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Third Component of Human Complement: Appearance of a Sulfhydryl Group following Chemical or Enzymatic Inactivation†

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ABSTRACT: Treatment of human C3 with hydroxylamine or hydrazine at physiological pH and ionic strength totally abrogates the intrinsic ability of this protein to sustain classical pathway induced hemolysis of sheep red blood cells. Concomitant with the loss of this function the appearance of a single sulfhydryl group can be followed by titration with the sulfhydryl-specific reagents *p*-(chloromercuri)benzoate, [1-^{14}C]iodoacetamide, 2,2′-dipyridyl disulfide, and 5,5′-dithiobis(2-nitrobenzoic acid). These reagents have also been used to follow the appearance of a free sulfhydryl group on conversion of C3 to C3b with bovine trypsin. Autoradiography of the electrophoretogram of separated α-, α'-, and β-polypeptide chains of inactivated, [1-^{14}C]carboxamidomethylated C3 samples has shown that the reactive sulfhydryl group is present in the α chain of C3 and in the α' chain of C3b, respectively. Digestion of the radiolabeled protein with porcine elastase has localized this sulfhydryl group to a 28 000-dalton fragment of the α chain with immunochemical and functional reactivities of the C3d domain. Autoradiographic analysis of a hydrolysate prepared from radioalkylated C3 and subjected to high-voltage paper electrophoresis has shown the labeled amino acid to be [1-^{14}C]-*S*-(carboxymethyl)cysteine. The susceptibility of native C3 to rapid and irreversible inactivation by nitrogen nucleophiles with the parallel appearance of a cysteinyl residue may indicate the presence of an internal thiol ester. The relationship of the proposed thiol ester to the ability of nascent C3b to acylate cell surface components and carbohydrate polymers is discussed within the context of a transesterification reaction.

The third component of human complement, C3, contributes importantly to immune surveillance and immune response pathways. The activation of C3 and subsequent binding of nascent C3b to a foreign cell or particle surface strengthen interactions with cellular elements operative in host defense. Complement (C) receptors specific for stable binding sites present on C3b, C3bi, and C3d have been detected on peripheral blood cells of many vertebrate species and include the neutrophil, eosinophil, monocyte, B-lymphocyte, and primate erythrocyte. Three distinct receptors have been identified and shown to bind to different regions of the C3 molecule (Gigli & Nelson, 1968; Ross & Polley, 1975; Ross & Rabellino, 1979). The importance of this class of protein–protein receptor interaction to defense mechanisms is highlighted by two sets of observations: (1) the studies of Alper et al. (1972) of a patient with inherited C3 deficiency indicated that her serum could not sustain opsonization of bacteria and she was, therefore, subject to severe and recurrent bacterial infections and (2) the studies of Ehlenberger & Nussenzweig (1977) on the respective roles of IgG and C3 for phagocytosis of sheep erythrocytes by monolayers of human monocytes and neutrophils indicated separate but synergistic roles for these two opsonins. The primary role of C receptors on these cells was to facilitate contact with the opsonized erythrocyte, and the role of the Fc receptor was primarily one of triggering the ingestive processes. Studies by Lewis et al. (1977) of immune response pathways have shown an obiligatory C3 requirement for complement receptor positive β-lymphocytes to respond in vivo or in vitro to T-cell-dependent antigens, suggesting that C3 receptors may be required for T-cell–B-cell cooperative responses, perhaps at the macrophage level. Therefore, in addition to their specific involvement in immune clearance pathways, these receptors may well be required for the modulation of the B-lymphocyte response to T-dependent antigens.

In consideration of the positive control exerted by C3 on bacterial pathogens, it is important to elucidate the mechanism by which nascent C3b binds to cell surface structures. The association of C3b with components present on all cell types (Götze & Müller-Eberhard, 1970; Müller-Eberhard, 1975) and many carbohydrate polymers including zymosan (Nicholson et al., 1974), dextran (Arnaout et al., 1979), and agarose (Goldstein et al., 1976; Capel et al., 1978) following "classical" or "alternative" pathway activation has been de-

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0006-2960/80/0419-4471$01.00/0

termined to proceed through a labile binding site with an apparent half-life of milliseconds (Muller-Eberhard et al., 1966). Disassociation of C3b cannot be effected by high salt, extremes of pH and temperature, reducing agents, or general protein denaturants; therefore, this interaction has been considered irreversible and probably covalent in nature. Law & Levine (1977) have shown, however, that nucleophilic reagents such as hydroxylamine or ammonia can effect the disassociation of C3b from membrane and particle surfaces, prompting the speculation that attachment of this protein involves the formation of an ester bond. Further studies by Law et al. (1979a,b) have shown that the site of C3b attachment resides in the C3d domain and the kinetics of disassociation under alkaline conditions are consistent with that of an ester bond. The detection of 0.6–0.7 mol of hydroxamate per mol of hydroxylamine-released C3d further indicated that the protein contributed the acyl group to the proposed ester bond.

The present report describes a systematic study of the effects of nitrogen nucleophiles, chaotropes, and protein denaturants on the functional and structural properties of highly purified C3. In conjunction with the accompanying paper (Janatova et al., 1980), we are presenting evidence that loss of the hemolytic function upon treatment of C3 with inactivating agents results in (1) the appearance of a free SH residing in the C3d fragment and (2) changes which preclude cleavage of C3 by the classical pathway C3-converting enzyme.

