FORMATION OF THE INITIAL C3 CONVERTASE

OF THE ALTERNATIVE COMPLEMENT PATHWAY

Acquisition of C3b-like Activities by Spontaneous
Hydrolysis of the Putative Thioester in Native C3*

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We present evidence suggesting that nonenzymatic, spontaneous hydrolysis of a thioester bond in native C3 constitutes the initial event in the activation of the alternative pathway.

It has been known since the original description of C3 that the hemolytic activity of the isolated protein decays spontaneously in aqueous solution at a slow rate (1). This decay was greatly accelerated by chaotropic agents (2). It has also been known that treatment of C3 with simple amines such as hydrazine abolished its hemolytic activity (3). Recently, evidence has accumulated that strongly supports the existence of an intramolecular thioester bond in native C3 (4–8). Modification of the thioester by incorporation of 1 mol of methylamine destroyed the potential of C3 to bind to biological targets of complement, induced conformational changes in the molecule (9) and led to the expression of functional binding sites for complement proteins resembling those of enzymatically generated C3b (4, 10). Methylamine-modified C3 $[C3(CH_3NH_2)]^1$ was shown (4) to bind Factor B and properdin and was susceptible to cleavage and inactivation by Factors H and I.

The present study was performed to investigate the spontaneous decay of the binding potential of native C3 and to determine whether hydrolysis of the thioester bond in C3 generates, without any further chemical or enzymatic modification, C3b-like functional properties. Because chaotropic agents facilitate access of water to relatively concealed groups by perturbing the tertiary structure of proteins, these agents were used to accelerate the spontaneous decay of C3. Chaotrope-treated C3 that has acquired C3b-like functions on the basis of this treatment will be tentatively designated $C3(H_2O)$. This denotation proposes that this form of C3 contains, instead of the thioester bond, a free sulfhydryl and carboxyl group.

[1] *Abbreviations used in this paper:* $C3(CH_3NH_2)$, C3 hemolytically inactivated by incorporation of methylamine; $C3(H_2O)$, C3 hemolytically inactivated by hydrolysis of the thioester bond; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); SDS, sodium dodecyl sulfate; SH, sulfhydryl; VBS, veronal-buffered saline.

Materials and Methods

Purified Proteins. C3 (11), Factor B (12), Factor D (13), Factor H (14), Factor I (14), properdin (15), and nephritic factor (16) were purified from human plasma or serum as previously described. L-(Tosylamido 2-phenyl)ethyl chloromethyl ketone-treated trypsin was obtained from Worthington Biochemical Corp., Freehold, N. J. Soybean trypsin inhibitor was obtained from Sigma Chemical Co., St. Louis, Mo.

Reagents. Organomercurial substituted agarose (Affi-Gel 501) and dithiothreitol were purchased from Bio-Rad Laboratories, Richmond, Calif. Guanidine (grade I), Trizma base (Tris), methylamine, and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical Co. Sodium dodecyl sulfate (SDS) and iodoacetamide were purchased from Pierce Chemical Co., Rockford, Ill. KSCN and urea were purchased from Mallinckrodt, Inc., St. Louis, Mo.

Buffers. Buffers used were: sodium veronal (10 mM) -buffered saline (140 mM), pH 7.3, (VBS); VBS containing 0.1% gelatin; GVB containing 10 mM EDTA; sodium phosphate (10 mM) -buffered saline (140 mM), pH 7.3; Tris (10 mM) -buffered saline (140 mM), pH 7.3.

Preparation of Modified Forms of C3. Native C3 was prepared from isolated C3 (11) by passage over organomercurial agarose. This C3 was used for all of the following modified C3 preparations and each had a final C3 concentration of 0.5 mg/ml. C3b was prepared by treatment with 5 μg/ml trypsin (3 min, 37°C), followed by 10 μg/ml soybean trypsin inhibitor. C3 was rendered hemolytically inactive by freezing (−35°C) and thawing (21°C) slowly eight times. C3 was treated with 0.33 M KSCN, 0.8 M guanidine, 2.8 M urea, or 0.1 M methylamine at pH 7.3 in VBS for 60 min at 37°C and then dialyzed against VBS.

Preparation of Affinity-purified Anti-C3a. Antiserum was raised to purified human C3a (17) in a goat. Affinity-purified anti-C3a was prepared by passage of the antiserum over a Sepharose column containing covalently attached, purified C3a (4). The bound antibody was eluted with 0.1 M glycine-HCl, pH 2.3.

