Structural Requirements for Thioester Bond Formation in Human Complement Component C3

REASSESSMENT OF THE ROLE OF THIOESTER BOND INTEGRITY ON THE CONFORMATION OF C3*

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Lourdes Isaac‡ and David E. Isenman§

From the Department of Biochemistry, University of Toronto, Toronto, Ontario M5S 1A8, Canada

A unique thioester bond, formed between the side chains of neighboring C and Q residues, is present in complement components C3 and C4 and the protease inhibitor α2-macroglobulin. This structure is essential for mediating covalent attachment to target acceptors and also for maintaining these proteins in their native conformation. An examination of the residues in the immediate vicinity of the C and Q reveals a very high degree of sequence similarity among the three proteins which crosses species barriers. The following is the sequence flanking the thioester residues in C3, the highly conserved amino acids being underlined and the the thioester-forming residues being indicated by italics: 1008S-V-T-P-S-G-C-G-E-Q-N-M-I-G-M-T-P-T1021. Through a site-directed mutagenesis and cDNA expression approach, we have examined the importance of the conserved amino acids in the formation, stability, and function of the thioester bond in C3. The behavior of the mutants fell into three categories. The potential loss in peptide backbone flexibility by the replacement of Q1009 by A or S was permissive to thioester formation and function as was replacement of M1015 by the still fairly bulky residue F. In contrast, replacement of M1015 by A resulted in an α-chain which was highly unstable toward proteolytic degradation. The third category, which included mutant molecules P1007G, P1020G, E1012Q, and Q1013N, displayed an unusual phenotype in which both the autolytic fragmentation and the hemolytic activity characteristics of thioester-intact molecules were absent. However, like their wildtype counterparts, these molecules retained the ability to be cleaved by C3 convertase (C4b2a), a conformation-dependent property that is normally lost in the conversion of native C3 to thioester-hydrolyzed C3(H2O). Since an identical functional profile was obtained when the thioester was deliberately prevented from forming in the mutant C1010A, we conclude that if a stable thioester fails to form during biosynthesis, at least parts of the mature protein can adopt a more native-like conformation than is the case when the thioester is first formed and then hydrolyzed in the mature protein. In view of these new findings, the interpretation of the previously observed correlation between the loss of thioester integrity and the adoption of a C3b-like conformation must be reassessed.

Proteolytic activation of C3 and C4 to C3b and C4b by their respective complement pathway convertases results in the exposure of an intramolecular thioester bond formed between a cysteinyl sulfhydryl and a glutaminyl γ-carbonyl, these amino acids being separated by only 2 residues (Gly and Glu) in the primary structure (reviewed in Tack, 1983). The reactive carbonyl group of the thiolactone ring can serve as an acyl donor to hydroxyl or amino group nucleophiles on a target surface, thereby covalently anchoring these molecules to the surface via ester or amide linkages, respectively. The transient ability of nascently activated complement components C3 and C4 to covalently attach to the surface of the target which bears the activating enzyme, while losing this attachment potential within milliseconds as a result of water hydrolysis of the exposed thioester in the course of diffusion through the aqueous milieu, provides an important control point in the complement cascade. This mechanism effectively limits complement attack to the activating substance, while sparing host tissue from the deleterious effects of complement activation.

An identical intrachain thioester structure is also present in the serum protease inhibitor α2-macroglobulin (α2-m)1 and in a similar manner is responsible for mediating the covalent binding of α2-m to the activating protease (Howard et al., 1983). As an indication of the high reactivity of the thioester carbonyl in C3, C4, and α2-m, heating of these proteins in the presence of denaturant will cause a portion of the molecules to undergo an autolytic cleavage reaction. In this reaction the peptide bond nitrogen of the glutamine contributing the carbonyl of the thioester attacks its own γ-carbonyl, leading to cyclization (pyrrolidine carboxylic acid formation), cleavage of the thioester bond, and concomitant scission of the peptide bond (Sim and Sim, 1983).

