Native conformations of human complement components C3 and C4 show different dependencies on thioester formation

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The thioester bond in complement components C3 and C4 and the protease inhibitor α_2 -macroglobulin have traditionally been thought of as fulfilling the dual roles of mediating covalent attachment and maintaining the native conformational states of these molecules. We previously reported that several human C3 thioester-region mutants, including variants E1012Q and C1010A, in the latter of which thioester-bond formation is precluded, display an unexpected phenotype. Despite the lack of a thioester bond in these mutants, they appear to adopt a native-like conformation as suggested by the finding that they are cleavable by the classical pathway C3 convertase, C4b2a, whereas the C3b-like C3(H₂O) species is not. Subsequently, a species referred to as C3(NH₃)* was described which potentially could account for the observations with the above mutants. C3(NH₃)* is a transient species formed on aminolysis of native C3 that can spontaneously re-form the thioester bond. Importantly, it has a mobility on cation-exchange HPLC that is distinct from both native C3 and C3(H₂O), but like the native molecule, it is cleavable by an alternative-pathway C3 convertase. In this study

we showed by using cation-exchange HPLC as an additional conformational probe that C3 C1010A and E1012Q mutant proteins did not resemble C3(NH₃)*. Instead they displayed a chromatographic behaviour that was indistinguishable from that of native C3. To assess the general applicability of these observations, we engineered the equivalent mutations into human C4, specifically C4 C1010A and C4 E1012Q. As expected, thioester-bond formation did not occur in either of these C4 mutants, but in contrast with the results with C3 we found no evidence for the formation of a stable native-like conformation in either C4 mutant, as assessed using cleavability by C1s as the conformational probe. A possible interpretation of our data is that the adoption of the native conformational state during biosynthesis of C3 and C4 is an energetically permissible process, even if it is not locked in via thioester-bond formation. Whereas this conformational state is stable in mature C3, it is unstable in mature C4, perhaps reflecting the additional post-translational cleavage of C4 before its secretion.

INTRODUCTION

The complement proteins C3 and C4 and the plasma protease inhibitor α_2 -macroglobulin possess an intramolecular thioester bond that is formed between the side chains of cysteine and glutamine within the sequence CGEQ. This thiolactone ring structure, which is similarly positioned within the primary sequences of the three molecules (residues 1010–1013 for both prepro-C3 and prepro-C4 and residues 972–975 for prepro- α_2 -macroglobulin) is essential for the binding of activated forms of these proteins to their targets. For the complement proteins, this involves covalent attachment via a transacylation mechanism to a nucleophile on the surface of a micro-organism or immune complex (reviewed in [1]). Whereas covalent attachment between the target protease and α_2 -macroglobulin can also occur, this is not obligatory for the inactivation of the protease. Instead, the protease is 'trapped' as a result of a conformational change in the molecule subsequent to proteolysis in the 'bait' region (reviewed in [2]). Similarly, conformational changes in C3 and C4 accompany activation by their respective convertases and result in acquisition of numerous protein-binding sites not present in the native molecule. Since for all three molecules essentially

the same conformational changes were induced by direct thioester cleavage in the absence of proteolysis, this led to the hypothesis that the thioester was essential for maintaining what amounted to a quasistable conformational state in the native molecules (see for example [3,4]).

The nature of the conformational changes in C3 and C4 that accompany thioester-bond cleavage has been characterized by biophysical, biochemical and immunological criteria. For example, whereas native C3 and C4 are cleavable by their respective convertases (C4b2a or C3bBb for C3 and C1 for C4), this property is lost on direct thioester cleavage by either a small nucleophile such as methylamine or solvolysis after treatment with mild chaotropic agents such as KBr [5–7]. Similarly, such treatments lead to changes in the respective near-UV CD spectra and in the surface hydrophobicities of both C3 and C4, the latter property being monitored by the binding of the environment-sensitive fluorescent probe 8-anilino-1-naphthalene sulphonate [3]. By either of these spectral criteria, the kinetics of the conformational change after thioester scission were complex and suggested the presence of intermediate conformational states. In the case of C3, the presence of conformational intermediates was also observed using cation-exchange HPLC at a pH near the

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum.

