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Spin-Label and Fluorescence Labeling Studies of the Thioester Bonds in Human α_2-Macroglobulin†

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ABSTRACT: Upon cleavage of the reactive thioester bonds (Cys-949-Glx-952) of tetrameric human α_2-macroglobulin (α_2M) by methylamine, one sulfhydryl group per α_2M subunit is exposed. These identical sulfhydryl group sites were labeled with the thiol-specific nitroxide spin-labels (1-oxy-2,2,5,5-tetramethyl-3-pyrrolin-3-yl)methyl methanethiosulfonate and (1-oxy-2,2,6,6-tetramethyl-4-piperidinyl)methyl methanethiosulfonate, a homologous series of maleimide spin-labels, and the thiol-specific fluorescent probe 2-[(4-maleimidophenyl)amino]naphthalene-6-sulfonic acid sodium salt (MANS). The ESR and fluorescence results showed that these sulfhydryl group sites were at the base of a narrow crevice that is ≥8 Å deep. Although the bound MANS fluorophore was slightly blue shifted with an enhanced quantum yield vs the free label in water, the environment of the sulfhydryl site appeared to be of a polar nature when compared with the emission maxima in several solvents of varying polarity. The Glx residue participating in the thioester linkage in the intact protein was labeled with 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl. The distance between the Glx and Cys moieties was estimated at ≥10-25 Å from double spin-labeling experiments.

The plasma protein α_2-macroglobulin (α_2M)[1] has a series of unique biochemical properties (Harpel & Brower, 1983). It is a M_r 725 000 glycoprotein composed of two noncovalent bound dimers, each of which contains two identical "subunits" linked together by disulfide bridges (Jones et al., 1972). Each subunit contains 1451 amino acid residues with 8 asparagine-linked oligosaccharide groups (Sottrup-Jensen et al., 1984). Of most interest, however, is the occurrence in each subunit of a labile peptide bond in the "bait region" that is cleaved upon interaction with serine proteinases (Harpel, 1973; Swenson & Howard, 1979). Upon covalent incorporation of methylamine at a thioester linkage about 240 residues away (Glx-952) resulting in the exposure of one sulfhydryl group (Cys-949) per subunit, a conformational change of the whole tetramer occurs which closely mimics that which occurs upon cleavage of the bait region (Larsson et al., 1985; Eccleston & Howard, 1985). The thioester sites are also in close proximity to the proteinase binding sites (Pochon et al., 1981; Feldman et al., 1985). This inhibition mechanism has been proposed to occur via a "trap" mechanism (Barrett & Starkey, 1973; Van Leuven, 1982), which appears to be unique among proteinase inhibitors. That is, a limited proteolysis in the bait region of an α_2M subunit is followed by a conformational change, during which the reactive thioester bonds are cleaved.

Several thiol-specific nitroxide spin-labels have been synthesized that bind covalently to cysteine residues, resulting in

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[1] Abbreviations: α_2M, α_2-macroglobulin; CPK, Corey-Pauling-Koltun; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); ESR, electron spin resonance; 4-amino-TEMPO, 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; MANS, 2-[(4-maleimidophenyl)amino]naphthalene-6-sulfonic acid sodium salt; maleimide spin-label **1**, 4-maleimido-TEMPO; maleimide spin-label **2**, 3-(maleimidomethyl)proxyl; maleimide spin-label **3**, 3-[(2-maleimidoethyl)carbamoyl]proxyl; maleimide spin-label **4**, 3-[(3-maleimidopropyl)carbamoyl]proxyl; maleimide spin-label **5** 3-[[2-(2-maleimidoethoxy)ethyl]carbamoyl]proxyl; piperidine thiol spin-label, (1-oxy-2,2,6,6-tetramethyl-4-piperidinyl)methyl methanethiosulfonate; proxyl, 2,2,5,5-tetramethylpyrrolidine-1-oxyl; proxyl thiol spin-label, (1-oxy-2,2,5,5-tetramethyl-3-pyrrolin-3-yl)methyl methanethiosulfonate; STI, soybean trypsin inhibitor; Tos-Arg-OMe, *p*-tosylarginine methyl ester; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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an ESR spectrum that is extremely sensitive to protein conformation and environment (Berliner, 1983; Berliner et al., 1981). We report here a conformational study *in solution* of the structural environment of this unique thioester linkage by ESR and fluorescence techniques.

