-Eberhard, 1969) or the alternative (Müller-Eberhard & Schreiber, 1980) pathway through a series of cascading reaction steps which are dependent upon the conversion of serum zymogens to serine active site, trypsin-like esterase enzymes (Porter & Reid, 1979; Götze, 1975). Activation of either C pathway results in the expression of

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[1] Abbreviations: Nomenclature for complement proteins follows that set forth by the World Health Organization (1982); C, complement; EDTA, ethylenediaminetetraacetate; TEMED, *N,N,N′,N′*-tetramethylenediamine; NHS, normal human serum; NHSE, normal human serum containing 10 mM EDTA; PBS, phosphate-buffered saline containing 5 mM potassium phosphate and 145 mM NaCl, pH 7.4; PBSE, PBS containing 10 mM EDTA; $NaDodSO_4$, sodium dodecyl sulfate; DOC, deoxycholate; ATIII, antithrombin III protease inhibitor; protein A, *Staphylococcus aureus* protein A; HSA, human serum albumin; SC5b-9, C5b-9 complexes containing S protein assembled in NHS upon complement activation; MC5b-9, C5b-9 complexes assembled on biological membranes upon complement activation; Cl_3CCOOH, trichloroacetic acid; Gdn·HCl, guanidine hydrochloride.

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multiple biological activities which include (1) deposition of C3b molecules on the surface under C attack, resulting in particle opsonization and clearance by C3b receptor, CR_1 positive neutrophils, monocytes, or macrophages (Lay & Nussenzweig, 1968; Huber et al., 1968; Ross, 1980), (2) production and release of C3a and C5a anaphylatoxins (Hugli & Müller-Eberhard, 1978), and (3) assembly of the C5b-9 membrane attack complex which is responsible for the irreversible target cell membrane damage asssociated with C activation (Lachman & Thompson, 1970; Götze & Müller-Eberhard, 1970; Kolb et al., 1972).

Assembly of the membrane bound C5b-9 complex (MC5b-9 complex) is initiated by the limited proteolytic cleavage of C5 to C5b and C5a fragments by the C5 convertase enzymes of either C pathway. The nascent C5b fragment combines with C6 and C7, resulting in the formation of a C5b,6,7 trimolecular complex (Kolb et al., 1972; Podack et al., 1977) capable of binding phospholipids, which results in the firm attachment of this intermediate complex to the target membrane surface (Podack & Müller-Eberhard, 1978; Podack et al., 1979). Binding of C8 and C9 to MC5b-7 complexes dramatically increases the expression of hydrophobic binding sites within the MC5b-9 complex proteins, enabling the complex to become at least partially embedded within the membrane interior (Esser et al., 1979; Podack et al., 1979; Hammer et al., 1977; Shin et al., 1977). As a result of this hydrophobic insertion, MC5b-9 complexes affect the loss of target cell osmotic balance and behave as integral membrane components requiring detergent extraction for solubilization (Bhakdi et al., 1975; Ware et al., 1981). Detergent-solubilized MC5b-9 complexes constitute a heterogeneous population of oligomeric structures (Ware et al., 1981) with the major component representing a $(1.7–1.9) \times 10^6$ molecular weight, MC5b-9 complex dimer (Biesecker et al., 1979; Ware et al., 1981) having a sedimentation coefficient of 26–29 S (Bhakdi & Tranum-Jensen, 1981; Ware et al., 1981).

When C is activated in human serum in the absence of C5b-9 complex surface receptors, a soluble, fluid-phase, C5b-9 complex is formed (Kolb & Müller-Eberhard, 1973) which contains, in addition to the five terminal C components, serum derived S protein (SC5b-9 complex) (Kolb & Müller-Eberhard, 1975; Podack & Müller-Eberhard, 1979). In contrast with MC5b-9 complexes, the soluble SC5b-9 complexes represent a homogeneous population expressing a molecular weight of 1×10^6 and a sedimentation coefficient of 23–24 S (Kolb & Müller-Eberhard, 1973). The S protein is incorporated into C membrane attack complexes in conjunction with, or immediately after, the interaction of C5b,6 complex with C7 (Podack et al., 1977, 1978), resulting in the formation of an SC5b-7 complex which has lost the ability to bind to biological membranes and induce target cell cytolysis in the presence of C8 and C9 (Podack et al., 1977). Therefore, S protein appears to be a naturally occurring regulator of C membrane attack complex assembly and expression of membranolytic activity.

Antithrombin III (ATIII), an inhibitor of activated serine protease enzymes of the coagulation system (Rosenberg & Damus, 1973; Harpel & Rosenberg, 1976), also has been reported to inhibit C5b,6,7 complex binding to biological membranes by a mechanism which appears to be similar to the one described for S protein (Curd et al., 1978). In an effort to further document and clarify the interaction between ATIII and the terminal complement proteins, we report and characterize the firm binding of ATIII to SC5b-9 complexes assembled in NHS upon C activation. The SC5b-9 complex associated ATIII was present in a modified form which exhibited an apparent increased molecular weight of 5000–7000 as compared to native, unincorporated ATIII when analyzed by $NaDodSO_4$–polyacrylamide slab gel electrophoresis under nonreducing conditions. Since ATIII is similarly modified subsequent to the formation of stable protease–antiprotease complexes with thrombin (Jesty, 1979), activated factor X (Jesty, 1979), or trypsin (Wong et al., 1982), it appears that ATIII binds to an SC5b-9 subcomponent which expresses proteolytic enzymatic activity during complex assembly. C6 and/or C7 are (is) the most likely site(s) for ATIII binding since C6 and C7 have been characterized recently as serine esterase enzymes (Kolb et al., 1982; W.P. Kolb et al., unpublished results).