C3, C4, and α2-m are all synthesized as 190-kDa precursor polypeptides and alignment of their primary structures reveals significant sequence identity, especially within the thioester region. Based on these alignments, it has been suggested that the three proteins have evolved from a common ancestral
gene (Sottrup-Jensen et al., 1985). In addition to their common covalent attachment mechanism, a further similarity among the three proteins is the role that the thioester bond plays in the maintenance of their respective native conformational states. Specifically, when the thioester bond is cleaved, even in the absence of proteolysis, a major conformational transition ensues (Isenman, 1983; Straight and McKee, 1983). The thioester bond may be cleaved in the absence of proteolysis by either direct attack by the small hydrophobic nucleophile CH3NH2 or by chaotropes such as KBr which are thought to "loosen" the folding of the macromolecule sufficiently to allow solvent water molecules to nuclophilically attack the thioester carbonyl. Whereas thioester hydrolysis resulting from either proteolytic activation or chaotrope treatment gives rise to a rapid conformational change in C3 and C4, aminolysis by CH3NH2 produces a very similar conformational end state, but at a much slower rate (Isenman et al., 1981; Isenman and Kells, 1982). For both complement proteins the conformational change is accompanied by a loss in the ability of the authentic convertases (C1s for C4 and C4b2a for C3) to remove their respective activation peptides (Janatova and Tack, 1981; Janatova et al., 1989). On the other hand, the conformational change in all three proteins results in the exposure or formation of binding sites for other serum macromolecules or blood cell receptors (Isenman, 1983; Pangburn and Müller-Eberhard, 1983, Gonzias and Pizzo, 1983). This is of particular physiologic importance in the case of C3, since its thioester-hydrolyzed form, C3(H2O), adopts a conformation that is capable of binding factor B (Pangburn and Müller-Eberhard, 1983), a prerequisite for cleavage of the latter by factor D. The spontaneous hydrolysis rate of C3 under physiologic solvent conditions and temperature is about 1%/h and thus, the C3b-like properties of C3(H2O) provide for continuous low level initiation of the alternative pathway of complement activation (Pangburn and Müller-Eberhard, 1983). On the other hand, C3(H2O) is not present indefinitely since, like C3b, it displays affinity for factor H and the C3(H2O)-H complex can then be cleaved by factor I into IC3(HO). It is interesting that the thioester bond plays a similar role in maintaining the native precursor states of C3, C4, and αm despite significant differences in post-translational chain processing and association of subunits. Thus, our previous studies on the conformational role of the thioester bond in C3 and C4, and those of others on αm, have prompted the suggestion that this bond is essential for maintaining an energetically unfavorable conformational state in the native proteins.

Given the apparently energetically unfavorable nature of the conformation which it stabilizes, the question arises as to how the thioester bond forms during biosynthesis of C3, C4, and αm. Karp (1983) has shown that the thioester is formed in mouse C4 prior to proteolytic processing of the single chain proenzyme into the mature three-chain form, but probably after core N-glycosylation. Recently, Auerbach et al. (1990) have proposed the involvement of an accessory molecule in the formation of the thioester bond in guinea pig C3. Their data suggest that the accessory molecule mediating thioester bond formation in C3 is distinct from the one(s) mediating thioester formation in C4 and αm. Nevertheless, even with the potential involvement of accessory molecules, one would expect that the amino acids flanking the Cys and Gln (whose side chains form the thioester) would play a crucial role in: (a) the packing of side chains resulting in the water exclusion presumably necessary for thioester formation; (b) the relative stability of the thioester toward spontaneous hydrolysis in the native molecule; and (c) its transacylation reactivity upon exposure to the external aqueous milieu. Fig. 1 is a compilation of amino acid sequences flanking the thioester bond in C3, C4, and αm in various species including, in the case of αm, three of invertebrate origin. Beside the obvious conservation of Cys-1010 and Gln-1013 which actually form the thioester bond, there are 5 other residues, indicated in boldface, which are either absolutely conserved, or for which there is only one variation among these sequences. Given that some of these conserved amino acids are ones which are uniquely able to impart certain structural or functional properties, we were interested in determining their relative importance. For example, is the rigidity imparted by prolines at positions 1007 and 1020 required for thioester formation and function? Similarly, the conservation of glycine at a given position may be indicative of a requirement for flexibility in the α-carbon backbone at that position. Moreover, except for the side chains of Cys-1016 and Gln-1015 which form the thioester, the only other functional group in this sequence comes from the γ-carboxylate of Gla-1012, an entity which has been previously postulated to fullfill a general base catalytic role in the transacylation reaction (Thomas et al., 1982; Davies and Sim, 1981; Howard, 1981).

In this study we have employed site-directed mutagenesis to explore the importance of some of the evolutionarily conserved amino acids in the vicinity of Cys-1010 and Gln-1013 in the formation and reactivity of the thioester bond in human C3. Besides identifying chemical features of this region which appear to be prerequisites for thioester formation and function, we have encountered an unexpected mutant phenotype which lacks a thioester, but nevertheless has native-like conformational properties. This has caused us to reassess the interpretation of our previously observed correlation (Isenman, 1983) between the absence of an intact thioester bond and the adoption of a C3b-like conformation.

**MATERIALS AND METHODS**

**Buffers**—The following diethyl barbiturate (veronal)-NaCl buffers were used (Rapp and Boros, 1963): VB, 4 mM veronal, pH 7.2, 0.15 M NaCl; VBE, 4 mM veronal, 0.5 mM MgCl2 (µ = 0.15); GVBE, 4 mM veronal, 0.1% gelatin; GVBE, GVBE made 10 mM in EDTA; SGVB, low ionic strength GVBE made isotonic with sucrose (µ = 0.06).

