RELATION OF A PUTATIVE THIOESTER BOND IN C3 TO ACTIVATION OF THE ALTERNATIVE PATHWAY AND THE BINDING OF C3b TO BIOLOGICAL TARGETS OF COMPLEMENT*

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Formation of the initial enzyme of the alternative pathway and generation of the metastable binding site of C3b may be intimately linked to the presence of a thioester bond in the C3 molecule. The metastable binding site of C3b was described in 1966 (1), but its chemical basis remained obscure until recently. The site effects the firm attachment of the activated metastable C3b to a wide variety of biological particles. It thereby enables C3b to fulfill its functions in the assembly of both activation pathways on the surface of target cells and in opsonization reactions (2).

Recently, two observations helped to clarify the chemical nature of the metastable binding site. It was shown that bound C3b is probably linked to targets via a covalent bond in which a carbonyl group of C3b forms an ester with a hydroxyl group of the target surface (3, 4). Secondly, C3b was shown to contain a sulfhydryl group not detectable in native C3 (5). Both the sulfhydryl and the reactive carbonyl groups were found to be located in the d-domain of the alpha-chain, which is known to contain the binding site of C3b. It may be postulated, therefore, that the two groups form an internal thioester in native C3 (6, 7).

That C3 loses its hemolytic activity upon treatment with amines such as hydrazine is known (8). In view of the described recent findings, the amine sensitivity of C3 assumes a new significance. In the present study, native C3 was treated with methylamine because this amine is available in radiolabeled form. Concomitant with loss of C3 hemolytic activity, 1 mol of methylamine became bound and 1 mol of sulfhydryl became exposed per mol of C3.

It is the purpose of this paper to report that methylamine-treated C3 behaves functionally like C3b with respect to (*a*) Factor B and β1H binding, (*b*) susceptibility to cleavage by C3b inactivator, and (*c*) the ability to form a fluid-phase C3 convertase in presence of Factors B and D, and Mg^{++}. The possibility is raised that C3b-like C3 may be produced under physiological conditions by the reaction of C3 with water. We wish to propose that C3b-like C3 arises by nonenzymatic spontaneous thioester-bond hydrolysis of C3 and thereby effects formation of a steady-state concentration of the initial C3 convertase of the alternative pathway of complement activation.

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Materials and Methods

Purified Components and Reagents. Highly purified C3 (9), Factor B (10), Factor D (11), β1H (12), C3b inactivator (12), activated properdin (13, 14), cobra venom factor (CoF)[1] (15), and partially purified nephritic factor (16) were prepared as described. C3b was generated from purified C3 (3 mg/ml) either by 5-min incubation with 2 μg/ml trypsin (TPCK treated; Worthington Biochemical Corp., Freehold, N.J.) followed by 4 μg/ml soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, Mo.) or by a 10-min incubation at 37°C with 30 μg/ml Factor B and 2 μg/ml Factor D in the presence of 5 mM $MgCl_2$. C3 hemolytically inactivated by incorporation of methylamine [$C3(CH_3NH_2)$] was prepared by 30-min incubation at 37°C with 0.2 M CH_3NH_2 at pH 8. Excess methylamine was removed by dialysis.

[^{14}C]methylamine hydrochloride (50.1 mCi/mmol) and [1-^{14}C]iodoacetamide (24.9 mCi/mmol) were obtained from New England Nuclear, Boston, Mass. as solutions in ethanol. Unlabeled methylamine hydrochloride was obtained from Sigma Chemical Co. Radioiodination of proteins was performed according to the method of Fraker and Speck (17) using Iodogen (Pierce Chemical Co., Rockford, Ill.). Sheep erythrocytes (E_S) that bore specifically attached C3b (E_SC3b) were prepared as described previously (18).

Buffers. The buffers were as follows: isotonic veronal-buffered saline, pH 7.4 (VB); VB containing 0.1% gelatin and 1.2 mM $MgCl_2$ (Mg-GVB); VB containing 0.1% gelatin and 10 mM EDTA (GVBE); and one-half-ionic strength VB containing 0.1% gelatin, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 2.5% glucose (DGVB).

Preparation of Anti-C3a-Sepharose. Antiserum was raised to purified human C3a (19) in a goat. The protein precipitated from this antiserum with 40% saturated ammonium sulfate was coupled to CNBr-activated Sepharose. Affinity chromatography-purified anti-C3a antibodies were isolated from the antisera by passage over C3a lacking the C-terminal arganine residue (iC3a)-Sepharose.

