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REVIEW

# The internal thioester and the covalent binding properties of the complement proteins C3 and C4

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**Abstract**

The covalent binding of complement components C3 and C4 is critical for their activities. This reaction is made possible by the presence of an internal thioester in the native protein. Upon activation, which involves a conformational change initiated by the cleavage of a single peptide bond, the thioester becomes available to react with molecules with nucleophilic groups. This description is probably sufficient to account for the binding of the C4A isotype of human C4 to amino nucleophiles. The binding of the C4B isotype, and most likely C3, to hydroxyl nucleophiles, however, involves a histidine residue, which attacks the thioester to form an intramolecular acyl-imidazole bond. The released thiolate anion then acts as a base to catalyze the binding of hydroxyl nucleophiles, including water, to the acyl function. This mechanism allows the complement proteins to bind to the hydroxyl groups of carbohydrates found on all biological surfaces, including the components of bacterial cell walls. In addition, the fast hydrolysis of the thioester provides a means to contain this very damaging reaction to the immediate proximity of the site of activation.

**Keywords:** complement; covalent binding; thioester

*The complement system*

Complement is the major defense effector in blood plasma. Like clotting, it is potentially extremely hazardous and has therefore evolved into a very complex system, comprising some 20 soluble components, together with cell surface inhibitors that limit damage to self, and receptors on a range of cell types with which the system interacts. It is active in both the acquired and innate immune systems. In acquired immunity, it is activated by antibody bound to foreign material (classical pathway). In innate immunity, the system can be activated, in a similar manner, by the mannan binding lectin (MBL), which recognize foreign carbohydrate structures (MBL pathway), and also by a mechanism that involves the differential interaction of a range of inhibitors and cofactors with self and non-self surface structures (alternative pathway). There is not space in this short article to give a complete description of the system, which has been reviewed elsewhere (Law & Reid, 1995). The central component of complement is C3, the covalent binding of this protein to activating surfaces is responsible for initiating most of the effects of the system (Fig. 1). A second component of the system, C4, is involved in the activation of C3 via the antibody and MBL pathways. It has a similar overall structure to that of C3, although the mature form of the protein is processed into three chains, as opposed to the two chains of C3. C4 also binds covalently to target cell surfaces.

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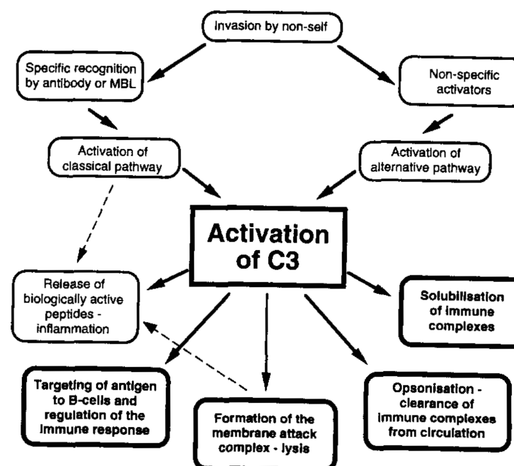


**Fig. 1.** Central role of C3 in complement-mediated activities. Activation of complement by the three pathways converge on the activation of C3. Except for the inflammatory activity mediated by the C3a, all others require the covalent binding of C3 to target cells or immune complexes (in bold).

*Activated fragments of C3 and C4 and their biological activities*

Native C3 and C4 circulate in the plasma. They are inert and do not bind to any receptors (or if they do, they do so with low affinity and do not trigger any response). Activation is brought about by the cleavage of a single peptide bond, which converts C3 into C3a and C3b, and C4 into C4a and C4b (Fig. 2). C3a and C4a are anaphylatoxins and consist of 77 residues (human) from the N-termini of the $\alpha$-chains of C3 and C4, respectively. C3b and C4b are the major cleavage products that bind to targets. The binding reaction is not efficient: typically, only about 10% of the C3b and C4b generated binds to targets, the other 90% remains in the fluid phase, where it is inactivated rapidly. Once generated, C3b is processed rapidly into iC3b and C3f by the regulatory protein factor I and its co-factors including factor H. In turn, iC3b is cleaved into C3c and C3dg, and C3dg into C3d and C3g. If C3b is surface-bound, the fragments remaining bound after subsequent processing are iC3b, C3dg, and C3d. C4b is cleaved into C4c and C4d, and the fragment remaining bound is C4d.

Surface-bound C4b and C3b, in combination with proteolytic components, are constituents of complex enzymes that activate C3 and C5. C4b combines with the C2a fragment to form the C4b2a complex, the C3-convertase of the classical activation pathway. In an analogous fashion, C3b combines with the Bb fragment to form C3bBb, the C3-convertase of the alternative pathway. Both of these enzyme complexes combine with an additional C3b fragment, to form the C4b2a3b and $C3b_2Bb$ complexes, respectively,