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isoelectric point [8]. More recently, a molecule referred to as C3(NH₃)* was found to have a characteristic migration on cation-exchange HPLC that was distinct from both native C3 and end-state conformation thioester-cleaved C3. C3(NH₃)*, produced on aminolysis of the thioester, was able to refold to the native conformational state and re-form the thioester bond after removal of the ammonia nucleophile [9]. In contrast, it is not possible to re-form the thioester if one starts with the end-state conformational species produced by aminolysis [i.e. C3(NH₃)*]. One interpretation of these observations is that C3(NH₃)* represents a conformational species that is almost native-like, and that, once this conformation is achieved, thioester-bond formation can occur spontaneously. The fact that the C3(NH₃)* species is cleavable by a nephritic-factor-stabilized alternative-pathway C3 convertase (M. K. Pangburn, unpublished work) lends further support to this species having a native-like conformation.

In a previous study on the structural requirements for formation of the thioester bond in C3 [10], we employed site-directed mutagenesis to produce several variant C3 molecules in which highly conserved amino acids in the vicinity of the thioester bond were mutated. Some of these mutants, including C1010A in which thioester formation was formally precluded, possessed an unexpected phenotype. Although these recombinant molecules lacked haemolytic activity and the ability to form denaturation-induced autolytic fragments, as would be expected for C3 molecules lacking a thioester bond [11], they could still be cleaved by the fluid-phase C3 convertase C4b2a. Of the human thioester-bond-containing molecules, this was the first example of an 'uncoupling' between the previously strict relationship of thioester-bond integrity and maintenance of the native conformational state. We proposed at the time that, if the thioester bond fails to form during C3 biosynthesis, a conformational end state could still be achieved that was sufficiently native-like to permit cleavage of the C3 α -chain by C3 convertase. In view of the subsequently described behaviour of the C3(NH₃)* species, it seemed possible that the conformation adopted by the thioester-deficient recombinant molecules such as C3 C1010A might resemble the C3(NH₃)* species, rather than the true native conformational state.

In this study we employed cation-exchange HPLC as an additional probe that might further define the nature of the conformation adopted by the C3 mutant molecules C1010A and E1012Q. In addition, we asked whether introducing the same mutations into C4 would give rise to a similar native-like phenotype, even in the absence of thioester-bond formation. Our results indicate that C3 mutants C1010A and E1012Q adopt a conformation that is indistinguishable from that of the native molecule by the additional criterion of cation-exchange HPLC. However, this does not appear to be a general phenomenon as the introduction of similar mutations into a C4 background yields a phenotype that is inconsistent with the mutant molecule having adopted a native-like conformation.

EXPERIMENTAL

Expression of recombinant C3 and C4B

The construction of C3 thioester-region mutants C1010A and E1012Q in the expression vector pSV-C3 has been described previously [10]. The equivalent C4 mutants were constructed in the expression plasmid pSVC4B (i.e. a cDNA coding for the B isotype of C4) via the gapped plasmid method [12,13]. For stable expression of recombinant C3, the full-length cDNAs were excised with *Hind*III and *Xho*I and blunt-end cloned into the

*Bam*HI site of the expression vector pKG5-En [14]. Linearized versions of these plasmids were transfected into the mouse myeloma cell line J558L and stable transfectants were selected and grown as previously described [14]. For the use of culture supernatants as a source of recombinant C3 in HPLC experiments, 2×10^6 cells were resuspended in 2 ml of Dulbecco's modified Eagle's medium (DMEM) containing 2 mM glutamine, 100 U/ml penicillin, 1 % Nutridoma (Boehringer-Mannheim Biochemicals) and 2 % fetal calf serum (FCS), and supernatants were harvested after 48 h. For some experiments, to avoid cleavage of C3b into iC3b by bovine factor I and cofactor H, K76COOH-treated FCS (K76COOH from Otsuka Pharmaceutical Co., Japan) was used as the serum supplement of the culture medium [14], as such treatment of the serum irreversibly inactivates bovine factor I. Recombinant C4 molecules were transiently expressed in COS-1 cells as described [15], except that, where noted, K76COOH-treated FCS was used in the culture medium instead of FCS that had only been heat-inactivated.

