Third Component of Human Complement: Structural Requirements for Its Function†

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ABSTRACT: Loss of the hemolytic function of C3 is accompanied by the stoichiometric appearance of a free SH group. We report here that the inactivation of C3 results in a profound change in its chromatographic behavior. Consequently, hemolytically inactive forms containing 1 SH group/C3 molecule can be separated from the native, hemolytically active form of C3 by chromatography on QAE-Sephadex A-50. All forms exhibit the same $\alpha\beta$ polypeptide chain structure and the N terminus of either α or β chain remains unchanged upon inactivation. Unlike native C3, hemolytically inactive forms do not undergo spontaneous α-chain fragmentation in the presence of Gdn·HCl or $NaDodSO_4$. The stage at which the capability of C3 to sustain further complement activation is affected by treatment with nucleophiles and chaotropes or upon spontaneous inactivation has been determined. Only native hemolytically active C3 molecules are cleaved by $\overline{C42}$, the classical pathway C3 convertase, resulting in the binding of C3b to the erythrocyte membrane. Hemolytically inactive forms of C3 are neither cleaved nor bound. These observations are indicative of the physicochemical changes occurring during the inactivation process, being expressed not only by a titratable SH group and by different chromatographic behavior but also because they seem to abolish the conformational requirements for C3 to act as the only known protein substrate of $\overline{C42}$ enzyme. Moreover, the inactivated C3 does not seem to participate in the activation of C5 as no hemolysis occurs in the presence of all classical pathway complement proteins when native C3 is substituted with the inactivated C3. These results are interpreted to mean that the proposed internal thiol ester serves to stabilize the conformation of the native C3 which is necessary for the interaction with C3 convertase, a prerequisite step for the subsequent activation of C3. This process is essential for C5 activation, the initiating step in the formation of the cytolytic complex C5b-9.

The complement system is the main effector of the humoral immune response and as such plays an important role in defending the body against infections. It operates via two pathways, the classical and the alternative. Both pathways are subject to intrinsic and extrinsic controls. Many complement proteins are present in serum in precursor forms. In the classical pathway, activation of the complement "cascade" follows an antibody–antigen interaction; in the alternative pathway, activation can be caused by a wide variety of biological substances. The two pathways proceed by sequential self-assembly of distinct multimacromolecular complexes with highly specific enzymatic activities; both of these have as their final substrates C3 and then C5. The ultimate product of each is the stable C5b-9 complex, which is potentially membranocytolytic. Several recent reviews are available dealing with the chemistry and function of the complement system (Müller-Eberhard, 1975; Götze & Müller-Eberhard, 1976; Porter, 1977; Barkas, 1978; Brade, 1978; Whicher, 1978; Lachmann, 1979; Stroud et al., 1979; Porter & Reid, 1979).

The third component of complement, C3, plays a central role as it is the protein at which the classical and alternative pathways of activation converge. The activation of C3 is accompanied by enzymatic cleavage of C3 by C3 convertase, either $\overline{C4b2a}$ or $\overline{C3bBb}$,[1] into the smaller C3a fragment with anaphylatoxic activity and the larger C3b fragment. "Nascent" C3b generated by either pathway is capable of binding to cell surfaces via its labile binding site. In both pathways the surface-bound C3b with "the active configuration" (Vogt et al., 1978; von Zabern et al., 1979a) participates in the activation of C5. Surface-fixed C3b does not modulate $\overline{C42}$ enzyme but does affect the configuration of C5 to make it accessible for the cleavage either by surface-bound C3 convertase or by an unbound version of this enzyme (Vogt et al., 1978; von Zabern et al., 1979a; Isenman et al., 1980). The cleavage of C5 into C5a and C5b represents the initiating step in the formation of the cytolytic complex C5b-9.

With respect to this important role of C3, it is essential to elucidate the nature and mechanism of reaction leading to the binding of C3b to cell surfaces. It has already been suggested that C3b–membrane binding occurs both by hydrophobic interactions and by a covalent bond, such as an ester (Law & Levine, 1977; Law et al., 1979b). The hypothesis of ester bond formation as the nature of C3b–membrane interaction presupposes the existence of chemical or enzymatic properties in native C3 (or C4) which are responsible for the appropriate covalent bond formation. It has been shown that C3b binds to receptor surfaces via the C3d fragment (Law et al., 1979a), and the existence of a reactive acyl group in C3d has recently been postulated (Law & Levine, 1977; Law et al., 1979b).

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[1] Abbreviations used: C3(n), native hemolytically active form of C3; C3(i), hemolytically inactive form of C3, containing 1 SH group/molecule; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); 2-PDS, 2,2′-dipyridyl disulfide; Gdn·HCl, guanidine hydrochloride; $NaDodSO_4$, sodium dodecyl sulfate; NP-40, Nonidet P-40, alkylphenol ethoxylate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; QAE, diethyl(2-hydroxypropyl)aminoethyl; EAC1, EAC14, EAC142, and EAC1423, cell surface–complement intermediates with the designated complement proteins attached to the cell; $\overline{C42}$ and $\overline{C3bBb}$, the C3 convertases.