Electrophoresis. Electrophoresis in the presence of SDS was performed in 9% polyacrylamide gels using the Canalco SAGE electrophoretic system from Miles Laboratories, Inc., Elkhart, Ind. Immunoelectrophoresis was performed in 1.2% agarose and a buffer containing Tris (38 mM), glycine (100 mM), and EDTA (10 mM) at pH 8.9.

Assays. C3 assays were performed by effective molecule titration described previously (18). The activity of the fluid-phase nephritic factor-stabilized C3 convertase was determined by the rate of C3 consumption at 37°C. Sulfhydryl titrations using Ellman's reagent (DTNB) were performed in 50 mM sodium phosphate, pH 7.4, using a Cary 219 spectrophotometer (Varian Associates, Inc. Palo Alto, Calif.). The reaction of DTNB with free sulfhydryl groups was followed at 412 nm and calculations used the reported extinction coefficient, 1.36×10^4 M^{-1} cm^{-1} (19).

Results

Rate of Spontaneous Decay of C3 Hemolytic Activity. As demonstrated in Fig. 1, native C3 slowly loses hemolytic activity when incubated at 37°C. The rate was determined to be between 0.2 and 0.4%/h (Table I) in the presence or absence of metal ions, azide, or the protease inhibitor benzamidine. The rate was significantly increased in the presence of 0.1 M imidazole, but it is not clear whether imidazole acts nucleophilically or by another mechanism. In the presence of physiological concentrations of Factors B, D, and Mg^{++}, formation of C3 convertase was uncontrolled and all C3 hemolytic activity was lost in less than 2 min. The presence of Factors H and I, however, provided an efficient control system and a mixture containing physiological concentrations of all five components (C3, B, D, H, and I) exhibited a rate of C3 inactivation of 1%/h (Fig. 1).

Enhancement of Spontaneous Decay of C3 Hemolytic Activity by Chaotropic Agents. Fig. 2 documents the effect of low concentrations of KSCN on C3 hemolytic activity. In the presence of 0.33 M KSCN, the rate of C3 inactivation was 6.25%/min compared with
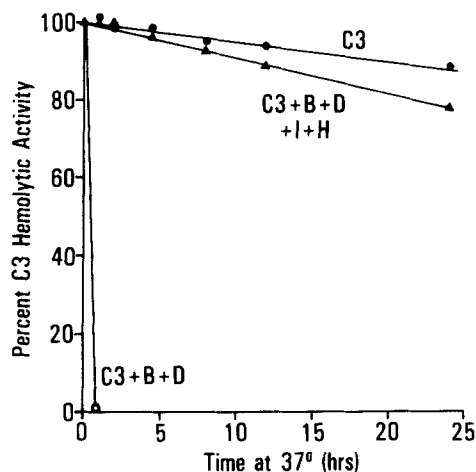
Fig. 1. The rate of inactivation of C3 hemolytic activity in water. Hemolytic activity was determined after long-term incubation at 37°C in VBS containing 0.5 mM magnesium. C3 was incubated alone (●) at physiological concentration (1,200 μg/ml) or in the presence of Factors B (200 μg/ml) and D (2 μg/ml) (○). Incubations containing the complete five-protein system (▲) were prepared by inclusion of physiological concentrations of the control proteins C3b inactivator (Factor I, 34 μg/ml) and β1H (Factor H, 465 μg/ml) in the mixture containing C3 and Factors B and D.

TABLE I

Rate of Loss of C3 Hemolytic Activity during 66 Hours at 37°C in Water

Buffer	Activity loss
	%/h
VBS, Mg^{++}	0.4
VBS, Mg^{++}, Ca^{++}, N_3^-	0.2
VBS, Mg^{++}, Ca^{++}, N_3^+, benzamidine	0.2
VBS, EDTA, N_3^-, benzamidine	0.3
VBS, EDTA, N_3^-, benzamidine, imidazole	0.8

Concentrations: C3, 1.2 mg/ml; Mg^{++} alone, 1.5 mM; Ca^{++} and Mg^{++}, 0.15 and 0.5 mM, respectively; EDTA, 10 mM; sodium azide, 0.1%; benzamidine, 10 mM; imidazole, 0.1 M. In the experiment with magnesium alone, the last sample analyzed was taken after only 24 h.

a rate of 0.005%/min in buffer (Table I). The rate of inactivation was therefore 1,250-fold enhanced under these conditions. Similar effects were seen with nonionic (urea) and cationic (guanidine) chaotropic agents (Fig. 3). The fact that all three chaotropes had similar effects suggests that inactivation was not the result of a specific sensitivity of C3 to a particular agent. The low concentrations found to be effective do not usually cause irreversible denaturation of secondary or tertiary protein structure.