Experimental Procedures

Material. Platelet-poor plasma from human blood was obtained from the Missouri–Illinois Regional Red Cross Blood Program (St. Louis, MO). Gel filtration and ion-exchange chromatographic media, as well as other chemicals used in the purification of C3, were as described earlier (Tack & Prahl, 1976). Gdn·HCl[1] and β-mercaptoethanol were from Pierce Chemical Co. $NaDodSO_4$ was a product of British Drug House Ltd. Bio-Rad Laboratories was the source of reagents for polyacrylamide gel electrophoresis. Ultrapure urea was purchased from Schwarz/Mann (Division of Becton, Dickinson and Co.). QAE-Sephadex A-50, Sephadex G-25, EDTA, Tris, DFP, DTNB, hydroxylamine hydrochloride ($NH_2OH·HCl$), PCMB, and trichloroacetic acid were obtained from Sigma. Hydrazine was supplied by Eastman. Aldrich Co. was the supplier of 2-PDS and MMTS. [^{14}C]Iodoacetamide and [^{14}C]NEM were purchased from New England Nuclear. Amersham Corp. was the source of phase combining system. STI was obtained from Worthington. Hemolysin and sheep blood were purchased from Cordis Laboratories. All other chemicals and reagents used were of the highest grade available.

Isolation of C3. The isolation of C3 was carried out as described by Tack & Prahl (1976). Further ion-exchange chromatography on QAE-Sephadex A-50 was used as a final step in C3 purification for the separation of hemolytically inactive and active forms of C3 when the degree of spontaneous inactivation exceeded 10–12% on long-term storage (Janatova et al., 1980).

Protein Determination. The concentration of C3 solutions was determined routinely by using an extinction coefficient ($E_{1cm}^{1\%}$ at 280 nm) of 9.7 (Tack & Prahl, 1976).

Hemolytic Assay. Titration of C3 hemolytic activity was carried out as described by Tack & Prahl (1976). The degree of lysis was determined spectrophotometrically at 415 nm following removal of unlysed cells and cellular debris by centrifugation.

Nucelophile Inactivation. Treatment of C3 with nitrogen nucleophiles was conducted as follows. Native C3 was transferred to 0.1 M sodium phosphate buffer (pH 7.0) either by centrifugation through Sephadex G-25 or by dialysis. The protein solution (6–10 mg/mL) was mixed with a volume of reagent stock solution (pH 7.0) required to give the desired concentration for a final reaction mixture volume of 2 mL. After incubation at 37 °C for 1 h, the reaction was terminated by centrifugation through Sephadex G-25, preequilibrated in 0.1 M sodium phosphate buffer (pH 7.0) containing 2 mM EDTA. The samples were immediately examined for (1) protein content, (2) hemolytic activity, (3) SH content, and (4) chain structure by polyacrylamide gel electrophoresis.

Inactivation of C3 with KBr. This procedure was carried out as described by Dalmasso & Müller-Eberhard (1964) for the depletion of C3 hemolytic activity in serum. Following the incubation of C3 with an equal volume of saturated KBr for 18 h at 4 °C, KBr was removed by three successive centrifugations through Sephadex G-25.

Inactivation of C3 by Trypsin. Enzymatic inactivation of C3 was carried out with β-trypsin at a substrate/enzyme molar ratio of 13.3:1 for 1–3 min at 37 °C in PBS (pH 7.4). The reaction was stopped by the addition of a threefold weight excess of STI relative to trypsin. For alkylation of the resultant SH with [^{14}C]iodoacetamide the trypsin–C3b was transferred into 0.2 M Tris-HCl (pH 8.2) containing 2 mM EDTA by centrifugation through Sephadex G-25. The release of an SH during the incubation of C3 with trypsin was followed directly as well by spectrophotometric titration with 2-PDS or DTNB.

Removal of Reagents from Protein Samples. A modification of the method reported by Cefka et al. (1971) for centrifugation through Sephadex G-25 was used in these studies. In our modification, the Sephadex gel was supported in a 5- or 30-mL Jelco disposable syringe fitted with a porous polypropylene disk. The protein solution (0.5–2 mL) was applied to the top of the preequilibrated gel (15 mL) following centrifugation for 6 min at 2500 rpm. After a 2–5-min period of equilibration, the protein solution free of reagent was centrifuged out of the Sephadex gel for 6 min at 2500 rpm. Although the degree of "desalting" was high (98–99%) and sufficient in most instances, this procedure has been repeated when reagent concentrations exceeded 200 mM to avoid interference with subsequent hemolytic and SH determinations.

Polyacrylamide Gel Electrophoresis. Samples of C3, before and after chemical or enzymatic inactivation, were examined by polyacrylamide gel electrophoresis in the presence of 0.1% $NaDodSO_4$ according to the procedure by Laemmli (1970) using a slab gel apparatus (Reid & Bieleski, 1968). Prior to gel electrophoresis, samples were equilibrated by centrifugation through Sephadex G-25 or by dialysis into PBS (pH 7.4), diluted 2:1 with Tris–glycine buffer containing 9% $NaDodSO_4$, 30% glycerol, and 15% β-mercaptoethanol, and heated in a boiling water bath for 1 min. For autoradiofluorography, 20 μg of protein containing between 3000 and to 4000 cpm of ^{14}C was applied to each well. After electrophoresis, the left half of the gel was stained with 0.25% Coomassie Brilliant Blue R-250 in 50% methanol–10% acetic acid. The right half was

[1] Abbreviations used: PCMB (or PMB), *p*-(chloromercuri)benzoate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NTCB, 2-nitro-5-thiocyanobenzoic acid; 2-PDS, 2,2′-dipyridyl disulfide; NEM, *N*-ethylmaleimide; MMTS, methyl methanethiolsulfonate; Gdn·HCl, guanidine hydrochloride; $NaDodSO_4$, sodium dodecyl sulfate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; QAE, diethyl(2-hydroxypropyl)aminoethyl; STI, soybean trypsin inhibitor; [^{14}C]SCM, *S*-carboxymethyl labeled with ^{14}C; DFP, diisopropyl phosphorofluoridate.

treated with Me_2SO and PPO as described by Bonner & Laskey (1974) and dried down on WW-3 chromatographic paper, and the gel was placed on X-ray film (XR-5, X-Omat R film by Kodak) for 3–4 days at −70 °C.