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Nucleophilic reagents such as hydroxylamine or hydrazine have long been known to convert C3 (and also C4) in human serum to hemolytically inactive forms (Müller-Eberhard & Biro, 1963; Budzko & Müller-Eberhard, 1969). However, the mechanism for this inactivation has not been known. Our previous work (Janatova et al., 1980) and this study using purified proteins have shown that the inactivation by nucleophiles and chaotropes is a nondegradative process, which is accompanied by stoichiometic appearance of a free SH group. This group is located in the part of the molecule (C3d) which is also bound to the cell surface (Law et al., 1979a). Our recent results have indicated that the inactivation of C3, in addition to exposing the SH group, involves modification of a chemically reactive group. This type of behavior is displayed by thiol ester bonds (Torchinskii, 1974; Liu, 1977). In the C3 protein the SH group and the reactive carbonyl group are present in the same relatively small tryptic peptide. In this communication, we present further studies probing the chemical and structural requirements for the C3 capability to function in hemolysis.

Experimental Procedures

Materials. Platelet-deficient plasma from human blood was obtained from the Missouri–Illinois Regional Red Cross Blood Program (St. Louis, MO). Gel filtration and ion-exchange chromatography media, as well as other chemicals used in purification, inactivation, and characterization of native and inactivated C3, were as described earlier (Tack & Prahl, 1976; Tack et al., 1979; Janatova et al., 1980). Sequencer reagents were from Pierce Chemical Co. Solvents for sequencer and LC were supplied by Burdick & Jackson Laboratories, Inc. Several batches of iodine-125 were supplied as sodium iodide by Amersham Corp. Immobilized lactoperoxidase and STI were obtained from Worthington. Hemolysin and sheep blood were purchased from Cordis and/or Flow Laboratories. All other chemicals and reagents used were of the highest grade available.

The isolation of C3 was carried out as described by Tack & Prahl (1976). Further ion-exchange chromatography on QAE-Sephadex A-50 allowed separation of hemolytically inactive and active forms of C3. Recommended conditions for the isolation of hemolytically active C3, i.e., C3(n), are shown in Figure 2a. Ultrafiltration over a PM10 Diaflo membrane was used for concentration of C3.

Hemolytic activity of C3 samples was determined quantitatively by titration as described by Tack & Prahl (1976).

Protein concentration of C3 solutions was determined routinely by measuring extinction at 280 nm, using an extinction coefficient ($E_{1\%}^{1cm}$ at 280 nm) of 9.7 and a molecular weight of 187 500 (Tack & Prahl, 1976).

Determination of SH group content was carried out by alkylation with [^{14}C]iodoacetamide or by spectrophotometric titrations with either DTNB or 2-PDS (Janatova et al., 1980). Treatment of C3 with inactivating reagents, removal of reagents from protein samples by centrifugation through Sephadex G-25, and polyacrylamide gel electrophoresis are also described in the preceding communication (Janatova et al., 1980).

Automated Edman degradation of 8 mg of KBr–NH_2OH-treated C3 and of spontaneously inactivated C3 was carried out on the Beckman 890C sequencer using a protein–0.2 M quadrol program (122974) and the Sequemat P-6 autoconverter. Phenylthiohydantoin (PTH) amino acids were analyzed by LC (Hewlett-Packard Model 1084B).

Radioiodination. Highly purified C3 (e.g., C3(n) from Figure 2a) was radioiodinated with ^{125}I by the lactoperoxidase (LPO) method (David & Reisfeld, 1974). The activity of immobilized LPO was determined prior to iodination by the procedure recommended by Worthington Co., and the concentration of H_2O_2 was calculated from the absorbance of 0.03% H_2O_2 at 230 nm by using the value 72.4 as a molar extinction coefficient. Na^{125}I in dilute NaOH (pH 7–11) at 5 mCi/50 μL was supplied by Amersham. C3(n) protein was transferred to 0.1 M sodium phosphate (pH 7.0) free of EDTA. LPO–Sepharose beads were washed with the same buffer. Solutions of 1 mM KI and 8.6 mM H_2O_2 were prepared fresh prior to iodination. In all iodination experiments the volumes of LPO–Sepharose beads (70 μL), 1 mM KI (10 μL), and 8.6 mM H_2O_2 (10 μL) were constant; the volume of Na^{125}I varied between 15 and 50 μL (1.5–5 mCi); the protein solution with 1–5 mg of C3 was adjusted so that the final volume of the reaction mixture was 1 mL. The reagents were added in the following order: LPO–Sepharose beads were rewashed and suspended in the C3 solution; then KI was added, followed by Na^{125}I (1 mCi/10 μL). The addition of fresh H_2O_2 initiated the reaction which proceeded for 30–40 min at 22 °C. Ten-microliter aliquots were removed for counting (89% of the cpm were precipitable with trichloroacetic acid), and most of the unbound iodide was removed by an immediate centrifugation through 4 g of coarse Sephadex G-25, preequilibrated in 0.1 M sodium phosphate (pH 7.0). Nonspecific binding of ^{125}I-labeled protein to Sephadex was reduced by prior centrifugation of 1 mL of buffer containing 6 mg of "cold" C3 through the gel. After this procedure 97–98% of ^{125}I was precipitable with trichloroacetic acid. The remaining unbound ^{125}I was removed by dialysis in 0.1 M phosphate (pH 7.0). The resulting specific activity was proportional to the input ratio of ^{125}I to protein. As an example, radioiodination of 2 mg of C3 with 3.5 mCi yielded 1.11×10^6 cpm/μg and when 4.87 mg of C3 was reacted with 5 mCi (in 1 mL) the final specific activity of native C3 was 6.61×10^5 cpm/μg.