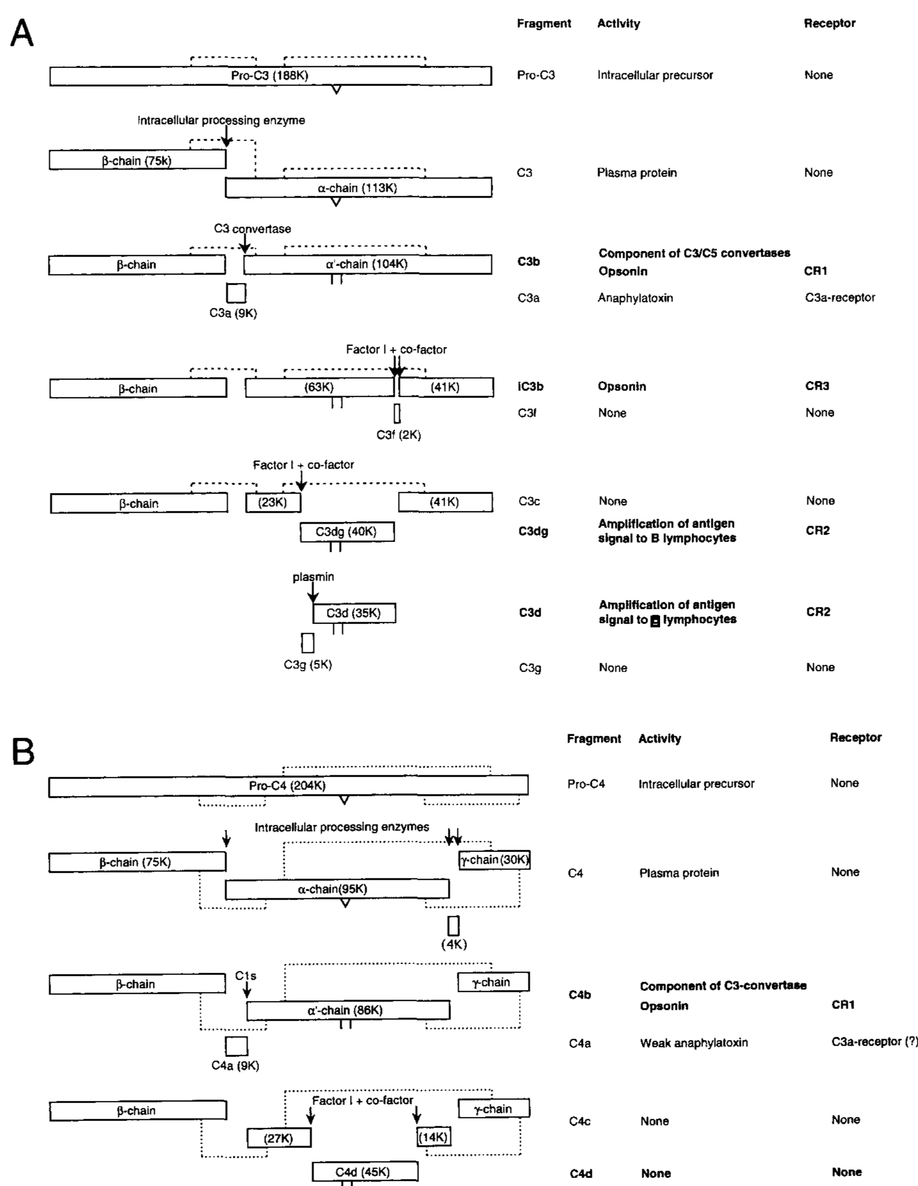


**Fig. 2.** Polypeptide structures of C3 (**A**) and C4 (**B**) and their activation products. Inverted triangles represent the internal thioester, which is broken upon conversion of C3 into C3b, and C4 into C4b (open parallel lines). Covalently bound components, and the activities they mediated, are indicated in bold. Dotted lines represent interchain disulfide bonds.

to activate C5 and initiate the assembly of the lytic components of complement. In addition to their roles in the activation of complement, the fragments of C3 and C4 mediate a wide range of other activities (Fig. 1). Apart from the inflammatory activities of C3a and C4a, all require the covalent binding of C3 and C4 fragments onto target cells or immune complexes. The fragments released into the fluid phase by proteolytic cleavage of bound C3b and C4b, i.e., C3f, C3c, C3g, and C4c, are not known to have any biological activities.

### *Covalent binding of C3 and C4 to targets*

It was recognized by the 1960s that the binding of C3b and C4b to cell surfaces was extremely tight (Dalmasso & Müller-Eberhard, 1967). Once bound to cells, they could not be removed except by reagents that also disrupted the integrity of the membrane structure. Using lipopolysaccharide-coated heavy-paraffin oil droplets as targets to activate the alternative pathway of complement, Stossel et al. (1975) demonstrated that bound C3 fragments could not be removed under extreme conditions except by proteases or by boiling in SDS. This was interpreted as indicating that the interaction between activated C3 fragments and the target surface components was a very strong hydrophobic one. Another phenomenon, which was not addressed in a coherent manner, was the means by which C3b could bind to so wide a range of unrelated surfaces, including cell membranes (Müller-Eberhard et al., 1966; Dalmasso & Müller-Eberhard, 1967), bacterial cell wall components (Johnston et al., 1969), immune aggregates (Theofilopoulos et al., 1974; Czop & Nussenzweig, 1976), zymosan (a large mannan complex from yeast cell walls) (Nicholson et al., 1974), and the artificial particle Sepharose (Goldstein et al., 1976). The key experiment, which provided the first evidence for the covalent binding of C3, was the analysis, by SDS-PAGE, of the membrane polypeptides of sheep erythrocytes after they had been treated sequentially with antibody, C1, C4, C2, and $^{125}$I-labeled C3. In addition to the two well-established $\alpha'$ and $\beta$ chains of C3b, an array of high molecular weight radioactive bands was evident. It was concluded that these were complexes of one of the chains of C3b with an assortment of erythrocyte membrane proteins. The nature of the bond was most likely to be covalent, but not disulfide, because it survived boiling in SDS and urea in the presence of reducing agents such as $\beta$-mercaptoethanol (Law & Levine, 1977). When the complexes were treated with alkaline hydroxylamine (1 M, pH 9.0, at 37 °C, for 1 h), conditions that would break ester bonds, the high molecular weight complexes dissociated and the radioactivity was recovered in the $\alpha'$ chain of C3b. Thus, the binding site on C3b was located to the $\alpha'$-chain (Law & Levine, 1977).

The orientation of the ester bond was established: the acyl group was located on C3b (Law et al., 1979). Thus, the reaction must be that of an activated acyl group in C3b with hydroxyl groups on the cell surface molecules. C4b was also found to bind to target cells by covalent bonds, but with a difference; it bound by hydroxylamine-resistant amide bonds as well as by ester bonds (Campbell et al., 1980; Law et al., 1980). These results satisfactorily answer the question of the universality of the binding reaction: by reacting with either hydroxyl or amino groups, C3b and C4b can potentially bind to all cell surfaces and biological molecules.

### *The discovery of the internal thioester*

The earliest observation that suggested that the complement protein C4 was unusual was that of Gordon et al. (1926), who reported its inactivation by ammonia, methylamine, and ethylamine. Similar findings were extended to C3 using hydrazine (Pillemer et al., 1953). The key experiment in establishing the presence of an internal thioester was the stoichiometric appearance of a free thiol group when C3 was inactivated by nitrogen nucleophiles, chaotropes, or by trypsin (Janatova et al., 1980a). When C3 was inactivated using $^{14}$C-methylamine, this became covalently bound to the molecule (Pangburn & Müller-Eberhard, 1980; Tack et al., 1980). If $^{3}$H-iodoacetic acid was used to label the released thiol group, both radioactive labels could be recovered in a single tryptic peptide. The two labeled residues were found in the sequence -Cys*-Gly-Glu-Glx*- (Tack et al., 1980), suggesting that there was a thioester between the thiol of the cysteine residue and the acyl group of the second glutamyl residue, forming a 15-member thiolactone ring (Fig. 3A).

It was established within the same period that a similar internal thioester was present in C4 (Campbell et al., 1981; Harrison et al., 1981) and $\alpha_2$-macroglobulin (Howard, 1981; Sottrup-Jensen et al., 1980, 1981). $\alpha_2$-Macroglobulin is a protease inhibitor that binds covalently to proteases as part of its inhibitory mechanism (see reviews by Sottrup-Jensen, 1987; Chu & Pizzo, 1994) and has a similar overall structure to C3 and C4 (Sottrup-Jensen et al., 1985). It was established by cDNA sequencing that the glutamyl residues of the thioesters in C3 (de Bruijn & Fey, 1985), C4 (Belt et al., 1984), and $\alpha_2$-macroglobulin (Kan et al., 1985) were synthesized as glutamines. The four residues, -Cys-Gly-Glu-Gln-, forming the thiolactone ring (Fig. 3A) are highly conserved, with the exception that the Gly at the second position is substituted by an Ala in mouse C4 (Nonaka et al., 1985) and in cattle C4 (Ren et al., 1993).

### *Conformational change and activation*

In order to transform from an inert circulating protein with an internal thioester, accessible only to small nucleophiles, into one having the capability to bind to targets, the protein must undergo a major conformational change during activation. It was therefore no surprise that C3 and C3b have gross conformational differences as determined by CD at near UV and by binding to the hydrophobic dye 8-anilino-1-naphthalenesulfonate. The change is instantaneous upon activation (Isenman et al., 1981). What was surprising was that, when C3 was inactivated with methylamine, i.e., by breaking the thioester, the conformational change took place over a long period of time, in the order of 10 h (Isenman et al., 1981). Similar results were obtained for C4 (Isenman & Kells, 1982). This suggests that, although the thioester contributes to the stability of the molecule, it is not the structural feature primarily responsible for maintaining the inert conformation of the native molecule; on the contrary, it is probably the conformation that protects the thioester from hydrolysis (see section on biosynthesis).