Spectrophotometric Tritration of SH Content with PCMB. These studies were performed in 0.05 M phosphate buffer (pH 7.4) in principle as described by Riordan & Vallee (1972). The absorbance increase was measured at 250 nm with a Cary 118 spectrophotometer. A dialyzed C3 solution was adjusted to 1.5 mg/mL, and 10-μL aliquots of 0.1 mM PCMB solution (pH 7.4) were added to the protein solution and buffer blank, 1 mL each. After mixing, the ΔA_{250} was recorded for 3–5 min. The end point of the titration was estimated graphically, and after correction of ΔA_{250} for the dilution factor the SH content of the C3 sample was calculated as nanomoles of PCMB at the end point divided by nanomoles of the protein in solution.

Determination of SH Content by Radioalkylation. Alkylation of C3 with [^{14}C]iodoacetamide was performed in the following manner. Fifty-microliter aliquots containing 5 μCi each of [^{14}C]iodoacetamide were dried down in 12 × 75 mm tubes under N_2 or vacuum and dissolved in 100 μL of 0.2 M Tris-HCl (pH 8.2) containing 2 mM EDTA. In specific instances the [^{14}C]iodoacetamide was dissolved in 0.2 M Tris-HCl buffer (pH 8.2) containing 2 mM EDTA and $NaDodSO_4$ or Gdn·HCl. An amount of C3 corresponding to 3 mg was then added, and the reaction was allowed to proceed for 2–6 h at room temperature. Excess [^{14}C]iodoacetamide was removed by dialysis against 5 mM Tris-HCl buffer (pH 8.2) containing 0.15 M NaCl. The molar ratio of [^{14}C]iodoacetamide to C3 in these studies was 20:1. Aliquots containing 300–500 μg of protein was added to 10 mL of phase combining system and counted in a Beckman liquid scintillation counter (LS-233). The specific activity of [^{14}C]iodoacetamide used was 15.76 mCi/mmol. On the basis of a molecular weight for C3 of 187 500 (Tack & Prahl, 1976), a specific activity of 1.85×10^5 dpm/mg of C3 corresponded to the incorporation of 1 mol of [^{14}C]iodoacetamide per mol of protein.

Spectrophotometric Titration of SH Content with DTNB. This procedure was performed according to the method of Ellman (1959) as modified by Janatova et al. (1968). Determinations were carried out in 50 mM sodium phosphate buffer (pH 7.0) in the presence of 2 mM EDTA. In a typical assay 400 μL of a C3 solution (3.5 mg/mL) was mixed with 50 or 100 μL of 0.01 M DTNB and the reaction was followed by measuring the absorbance at 412 nm. The SH content was calculated from the ΔA_{412} and known C3 concentration by using 13 600 M^{-1} cm^{-1} for the molar extinction coefficient of TNB ion and 187 500 for the molecular weight of C3. In the experiments with KBr, the starting concentration of C3 was 7 mg/mL as samples were diluted with an equal volume of saturated KBr.

Spectrophotometric Titration of SH Content with NTCB. Titrations with NTCB were conducted in the same manner as described earlier for DTNB. The NTCB reagent was prepared from DTNB as described by Degani et al. (1970). A 10 mM NTCB solution in 0.1 M phosphate buffer (pH 7.0) containing 2 mM EDTA was used in these assays.

Spectrophotometric Titration of SH Content with 2-PDS. The procedure of Grassetti & Murray (1967) was carried out in a manner similar to the determination with DTNB. Due to the lower molar extinction coefficient of 2-thiopyridone (7.06×10^3 at 343 nm), higher concentrations of C3 were used in this assay. The reagent (1.25 mM 2-PDS in H_2O) was prepared according to Brocklehurst & Little (1973). This reagent was stable and gave low blank absorbance values in buffer systems over a wide pH range. The 2-PDS has been found to be an especially suitable reagent for following the release of the sulfhydryl group in Gdn·HCl.

Determination of SH Content with [^{14}C]NEM. These studies were performed as suggested by Riordan & Vallee (1972). Aliquots of native and KBr-treated C3 containing 2.6 mg of protein each were reacted with a 15.2-fold molar excess of [^{14}C]NEM in 0.1 M sodium phosphate buffer (pH 7.0) for 4 h in the dark at room temperature. After thorough dialysis, 250-μg aliquots of C3 were counted in 10 mL of phase combining solvent. The specific activity of [^{14}C]NEM used in these studies was 23.7 mCi/mmol. A specific activity of 2.78×10^5 dpm per mg of C3 corresponded to the incorporation of 1 mol of [^{14}C]NEM per mol of protein.

Determination of SH Content with MMTS. This determination was performed in a manner similar to that of Smith et al. (1975). Native or inactivated C3 was treated with a 10–30-fold molar excess of MMTS in 0.02 M glycine–NaOH buffer (pH 8.25) containing 75 mM NaCl and 2 mM EDTA in an ice bath for 30 min.

Tryptic Digestion of Radioalkylated C3. Between 4 and 6 mg of (1) trypsin–C3b and (2) 200 mM NH_2OH- and (3) KBr-treated C3 samples were alkylated with [^{14}C]iodoacetamide. The samples were precipitated with equal volumes of 20% trichloroacetic acid and after 1 h were centrifuged at 10 000 rpm for 15 min. The precipitates were washed sequentially with 5% trichloroacetic acid, ethanol–ethyl ether (1:1), and ethyl ether. The pellets were dried, suspended in 1 mL of 0.1 M NH_4HCO_3 (pH 8.5), and incubated with 600 μg of β-trypsin at 37 °C for 2 h. The trypsin digests were lyophilized and dissolved in 500 μL of 20% formic acid. About 85% of the total counts per minute were solubilized by this procedure and applied to a Sephadex G-25 column (1.5 × 50 cm) equilibrated in 10% acetic acid. Eluates (0.5 mL/tube) were monitored for absorbance at 280 nm, and 10-μL aliquots were taken for determination of radioactivity. The ^{14}C-containing peptide pools were dried and redissolved in 200 μL of 10% acetic acid.