Synchronous Appearance of a Sulfhydryl (SH) Group in C3 with Loss of C3 Hemolytic Activity During Treatment with Chaotropic Agents.   It has previously been shown (4, 6, 8) that the reaction of C3 with methylamine results in the simultaneous loss of hemolytic activity and the incorporation of 1 mol of methylamine per mol of C3. This reaction has been shown to expose a titratable SH group. Fig. 4 demonstrates that inactivation by methylamine and exposure of a single SH group are simultaneous events. Treatment of native C3 with KSCN to enhance the spontaneous decay resulted in the simultaneous appearance of SH and loss of hemolytic activity (Fig. 5). Although the
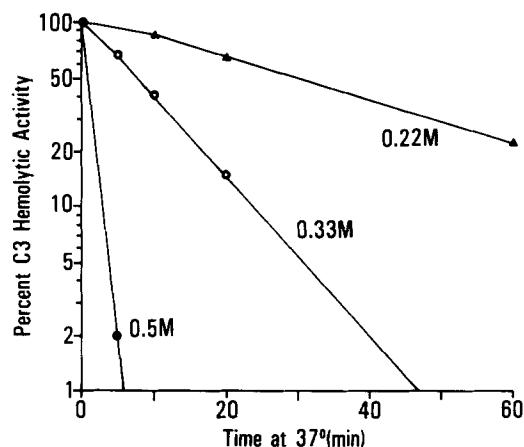
FIG. 2.   Enhanced inactivation of C3 in the presence of the chaotropic agent KSCN. Native C3 was incubated with 0.22 M (▲), 0.33 M (O), or 0.5 M (●) KSCN in VBS at 37°C. Samples were removed at various times, diluted 100-fold in cold VBS containing gelatin and EDTA, and assayed for C3 hemolytic activity.
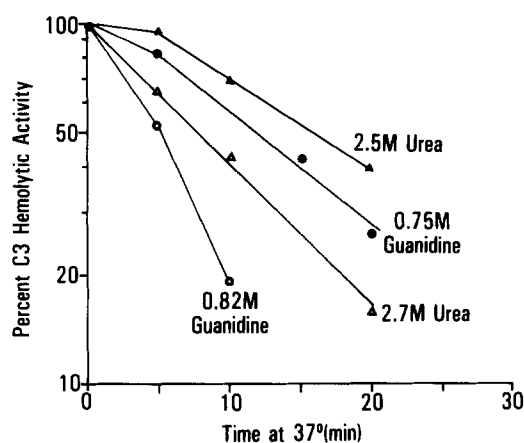

FIG. 3.   Enhanced inactivation of C3 in the presence of urea or guanidine. Native C3 was incubated with 2.5 M urea (▲), 2.7 M urea (△), 0.75 M guanidine (●), or 0.82 M guanidine (O) in VBS at 37°C. Samples were removed at the indicated times, diluted 100-fold in cold VBS containing gelatin and EDTA, and assayed for C3 hemolytic activity.

maximum exposure of SH per mol C3 was less than 1 when a relatively high concentration of KSCN was used (0.5 M), the kinetics of inactivation and SH appearance were identical, i.e., 50% inactivation corresponded to exposure of half of the maximum amount of SH observed. Table II compares the hemolytic activity and free SH content of C3 subjected to six different treatments that result in loss of C3 hemolytic activity. Proteolytic conversion of C3 to C3b liberated 1 mol SH per mol C3b. Repeated freezing and thawing also exposed 1 mol SH per mol C3. Low concentrations of KSCN or guanidine liberated 0.75 and 0.72 mol SH per mol protein, respectively. Treatment with urea led to inactivation of hemolytic activity but no SH appearance, possibly because of further denaturation.