It has long been known that chaotropes, such as KBr, are able to render C3 inactive (Dalmasso & Müller-Eberhard, 1966). When C3 was incubated with $^{3}$H-glycerol in the presence of KBr, covalent binding to the glycerol was observed (Law, 1983). This reaction can also be induced by other reagents known to inactivate C3, such as KSCN, and low concentrations of guanidine hydrochloride (1 M) and urea (3 M). These results suggest that this mode of inactivation is a process similar to the natural activation of C3. Furthermore, the proteolytic cleavage of C3 into C3a and C3b is not necessary; a conformational change, as induced by the various reagents, is sufficient to bring about the covalent binding reaction.

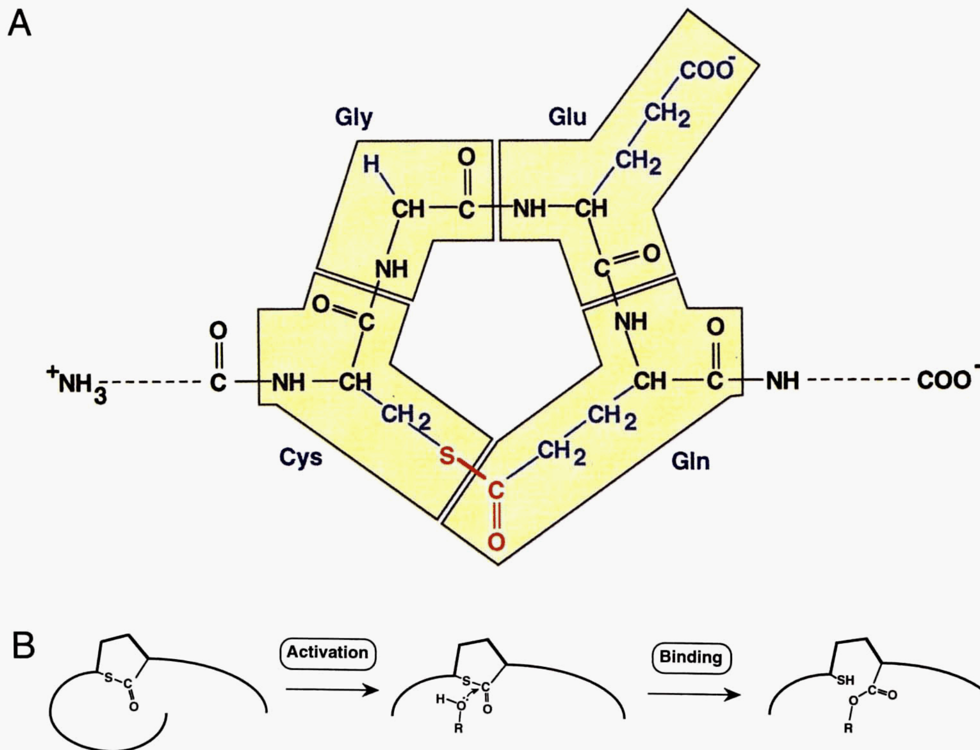


**Fig. 3. A:** Fifteen-member thiolactone ring composed of the four residues -Cys-Gly-Glu-Gln-. The polypeptide backbone is in black, side chains are in blue, and thioester bond is in red. Gly may be replaced in Ala as found in mouse C4 and cattle C4B. N-terminal and C-terminal extensions are shown. **B:** Putative covalent binding reaction of C3 and C4.

Based upon the evidence described thus far, it was reasonable to propose that the activation process involved a conformational change, induced by the cleavage of the $\alpha$-chain. This led to the exposure of the thioester, which became liable to attack by nucleophiles in its immediate surroundings (Hostetter et al., 1982). Some molecules would bind to nearby macromolecules, usually molecules on the target surfaces on which complement activation was occurring, but the majority would be hydrolyzed and remain in solution, thus providing an explanation for the low efficiency of the reaction (Fig. 3B).

*The autolytic cleavage reaction*

Another property, unique to the thioester proteins and first described in $\alpha_2$-macroglobulin, is an autolytic cleavage reaction (Fig. 4) that occurs under extreme denaturing conditions (Harpel et al., 1979). An intact thioester is required because pretreatment of the protein with nucleophilic amines, or activation of the protein by proteases, resulting in the hydrolysis of the thioester, abolishes the reaction. The reaction is temperature and pH dependent and is induced by treating the proteins at elevated temperature (close to 100 °C) and at alkaline pH (Sim & Sim, 1981).

Khan and Erickson (1981, 1982) synthesized the thiolactone ring of the thioester proteins (Fig. 3A) and showed that the hydrolysis of the peptide bond (autolytic cleavage) is the preferred reaction over the hydrolysis of the thioester. Thus, the overall structure of the thioester proteins must play a role in restricting the autolytic cleavage reaction. By incubating C3 with $^3$H-glycerol in the presence of different concentration of guanidine hydrochloride, it was shown that covalent binding to glycerol was induced between 0.5 M and 1.5 M guanidine hydrochloride (Law, 1983). Above 1.5 M guanidine hydrochloride, a decline in the binding reaction was observed and in its place the autolytic cleavage reaction became evident. Only the autolytic cleavage reaction was observed at guanidine hydrochloride concentrations over 3.0 M. Thus, the autolytic cleavage reaction, although an intrinsic property of the thiolactone ring, only proceeds when the protein is denatured sufficiently. It is therefore of no biological significance in the functions of the thioester proteins. However, it is extremely useful in the laboratory for testing for the presence of a thioester protein. A sample is added to preheated (~95 °C) Laemmli (1970) gel buffer (1% SDS with 8 M urea) for 5 min before reduction and electrophoresis in SDS-polyacrylamide gel. The appearance of lower molecular weight bands indicate the occurrence of autolytic cleavage and therefore the presence of an intact thioester in the protein. In the case of C3, the $\alpha$-chain of 110 kDa is split into two fragments of 67 kDa and 43 kDa.

*The two isotypes of human C4, C4A, and C4B*

As noted above, when the rate of hydrolysis of the thioester in the synthetic thiolactone ring was determined, it was found to be very slow, with a half life of approximately 10 h (Erickson & Khan, 1983). It has long been known that the covalent binding and hydrolysis reactions of nascent C3b are very fast (Müller-Eberhard et al., 1966), with a $t_{1/2}$ of considerably less than 1 s (Sim et al., 1981). The huge difference in the rate of hydrolysis of the thioester in the synthetic thiolactone and within the protein suggested that the covalent binding reaction in the complement proteins may be catalyzed. It would have been impossible to determine which res-