Incorporation of [^{14}C]Methylamine into C3d Released from Zymosan. Zymosan was incubated for 2 h at 37°C with normal human serum that contained a 1:20 dilution of 0.1 M $MgCl_2$ that contained 0.1 M EGTA (MgEGTA). After a thorough washing, the zymosan bearing the inactivated form of C3b generated upon cleavage by C3b inactivator (iC3b) was treated with 5 μg/ml trypsin for 20 min at 37°C to convert iC3b to C3d. The washed particles were then incubated for 6 h at 37°C with 4 M guanidine, 2 mM [^{14}C]methylamine (10 μCi), and 0.1 M sodium phosphate buffer, pH 8. Unbound label was removed by passage over Sephadex G-25 in 6 M guanidine. The labeled protein fraction was dialyzed against 10% acetic acid, lyophilized, and subjected to polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS).

Assays. C3 assays were performed by the effective molecule titration method described earlier (20). The activity of the fluid-phase properdin-stabilized C3 convertases formed using C3b or $C3(CH_3NH_2)$ was determined by the rate of C3 consumption at 37°C. Binding assays for the measurement of the interaction between ^{125}I-β1H and E_SC3b were performed as previously described (18). Competition between cell-bound C3b and fluid-phase C3, C3b, or $C3(CH_3NH_2)$ for ^{125}I-factor B was measured in DGVB containing 4 mM $MgCl_2$. The assay mixtures contained 5×10^7 E_SC3b (bearing 80,000 C3b/cell), 2 μg ^{125}I-factor B (10 nCi/μg), and various amounts of C3, C3b, or $C3(CH_3NH_2)$ in a total vol of 100 μl. The samples were incubated 10 min at 21°C and centrifuged in a Beckman microfuge (Beckman Instruments, Inc., Fullerton, Calif.). The amount of radiolabeled Factor B bound was determined by its depletion from the supernate. Quantitation of functional C3b bound to E_S was performed by incubation of 1.5×10^7 cells with 300 ng Factor B and 10 ng Factor D in 20 μl Mg-GVB for 3

[1] *Abbreviations used in this paper:* $C3(CH_3NH_2)$, C3 hemolytically inactivated by incorporation of methylamine; $C3(H_2O)$, C3 hemolytically inactivated by water hydrolysis of the thioester bond; C3bINA, C3b inactivator; CoF, cobra venom factor, a C3b-like protein isolated from cobra venom; GVBE, veronal-buffered saline that contained 0.1% gelatin and 10 mM EDTA; DGVB, half-ionic strength veronal-buffered saline containing 0.1% gelatin, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 2.5% glucose; E_S, sheep erythrocytes; E_SC3b, sheep erythrocytes that bear specifically bound C3b; iC3a, C3 lacking the C-terminal arginine residue; iC3b, inactivated form of C3b generated upon cleavage by C3bINA; MgEGTA, 0.1 M $MgCl_2$ containing 0.1 M EGTA; MgGVB, veronal-buffered saline containing 0.1% gelatin and 1.2 mM $MgCl_2$; SDS, sodium dodecyl sulfate; VB, veronal-buffered saline.

min at 37°C to allow cell-bound C3-convertase formation. Lysis was determined after addition of 1 ml of guinea pig serum (2% in GVBE) and incubation for 10 min at 37°C.

C3 Consumption in Serum. Activation of the alternative pathway in serum by the addition of C3b or C3(CH_3NH_2) was measured as C3 consumption after 20 min at 37°C. The reaction mixtures contained 25 μl normal human serum, 5 μl 0.1 M MgEGTA, and various amounts of C3, C3b, C3(CH_3NH_2) (1.9 mg/ml), or buffer to yield a final volume of 110 μl. C3 activity was assayed as described above. Samples that recieved additions of native C3 were compared with controls that contained EDTA; the amount of C3 consumed was calculated as if all of the consumption represented consumption of the serum-derived C3.

Electrophoresis and Scintillation Counting. Analysis of intermediates formed during proteolytic digestion of [^{14}C]methylamine-labeled C3 was performed by SDS polyacrylamide gel electrophoresis using the method of Laemmli (21). The gel slices were solubilized with an NCS tissue solubilizer (Amersham Corp., Arlington Heights, Ill.) and analyzed with a Beckman LS-8000 liquid scintillaltion counter (Beckman Instruments, Inc.) using the toluene-based scintillant Scinti Verse (Fisher Scientific Co., Pittsburgh, Pa.) that contained 5% acetic acid and 8% water.

Results

Stoichiometry of the Reaction of Methylamine with C3. Incubation of native human C3 with radiolabeled methylamine led to inactivation of C3 hemolytic activity and concomitant incorporation of methylamine into the protein (Fig. 1). C3 at a concentration of 2.7 mg/ml was incubated at 37°C and pH 8.0 with 12.6 mM [^{14}C]-methylamine (15.9 mCi/nmol), 20 mM sodium phosphate, and 0.15 M sodium chloride. Samples were removed at time intervals and diluted 15-fold in cold GVBE. Part of the diluted sample was used for titration of C3 hemolytic activity and the remainder passed over Sephadex G-25 to remove unbound methylamine. The rate of loss of hemolytic activity was identical to the rate of methylamine incorporation. Uptake ceased at 0.87 mol of methylamine/mol of C3. Similar treatment of C3b resulted in uptake of only 0.03 mol of methylamine/mol of C3b.