Separation of Native C3 from Hemolytically Inactive, SH-containing C3 Derivatives on Organomercurial Agarose.   The majority of the hemolytically inactive forms of C3
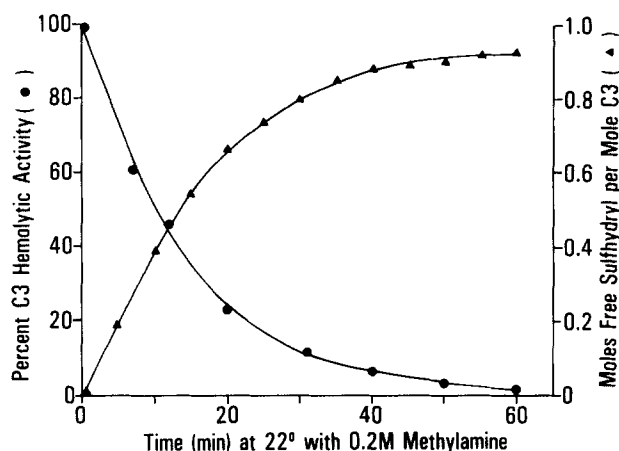
FIG. 4. Synchronous appearance of a free SH group in C3 and inactivation of hemolytic activity during incubation with methylamine. C3 (1 mg/ml) was incubated with 100 mM methylamine in 50 mM sodium phosphate buffer, pH 8.0, at 22°C. The reaction with DTNB (14 μM) was followed continuously at 412 nm. Aliquots were removed at the indicated times, diluted 100-fold in cold VBS containing gelatin and EDTA, and assayed for C3 hemolytic activity.
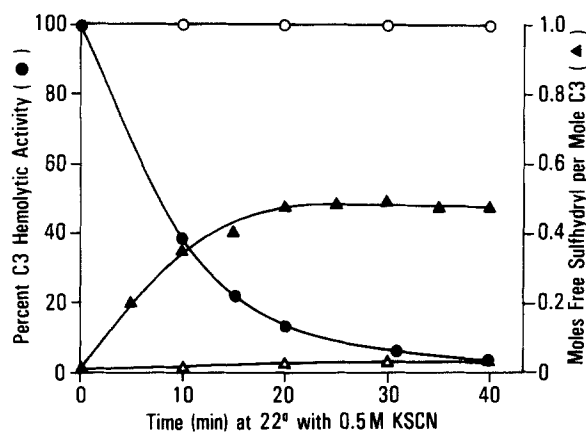

FIG. 5. Appearance of a free SH group during chaotrope-accelerated hemolytic inactivation of C3. C3 (1 mg/ml) was incubated with 0.5 M KSCN in 50 mM sodium phosphate buffer, pH 7.4, at 22°C (closed symbols). The reaction with DTNB (14 μM) was followed continuously at 412 nm. Aliquots were removed at the indicated times, diluted 100-fold in cold VBS containing gelatin and EDTA, and assayed for C3 hemolytic activity. The control experiment (open symbols) lacking KSCN was performed in a similar manner.

generated during purification, storage, freezing and thawing contain a single free SH group. Complete removal of this material from hemolytically active, native C3 was achieved by passage of the protein through agarose bearing a covalently coupled p-mercurianiline derivative.[2] Native C3 was not bound to the organomercurial agarose. It eluted immediately behind a small amount of inactive, aggregated C3 (Fig. 6). The specific hemolytic activity of the C3 sample applied to the column had decreased during storage at −35°C to 23% of that of native C3. The specific activity of the

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[2] Two forms of organomercurial agarose are commercially available. Affi-Gel 501 from Bio-Rad Laboratories, has a spacer six atoms longer than the product from Sigma Chemical Co., and only the Bio-Rad material binds SH-containing derivatives of C3.

TABLE II

Hemolytic Activity and Free SH Content of Freeze-thawed or Chaotrope-treated C3*

	C3 (Native)	C3b	C3 (Freeze-thawed)	C3 (KSCN)	C3 (Guana-dine)	C3 (Urea)	C3 (CH₃NH₂)
Hemolytic activity‡	95	0	6	0	0	0	0
Moles SH/mole C3§	0.15	1.01	1.05	0.75	0.72	0.13	0.90

