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Fourth Component of Human Complement: Studies of an Amine-Sensitive Site Comprised of a Thiol Component†

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ABSTRACT: Studies of human C3 following treatment with nitrogen nucleophiles revealed the presence of an amine-sensitive bond whose properties were consistent with an internal thiol ester [Janatova, J., Lorenz, P. E., Schechter, A. N., Prahl, J. W., & Tack, B. F. (1980a) *Biochemistry 19*, 4471–4478; Janatova, J., Tack, B. F., & Prahl, J. W. (1980b) *Biochemistry 19*, 4479–4485; Tack, B. F., Harrison, R. A., Janatova, J., Thomas, M. L., & Prahl, J. W. (1980b) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5764–5768]. The selected studies revealing the generation of a reactive carbonyl and a sulfhydryl group in C3 were extended to C4 and C5. As with C3, the reaction of methylamine with native C4 is stoichiometric, covalent, accompanied by the appearance of a sulfhydryl group, and results in the loss of C4 hemolytic activity. Electrophoretic and autoradiofluorographic analyses of radiolabeled ([^{14}C]-methylamine or [^{14}C]iodoacetamide) C4 samples have shown that the reactive carbonyl and resultant sulfhydryl group both reside in the α-polypeptide chain. A sulfhydryl group was also detected following cleavage of native C4 by $C1\bar{s}$, a serine protease responsible for activation of the C2 and C4 components, and was shown to be present in the α' chain of C4b. Inactivation of C4 by treatment with nitrogen nucleophiles or as it naturally occurs on storage is apparently accompanied by physicochemical changes in the molecule. Hemolytically inactive forms of C4 can be separated from native C4 by chromatography on diethyl(2-hydroxypropyl)aminoethyl-Sephadex A-50, and all chromatographic forms exhibit the same $\alpha\beta\gamma$ polypeptide chain structure. In contrast to native C4, hemolytically inactive forms are not cleaved by $C1\bar{s}$ and fail to undergo a denaturant-induced α-chain fragmentation. The autolytic cleavage of the C4 α chain, accompanied by the expression of a sulfhydryl group, is a property of native C4 seen when the protein is incubated in sodium dodecyl sulfate or guanidine hydrochloride. The cleavage reaction results in two fragments, $C4_\alpha$-40 000 and $C4_\alpha$-54 000, with the sulfhydryl group present in the N-terminal, lower molecular weight fragment. This reaction can be prevented either by the presence of β-mercaptoethanol or by prior nucleophile or spontaneous inactivation. In contrast to C3 and C4, the hemolytic function of C5 is not affected by treatment with amines, and C5 does not undergo autolytic cleavage. Data presented here are interpreted to indicate the presence of an internal thiol ester in C4 and the absence of such a bond in C5. The relationship between this site and the covalent association of C4b with red cell membranes and immune aggregates is discussed within the context of a transesterification reaction.

The human complement proteins C4, C3, and C5 are present in plasma in precursor forms as disulfide-bridged subunit structures. A three-polypeptide chain structure, $\alpha\beta\gamma$, exists in C4 (Schreiber & Müller-Eberhard, 1974), while C3 and C5 are comprised of two polypeptide chains each, α and β (Nillsson & Mapes, 1973). Chemical and biological properties of these proteins have been reviewed in detail (Müller-Eberhard, 1975; Porter & Reid, 1979; Stroud et al., 1979). The activation of the native C4, C3, and C5 is a sequential enzymatic process in which the proteins are first converted at the membrane surface by a limited proteolysis into a transient "activated" state. This activation step results in production of two fragments. The smaller one (C4a, C3a, and C5a) is released from the N terminus of the respective α chain and is referred to as an activation peptide with anaphylatoxic activity (Gorski et al., 1979; Bokisch et al., 1969; Hugli et al., 1975; Fernandez & Hugli, 1978). The larger fragment (C4b or C3b) contains the labile binding site, and consequently can form a covalent association with receptor(s) on a plasma membrane, cell wall, or immune aggregate. The surface-bound fragment with "active" configuration functions to recruit and participate in the activation of the next complement component in the reaction sequence. C4b and C3b are subcomponents of the classical pathway C3- and C5-converting enzymes ($\overline{C4b2a}$ and $\overline{C4b2a3b}$, respectively) which are assembled on the particle surface (Müller-Eberhard et al., 1967). C3b further functions as a subcomponent of the C3 and C5 con-

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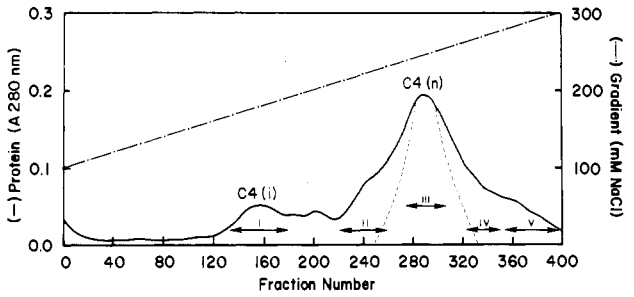

FIGURE 1: Elution profile of C4 following long-term storage at −70 °C on a 1.4 × 15.5 cm column of QAE-Sephadex A-50 and DEAE-Bio-Gel A mixed in a 3:1 (v/v) ratio. The column was equilibrated with 20 mM sodium phosphate buffer (pH 7.0) containing 100 mM NaCl and 2 mM EDTA. A sample of 80 mg of protein (11.5 mL) was applied in the same buffer. After a wash with the starting buffer (12 mL), a linear gradient of NaCl from 100 to 300 mM (1200 mL total) was developed. Fractions (3 mL) were collected at a flow rate of 30 mL/h, and the absorbance was monitored at 280 nm. Protein-containing fractions were pooled as indicated, concentrated by ultrafiltration, and assayed for protein content, polypeptide chain structure, hemolytic activity, and SH group content. The $\alpha\beta\gamma$ polypeptide chain structure, characteristic of native C4, was observed in all pools. However, only native C4, i.e., C4(n), present in pool III, was capable of mediating hemolytic activity. The highest SH content was determined in C4(i), pool I. All C4 pools were also tested for the susceptibility to cleavage by C1$\bar{s}$-converting enzyme and for the ability to generate α-40 and α-54 (M_r = 40 000 and 54 000, respectively) fragments from the C4 α chain in the presence of NaDodSO$_4$ (see Figure 2A,B).

vertases ($\overline{C3bBb}$ and $\overline{C3b_nBb}$, respectively) in the alternative pathway (Müller-Eberhard & Götze, 1972; Medicus et al., 1976; Daha et al., 1976; Vogt et al., 1978; von Zabern et al., 1979). Activation of C5 results in the assemblage of the C5b-9 cytolytic complex (Kolb et al., 1972; Mayer, 1972; Kolb & Müller-Eberhard, 1975; Podack et al., 1976) initiated by the recruitment of C6 and C7 by C5b (Lachmann & Thompson, 1970; Arroyave & Müller-Eberhard, 1973; Goldlust et al., 1974).