Metabolic labelling

Cell cultures of stable C3 transfectants in J558L were expanded in DMEM supplemented with 4 mM glutamine, 10 % heat-inactivated FCS, 100 U/ml penicillin and 100 μ g/ml streptomycin. Before metabolic labelling, 2×10^6 cells were transferred to 1 ml of methionine- and cysteine-free DMEM (ICN) containing 10 % heat-inactivated FCS, 4 mM glutamine, 1 % Nutridoma, 100 U/ml penicillin and 100 μ g/ml streptomycin. After a 1 h incubation to deplete endogenous methionine and cysteine, the medium was supplemented with 100 μ Ci of [³⁵S]methionine and [³⁵S]cysteine (Tran³⁵S-label; ICN). After 5 h, 1 ml of complete DMEM was added and incubation was continued for 18 h. Culture supernatants were split into four equal volumes for the following treatments: (a) direct immunoprecipitation; (b) treatment with C3 convertase (C4b^{oxy}2a) followed by immunoprecipitation; (c) pretreatment with KBr followed by immunoprecipitation; (d) pretreatment with KBr, and with C3 convertase, followed by immunoprecipitation. The treatment with KBr consisted of incubating the samples with 2 M KBr for 3 h at 37 °C, followed by dialysis against 4 mM sodium barbital, pH 7.2, containing 150 mM NaCl, 0.15 mM CaCl₂ and 0.5 mM MgCl₂. For the untreated and KBr-treated samples that were immunoprecipitated, boiling in SDS sample buffer was first carried out under non-reducing conditions to induce autolytic fragmentation [10]. Immunoprecipitations were carried out as described previously, as was the treatment of the samples with fluid-phase C4b^{oxy}2a [10]. All samples were analysed by SDS/PAGE on 8 % gel under reducing conditions [16]. The stained and fixed gels were impregnated with 1 M sodium salicylate and bands were visualized autofluorographically. Where noted in the text, similar immunoprecipitations were carried out on recombinant C3-containing supernatants in which the media employed contained 2 % K76COOH-treated FCS and 1 % Nutridoma in place of the standard 10 % FCS.

Metabolic labelling of COS-1 cells transiently transfected with pSVC4B derivatives was performed as described previously [15]. Radiolabelled culture supernatants were subjected to the analogous four immunoprecipitation pretreatments described above for C3, except that the convertase employed was C1. C1 treatment was with 5 μ g/0.5 ml of culture supernatant for 1 h at 37 °C.

Cation-exchange HPLC chromatography

The concentration of recombinant C3 in the culture supernatants was determined by RIA [10], and a volume corresponding to

200 ng (usually about 200 μ l) was diluted 20-fold with 50 mM sodium phosphate, pH 6.0. After passage through a 0.45 μ m Acrodisc-3 filter (Gelman Sciences) to remove particulate matter, the sample was injected on to a Mono S HR 5/5 FPLC column (Pharmacia Biosystems) equilibrated in 50 mM sodium phosphate (pH 6.0)/75 mM NaCl and running at 1.0 ml/min on a Beckman System Gold HPLC. On sample injection, a solvent program was initiated which washed the column with starting buffer for 5 min and generated a linear NaCl gradient from 75 to 375 mM over a period of 20 min. This was followed by a sharp step to 500 mM NaCl for 5 min and finally re-equilibration with the starting buffer (i.e. 75 mM NaCl). Where noted in the text, a modified version of this gradient was used in which the starting buffer was 50 mM sodium phosphate, pH 6.0, with no NaCl, and after a 5 min wash, a linear gradient from 0 to 375 mM NaCl was generated over a period of 20 min. In all cases, the flow rate was 1 ml/min and sixty 0.5 ml fractions were collected for analysis by capture ELISA.

The Mono S column was calibrated with plasma-derived purified native C3 as well as with this protein after treatment with trypsin to generate C3b (1%, w/w; 37 °C; 5 min; followed by an equal weight of soya-bean trypsin inhibitor) and KBr (as described in a previous section) to generate C3(H₂O). C3(NH₃)* was generated by treatment of native C3 with 100 mM NH₄Cl/100 mM NaCl, pH 8.2, at 4 °C for 40 min. Each C3 species (125 μ g) was loaded on to the column, fractions were collected and the elution profiles were monitored at 230 nm. Because of the sensitivity of the ELISA to residual background from these calibration runs, before analysis of culture supernatants containing the recombinant proteins, the column was stripped with the following sequence of solvents (500 μ l of each): 2 M NaCl; 0.5% SDS in 0.5% NaOH; 2 M NaOH; 75% acetic acid; 50 mM sodium phosphate (pH 6); 500 mM NaCl. Finally the column was re-equilibrated with 10 column vol. of 50 mM sodium phosphate (pH 6)/75 mM NaCl.