Peptide Mapping. Two-dimensional thin-layer maps were developed as described by Gracy (1977). Samples of ^{14}C-labeled peptide pools (4000 cpm each) were applied to 20 × 20 cm TLC plastic sheets precoated with cellulose, divided in half. C3b was used as a control. Electrophoresis in pyridine–glacial acetic acid–H_2O (1:10:89) buffer (pH 3.7) was conducted at 400 V for 3 h or until methyl green migrated 12 cm. The plates were air-dried at room temperature and cut in half in the direction of electrophoresis and subsequently chromatographed in a solvent mixture composed of 1-butanol–pyridine–glacial acetic acid–H_2O in a volume ratio of 26:20:4:16. After air-drying at room temperature, the TLC sheets were placed on Kodak X-Omat R film for 1 week at −70 °C.

High-Voltage Electrophoresis. The 6 N HCl hydrolysates of [^{14}C]C3 samples (6000 cpm each) were electrophoresed on Whatman 3MM chromatographic paper in pyridine–acetate buffer (pH 3.5) at 3000 V for 80 min. The center portion of the electrophoretogram which corresponded to radiolabeled C3 hydrolysates was cut out and placed on X-ray film for several weeks. Side strips with SCM-Cys standards were developed with cadmium–ninhydrin stain.

Results and Discussion

Ecker et al. (1943) first reported the loss of C4 hemolytic activity on treatment of human serum with hydrazine. Müller-Eberhard & Biro (1963) later purified C4 and demonstrated that the ability of this component to combine with EACl was lost on treatment of the protein with 15 mM hy-

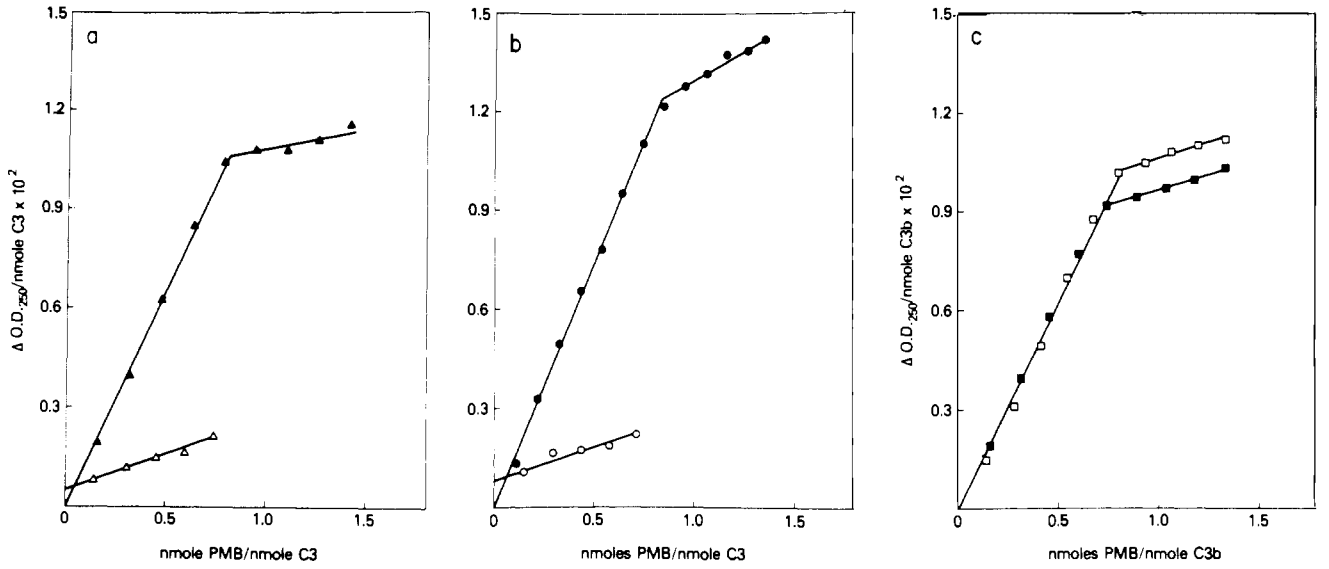

FIGURE 1: Spectrophotometric titrations with PMB following inactivation of C3 with 1 M hydroxylamine (a, ▲), 5 mM hydrazine (b, ●), and 1% (w/w) bovine trypsin (c, □). The reactivity of control samples of C3 used in the hydroxylamine and hydrazine studies are indicated by △ and ○, respectively. The SH content of C3b prepared with trypsin was also determined following treatment with 1 M hydroxylamine (c, ■).

drazine. In addition, changes in the immunoelectrophoretic mobility and sedimentation coefficient of C4, accompanying its inactivation, were apparent from these studies. West et al. (1966) extended these observations to C3, showing that a C3 conformational determinant (B-antigen) was lost on treatment of human serum with 15 mM hydrazine or 30 mM ammonia and that the immunoelectrophoretic mobility of the α_{2D} (C3d) fragment was specifically affected. More recently, Müller-Eberhard & Götze (1972) identified C3 as the hydrazine-sensitive factor of the alternative pathway. The mechanism by which inactivation of C3 and C4 proceeds, however, has remained unclear.

Treatment of C3 with 1 M hydroxylamine (pH 7.0) for 1 h at 37 °C resulted in the appearance of an SH reactive with PMB, and a titration end point of 0.82 nmol of PMB per nmol of protein was graphically estimated (Figure 1a). A control sample of C3 by comparison bound 0.07 nmol of PMB per nmol of protein. In a parallel experiment C3 treated with 5 mM hydrazine (pH 7.0) for 1 h at 37 °C was titrated with PMB and an end point corresponding to the consumption of 0.84 nmol of PMB per nmol of C3 was determined (Figure 1b). A similar reactivity was also apparent for trypsin-generated C3b; an end point of 0.81 nmol of PMB per nmol of protein was observed (Figure 1c). Treatment of C3b with 1 M hydroxylamine prior to PMB titration did not significantly affect the titration end point.