The association of C3b and C4b with the red cell membrane and the interaction of C3b with yeast cell wall components (e.g., zymosan) have been shown to occur both by hydrophobic interactions and by covalent bonding (Law & Levine, 1977; Law et al., 1980). Studies involving the interaction of C4b with immune aggregates (Goers & Porter, 1978; Campbell et al., 1980) have further revealed the formation of a covalent link between the α' chain of C4b and an amino acid residue(s) present in the Fd region of the antibody heavy chain. Although strong interactions of C5b with the membrane bilayer have been recognized (Hammer et al., 1975), the association is not covalent in nature (Law et al., 1980). A mechanism of covalent bonding of C4b and C3b to structures present at sites of complement activation is at this time unresolved. However, it has been proposed that the bond between the protein and surface receptor(s) on the red cell membrane or cell wall is an oxygen ester. This is based on data of the chemical reactivity of the bond in question with hydroxylamine, or at alkaline pH (Law & Levine, 1977; Law et al., 1979b). The identification of 0.6–0.7 mol of hydroxamate per mole of hydroxylamine-released C3d fragment has further indicated that the protein contributes the acyl group to the ester bond (Law et al., 1979b). The mechanism of covalent attachment has been suggested to be the result of a transesterification reaction (Law et al., 1979b). Support for this hypothesis has been derived from the identification of an internal thiol ester bond in native human C3 (Tack et al., 1980b). In this paper, we present evidence for the presence of an analogous reactive site, also comprised of a thiol ester, in human C4.

Experimental Procedures

Materials. Unless specified otherwise, the human plasma and the gel filtration and ion-exchange chromatographic media, as well as other chemicals used in purification, inactivation, and characterization of native and inactivated complement proteins, were as described earlier (C3, Tack & Prahl, 1976; Tack et al., 1979b, 1980b; Janatova et al., 1980a; C4, Bolotin et al., 1977; C5, Tack et al., 1979a). [1-^{14}C]Iodoacetamide was supplied by New England Nuclear and [^{14}C]methylamine hydrochloride by Amersham. Unlabeled methylamine hydrochloride was obtained from Sigma. Fresh sheep blood, in 3.8% sodium citrate, was purchased from Richards Beard Richards, Inc., Pleasant Grove, UT. All other chemicals and reagents used were of the highest grade available.

Isolation of Complement Components. The C3 protein was isolated as previously described (Tack & Prahl, 1976) with the exception that DEAE-Sephacel[1] was used in place of DEAE-cellulose. Further chromatography on QAE-Sephadex A-50 has been implemented as the last step in C3 purification (Janatova et al., 1980b). The C4 component was isolated according to Bolotin et al. (1977) and submitted to further fractionation on activated thiol–Sepharose 4B (Brocklehurst et al., 1973), in order to separate SH-containing C4 from hemolytically active C4. A sample of 20–60 mg of C4 (1–3 mL), containing, e.g., 0.36 mol of SH/mol of protein, was applied to a 1.2 × 10 cm column of a Sepharose–(reduced glutathione 2-pyridyl disulfide) conjugate preequilibrated with 50 mM Tris-HCl buffer (pH 8.2) and 1 mM EDTA. The concentration of NaCl was increased from 0.15 M while equilibrating to 0.5 M in the washing buffer. The eluate (3 mL/fraction) was monitored by measuring the absorbance at 280 and 343 nm. Fractions containing native C4 (usually no. 4–8) were pooled, dialyzed, and characterized by hemolytic assay and by radioalkylation or spectrophotometric titration of the SH group. Hemolytically active C4 obtained by this procedure contained 0.03–0.07 mol of SH/mol of C4. The content of SH could be increased to 1 mol of SH/mol of C4 by treatment with, e.g., C1$\bar{s}$. The C4 protein bound to thiol–Sepharose was eluted with the above buffer containing, in addition, 50 mM L-cysteine. Older C4 preparations were fractionated by ion-exchange chromatography (see Results). The procedure of Tack et al. (1979a) was used for isolation of the C5 component. In some instances, chromatography on QAE-Sephadex A-50 was used in place of the protein A–Sepharose CL-4B column to remove IgG proteins from post-hydroxylapatite C5. Conditions were the same as those described in Figure 1 for C4, except that only the first gradient (100–200 mM NaCl) was applied. IgG eluted before C5. Proteins were concentrated by ultrafiltration at 4 °C.

Quantitative Hemolytic Assays. Hemolytic titrations were carried out as previously reported (Tack & Prahl, 1976; Bolotin et al., 1977; Tack et al., 1979a). The degree of lysis was

[1] Abbreviations used: C4(n), native hemolytically active form of C4; C4(i), hemolytically inactive form of C4, containing a free SH group; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); 2-PDS, 2,2′-dipyridyl disulfide; NTCB, 2-nitro-5-thiocyanobenzoic acid; ICH_2CONH_2, iodoacetamide; Gdn·HCl, guanidine hydrochloride; NaDodSO$_4$, sodium dodecyl sulfate; NH_2OH, hydroxylamine; CH_3NH_2, methylamine; NH_2NH_2, hydrazine; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; QAE, diethyl(2-hydroxypropyl)aminoethyl; EAC1, EAC14, EAC142, and EAC1423, sheep red blood cells (E), sensitized with antibody (A) and reacted with the indicated complement proteins; $\overline{C42}$, the C3 convertase in the classical pathway of the complement; C1$\bar{s}$, a serine protease responsible for activation of the C2 and C4 components; PBS, phosphate-buffered saline.

determined by measuring the absorbance of the fluid phase at 415 nm with a Gilford Model Stasar II spectrophotometer after centrifugation (Clay Adams, Sero-Fuge II centrifuge, 3 min).

Protein Concentrations. A modification of the Lowry method (Hartree, 1972) was used to determine the protein concentration of C4 and C5 samples. The protein concentration of C3 solutions was determined by measuring the absorption at 280 nm, using an absorption coefficient ($A^{1\%}_{1cm}$ at 280 nm) of 9.7 and a carbohydrate-free molecular weight of 185 000 (Tack & Prahl, 1976). The C4 concentration has also been measured spectrophotometrically by using an absorption coefficient of 8.3 (Nagasawa & Stroud, 1977) and 198 000 as a molecular weight (Bolotin et al., 1977).

Content of Sulfhydryl Group. Determinations of SH group content were carried out by alkylation with [1-^{14}C]iodoacetamide or by spectrophotometric titration with DTNB, NTCB, or 2-PDS as described earlier by Janatova et al. (1980a).