Localization of Methylamine in the d-Domain of the α-Chain. To determine the location of the amine in C3(CH_3NH_2), the protein was enzymatically converted to C3b and iC3b, and to C3c and C3d. C3(CH_3NH_2) and its fragments were analyzed by SDS polyacrylamide gel electrophoresis under reducing conditions. As shown in Fig. 2, the

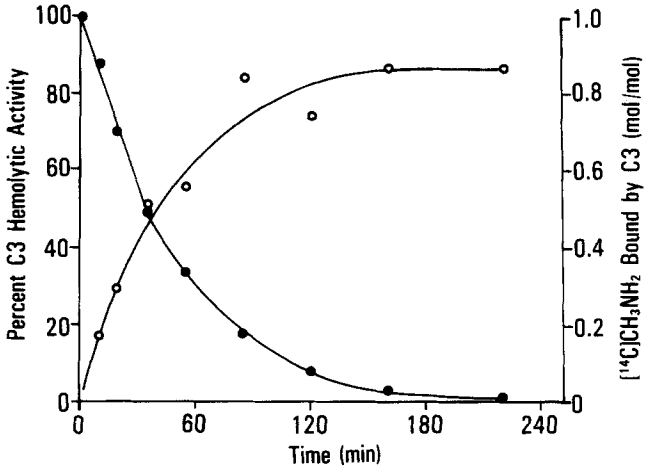

FIG. 1. Stoichiometry of the incorporation of radiolabeled methylamine by C3 (○) and correlation of incorporation with the loss of hemolytic function (●).

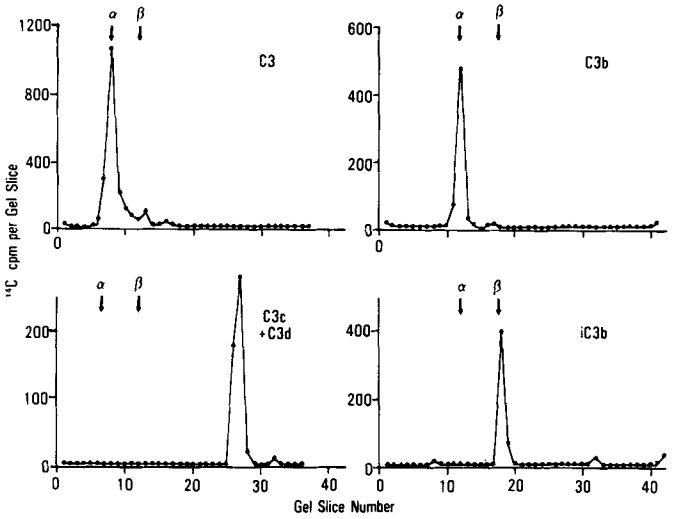

FIG. 2. Localization of the methylamine-reactive site to the C3d portion of C3. Polyacrylamide gel electrophoresis in the presence of SDS and dithiothreitol of C3(CH_3NH_2) and its breakdown products. [^{14}C]methylamine-labeled C3 (100 μg in 100 μl) was converted to C3b by mild trypsinization (1 μg, 5 min, 37°C followed by 2 μg soybean trypsin inhibitor). iC3b was produced by treatment of C3b with 5 μg/ml C3bINA and 50 μg/ml βlH for 20 min at 37°C. Subsequent addition of 3 μg trypsin generated the C3c and C3d fragments. Only the C3d fragment contained radiolabel. The arrows indicate the positions of the C3 α-chain (M_r 110,000) and C3 β-chain (M_r 75,000).

TABLE I

Quantitation of Free Sulfhydryl Groups in C3, C3b, and C3(CH_3NH_2)

	Mol iodoacetamide incorporated/mol C3	
	In buffer	In 0.45 M guanidine
C3	0.04	0.22
C3b	0.74	0.91
C3(CH_3NH_2)	0.31	1.22

radiolabel was found in the α-chain of C3, the α′-chain of C3b (α-chain of C3b minus C3a), the α′-67,000 piece of iC3b, and C3d (M_r = 31,000). Methylamine released C3d bound to zymosan particles and in the process was incorporated into the protein. The release reaction was performed in presence of 4 M guanidine hydrochloride.

Appearance of an Iodoacetamide Reactive Group after Methylamine Treatment of C3. Table I shows the results of reacting C3(CH_3NH_2), C3, and C3b with [^{14}C]iodoacetamide. The protein preparations were incubated at pH 7.2 and 37°C for 30 min with a sevenfold molar excess of iodoacetamide. Native C3 incorporated less than 0.04 mol of the sulfhydryl reagent/mol protein. C3b and C3(CH_3NH_2) bound 0.74 and 0.31 mol/mol, respectively. In the presence of 0.45 M guanidine, binding of iodoacetamide to C3b and C3(CH_3NH_2) increased to 0.91 and 1.22 mol, respectively. More than 80% of the label was found in the α-chain of C3(CH_3NH_2), in the α′-67,000 piece of iC3b, and in C3d, both derived from the modified protein.