C3 capture ELISA

Microtitre plates were coated with 100 μ l or rabbit anti-(human C3c) IgG (Sigma) at 10 μ g/ml in 10 mM NaHCO₃, pH 9.8, for at least 12 h at 4 °C. The plates were blocked with 250 μ l/well PBS/Blotto/Tween [10 mM sodium phosphate, 150 mM NaCl, 0.05% Tween 20, 0.02% NaN₃ and 5% (w/v) skimmed milk powder] for 90 min at 37 °C. The wells were washed five times with PBS/Blotto/Tween and then incubated with 200 μ l of each column fraction for 2 h at 37 °C. The wells were washed five times with PBS/Blotto/Tween and then incubated with 100 μ l of goat anti-(human C3c) IgG (Quidel; diluted 1:1000 in PBS/Blotto/Tween) for 2 h at 37 °C. The plates were again washed five times with PBS/Blotto/Tween and incubated with 100 μ l/well of alkaline phosphatase-conjugated rabbit anti-goat IgG (Jackson Immunologicals; diluted 1:2000 in PBS/Blotto/Tween) for 2 h at 37 °C. The plates were washed five more times as above and then 200 μ l of 0.5 mg/ml *p*-nitrophenyl phosphate diluted in 100 mM diethanolamine/0.5 mM MgCl₂/0.02% NaN₃, pH 9.8, was added to each well. After approx. 15 min, the colour change was read at 405 nm on a Bio-Rad microtitre plate reader.

Haemolytic assays

C3- and C4-specific haemolytic assays were performed as described previously [14,15].

RESULTS

Characterization of wild-type C3 and the thioester-region mutants C1010A and E1012Q when stably expressed in J558L cells

We have previously characterized a series of thioester-region mutants (C1010A, E1012Q, P1007G, Q1013N and P1020G), which, despite lacking a thioester bond, displayed the unusual property of being cleavable by classical-pathway C3 convertase (C4b^{oxy}2a) [10]. Since this is a property that is normally associated with thioester-intact native C3, but not C3 in which the thioester has been deliberately cleaved, it suggested that, even in the absence of a thioester, this series of mutant C3 molecules possessed a native-like conformation. The ability to separate conformational isomers of C3 using a Mono S cation-exchange column [8,9,17] provided an alternative approach to assessing the conformational state of the above series of thioester-region mutants of C3. To have sufficient and continuously available quantities of recombinant protein for such analyses, it was necessary to generate stable cell lines expressing these C3 molecules. We have therefore subcloned wild-type, C1010A and E1012Q C3 cDNAs into the neo cassette-containing plasmid pKG5-En [14], and these vectors were used to transfect mouse myeloma J558L cells as previously described. To confirm that the protein phenotypes were the same as we observed previously for protein derived from transiently transfected COS-1 cells, the stable cell lines were metabolically labelled and the supernatants were subjected to a number of treatments, followed by immunoprecipitation with rabbit anti-(human C3) IgG (Figure 1). The recombinant C3 proteins stably expressed by transfected J558L cells behaved very much as described for transfected COS-1 cells inasmuch as the 47 kDa autolytic fragment was exclusively seen in the C3 wild-type supernatant (the 72 kDa autolytic fragmentation product co-migrates with β -chain and could therefore

Figure 1 Functional profiles of wild-types C3 and its thioester-region mutants C1010A and E1012Q stably expressed in J558L cells

Metabolically labelled culture supernatants, harvested in medium containing non-K76C00H-treated FCS, were either directly immunoprecipitated with rabbit anti-(human C3c) serum or immunoprecipitated after pretreatment with C4b2a. The same procedures were also performed on supernatants that had been pretreated with 2 M KBr to deliberately hydrolyse the thioester bond (for details see the Experimental section). SDS was incorporated into the immunoprecipitated material under autolytic conditions, after which the samples were reduced and analysed by SDS/PAGE (8% gel), followed by autofluorography.