Total and trichloroacetic acid precipitable counts were determined as follows. Ten-microliter samples of ^{125}I-containing solution was diluted 1:100 in 0.1 M phosphate buffer. Thirty microliters of the diluted sample was added to 300 μL of cold C3 (2 mg/mL). Then two 100-μL aliquots of "hot/cold" C3 solution were precipitated with 100 μL of 20% trichloroacetic acid. The precipitates were counted before and after three washes with 10% trichloroacetic acid. The concentration of [^{125}I]C3 was determined directly from the absorbance at 280 nm, measured in cuvettes with a 2-mm path length, assuming a similar absorption coefficient for hot and cold C3. The hemolytic activity of C3(n) remained unchanged (within the experimental error of the hemolytic assay) after radioiodination.

Inactivation of C3 (125I-Labeled C3).* Three 400-μL samples of native [^{125}I]C3 (3.64 mg/mL) were pipetted into 5-mL sterile disposable plastic tubes and diluted with 400 μL of phosphate buffer, saturated KBr, or 1 M NH_2OH (pH 7.0). Native C3* and 500 mM NH_2OH-treated C3* were incubated at 37 °C for 70 min; the KBr treatment of C3* proceeded at 4 °C for 25 h. The samples were thoroughly dialyzed in preboiled dialysis tubing in separate 500-mL plastic containers at 4 °C against PBS (pH 7.4) (total of 2 L each). Dialyzed samples were transferred into tubes. Their volume, protein concentration (1 mg of C3 per mL), and radioactivity were determined, and the final specific activity of the samples was found to be 6.61×10^5 cpm per μg of native C3*, 5.65×10^5 cpm per μg of KBr-treated C3*, and 5.72×10^5 cpm per μg of 500 mM NH_2OH treated C3*. In other experiments, native

C3* was treated with 5 mM NH_2NH_2 (at 37 °C for 90 min) and C3(i) (spontaneously inactivated C3) was radiolabeled. Their specific activities were 6.30×10^5 and 6.23×10^5 cpm per μg, respectively. All radiolabeled native and inactivated C3* samples were assayed for hemolytic activity prior to further experimentation.

Erythrocyte–Complement Intermediates. EA cells, erythrocytes sensitized with hemolysin, were prepared according to Nelson et al. (1966). $EACl_{gp^4hu^2hu}$ intermediates were prepared as follows. Ten milliliters of EA cells at 1×10^9 cells/mL was incubated with 0.5 mL of C4-deficient guinea pig serum at 0 °C for 10 min. After three washes with glucose–GVB^{2+} at 0 °C the cell suspensions were adjusted to the original volume and incubated for 30 min at 0 °C with 0.5 mL of KBr-treated human serum to which 225 μg of human C4 had been added. They were washed twice with glucose–GVB^{2+} buffer at 0 °C, and after adjustment to a concentration of 4×10^8 cells/mL, they were held at 0 °C.

Radioactive Binding Assay. Aliquots (0.5 mL) of freshly prepared EAC142 cells were added to 0.5-mL samples containing 50, 25, 12.5, 6.25, 3.125, 0.78, and 0.39 μg of [^{125}I]C3 in GVB^{2+} and were incubated at 37 °C for 1 h (10-μL samples were withdrawn for counting at the beginning of the incubation).

After incubation with radiolabeled native and inactivated C3*, cellular intermediates were sedimented by centrifugation and the supernatants were transferred into clean tubes. Cell pellets were resuspended in 200 μL of GVB^{2+} and washed 3 times each with 3 mL of GVB^{2+}. After the third wash the cells were resuspended in 200 μL of GVB^{2+} and 100-μL aliquots, containing 1×10^8 cells, were transferred into clean tubes. After three more washes of 3 mL of GVB^{2+} each, the cells were resuspended in 100 μL of GVB^{2+} and the tubes containing the cells were counted in a Packard Auto-Gamma scintillation spectrometer, Model 5210.

The number of molecules bound per cell was calculated from cell-associated radioactivity by using the determined specific activities (as described above), Avogadro's number, and a molecular weight of 187 500 (1 μg of C3 represents 3.25×10^{12} molecules). Experiments were performed in triplicate. As a control for nonspecific binding of protein, C3* samples were incubated with plain EA cells.

An examination of the liquid phase which was collected after the reaction of native C3* or inactivated C3* samples with EAC142 or with EA cells was done by polyacrylamide gel electrophoresis in the presence of β-mercaptoethanol and $NaDodSO_4$. Immediately after removal of the supernatants, samples were withdrawn and prepared for determination of radioactivity. Samples for gel electrophoresis were diluted into buffer containing β-mercaptoethanol and $NaDodSO_4$, heated at 95 °C for 1 min, and frozen. Gel electrophoresis was performed on the following day (with samples containing 3000 cpm) in the manner described earlier (Janatova et al., 1980), and the gels were inspected by autoradiofluorography.

Results

Separation of Active and Inactive Forms of C3 by Chromatography on QAE-Sephadex A-50 and Their Characterization. C3 was isolated from platelet-poor human plasma by a multistep procedure which included sequential PEG fractionation, plasminogen depletion, ion-exchange chromatography, and gel filtration (Tack & Prahl, 1976). While subjecting C3 to ion-exchange chromatography as a further purification of the posthydroxylapatite C3, it was observed that on DEAE-Bio-Gel A or QAE-Sephadex A-50 some preparations gave rise to two peaks of protein, C3(i) and C3(n), both of which were C3 by the criteria of immunodiffusion against anti-C3, immunoelectrophoresis, and gel electrophoresis in $NaDodSO_4$ with β-mercaptoethanol. Only the C3(n) peak, however, was capable of mediating hemolytic activity. This fractionation, normally carried out in the presence of 1–5 mM EDTA, could be negated by replacing the EDTA with 2 mM Ca^{2+}. The hemolytically inactive C3 was usually not seen in freshly purified C3 but appeared upon storage at −70 °C. Because of these observations, we have turned to further ion-exchange chromatography on QAE-Sephadex A-50 as the last step in the purification of human C3. The ion-exchange pattern of freshly prepared C3 is presented in Figure 1a. The native, hemolytically active C3 molecules are eluted only in the C3(n) peak. C3(i) protein corresponds to a hemolytically inactive form of C3.