**Cell Culture Media**—Tissue culture products were from GIBCO. The high glucose formulation of Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 50 units/ml penicillin, and 100 µg/ml streptomycin was the basal tissue culture medium used in this study. The pH of the medium was maintained by 5% CO2 in a humidified incubator. COS-1 cells were maintained in DMEM supplemented with 10% heat-inactivated fetal calf serum (DMEM-10% FCS). DMEM-DEAE-Dextran transfection medium consisted of serum-free DMEM containing 50 µM Tris, pH 8.0, and 0.5 mg/ml DEAE-Dextran (Sigma). The chloroquine concentration in DMEM-10% FCS-chloroquine was 100 µM.

**Purified Complement Components**—C1s (Gigli et al., 1976), C2 (Nagasawa and Stroud, 1977), C3 and C5 (Tack and Prahm, 1976), and C4 (Bolotin et al., 1977) were purified from fresh frozen human plasma as previously described. A functionally pure human C1r-reagent, used in the preparation of complement component cellular intermediates, was prepared from a euglobulin precipitation of whole human serum (Cooper and Müller-Eberhard, 1968). Guinea pig complement (GIBCO) was treated with potassium isothiocyanate and hydrazine hydrate to obtain a C6-9 reagent (Cooper and Müller-Eberhard, 1970).

**Construction of Expression Vectors Encoding Wildtype and Mutant C3**—The construction of a full-length cDNA expression plasmid for human C3, pSV-C3, has been previously described (Taniguchi-Sidle and Isenman, 1982). Selected amino acid codons within the region encoding the thioester residues were altered directly in the expression vector by the gapped-plasmid method of site-directed mutagenesis (Inouye and Inouye, 1987). The anti-sense mutagenic oligonucleotides were designed to have a minimum melting temperature to the wildtype nucleotide sequence of 50 °C, based on the formula of 2 °C for A or
T matches and 4°C for G or C matches for hybridization in 6×SSC (0.9 M NaCl, 0.09 M sodium citrate, pH 7.0) (Suggs et al., 1981). The sequences were confirmed by strand-denaturation deoxysequencing of the plasmid (Sanger et al., 1977; Mierendorf and Pfeifer, 1987) using a 50-mer sequencing reagents (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) and an anti-sense oligonucleotide primer which hybridizes approximately 35 bases downstream of the 3′-most mutation made in the cDNA.

Expression of Recombinant C3—C3 cDNA expression plasmids were transfected into COS-1 cells by a modification of the DEAE-Deoxypolyribose procedure for transient expression (Ogilby et al., 1987). Briefly, 60-mm diameter culture dishes were seeded with 4×10^5 COS-1 cells 16-24 h prior to transfection and allowed to adhere in DMEM-10% FCS. The medium was removed, the cells were washed in Hank's buffered salt solution (GIBCO) and 1.5 ml of DMEM-DEAE-Deoxypolyribose medium containing 5 μg of pSV-C3 were added. After 40 min at 37°C, the transfection medium was replaced with DMEM-10% FCS-chloroquine followed by a 3-h incubation at 37°C. The chloroquine-containing medium was removed, the cells were washed in Hank's buffered salt solution, 3 ml of DMEM-10% FCS were added, and the cells were returned to the incubator. Supernatants to be assessed for hemolytic activity were harvested after 72 h and dialyzed against VBS.

To assess the biosynthetic processing of C3 and determine whether the molecule displayed characteristics consistent with the presence of an intact thioester bond, the transfected cells were metabolically labeled for 24 h post-transfection, and the secreted C3 was immunoprecipitated from the culture supernatants, with or without various treatments. For biosynthetic labeling, the culture medium was replaced with 1.5 ml of methionine- and cysteine-free DMEM-10% FCS (ICN Biomedicals, Inc., Irvine, CA), and after 1 h to allow internal methionine depletion, the medium was supplemented with approximately 150 μCi of [35S]methionine/[35S]cysteine (Translabel, ICN). Following a 5-h incubation at 37°C, 1.5 ml of methionine-containing medium was added and the incubation was continued overnight. The supernatants were harvested and usually divided into 4 aliquots. C3 was immunoprecipitated from an untreated sample using a rabbit polyclonal IgG anti-human C3c (Sigma). Prior to immunoprecipitation, the second sample was incubated for 60 min at 37°C with fluid-phase C3 convertase (C4b2a2a). The latter was prepared by treating 15% (w/w) for 3 min (Polley and Muller-Eberhard, 1967). Four C4b equivalents of C4b2a2a were used per sample. The 2 remaining aliquots were treated with 2.0 M KBr at 37°C for 3 h. After dialysis against VBS, C3 was immunoprecipitated from the third sample, while the fourth was treated with C4b2a2a as above prior to immunoprecipitation. In some experiments, C3-containing culture supernatants were reacted with 0.1 M methanolamine, 0.05 M Tris-HCl, pH 7.4 at 37°C, followed by dialysis against VBS prior to subsequent treatments. Autolytic fragmentation of immunoprecipitated C3 was induced by boiling the samples in 2% SDS, 6 M urea, 0.1% SDS-PAGE (Laemmli, 1970) under reducing conditions. The gels were stained with Coomassie Blue, treated with Autofluor (National Diagnostics, Manville, NJ) intensifying solution, dried, and exposed to Kodak XAR film at -70°C.