Materials and Methods

Chemicals. The proxyl and piperidine thiol spin-labels were Reanal (Budapest) products, which were generous gifts from Dr. K. Hideg, University of Pecs, Pecs, Hungary. The maleimide spin-labels **1–5**, 4-amino-TEMPO, Tos-Arg-OMe (lot 610-0550), and STI (lot 93F-8056) were purchased from Sigma Chemical Co. MANS was from Molecular Probes, Inc. Zinc-chelating Sepharose and Sephadex G-200 were from Pharmacia. DTNB and Hepes were from Nutritional Biochemicals, Inc. All other chemicals were of reagent grade.

Proteins. α_2M was prepared according to the methods of Kurecki et al. (1979) and Hall and Roberts (1978) with slight modifications. Outdated plasma (obtained from the central Ohio Red Cross Blood Services) was centrifuged twice at 4200*g* for 10 min, brought to 50% ammonium sulfate, and centrifuged at 8000*g* for 40 min. The precipitate was resuspended in 50% ammonium sulfate, stirred for 1 h, and centrifuged at 8000*g* for 40 min. The precipitate was dissolved in distilled water and dialyzed against distilled water for 3 days. After centrifugation at 8000*g* for 40 min, the supernatant was then brought to 40% saturated ammonium sulfate by addition of solid ammonium sulfate, stirred for 1 h, and then centrifuged again (8000*g*, 40 min). The supernatant was then brought to 55% ammonium sulfate saturation by addition of solid ammonium sulfate, stirred for 1 h, and centrifuged again (8000*g*, 40 min). The precipitate was then dissolved in 0.02 M sodium phosphate–0.15 M NaCl, pH 6.0, and dialyzed against the same buffer. The resultant crude α_2M was purified on a 2.5 × 16 cm zinc-chelating Sepharose column with a linear pH gradient (pH 6.0–4.7). Each fraction was assayed for α_2M activity. The α_2M peak eluted at ca. pH 5.7. The pooled α_2M fractions were precipitated with 50% ammonium sulfate and rechromatographed. The purified α_2M was shown to yield only a single band by SDS–PAGE.

Methods. Protein concentration was estimated spectrophotometrically by using $\epsilon_{280}^{1\%} = 0.89$ mg/mL for α_2M with a M_r of 725 000. The activity of α_2M was measured by a modification of the procedure of Ganrot (1966) with Tos-Arg-OMe as the trypsin substrate. Aliquots of each α_2M elution fraction were incubated in a mixture containing 85 μL of 0.1 M Hepes buffer (pH 8.3) and 5 μL of trypsin stock solution (100 μg/mL). After 3 min, 10 μL of STI (200 μg/mL) was added. The sample was then assayed by trypsin hydrolysis of Tos-Arg-OMe at 247 nm on a Perkin-Elmer Lambda 5 spectrophotometer at 30 °C.

Sulfhydryl group content was assayed spectrophotometrically with DTNB by the procedure of Ellman (1959).

Spin-Labeling of α_2M. In a typical experiment, 500 μL of 7 μM α_2M (0.1 M Hepes–0.15 M NaCl, pH 8.3) was treated with 200 mM methylamine for 2 h. Then 20 μL of 5.3 mM appropriate spin-label in acetonitrile was added, and the resultant mixture was incubated for 1–2 h. The sample was chromatographed on Sephadex G-200 (1 × 8 cm) and then dialyzed vs 0.02 M phosphate–0.15 M NaCl buffer (pH 6.0, 4 °C) until no ESR signal was detected in the dialysate. Alternatively, initial spin-labeling with 4-amino-TEMPO was accomplished by incubating native α_2M with a 30-fold molar excess of the spin-label for 3 days (in the standard Hepes buffer described above at room temperature), followed by exhaustive dialysis.

ESR spectra were measured on a Varian E-4 spectrometer in quartz flat cells. Spin-label stoichiometry was determined by double integration with an E-935 data system or by the "spin count" technique (Berliner, 1981).