Experimental Procedures

Materials

Freshly drawn human plasma, containing 10 mM citrate as an anticoagulant, was obtained from consenting healthy human volunteers at the Medical Center Hospital Blood Bank, The University of Texas Health Science Center, San Antonio, TX. Serum was obtained from freshly drawn plasma upon addition of $CaCl_2$ to a final concentration of 20 mM followed by a 1–2-h incubation at 37 °C. EDTA, guanidine hydrochloride (Gdn·HCl), sodium phosphate, sodium metabisulfite, and acetonitrile were purchased from Fisher Scientific Co., Fairlawn, NJ. Sepharose CL-4B, Ultragel AcA-22, and staphylococcal protein A (protein A) were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden; CNBr, Kodak X-Omat AR film, and Coomassie Brilliant Blue G-250 were purchased from Eastman Kodak Co., Rochester, NY. Agarose, electrophoresis grade acrylamide, *N,N'*-methylenebis-(acrylamide), TEMED, ammonium persulfate, and Bio-Gel A-15M were purchased from Bio-Rad Laboratories, Richmond, CA. Nitrocellulose sheets (0.40-μm pore size in roll form) were obtained from Schleicher & Schuell, Keen, NH. Goat anti-human antithrombin III (ATIII) and anti-human serum albumin were purchased from Behring Diagnostics, Somerville, NJ; goat anti-human C5, C6, C7, C8, and C9 and factor B were prepared as described (Wetsel et al., 1980; Kolb et al., 1982; Götze & Müller-Eberhard, 1971); $Na^{125}I$ (sp act. 17 Ci/mg) was purchased from ICN, Irvine, CA; amido black was from J. T. Baker Co., Phillipsburg, NJ. Chloramine T, zymosan, and powdered dalia root inulin were obtained from Sigma, St. Louis, MO. Sodium azide was purchased from MCB Manufacturing Chem., Inc., Cincinnati, OH. Highly purified ATIII was provided by the Plasma Derivatives Laboratory of the American Red Cross, Bethesda, MD.

Methods

MC5b-9 and SC5b-9 Complex Purification. MC5b-9 complexes were isolated in highly purified form by selective detergent extraction of complement-lysed sheep erythrocyte membranes as described previously (Ware et al., 1981). SC5b-9 complexes were isolated from inulin-activated NHS by goat anti-human C5 immunoadsorbent, affinity column chromatography as described (Ware et al., 1981). SC5b-9 complexes also were isolated in highly purified form from inulin-activated NHS by conventional chromatographic procedures as described by Kolb & Müller-Eberhard (1975) with the following modification. A second gel filtration chromatographic step was included as the last step in the isolation procedure in which the SC5b-9 pool obtained after Pevikon-block electrophoresis was applied to a Bio-Gel A-15 M column (2.5×120 cm) equilibrated with PBSE buffer containing 0.5

M NaCl plus 0.1% Triton X-100. This replaced the CsCl flotation step since gel filtration in the presence of detergent effectively removed the contaminating serum lipoproteins.

Radioiodination. Highly purified C6, isolated according to the procedure of Kolb et al. (1982), was radioiodinated by a chloramine T procedure which was similar to the one described by McConahey & Dixon (1966). After dialysis against 50 mM sodium phosphate buffer, pH 7.0, 1 mg of C6 was mixed with 2 mCi of ^{125}I on ice in a total volume of 1 mL. Three, 10-μL aliquots of chloramine T (1 mg/mL water) were added slowly at 5-min intervals with continuous mixing. The reaction was terminated upon the addition of 5 μL of sodium metabisulfite (10 mg/mL water), and unbound ^{125}I was removed by dialysis with 500 mL (30 min), 1000 mL (2 h), and 2000 mL (16 h) of PBS at 4 °C. The ^{125}I-labeled C6 preparations were labeled to a specific activity of (0.4–1.2) $\times 10^6$ cpm/μg with retention of 50–70% specific hemolytic activity. Staphylococcal protein A was radioiodinated in a similar manner. After dialysis against 50 mM sodium phosphate buffer, pH 7.0, 500 μg of protein A was mixed with 200 μCi of ^{125}I on ice in a total volume of 500 μL. Five microliters of chloramine T (37.6 mg/mL in 50 mM phosphate buffer, pH 7.0) was added slowly with continuous mixing for 10 min on ice. Excess sodium metabisulfite was added to stop the radiolabeling reaction, and unbound ^{125}I was removed by exhaustive dialysis as described above for radiolabeled C6. ^{125}I-Labeled protein A was radiolabeled to a specific activity of (5–8) $\times 10^5$ cpm/μg.

$NaDodSO_4$–Polyacrylamide Slab Gel Electrophoresis. Polyacrylamide slab gel electophoresis (7.5% polyacrylamide running gel and 5% stacking gel) in the presence of $NaDodSO_4$ was conducted as described by Laemmli (1970). Following electrophoresis, the gels were fixed in Cl_3CCOOH–20% methanol, stained in 25% methanol–10% acetic acid containing 0.25% Coomassie Brilliant Blue, and destained in 10% methanol–10% acetic acid. Molecular weight estimates were made in $NaDodSO_4$–polyacrylamide slab gels under nonreducing conditions by utilizing the following reference proteins and indicated molecular weight values: C5b (185 000); C6 (115 000); C7 (98 000), C8 α-γ (95 000); C9 (71 000); C8 β (68 000).

Immunoreplication Procedure. $NaDodSO_4$–polyacrylamide slab gels were run for 18 h at a constant current setting of 5–8 mA. The unfixed gels were soaked in transfer tank buffer (25 mM Tris base–192 mM glycine in 20% methanol) at 4 °C for 20 min. The gel was sandwiched between two sheets of nitrocellulose as described by Tobin et al. (1979) and placed into a recirculating gel destaining apparatus (MRA, Clearwater, FL, model M159) in transfer tank buffer precooled to 4 °C. The protein transfer was conducted at 0.6 A for 5 h at 4 °C and was judged to be quantitative since no remaining protein bands could be visualized within the $NaDodSO_4$ slab gel upon fixation and Coomassie Blue staining subsequent to completion of the electrotransfer procedure. All protein bands were transferred from the $NaDodSO_4$–polyacrylamide slab gel to the anode-orientated nitrocellulose sheet as determined upon protein staining of the nitrocellulose sheets placed on either side of the gel during the transfer process. The nitrocellulose blots were stained for protein by incubation in 0.1% amido black in 45% methanol–10% acetic acid at 22 °C for 5 min. The protein-stained nitrocellulose blots were rinsed briefly in water and destained in 90% methanol–2% acetic acid. Unstained nitrocellulose blots were prepared for immunological detection of specific antibody-reactive protein (immunoreplication procedure) by soaking in nitrocellulose saline (10 mM Tris buffer, pH 7.4, plus 155 mM NaCl) containing 3% bovine serum albumin for 1 h at 40 °C. The nitrocellulose blots were rinsed briefly in nitrocellulose saline and incubated in specific antibody (usually a 1:10 final dilution) diluted in nitrocellulose saline plus 3% bovine serum albumin for 16–20 h at 22 °C. Unbound antiserum proteins were removed by five washing steps (5–10 min each) in 150-mL volumes of nitrocellulose saline. The washed nitrocellulose blots were then incubated in 60 mL of nitrocellulose saline plus 3% bovine serum albumin containing 100 μg of ^{125}I-labeled staphylococcal protein A at 22 °C for 30–60 min with gentle mixing on a rocking platform (Bellco Glass Co., Vineland, NJ). ^{125}I-Labeled protein A was always utilized within 72 h after labeling. Unbound ^{125}I-labeled protein A was removed by extensive washing as described above for removal of unbound antiserum proteins, the nitrocellulose blots were dried, and autoradiography was conducted at 22 °C by employing Kodak X-Omat AR film for 2–16 h as required.