Figure 2 Separability of C3 species on a Mono S column

Human C3 that had been purified from plasma was treated with trypsin to generate C3b, with KBr to generate C3(H₂O), or NH₄Cl to generate C3(NH₃)^{*}. Untreated or treated samples of purified human C3 were diluted 20-fold in 50 mM phosphate buffer, pH 6.0, and analysed on a Mono S column. Sixty 0.5 ml fractions were collected and the absorbance of each fraction was measured at 230 nm (see the Experimental section for further details of treatments and chromatographic conditions). Native C3 was eluted at 6.0 ml, C3b at 4.0 ml, C3(H₂O) at 23.5 ml and C3(NH₃)^{*} at 16.0 ml.

not be unambiguously detected). The 47 kDa autolytic fragment was not detected in C3 wild-type if the thioester bond was deliberately hydrolysed as a result of pretreatment with KBr. We also studied the cleavability of these molecules by fluid-phase C3 convertase, with and without prior KBr treatment. In keeping with the previous results with COS-1 cells, the α -chains of this series of mutant C3 molecules were cleavable by C4b^{oxy}2a (Figure 1). In addition to the presence of the α' -chain in the C3 convertase-treated samples, there were also bands at 67 and 40 kDa which formed as a result of secondary cleavage by factor I and cofactor H which is present in FCS-supplemented medium. The 40 kDa band present in even the untreated samples reflects the presence of C3 with intact peptide chains which has undergone a conformational change to the C3b-like form and is thus also cleaved by bovine factor I and cofactor H (the other product of this cleavage co-migrates with β -chain). One minor difference seen when comparing the proteins expressed in J558L and COS-1 cells is that the extent of conversion of pro-C3 into mature two-chain C3 is more complete for both wild-type and mutant proteins in J558L transfectants.

Cation-exchange HPLC analysis of wild-type and thioester-region mutant C3

To use Mono S column chromatography as a conformational probe of the thioester-region C3 mutant molecules, it was necessary to first calibrate the specific column employed using the appropriate derivatives of purified human C3. The elution positions of native C3, C3b (produced by C4b2a treatment), C3(H₂O) (produced by KBr treatment) and the intermediate state C3(NH₃)^{*} (produced by NH₄Cl treatment), as monitored by A_{230} , are shown in Figure 2 (specific elution volumes are given in the legend). These chromatograms confirm the separability of the various species and are in agreement with those published previously [8,9,17]. Thus, with this knowledge of the elution positions of the various C3 species, it was possible for us to study the recombinant C3 molecules and to assign them to distinct conformational categories.

For the analysis of the various recombinant molecules, appropriately treated culture supernatants were loaded on to the Mono S column and the elution positions of the various species monitored by capture ELISA. Wild-type recombinant C3 species [i.e. untreated C3, C3b and C3(H₂O)] were eluted at positions identical with those observed for their purified C3 counterparts (Figure 3). The conformations of the thioester-region mutant C3 molecules C1010A and E1012Q were investigated by the same methodology and the data are shown in Figure 3 for the three conditions employed. It can be seen that the wild-type and mutant C3 molecules showed similar elution profiles under all three conditions. It is particularly noteworthy in the profiles for the untreated molecules that neither of the two mutant molecules showed clear-cut peaks at the position expected for the C3(NH₃)^{*} species.

Although the data shown in Figure 3 were highly suggestive that, in the absence of thioester-bond formation during biosynthesis, the molecules nevertheless adopted a native-like conformational state, at least in the case of the untreated C1010A mutant there was a small shift in the elution position relative to the wild-type protein (peak position 5 ml compared with 6 ml). Similarly, there was lack of perfect peak coincidence for the KBr-treated samples. One possible explanation for the case of the untreated C1010A protein is that the observed elution profile did not represent a single species, but there was also some contribution from iC3(H₂O) present in the sample. In the presence of bovine factors H and I in the medium, this species would form

Figure 3 Elution profiles of wild-type and thioester-region mutant recombinant C3 proteins on Mono S cation-exchange chromatography

J558L cell transfectants were grown in culture medium supplemented with standard FCS. Supernatants containing wild-type C3 or the C3 mutant proteins C1010A or E1012Q were treated with C4b2a to generate C3b or KBr to generate C3(H₂O). Untreated or treated samples containing 200 ng of the recombinant protein were diluted 20-fold in 50 mM sodium phosphate, pH 6.0, and injected on to a Mono S column. Sixty fractions of 0.5 ml were collected using the standard elution conditions described in the Experimental section, and the presence of C3 in each fraction was detected by capture ELISA (absorbance at 405 nm). The chromatography experiments were run in triplicate and the data shown represent the mean of these measurements.