* Preparation of the various forms of C3 is described in Materials and Methods.
‡ Percent hemolytic activity compared with C3 in normal human serum.
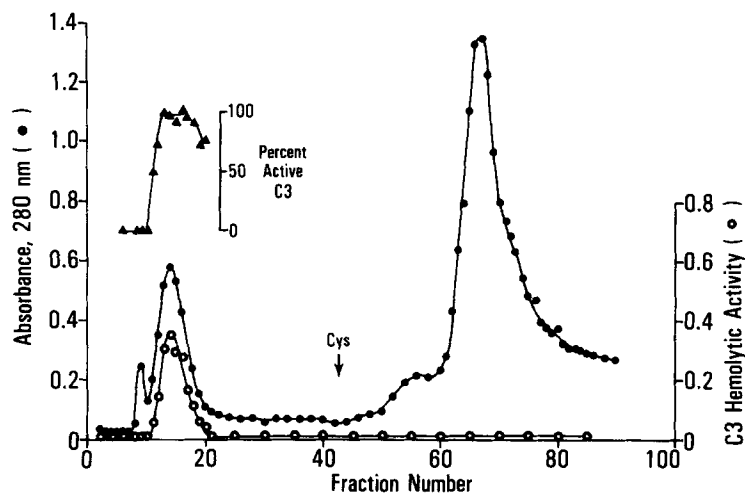§ Measured by spectral change at 412 nm after treatment with DTNB.

FIG. 6.   Separation of native C3 from SH-containing, hemolytically inactive forms of C3 and C3b by chromatography on organomercurial agarose. A 1 ml sample of C3 (10 mg) that had become 77% inactive during storage at −35°C was applied to a 1.5- × 18-cm column of organomercurial agarose (Affi-Gel 501, Bio-Rad) equilibrated in VBS. The column was washed with 2 column vol of VBS followed by 2 vol of VBS containing 10 mM cysteine. All of the applied hemolytic activity eluted just behind a small peak of inactive material gel filtering in the breakthrough. The specific hemolytic activity of the native C3 peak was 97% of the activity of C3 in fresh normal human serum and this peak contained 26% of the applied protein. The three peaks of protein were examined by polyacrylamide gel electrophoresis in SDS and exhibited the characteristic α and β chain pattern of native C3. The cysteine-treated column was regenerated by treatment with 10 mM HgCl₂ according to the manufacturer's directions.

eluted material was that of native C3 and all of the applied activity was recovered. Binding of inactive C3 must have occurred via the free SH group because cysteine eluted the bound material and pretreatment of the protein with iodoacetamide prevented binding. Organomercurial agarose also binds other SH-containing C3 derivatives, such as C3(CH₃NH₂), C3b, C3bi, and C3d.

Demonstration of C3b-like Functions of C3(H₂O) Generated with Chaotropic Agents. C3 altered by chaotropic agents, by freezing and thawing, or by methylamine treatment acquired both structural and functional properties similar to proteolytically generated C3b. Fig. 7 shows that C3 modified by repeated freezing and thawing or treatment with KSCN, guanidine, or methylamine was capable of forming an alternative pathway C3 convertase. Although the enzyme could be formed with factors B and D and magnesium alone, the presence of properdin or nephritic factor facilitated its
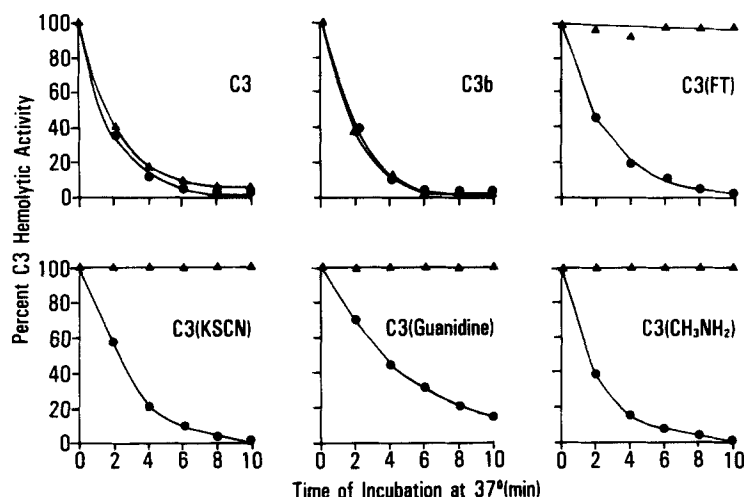
FIG. 7.   Formation of C3 convertase by chaotrope-treated and freeze-thawed C3 and inhibition of the convertases by antibodies to the C3a domain. The time-course of C3 consumption by the preformed convertases is shown. The enzymes were formed by incubating C3 (treated as indicated) with Factors B and D and nephritic factor in the presence of 0.5 mM $MgCl_2$ for 3 min at 37°C. Further enzyme formation was blocked by the addition of EDTA. C3b and native C3, because of rapid conversion to C3b, were not affected by subsequent addition of antibodies to C3a (▲), whereas the other convertases were inhibited. This inhibition demonstrates that these convertases contain molecules bearing the C3a domain.