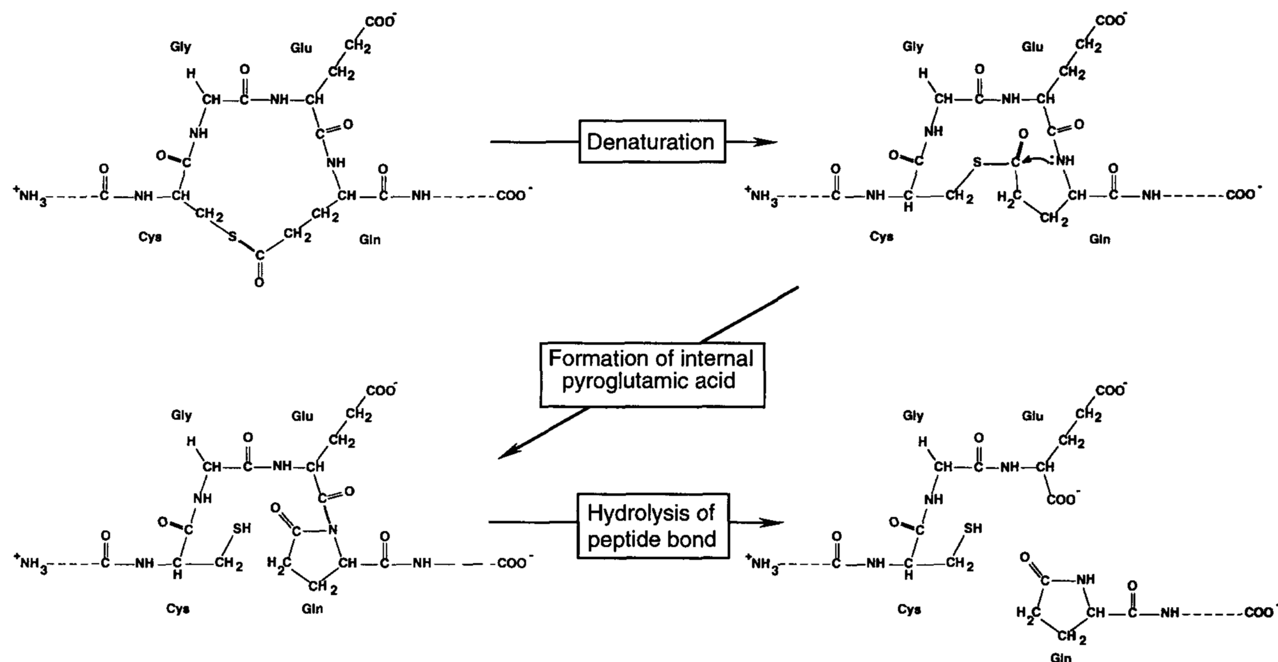


**Fig. 4.** Autolytic cleavage reaction. Note that this reaction only takes place if the protein is denatured extensively, for example, in SDS or high concentration of guanidine hydrochloride (>3 M). It is also the predominant reaction in the chemically synthesized thiolactone ring without the constraints of the rest of the protein (Erickson & Khan, 1983).

idues were involved in catalysis, other than those of the thioester, if not for the fact that humans have two C4 isotypes, which bind differently to hydroxyl and amino groups.

In the 1970s, it was observed that there was an increased incidence of some diseases, especially of an autoimmune nature, in individuals with certain histocompatibility types (for a recent review, see Campbell & Milner, 1993). Early studies focused on the human leukocyte class I and class II antigens, and their genes were mapped to the major histocompatibility complex (MHC), which is located on the short arm of chromosome 6 in humans. The class I genes and class II genes are at different ends of the MHC, and the region in between, designated class III, contains about 1 Mb of DNA. A number of polymorphic markers, one of which was C4, were mapped to the class III region. There are two C4 gene loci, arranged in tandem, coding for the isotypic proteins C4A and C4B (O'Neill et al., 1978; Carroll et al., 1984). Both C4A and C4B are highly polymorphic, with about 35 different allotypes described by the early 1980s (see Mauff et al., 1993). There are very few crossovers within the MHC, and the genes in the class I, II, and III regions are almost always inherited as haplotypes (Awdeh et al., 1983). There was speculation that there could be some selective advantage in certain haplotypes because specific combination of class I, II, and III allotypes were "compatible" with each other in some immune reactions (Porter, 1983, 1985). In particular, C4 null alleles (i.e., alleles corresponding to no expression of the protein in plasma) for both C4A and C4B are common, each with a frequency in the order of 10–15%. The presence of null alleles have been shown to correlate with the elevated incidence of certain autoimmune disorders, including systemic lupus erythematosus (see Hauptmann et al., 1988). Thus, there was great interest in studying the activity and determining the structure of the many C4 allotypes.

Awdeh and Alper (1980) first reported the higher specific hemolytic activity of C4B over C4A. Using purified proteins, the difference was determined to be about three- or fourfold (Isenman & Young, 1984; Law et al., 1984a). The basis of this difference in activity was shown to lie in the more efficient binding of C4B, when compared with C4A, to the hydroxyl groups of the carbohydrate-rich sheep erythrocytes, the standard target cell for assessing complement activity. Interestingly, when binding to the amino groups of the protein-rich immune complexes were studied, the reverse situation was found: C4A bound more efficiently than did C4B, indicating that the thioester of the two C4 isotypes mediated different kinds of binding reaction.

Studies of C3 and C4 binding to large surfaces, such as erythrocytes or antibody–antigen complexes, can give only qualitative data. It is not feasible to calculate kinetic parameters, because the effective concentration of the acceptor molecules on the target surface is impossible to determine. We therefore studied the covalent binding reaction in a fluid phase system, where C4 was activated in the presence of a small molecule by the complement component C1s (Law et al., 1984b; Dodds et al., 1985). Glycerol and glycine were chosen as the representative substrates bearing hydroxyl- and amino-groups, respectively. It was found that, although C4A bound very efficiently to glycine, C4B only did so with an efficiency of about 1% of that of C4A. Conversely, C4A binding to glycerol was difficult to detect, whereas C4B binding was detected easily. The $k_2/k_0$ values (see footnote b of Table 1) for C4A and C4B binding to glycine and glycerol are listed in Table 1.

It was fortuitous that we came across a monoclonal antibody, LOO3. When a mixture of C4A and C4B was loaded onto the LOO3-Sepharose column, C4A was eluted before C4B in a pH gradient (Dodds et al., 1985). We were thus able to purify many C4A and C4B allotypes and determine their binding efficiencies to glycine and glycerol. The main conclusion that could be drawn was that all C4A allotypes have the same binding specificities with respect to glycine and glycerol, as do all C4B allotypes (Dodds et al.,

**Table 1.** *Covalent binding properties of natural thioester proteins and engineered C4 variants*[a]

| | Residues 1101–1106 | $k_2/k_0$ ($M^{-1}$)[b] Glycine | Glycerol | Covalent linkage[c] |
|---|---|---|---|---|
| **Plasma proteins** | | | | |
| C4A (human) | PCPVLD | 13,400 | 1.3 | Amide |
| C4A (cattle) | PFPVMD | 17,700 | 2.0 | |
| C4B (human) | LSPVIH | 119 | 15.5 | Ester |
| C4B (cattle) | PCPVIH | 126 | 17.6 | |
| C4 (mouse) | PCPVIH | 136 | 12.3 | |
| C4 (guinea pig) | PCPVIH | 255 | 22.5 | |
| C4 (fin whale) | PHPVIH[d] | 575 | 13.6 | |
| C3 (human) | DAPVIH | 0 | 23.0 | Ester |
| $\alpha_2$M (human) | SGSLLN | 206 | 1.2 | Amide |
| $\alpha_1$I3 (rat) | SGSLLN | 5,500 | 1.5 | Amide |
| **Expressed C4 (on a C4B background)** | | | | |
| LS1101PC[e] | PCPVIH | 220 | 20.0 | Amide |
| LS1101DA[e] | DAPVIH | 125 | 15.0 | |
| LSPVIH1101SGSLLN[e] | SGSLLN | 15,000 | 1.0 | |
| L1101P | PSPVIH | | | Ester |
| S1102C | LCPVIH | | | Ester |
| H1106D | LSPVID | | | Amide |
| I1105L | LSPVLD | | | Amide |
| L1101P/H1106D | PSPVID | | | Amide |
| I1105A | LSPVAH | | | Amide |
| LS1101PC/H1106A | PCPVLA | 20,500 | 3.3 | |
| H1106A | LSPVIA | 22,400 | 3.3 | Amide |
| H1106K | LSPVIK | 65 | 0.3 | |
| H1106R | LSPVIR | 1,500 | 0.1 | |
| H1106S | LSPVIS | 5,500 | 0.7 | |
| H1106Y | LSPVIY | 850 | 6.5 | |