Treatment with Nucleophiles and Removal of Reagents. Both procedures were performed, unless specified otherwise, as reported in a previous paper (Janatova et al., 1980a). In experiments with methylamine, the composition of reaction mixtures for individual samples was as follows: (a) native protein in buffer only, (b) native protein treated with [^{14}C]-iodoacetamide, (c) native protein treated with 25 mM methylamine in the presence of [^{14}C]iodoacetamide, and (d) native protein treated with 25 mM [^{14}C]methylamine. With the exception of sample (d), aliquots of 500 μg of protein were adjusted either with plain buffer or with buffer containing the reagent to a final volume of 500 μL and incubated in glass tubes for 4 h at 37 °C. The protein samples used in these studies contained 0.020 M sodium phosphate (pH 7.0), 0.18–0.24 M NaCl, and 2 mM EDTA. The pH of the final reaction mixture was brought to 7.4 by the addition of 0.1 M sodium phosphate (pH 7.48) containing 0.15 M NaCl and 0.005 M EDTA. In sample (d), the final volume containing 500 μg of protein was adjusted to 200 μL. After the incubation period, sample (d) was diluted with pH 7.48 buffer to a volume of 500 μL. Following incubation, each of the samples (i.e., a, b, c, and d) was dialyzed in prewashed dialysis tubing at 4 °C against PBS (pH 7.4) in separate containers. The protein concentration in dialyzed samples was also determined spectrophotometrically; the same $A^{1\%}_{280}$ value was used for native and chemically treated protein. The concentration of C5 was determined by using an assumed value of 10.0 for $A^{1\%}_{280}$. These experiments were performed in parallel with identical conditions for each protein under study. All samples were assayed for hemolytic activity, bound radioactivity, chain structure, and site of radiolabel incorporation.

Treatment of C4 with C1$\bar{s}$. Post-thiol–Sepharose C4 in 10 mM Tris-HCl (pH 7.8), containing 2.5 mM EDTA and 500 mM NaCl, was incubated with C1$\bar{s}$ (w/w; 100:1) for 30 min at 37 °C. This was followed by immediate alkylation with [^{14}C]iodoacetamide for determination of SH content. The appearance of an SH group during the incubation of C4(n) with C1$\bar{s}$ was also followed directly by spectrophotometric titration with DTNB in PBS (pH 7.4). The SH content reached a plateau within the first few seconds of mixing. No further increase was observed with time.

Polyacrylamide Gel Electrophoresis. Preparation of samples for electrophoretic analysis before and after chemical or enzymatic inactivation was carried out as described earlier (Janatova et al., 1980a). Electrophoresis was performed in a discontinuous buffer system (Laemmli, 1970) in the presence of NaDodSO$_4$. Protein bands were visualized by staining with Coomassie Brilliant Blue R-250. The gels were then prepared for autoradiofluorography according to the procedure of Bonner & Laskey (1974).

High-Voltage Electrophoresis. HCl (6 N) hydrolysates of [^{14}C]C4 samples were electrophoresed with a "flat-bed" apparatus on Whatman 3MM chromatographic paper in 7% formic acid (pH 1.7) at 2000 V for 105 min. Side strips with SCM-Cys standards were developed with Cd–ninhydrin stain. Strips (1 cm) from the middle part of the electrophoretogram, corresponding to hydrolysates of [^{14}C]C4 samples, were cut out and assayed for radioactivity.

Results

Separation and Characterization of Hemolytically Active and Inactive Forms of the C4 Component. Several preparations of C4 from human plasma were obtained by the method of Bolotin et al. (1977). Even relatively fresh C4 samples contained detectable amounts of hemolytically inactive molecules which exhibited an $\alpha\beta\gamma$ polypeptide chain structure identical with that of native C4. The hemolytically inactive C4 component in these samples possessed a free SH group detectable either by titration with DTNB or by alkylation with [^{14}C]iodoacetamide. Sulfhydryl-containing molecules could be separated from native C4 by covalent chromatography on activated thiol–Sepharose as described under Experimental Procedures. The appearance of hemolytically inactive molecules *without* a detectable SH group has, however, been observed on storage of C4 samples at −70 °C. The studies to be described required that the starting protein be hemolytically active. Since the hemolytically inactive component without titratable SH could not be separated from the active form on thiol–Sepharose, it was necessary to develop an additional method of C4 fractionation.

A chromatographic procedure for the separation of hemolytically active (n) and inactive (i) forms of C4 by using a modification of the method described earlier for fractionation of C3 preparations (Janatova et al., 1980b) is shown in Figure 1. Material from three C4 preparations was pooled, dialyzed against starting buffer, and applied to a column of QAE-Sephadex A-50–DEAE-Bio-Gel A mixed in a 3:1 volume ratio. A linear salt gradient from 100 to 200 mM NaCl was applied at a flow rate of 30 mL/h, and 3-mL fractions were collected. As the elution parameters were quite different from those of C3, the first gradient was followed by a second which extended the NaCl concentration to 300 mM. A plot of the absorbance profile is shown in Figure 1. After the quantitative determination of C4 hemolytic activity in solutions from selected tubes, individual fractions were pooled as indicated and analyzed by polyacrylamide gel electrophoresis in the presence of NaDodSO$_4$ and β-mercaptoethanol. Protein material in each C4 pool (I–V) exhibited an $\alpha\beta\gamma$ polypeptide chain structure characteristic of native C4; however, only pool III contained C4 which was functionally active by hemolytic assay. Spectrophotometric titration of C4 pools with DTNB resulted in SH values (expressed as mol of SH/mol of C4) as follows: pool I, 0.48; pool II, 0.39; pool III, 0.10; pool IV, 0.26; pool V, 0.19. These results indicated that the separation of the C4 chromatographic forms is not due to the presence or absence of a detectable SH group; furthermore, they revealed the heterogeneity within each C4 pool.

Earlier studies of C3 (Janatova et al., 1980a,b) had shown that only the native hemolytically active protein could be cleaved by the classical pathway convertase ($\overline{C4b2a}$) and, further, that this active C3(n) form of C3 fragmented when diluted into NaDodSO$_4$ or Gdn·HCl. Incubation of the C4 pools I–IV with C1$\bar{s}$ (Figure 2A) indicated that while the