Figure 4 Immunoprecipitation of metabolically labelled wild-type, C1010A and E1012Q recombinant C3 from culture medium supplemented with K76COOH-treated FCS

Culture supernatants were either directly immunoprecipitated with rabbit anti-(human C3c) serum or immunoprecipitated after pretreatment with C4b2a. The same procedures were also performed on supernatants that had been pretreated with 2 M KBr to deliberately hydrolyse the thioester bond (for details see the Experimental section). Analysis was on SDS/PAGE (8% gel), run under reducing conditions, followed by autoradiography.

as a result of factor I-mediated cleavage of end-state C3(H₂O)-like molecules that are present. In fact, the presence of a considerable amount of α -40 fragment, which is visible in Figure 1, for all of the untreated samples clearly indicates that this species is indeed present and may have caused broadening of all of the peaks. Since it is possible to prevent factor I-mediated cleavage by harvesting the recombinant proteins in medium supplemented with FCS pretreated with K-76COOH, a procedure that irreversibly inactivates factor I, we repeated the crucial experiments using wild-type and C1010A C3 proteins that were secreted into K-76COOH-treated medium. As can be seen in the metabolic labelling/immunoprecipitation experiment shown in Figure 4, there are now no α -40 bands in any of the sample lanes, and the α' -chain is the stable end product of C3 convertase cleavage; both of these observations indicate that factor I has been inactivated.

Wild-type and C1010A C3 proteins produced in the presence of K76COOH-treated FCS were then reanalysed by Mono S chromatography using a slightly altered elution protocol in which binding to the column was carried out at lower ionic strength. The effect of this modification was to shift the elution position of the native wild-type protein to a later point in the salt gradient (compare elution position in Figure 5 with that in Figure 2). This did not, however, affect the order in which the different species were eluted. As can be seen in Figure 5, untreated samples of wild-type and C1010A C3 now co-eluted as sharp peaks, as did the KBr-treated derivatives of the same molecules. Thus, by both the criteria of cleavability by classical-pathway C3 convertase and elution position on a Mono S column, wild-type and C1010A C3 were conformationally indistinguishable.

Conformation of the equivalent thioester-region mutants of C4

Considering the high degree of sequence identity between C3 and C4 in the vicinity of the thioester, we wished to determine the

Figure 5 Elution profiles on Mono S cation-exchange chromatography of wild-type and C1010A recombinant C3 that had been harvested in culture medium supplemented with K76COOH-treated FCS

The elution conditions in this experiment differed slightly from those used in the experiments depicted in Figures 2 and 3. The equilibration buffer was 50 mM sodium phosphate, pH 6, as before. After sample injection, the column was washed at 1 ml/min with starting buffer for 5 min, at which time a linear NaCl gradient from 0 to 375 mM was generated over a period of 20 min. This was followed by a sharp step to 500 mM for 5 min, followed by re-equilibration with starting buffer. Sixty fractions of 0.5 ml were collected and analysed by C3 capture ELISA (absorbance at 405 nm). Calibration runs with purified C3 derivatives run under these conditions yielded the following elution volumes: native C3, 16 ml; C3(NH₃)₃⁺, 22 ml; C3(H₂O), 27 ml.

effect of making mutations in human C4 that were equivalent to those in human C3 which gave the native-like conformational phenotype, despite preventing thioester-bond formation. As is the case in C3, replacement of C4 residue Cys-1010 by alanine precludes the possibility of thioester formation. To determine whether C4 could attain a native-like conformational state in the absence of thioester-bond formation during biosynthesis, we employed C1 cleavability as a conformational probe. Just as post-synthetic thioester cleavage in C3 results in the acquisition of a C3b-like conformational state that is not susceptible to cleavage by C3 convertase, so too does post-synthetic cleavage of the thioester of C4 result in transformation of this molecule to a C4b-like conformational state that is not cleavable by its convertase, namely C1 [5]. When wild-type, C1010A and E1012Q derivatives of C4 were transiently expressed in COS-1 cells, haemolytic activity could only be detected for the wild-type species (results not shown), suggesting that thioester-bond formation had not occurred in either of the two mutant molecules. When the conformation of these molecules was probed by the criterion of C1 cleavage susceptibility, whereas C1 readily cleaves

Figure 6 Dependence of cleavage of C4 α -chain by C1 on thioester-bond integrity

COS-1 cells were transiently transfected with pSVC4B-WT, pSVC4B-C1010A or pSVC4B-E1012Q. Metabolically labelled culture supernatants were harvested in medium supplemented with K76COOH-treated FCS. Supernatants were either directly immunoprecipitated with rabbit anti-(human C4) serum or immunoprecipitated after pretreatment with C1. The same procedures were also performed on supernatants that had been pretreated with 2 M KBr to deliberately hydrolyse the thioester bond (for details see the Experimental section). Analysis was by SDS/PAGE (8% gel), run under reducing conditions, followed by autoradiography. Recombinant C4 α -chain is often a doublet, where the upper band represents the initially secreted form (α_s) and the lower band (α_p) is due to post-secretion trimming at the C-terminus by a plasma protease [18].