Reactivity of C3 with Nucleophiles Other than Methylamine. The sensitivity of C3 to a

series of nucleophilic compounds bearing increasingly bulky alkyl-side chains was examined. Fig. 3 shows that C3 was inactivated by methylamine 73 times faster than by ethylamine, and 750 times faster than by isopropylamine. No inactivation was observed with t-butylamine. The small differences in ionization constants (pK_a) and nucleophilicity of these compounds do not account for the rate differences observed. Therefore, a sterically restricted site is postulated for the amine-sensitive group in C3.

To examine the probable physiological mechanism of modified C3 production, several nucleophilic compounds found in plasma were tested. Water at pH 7.3 and 37°C effected a loss of C3 hemolytic activity of 0.5%/h. Loss of activity was much accelerated in the presence of low concentrations of chaotropic agents. For instance, 0.33 M KSCN caused 50% inactivation in 8 min, and 0.75 M guanidine effected 50% inactivation in 12 min. Ammonia at 0.05 M or ethanolamine at 0.2 M caused 50% inactivation in 1 h. The results suggest that at normal plasma concentrations of ammonia (12 μM), ethanolamine (1.7 μM), and methylamine (0.1 μM), these nucleophiles cause inactivation of only 0.005% C3/h. It appears that direct hydrolysis of C3 by water probably plays a greater role in generating C3b-like C3 in plasma than do amines.

Presence of the C3a Domain on C3(CH_3NH_2) and Relative Resistance of the Modified Protein to Cleavage by C3 Convertases. The possibility was considered that in the course of methylamine modification of C3, the C3a domain was removed from the molecule. This possibility could be ruled out because C3(CH_3NH_2) was bound by anti-C3a-Sepharose and this binding could be inhibited by purified C3a. Binding could also be prevented by prior treatment of C3(CH_3NH_2) with trypsin, which dissociates C3a by poteolytic cleavage. C3 convertases cleaved C3(CH_3NH_2) less efficiently than C3. At low enzyme:substrate ratios, cleavage of C3(CH_3NH_2) was negligible. When a sixfold molar excess of CoF-dependent C3 convertase was employed, definite cleavage of C3(CH_3NH_2) occurred, but the reaction did not go to completion in 30 min at 37°C. Similar results were obtained with the classical and alternative C3 convertases.

Interaction of C3(CH_3NH_2) with Factor B and β1H. The binding of Factor B to cell-bound C3b can be inhibited by fluid-phase C3b. Fig. 4 shows that C3(CH_3NH_2), unlike native C3, can also inhibit Factor B binding to cell-bound C3b, indicating that

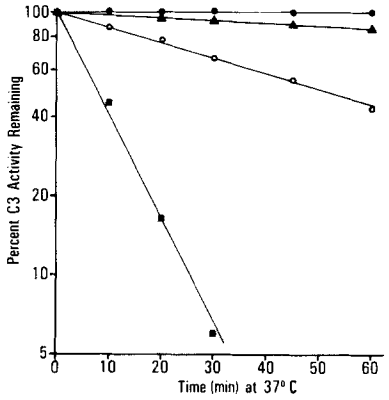

FIG. 3. Inactivation of C3 hemolytic activity at pH 8 by 0.025 M methylamine (■) and 0.2 M ethylamine (○), isoproplylamine (▲), or t-butylamine (●). Control incubations in 20 mM sodium phosphate buffer alone showed no inactivation.

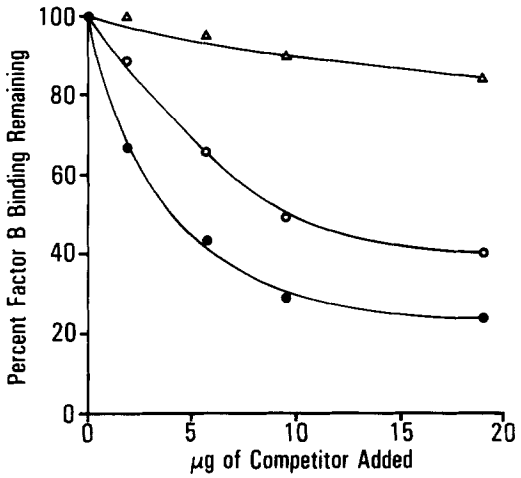

FIG. 4. Acquisition of affinity for Factor B by methylamine treatment of C3. Competition by fluid-phase C3 (Δ), C3(CH_3NH_2) (○), and C3b (●) with the binding of ^{125}I-Factor B to E_SC3b. The assays contained 5 × 10^7 E_SC3b and 2 μg ^{125}I-Factor B in a final volume of 100 μl DGVB that contained 4 mM $MgCl_2$. Binding was determined after 10 min at 21°C by measuring the decrease of radioactivity in the fluid phase after removal of the cells.