Fluorescence Labeling Experiments. In a typical experiment, 1 μM α_2M (0.1 M Hepes–0.15 M NaCl, pH 8.3) was reacted with 200 mM methylamine for 2 h and then reacted with MANS (30 μM) for 24 h. The sample was then dialyzed for 3 days in 50 mM phosphate–0.15 M NaCl buffer, pH 6.0. The stoichiometry of bound MANS to α_2M was estimated spectrophotometrically by using $\epsilon_{355nm} = 4700$ M^{-1} cm^{-1} (Gupte & Lane, 1979). The fluorescence spectra were measured on a Perkin-Elmer MPF-44A spectrofluorometer at 25 °C. Quantum yields were measured against quinine sulfate (in 0.1 M H_2SO_4) as a standard, where a value of 0.70 was assumed (Martin & Richardson, 1979).

Results

Spin-Labeled α_2M Sulfhydryl Groups. The variety of thiol-specific labels used in this study are shown in Figure 1A. They include the fluorescent probe 2-[(4-maleimidophenyl)-amino]naphthalene-6-sulfonic acid sodium salt (MANS), which has been shown to bind covalently to sulfhydryl groups and is sensitive to the polarity of its environment on a protein (Weber & Farris, 1979; Gupte & Lane, 1979; Lakowicz, 1983). Figure 1B shows the structures of a series of maleimide spin-labels (**1–5**) of increasing molecular length. The exposed sulfhydryl groups of (methylamine-treated) α_2M were covalently modified with a series of seven thiol-specific nitroxide spin-labels. In each case the stoichiometry of exposed thiol groups was measured with DTNB before and after labeling. The stoichiometry of bound nitroxide was also measured by double integration. Typically, our α_2M preparations yielded 3.6–3.7 SH groups per (tetrameric) α_2M by either DTNB or spin concentration measurements. The ESR spectra for each maleimide label bound to the (identical) thiol group(s) of α_2M are shown in Figure 2. Note the very strong immobilization for the shortest label, **1**, characterized by the hyperfine extrema splitting, $2T_{\parallel} = 67.6 \pm 0.3$ G, which is close to that observed for a powder or rigid glass spectrum (Berliner, 1981). As the molecular length increases, the label mobility slowly increases with a sharp break to a highly mobile environment between labels **3** and **4**, as evidenced by the almost complete shift of the two extrema (marked in spectrum 1) to the narrow line triplet component (most obvious in spectrum **5**). That is, the apparent "depth" of the thiol binding site in α_2M must be ≥12.8 Å, when one considers the sharp increase in mobility for label **4**. Actually, if one considers the distance from the maleimido group to the substituent carbon on the nitroxide ring, where the first single-bond rotation occurs between the nitroxide ring and its tether to the protein, this depth decreases to ≥8 Å if the rigid nitroxide ring protruded out of the pocket.

The strongly immobilizing environment at the bottom of the sulfhydryl site is also exemplified by the ESR spectra in Figure 3 for the two reversible proxyl and piperidine thiol-specific spin-labels, respectively. Again here, as with maleimide spin-label **1**, the hyperfine extrema separations, were large, $2T_{\parallel} = 65.9 \pm 0.3$ and 63.8 ± 0.3 G for the proxyl and piperidine thiol spin-labels, respectively. Similar spectra were also observed for protease-inactivated α_2M (Zhao and Berliner, unpublished results).

Spin-Labeling with a Nitroxide Amine. Upon inactivation of α_2M by methylamine, a methylamide is formed at Glx-952, in concert with the cleavage of the thioester linkage to Cys-949 (Sottrup-Jensen et al., 1984). Larsson and Björk (1984) reported that increasing the size of the primary amine inactivator