Figure 7 Assessment of thioester-bond formation in recombinant C4 molecules by autolytic fragmentation

Autofluorograph of the β -chain to γ -chain region of an SDS/PAGE (8% gel; reducing conditions) analysis of C4 immunoprecipitated from the culture supernatants of COS-1 cells that had been transfected with pSVC4bWT, pSVC4B C1010A or pSVE1012Q. Before reduction, SDS was incorporated under autolytic conditions. The metabolic labelling and growth conditions of the experiment were exactly as in Figure 6, except that media were supplemented with FCS that had not been treated with K76COOH. The 40 kDa N-terminal autolytic fragment is indicated. The larger C-terminal fragments, 60 and 54 kDa for α_s and α_p respectively, are occluded by other degradation products.

wild-type C4 α -chain to α' -chain, this cleavage did not occur in either C4 C1010A or C4 E1012Q (Figure 6). That the cleavage of the wild-type protein depended on the conformational state in thioester-intact C4 was shown by the loss of cleavability after deliberate hydrolysis of the thioester with KBr.

Although autolytic fragmentation is normally a direct indicator of thioester-bond formation, for reasons that we do not fully understand autolytic fragmentation has been difficult to demonstrate for both wild-type C3 and C4 secreted into medium containing K76COOH-treated FCS. Thus, as can be seen in Figure 6, autolytic fragmentation was not detectable even in the wild-type C4 'positive control'. To circumvent this problem, we performed a separate experiment in which metabolically labelled recombinant C4 molecules harvested in non-K76COOH-treated medium were subjected to immunoprecipitation under autolytic conditions. Figure 7 represents the β to γ segment of the autofluorograph, which has been deliberately overexposed to detect any faint presence of autolytic fragments in the mutant C4 proteins. It can be seen that an α -40 N-terminally derived autolytic fragment is exclusively present in the untreated wild-type C4 sample, showing the expected sensitivity of autolytic fragmentation to KBr and C1 treatments. There was no evidence of autolytic fragmentation when samples of C4 E1012Q and C4 C1010A were similarly analysed, confirming the lack of thioester-bond formation in these mutant molecules.

DISCUSSION

Before the discovery of a thioester structure in the proteins C3, C4 and α_2 -macroglobulin, it had been assumed that proteolytic cleavage, either of the peptide bond C-terminal to residue 77 in the case of C3 and C4 or within the 'bait' region of α_2 -macroglobulin, was the trigger for the initiation of conformational changes within these molecules. These conformational changes were in turn responsible for transforming the molecules from their respective precursor native forms to states in which they stably expressed various protein-binding functions. Subsequent to the discovery of the thioester structure, a series of reports showed a strict correlation in human C3, C4 and α_2 -macroglobulin between thioester integrity and the molecule being in its native conformational state [19–25]. This led to the hypothesis that the thioester bond was required for maintenance of the native conformation in these molecules and that it was its scission, rather than scission of the peptide bond, that was the triggering event for the ensuing conformational change [3,4]. Indeed Fothergill [26] coined the phrase 'spring in the molecular mousetrap' to describe the role of the thioester. By inference, one assumed that the achievement of the native conformational state in these molecules during biosynthesis would also depend on formation of the thioester bond. However, the attainment of a conformational state in C3 in the absence of thioester-bond formation (e.g. in C3 C1010A) that was sufficiently native-like to be cleaved by the conformationally sensitive protease C4b2a [10] brought into question the importance of the thioester in maintaining the native conformational state. Subsequent to C3 convertase cleavage, such thioester-deficient molecules acquired binding sites for factors H and I, this refocused attention on the peptide-cleavage event as being the actual trigger for the conformational change. Nevertheless, it was possible that the conformation of the C3 molecules in which the thioester did not (or could not) form during biosynthesis represented an intermediate state that was neither fully native nor fully C3b-like, but which was nevertheless cleavable by C3 convertase. The C3(NH₃)* species described by Pangburn [9] as a conformational intermediate capable of spontaneous re-formation of the thioester had exactly these properties. However, in the present study, the elution positions of both C3 C1010A and E1012Q on Mono S HPLC were decidedly different from that of C3(NH₃)* and were in fact indistinguishable from that of wild-type native C3. Thus, by two conformational criteria, C3 molecules biosynthesized