TABLE II

Inhibition of Factor B and β1H Binding to E_zSC3b by Fluid-Phase C3(CH_3NH_2) or C3b

Competitor	Amount of competitor required for 50% inhibition of component binding*	
	B binding	β1H binding
	μg	
C3	494	205
C3b	5	19
C3(CH_3NH_2)	9	6

* Factor B binding to E_SC3b in the presence of fluid-phase competitors was measured as described in Materials and Methods. β1H binding to E_SC3b was measured as previously described (18), except for the presence of fluid-phase competitors in the reaction mixture.

C3(CH_3NH_2) behaves in this respect like C3b. Similar results were obtained with β1H (Table II), indicating the existence of β1H- and Factor B-binding sites on C3(CH_3NH_2).

Cleavage of C3(CH_3HN_2) by C3b Inactivator and β1H. Fig. 5 shows the results of exposing C3(CH_3NH_2) to C3b inactivator (C3bINA) and β1H. SDS polyacrylamide gel electrophoresis analysis under reducing conditions showed that the α-chain of C3(CH_3NH_2) was cleaved directly without prior conversion of the protein to C3b. In comparison with similarly treated C3b, which yielded the known α′-67,000 and α′-40,000 fragments and the 75,000 mol wt β-chain, C3(CH_3NH_2) yielded the same fragments, except that the larger of the two α-chain pieces was ~10,000 dalton heavier. This fragment, therefore, migrated together with the beta-chain and, in all probability, contains the C3a domain.

Activation of the Alternative Pathway in Serum by C3(CH_3NH_2). To assess whether

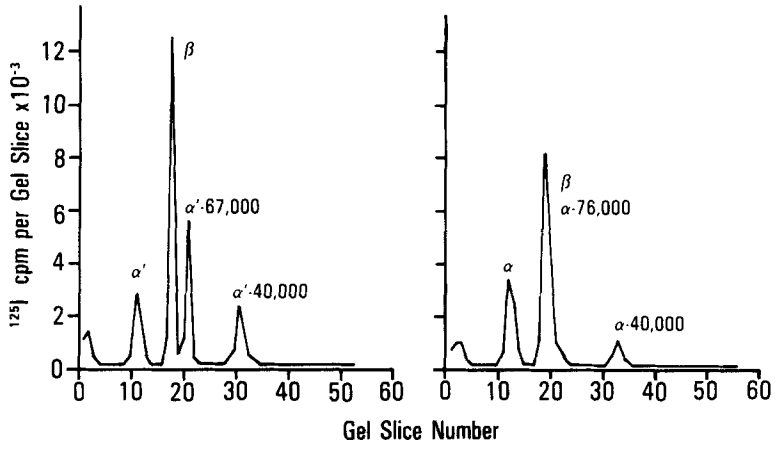

FIG. 5. Cleavage of C3(CH_3NH_3) by C3bINA and βlH. (Left) Polyacrylamide gel electrophoresis in the presence of SDS and dithiothreitol of ^{125}I -C3b after a brief incubation with C3bINA and βlH. The two α'-chain cleavage products, α'-67,000 and α'-40,000, are indicated. (Right) Similar treatment of ^{125}I-C3(CH_3NH_2) showed only the α'-40,000 and a proportional increase in radioactivity in the 75,000 M_r β-chain peak. The α-76,000 chain appears to be composed of α'-67,000 and covalently linked C3a.

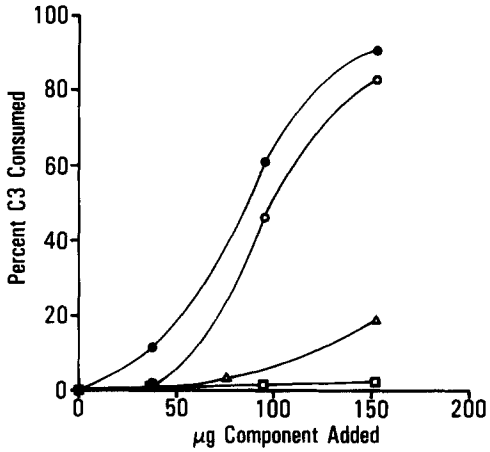

FIG. 6. C3 consumption in human serum upon addition of C3(CH_3NH_2). C3 consumption occurred via alternative pathway activation in MgEGTA containing serum after the addition of C3b (●), C3(CH_3NH_2) (○), or native C3 (△). Control, (□). Experimental details are given in Materials and Methods.

altered C3b-like C3 can form a C3 convertase in serum and, therefore, might be responsible for the initial C3b deposition that triggers alternative pathway activation, high concentrations of C3(CH_3NH_2) were added to normal human serum in which the classical complement pathway had been blocked. Fig. 6 shows that like C3b, C3(CH_3NH_2) was capable of initiating C3 consumption via the alternative pathway. Similar results were obtained with C3 treated with 1 M KSCN or subjected to repeated freeze thawing. The latter two treatments presumably resulted in hydrolysis of the thioester bond.