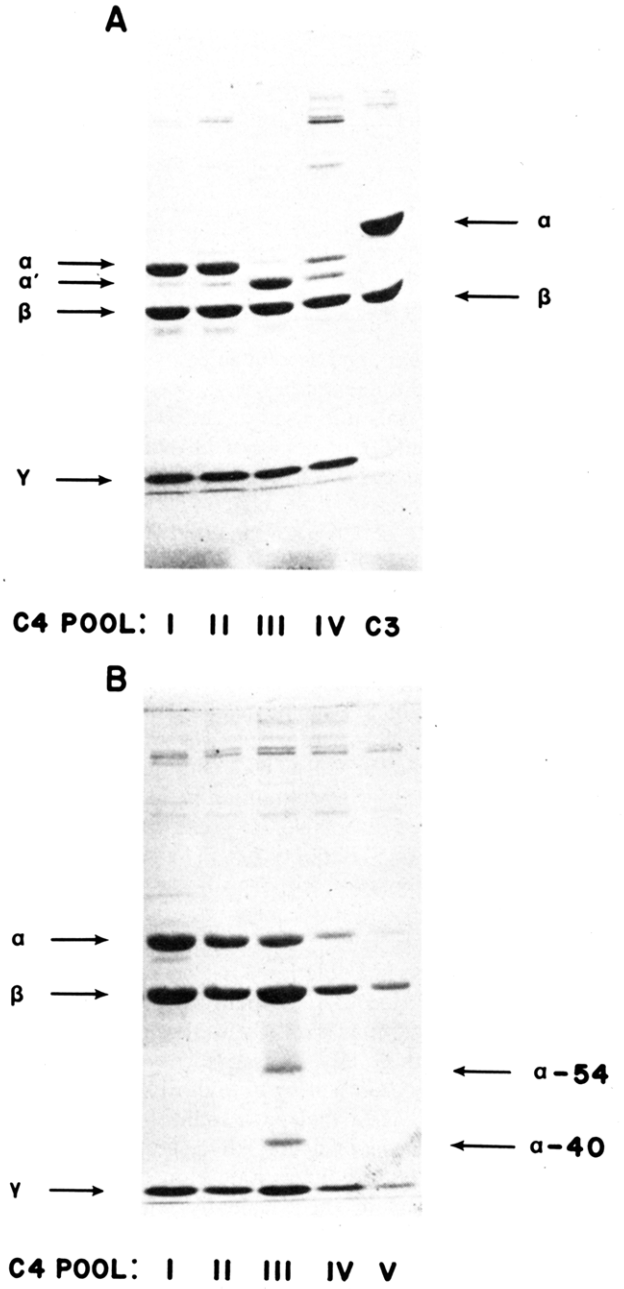

FIGURE 2: Polyacrylamide gel electrophoresis of C4 pools I–V (Figure 1) in the presence of $NaDodSO_4$ and β-mercaptoethanol following pretreatment of the protein samples with (A) $C1\bar{s}$ or (B) $NaDodSO_4$. Samples shown in the upper gel (A) were incubated with $C1\bar{s}$ (100:1) for 30 min at 37 °C; those samples presented in the lower gel (B) were incubated in 0.2% $NaDodSO_4$ (in the absence of a reducing agent) for 5 h at 37 °C followed by 12 h at room temperature. Both treatments were carried out in sodium phosphate buffer (pH 7.0) containing 2 mM EDTA. Prior to electrophoresis, each sample was reduced with β-mercaptoethanol in the presence of $NaDodSO_4$.

hemolytically active form of C4 [C4(n) present in pool III] was converted to C4b, hemolytically inactive forms of C4 (pools I, II, and IV) were refractory to hydrolysis by this enzyme. Similarly, treatment of these same C4 pools (I–V) with $NaDodSO_4$ in the absence of a reducing agent indicated that only C4(n) from pool III exhibited a denaturant-induced α-chain fragmentation (Figure 2B). This indicates that, as with C3, only the hemolytically active form of C4 [i.e., C4(n) present in pool III] could be cleaved in solution by the physiological enzyme; furthermore, this material exhibits a denaturant-induced autolytic cleavage of the α chain, producing fragments with molecular weights of 40 000 and 54 000.

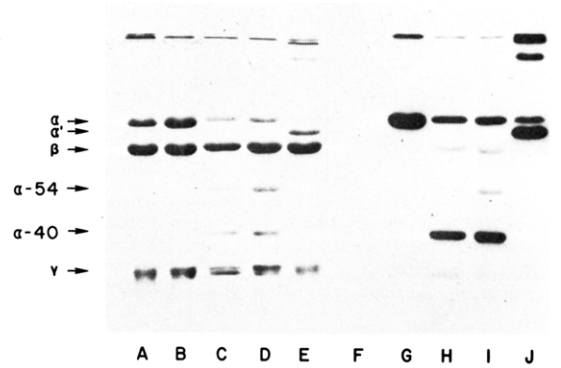

FIGURE 3: Electrophoretic and autoradiofluorographic analyses indicating the chain structure (tracks A–E) and location of the radioalkylated SH group (tracks F–J) in human C4 before (A and F) and after treatment with 500 mM NH_2OH (B and G), 6 M Gdn·HCl (C and H), 0.2% $NaDodSO_4$ (D and I), and $C1\bar{s}$ (E and J). Prior to electrophoresis, the samples of C4 were treated as follows: (A and F) in this control sample, native C4 was incubated only with [^{14}C]-iodoacetamide; (B and G) native C4 was treated with 500 mM NH_2OH (pH 7.4) at 37 °C for 1 h, and prior to radioalkylation the reagent was removed by centrifugation at 4 °C through Sephadex G-25 in Tris-HCl buffer (pH 8.2); (C and H, D and I) native C4 was diluted into denaturant in 0.2 M Tris-HCl buffer (pH 8.2) containing 5 mM EDTA and 5 μCi of [^{14}C]iodoacetamide; (E and J) native C4 was incubated with $C1\bar{s}$ (100:1) at pH 7.8 (30 min, 37 °C). In each instance, the pretreated sample was radioalkylated with 5 μCi of [^{14}C]iodoacetamide (specific activity 15.76 mCi/mmol); a 1-h incubation period at 37 °C was followed by an 18-h one at room temperature. Excess alkylating reagent and the denaturant, respectively, were removed by dialysis against saline buffered with either phosphate (pH 7.4) or Tris-HCl (pH 8.2) containing 2–5 mM EDTA. Samples were then reduced with β-mercaptoethanol in the presence of $NaDodSO_4$.

Unless otherwise specified, only native C4 isolated by the above procedures was used in the following studies.

Treatment of Native Components with Hydroxylamine or Denaturants. Treatment of native C3 with hydroxylamine, hydrazine, and denaturants has been shown to result in the loss of hemolytic function and the appearance of an SH group (Janatova et al., 1980a). Since structural and functional homologies between C4, C3, and C5 have been observed (Müller-Eberhard, 1975; Stroud et al., 1979; Porter & Reid, 1979), it was of interest to extend our earlier nucleophile inactivation studies on C3 to C4 and C5.

The results of radioalkylation experiments with [^{14}C]iodoacetamide following chemical or enzymatic treatment of native C4 were as follows: as with C3, one SH group per C4 molecule was expressed on treatment with 500 mM NH_2OH at pH 7.4 (0.80 mol of SH/mol), after dilution into denaturants, such as 6 M Gdn·HCl (0.93 mol of SH/mol) or 0.2% $NaDodSO_4$ (0.76 mol of SH/mol), or after cleavage with $C1\bar{s}$ (1.00 mol of SH/mol). Control samples of native C4 bound 0.03 mol of [^{14}C]iodoacetamide per mole of protein.

Radioalkylated C4 samples were subjected to polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ and β-mercaptoethanol. The *chain structure* of native and treated C4 samples is shown in the left panel (tracks A–E) of Figure 3. A control sample of native C4 (track A) exhibited the characteristic αβγ polypeptide chain structure with respective molecular weights of 94 000, 70 000, and 32 000. Treatment of native C4 with 500 mM NH_2OH (track B) or 300 mM NH_2NH_2 (Bolotin et al., 1977) did not result in any detectable change in the size of the constituent chains. A specific cleavage of the α chain with the production of two fragments with molecular weights of 40 000 and 54 000 was observed, however, when native C4 was treated with 6 M Gdn·HCl (track C) or 0.2% $NaDodSO_4$ (track D) in the absence of a reducing agent.