Our initial results indicated that C3 treated with hydroxylamine and hydrazine acquired a stoichiometric reactivity with PMB, suggesting the presence of an amine-sensitive bond in the native protein comprised of an SH component. The observation that hydrazine elicited a dose-dependent inactivation of C3 with concomitant SH expression in the concentration range of 1–5 mM would imply reaction with a specific site. Hydrazine at these low concentrations is unlikely to produce this effect through nonspecific denaturation of the protein thereby exposing a buried SH. The expression of an SH in C3b following treatment of C3 with trypsin has been interpreted to indicate the existence of an internal mechanism for hydrolysis of this bond facilitated by cleavage of the Arg–Ser peptide bond positioned at 77–78 in the α-polypeptide chain (Tack et al., 1979). In order to test these initial conclusions, we performed the following experiments: (1) the SH content following inactivation was quantitatively evaluated with other SH-reactive reagents including [^{14}C]iodoacetamide, [^{14}C]NEM, 2-PDS, DTNB, NTCB, and MMTS; (2) the correlation between the degree of hemolytic inactivation and the expression of the SH component was investigated; (3) the effects of general protein denaturants (urea, Gdn·HCl, and $NaDodSO_4$) and the chaotrope KBr on the expression of the SH component were evaluated; (4) the identity and location of the residue contributing the SH was established.

Table I: Treatment of Human C3 with Hydroxylamine and Hydrazine

reagent	concn (mM)	mol of SH per mol of C3[b]	% inactivation[c]
hydroxylamine[a]	5	0.07	0
	50	0.44	48
	200	0.82	88
	500	1.00	100
hydrazine[a]	0.1	0.15	10
	1.0	0.48	45
	2.5	0.70	78
	5.0	0.86	92

[a] Native C3 was treated with the indicated concentrations of hydroxylamine and hydrazine in 100 mM phosphate buffer (pH 7.0) containing 2 mM EDTA at 37 °C for 1 h. The reagent was removed by centrifugation at 4 °C through Sephadex G-25 in the same buffer or in Tris-HCl (pH 8.2). The samples were immediately assayed for SH content and hemolytic activity. [b] The SH concentration was determined by DTNB and 2-PDS titrations and by alkylation with [^{14}C]iodoacetamide. Control samples of C3 held at 37 °C for 1 h consistently titered 0.06 mol of SH per mol of protein. [c] The % hemolytic inactivation was determined by comparison with the activity (CH_{50} units/milligram) of a control sample of C3 held at 37 °C for 1 h and centrifuged through Sephadex G-25.

Treatment of Native C3 with Nitrogen Nucleophiles. Although PMB is considered a highly selective reagent for SH determination when the extent of reaction is followed spectrophotometrically at 250 nm, it was necessary to obtain confirmation with other sulfhydryl titrants. The results of SH quantitation with [^{14}C]iodoacetamide, 2-PDS, and DTNB following treatment of the protein with hydroxylamine (5–500 mM) and hydrazine (0.1–5 mM) are presented in Table I.

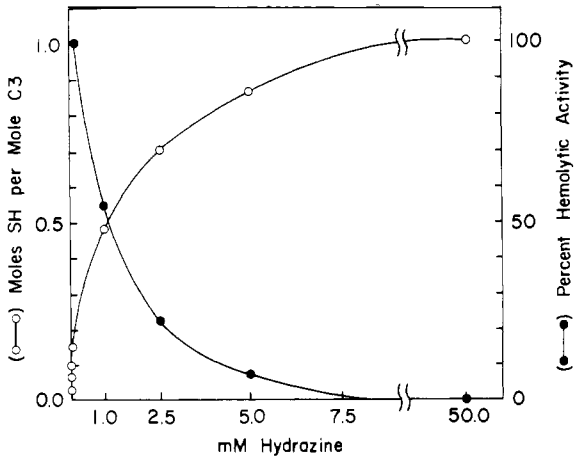

FIGURE 2: A plot of functional activity and corresponding SH content following inactivation of C3 with hydrazine (1 h; 37 °C) at final concentrations of 1–50 mM. SH determinations were performed spectrophotometrically with DTNB at 412 nm. The specific hemolytic activity of C3 (CH_{50} units/milligram) incubated for 1 h at 37 °C in the absence of hydrazine was taken as the 100% value.

Parallel experiments were also performed to assess the effect of nucleophile treatment on the hemolytic function of C3. A plot of the data obtained in a separate set of experiments with hydrazine where the SH component was followed with DTNB at 412 nm is shown in Figure 2. In each instance comparable results were obtained with the above SH reagents and a 1:1 correspondence with the loss of functional activity was observed. It is apparent from these results that the final concentrations of hydroxylamine and hydrazine required to achieve comparable degrees of inactivation are significantly different. At pH 7.0 hydrazine was observed to be 50–100-fold more effective than hydroxylamine. The reactivity of trypsin-generated C3b has, as well, been studied with the above SH reagents. The extent of reaction with each was determined to be 0.97–1.04 mol of SH per mol of C3b.

Treatment of Native C3 with Chaotrope or Denaturants. Chaotropic reagents such as KBr and KSCN have been reported to block C3 hemolytic function in human serum (Dalmasso & Müller-Eberhard, 1964). Treatment of the native protein with a half-saturated solution of KBr for 19 h at 4 °C resulted in total hemolytic inactivation and an acquired 1:1 reactivity with [^{14}C]iodoacetamide (0.94–1.06), [^{14}C]NEM (0.93), 2-PDS (0.96), and DTNB (0.88–0.99). When the course of reaction was followed with DTNB at 412 nm, the appearance of the SH was rapid and reached a plateau within 90 min. Control experiments with half-saturated solutions of NaCl indicated retention of full hemolytic function and less than 0.1 mol of titratable SH per mol of protein following a similar incubation period.