demonstration. In each case, the enzyme was allowed to form during a 3-min incubation period after which EDTA was added to prevent subsequent enzyme formation. Where anti-C3a was used, it was added together with the EDTA. Substrate C3 was then added to the reaction mixture and samples were withdrawn at various times for analysis of C3 consumption. The activity of the preformed enzyme could be inhibited by antibodies specific for the C3a domain in every case except C3b and native C3. C3b was not affected because it does not contain the C3a domain. Native C3 was not affected because it was rapidly converted to C3b. Conversion was probably initiated by the C3 convertase formed from $C3(H_2O)$, traces of which are always present in native C3. Thus the prevalent enzyme measured was the C3b-containing C3 convertase. On the other hand, $C3(H_2O)$ is very slowly cleaved by C3 convertases (4, 5, 10, 20), and thus it is not converted to C3b and retains the C3a domain. C3 inactivated in the presence of 2.8 M urea did not form a detectable amount of C3 convertase.

Susceptibility of $C3(H_2O)$ to Cleavage by Factors H and I.   Fig. 8 demonstrates that $C3(H_2O)$ prepared by various treatments exhibits the α and β chain structure of native C3. Treatment with Factors H and I (previously called $\beta1H$ and C3b inactivator) does not affect the structure of native C3 but it results in cleavage of the α' chain of C3b into two fragments of 67,000 and 40,000 mol wt (α'-67 and α'-40) (14). This cleavage abolishes all of the activities of C3b. In contrast, $C3(H_2O)$ prepared by freezing and thawing or by KSCN or guanidine treatment was sensitive to Factors H and I, but the fragmentation pattern was different from that of C3b. Cleavage yielded fragments of 76,000 and 40,000 mol wt (α-76 and α-40), indicating that an intact α chain bearing the amino terminal 9,000 mol wt C3a domain had been cleaved. A trace of these fragments was seen in the native C3 preparation, probably
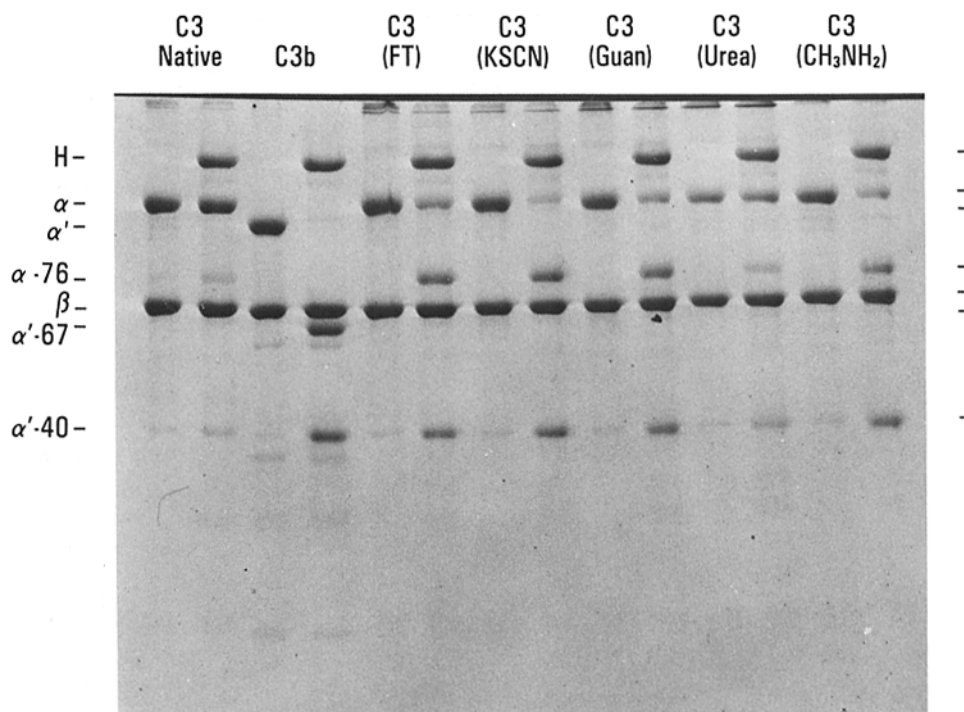
FIG. 8.   Gel electrophoresis in SDS of C3, C3b, and C3 hemolytically inactivated by freeze-thaw (FT), chaotropic agents, and methylamine. Two samples (10 μg in 60 μl) of each protein are shown, the second was treated for 30 min at 37°C with C3b inactivator (Factor I, 17 μg/ml) and β1H (Factor H, 33 μg/ml). Treatment of C3b with Factors I and H yielded two fragments of the α' chain with molecular weights of 67,000 and 40,000 (α'-67 and α'-40). Cleavage of other hemolytically inactive preparations of C3 yielded α-76 and α-40 fragments, indicating a susceptibility to Factors I and H in spite of the presence of the C3a domain on the amino terminal of the α'-67 fragment. This result has been interpreted as an indication that a C3b-like conformation was acquired without peptide bond scission during the various treatments.