Formation of C3 Convertase with C3(CH_3NH_2). To investigate whether a C3 conver-

tase can be formed with C3(CH_3NH_2) as with C3b, 5 μg of C3(CH_3NH_2) was incubated with 8 μg Factor B, 2 μg activated properdin, and 0.5 μg Factor D in the presence of 2 mM Mg^{++} for 3 min at 37°C. Continued formation of convertase was blocked by addition of EDTA before the substrate, native C3, was added. C3b was used for comparison. Fig. 7 shows that C3(CH_3NH_2), like C3b, was capable of forming a C3 convertase, as evidenced by consumption of C3.

Deposition of E_SC3b by Fluid-Phase C3 Convertase that Contains C3(CH_3NH_2). To determine whether the fluid-phase C3 convertase formed by C3(CH_3NH_2) was capable of catalyzing C3b deposition onto cells and, thus, sustaining alternative pathway initiation, the following experiment was performed. Reaction mixtures were made containing 1.5×10^7 sheep erythrocytes, various amounts of native C3, and constant amounts of the preformed C3 convertase prepared with 0.5 μg of C3(CH_3NH_2). The total reaction volume was 100 μl. A parallel set of samples contained C3b instead of C3(CH_3NH_2). The reaction was allowed to proceed for 15 min at 37°C. The cells were washed and then assayed for the presence of functional C3b on their surface as described in Materials and Methods. No C3b deposition was observed in the absence of C3(CH_3NH_2) or C3b. As Fig. 8 demonstrates, in depositing C3b from the fluid phase onto the cells, the C3(CH_3NH_2) containing C3 convertase was as efficient as the C3b containing C3 convertase.

Discussion

It is known that the ability of the alternative pathway to proceed on the surface of activating particles depends entirely on the initial deposition of C3b molecules (22, 23). Deposition requires enzymatic cleavage of C3 in the fluid phase and generation of metastable C3b that is capable of binding to biological particles. Therefore, the obvious question has been: What is the nature of the initial C3 convertase? If the C3-derived subunit of this enzyme were C3b, an additional protease would have to be postulated as a constituent of the alternative pathway. It has been established that the initial C3 convertase of the alternative pathway is dependent on C3, Factors B

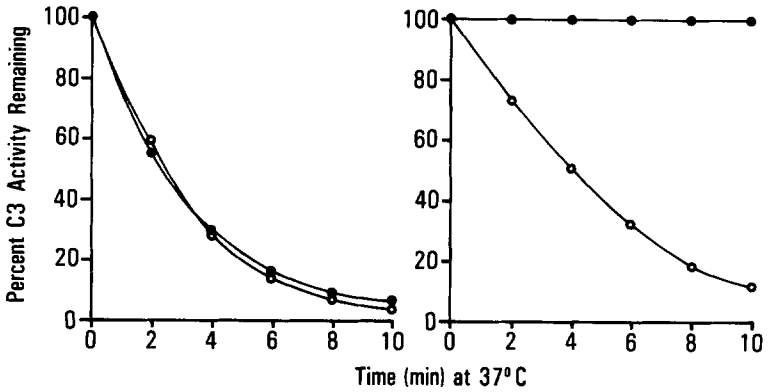

FIG. 7. Demonstration that C3(CH_3NH_2) (○, right panel) forms a functional C3 convertase with Factors B and D and properdin. For comparison, the C3 convertase formed using C3b is shown (○, left panel). C3 consumption by the preformed, C3b containing convertase was not inhibited by the addition of antibodies to C3a (●, left panel), whereas the C3(CH_3NH_2) containing convertase was completely inhibited by antibodies to C3a (●, right panel), demonstrating that only the latter convertase contains a molecule bearing the C3a domain.

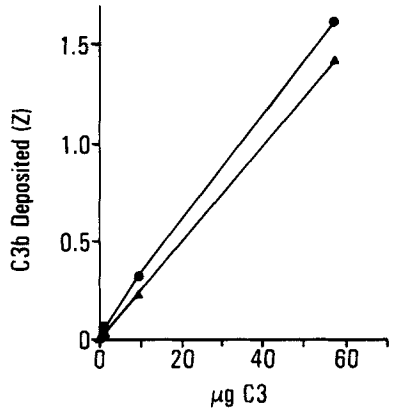

FIG. 8. Deposition of C3b on sheep erythrocytes (E_S) by a fluid-phase C3 convertase containing $C3(CH_3NH_2)$. Fluid-phase conversion of various amounts of native C3 to C3b in the presence of E_S was catalyzed by properdin(P)-stabilized C3 convertases that contained either $C3(CH_3NH_2)$ or C3b. Deposited C3b was assayed as described in Materials and Methods. ●, C3b, Bb, P; ▲, $C3(CH_3NH_2)$, Bb, P.