[a]Information compiled from Dodds and Law (1988, 1990), Carroll et al. (1990), Sepp et al. (1993), and Ren et al. (1993, 1995).
[b]Binding efficiency (BE), defined as the fraction of thioester protein bound with glycine or glycerol, was determined from experiments at concentrations of glycine or glycerol that give intermediate binding. $k_2$ is the second-order binding rate of the thioester proteins to glycine or glycerol and $k_0$ the first-order hydrolysis rate of the thioester. Values of $k_2/k_0$ are calculated by the formula $k_2/k_0 = BE/[G](1 - BE)$, where [G] is the concentration of glycine or glycerol (Law et al., 1981; 1984b).
[c]Determined by binding of the thioester proteins to sheep erythrocytes and tested for hydroxylamine sensitivity of the covalently bound complexes. Ester bonds are sensitive to hydroxylamine treatment whereas amide bonds are resistant (Carroll et al., 1990).
[d]S.K.A. Law (unpubl. information). Fin whale DNA was a generous gift from Dr. Bill Amos, Department of Genetics, University of Cambridge.
[e]The convention for the variants are the normal sequence followed by the position of the first residue followed by the variant sequence. The residues 1101–1106 of the LS1101PC variants are those found in mouse and guinea pig C4 and cattle C4B; the residues of LS1101DA are those in human C3, and the residues of LSPVIH1101SGSLLN are those in most $\alpha$-macroglobulins, including human $\alpha_2$-macroglobulin ($\alpha_2$M) and rat $\alpha_1$-inhibitor 3 ($\alpha_1$I3). Note that the binding properties of LS1101DA are different from human C3, and those of LSPVIH1101SGSLLN are different from human $\alpha_2$M but similar to rat $\alpha_1$I3. The low binding of human $\alpha_2$M may be attributed to its being a tetramer (Ren et al., 1995).

1985, 1986). DNA sequencing of a number of human C4 allotypes showed that there are polymorphic residues at 12 different positions, but only four of these segregate with the C4A versus C4B classification (Belt et al., 1985; Yu et al., 1986). These four residues are found in the hexapeptide sequence located in positions 1101 to 1106 (pro-C4 numbering): the sequence for C4A is PCPVLD, and that for C4B is LSPVIH. The primary structure of mouse C4 was also determined; it has a hybrid of the human C4A and C4B sequences in these six residues, PCPVIH (Nonaka et al., 1985). When the binding of mouse C4 to glycine and glycerol was studied, it was found to be indistinguishable from C4B (Dodds & Law, 1988). Thus, the residues responsible for the difference in the binding reactions were narrowed to two, IH in C4B and LD in C4A. Because it was unlikely that the hydrophobic residues, Ile and Leu, would contribute to a chemical reaction, it was most likely that the His/Asp substitution was accountable for the different binding properties of C4B and C4A. C4 was isolated from a number of mammalian species and their binding properties studied (Dodds & Law, 1990). In all cases, C4 with C4B-like binding properties were found. In addition, proteins with C4A-like binding properties were found in the primates, sheep, and cattle. For the proteins whose sequences, equivalent to positions 1101–1106 of human C4, are known, the binding data are shown in Table 1.

However, because the entire coding sequences of the various C4 types were not determined, it was possible that other residues in the undetermined regions may affect the binding reaction. That the Asp/His substitution was the only important change was proved by site-directed mutagenesis. When two C4 molecules, with a single Asp/His difference at position 1106, were expressed, they were shown to behave as C4A and C4B, respectively, as determined by erythrocyte binding (Carroll et al., 1990) and by glycine/glycerol binding (Sepp et al., 1993).

*There is only one catalyzed reaction*

How does the residue at position 1106 affect the binding specificity of the thioester, which is located at position 991–994? Originally, it was proposed that the His in C4B could act as a base to catalyze hydroxyl group binding, and that the Asp in C4A could promote amino group binding by deprotonation (Law et al., 1984a). This proposal was conceptually difficult to accept: it required two different amino acids, when replacing each other at the same position, to mediate two different reactions. However, when C4 variants, with an alanine at position 1106 (LS1101PC/H1106A and H1106A in Table 1), or in which the six residues of 1101–1106 were replaced by those of $\alpha_2$-macroglobulin, SGSLLN (LSPVIH 1101SGSLLN in Table 1), were constructed, they were found to have C4A-like binding properties (Sepp et al., 1993). Thus, the Asp of C4A is not necessary and it was argued that there was only one catalytic reaction, that mediated by the His at position 1106, for the reaction of C4B with hydroxyl nucleophiles, including water. When the His was replaced by an acidic residue, such as Asp, or neutral residues such as Ala and Asn, binding of hydroxyls to the thioester was not catalyzed and, as a consequence of the slower reaction with water, the binding of the more nucleophilic glycine appears to be more efficient. This interpretation, with catalyzed hydrolysis in C4B, predicts a difference in the hydrolysis rate of the thioester in C4A and C4B during activation (i.e., the value for $k_0$). This was demonstrated subsequently; the thioester hydrolysis rate in C4A was determined to be 0.068 $s^{-1}$ ($t_{1/2} \sim$ 10 s), and that of C4B was too fast to be determined ($t_{1/2} < 1$ s) (Sepp et al., 1993).

*The role of histidine 1106*

What is the role of the His at position 1106 in the hydrolysis and hydroxyl group binding of the thioester? His could act either as a base or a nucleophile. We therefore prepared a series of C4 variants with basic/nucleophilic residues at position 1106 (Ren et al., 1995). Of particular interests are two variants in which Tyr or Lys were substituted for the His. C4-H1106Y binds glycerol, although not quite as efficiently as C4B (Table 1). Thus, whatever mechanism we propose, it must satisfy the condition that it can be mediated by either His or Tyr. It is unlikely that His and Tyr could fulfill the same role in an acid/base reaction as the side chain $pK_a$ of the two residues are on opposite sides of neutrality. Fortuitously, C4-H1106Y has a very slow binding reaction, with a $t_{1/2}$ of about 90 s. This was slow enough that we were able to show that the free thiol of the thioester appeared before the binding to substrate took place, suggesting that the reaction proceeded through the formation of an intermediate. Using the C4-H1106K variant, which has very low binding activities, we were able to show that the $\epsilon$-amino group of the Lys attacked the thioester to form an intramolecular amide bond. Tryptic peptides were isolated and the covalent crosslinking of the Lys to the Gln residue of the thioester was confirmed by sequencing. We therefore concluded that the intermediate is formed by the nucleophilic attack on the thioester by the side chain of residue at position 1106 (Dodds et al., 1996).