because it had been frozen and thawed once before electrophoresis. Although all preparations contained a small proportion of protein that was resistant to cleavage, the urea-treated sample was the most resistant, which is consistent with the failure of this material to form a C3 convertase. In view of these results, it may be concluded that C3(H2O) resembles C3(CH3NH2) with respect to all C3b-like functions, including susceptibility to attack by Factors H and I.

Discussion

This investigation (a) examines the spontaneous transition of C3 to C3b-like C3, (b) describes procedures to accelerate this transition, (c) presents evidence that the alteration is due to thioester hydrolysis, and (d) demonstrates that the product is capable of interacting with alternative pathway components to form a functional C3 convertase. These observations support the hypothesis (4) that C3(H2O) is generated spontaneously in plasma and forms a C3 convertase that proteolytically produces metastable C3b capable of binding covalently to biological particles.

The spontaneous decay of C3 hemolytic activity in buffer was found to proceed at a rate of 0.2–0.4%/h at 37°C. In the presence of Factors B and D, consumption of C3

was complete in 2 min because of uncontrolled formation of fluid-phase C3 convertase. The presence of physiological concentrations of the control proteins, Factors H and I, prevented rapid amplification but allowed C3 consumption to increase to 1%/h. Low concentrations of chaotropic agents enhanced the rate of spontaneous C3 decay, which correlated with the rate of SH group liberation. The results support the conclusion that treatment with chaotropes caused the hydrolysis of the thioester in native C3 (21). The proof that water is in fact incorporated into native C3 during conversion to the functionally C3b-like form must await the demonstration that oxygen-18 is incorporated from $H_2^{18}O$.

Native C3 could be separated from $C3(H_2O)$ and C3b by chromatography on organomercurial-derivatized agarose. This method takes advantage of the presence of a free SH group in $C3(H_2O)$ and C3b. The method has several advantages over published methods that employ ion-exchange chromatography to separate inactive from native C3 (5, 20). The present method differs from other procedures in that native C3 is not adsorbed to the column, allowing yields approaching 100%. The column size has been scaled down to process microgram quantities of protein and the elution of native C3 required only minutes. This method should also be applicable to C4 and α_2-macroglobulin, which probably contain similar thioester moieties (22, 23).

The exploration of the effect of chaotropes on C3 properties was prompted by the previous finding (4) that $C3(CH_3NH_2)$ exhibited C3b-like functional characteristics, including the ability to form a fluid-phase C3 convertase with Factors B, D, and P. The modified protein was susceptible to cleavage and inactivation by Factors H and I. Further, the $C3(CH_3NH_2)$-containing fluid-phase enzyme was capable of depositing C3b on the surface of receptive particles. A kinetic analysis of the binding of CH_3NH_2 and the resulting conformational transition and appearance of functional sites showed the following sequence of events (9). Although methylamine binding and metastable binding-site inactivation were rapid ($t_{1/2}$, 5.5 min), the conformational rearrangement of the modified protein was markedly slower. The appearance of the binding site for Factor B was also slower than CH_3NH_2 binding ($t_{1/2}$, 25 min) and the appearance of susceptibility to cleavage by Factors H and I was even further delayed ($t_{1/2}$, 35 min). These observations suggest that the fluid-phase C3 convertase may be formed before the modified protein is fully subject to control.