For pulse-chase experiments, three replicate transfections (corresponding to the number of time points to be sampled) were each depleted of endogenous methionine as described above and pulse-labeled for 1 h using 150 μCi of [35S]methionine/[35S]cysteine. Following removal of the radioactive medium, the cells in two of the dishes were allowed to grow in an equal volume of non-radioactive medium (i.e., 0.2, and 24 h). At the chase time (i.e., 0, 2, and 24 h), the culture supernatant was harvested, and the cells were washed twice in Hank's buffered salt solution and then lysed by freeze-thaw cycles in 1 ml of 0.5% Triton X-100, 0.25% sodium deoxycholate in phosphate-buffered saline. Cell debris was removed from the lysates by centrifugation, and metabolically labeled C3 was immunoprecipitated from both lysate and supernatant samples as described below.

Immunochemoal Methods—The concentration of recombinant C3 antigen in the culture supernatants of transfected cells was determined by a competitive solid-phase radioimmunoassay (Harlow and Lane, 1988) using [3H]human C3c and anti-human C3c (Sigma) as the capture antibody on flexible polyvinylchloride microtiter wells (Falcon, Oxnard, CA). Purified plasma C3 was used to obtain a standard curve.

C3 was immunoprecipitated from treated or untreated culture supernatants, and in the case of pulse-chase experiments from intracellular lysates as well, using the above antibody, a Staphylococcus aureus suspension (IGI, The Enzyme Center, Malden, MA) and the previously described buffers, preclearing step, and washing procedures (Cole et al., 1986).

Hemolytic Assay—The hemolytic activity of recombinant C3 in transfection supernatants was determined using EAC4′72 in conjunction with purified human C5 and a guinea pig C6-9 reagent as described by Cooper and Müller-Eberhard (1970). Comparisons were made on the basis of Z units/tg of immunochemoal determined recombinant C3 antigen, where Z = −ln (1-fractional lysis) and is physically equal to the number of "hemolytically effective" molecules/erythrocyte.

RESULTS

Criteria for Thioester Integrity and Function—In order to determine whether mutations which we introduced into human C3 at the conserved positions (as indicated in Fig. 1, lower panel) had a detrimental effect or were neutral toward thioester formation and function, we used three criteria to assess whether the thioester bond had formed in the mutant recombinant C3 molecules. These were the molecule's ability to undergo denaturation-induced autolytic fragmentation, its ability to be proteolytically cleaved by the classical pathway C3 convertase (C4b2a2a), and its ability to transacylate onto target surfaces. The last property was monitored via hemolytic activity. The first two properties were assessed via the appearance of diagnostic bands on SDS-PAGE autoradiograms of metabolically labeled recombinant C3 molecules that had been subjected to various treatments before, or in conjunction with immunoprecipitation.