Treatment of C3(n) with 200 mM hydroxylamine at 37 °C for 1 h abolished the hemolytic activity of C3 and converted C3(n) into C3(i), the hemolytically inactive form of C3 (see Figure 1b). However, the chain structure, as judged by gel electrophoresis in $NaDodSO_4$ with β-mercaptoethanol, remained unaltered. An example of a typical electrophoretogram, showing the αβ chain structure of C3(n) before (A_0) and after (C_0) hydroxylamine treatment, is given in Figure 5.

N-Terminal sequences of C3(n) before and after its inactivation with either 500 mM NH_2OH or half-saturated KBr, as well as that of C3(i), were inspected by automated Edman degradation. In all instances the same double sequence was observed for the first 10 residues: (1) Ser-Ser, (2) Val-Pro, (3) Gln-Met, (4) Leu-Tyr, (5) Thr-Ser, (6) Glu-Ile, (7) Lys-Ile, (8) Arg-Thr, (9) Met-Pro, and (10) Asx-Asx. These results indicate that inactivation by nucleophiles and chaotropes or spontaneous inactivation [e.g., C3(i) in Figure 2a] does not result in alteration of the N-terminal end of either of the two chains. However, there has been one difference seen between the data presented here and those reported earlier for individual α and β chains (Tack et al., 1979). In this paper, residues Lys and Ile have been consistently detected by the LC method at position number 7 of the C3 double sequence, while Lys and Gly were determined earlier by the back hydrolysis method. Therefore, N-terminal sequence analyses of separate α and β chains of C3 have been repeated and the seventh residue in β chain has been determined by the LC method as Ile.

In agreement with previous results indicating that the loss of hemolytic activity is accompanied by the appearance of an SH group, hemolytically inactive C3(i) possessed one free SH group. This group was easily accessible to and reacted virtually immediately with various sulfhydryl reagents in the absence of denaturants. It should be noted that this SH group was found to be relatively labile and its content decreased with time when inactivated samples were stored at +4 °C. The rate of SH loss seems to be somehow connected to the means of C3 inactivation.

Apart from the two major peaks, C3(n) and C3(i), further components have been observed in some C3 preparations. The distribution of the C3 protein into one, two, or more peaks appears to be related to the history of the preparations and storage of the C3 (see Figure 2). Chain structure, the presence of α and β chains seemed to be the same in all peaks of the proteins as judged by gel electrophoresis. However, only C3(n) was capable of mediating hemolytic activity. While a free SH group could be detected in all inactive forms of C3, none was detectable in C3(n) with a wide variety of SH reagents (Janatova et al., 1980). The alkylation of an SH group does