Rocket Immunoelectrophoresis. Rocket immunoelectrophoresis buffer contained 38 mM Tris base, 100 mM glycine, and 10 mM Na_2EDTA (pH 8.8). Agarose stocks were prepared by melting electrophoresis-grade agarose (Bio-Rad Labs, Richmond, CA) in rocket immunoelectrophoresis buffer at a final agarose concentration of 1%, and the melted agarose was divided into 15-mL portions and stored at 4 °C until used. After being melted in a boiling water bath, the required amount of agarose was mixed with the indicated volume of prewarmed antiserum to a final total volume of 5 mL. Three and one-half milliliters of each antibody–agarose mixture was layered onto a 1 × 3 in. glass microscope slide. The indicated antigen samples were applied into 4-mm diameter wells, and the individual plates were subjected to constant-voltage electrophoresis (8 V across each slide) for 16 h at 22 °C. The slides were washed exhaustively with 150 mM NaCl plus 0.1% sodium azide, followed by a water wash, and the slides were dried while covered with lens paper (Arthus H. Thomas Co., Philadelphia, PA). The slides were stained for 5 min in 40% ethanol–10% acetic acid plus 0.05% amido black and destained in 90% methanol–5% acetic acid.

Results

^{125}I-Labeled C6-radiolabeled SC5b-9 complexes were assembled upon addition of 25 g of powdered inulin to 1 L of freshly drawn NHS containing 100 μg of ^{125}I-labeled C6. After incubation at 37 °C for 3 h with stirring, the particulate inulin was removed by centrifugation (5000*g*, 30 min), and the ^{125}I-labeled C6-radiolabeled SC5b-9 complexes were concentrated and partially purified upon the addition of ammonium sulfate, at 4 °C with stirring, to a final concentration of 40% of saturation. The ammonium sulfate precipitated proteins were collected by centrifugation (5000*g*, 30 min), resuspended to 120 mL with PBS, and applied to an Ultragel AcA-22 column. The column profile, as seen in Figure 1, clearly indicated the production of the fluid-phase SC5b-9 complex by the incorporation of a portion of the ^{125}I-labeled C6 into a million molecular weight complex present in fractions 220–260 [see Kolb & Müller-Eberhard (1973, 1975) for a detailed description of SC5b-9 complex formation, isolation, characterization, and gel filtration behavior]. Portions of various column fractions also were examined by row–Ouchterlony analysis against goat anti-human ATIII as indicated in the inset panel of Figure 1. After being stained, the row–Ouchterlony pattern indicated that column fractions containing SC5b-9 complexes also expressed antithrombin III antigenic determinants. When inulin was incubated with ^{125}I-labeled C6-containing NHS in the presence of 20 mM

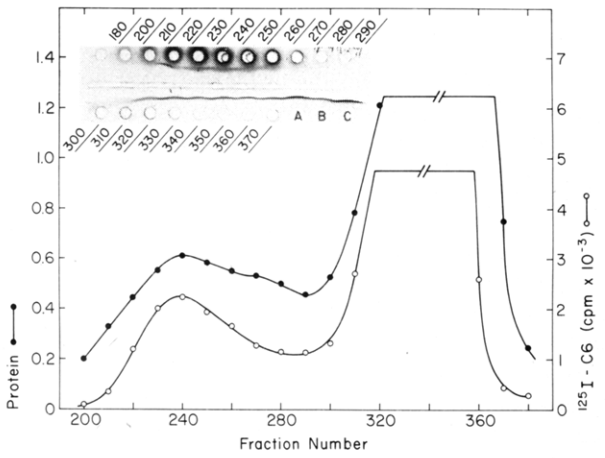

FIGURE 1: Expression of antithrombin III antigenic determinants in SC5b-9 complex containing fractions obtained upon gel filtration column chromatography. One liter of ^{125}I-labeled C6-containing NHS was activated with inulin, and the SC5b-9 complexes were concentrated and partially purified by ammonium sulfate precipitation as outlined in the text. The precipitate was resuspended in 120 mL of PBS and applied to an Ultragel AcA-22 column (5 × 100 cm) equilibrated with PBS at 10 mL/h, collecting 4-mL fractions. Every tenth fraction was assayed for ^{125}I-labeled C6 (1 mL) and protein (A_{280}). In addition, as indicated by the insert in the upper left corner, 8 μL of every tenth fraction was examined by row–Ouchterlony analysis (2-mm diameter wells) against goat anti-human ATIII antiserum. Well A received 8 μL of inulin-activated NHS before ammonium sulfate precipitation, well B received 8 μL of the supernatant after ammonium sulfate fractionation, and well C received 8 μL of the resuspended 40% ammonium sulfate precipitate.

EDTA, no SC5b-9 complexes were formed, and no antithrombin III antigenic determinants were detectable in the column fraction containing 1 000 000 molecular weight proteins or protein complexes (row–Ouchterlony data not shown, see Figure 3).

In an effort to further characterize the SC5b-9 complex associated ATIII antigenic determinant(s), ^{125}I-labeled C6-containing NHS was activated with zymosan and subjected to gel filtration on a Bio-Gel A-15 M column. Samples from various column fractions, as indicated in Figure 2, were subjected to NaDodSO$_4$–polyacrylamide slab gel electrophoresis under nonreducing conditions and analyzed for the presence of ATIII antigens by immunoreplication procedures as outlined under Experimental Procedures. The proteins present within the NaDodSO$_4$–polyacrylamide slab gel were electrophoreticaly transferred to a sheet of nitrocellulose which was incubated subsequently in a 1:10 dilution of monospecific goat anti-human ATIII antiserum for 20 h at 22 °C. After the nitrocellulose blot was washed extensively, it was incubated with radioiodinated protein A, washed again, and exposed to X-ray film for autoradiography. As seen in Figure 2, the immunoreplica lanes corresponding to the SC5b-9 complex containing fractions (fractions 220–260) exhibited a band of radioactivity corresponding to radioiodinated C6 incorporated into assembled SC5b-9 complexes and a second, lower molecular weight band corresponding to the SC5b-9 complex associated ATIII. Control nitrocellulose blots incubated with normal goat serum followed by ^{125}I-labeled protein A, or with ^{125}I-labeled protein A alone, exhibited only the SC5b-9 complex, ^{125}I-labeled C6 protein band upon autoradiography (data not shown). Figure 2 also shows that gel filtration column fractions containing IgG immunoglobulin demonstrated high background levels of ^{125}I-labeled protein A binding. When a portion of the same ^{125}I-labeled C6-containing NHS pool utilized for the experiments described in Figure 2 was activated with zymosan in the presence of 20 mM EDTA, no SC5b-9