Culture media supernatants of COS-1 cells transiently transfected with wildtype human C3 cDNA expression vector pSV-C3 displayed C3 hemolytic activity which, on a per weight of antigen basis, was approximately 60% that of plasma-purified C3. Immunoprecipitations with anti-C3 from supernatants of transiently transfected cells that had been metabolically labeled with [35S]methionine/[35S]cysteine re-
vealed the presence of specific bands corresponding to mature C3 α- (115 kDa) and β-chains (75 kDa) as well as a band corresponding to secreted pro C3 (Fig. 2). Also apparent in the “direct” immunoprecipitation lane (lane b) is a band at about 47 kDa that arises from denaturation-induced autolytic fragmentation and represents the COOH-terminal portion of C3 α-chain. A band corresponding to the NH₂-terminal autolytic fragment cannot be visualized as it comigrates with the β-chain. In our hands the extent of autolytic fragmentation is similar regardless of whether the S. aureus-bound material is directly boiled in SDS-PAGE sample buffer containing reducing agent (lane b), or heated first in SDS under non-reducing conditions (i.e. autolytic conditions) and then reduced (lane c). Finally, since some of the secreted wildtype material would have undergone spontaneous thioester hydrolysis, and since bovine factors H and I are present in the FCS-containing culture medium, this would result in the appearance of the constituent bands of iC3(H₂O), specifically α-40 and α-75, the latter comigrating with the β-chain. Some of the α-40 band may also have arisen from factor I-mediated cleavage of thioester-hydrolyzed pro-C3. This cleavage would also produce a β-α75 species which is the likely identity of the band migrating in between pro-C3 and α-chain. A similar pattern of bands for immunoprecipitations of metabolically labeled recombinant C3 from transfected J558L mouse plasmacytoma cells has been reported by us previously (Taniguchi-Sidle and Isenman, 1992). Treatment, prior to immunoprecipitation with anti-C3, of the wildtype transfection supernatant with C4b2a (i.e. autolytic conditions) after which reducing agent was added; d, pretreatment of the supernatants with C4b2a followed by immunoprecipitation with anti-C3 under autolytic conditions; e, pretreatment of the supernatant with 2 M KBr followed by direct immunoprecipitation with anti-C3; f, pretreatment of the supernatant with 2 M KBr followed by autolytic conditions immunoprecipitation with anti-C3; g, direct immunoprecipitation with anti-C3 of supernatant pretreated first with 2 M KBr and, following dialysis, with C4b2a.

**FIG. 2.** Assessment of thioester integrity and the extent of native-like conformation in wildtype (WT) recombinant C3. Autoradiographs of 9% SDS-PAGE (reducing conditions) of C3 immunoprecipitated from the culture supernatants of metabolically labeled COS-1 cells transfected with wildtype pSV-C3. Specificity controls and combinations of KBr and C4b2a treatments used to assess thioester integrity are indicated by the following key: a, non-specific immunoprecipitation using anti-C4; b, “direct” immunoprecipitation with anti-C3 of a pSV-C3 transfection supernatant and of a sham transfection supernatant (No DNA, but metabolically labeled, right side lane), “direct” refers to the fact that there has been no deliberate attempt to induce autolytic fragmentation; c, immunoprecipitation with anti-C3 followed first by boiling in SDS sample buffer without reducing agent to induce maximal autolytic fragmentation (referred to as "autolytic conditions") after which reducing agent was added; d, pretreatment of the supernatants with C4b2a followed by immunoprecipitation with anti-C3 under autolytic conditions; e, pretreatment of the supernatant with 2 M KBr followed by direct immunoprecipitation with anti-C3; f, pretreatment of the supernatant with 2 M KBr followed by autolytic conditions immunoprecipitation with anti-C3; g, direct immunoprecipitation with anti-C3 of supernatant pretreated first with 2 M KBr and, following dialysis, with C4b2a.

**TABLE I**

| Recombinant C3 molecule | Presence of autolytic fragment | Cleavage by C4b2a | Cleavage by C4b2a after KBr | Relative hemolytic activity |
|-------------------------|-------------------------------|------------------|----------------------------|----------------------------|
| Wildtype                | Yes                           | Yes              | No                         | 1.0                        |
| G1009A                  | Yes                           | Yes              | No                         | 0.9                        |
| G1009S                  | Yes                           | Yes              | No                         | 0.9                        |
| M1015F                  | Yes                           | Yes              | No                         | 0.7                        |
| P1007G                  | No                            | Yes              | No                         | 0.07                       |
| C1010A                  | No                            | Yes              | No                         | Undetectable              |
| E1012Q                  | No                            | Yes              | No                         | Undetectable              |
| Q1013N                  | No                            | Yes              | No                         | Undetectable              |
| P1020G                  | No                            | Yes              | No                         | Undetectable              |
| M1015A                  | No                            | No               | ND*                        | Undetectable              |

* ND, not done.
Requirements for Thioester Bond Formation in C3

FIG. 3. Substitutions permissive to thioester formation. Autoradiographs of 9% SDS-PAGE (reducing conditions) of C3 immunoprecipitated from the culture supernatants of metabolically labeled COS-1 cells transfected with wildtype C3 expression plasmid, and with expression plasmids for mutant C3s G1009S, G1009A, and M1015F, as indicated above each panel. G1009A was run on a separate gel from the others. All immunoprecipitations were done under autolytic conditions as explained in Fig. 2. a, anti-C3; b, pretreatment with C4b2a, followed by anti-C3; c, pretreatment with first KBr and then C4b2a, followed by anti-C3; d, nonspecific immunoprecipitation control (anti-C4).