However, a base is still required to catalyze the attack by substrates or water on the acyl-intermediate. But we have a base: the thiolate anion on the Cys of the thioester freed by this intramolecular nucleophilic attack. That the thiolate anion is the base was demonstrated by the ability of iodoacetamide, which reacts specifically with the thiolate anion, to inhibit the binding reaction of the very slowly reactive C4-H1106Y variant. Unfortunately, iodoacetamide inhibition could not be demonstrated in C4B; the very short half life of the intermediate in this protein ($t_{1/2} < 1$ s) meant that the reaction between iodoacetamide and the released thiol was too slow to have any effect. Iodoacetamide also had no effect on the binding reaction of C4A because no intermediate is formed in this reaction. The thiol is released concurrently with substrate binding, and therefore has no role in the reaction (Dodds et al., 1996). The binding reaction of C4B is illustrated in Figure 5. It should be noted that we have used the terms thiol and thiolate anion interchangeably, and indeed the two forms equilibrate, with a $pK_a$ of about 8, disregarding local environmental effects. It is the thiolate anion that acts as a base in the proposed reaction mechanism and it is also this form that reacts with iodoacetamide.

Iodoacetamide also inhibits glycine binding to C4-H1106Y. This may be accounted for by postulating that glycine binds both directly to the exposed thioester and to the acyl-phenol intermediate, in which case it is catalyzed by the thiolate anion. The binding of glycine to C4B and C4-H1106K is of similar efficiency, and this is likely to be due to the direct reaction of glycine with the thioester in competition with the residue at position 1106. Experiments are being performed to clarify these points.

Human C3 (and C3 from all other species sequenced to date) has a His at the position equivalent to residue 1106 of C4 (de Bruijn & Fey, 1985; Lambris et al., 1993). C3 forms predominantly ester bonds and reacts with glycerol with similar efficiency to that of C4B. However, C3 binding to glycine was only detectable at more alkaline pH (Law et al., 1984b), or at much higher glycine concentrations (Law et al., 1981). We propose that C3 binding involves an intermediate similar to that formed during C4B activation.

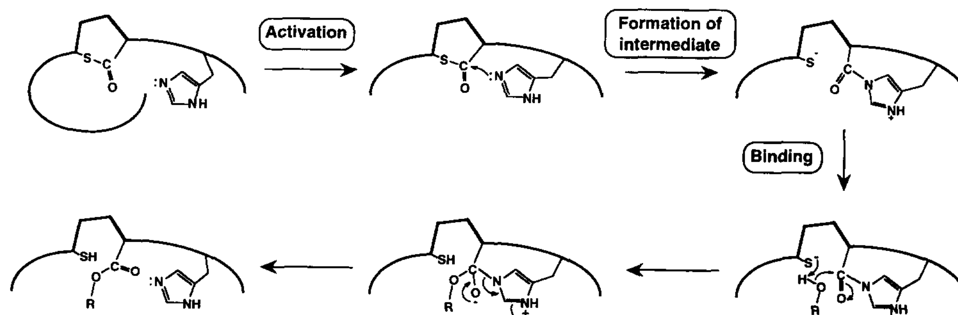


**Fig. 5.** Covalent binding reaction of C4B and most likely that of C3 (Dodds et al., 1996). The binding reaction of C4A and the $\alpha$-macroglobulins is more closely represented by that shown in Figure 3B.

That it has very poor binding to glycine may reflect a faster rate in the formation of the intermediate. Preliminary data have been obtained in support of this conjecture. A C3-H1126D variant was constructed and expressed, and was shown to bind glycine at very low concentrations, but to bind glycerol poorly (unpubl. obs.). Thus, by changing the His to an Asp, we have converted C3 from a molecule with C4B-like binding properties to one that is C4A-like.

*The dual role of the thioester*

It is a common experience among researchers on the thioester proteins that, after activation, the free thiols of C3 and C4 are very difficult to detect. It requires the immediate presence of thiol-specific reagents during activation to give a quantitative titration. An experiment was re-enacted to emphasize this point. The thiol-specific reagent, $^3$H-iodoacetic acid, was added at different times after the activation of C4 by C1s. More than 60% of the free thiol became undetectable after 15 min (Fig. 6). Thus, the thioester, in addition to keeping the acyl group activated, also serves the purpose of protecting the thiol group from oxidation. The thiol is only released upon activation, when the His at position 1106 attacks the thioester to form an acyl-imidazole intermediate. The thiolate anion then acts as a base, within a fraction of a second, to catalyze the hydrolysis and hydroxyl binding reactions (Dodds et al., 1996).

*Binding selectivity of C3*

Thus far, we have focused on C3 and C4 binding reactions at the primary level in that they react universally with nucleophilic groups (mainly hydroxyl and amino groups on biological targets). A question we must address is whether there is selectivity of the binding at higher levels. The next level of selectivity was examined by studying the effect of molecular structures immediate to the hydroxyl or amino groups. Studies using alcohols, monosaccharides, and amino acids, either by direct binding (Law et al., 1981, 1984b) or by inhibition (Law et al., 1984b; Sahu et al., 1994), showed that C3 binds differently to the various simple molecules (Table 2). The binding to primary alcohols is preferred over secondary alcohols, and the binding to $t$-butanol is difficult to detect. This general trend is also observed when studying the binding of monosaccharides; removal of the primary hydroxyl group at $C_6$ leads to less efficient binding, and inositol, bearing only secondary hydroxyl groups, is a very poor acceptor molecule. However, this formulation does not apply in the case of the amino acids serine and threonine, where the secondary hydroxyl group of threonine appears to bind more effectively than the primary hydroxyl group of serine (Law et al., 1981; Sahu et al., 1994). Furthermore, the phenolic group of tyrosine appears to be even more potent (Sahu & Pangburn, 1995). A much more complex pattern of binding was observed when a series of peptides containing various combinations of serine, threonine, and tyrosine (Sahu & Pangburn, 1995), and other synthetic compounds (Sahu & Pangburn, 1996), were used, and it is difficult to generalize upon the preferred features to which activated C3 binds.

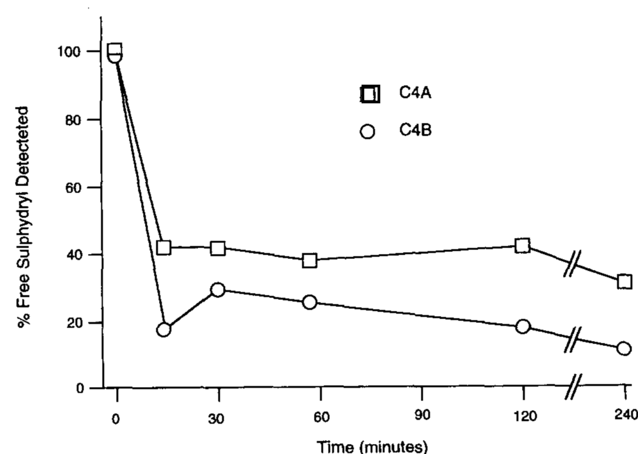


**Fig. 6.** Level of detectable free thiol after the activation of C4A and C4B. C4A and C4B were incubated with C1s under conditions such that the $t_{1/2}$ of activation is about 3 s (Sepp et al., 1993). $^3$H-iodoacetic acid was added to label the free thiol after the indicated period of time. Radiolabeled iodoacetic acid was included in the sample of $t$ = 0 s (for 100% detection) before the addition of C1s. More than 60% of the free thiol became undetectable after 15 min.