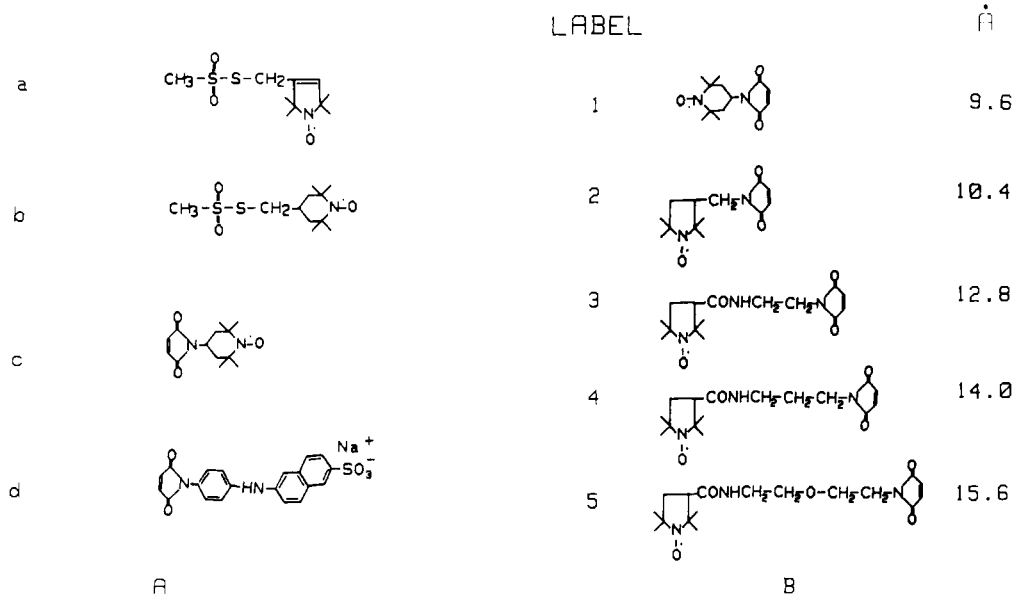

FIGURE 1: (A) Structures of a variety of thiol-specific labels. (B) Structures of the maleimide spin-labels used in this study and their length determined from CPK molecular models.

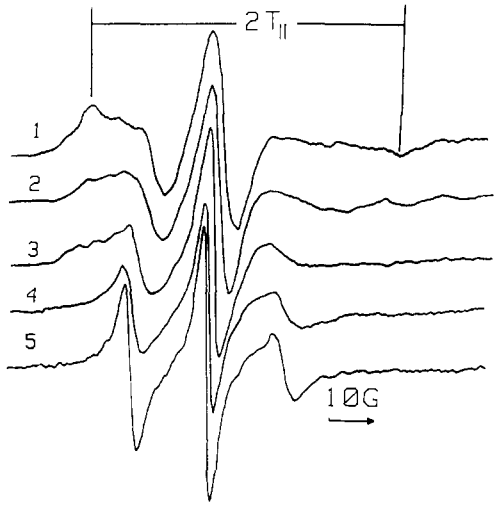

FIGURE 2: X-band ESR spectra of spin-labeled α_2M with maleimide spin-labels **1–5**. Conditions: 7 μM spin-labeled α_2M and 0.02 M phosphate–0.15 M NaCl, pH 6.0. ESR parameters: scan range 100 G, field set 3400 G, modulation amplitude 2 G, microwave power 20 mW, and scan time 2 min (each spectrum was averaged for 50 scans).

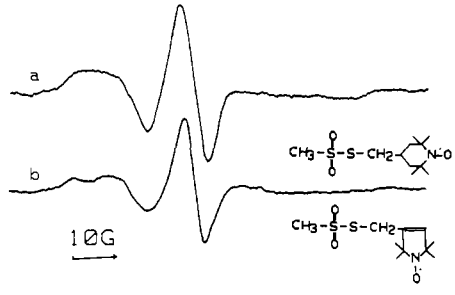

FIGURE 3: X-band ESR spectra of α_2M spin-labeled after reaction with methylamine with the proxyl and piperidine thiol spin-labels, respectively. All conditions were as in Figure 2.

decreased the rate of thiol group exposure. Furthermore, branched alkylamines inactivated α_2M at close to insignificant rates. We nonetheless treated α_2M with a 30-fold excess of 4-amino-TEMPO, a cyclic piperidine nitroxide containing an amino substituent at C-4. Although the reaction was extremely slow, of the order of days for 68% incorporation on

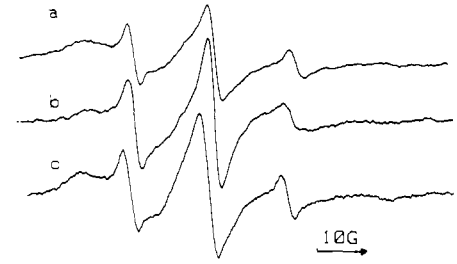

FIGURE 4: X-band ESR spectra of α_2M spin-labeled with (a) 4-amino-TEMPO and (b) 4-amino-TEMPO plus maleimide spin-label **1** and (c) computer summation of spectrum a and spectrum 1 of Figure 2 normalized to the same label concentration on each site.