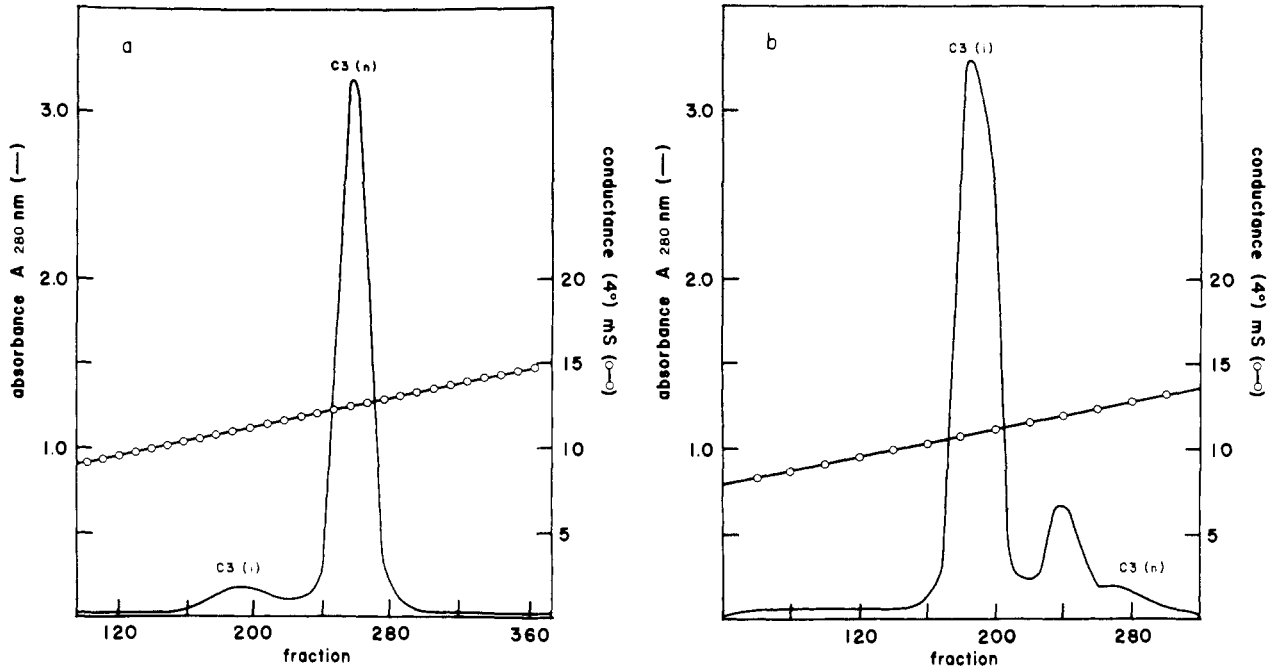

FIGURE 1: (a) Chromatography on QAE-Sephadex A-50 as the last step in C3 purification. In this instance a 120-mL solution, containing 1130 mg of the freshly prepared C3 (Tack & Prahl, 1976), was applied to a 5.0 × 44 cm gel bed of QAE-Sephadex A-50 preequilibrated in 25 mM Tris-HCl (pH 7.8) containing 2 mM EDTA and 100 mM NaCl. After a short wash with the same buffer, a linear gradient of NaCl to 300 mM (7 L total) was developed. The column was operated under a hydrostatic pressure of 65 cm of buffer resulting in a flow rate of 50–60 mL/h. 15 mL of eluate per tube was collected. The eluted fractions were inspected by measurement of A_{280} (—) and conductivity (O) at +4 °C as shown above. The protein in both peaks was C3. Hemolytically active C3 molecules were eluted only in the C3(n) peak. The C3(i) protein corresponds to a hemolytically inactive form of C3, which possesses one free SH group. (b) The isolation of C3(i) protein, the hemolytically inactive form of C3, from hydroxylamine-treated C3(n) by chromatography on QAE-Sephadex A-50. The C3(n) protein was incubated in 0.1 M sodium phosphate (pH 7.0) with 200 mM NH_2OH for 1 h at 37 °C. After pH adjustment to 8.0, the SH group was blocked by alkylation with iodoacetamide at a 20-fold molar excess. The alkylated sample was dialyzed and fractionated under the conditions as described in part a of this figure.

not affect the elution parameters of C3(i), which are the same as those of spontaneously inactivated C3.

Chemically or Spontaneously Inactivated Forms of C3 Are Not Cleaved by $\overline{C42}$ and Are Incapable of Being Bound to EAC142 Cells. C3 must be cleaved by a C3 convertase and C3b must bind to a cell membrane where it participates in the activation of C5 in order to be active in hemolysis. For determination of the stage of this process at which the capability of C3 to sustain further complement activation is affected by treatment with nucleophiles and chaotropes, radioiodinated native and inactivated (NH_2OH and KBr) forms of C3 (Figure 3) were incubated with EAC142 cells and their potential for cleavage (Figure 5) and binding (Figure 4) was examined. In all cases, only native C3 molecules were cleaved to C3b and became bound to the cell membrane. All hemolytically inactive forms of C3 (e.g., Figure 3) were neither cleaved by $\overline{C42}$ (Figure 5) nor bound to EAC142 (Figure 4). Similar experiments were carried out with hydrazine and spontaneously inactivated C3, and neither of these forms of C3 was cleaved or became bound to cell membranes (results not shown). All C3 samples were examined by the usual hemolytic assay as shown in Figure 3 before these experiments were performed.

Stability of Hemolytically Active and Inactive Forms of the C3 Molecule. C3(n) was found to be relatively stable with respect to retention of its *hemolytic function* when incubated, under sterile conditions, at 37 °C in 0.1 M sodium phosphate in the pH range 5.5–7.5. No change was detected after 3, 6, 25 and 48 h, and even after 4 weeks of incubation up to 61–75% of protein molecules retained C3 hemolytic activity.

However, we have observed a complete loss of hemolytic activity of C3(n) after incubation in 0.1 M sodium acetate, pH 4.0, at 37 °C for 90 min. The concomitant appearance of an SH group was followed by titration with 2-PDS. The rate of inactivation at low pH seems to be maximal at about pH 4.0 and decreases rapidly in both directions. At higher pH, e.g., pH 9.0 or 9.5, we have found that C3 is apparently more stable in phosphate than in Tris-HCl buffer. Overnight incubation at 37 °C in 200 mM Tris-HCl, pH 9.0 and 9.5, led to substantial inactivation (66%), and 0.68 mol of SH per mol of C3 was detected. We do not know as yet whether the inactivation in this pH range is due to the effect of pH or rather to the effect of Tris, which is a primary amine.

The stability of the *αβ chain structure* of C3(n) has also been investigated. No fragmentation (in the *absence* of denaturants) has been detected even after 1–4 weeks of incubation at 37 °C in the pH range 5.5–9.0. No degradation of α or β chain has been observed after chemical treatment of C3(n) with concentrations of NH_2OH up to 500 mM, with various concentrations of NH_2NH_2 up to 200 mM, or with half-saturated KBr. Also, no cleavage has been seen after 18–42 h of incubation at 37 °C in the presence of half-saturated NaCl, 1% NP-40, and 1% Triton.

The α chain of C3(n) is, however, cleaved specifically in the presence of denaturants (>2 M Gdn·HCl, >0.05% Na-$DodSO_4$) into two fragments with concomitant production of a free SH group, localized to the smaller (M_r ~46 000) fragment. The larger (M_r ~68 000) fragment migrates in the 7.5% gel of our electrophoretic system in front of β chain [see Figure 4 of Janatova et al. (1980)].