**Table 2.** *Substrate selectivity of C3 covalent binding*

| Substrates | $k_2/k_0$ ($M^{-1}$)[a] | Relative[a] reactivity |
|---|---|---|
| **Alcohols** | | |
| Methanol | 16.8 | 404 |
| Ethanol | 17.0 | 314 |
| 1-Propanol | 18.8 | 492 |
| 1-Butanol | 18.0 | 419 |
| Glycerol | 15.0 | 579 |
| Ethylene glycol | | 377 |
| 2-Propanol | 4.2 | 141 |
| 2-Butanol | 3.3 | |
| $t$-Butanol | 0.0 | 55 |
| **Monosaccharides** | | |
| Glucose | 5.5 | 336 |
| 2-Deoxyglucose | | 618 |
| 6-Deoxyglucose | | 236 |
| Mannose | 10.0 | 491 |
| Rhamnose (6-deoxymannose) | | 347 |
| Galactose | 2.6 | 360 |
| Fucose (6-deoxygalactose) | 4.9 | 179 |
| Inositol | 0.8 | 56 |
| **Amino acids** | | |
| Serine | 8.2 | 366 |
| $O$-Methylserine | 1.9 | 0 |
| Threonine | 19.7 | 1,575 |
| $O$-Methylthreonine | | 87 |
| Tyrosine | | 17,273 |

[a]See Table 1 for definition of $k_2/k_0$. Data compiled from Law et al. (1981, 1984b)

[b]Relative reactivity to water, data compiled from Sahu et al. (1994), and Sahu and Pangburn (1994, 1995). The value of relative reactivity is that to water and is related to $k_2/k_0$ ($M^{-1}$) by a factor of 55 (M) for the concentration of water.

At the macromolecular level, the covalent binding of C3 to immune complexes had been studied. It was shown that C3b mainly bound to the antibodies (Gadd & Reid, 1981) and at multiple sites (Anton et al., 1989, 1994). Using heat-aggregated IgG, obtained from a myeloma patient, Shohet et al. (1993) showed that C3b, activated via the alternative pathway, bound to one major region of the heavy chain of IgG containing six potential acceptor sites, in the form of serine and threonine residues. Sahu and Pangburn

(1994) synthesized variants of this peptide and demonstrated that all six potential sites contributed to C3b binding with varying degree of efficiency. C4 (a mixture of C4A and C4B) binding to ovalbumin/anti-ovalbumin complexes was also studied (Alcolea et al., 1987). It was found that C4 bound to both the heavy and light chains of the antibody. Both hydroxylamine-resistent (64%) and hydroxylamine-sensitive (36%) covalent complexes were detected, as expected from a C4A and C4B mixture. Taken together, these experiments suggest that C3 and C4 do not bind to a single specific residue on the antibody molecule.

It may be expected that C3b and C4b should have a specific interaction in the formation of the C5-convertase, the C4b2a3b complex, of the complement classical pathway. C3 is cleaved by the C4b2a enzyme on the target surface, and some is incorporated to form the C4b2a3b complex. It was demonstrated that C3b binds covalently to C4b and that the binding is specific to a single serine residue (Kozono et al., 1990; Kim et al., 1992). However, it was also demonstrated that this particular binding is not necessary. A mutant C4, in which the serine residue was replaced so that the particular covalent bond between C3b and C4b could not be formed, could nonetheless form a functional C5-convertase (Kim et al., 1992). Thus, at least in vitro, the formation of the C5-convertases does not depend on covalent binding between C4b and C3b in the C4b2a3b complex. The covalent bond is formed because it is possible, not because it is necessary. Similarly, the $C3b_2Bb$ complex, the C5-convertase of the alternative pathway, was shown to contain two covalently bound C3b molecules. The residue on the acceptor C3b has not been identified (Kinoshita et al., 1988; Hong et al., 1991).

*The natural release of covalently bound C3b from targets*

When the covalent nature of the bond between C3b and target cell macromolecules was first proposed (Law & Levine, 1977), it was greeted with mixed enthusiasm. Although it was consistent with our key experimental data and with data obtained previously by others on the very tight nature of the binding, there was nonetheless the puzzling result that C3b can be released from cell surfaces (Bhakdi et al., 1974). If sheep erythrocytes bearing C3b were incubated at 37 °C, there was a slow release into the supernatant of C3b, but not in the form of high molecular weight covalently linked complexes (Venkatesh et al., 1984). Using C3b-glycerol complexes as the starting material, it was found that the release was prevented when the protein was denatured. The release was pH and temperature dependent, and only occurred in ester-bonded complexes (Venkatesh et al., 1984; Venkatesh & Levine, 1988). Similar rates of release were observed for C3b covalently bound to IgG aggregates or to tyrosine (Sahu & Pangburn, 1995). With the elucidation of the binding mechanism, we can speculate that this release is analogous to the binding reaction. The substrate could be released from C3b by the attack of the His at the position equivalent to C4-1106 on the ester bond.

*Biosynthesis of the internal thioester*

How is the thioester synthesized? Is the folding of the native protein sufficient to bring about the condensation of Cys and Gln to form the thioester, or are other molecules required to catalyze the process? Both alternatives have found support from experimental evidence, but they are not mutually exclusive. Treatment of the thioester proteins with ammonia breaks the thioester bond, to give the original Cys and Gln residues. The accompanying conformational change, to a C3b-like state, however, occurs over 10 h (Isenman et al., 1981). Pangburn (1992a) was able to remove the excess ammonia from C3 before the conformational change became extensive, and isolate an intermediate conformer that was able to reform the thioester. These experiments were repeated, although less successfully, for C4 and $\alpha_2$-macroglobulin (Pangburn, 1992b). Thus, it was concluded that the condensation of the Cys and Gln can take place in the absence of other proteins.

Isaac and Isenman (1992) studied the formation of the thioester in C3 by site-directed mutagenesis of residues near the thioester site. Four types of products were found (Table 3). Two of these were trivial: they either behaved like the wild-type C3 or were unstable. The third and fourth types of mutants could not form a thioester. However, because they could be cleaved by the C4b2a enzyme, which is known to cleave C3 into C3a and C3b only if the C3 has a native conformation (Janatova et al., 1980b), it was concluded that the convertase cleavage sites of these proteins had been folded correctly and that the gross structure of the proteins was probably C3-like rather than C3b-like. The third group could not form the thioester because either the Cys or the Gln had been changed. Results from these suggest that the thioester is not necessary to maintain the native conformation of the proteins, although this contradicts the observation that a slow conformational change occurs when the thioester is cleaved in the native protein. It is therefore probable that, although the C3 convertase cleavage site is folded correctly, the conformation of these mutants is slightly different from that of the native molecule. From these observations, it is not surprising to find that some members of this family do not have a thioester. For example, ovostatin, an $\alpha$-macroglobulin of the chicken, does not have an internal thioester because the Cys of the CGEQ sequence is replaced by an Asn (Nielsen et al., 1993). It is, however, able to act as a protease inhibitor by a protease-initiated conformational change, and so probably also has a native-like conformation (Nagase & Harris, 1983). Members of the fourth group have the CGEQ sequence, but the thioester is not formed. This suggests that folding the protein into the correct conformation is not sufficient for thioester formation to occur. When considered in conjunction with the reformation of the thioester upon limited unfolding (Pangburn, 1992a, 1992b), this further suggests that local residues are crucial in guiding the Cys and Gln to a suitable spatial orientation, and/or play a critical part in the chemical mechanism of the condensation reaction. All proteins in the third and fourth groups, although folded correctly at least partially, are hemolytically inactive, thus re-emphasizing the absolute necessity for the thioester and covalent binding in the biological functions of the complement proteins.