Table I: Fluorescence Parameters of MANS in Various Solvent Systems

solvent	λ_{ex} (nm)	λ_{em} (nm)	quantum yield
water	335	450	0.002
α_2M	333	445	0.041
ethanol	330	440	0.133
DMF	320	430	0.166
chlorobenzene	310	405	0.228

the basis of both spin concentration and thiol group exposure, we obtained the highly immobilized ESR spectrum shown in Figure 4a. Evidently, the environment around Glx-952 must also be quite restricted, similar to that observed for the Cys-949 environment above (Figures 2 and 3). Spectrum 4b depicts the ESR spectrum after subsequently labeling the exposed thiol with maleimide spin-label **1**. In order to clearly distinguish if any spin–spin interactions occurred between these two labeled sites, we compared spectrum b of Figure 4 with a computer-summed spectrum of 4-amino-TEMPO, α_2M, and maleimide spin-label **1** minus α_2M alone (Figure 4c). A close examination of spectra b and c of Figure 4 and their difference (not shown) showed little or no evidence for either dipolar or exchange interactions between the two electrons on the amino-TEMPO and maleimide nitroxide labels.[2]

Fluorescence Studies of the Sulfhydryl Site. α_2M was labeled with the maleimido fluorophore MANS with a stoichiometry of ca. 3.5/α_2M (tetramer). The emission spectrum

[2] However, labeling at the thiol group induced a slight increase in the more mobile narrow line components (Figure 4a,b).

of the labeled protein was compared with emission spectra of the free label in solvents of varying polarity (H_2O, ethanol, DMF, and chlorobenzene, respectively). The fluorescence parameters and quantum yields are summarized in Table I. The polarity of the environment around Cys-949 appears to fall between that of ethanol and water, i.e., a somewhat hydrophobic yet solvent-accessible environment.

Discussion

A tentative model of α_2M structure was recently proposed by Feldman et al. (1985), who depicted the molecule as an overall hollow cylindrical structure comprised of two identical functional halves resembling the shape of a cyrillic Ж. They further noted that the sulfhydryl sites, which form after methylamine inactivation, were located somewhat deep into the structure, by a hollow core accessible to solvent. Several thiol-specific spin-labels were covalently incorporated into α_2M with a stoichiometry that agrees closely with that known for Cys-949 on each of the four identical subunits that comprise a single α_2M tetramer. While we have not specifically identified the labeled thiol, all previous biochemical evidence dictates Cys-949 as the only possibility. The ESR results are consistent with either a narrow sterically restricted cavity of $\geq$8 Å in depth or a cavity or groove on the surface of the α_2M molecule, formed by hydrophobic van der Waals interactions with nonpolar residues lining same.

When the fluorescence results, which were consistent, possibly, with a solvated environment (Table I), and the gradual increase in mobility from spectra **1** to **3** are considered, the cavity model appears to be more consistent with the combined ESR, fluorescence (Table I), and cylindrical model structure of Feldman et al. (1985), who postulated that the thioester sites are located at the base, or hinge, of the "trap arms" that swing closed to entrap the proteinase. While it is clear that the cavity in α_2M which entraps a proteinase must be of dimensions of the order of 40–70 Å (Feldman et al., 1985; Crews et al., 1987), the structural environment *at* the thiol group locus is a much narrower cleft.

Recent results by Larsson et al. (1987) with the thiol-specific fluorophore 8-[(*N*-acetylamino)ethyl]naphthalene-1-sulfonic acid were concluded to be consistent with a buried SH group by virtue of an emission blue shift, enhanced quantum yield, and reduced lifetime, generally in agreement with our studies with MANS.

The depth of the substituted glutamide binding site (Glx-952) was not estimated since a homologous series of amino nitroxides was not available; however, the Glx-952 side chain probably occupies the same hollow cavity as does the thiol group (Feldman et al., 1985). What was significant at this juncture, however, was the ability of the amino-TEMPO label to inactivate and become incorporated at the thioester linkage. The lack of any observable spin–spin interactions between the amino-TEMPO and maleimide **1** nitroxide moieties yields a lower limit of the interatomic distance between the free electrons on each N–O moiety (i.e., not between the thiol and amido group of the cleaved thioester bond). If the distance between the N–O group to the functional group on each label is estimated at ca. 9.6 and 6 Å, respectively, an additional distance of about 15 Å could enter into the separation between the two residues when the N–O moieties were at a minimum separation from one another.