In addition to nucleophilic and chaotropic reagents, the effects of treatment with urea, Gdn·HCl, and $NaDodSO_4$ were studied. The results of urea (3 and 1.5 M) and Gdn·HCl (1 and 0.5 M) treatment are shown in Figure 3 where the course of reaction was followed with 2-PDS at 343 nm. In the presence of 1 M Gdn·HCl the reactivity with 2-PDS reached a plateau at 20 min corresponding to 0.96 mol of SH per mol of protein. The same final degree of reaction was apparent in 3 M urea; however, an incubation period of 1 h was required. In 0.5 M Gdn·HCl and 1.5 M urea the kinetics of SH release were slower with both samples reaching a value corresponding to 0.52 mol of SH per mol of protein following a 3-h incubation. A summary of results pertaining to urea, Gdn·HCl, and $NaDodSO_4$ treatment of C3 is shown in Table II. The SH content was determined following an incubation period

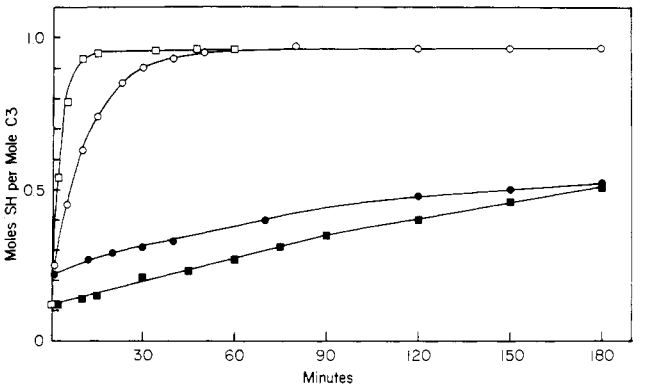

FIGURE 3: Titration of an SH in C3 on treatment with Gdn·HCl (1 M, □; 0.5 M, ■) and urea (3 M, ○; 1.5 M, ●) as followed spectrophotometrically with 2-PDS at 343 nm.

Table II: Treatment of C3 with Denaturants

		mol of SH/mol of C3		
denaturant	concn	DTNB[a]	2-PDS[a]	ICH_2CONH_2[b]
Gdn·HCl	1.0–5.0 M	ND[c]	0.96–1.00	ND
	6.0–7.0 M	0.91–0.96	ND	0.80–0.90
urea	3.0–6.0 M	ND	0.90–1.00	ND
$NaDodSO_4$	0.2%	0.09	0.43 rising to 1.01	0.81–0.83

[a] Native C3 was diluted into denaturant in 0.1 M sodium phosphate (pH 7.0) containing 2 mM EDTA and reaction with DTNB or 2-PDS was followed spectrophotometrically at 412 or 343 nm, respectively. [b] Native C3 was diluted into denaturant in 0.5 M Tris-HCl (pH 8.2) containing 5 mM EDTA and 5 μCi of [^{14}C]ICH_2CONH_2. After a 2–5-h incubation period the denaturant and excess alkylating reagent were removed by dialysis. Control samples of C3 incubated for equivalent time periods in the absence of a denaturing agent titered 0.05–0.10 mol of SH per mol of protein by all methods. [c] ND = not determined.

of 1 h at 37 °C with DTNB, 2-PDS, and [^{14}C]iodoacetamide. The failure of DTNB to react with the SH in the presence of 0.2% $NaDodSO_4$ is probably attributable to charge repulsion between the carboxylate anion of the reagent and the $NaDodSO_4$–protein complex.

Treatment of Native C3 with SH-Reactive Reagents. In contrast to the report by Polley & Müller-Eberhard (1967) that native C3 contains 1–2 free SH/molecule, we consisitently titrate less than 0.1 SH/molecule with a wide range of SH reagents. The most straightforward explanation for this difference would be to question the selectivity of [^{14}C]PMB used in their studies. It is known that this reagent binds to sites in proteins other than a free SH (Glazer, 1976). An assessment of the SH content of protein based on the specific activity following incubation with [^{14}C]PMB could therefore be misleading. In our studies the binding of PMB was based on the ΔE_{250} which is a property of the chromophore seen only on association with a free SH. In addition to [^{14}C]iodoacetamide, DTNB, and 2-PDS, incubation of native C3 with NTCB, [^{14}C]NEM, or MMTS has not resulted in detection of an SH or the loss of hemolytic activity. However, subsequent treatment of these samples with an inactivating agent resulted in the stoichiometric appearance of an SH detectable with these SH reagents which differ with respect to size, charge, and hydrophobicity.

Localization of the Sulfhydryl Group. Studies by Bolotin et al. (1977) have indicated that treatment of C4 with hydrazine does not result in an apparent change in size of either the α, β, or γ chain. Treatment of C3 with hydroxylamine, however, has been reported to be accompanied by cleavage of the α chain (Budzko & Müller-Eberhard, 1969; Budzko