The biosynthesis of the thioester may require residues outside the immediate proximity of the thioester. C4d is a natural degradation product of C4, which becomes detached upon cleavage (Fig. 2B) and therefore may be presumed to have a self-contained structure. We constructed a cDNA of C4d and expressed it in the Chinese hamster ovary cells. Because we have expressed functional C4 in this system (Sepp et al., 1993; Ren et al., 1995), it may be assumed that all of the necessities for thioester biosynthesis are present. C4d was purified and the tryptic peptide containing the thioester site was isolated. The sequence GCEQTMIYL was detected. Significantly, we could not detect the GCEETMIYL sequence with a glutamic acid at the fourth position instead of a glutamine, which would have been the case if the thioester had been formed and then hydrolyzed. Thus, if the thioester bond is

**Table 3.** *C3 thioester region mutants and their properties*[a]

Human C3 sequence from positions 1007 to 1020

| 1007 | 1008 | 1009 | 1010 | 1011 | 1012 | 1013 | 1014 | 1015 | 1016 | 1017 | 1019 | 1020 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| P | S | G | C | G | E | Q | N | M | I | G | M | P |

| Recombinant C3 molecules | Internal thioester | C4b2a[b] sensitivity | Hemolytic activity |
|---|---|---|---|
| **I Normal C3** | | | |
| Wild type | Yes | Yes | 1.0 |
| G1009A | Yes | Yes | 0.9 |
| G1009S | Yes | Yes | 0.9 |
| M1015F | Yes | Yes | 0.7 |
| **II Unstable C3**[c] | | | |
| M1015A | No | No | Undetectable |
| **III Defective C3 mutants with modified C1010 or Q1013** | | | |
| C1010A | No | Yes | Undetectable |
| Q1013N | No | Yes | Undetectable |
| **IV Defective C3 mutants with CXXQ sequence retained** | | | |
| P1007G | No | Yes | 0.07 |
| E1012Q | No | Yes | Undetectable |
| P1020G | No | Yes | Undetectable |

[a]Results from Isaac and Isenman (1992), the modified cDNA clones of C3 were transfected into COS cells and the secreted products were analyzed.
[b]Only C3 with the wild-type conformation can be cleaved by the C4bC2a enzyme complex.
[c]SDS-PAGE analysis of the C3 immunoprecipitate from the culture supernatant did not show the two chain pattern of C3. The protein was probably folded incorrectly and degraded extensively before secretion.

formed at all, we estimate the efficiency to be less than 0.1% (unpubl. obs.).

Although the thioester can be reformed in ammonia-treated C3 (Pangburn, 1992a), it is not clear whether the intermediate conformer lies in the biosynthetic pathway of the internal thioester. In addition, even if it does, it does not preclude the requirement for other proteins, perhaps in the form of chaperones, for attaining the structure of the intermediate conformer. C3 deficiency has been reported in the guinea pig. It was found that their C3 mRNA has the wild-type coding sequence (Auerbach et al., 1990), suggesting that the deficiency is not due to a defect in the C3 gene, but in some other protein that is needed for the biosynthesis of a functional C3. It is interesting to note that the C3-deficient guinea pigs are not deficient in C4 or $\alpha$-macroglobulin (Auerbach et al., 1990). Thus, if there is a protein required for the formation of the thioester in C3, it is not the same protein for thioester biosynthesis in C4 and $\alpha$-macroglobulin.

### *The biological significance of the catalyzed hydrolysis reaction*

It is probable that the thioester proteins of the complement system evolved from an ancestral protein more similar to the present day $\alpha$-macroglobulins (Dodds & Day, 1993). $\alpha$-Macroglobulins have an uncatalyzed binding reaction, having an Asn at the residue equivalent to C4-1106. When proteases cleave the $\alpha$-macroglobulins, the thioester can react with the $\epsilon$-amino groups of the lysine residues on the proteases to form amide-linked complexes. The covalent binding of the $\alpha$-macroglobulin to proteases inhibits their activities by hindering their access to substrates. Most of the $\alpha$-macroglobulins are homodimeric or homotetrameric complexes and these are more efficient in "trapping" proteases when activated (see Chu & Pizzo, 1994). In fact, the covalent binding reaction is probably not necessary except in the monomeric $\alpha$-macroglobulins. This is well illustrated by chicken ovostatin, a tetrameric $\alpha$-macroglobulin (Nagase et al., 1983) that does not have a thioester (Nielsen et al., 1993), but-nonetheless functions as a protease inhibitor (Nagase & Harris, 1983). In these cases, the gross conformational change induced by proteolytic activation is more important in the function of the $\alpha$-macroglobulins. By treating human $\alpha_2$-macroglobulin with porcine pancreatic elastase, Chu et al. (1991) were able to follow the kinetics of the appearance of the free thiol, and determined the $t_{1/2}$ to be ~7 s.

In 1966, Müller-Eberhard and co-workers studied the binding of C3b to sheep erythrocytes bearing the classical pathway C3-convertase, the C4b2a enzyme. They found that the binding was only about 10% effective: i.e., only about 10% of the C3b generated became bound to the erythrocytes. However, if the C4b2a complex was added in the fluid phase, C3 conversion, although proceeding normally, did not lead to C3 deposition on bystander sheep erythrocytes (Müller-Eberhard et al., 1967). This led them to propose the concept of the "labile binding site." A time limit is imposed on the activated C3b molecule, and the rate of diffusion puts a limit on the effective range of the reactive protein from the site of activation.

Now we have determined the chemical reaction required for such a "labile binding site." After the divergence of the complement proteins from the present day $\alpha$-macroglobulins, they evolved to make use of a histidine residue to promote binding to hydroxyl groups on the carbohydrate moieties on surfaces of pathogenic microorganisms. Catalyzed hydrolysis in the medium became the restricting reaction. C4A does not have a histidine at position 1106 and binds predominantly to amino groups. However, C4B must be considered the more universal form of C4 because it is found in all mammals studied. C4A, on the other hand, is found in a limited

number of species and only in addition to C4B (Dodds & Law, 1990). It may therefore be regarded as a suboptimal C4 that has lost the histidine, and hence its catalytic activity with hydroxyl groups, and is only tolerated in species that also have genes for C4B (Law & Dodds, 1996).

The $t_{1/2}$ of activated $\alpha_2$-macroglobulin and C4A of ~10 s (Chu et al., 1991; Sepp et al., 1993) is extremely short in comparison with that of the synthetic thiolactone ring, which is ~10 h (Erickson & Khan, 1983). Thus, there are still some features missing to account for the high reactivity of the internal thioester. In the absence of further fortuitous clues, as was the case in C4A and C4B, the delineation of these features must await the 3D structural determination of the thioester proteins.


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