Table I: Incorporation of ^{14}C Label (Moles/Mole of Protein) following Treatment[a] of Native Protein with (b) [^{14}C]Iodoacetamide, (c) Methylamine and [^{14}C]Iodoacetamide, or (d) [^{14}C]Methylamine

sample:	b buffer, ICH_2*$CONH_2$	c CH_3NH_2, ICH_2*$CONH_2$	c–b ΔSH due to CH_3NH_2	d *CH_3NH_2, buffer	c–b/d ΔSH/*CH_3NH_2
C3(n)	0.28	0.77	0.49	0.50	0.98
C4(n)	0.21	0.83	0.62	0.64	0.97
C5(n)	0.11	0.11	0.00	0.19	0.00

[a] All samples were incubated for 4 h at 37 °C in sodium phosphate buffer (pH 7.4) containing NaCl and EDTA. The molar incorporation was calculated by using molecular weights for C3, C4, and C5 of 185 000, 198 000, and 185 000, respectively. The specific activity of [^{14}C]iodoacetamide in (b) and (c) was 19.1 mCi/mmol. Concentration of methylamine in (c) and (d) was 25 mM. The specific activity of [^{14}C]methylamine in (d) was 21.08 mCi/mmol.

The presence of β-mercaptoethanol in C4 samples prior to dilution into $NaDodSO_4$ was necessary in order to prevent the cleavage reaction (track A). An $\alpha'\beta\gamma$ chain structure characteristic of C4b was observed after the incubation of native C4 with $C1\bar{s}$ (track E).

The corresponding autoradiogram is shown in the right panel (tracks F–J) of Figure 3. As with C3, the ^{14}C-labeled SH group was present in the α chain of NH_2OH-treated C4 (track G) and the α′ chain of C4b (track J). Considerable radioactivity is also apparent in high molecular weight aggregates following enzymatic activation. On treatment with Gdn·HCl or $NaDodSO_4$, the ^{14}C label was incorporated into the residual α chain and the 40 000 molecular weight fragment (tracks H and I) of the α chain. Trace incorporation into the β chain and the 54 000 molecular weight fragment was also observed on inspection of the autoradiogram and is apparently due to sulfhydryl–disulfide interchange within the molecule.

We conclude from these results that the C4 component, like the C3 protein, contains a single sulfhydryl group located in the α chain which is unreactive in the native protein (track F) but can be titrated following treatment with hydroxylamine (track G), denaturants (tracks H and I), and the converting enzyme $C1\bar{s}$ (track J). Analysis of acid hydrolysates of 1-^{14}C-carboxamidomethylated C4 samples by high-voltage electrophoresis (data not shown) has indicated that the principal site of radioalkylation is a cysteinyl residue in both chemically (track G) or enzymatically (track J) treated C4.

In contrast to C4 and C3, we have been unable to demonstrate greater than 0.25 mol of [1-^{14}C]iodoacetamide incorporation per mole of either native (i.e., untreated C5 sample capable of mediating hemolysis), nucleophile-treated, or detergent-treated C5. On incubation of native C5 with 500 mM NH_2OH or $NaDodSO_4$ (0.2% and 2%), the incorporation of [^{14}C]iodoacetamide decreased from 0.25 to 0.15 and 0.03–0.07 mol per mol of protein, respectively. Only after treatment with 6 M Gdn·HCl was an increase in [^{14}C]iodoacetamide incorporation from 0.25 to 0.38 mol per mole of C5 observed. The chemical treatments described here did not result in detectable $C5_\alpha$- or $C5_\beta$-chain degradation as judged by gel electrophoresis in the presence of $NaDodSO_4$ under reducing conditions. Subsequent autoradiofluorography of these samples indicated that the ^{14}C label was present in the $C5_\alpha$ chain (data not shown). No attempt has been made to identify the ^{14}C-labeled residue(s) in C5. Assuming that we are dealing with a cysteinyl residue, it is not clear as yet whether this residue is located in the C5a fragment or in the region of the α′ chain corresponding to the one which is analogous to C3d or C4d domains. It also should be noted that the possible relationship between the presence of an alkylatable residue and the lack of C5 hemolytic activity has not been studied as yet.

Treatment of Native Components with Methylamine. Recent studies (Tack et al., 1980a,b) have shown that C3 hemolytic function is abrogated by treatment with methylamine. The reaction of native C3 with this reagent was stoichiometric, covalent, and accompanied by the appearance of an SH group. Similar studies have been performed on C4(n) and C5(n) pools and used a sample of C3(n) as a control. The percentage of hemolytically active molecules present in the C4(n) and C3(n) pools was evaluated by determination of the differential SH content with DTNB before and after treatment of C4(n) with $C1\bar{s}$, and C3(n) with half-saturated KBr. The starting SH values correspond to the amount of hemolytically inactive molecules with an SH group. The difference between final (after $C1\bar{s}$ or KBr treatment) and starting SH values corresponds to the fraction of hemolytically active molecules. By these methods, it was found that the C4(n) pool consisted of 68% hemolytically active C4 and 32% hemolytically inactive forms, of which 22% were devoid of an SH group. Similarly, the C3(n) pool contained 59% hemolytically active C3, 21% SH-containing molecules, and 19% inactive forms with no detectable SH group. The C5(n) pool contained 0.10 mol of SH/mol of C5. The results of [^{14}C]-iodoacetamide incorporation without (b) and with unlabeled CH_3NH_2 present (c) and the incorporation of [^{14}C]methylamine (d) in a separate but parallel experiment with each protein are presented in Table I. A 1:1 correspondence between the incorporation of [^{14}C]methylamine and the appearance of an SH group was clearly established for both C3 and C4 samples in this study. All samples were further analyzed for hemolytic function by quantitative hemolytic titrations (Figure 4). The polypeptide chain structure of each sample was investigated by polyacrylamide gel electrophoresis in $NaDodSO_4$. An autoradiogram developed from this gel indicated the location of the covalently bound radiolabel (Figure 5).

The information obtained from this set of experiments can be summarized as follows: (1) the proteins under study do not contain an accessible SH group in their hemolytically active state; (2) treatment of the native proteins with [^{14}C]-iodoacetamide does not affect the hemolytic activity of these proteins; (3) treatment of native C3 and C4 with methylamine or [^{14}C]methylamine results in a total loss of hemolytic function; (4) the thiol group reactive with [^{14}C]iodoacetamide following methylamine treatment is present in the α chain of both C3 and C4; (5) the reaction of C3 and C4 with [^{14}C]-methylamine is stoichiometric and covalent; (6) the incorporation of [^{14}C]methylamine is strictly into the α-polypeptide chains of C3 and C4; (7) unlike C3 and C4, native C5 does not react with methylamine, and the hemolytic activity is unaffected by methylamine treatment; (8) the polypeptide chain structures of these proteins do not appear to be affected by treatment with either iodoacetamide or methylamine.