and D, and Mg^{++}, but not on properdin or other factors (22–25). Lachmann et al. (26–28) envisioned, as the initiating event, a spontaneous tick-over of C3 that resulted in continuous low level C3b formation in vitro and in vivo without evoking the need for exogenous activation. Fearon and Austen (29) suggested that native C3 together with Factors B and D, and possibly properdin, can form a convertase capable of generating initial C3b. It was concluded in this laboratory that native C3, rather than C3b, is the critical subunit of the initial C3 convertase because experimental evidence indicated that the enzyme was a fluid-phase enzyme and that no extraneous proteases were involved in its formation (25, 30). The ability of native uncleaved C3 to initiate formation of the initial enzyme was attributed to the probable occurrence of reversible conformers of C3 that express affinity for Factor B (2).

The observations reported in this paper strongly suggest that a heretofore unrecognized form of C3, distinct from native C3, constitutes the C3-derived subunit of the initial enzyme. Incubation of native C3 with low molecular weight nucleophilic reagents results in loss of C3 hemolytic activity (1, 8, 15, 31). In the case of methylamine (6, 7, 32), a covalent incorporation of the amine has been demonstrated. $C3(CH_3NH_2)$ has been found in this study to be functionally C3b-like, although the C3a domain of the molecule remained covalently attached. The observations are as follows: (*a*) Fluid-phase $C3(CH_3NH_2)$ was shown to compete with cell-bound C3b for binding of Factor B and β1H. (*b*) $C3(CH_3NH_2)$ was cleaved by C3bINA in the presence of β1H. Of the two α-chain products, one was shown to be larger than the analogous α'-67,000 fragment of C3b (12) by approximately the molecular weight of C3a. (*c*) Addition of $C3(CH_3NH_2)$ to human serum caused C3 consumption to a similar extent as the addition of C3b. (*d*) $C3(CH_3NH_2)$ formed a C3 convertase in the presence of isolated Factors B and D and Mg^{++}, much like C3b. Passage over a Sepharose anti-C3a column removed the convertase-forming material from $C3(CH_3NH_2)$, but not from C3b. The preformed C3 convertase prepared with $C3(CH_3NH_2)$ was inhibited by purified anti-C3a, whereas the C3b containing enzyme

was unaffected. These experiments demonstrated conclusively that the C3(CH_3NH_2)-induced C3 convertase contained a C3a-bearing form of C3.

It is known that a low-rate spontaneous consumption of C3 occurs in nonactivated plasma. It is probable that a spontaneously arising, altered form of C3 is responsible. However, a role of low molecular weight amines in the formation of the altered C3 is doubtful because of their low plasma concentration. The most effective nucleophile in plasma is probably water because its concentration is 55 M. Direct hydrolysis of the putative thioester bond in C3 may provide, under physiological conditions, a steady source for C3b-like C3. The known effects of chaotropic reagents such as KBr or KSCN on C3 hemolytic activity and physiochemical properties (33) supports the view that C3 hemolytically inactivated by water hydrolysis of the thioester bond [C3(H_2O)] may be formed in a manner analogous to C3(CH_3NH_2). Chaotropic reagents perturb the secondary and tertiary structure of proteins (34) and, in the case of C3, may facilitate the reaction of water with the putative thioester.

The hypothesis that C3 contains an intramolecular thioester has evolved from the following evidence: (*a*) Inactivation of the plasma protease inhibitor α_2-macroglobulin by methylamine results from the covalent incorporation of the reagent as the methylamide of a glutamic acid residue (32). (*b*) A covalent reaction of methylamine also occurs with C3. (*c*) Simultaneously, a free sulfhydryl group appears in C3. (*d*) Compared with C3b, it was difficult to react the sulfhydryl group in C3(CH_3NH_2) with iodoacetamide without the aid of denaturing agents, suggesting proximity of the methylamine-reactive site to the sulfhydryl group. (*e*) A peptide sequence that contains the methylamine-reactive glutamyl residue of α_2-macroglobulin has the structure: Gly-Cys-Gly-Glu-Glu*-Asn-Met- (32), where the asterisk (*) indicates the residue that contained the methylamine. (*f*) The methylamine-substituted and carboxymethylated peptide of iodoacetamide-treated C3(CH_3NH_2) exhibited an identical seven amino acid residue sequence (B. F. Tack. Personal communication). Thus, the iodoacetamide-reactive cysteinyl residue and the methylamine-reactive glutamyl residue are separated only by two amino acid residues.