FIG. 4. Pulse-chase experiment demonstrating the susceptibility of the M1015A mutant to intracellular proteolysis. Autoradiographs of 9% SDS-PAGE (reducing conditions) of C3 immunoprecipitated from the culture supernatants and intracellular lysates of metabolically labeled COS-1 cells transfected with wildtype C3 expression plasmid (WT), and with that of mutant C3 M1015A. The times of the pulse and chase conditions are shown on each panel.

profile when the recombinant protein was subjected to our various assays for thioester integrity and functionality. The P1007G molecule was found to be almost devoid of hemolytic activity (~7% of wildtype), and when examined for its ability to undergo denaturation-dependent fragmentation, there was no evidence of the 47-kDa autolytic fragment band indicative of normal thioester function (Fig. 5, P1007G panel, lane a). Despite failing to fulfill two of our criteria for thioester integrity (i.e. hemolytic activity and autolytic fragmentation), the α-chain of the P1007G molecule was readily cleaved by C4b2a and moreover, pretreatment with KBr altered the conformation of this molecule such that it became resistant to this cleavage (Fig. 5, P1007G panel, lanes b and c). Similar functional profiles were also observed when the potentially catalytic carboxylate side chain of Glu-1012 was changed to a neutral Gln residue (E1012Q, Fig. 5), when Gln-1013, which contributes the carbonyl of the thioester, was changed to a shorter Asn residue (Q1013N, Fig. 5), or when Pro-1020 was mutated to Gly (P1020G, data not shown), the only notable difference being that none of the latter three mutant molecules displayed any detectable hemolytic activity.

In an attempt to account for the unexpected functional profiles of the latter four mutant molecules, we formulated several testable hypotheses. The first hypothesis (a) held that the thioester formed initially but was unstable toward solvolysis. However, the ensuing conformational change to a C3b-like structure was slow and therefore the molecule remained cleavable by C4b2a. A second possibility (b) was that the thioester formed, but did not display the chemical reactivity of the wildtype structure. Nevertheless, it stabilized a native-like conformation which was cleavable by C4b2a. A third possibility (c) was that the thioester either did not form at all, or it did so only transiently. However, the absence of a stable thioester bond at the pro-C3 stage of biosynthesis might result in the adoption of an intermediate conformation which was neither fully native nor C3b-like, but which had an accessible cleavage site for C4b2a.

Each of the above hypotheses were amenable to experimental verification as follows. If a were true, one would predict
that prolonged incubation of the sample at 37 °C would result in the completion of the conformational change and the accompanying acquisition of resistance to cleavage of the molecule by C4b2a. If b were correct then, although the chemical reactivity of the thioester may not be sufficient to permit transacylation or autolytic fragmentation, the thioester should nevertheless be susceptible to cleavage by methylamine, since even linear thioesters can be cleaved by small amino group nucleophiles (Noda et al., 1953). Therefore, after prolonged methylamine treatment one would expect a loss in the susceptibility to cleavage by C4b2a because of the conformational change which has been shown to accompany thioester disruption in C3 (Isenman et al., 1981). Finally, if c were true, then neither prolonged incubation at 37 °C nor treatment by methylamine should affect cleavage by C4b2a.

In order to distinguish among the three possibilities, the experiments described above were performed on culture supernatants containing metabolically labeled C3 of either recombining wildtype or E1012Q origin. As can be seen in Fig. 6, whereas prolonged incubation of the wildtype C3 molecule does not substantially affect its susceptibility to cleavage by C4b2a, treatment with methylamine does result in the α-chain becoming refractory to this cleavage. In marked contrast, with the E1012Q molecule susceptibility to cleavage by C4b2a remains unaffected both by the prolonged incubation and the treatment with methylamine. This behavior is therefore consistent with c, namely that despite the lack of an intact thioester bond, at least a part of the molecule adopts a conformation that is sufficiently similar to the thioester-intact native state to be recognized and cleaved by C4b2a. In order to further substantiate this hypothesis, we changed Cys1010 (i.e. the sulfhydryl donor to the thioester bond) to Ala, thereby ensuring that a thioester bond could not form, and compared the functional profile of C1010A C3 to those of the E1012Q, Q1013N, P1007G, and P1020G mutant molecules. The right side of Fig. 6 depicts these experiments and reveals that the C1010A molecule behaved identically to the above four mutants, both with respect to the absence of autolytic fragmentation products and susceptibility to α-chain cleavage by C4b2a, even after prolonged incubation at 37 °C. KBr treatment of the C1010A mutant did, however, abolish cleavage by C4b2a. Finally, as one would expect, the C1010A mutant molecule was totally devoid of hemolytic activity. The functional profiles of the various thioester region mutants engineered in this study are summarized in Table I.