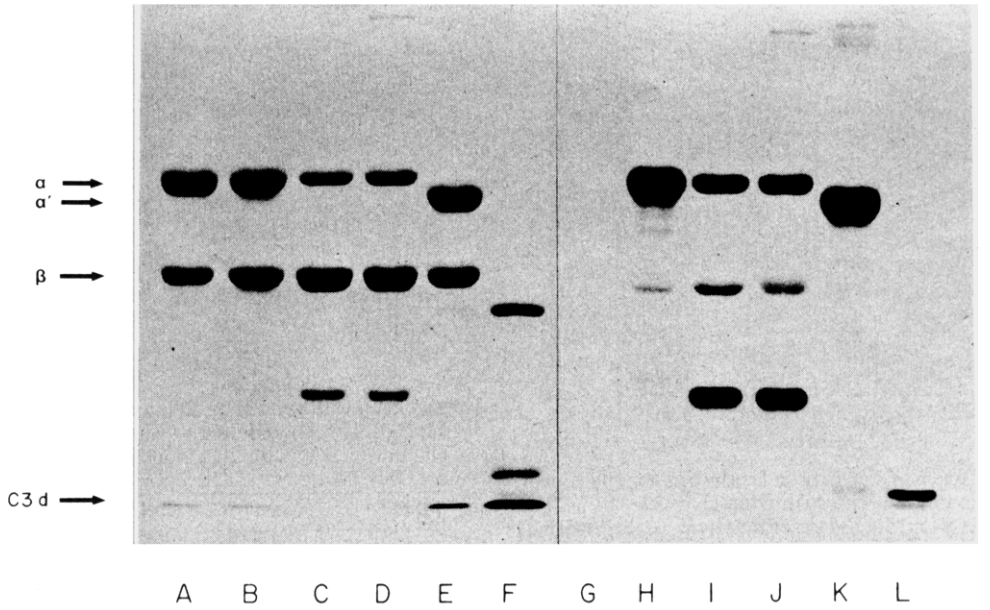

FIGURE 4: $NaDodSO_4$–polyacrylamide gel electrophoresis of C3 samples pretreated with 0.5 M hydroxylamine (B), 6 M Gdn·HCl (C), 0.2% $NaDodSO_4$ (D), 1% (w/w) trypsin (E), and 1% (w/w) porcine elastase (F). Prior to electrophoresis each sample was radioalkylated with [^{14}C]iodoacetamide and then reduced with β-mercaptoethanol. Samples in tracks A–F were stained with Coomassie Brilliant Blue. The corresponding autoradiogram of this gel (G–L) is shown to the right. A C3 control was run in tracks A and G.

et al., 1971). In this study, samples of C3 following treatment with 500 mM hydroxylamine, 6 M Gdn·HCl, 0.2% $NaDodSO_4$, and bovine trypsin were radioalkylated with [^{14}C]iodoacetamide and subjected to gel electrophoresis in the presence of $NaDodSO_4$ and β-mercaptoethanol (Figure 4). In the left panel (tracks A–F) the protein bands were visualized by staining with Coomassie Brilliant Blue (R-250), and in the right panel (tracks G–L) the presence and location of ^{14}C label was detected by autoradiography following PPO impregnation. A control sample of C3 (track A) and C3 treated with 500 mM hydroxylamine (track B) exhibited the characteristic α- and β-polypeptide chain structure. A partial fragmentation of the α chain was observed, however, for samples of native C3 when treated with 6 M Gdn·HCl or 0.2% $NaDodSO_4$ (shown in tracks C and D, respectively). An $\alpha'\beta$ chain structure characteristic of C3b was observed following trypsin treatment (track E). When an autoradiogram of corresponding samples was developed, it was apparent that (1) the incorporation of the radiolabel was strictly into the α chain following hydroxylamine inactivation (track H), (2) on treatment with Gdn·HCl or $NaDodSO_4$ the incorporation again is into the α chain [however, a partial fragmentation into bands of 68 000 and 46 000 daltons was apparent where the 46 000-dalton component retained the radiolabel (tracks I and J)], and (3) with C3b the radiolabel was uniquely associated with the α' chain (track K). When hydroxylamine-treated C3 was digested with porcine elastase, several α-chain derived fragmentation products were observed (track F) and the ^{14}C label was found to reside with a 28 000-dalton fragment (track L) subsequently shown to have antigenic, immunoelectrophoretic, and functional properties of the α_{2D} (C3d) fragment. The fragmentation pattern of the C3 α chain observed in these studies is comparable with that reported by Taylor et al. (1975), using human leukocyte elastase.

Identification of the Residue with a Reactive Sulfhydryl. The results of high-voltage electrophoresis of acid hydrolysates of radioalkylated C3 samples are shown in Figure 5 and include material inactivated with hydroxylamine, trypsin, and KBr. The original electrophoretogram is shown in part A; side strips were stained with cadmium–ninhydrin and indicate the positon of an SCM-Cys standard. The center portion of the electrophoretogram, corresponding to C3 hydrolysates, was autoradiographed, and the results are shown in part B. The major ^{14}C-labeled spot corresponded to SCM-Cys in all samples tested. The cathodal section is not shown as no radioactive components were detected in this area.

Peptide Mapping of Tryptic Digest. Samples of C3 that had been inactivated with hydroxylamine, trypsin, and KBr and subsequently radioalkylated with [^{14}C]iodoacetamide were incubated with β-trypsin at 37 °C for 2 h. Tryptic digests were fractionated by gel filtration, and ^{14}C-containing peptide pools were analyzed by a two-dimensional mapping procedure on cellulose plates. Electrophoresis at pH 3.7 was followed by ascending chromatography. Autoradiography of the peptide maps indicated the presence of a major ^{14}C-labeled peptide in each sample (Figure 6). The C3b digest was used as a control, and identical patterns were obtained in both runs A and B. The peptide migrated 48 mm on electrophoresis and 17 mm on ascending chromatography. While the pattern of KBr-inactivated C3 was superimposable with that of C3b, the ^{14}C-labeled peptide from hydroxylamine-treated C3 exhibited significant differences in both the first and second dimensions (electrophoresis, 37 mm; chromatography, 25 mm). It is apparent from these results that the hydroxylamine-sensitive site in C3 resides in the same tryptic peptide as the [^{14}C]-SCM-Cys residue. Studies are in progress to isolate by standard chromatographic procedures the radioalkylated peptide from each of the above C3 samples and to identify the hydroxyamine-reactive residue.

Proposed Form and Role for an SH in C3 Function. This study has shown that the hemolytic inactivation of C3 by nitrogen nucleophiles is a nondegradative process accompanied by the appearance of a single SH attributable to a cysteinyl residue present in the C3d fragment. Our results are in accordance with the properties of thiol ester bonds which have been recognized to be highly susceptible to cleavage by nitrogen nucleophiles, including hydroxylamine and hydrazine (Bruice & Benkovic, 1966; Torchinskii, 1974; Liu, 1977). The appearance of an SH following treatment of C3 with denaturants and KBr has been interpreted to result from confor-