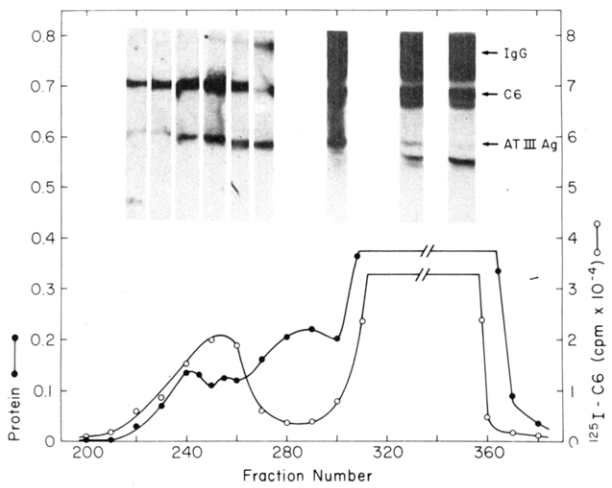

FIGURE 2: Antithrombin III immunoreplication analysis of zymosan-activated normal human serum subjected to gel filtration column chromatography. Ten milliliters of freshly drawn normal human serum containing 65 μg of ^{125}I-labeled C6 (50 × 10^6 cpm) and 0.25% Triton X-100 was activated with 100 mg of zymosan at 37 °C for 3 h with frequent mixing. After removal of the zymosan by centrifugation (5000g, 30 min), the sample was applied to a Bio-Gel A-15M column (2.5 × 120 cm) equilibrated with PBSE plus 0.1% Triton X-100 and 0.5 M NaCl at a flow rate of 1.5 mL/h, collecting 0.5-mL fractions. Every fifth fraction was assayed for ^{125}I-labeled C6 radioactivity (50-μL samples), and protein (A_{280}) and samples from various fractions were subjected to NaDodSO$_4$–polyacrylamide slab gel electrophoresis and ATIII immunoreplication as described in the text. The inset immunoreplica autoradiography strips (lanes 1 to 9 from left to right) represent analysis of the following samples: lane 1, fraction 220 (50 μL); lane 2, fraction 230 (50 μL); lane 3, fraction 240 (50 μL); lane 4, fraction 250 (50 μL); lane 5, fraction 260 (50 μL); lane 6, fraction 270 (50 μL); lane 8, fraction 300 (21 μL); lane 8, fraction 330 (8 μL); lane 9, fraction 350 (3 μL). The radioactive band present in lanes 8 and 9 which migrated below the SC5b-9 complex associated ATIII antigen expressed the same apparent molecular weight as that of native, unmodified ATIII.

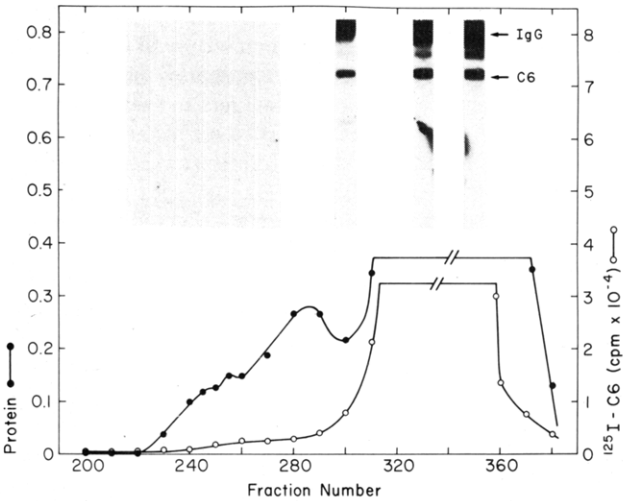

FIGURE 3: Concomitant inhibition of SC5b-9 complex formation and expression of high molecular weight antithrombin III antigenic determinants by EDTA. ^{125}I-Labeled C6-containing NHS was incubated with zymosan in the presence of 20 mM EDTA and subjected to Bio-Gel A-15M gel filtration column chromatography and ATIII immunoreplication analysis as outlined in the legend to Figure 2.

complexes were formed, and no ATIII antigenic determinants were demonstratable upon immunoreplication of samples obtained from the gel filtration column fractions expected to contain 1 000 000 molecular weight proteins or protein complexes (Figure 3).

To further verify the firm association between ATIII and SC5b-9 complexes, as suggested from the ATIII immunore-

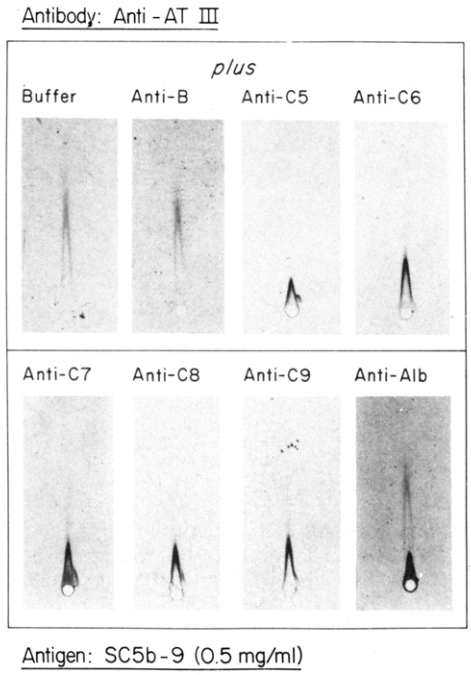

FIGURE 4: SC5b-9 rocket immunoelectrophoresis. Sixteen microliters of SC5b-9 (0.5 mg/mL PBS containing 1 mg of HSA/mL) was subjected to rocket immunoelectrophoresis (4-mm diameter wells) as described in the text and under Experimental Procedures. Each 3.5-mL agarose slab contained 150 μL of anti-ATIII antiserum plus the following indicated addition: rocket immunoelectrophoresis buffer alone (200 μL); anti-factor B (250 μL); anti-C6 (250 μL); anti-C7 (300 μL); anti-C8 (500 μL); anti-C9 (650 μL); anti-human serum albumin (500 μL). Different volumes of each antiserum were employed since the amount of specific antibody present per milliliter of each antiserum was variable.