Studies of the Autolytic Cleavage Reaction. The preceding results with C4 indicated that the native conformation is required not only for its hemolytic function, including its susceptibility to cleavage by $C1\bar{s}$, but also for the autolytic cleavage reaction which takes place in the presence of $NaDodSO_4$ or Gdn·HCl (Figure 2B and Figure 3). Spontane-

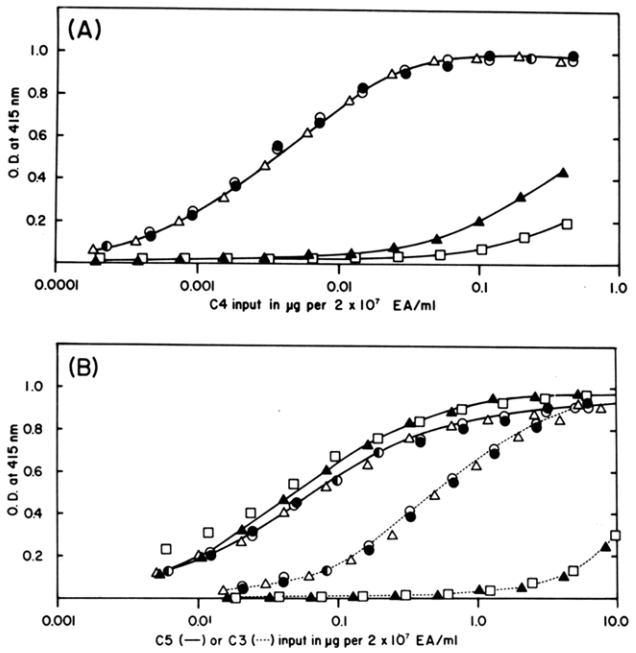

FIGURE 4: Quantitative determination of the hemolytic activity of (A) C4(n) and (B) C5(n) and C3(n) before and after a 4-h incubation at 37 °C in sodium phosphate buffer (pH 7.4) containing NaCl, EDTA, and either iodoacetamide or methylamine or both. The compositions of individual samples were as follows: native protein in buffer only (●); native protein treated with [^{14}C]iodoacetamide (△); native protein treated with 25 mM methylamine in the presence of [^{14}C]iodoacetamide (▲); native protein treated with [^{14}C]-methylamine (□). Samples (●) kept at 4 °C were used as controls for native, i.e., untreated, protein (○). Hemolytic assays containing 0.4 mL of EA cells (5×10^7 cells/mL), 0.5 mL of a protein sample diluted in $GVBS^{2+}$, and 0.1 mL of serum (diluted 1:10) deficient in a respective complement component were incubated at 37 °C for 1 h. After an addition of 1 mL of cold isotonic saline, the unlysed cells were pelleted by centrifugation, and the absorbance at 415 nm was measured.

ously inactivated forms of C4 and C4b prepared from native C4 by incubation with $C1\bar{s}$ failed to fragment under the same conditions. The following results (Figure 6) are in accordance with the supposition that the disruption of the same functional site present in native C4 is, at least primarily, responsible for the different properties seen between hemolytically active and inactive forms of C4.

Thus, [^{14}C]methylamine-treated C4 which was fully inactive by the hemolytic assay was no longer susceptible to cleavage by $C1\bar{s}$ (track 3), and also failed to undergo spontaneous fragmentation of the α chain (track 1). To address the possibility that the observed fragmentation was the direct result of proteolysis, native C4 and [^{14}C]methylamine-treated C4 were coincubated at pH 7.0 for 16 h at 37 °C in the presence of 0.2% $NaDodSO_4$. The results are shown in track 2 of Figure 6 and indicate that the α chain of the latter sample is resistant to fragmentation. The cleavage reaction was found to be time dependant when native C4 was incubated in 0.2% $NaDodSO_4$ at 37 °C. The maximum yield was reached at 4 h, and the fragmentation pattern remained unchanged on prolonged incubation. In accordance with C3 (Janatova et al., 1980a,b), only a partial fragmentation of the α chain has been observed. The extent of fragmentation does not appear to be influenced by pH (7.0 vs. 8.2) or buffer (phosphate vs. Tris). The presence of an SH-reactive reagent, e.g., iodoacetamide, does not affect the fragmentation of C4(n) in the presence of $NaDodSO_4$.

In order to ascertain the proximity of the autolytic cleavage site to the thiol component of the labile binding site, the

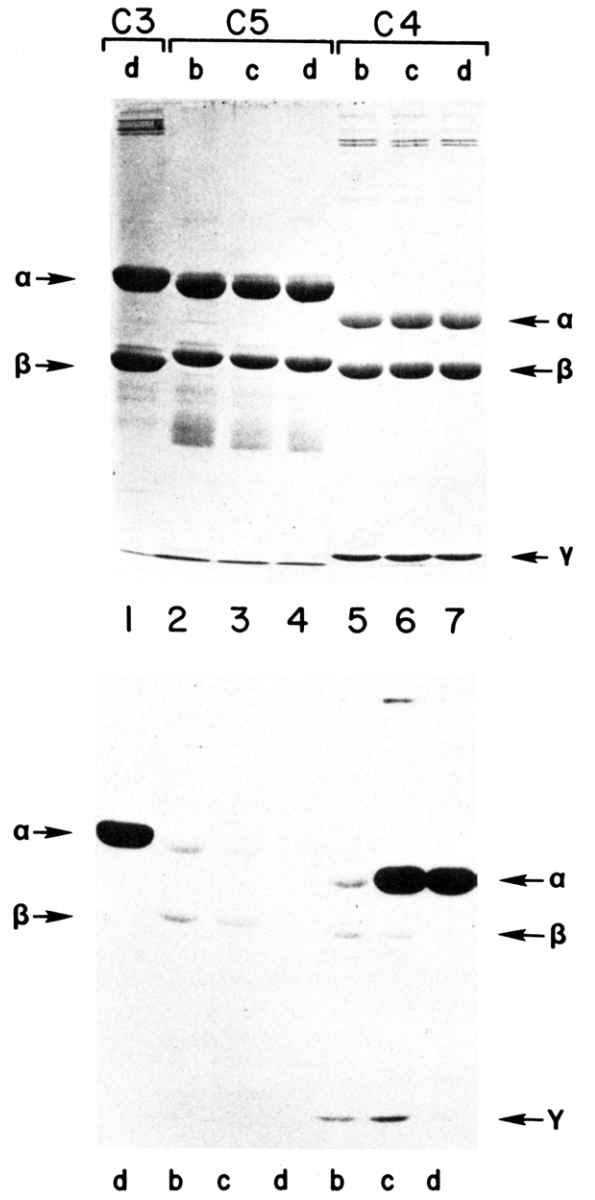

FIGURE 5: Electrophoretic and autoradiofluorographic analyses of C4(n) and C5(n) samples after treatment with $ICH_2{}^*CONH_2$ (b), CH_3NH_2 and $ICH_2{}^*CONH_2$ (c), and $^*CH_3NH_2$ (d). A $^*CH_3$-NH_2-treated C3(n) sample was run as a control in track 1. Prior to electrophoresis, each sample was reduced with β-mercaptoethanol in the presence of $NaDodSO_4$. The autoradiogram is shown beneath the Coomassie Blue stained gel.