**DISCUSSION**

There is a considerable body of evidence indicating that the thioester bond present in C3, C4, and α2-macroglobulin fulfills the dual functions of mediating covalent transacylation and maintaining the native precursor conformational state of these molecules (Isenman, 1983; Gonias and Pizzo, 1983; Straight and McKee, 1983; Björk and Fish, 1982; Sottrup-Jensen, 1989; Stoops et al., 1991). A search of the Swiss protein data bank for the sequences CEGEQ or CAEQ, the tetrapeptide sequences involved in forming the thiolactone ring in C3, C4, and α2-macroglobulin of various species, revealed that these two sequences occurred in quite a number of other proteins which were not known to display the characteristics of the thioester-containing proteins. For example, the CGEQ sequence is found in an **Escherichia coli** ATPase, an integrin β-chain of **Xenopus**, a human cell cycle regulatory protein, and a **Drosophila** salivary glue protein. Additionally, the CAEQ sequence forming the thioester in mouse C4 and Slp was found in fibrinogen γ-chains of various species. In none of these cases, however, was there significant sequence conservation in the regions immediately flanking the tetrapeptide sequence. This is in marked contrast to what is observed in the flanking sequences of the known thioester-containing proteins (Fig. 1), strongly suggesting that the conserved residues play an important role in thioester formation and function.

In the course of the present site-directed mutagenesis study on human C3, we have identified the conserved flanking sequence residues that are indispensable for normal thioester function. Thus, the rigidity that would be imposed on the peptide chain by the prolines at positions 1007 and 1020 appears to be necessary for stable thioester formation since the substitution of glycine at either proline position, which might be expected to permit considerable peptide chain flexibility, did not yield a thioester-intact native-like molecule. In contrast, the conserved glycine at position 1009 was not required for its flexibility characteristics as it could be replaced by either alanine or serine and still yield a thioester-intact molecule. The conserved methionine at position 1015 appears to be required for its contribution of a large hydrophobic side chain since its substitution by another bulky hydrophobic side chain, phenylalanine, was tolerated with respect to thioester formation and reactivity. However, replacement of M1015 by A resulted not only in the apparent absence of the thioester bond, but additionally in a major

**Fig. 6. Effect of CH3NH2 treatment on C4b2a cleavage.** Autoradiographs of 9% SDS-PAGE (reducing conditions) of C3 immunoprecipitated from the culture supernatants of metabolically labeled COS-1 cells transfected with expression plasmid for wildtype C3 (WT), and those for mutants E1012Q, and C1010A. All immunoprecipitations were done with anti-C3 under autolytic conditions as explained in Fig. 2. The pretreatments were: a, 0 °C; 24 h; b, 0 °C; 24 h followed by C4b2a; c, 37 °C; 24 h; d, 37 °C; 24 h followed by C4b2a; e, 100 mM CH3NH2, 37 °C; 24 h; f, 100 mM CH3NH2, 37 °C; 24 h followed by C4b2a; g, 2 M KBr; h, 2 M KBr and then C4b2a. The rightmost C1010A panel was derived from a separate gel.
altered in protein folding which rendered the $\alpha$-chain extremely susceptible to intracellular proteases. A further indication of the very strict spatial requirements for thioester formation comes from the observation that replacement of glutamic acid 1013 by asparagine, a substitution that moved the reactive amide side chain by only about 1.5 Å, was incompatible with stable thioester formation. Finally, although we wished to assess whether the carboxyl side chain of glutamic acid 1012 fulfilled a catalytic role in the transacylation reaction, as had been proposed previously (Thomas et al., 1982; Davies and Sim, 1981; Howard, 1981), we were unable to reach any conclusions on this point because replacement of the negatively charged carboxylate side chain (assuming a normal $pK_a$) by the isocysteine but non-ionizable amide side chain of glutamic acid also abrogated stable thioester formation.

The inability of the P1007G and P1020G mutants to form a stable thioester, on the one hand, indicates the structural importance of these highly conserved prolines in forming or stabilizing the thiolactone ring, but on the other hand is inconsistent with the replacement in rabbit C3 of P1007 by G and P1020 by H (Fig. 1). Either the "double mutation" is self-compensatory or one has to consider the possibility that the mutant C3 sequence in the thioester region is in error. Recent reports on the thioester region sequences of cobra C3 and lamprey C3 (Fritzinginger et al., 1991; Nonaka and Takahashi, 1991), species evolutionarily distant from mammals and from each other, show the conservation of P1007 and P1020 as well as the other highly conserved residues indicated in Fig. 1.

As mentioned above, replacement of G1009 by either A or S does not produce a change in thioester function that was detectable by any of the assays used. A noteworthy difference between either the wildtype G1009 or mutant G1009A molecules and the G1009S mutant is that treatment of the latter with KBr, while apparently cleaving the thioester as judged by the absence of an autolytic fragment after the treatment, only partially induces the conformational change which makes the molecule refractory to cleavage by C4b2a. It is possible that the apparently greater stability of the native-like conformation in the G1009S molecule is the result of an additional hydrogen bond that could be formed via the hydroxyl group of the serine side chain.