plication results presented above, we conducted a series of rocket immunoelectrophoresis experiments employing SC5b-9 complex preparations isolated by conventional column chromatographic procedures as outlined under Experimental Procedures. For these studies, human serum albumin (HSA) was added to the SC5b-9 complex preparations as an independent antigenic component to a final concentration of 1 mg/mL. Each agarose slab contained a constant amount of goat anti-human antithrombin III antiserum plus buffer, or an additional antisera specificity as indicated in Figure 4. After electrophoresis, the slides were washed and stained, and the results presented in Figure 4 indicated that (1) goat anti-ATIII antibodies clearly reacted with SC5b-9 complex preparations isolated by conventional chromatographic procedures (Kolb & Müller-Eberhard, 1975) (upper left panel, anti-ATIII plus buffer alone), (2) anti-factor B antibodies did not react with SC5b-9 complexes since a second rocket immunoprecipitate was not evident, and since inclusion of anti-factor B antiserum in the antibody-containing agarose slab did not influence the height of the rocket formed upon interaction of anti-ATIII antibodies with SC5b-9 complexes (upper left, anti-ATIII plus anti-factor B), (3) anti-HSA clearly reacted with the HSA added to the SC5b-9 complex preparations as an independent antigen–antibody system as evidenced by the formation of a second rocket immunoprecipitate which had no effect on the height of the rocket immunoprecipitate formed upon the interaction of anti-ATIII antibodies with SC5b-9 complexes (lower right, anti-ATIII plus antialbumin), and (4) inclusion of anti-C5, -C6, -C7, -C8, or -C9 antiserum in the antibody-containing slabs dramatically reduced the peak height of the rocket immunoprecipitates formed upon the interaction of anti-ATIII antibodies with SC5b-9 complexes. Additional rocket immunoelectrophoresis experiments also were conducted in which a constant volume of anti-C5, -C6, -C7, -C8, or -C9 antiserum was incorporated into the agarose slabs with increasing concentrations of anti-ATIII antibodies. The anti-ATIII antibodies were able to dramatically reduce the peak height of the rocket immunoprecipitates formed upon the interaction of antiterminal complement component antibodies with SC5b-9 complexes (data not shown). Collectively, these results clearly demonstrated that antibodies reactive with antithrombin III and antibodies reactive with the terminal components of complement all recognized the same molecular moiety present in SC5b-9 complex preparations.

During the course of these studies, it was observed that rocket immunoelectorphoresis patterns obtained with SC5b-9 complexes isolated by anti-C5 immunoadsorbent affinity column chromatography procedures (Ware et al., 1981) demonstrated variable and, at times, undetectable reactivity with anti-ATIII antisera. These results suggested the 4 M Gdn·HCl employed to dissociate and elute the SC5b-9 complexes from the anti-C5 column also effected partial and, in some cases, complete ATIII dissociation from SC5b-9 complexes. In an effort to examine this possibility, we activated ^{125}I-labeled C7-containing NHS (750 mL) with inulin (10 mg/mL) at 37 °C for 2 h and then applied it to an anti-C5 immunoadsorbent column (6 × 50 cm) as described (Ware et al., 1981). The anti-C5 column was washed with PBSE containing 1 M NaCl and 1% Triton X-100, and the SC5b-9 complexes were eluted with 400 mL of 3 M Gdn·HCl. The ^{125}I-labeled C7-containing fractions were pooled, exhaustively dialyzed vs. PBSE, and concentrated by positive pressure ultrafiltration. The concentrated SC5b-9 preparation was divided into two equal portions, and solid Gdn·HCl was added to a final concentration of 4 M to one sample and deoxycholate (DOC) was added to a final concentration of 10% (v/v) to the other. After incubation at 37 °C for 1lh, each sample was separately applied to a Bio-Gel A-15M column (1.5 × 120 cm) equilibrated with PBSE plus 0.5 M NaCl. The Bio-Gel A-15M column elution profile for the SC5b-9 complex sample preincubated with 4 M Gdn·HCl is seen in the top panel of Figure 5. Fifty-microliter portions from selected column fractions were analyzed on two $NaDodSO_4$–polyacrylamide slab gels run in parallel. One gel was stained for protein and is presented in the lower left panel of Figure 5 which shows a typical SC5b-9 complex gel filtration profile (fractions 89–110) with the presence of several lower molecular weight proteins in fractions 115–140. These latter proteins were tentatively identified as IgG (M_r 150 000), S protein (M_r 86 000), and C9 (M_r 71 000). The proteins present within the second gel were transferred to nitrocellulose and subjected to immunoreplication with anti-ATIII antibodies as described under Experimental Procedures. The autoradiograph of the anti-ATIII immunoreplica of this companion gel is shown in the lower right-hand panel of Figure 5 and indicates that in addition to the presence of ATIII in the SC5b-9-containing fractions (89–110) the majority of the SC5b-9 complex associated ATIII had been dissociated upon exposure to Gdn·HCl and was now found in the lower molecular weight containing gel filtration column fractions. Furthermore, it is clear that the ATIII present in these fractions was derived from SC5b-9 complexes since the anti-C5 imunoadsorbent columns will not bind ATIII antigen from NHS (Wetsel et al., 1980) or from NHS subsequent to incubation with zymosan or inulin in the presence of 20 mM EDTA. The $NaDodSO_4$–polyacrylamide slab gel and anti-ATIII immunoreplication patterns obtained for the Bio-Gel A-15M column profile of the SC5b-9

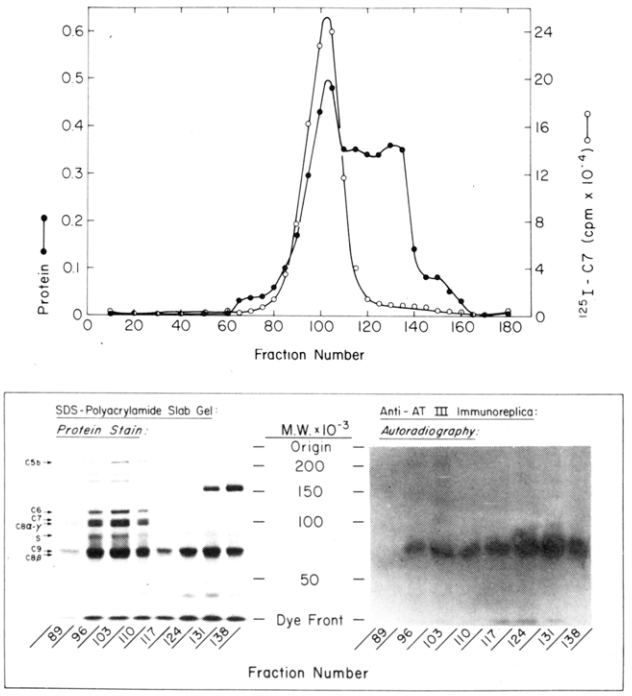

FIGURE 5: ATIII dissociation from SC5b-9 complexes by guanidine hydrochloride. SC5b-9 complexes were eluted from an anti-C5 immunoadsorbent column and preincubated with 4 M Gdn·HCl before being subjected to Bio-Gel A-15M gel filtration column chromatography. Fifty microliters of the indicated column fractions was analyzed by $NaDodSO_4$–polyacrylamide slab gel electrophoresis and anti-ATIII immunoreplication as outlined in the text.

complex preincubated with 10% DOC were the same as those presented in Figure 5 for the Gdn·HCl-treated sample (data not shown). These results indicated that both Gdn·HCl and DOC were able to effect selective ATIII dissociation from ATIII-containing SC5b-9 complexes, which explains why SC5b-9 complexes eluted from immunoadsorbent columns with Gdn·HCl express variable amounts of firmly bound ATIII. In addition, the results presented in Figure 5 also demonstrated that SC5b-9 complex associated ATIII had an approximate molecular weight of 70 000 and comigrated with C9, which explains why it has not been identified previously as an SC5b-9 subcomponent.