Fig. 9 shows the chemical reactions at the activated carbonyl site of C3 that the present hypothesis predicts. A thioester is shown for the reasons summarized above, but the same reactions would occur whether an ester (4), amide, or carbonyl group were present in a reactive form. The sensitivity of C3 to nucleophilic attack results from the scission of the reactive bond and incorporation of the nucleophile. The methylamine product is shown. Water hydrolysis of the thioester would yield −OH in place of the methylamide. As demonstrated in this paper, nucleophilically altered C3 can form an alternative pathway C3 convertase, which proteolytically converts C3 to metastable C3b by cleavage of peptide bond 77 of the α-chain and dissociation of C3a (35). The reactive site of metastable C3b, according to the present hypothesis, is a highly reactive form of the thioester capable of undergoing transesterification with −OH groups on carbohydrate-bearing surfaces. Failing to encounter an appropriate receptive surface, metastable C3b reacts with water, forming fluid-phase C3b. These reactions explain the formation of the initial C3 convertase and the mechanism of C3b deposition required by current theories of alternative pathway activation.

Summary

The reaction of [^{14}C]methylamine with native human C3 led to the stoichiometric incorporation of methylamine, loss of hemolytic activity, and the concomitant expo-

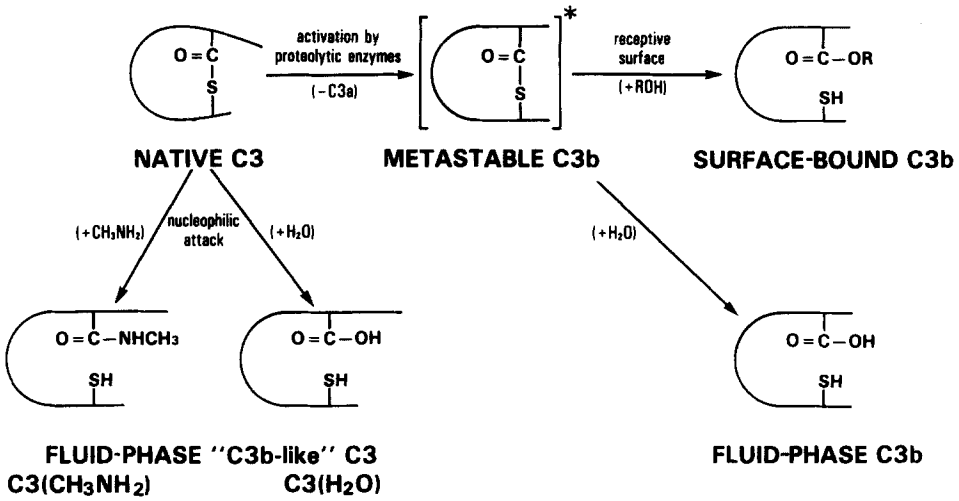

FIG. 9. Proposed chemical reactions at the activated carbonyl site of C3. Current evidence suggests that an intramolecular thioester bond in the α-chain of C3 becomes the reactive group of the metastable binding site of proteolytically produced C3b. C3 altered by nucleophilic attack or water hydrolysis of the thioester becomes functionally C3b-like without proteolytic release of C3a.

sure of a sulfhydryl group that could be labeled with [^{14}C]iodoacetamide. Both labeled sites were located in the C3d portion of the α-chain, which is known to contain the metastable binding site of C3b.

The methylamine-modified C3 [C3(CH_3NH_2)] was shown to exhibit many of the functional properties of C3b, although the C3a portion of the molecule remained covalently attached. C3(CH_3NH_2) bound Factor B and β1H, and could be cleaved by C3b inactivator in the presence of β1H. C3(CH_3NH_2) added to human serum caused activation of the alternative pathway and consumption of C3. In presence of Factors B and D and Mg^{++}, C3(CH_3NH_2) formed a C3 convertase. The convertase-forming material could be removed from solution by anti-C3a Sepharose and the preformed convertase was completely inhibited by purified antibody to C3a. This antibody did not affect the function of the C3 convertase that contained C3b. Similar functional properties were exhibited by C3 exposed for short periods of time to relatively low concentrations of chaotropic reagents, such as KSCN or guanidine. These results suggest that the initial C3 convertase of the alternative pathway may be formed from native C3, without proteolysis, by the attack of a variety of nucleophiles including water. The C3 convertase formed from this altered C3 then generates by proteolytic cleavage the initial metastable C3b that is capable of attaching to receptive surfaces. Conversion of C3 to C3b exposes one sulfhydryl residue as does modification of C3 with methylamine. When the C3d portion of C3b bound to zymosan particles via the metastable binding site was treated with radiolabeled methylamine, the fragment was released from the particles in radiolabeled form. These findings are consistent with the concept that native C3 contains an active carbonyl group, probably in the form of a thioester, which can either react with water to form functionally C3b-like C3 or, upon enzymatic conversion of C3 to C3b, allows C3b to form an ester bond with hydroxyl groups on the target surface.

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