This cleavage could not be prevented, either by further purification of C3, including the use of affinity adsorbents bearing anti-β1H or anti-C3bINA, or by the presence of chemical or polypeptide inhibitors or inactivators of protease activity. Only the presence of a reducing agent, such as β-

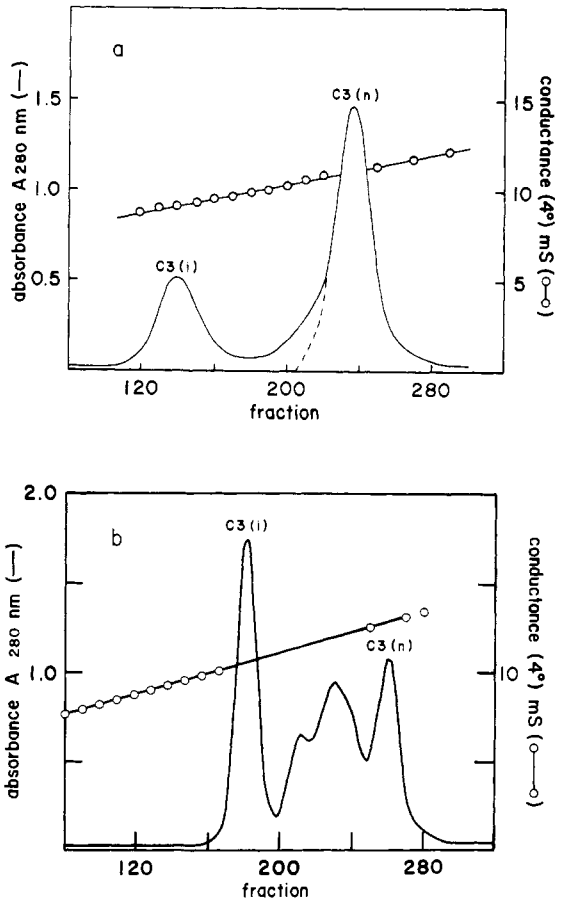

FIGURE 2: (a) Rechromatography of C3(n) protein on QAE-Sephadex A-50 after it had been stored at pH 7.0 in 20 mM PO_4, 2 mM EDTA, and 190 mM NaCl for 6 months at −70 °C. 35 mL of solution containing 360 mg of C3 was diluted with 20 mM PO_4 and 2 mM EDTA (pH 7.0) to obtain a conductivity corresponding to the starting buffer and was applied to a 2.5 × 43 cm gel bed, preequilibrated with 20 mM PO_4, 2 mM EDTA, and 100 mM NaCl (pH 7.0) (conductivity ~7.0 mS at 4 °C). A linear gradient from 100 to 250 mM NaCl (2 L total) in 20 mM PO_4 and 2 mM EDTA (pH 7.0) was used for the elution of proteins. The column was operated by hydrostatic pressure, yielding a flow rate of 30 mL/h. 80 drops (~5 mL) of eluate per tube was collected. Representative fractions were assayed for protein content, conductivity, hemolytic activity, and chain structure, and the pools were assayed for SH content. (b) Elution profile of an older C3 preparation after fractionation on QAE-Sephadex A-50 demonstrates the presence of further components in some C3 preparations. The chain structure seemed to be the same in all peaks of the proteins as judged by gel electrophoresis. However, only C3(n) protein was capable of mediating hemolytic activity. In this instance 402 mg of C3 in 84 mL was applied to a 2.5 × 42 cm gel bed of QAE-Sephadex A-50, equilibrated in 25 mL Tris-HCl, 2 mM EDTA, and 100 mM NaCl (pH 7.8). A linear gradient of increasing NaCl concentration from 100 to 300 mM (2 L total) was employed in the elution of C3 proteins. Apart from buffer system and gradient slope, the conditions of fractionation and evaluation of fractions were the same as those described in part a of this figure.

mercaptoethanol, would prevent the fragmentation of α chain when C3(n) is placed into Gdn·HCl or $NaDodSO_4$.

C3(i) samples, either spontaneously [e.g., C3(i) in Figure 2a] or chemically (e.g., half-saturated KBr, 250 or 500 mM NH_2OH, 10, 50 or 200 mM NH_2NH_2, pH 4.0, acetate, or 0.5 or 1 M Gdn·HCl) inactivated C3, were also tested for the ability to generate 46 000- and 68 000-dalton fragments from C3 α chain in the presence of Gdn·HCl or $NaDodSO_4$. In addition to other functional and structural dissimilarities between C3(n) and C3(i), C3(i) was found, unlike C3(n), not to undergo the specific fragmentation of α chain in the presence of Gdn·HCl (>2 M) or $NaDodSO_4$ (>0.05%).

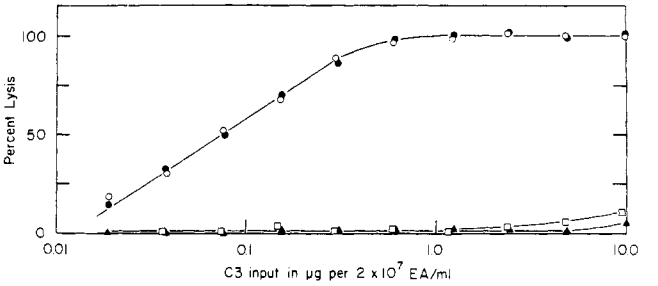

FIGURE 3: Quantitative determination of C3 hemolytic activity of C3(n) before (●) and after (○) radioiodination indicates that the hemolytic activity of native C3 was not noticeably affected by the radioiodination procedure. The treatment of radioiodinated C3(n) (○) with 500 mM NH_2OH (□) or half-saturated KBr (▲) resulted in a complete loss of hemolytic activity, as shown above. These samples were further examined for their potential for cleavage by $\overline{C42}$ (Figure 5) and for binding to $\overline{EAC142}$ (Figure 4).