following experiment was performed. The SH group expressed on treatment of native C4 with methylamine or half-saturated KBr was reacted with 0.01 M NTCB at pH 8.2 (0.1 M sodium phosphate and 2 mM EDTA). Subsequent overnight incubation at 37 °C of the cyanylated protein in 0.2% $NaDodSO_4$ at pH 8.2 (0.1 M sodium phosphate and 2 mM EDTA) resulted in cleavage of the peptide bond at the *S*-cyanocysteinyl residue (Jacobson et al., 1973) with the production of two fragments with molecular weights of 40000 and 54000. These fragments corresponded in size to the fragments seen on autolytic cleavage of native C4. While the radioalkylated SH group had been localized to the smaller fragment following autolytic cleavage (Figure 1, tracks H and I), the radiolabel from NT*CB was present on the larger fragment after cleavage at the *S*-cyanocysteinyl residue. These results suggest that the smaller fragment must correspond to the N-terminal portion of the α chain and that the autolytic cleavage site must be located only a few residues from Cys toward the C terminus

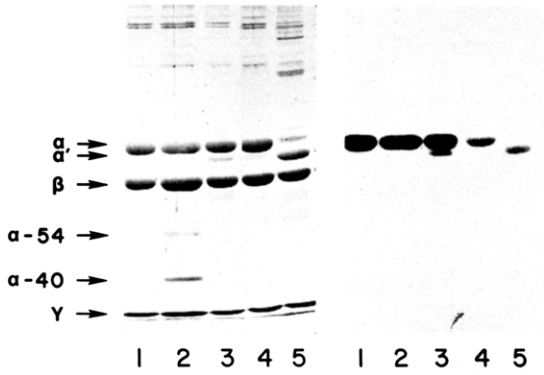

FIGURE 6: Electrophoretic and autoradiofluorographic analyses of $*CH_3NH_2$-inactivated C4 (track 7 in Figure 5) after incubation with 0.2% $NaDodSO_4$ at pH 7 for 16 h (track 1), 0.2% $NaDodSO_4$ in the presence of untreated C4(n) at pH 7 for 16 h (track 2), and with $C1\bar{s}$ at pH 7.4 for 30 min at 37 °C (track 3). Tracks 4 and 5 show the results of the incubation of C4(n) at 37 °C with 10 mM NH_2NH_2 at pH 7.4 for 2.5 h or $C1\bar{s}$ (100:1) at pH 7.4 for 1 h followed by a 4-h incubation with $*CH_3NH_2$. Prior to electrophoresis, each sample was reduced with β-mercaptoethanol in the presence of $NaDodSO_4$. The autoradiogram is shown in the right panel.

of the α chain. The results of polyacrylamide gel electrophoresis in $NaDodSO_4$ without prior reduction with β-mercaptoethanol indicated that both fragments (either from autolytic cleavage or from cleavage of the S-cyanylated protein) are disulfide bonded in an undetermined manner to the rest of the C4 molecule. Similar results were also observed in the case of the C3 protein, and they will be presented in another paper, including a more detailed investigation of the autolytic cleavage reaction (J. Janatova and B. F. Tack, unpublished data).

Miscellaneous Observations. Both hydrazine and $C1\bar{s}$ were reported to inactivate C4 in the fluid phase (Müller-Eberhard & Lepow, 1965). In order to show that inactivated C4 does not react with [^{14}C]methylamine, aliquots of native C4 were pretreated first with either 0.01 M hydrazine for 2.5 h or $C1\bar{s}$ (100:1) for 1 h (pH 7.4, 37 °C), and then the incubation was continued for another 4 h after the addition of [^{14}C]methylamine (25 mM). Samples were analyzed (after thorough dialysis) for hemolytic activity, bound radioactivity, polypeptide chain structure, and radiolabel location. As expected, both samples were completely inactive with respect to C4 hemolytic function. A certain amount of bound radioactivity was detected in both samples, and a partial incorporation of the radiolabel into the α or α' chain was observed. However, only 0.15 mol of methylamine/mol of C4 in hydrazine-pretreated C4, and 0.37 mol of methylamine/mol of C4 in $C1\bar{s}$-pretreated C4, was incorporated in comparison with 0.64 mol/mol of C4 in the control sample treated only with [^{14}C]methylamine.

Several experiments were directed at establishing the stoichiometry between the gradual loss of C4 hemolytic function and the appearance of a free SH group upon treatment with methylamine. When the conditions were the same as those used for C3 [Figure 1 in Tack et al. (1980b)], full inactivation of C4 was observed after the incubation with only 10 mM methylamine (pH 7.4; 1 h; 37 °C). When native C4 was incubated with a lower concentration of methylamine (5 mM), the content of hemolytically active molecules dropped from 68% to 14% during the first 10 min of incubation. These experiments indicated the larger sensitivity of C4 to the treatment with amines in comparison with C3.

Discussion

Nucleophile reagents, such as amines, have been known to convert C3 and also C4 into hemolytically inactive forms (Budzko & Müller-Eberhard, 1969; Ecker et al., 1943; Müller-Eberhard & Biro, 1963; Seifter et al., 1963). Our previous studies on C3 (Janatova et al., 1980a,b; Tack et al., 1980a) and this study on C4, using highly purified proteins, have shown that the inactivation by nucleophiles (with respect to their hemolytic function) is a nondegradative process which is accompanied by the appearance of a single SH group, which is contributed by a cysteinyl residue located in the α chain of both proteins. Activation of C3 and C4 by limited proteolysis with bovine trypsin and $C1\bar{s}$, respectively, has also been shown to affect the expression of an SH group. More recently, we have sequenced a tryptic peptide comprising the methylamine-reactive site in native C3 (Tack et al., 1980b) and have concluded that the properties of this site are consistent with an internal thiol ester. Identical primary structure at the methylamine-reactive site between the C3 tryptic peptide and the chymotryptic peptide from methylamine-inactivated α_2-macroglobulin (Swenson & Howard, 1979) indicates that these two proteins share a common functional site by which they are capable of covalent bonding.

The separation of different chromatographic forms of C4 and their subsequent analysis as reported in this study have shown the necessity of the native conformation for cleavage by $C1\bar{s}$ and also for the autolytic cleavage in the presence of $NaDodSO_4$. The observation that inactivated C4 (hydrazine treated) does not bind to EAC1 has already been reported by others (Müller-Eberhard & Lepow, 1965). Consequently, the C3-converting enzyme, $\overline{C4b2a}$, cannot be formed on the cell surface. Whether C4(i) is capable of binding native C3 in the fluid phase is not clear as yet, although limited data suggesting the participation of C4(i) in the formation of a C3 convertase have been reported (von Zabern et al., 1980).