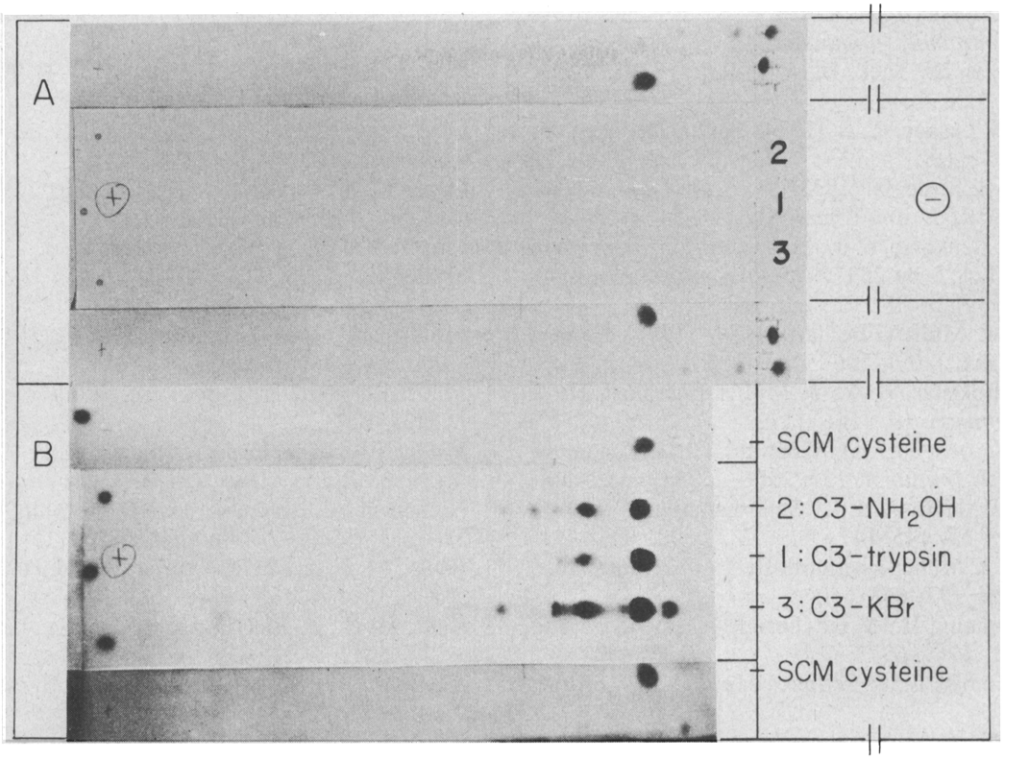

FIGURE 5: Results of high-voltage electrophoresis of acid hydrolysates of radioalkylated ([^{14}C]iodoacetamide) C3 samples pretreated with hydroxylamine, KBr, and trypsin are shown in two parts. Part A represents the original electrophoretogram where an SCM-Cys standard was included and stained with cadmium–ninhydrin, and part B is the corresponding autoradiogram.

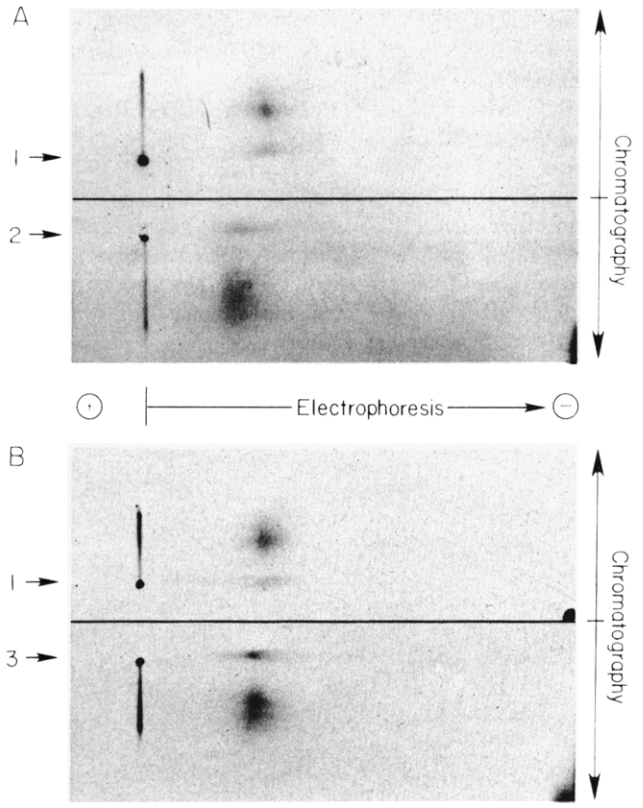

FIGURE 6: Autoradiograms of two-dimensional tryptic peptide maps of radioalkylated ([^{14}C]iodoacetamide) C3 samples pretreated with hydroxylamine, Kr, and trypsin. The tryptic digests of hydroxylamine-inactivated C3 (2) and C3b (1) were electrophoresed separately on plate A and that of KBr-inactivated C3 (3) along with C3b (1) on plate B.

mational destabilization of the protein with subsequent labilization of the thiol ester bond. This conclusion is supported in part by the observation that the conversion of native C3 to C3b is accompanied by the appearance of a free SH. It is tempting to speculate that a conformational change in C3 resulting from the action of denaturing agents or proteolytic enzymes positions the imidazole group of a histidyl residue for nucleophilic displacement at the carbonyl carbon of the thiol ester. Precedence for such a reaction has come from kinetic studies of the hydrolysis of 1-propyl-γ-(4′-imidazolyl) thiolbutyrate where the steric relationship of the ester bond to the imidazolyl group served to increase by a factor of 10^6 the rate of hydrolysis of the thiol ester bond at neutral pH (Bruice, 1959). Until further structural data are obtained, however, such considerations must remain speculative.

In summary, we propose the existence of an internal thiol ester bond in native C3. A functional center of this nature could provide the energy requirement for the formation of a covalent bond between C3b and cell surface structures. A transesterification reaction has been recently proposed by Law et al. (1979b) as one possible model for acyl group transfer. The concept of an internal thiol ester bond lends considerable support to this hypothesis.

Acknowledgments

We express our appreciation to Laurent E. Wanner for his technical assistance throughout the course of a large part of this work and to Donald G. Morse for the preparation of figures. We thank Dr. William R. Gray and Carol Bolotin for their interest and advice during the prepartion of this manuscript. We also express our gratitude to Yolanda Gooden, ViviAnn Rose, and Edie Vetter for their secretarial assistance.

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