without an intact thioester had been found to adopt a conformation that was indistinguishable from that of the thioester-intact wild-type molecule. This suggests that folding to a native-like conformational state in single-chain pro-C3 is an energetically permissible process which does not require stabilization through formation of the intramolecular thioester.

In this study we have also shown that, by the criteria of autolytic fragmentation and haemolytic activity, a thioester bond is not formed in either C4 C1010A (where it is formally precluded) or the isosteric point mutant C4 E1012Q. As found for the equivalent C3 mutant (C3 E1012Q), it may be that the carboxylate side chain of E1012 has a catalytic role in thioester-bond formation. However, the conformations of both of these mutant C4 molecules were judged to be non-native as they were not cleaved by C1. Such behaviour is in fact consistent with them having adopted a C4b-like conformation. A similar conformational dependence on thioester-bond integrity was also seen in a human α_2 -macroglobulin mutant, C949S (C972S, prepro numbering), in which thioester-bond formation was precluded [27]. This thioester-deficient variant showed conformational properties that were indistinguishable from the wild-type protein in which the thioester bond had been nucleophilically cleaved with methylamine. Specifically, it migrated as a 'fast' species on non-denaturing PAGE, it was resistant to cleavage in the bait region and thus was non-inhibitory towards proteases, and it expressed the receptor-binding domain which is normally sequestered in the native molecule but is utilized for clearance of α_2 -macroglobulin that becomes complexed to proteases [28]. Similar results were found by Van Rompaey et al. [29] when they examined the conformational properties of an expressed version of a naturally occurring thioester-region variant of α_2 -macroglobulin, C949Y. Thus, for both human C4 and human α_2 -macroglobulin, an intact thioester bond appears to be more important for achieving and/or maintaining the native conformational state of the molecule during the course of biosynthesis than is the case for human C3. In the case of C4, this may reflect an instability introduced by the additional post-translational cleavage that splits the equivalent of the C3 α -chain into two chains, α and γ . In the case of α_2 -macroglobulin, it may reflect conformational constraints imposed by either covalent dimer formation or non-covalent tetramer formation.

Although our observation that a thioester-deficient variant of C3 could have a native-like conformational state was the first exception to the rule that 'coupled' thioester integrity with maintenance of the native conformation among the three human proteins, there is precedence for such 'uncoupling' among certain species of α_2 -macroglobulin. In bovine α_2 -macroglobulin, direct cleavage of the thioester by reaction with methylamine does not give rise to the conformational change that produces the 'slow' into 'fast' electrophoretic mobility conversion seen in most other α_2 -macroglobulins [24,30]. This conversion does, however, occur after trypsin cleavage within the bait region of the methylamine-treated protein. Nevertheless, the conformation of the methylamine-treated molecule is not completely native as it expresses the receptor-recognition site. In rat α_2 -macroglobulin, even less of a conformational change is induced by direct thioester scission with methylamine [31]. In this case, virtually all of the properties of the native protein are retained in the thioester-cleaved molecule, including the ability to trap proteases on bait-region cleavage, non-exposure of the receptor-binding domain and retention of the 'slow' electrophoretic mobility until after bait-region cleavage. At the far end of this spectrum of behaviour is ovostatin (also known as ovomacroglobulin) which most closely resembles the phenotype seen in C3 C1010A. Ovostatin is a tetrameric protease inhibitor in chicken egg white that shows

approx. 40% sequence identity with mammalian α_2 -macroglobulins [32]. Although it contains a bait region and inhibits proteases via the trap mechanism [33], because of the substitution of asparagine for cysteine at residue 949 within an otherwise highly conserved thioester region sequence, it does not form a thioester. Clearly these examples re-emphasize the role of peptide-bond cleavage in providing the primary trigger for the conformational changes in this family of related proteins. Nevertheless, the conformational change that ensues as a result of spontaneous thioester cleavage in C3 remains important because the C3b-like C3(H₂O) species is essential for the initiation of the alternative pathway [34].

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