Immunoreplication procedures also were utilized to analyze highly purified, membrane-derived C5b-9 complexes (MC5b-9 complexes) for the presence of ATIII. The left panel of Figure 6 shows the $NaDodSO_4$–polyacrylamide slab gel protein-staining profile for highly purified antithrombin III (left lane), MC5b-9 complexes (middle lane), and SC5b-9 complexes (right lane). The nitrocellulose blot stained with amido black, as seen in the center panel of Figure 6, indicated the high fidelity of protein transfer attainable by this technique. The autoradiograph of the anti-ATIII immunoreplica obtained from an unstained nitrocellulose blot run in parallel (Figure 6, right panel) unequivocally demonstrated the ability of anti-ATIII antibodies to react with highly purified ATIII and with SC5b-9 complex associated ATIII. However, the membrane-derived C5b-9 complexes were devoid of detectable quantities of ATIII. In addition, the results presented in Figure 6 indicated that SC5b-9 complexes contained a modified form of ATIII ($ATIII_M$) which had an apparent molecular weight on nonreducing $NaDodSO_4$–polyacrylamide gels of 70 000 which was approximately 7000 daltons greater than the molecular weight of 63 000 expressed by native ATIII (Nordenman et al., 1977).

The results presented in Figure 6 further indicated the anti-ATIII antiserum utilized for these studies did not cross-react with terminal complement component proteins within nonreduced $NaDodSO_4$–polyacrylamide slab gels since no lines of reactivity were visualized in MC5b-9 complex preparations upon anti-ATIII immunoreplication and subsequent autoradiography (Figure 6, right panel). Rocket immunoelectrophoresis also was employed to demonstrate that the results presented in Figures 1 and 4 were not due to an interaction between anti-ATIII antiserum and native terminal complement components. As seen in Figure 7, when NHSE was subjected to rocket immunoelectrophoresis in agarose slabs containing a constant amount of anti-ATIII antiserum mixed with various antisera to the individual terminal complement components, no evidence of anti-ATIII antibody interaction

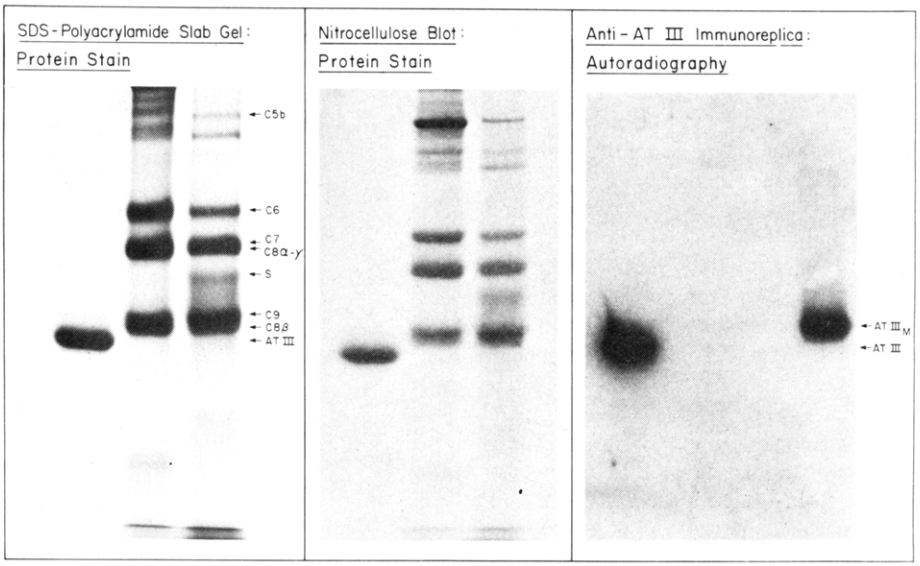

FIGURE 6: Anti-ATIII immunoreplication analysis of MC5b-9 complexes. Triplicate patterns of highly purified antithrombin III (10 μg, lane 1), MC5b-9 complexes (25 μg, lane 2), and SC5b-9 complexes (25 μg, lane 3) were analyzed on the same 7.5% $NaDodSO_4$–polyacrylamide slab gel. After electrophoresis, the gel was cut into three equal portions. The first portion was fixed in 10% Cl_3CCOOH and stained for protein with Coomassie Brilliant Blue G-250 (left panel). The protein profiles within the second and third portions were simultaneously electrotransferred to nitrocellulose and either stained for protein with amido black (center panel) or subjected to anti-ATIII antibody immunoreplication (right panel) as outlined under Experimental Procedures.

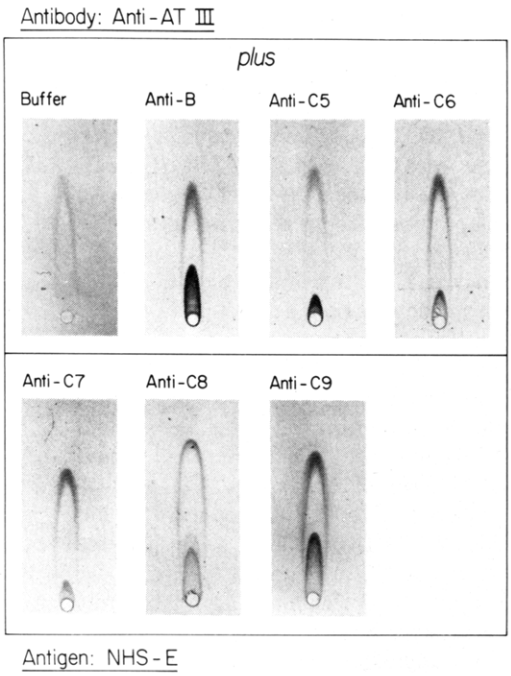

FIGURE 7: Antithrombin III rocket immunoelectrophoresis. Eight microliters of normal human serum containing 10 mM EDTA (NHSE) was subjected to rocket immunoelectrophoresis (2-mm diameter wells) as described under Experimental Procedures. Each 3.5-mL agarose slab contained 100 μL of goat anti-human antithrombin III antiserum plus the following indicated addition: anti-factor B (300 μL); anti-C5 (150 μL); anti-C6 (250 μL); anti-C7 (250 μL); anti-C8 (300 μL); and anti-C9 (250 μL). The same antisera lot numbers were employed for this study as utilized in Figures 1 and 4 where indicated.

with native terminal complement component could be demonstrated. That is, clearly distinct, unrelated rocket immunoprepcipitates were formed in all cases, and the presence of relatively high concentrations of antiterminal complement component antiserum in the antibody-containing agarose slabs had no effect on the interaction between anti-ATIII antibodies and ATIII present in normal human serum.