Summarizing, the sulfhydryl group of the cleaved thioester linkage of α_2M resides in a narrow, highly solvent accessible cleft of $\geq$8 Å in depth. The Glx-952 side chain appears also to lie in a sterically restricted environment separated by at least 10–25 Å from the thiol moiety.

Added in Proof

After submission of our work, a similar spin-label study was submitted (Gettins et al., 1988), which reconfirmed the minimum depth of the thiol group sites as ca. 9 Å by an analysis identical with our methodology.

Registry No. Cys-Glu thioester, 105580-05-0; Cys-Gln thioester, 114583-41-4.

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Comparison between Complement and Melittin Hemolysis: Anti-Melittin Antibodies Inhibit Complement Lysis†

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ABSTRACT: A comparison is made between the hemolytic actions of melittin and the ninth component of complement (C9). Melittin and C9 produce "pores" of similar effective radius in erythrocytes under standardized conditions, and their hemolytic action is suppressed by metal ions at similar concentrations, suggesting a common mechanism. Polyclonal anti-melittin immunoglobulin G (IgG) produced in rabbits retards hemolysis mediated by human C9 in a specific manner. Such antibodies react in several immunoassays with human and monkey C9 but not with C9 from lower animals, and no inhibition of lysis mediated by C9 molecules from these animals is observed. Thus, it is unlikely that anti-melittin IgG reacts with a structural element, such as an amphipathic helix, on human C9 since such structures are also predicted to exist in other C9 molecules. Human C9 and melittin block cross-reactivity in a dose-dependent manner, and anti-melittin IgG recognizes an epitope located between amino acid residues 245 and 390 of human C9 on "Western" blots. Comparison of the melittin and human C9 sequences indicates two regions of complete homology, a tetrapeptide at positions 292–295, and a pentapeptide at positions 527–531 in human C9, corresponding to residues 8–16 in melittin. Inhibition of hemolysis is not caused by blocking of C9 binding to the C5b-8 complex; rather the antibody must dissociate from the bound C9 before lysis ensues, indicating that it interferes with a postbinding event. It is proposed that anti-melittin binds to a conformational epitope on native, folded human C9 and thereby retards unfolding of the molecule, which is required for membrane insertion and hemolysis.

The membrane attack complex (MAC)[1] mediates the cytotoxic effects of complement. It assembles from five water-soluble precursor glycoproteins, C5, C6, C7, C8, and C9, on a target membrane after cleavage of C5 into C5a and C5b and binding of the trimolecular C5b-7 complex. C9, the protein acting last, is responsible for the high efficiency of complement-mediated cytolysis. Although the rough details of the assembly process are understood, the actual mechanism(s) through which the MAC kills cells are still obscure and are a matter of considerable debate (Esser, 1982; Mayer, 1982; Bhakdi & Tranum-Jensen, 1983; Müller-Eberhard, 1984). The primary models for MAC function that are debated are the "leaky patch" model and the "doughnut" model (Esser, 1982). These two models originated from different experimental approaches that were used to study complement-mediated cytolysis. The doughnut model has its roots in the morphological description of the complement lesion as visualized in the electron microscope (Humphrey & Dourmashkin, 1969; Bhakdi & Tranum-Jensen, 1978), whereas the leaky patch model is based on biochemical and biophysical studies aimed at describing the molecular interactions between the terminal complement proteins and target lipid bilayers (Kinsky, 1970; Esser et al., 1979a,b; Sims & Lauf, 1978, 1980). The former envisions formation of protein-walled channels that cause cell death, and the latter postulates strong protein–lipid interactions that reorient lipid bilayers, thereby causing general weakening of the membrane barrier.

We reasoned that a direct comparison between complement-mediated hemolysis and hemolysis resulting from the action of agents that are believed to cause lysis by leaky patch formation might allow a better distinction between the two models for immune lysis. Esser et al. (1979b) had used this approach earlier to compare the efficiency to cause virolysis between channel formers, such as nystatin, and complement. Since formation of nystatin channels across the viral membrane had no effect on infectivity, they concluded that complement

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[1] Abbreviations: MAC, membrane attack complex; EDTA, ethylenediaminetetraacetate; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; TBS, Tris-buffered saline; EA, antibody-sensitized erythrocytes; EAC1–8, EA carrying complement proteins C1 through C8; kDa, kilodalton(s). Complement proteins are named in accordance with recommendations in *Bulletin of the World Health Organization* (1968).

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