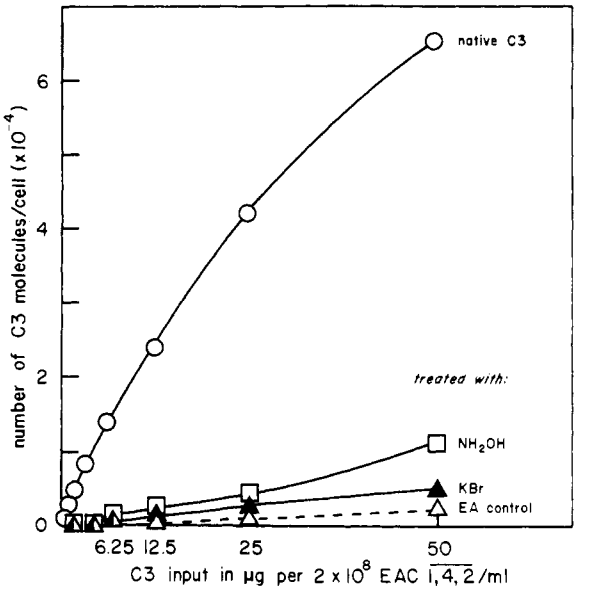

FIGURE 4: Examination of the binding potential of the inactivated form(s) of C3. ^{125}I-Iodinated native (○), inactivated $\underline{NH_2OH}$ (□), and KBr (▲) forms of C3 were incubated with $\overline{EAC142}$ cells. The number of bound C3 molecules was determined from the cell-associated radioactivity and plotted against the input of C3. The results presented in this figure indicate that only native C3 molecules, capable of mediating hemolytic activity, can participate in the formation of $\overline{EAC1423}$ intermediate. On the other hand, inactive forms of C3 were not bound to $\overline{EAC142}$ cells. Incubation with EA cells (△) served as a control for nonspecific binding; approximately the same low values were obtained for native as well as for inactivated forms of C3.

Our results indicate that the observed cleavage does not arise from contaminating proteases and that it is, in fact, an abnormal manifestation of an inherent property of C3(n), and not that of C3(i), expressed because of the conformational changes induced by a denaturant. The possibility that a nascent SH group is involved in this "autolytic" cleavage of C3 α chain is being presently investigated.

Discussion

To date, in all freshly inactivated C3 samples the loss of hemolytic activity has been accompanied by stoichiometric appearance of an SH group; thus appearance of an SH group can serve as a convenient probe for inactivation using either DTNB or 2-PDS as SH-reactive reagents. In addition to the appearance of an accessible SH group (Janatova et al., 1980), the chemical or spontaneous inactivation of C3 results in profound changes in chromatographic behavior (see Figures

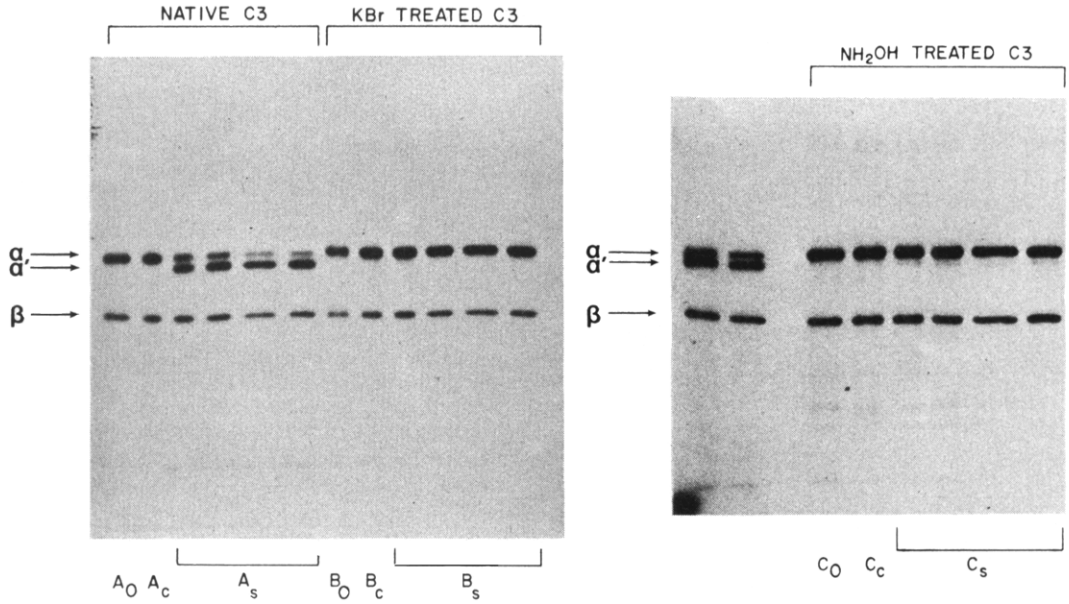

FIGURE 5: Examination of the ability of $C\overline{42}$ to cleave hemolytically inactive forms of C3. Autoradiofluorograms showing the chain structure of the radioiodinated native (A), KBr (B), and NH_2OH (C) treated C3 after incubation with EA and $EAC\overline{142}$ cells. A_0, B_0, and C_0 are the respective starting materials, and A_c, B_c, and C_c are the fluid phases of these samples after incubation with 2×10^8 EA cells. A_s, B_s, and C_s show the fluid phases of samples after incubation of 2×10^8 $EAC\overline{142}$ cells with C3 at concentrations of 25, 6.25, 1.56, and 0.39 μg/mL, respectively. Only native C3 molecules, capable of mediating hemolytic activity were cleaved to C3b (see α' in A, this figure). Inactive forms of C3 were not cleaved by $C\overline{42}$ (note uncleaved α chain in B_s and C_s).