Discussion

Several different experimental approaches were employed in these studies to demonstrate the firm binding of antithrombin III to SC5b-9 complexes assembled upon C activation of NHS: (1) SC5b-9 complexes subjected to gel filtration column chromatography in the presence of physiologic ionic strength buffers or in the presence of 0.5 M NaCl and 0.1% Triton X-100 expressed ATIII antigenic determinants when examined by double immunodiffusion against anti-ATIII antiserum (Figure 1) or by anti-ATIII immunoreplication procedures (Figure 2); (2) rocket immunoelectrophoresis demonstrated that antibodies reactive with ATIII and antibodies reactive with the individual terminal C components reacted with the same macromolecular entity present in the highly purified SC5b-9 complex preparations (Figure 4); and (3) SC5b-9 complexes incubated with 4 M Gdn·HCl for as long as 16 h at 37 °C still retained detectable amounts of bound ATIII (Figure 5). In this regard, the anti-ATIII immunoreplication procedure was found to be significantly more sensitive in detecting SC5b-9 complex associated ATIII than either double immunodiffusion or rocket imunoelectrophoretic procedures.

It appears unlikely that the ATIII bound to highly purified SC5b-9 complexes was present in the form of a preexisting ATIII–coagulation protease complex which associated with SC5b-9 complexes subsequent to C activation of normal human serum and SC5b-9 complex assembly for the following reasons: (1) the ATIII associated with highly purified SC5b-9 complexes was resistant to dissociation by a variety of experimental manipulations as summarized above; (2) we have been unable to detect the presence of thrombin antigenic determinants in the SC5b-9 complex preparations utilized for these studies (data not shown); and (3) Curd et al. (1978) demonstrated a direct interaction between highly purified C5b,6 complex and ATIII in the presence of C7 , resulting in the formation of a stable and soluble ATIII–C5b-7 complex.

The inhibition of proteolytic enzymes by antithrombin III is typical of protease–antiprotease reactions in which the enzyme active site becomes sterically blocked by stoichiometric amounts of inhibitor (Rosenberg & Damus, 1973). Several studies have been published which suggest that a covalent bond is formed between antithrombin III and the inhibited protease during the reaction (Rosenber & Damus, 1973; Owen et al., 1976; Chandra & Bang, 1977). The evidence for covalent bond formation is based on the resistance of antithrombin III–thrombin complexes to dissociate upon prolonged treatment with $NaDodSO_4$ (Rosenberg & Damus, 1973) or 6 M Gdn·HCl (Owen et al., 1976). Effective antithrombin III–thrombin complex dissociation required treatment with hydroxylamine, dilute alkali, or ammonia in the presence of a protein denaturant (Owen et al., 1976; Fish & Björk, 1979) which suggested the bond formed between ATIII and the complexed protease was a carboxylic ester (acyl bond). In all examples reported to date, dissociation of acyl-bonded ATIII–protease complexes resulted in the release of a modified form of ATIII ($ATIII_M$) which had lost inhibitor activity (Jesty, 1979; Fish et al., 1979; Griffith & Lundblad, 1981; Wong et al., 1982). $ATIII_M$ is composed of two disulfide-linked polypeptide fragments (Jesty, 1979; Fish & Björk, 1979) resulting from the cleavage of ATIII at a single Arg–Ser peptide bond located at position 384 which is 39 amino acid residues from the carboxyl terminus of the ATIII polypeptide chain (Jornvall et al., 1979; Carrel et al., 1980). The proteolytically modified form of ATIII expresses a molecular mass which is 5000–7000 daltons greater than that of native, unmodified ATIII upon $NaDodSO_4$–polyacrylamide gel analysis under nonreducing conditions (Jesty, 1979; Wong et al., 1982). The subunit structure of $ATIII_M$ bound to SC5b-9 complexes was not determined in the present studies since anti-ATIII antibodies would not react with $ATIII_M$ after reduction in the presence of $NaDodSO_4$.

Antithrombin III–protease complexes are subject to varying degrees of spontaneous dissociation at neutral pH and physiologic conditions. The extent of spontaneous dissociation under these conditions appears to be dependent upon the proteolytic enzyme present in the complex. For example, spontaneous dissociation of ATIII–thrombin complexes requires several days (Danielsson & Björk, 1980) while active trypsin spontaneously dissociates from ATIII–trypsin complexes with a half-time of approximately 15 min (Wong et al., 1982). However, rapid and complete dissociation can be achieved, irrespective of the proteolytic enzyme present in the ATIII–protease complex, with the Tris buffer system, pH 8.8–9.0, employed in the $NaDodSO_4$–polyacrylamide slab gel system of Laemmli (1970) (Jesty, 1979). This observation explains why the ATIII bound to SC5b-9 complexes, which have a molecular weight of 1 000 000 upon gel filtration column chromatography, was visualized as uncomplexed $ATIII_M$ in the Laemmli gel system employed in these studies.

The modified ATIII dissociated from SC5b-9 complexes was indistinguishable from $ATIII_M$ dissociated from ATIII–protease complexes as described previously and discussed above. Namely, (1) ATIII was firmly bound to SC5b-9 complexes assembled in NHS upon C activation, (2) evidence for spontaneous dissociation of $ATIII_M$ from SC5b-9 complexes was observed (Figure 4), although the contribution of Gdn·HCl or DOC to accelerating $ATIII_M$ dissociation was not fully determined, and (3) $ATIII_M$ dissociated from SC5b-9 complexes exhibited an increase in the apparent molecular mass of 5000–7000 daltons as compared to unincorporated, native ATIII when analyzed by $NaDodSO_4$–polyacrylamide slab gel electrophoresis under nonreducing conditions. These results strongly suggest that antithrombin III binds to a terminal C component which expresses proteolytic activity during assembly of SC5b-9 complexes in free solution. However, ATIII does not bind to the corresponding terminal C component(s) participating in the assembly of functionally active C5b-9 complexes on the surface of a target cell membrane under C attack (Figure 6). Thus, ATIII and S protein exhibit similar patterns of binding reactions with fluid-phase and membrane-bound C5b-9 complexes (Kolb & Müller-Eberhard, 1975; Curd et al., 1978; Podack & Müller-Eberhard, 1979).