The first compelling evidence that C3b and C4b are bound to cell surfaces by a covalent, hydroxylamine-sensitive, and therefore an esterlike bond has been provided by the studies of Law & Levine (1977) and Law et al. (1980). Their findings that the site of C3b attachment resided in the C3d domain (Law et al., 1979a) and that the protein contributed the acyl group to the proposed ester bond prompted the speculation that the reactive acyl group could be transferred via a transesterification reaction (Law et al., 1979b). Our concept of an internal thiol ester bond as a functional group in native C3 and C4 lends considerable support to the proposed transacylation mechanism. Further support for such an acylation mechanism has come from experiments showing that the binding of C3b to trypsin–Sepharose can be inhibited either by the presence of carbohydrates and IgG (Capel et al., 1978) or by the presence of various nucleophiles (Sim et al., 1981). The later reagents (in their radiolabeled forms) were used for the covalent labeling of the C3b-labile binding site. Recent studies by Law et al. (1980) suggested that C4b is bound to similar molecules on cell surfaces as C3b, and that the respective bond(s) can be disrupted by the effect of hydroxylamine or under alkaline conditions. Campbell et al. (1980), however, reported that only partial dissociation of bound C4b could be achieved by the effect of hydroxylamine. This and their previous study on the binding of C4b to antibody–antigen aggregates and antibody-coated erythrocytes (Campbell et al., 1980; Goers & Porter, 1978) suggested that only C4b molecules bound to the antibody molecule can be effective in hemolysis. The bond between C4b and the IgG molecule appeared to be significantly more stable than the postulated C3b–polysaccharide ester bond at high pH or in hydroxylamine. These results suggest that the postulated active acyl group (apparently released from a labilized thiol ester in C4)

is reacting either with an amino acid residue in the Fd section of the heavy chain (Campbell et al., 1980) or with carbohydrate structures present on cell surfaces (Law et al., 1980). The binding of "activated" C4b to IgG aggregates could be inhibited by the covalent association between the C4b-labile binding site and [^{14}C]putrescine (Campbell et al., 1980).

In the course of our studies, the tendency of C4, upon incubation in denaturants, to undergo a specific cleavage of the α chain was observed. In all respects, this cleavage reaction was similar to that already described for C3 (Janatova et al., 1980b). The same observations with C3, C4, and α_2-macroglobulin have been made by Sim & Sim (1981), Howard (1980), and Howard et al. (1980). Some aspects of the denaturant-induced autolytic cleavage of the C4 α chain have been presented in this paper. On the basis of our observations, we can conclude that the specific fragmentation of the α chain of C4 or C3 does not arise from contaminating proteases and that it is in fact a unique property of a hemolytically active form of C4 or C3. Disruption of the proposed thiol ester bond in general prevents this cleavage. Our studies on the fragmentation of S-cyanylated C4 or C3, where the cysteinyl residue of the thiol ester was the point of cleavage, suggested that the site of autolytic cleavage was proximal to the position of the thiol ester bond. The recent study by Howard et al. (1980) provides direct evidence that in the case of α_2-macroglobulin the denaturant-sensitive bond is located within the methylamine-reactive site, which was proposed to be an internal pyroglutamyl residue. This reactive site assignment is in contrast to the internal thiol ester suggested by Tack et al. (1980a,b) for C3 and α_2-macroglobulin. We are conducting further structural studies on C3 in order to be able to define the mechanism of the autolytic cleavage reaction more precisely. However, it is apparent from our data that the site of an autolytic cleavage, and hence the site of the thiol ester, is located 46 000 daltons in C3 and 40 000 daltons in C4 from the N terminus of the α chain.

In summary, the results of our studies suggest that like native C3 native C4 also contains an internal thiol ester which (1) contributes to the stabilization of the native conformation prior to activation by the respective converting enzyme, (2) may serve as the source of an activated acyl group in a transesterification process, a proposed mechanism for the covalent binding of "nascent" C4b and C3b to cell surfaces, and (3) may be involved in the autolytic denaturant-induced cleavage of the C4α and C3α chains. Although there is considerable structural homology between C5a and C3a (Fernandez & Hugli, 1978), our results on the treatment of native C5 with hydroxylamine, methylamine, $NaDodSO_4$, or Gdn·HCl suggest that the mechanism of binding between C5b and cell surfaces must differ from that of C3b and C4b, as no effects of nucleophiles on C5 hemolytic activity have been observed. Moreover, no changes in the size of C5 $\alpha\beta$ chains were observed in the presence of $NaDodSO_4$ or Gdn·HCl.

Acknowledgments

We dedicate this work to the memory of Dr. James W. Prahl, our excellent teacher, colleague, and dearest friend. The isolation of some of the protein material used in this study as well as the preliminary experiment dealing with C4 inactivation was carried out in collaboration with the late Dr. J. W. Prahl. We express our appreciation to Laurent E. Wanner for technical assistance during protein isolation, to Matthew L. Thomas for reading the manuscript, to Donald G. Morse for the preparation of figures, and to Courtney Clayton and Edie Vetter for excellent secretarial assistance. We also gratefully acknowledge the availability of preprints of manuscripts by Sim & Sim (1981) and Sim et al. (1981) cited in the present work.

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Polymorphism of Brain Tubulin†

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ABSTRACT: Calf brain tubulin was subjected to isoelectric focusing and tryptic peptide map analysis. Results from isoelectric focusing experiments showed a total number of 17 well-resolved protein peaks. The number of peaks and the mass distribution under each peak remained the same when the concentration of protein or ampholyte was altered. When the protein was subjected to two-dimensional isoelectric focusing, a diagonal pattern was observed, indicating that the multiple peaks observed are not a manifestation of tubulin–ampholyte interaction. Further investigation by isolating these individual subspecies and subjecting them to isoelectric focusing yielded single peaks corresponding to the original ones without generating the initial pattern of multiple peaks. Tryptic peptide maps showed that among the subspecies of the α subunit there are 26 spots that are common among them. There are, however, 7 ± 1 spots that are unique in each subspecies. Similar observations were obtained for the subspecies of the β subunit although there are only 2 ± 1 unique spots in each subspecies. These results suggest that tubulin subunits probably consist of polypeptides with both constant and variable regions in their sequences. Identical results were obtained for canine and rabbit brain tubulin, indicating that tubulin polymorphism is common among brain tissues. Tubulin isolated by either the polymerization–depolymerization or the modified Weisenberg procedures yielded identical results. These results show that the same subspecies of tubulin are extracted by both isolation procedures.

Microtubules constitute a major portion of cytoplasmic proteins in nerve cells (Hiller & Weber, 1978). They have been implicated to play a central role in axonal transport, neurotransmitter release, neurite outgrowth, and synaptogenesis (Shelanski & Feit, 1972). A cell is able to control the polymerization and organization of microtubules, thus influencing the cellular processes with which microtubules seem to be associated. The mechanism of control is believed to be through an influence on the dynamic equilibrium of microtubules with its subunit protein, tubulin. For many years, tubulin isolated from various mammalian brain tissues has been shown to exist as a complex of two nonidentical polypeptide chains, α and β. These polypeptide chains have a molecular weight of about 54 000 (Lee et al., 1973) and have been shown to possess different amino acid sequences (Luduena & Woodward, 1973). Recently, however, there has been an increasing number of reports implying that brain tubulin exhibits extensive heterogeneity when subjected to isoelectric focusing (Gozes & Littauer, 1978; Gozes et al., 1979; Marotta et al., 1978; Feit et al., 1977; Forgue & Dahl, 1979; Dahl & Weibel, 1979; Nelles & Bamburg, 1979). The number of subspecies reported varies from three to nine. Although the cause for the reported heterogeneity remains unknown, it has been proposed that the number of subspecies

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