1 and 2). The conductivity at which C3(i) and C3(n), i.e., hemolytically inactive and active forms of C3, elute from the QAE-Sephadex A-50 column depends on the buffer system used. It should be noted that phosphate gives better resolution and C3(n) samples seem to be more stable in phosphate than in Tris-HCl buffer. Under the conditions as described in Figure 2a the C3b fragment elutes, as expected, after C3(n). There is a much smaller difference in elution parameters between C3(n) and C3b than between elution of C3(i) and C3(n), which is rather surprising. Moreover, the alkylation of an SH group with iodoacetamide prior to ion-exchange chromatography does not affect the elution of C3(i). It has not been possible as yet to draw any definitive conclusions whether differences in the chromatographic behavior of C3 reflect mainly changes in conformation and/or in charge. Our preliminary experiments with difference spectra of KBr, hydrazine, or pH 4.0 treated C3 samples suggest that conformational changes take place during chemical inactivation which may be similar to those reported by Isenman & Cooper (1979) for the enzymatic cleavage of C3 into C3a and C3b. The ability to inactivate C3 without cleavage and with the appearance of the same SH group that appears in C3b suggests that this is a possible model for investigating the conformational change accompanying the loss of activity.

The treatment of C3(n) by nucleophiles, chaotropes, and spontaneous inactivation yields a form(s) of C3, e.g., C3(i), which is no longer a substrate for the $C\overline{42}$ enzyme; as a consequence, inactivated C3 samples were neither cleaved by $C\overline{42}$ into C3b (Figure 5) nor bound to the cell membrane. On the other hand, when native C3, i.e., C3(n), was incubated with EAC142, practically all the C3 in the fluid phase was converted to C3b, and up to 60 000 molecules/cell were bound (Figure 4). All native and inactivated C3 samples were assayed for hemolytic activity; only in the case of native C3 samples was hemolysis observed. We can conclude from these results that the proposed "rupture" of a thiol ester bond (Janatova et al., 1980; Tack et al., 1980) upon treatment with inactivating agents leads to a different conformation. As a consequence, C3 is no longer able to serve as a substrate for the $C\overline{42}$ cleavage. We assume that as with inactive C3b ($C\overline{42}$ generated) in the fluid phase (Vogt et al., 1978; von Zabern et al., 1979a), the chemically or spontaneously inactivated C3 is apparently not capable of modulating C5, which in turn cannot be cleaved by C3/C5 convertase to give rise to C5b, which is necessary for the formation of the C5b-9 membranocytolytic complex. It has been shown recently that the ability to bind C5 is a property of surface-bound C3b with "the active configuration", while fluid-phase C3b (Vogt et al., 1978; von Zabern et al., 1979a,b) and native C3 appear to have no affinity for C5 (Isenman et al., 1980).

In our hands, nitrogen nucleophile treated C3 was hemolytically inactive; i.e., it was not cleaved by $C\overline{42}$, it did not bind to cell membranes, and it did not seem to participate in the activation of C5. As a consequence, no hemolysis occurred in the presence of all classical pathway complement proteins when native C3 was replaced with the inactivated C3 (Figure 3). Similar observations on amine-treated human C3 were recently reported by von Zabern et al. (1979b) and in principle agree with our studies except that they report that amine-treated C3 (and C4) samples still retain considerable activity in formation of fluid-phase C3/C5 convertases. This discrepancy could perhaps be explained by the fact that in their study apparently not all molecules were converted to the inactive forms.

At this point it should be emphasized that none of our chemical inactivations by nucleophiles, chaotropes, and extremes of pH were accompanied by cleavage of α chain. However, on several occasions, we have observed cleavage of α chain into C3a-like and C3b-like fragments on dialysis or standing of 500 mM NH_2OH treated C3 samples at +4 °C, 24 h and longer, although no degradation had been observed in these same samples when inspected by gel electrophoresis in $NaDodSO_4$ within a few hours after the inactivation process. This phenomenon could perhaps explain the discrepancies between our observations and the results presented by Budzko et al. (1971) on cleavage of α chain with NH_2OH (generation

of C3a-like fragment) and also those between our observations and the recent report by Law et al. (1979b) that NH_2OH-treated C3 is cleaved by C3 convertase.

In some respects the inactive forms of C3 resemble the fluid-phase C3b. The inactive forms of C3 exhibit the same SH group as that seen on C3b (Janatova et al. 1980), and in both the availability of the binding site for the reaction of C3 with $EAC\overline{42}$ and the capability to participate in the activation of C5 are apparently lost. Also the B antigenicity is lost in fluid-phase C3b and amine-treated C3 (von Zabern et al., 1979b). The question arises as to whether or not all of the biological functions attributed to fluid-phase C3b are abrogated in chemically inactivated C3 Whether these forms would bind factor B and form an alternative pathway C3 convertase, whether they would be cleaved by C3bINA·β1H, or whether the binding sites for the C3b or C3d receptors (Dierich & Bokisch, 1977) are available in inactivated C3 remains to be seen.

In addition to its proposed role in acyl group activation (Janatova et al., 1980), the SH group, present in the form of a thiol ester, may serve to stabilize the conformation of native C3 necessary for the interaction with $C\overline{42}$. Therefore, identification of a reactive acyl group as part of a proposed internal thiol ester and certain other aspects of C3 activation, such as conformational changes which apparently accompany activation and the loss of activity, remain to be elucidated.

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