In undertaking our study of the structural requirements for thioester formation in C3, it was important to decide upon the criteria that we would use to assess thioester formation. We chose two criteria, hemolytic activity and autolytic fragmentation, that were dependent on the transacylation properties of the thioester bond in C3 and one, susceptibility to cleavage by C4b2a, that was dependent on the role that the thioester had been shown to play in stabilizing the native conformational state of the protein. Because it had been proposed that E1012 may contribute to the chemical reactivity of the reactive amide group of Q1013, it seemed possible that in the E1012Q mutant we might encounter a phenotype in which the thioester would form, but that both hemolytic activity and autolytic fragmentation would be impaired. We therefore expected that the property that was sensitive to the native conformational state of C3 would allow us to distinguish between absence of stable thioester bond formation and formation of a thioester that displayed impaired reactivity in transacylation reactions. Indeed, the characteristics of the E1012Q mutant, namely absent transacylation function but native conformational state as judged by susceptibility to C4b2a cleavage, were fully consistent with the above predicted phenotype. However, one would not have predicted that exactly the same phenotype would have resulted from the proline to glycine mutations at positions 1007 and 1020, since prolines are unlikely candidates for affecting the electrophilicity of the carbonyl group of the thioester bond. Furthermore, methylamine treatment of the E1012 mutant molecule, under conditions that would be expected to cleave even non-hyper-reactive linear thioester model compounds (Noda et al., 1953), did not affect its susceptibility to cleavage by C4b2a. It therefore seemed more reasonable to conclude that, despite the native-like behavior toward the conformational probe in the E1012Q, P1007G, and P1020G molecules, there was no thioester bond present. The fact that the C1010A mutant, in which the thioester could never form because of the absence of the thiol component of the bond, displayed a functional profile identical to the above three mutants provides further evidence that P1007, E1012, and P1020 are either essential for thioester formation per se, or for it having more than a transient lifetime.

Our finding that C3 molecules in which the thioester bond does not stably form can nevertheless display conformational properties that, at least in parts of the molecule, are native-like requires that we re-evaluate the role that this bond plays in maintaining the native conformation of not only C3, but C4 and $\alpha_2$-m as well. The previous correlation between thioester scission by methylamine and the occurrence of a fairly major conformational change in C3, C4, and $\alpha_2$-m that could be detected by both biophysical (Isenman, 1983; Straight and McKee, 1983; Björk and Fish, 1982; Gonias and Pizzo, 1983) and biochemical (Pangburn and Muller-Eberhard, 1983; Gonias and Pizzo, 1983) probes of protein conformation led to the hypothesis (Isenman et al., 1981; Isenman, 1983) that the thioester was necessary for the stabilization of a conformationally strained state of protein folding in these molecules, and in fact the phrase "spring in the molecular mouse trap" was coined to describe its role (Fothergill, 1982). In view of the present findings, it is necessary to amend this hypothesis. As a working model, we propose that during biosynthesis local areas of the protein, such as those surrounding the site for C4b2a cleavage, can adopt a native-like conformation, even in the absence of stable thioester bond formation. Regardless of the initial presence or absence of a thioester bond, such local energy minima are not very stable in secreted two-chain C3, as evidenced by the fact that a native-like conformation does not reform after mild chaotrope treatment. The role of the thioester bond may be to stabilize one particularly unfavorable folding configuration in the mature protein that may be required for propagating a putative proteolytic activation-induced conformational change. When an intact thioester bond is cleaved in the native molecule, even in the absence of proteolysis it is possible that an alternative lower energy conformation for the "strained" region can only be reached via a pathway that disrupts other quasi-stable native regions in the protein. However, if thioester bond formation were not possible during biosynthesis, the putatively "strained" region might directly adopt the non-native lower energy state via a pathway that was nondeleterious to the native-like folding in other regions of the molecule. Interestingly, cleavage by C4b2a of molecules which lacked the thioester bond (e.g. C1010A) resulted in the molecule acquiring binding sites for bovine factors H and I that were present in the culture medium and the consequent further cleavage of the $\alpha$-chain into $\alpha^{*}$-67 and $\alpha$-43. This suggests that even in these molecules, C4b2a cleavage promoted the adoption of a C3b-like conformation in regions of the protein distinct from the C4b2a cleavage site. Future studies on the C1010A molecule using biophysical, biochemical, and immunochemical probes of protein conformation will be required to further define the differences
between its conformation and that of native C3 and thus potentially further clarify the conformational role of the thioester bond in the initial protein folding process.

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