It was hypothesized previously (4) that an analogous product of C3, $C3(H_2O)$, is formed by spontaneous hydrolysis of the internal thioester. The present results support this hypothesis and suggest that the initial enzyme of the alternative pathway is formed from spontaneously arising $C3(H_2O)$. As demonstrated in this study, $C3(H_2O)$ possesses the functional properties of C3b and, like fluid-phase C3b, has lost its potential to bind to biological particles. It is proposed, therefore, as schematically presented in Fig. 9, that the initial event in the alternative pathway is the spontaneous generation of $C3(H_2O)$, which is formed continuously at a slow rate. Due to the differential rate of appearance of binding sites during the transition to the C3b-like form of the molecule, the enzyme $C3(H_2O),Bb$ may form and generate metastable C3b before the convertase becomes fully susceptible to control by Factors H and I. Deposition of C3b onto biological particles permits the discriminatory ability of C3b to be expressed. On nonactivators of the alternative pathway, it is rapidly degraded by Factors H and I. On activators, the affinity of C3b for Factor H is reduced and
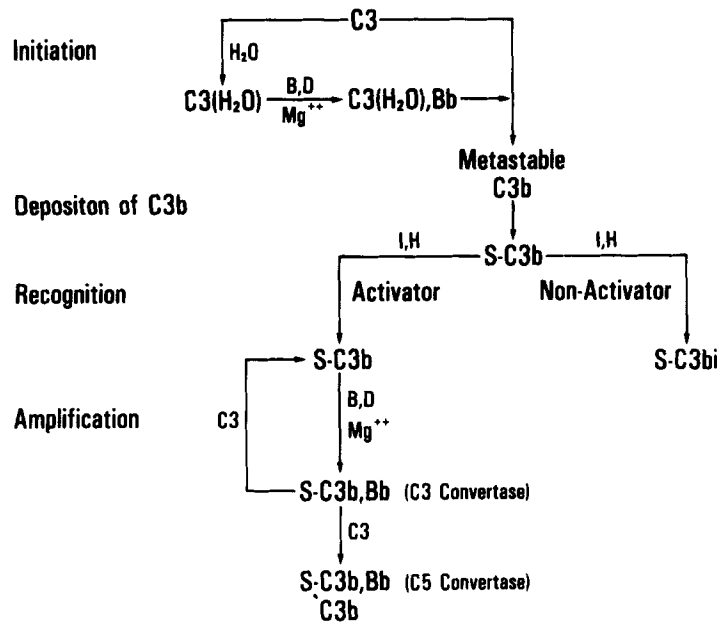
Fig. 9. A schematic representation of the proposed molecular dynamics of alternative pathway activation described in the text. Initiation results from spontaneous hydrolysis of the putative thioester in native C3, which produces a C3 molecule with C3b-like functional properties. The resulting C3 convertase [C3(H₂O),Bb] generates metastable C3b. Deposition of metastable C3b occurs randomly from the fluid phase, but only on activators does the bound C3b escape control sufficiently to mediate feedback amplification, additional C3b deposition, and C5 convertase formation.

degradation is sufficiently retarded to allow C3 convertase formation, amplification, and C5 convertase assembly.

The recognition of C3(H₂O) as a heretofore unknown functional form of C3 and the observation that C3(H₂O) arises spontaneously may resolve the long-standing question as to the manner in which the alternative pathway is initiated (24–27).

Summary

Activation of the alternative pathway of complement commences with the formation of an initial fluid-phase C3 convertase. Treatment of C3 with the nucleophilic reagent methylamine has previously been shown to result in the cleavage of an intramolecular thioester bond and to induce C3b-like properties, including the ability to form a fluid-phase C3 convertase. This report examines the hypothesis that spontaneous hydrolysis of the thioester generates a derivative of C3 that is responsible for the formation of the initial C3 convertase of the alternative pathway. The rate of spontaneous decay of C3 hemolytic activity in buffer was found to be between 0.2 and 0.4%/h. In the presence of other alternative pathway proteins, the rate of inactivation was 1%/h. The rate of spontaneous inactivation was greatly accelerated by low concentrations of chaotropic agents such as KSCN or guanidine. Liberation of a sulfhydryl group, not present in native C3, correlated with loss of hemolytic activity, indicating that exposure to chaotropic agents resulted in thioester hydrolysis. Unlike native C3, C3 bearing a single reactive sulfhydryl group was capable of

generating fluid-phase C3 convertase with Factors B, D, and P and was cleaved by Factor I (C3b inactivator) in the presence of Factor H (β1H). The fragmentation patterns indicated that the C3a domain was covalently associated with the functionally C3b-like C3. Organomercurial agarose was employed for the rapid removal of sulfhydryl-bearing, hemolytically inactive forms of C3 and C3b from native hemolytically active C3.

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