It is most likely that the C6 and/or C7 subcomponents represent(s) the ATIII binding site(s) within the SC5b-9 complex since (1) ATIII binds to nascent C5b,6,7 complexes in free solution during the assembly of C5b,6 complex with C7, resulting in the inhibition of C attack complex binding to membrane surfaces (Curd et al., 1978), and (2) C6 and C7 recently have been characterized as serine esterase enzymes (Kolb et al., 1982; W. P. Kolb, unpublished results). Unequivocal identification of the terminal C component enzyme(s) which bind(s) ATIII and the relationship between ATIII and S protein binding sites within nascent C5b-7 and C5b-9 complexes remain to be determined. Nevertheless, the data presented clearly indicate that the ability of antithrombin III to bind, and thereby modulate activated serine protease enzymes, is not restricted to the coagulation system since it appears that antithrombin III can also bind to an enzyme(s) present within the membrane attack complex of the complement system of plasma proteins.

Registry No. ATIII, 9000-94-6.

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Electron Paramagnetic Resonance and Optical Evidence for Interaction between Siroheme and Fe_4S_4 Prosthetic Groups in Complexes of *Escherichia coli* Sulfite Reductase Hemoprotein with Added Ligands†

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ABSTRACT: Janick & Siegel [Janick, P. A., & Siegel, L. M. (1982) *Biochemistry 21*, 3538-3547] showed that the EPR spectrum of the reduced Fe_4S_4 center ($S = {}^1/_2$) in fully reduced native ("unligated") *Escherichia coli* NADPH-sulfite reductase hemoprotein subunit (SiR-HP) is perturbed by interaction with paramagnetic ferrous siroheme ($S = 1$ or 2) to yield several novel sets of EPR signals: one set with all g values between 2.0 and 2.8, termed "$S = {}^1/_2$" type, and two sets with the lowest field g value between 4.7 and 5.4, termed "$S = {}^3/_2$" type. The present study has shown that EPR spectra of fully reduced SiR-HP are nearly quantitatively converted to the classical "$g = 1.94$" type typical of $S = {}^1/_2$ Fe_4S_4 clusters when the heme has been ligated by strong field ligands such as CO, CN^-, S^{2-}, and AsO_2^-, converting the ferroheme to $S = 0$. However, the exact line shapes and g values of the $g = 1.94$ differ markedly when different ligands are bound to the heme. Also, optical difference spectra taken between enzyme species in which the heme is kept in the same (Fe^{2+}) oxidation state while the Fe_4S_4 center is reduced or oxidized show that the optical spectrum of the ligated siroheme is sensitive to the oxidation state of the Fe_4S_4 cluster. These results indicate that the heme-Fe_4S_4 interaction of native SiR-HP persists even when the heme Fe is bound to exogenous ligands. We have also found that the g values of the exchange-coupled $S = {}^1/_2$ and $S = {}^3/_2$ type signals of native reduced SiR-HP can be significantly shifted by addition of potential weak field heme ligands—halides and formate—or low concentrations of certain chaotropic agents—guanidinium salts and dimethyl sulfoxide—to the fully reduced enzyme. Such agents can also promote interconversion of the $S = {}^1/_2$ and $S = {}^3/_2$ type signals. These effects are reversed on removal of the agent. Treatment of reduced SiR-HP with relatively large concentrations of chaotropes, e.g., 60% dimethyl sulfoxide or 2 or 3 M urea, leads to abolition of the $S = {}^1/_2$ and $S = {}^3/_2$ EPR signals and their replacement by signals of the $g = 1.94$ type.

Multielectron reduction reactions involve some of the most important and yet least understood enzymatic processes known. Two of these reactions, catalyzed by cytochrome *c* oxidase ($O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$) and nitrogenase ($N_2 + 8H^+ + 6e^- \rightarrow 2NH_4^+$), respectively, involve enzymes with multiple subunits and multiple prosthetic groups. The complexity of these enzymes has slowed progress in the analysis of the multielectron reduction processes catalyzed by them. In contrast, two other multielectron reduction reactions, sulfite reduction to sulfide ($SO_3^{2-} + 8H^+ + 6e^- \rightarrow H_2S + 3H_2O$) and nitrite reduction to ammonia ($NO_2^- + 8H^+ + 6e^- \rightarrow NH_4^+ + 2H_2O$), can be catalyzed by monomeric enzymes which contain only two prosthetic groups—an Fe_4S_4 center and a novel heme, termed "siroheme"—on a single polypeptide chain of M_r ~60 000 (Lancaster et al., 1979; Siegel et al., 1982; Krueger & Siegel, 1982a). Although sulfite and nitrite reductases are physiologically distinct proteins within a given organism (Krueger & Siegel, 1982a), each enzyme is capable of catalyzing both types of multielectron reduction reaction.

The NADPH-sulfite reductase of *Escherichia coli* consists of an oligomeric complex between four "hemoprotein" subunits of M_r 54 600, each of which contains 1 mol of siroheme and one Fe_4S_4 center, and an octameric "flavoprotein" which catalyzes electron transfer from NADPH, the physiological reductant, to the hemoprotein subunits, which serve as the sites of sulfite reduction (Siegel & Davis, 1974; Siegel et al., 1982). The complex can be dissociated in 4 M urea and the monomeric hemoprotein subunit (SiR-HP)[1] isolated free of the flavoprotein by DEAE-cellulose chromatography (Siegel & Davis, 1974). The SiR-HP subunit catalyzes both sulfite and nitrite reduction at high rates if supplied with a suitable artificial electron donor, such as reduced methylviologen (Siegel et al., 1982).

Christner et al. (1981) have shown by Mössbauer spectroscopy that the iron atoms of the heme and Fe_4S_4 prosthetic groups in SiR-HP are exchange coupled, i.e., they must be chemically linked by a bridging ligand in the oxidized enzyme as isolated. Janick & Siegel (1982) demonstrated that SiR-HP

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[1] Abbreviations: Dfl, 5'-deazaflavin; Fe_4S_4, tetranuclear iron-sulfur center; MV^+, methylviologen cation radical; SiR, sulfite reductase; SiR-HP, hemoprotein subunit of *E. coli* NADPH-sulfite reductase hemoflavoprotein complex; Me_2SO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; GdmCl, guanidinium chloride.

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