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A Chemical Approach to Immunoprotein Engineering: Chemoselective Functionalization of Thioester Proteins in Their Native State

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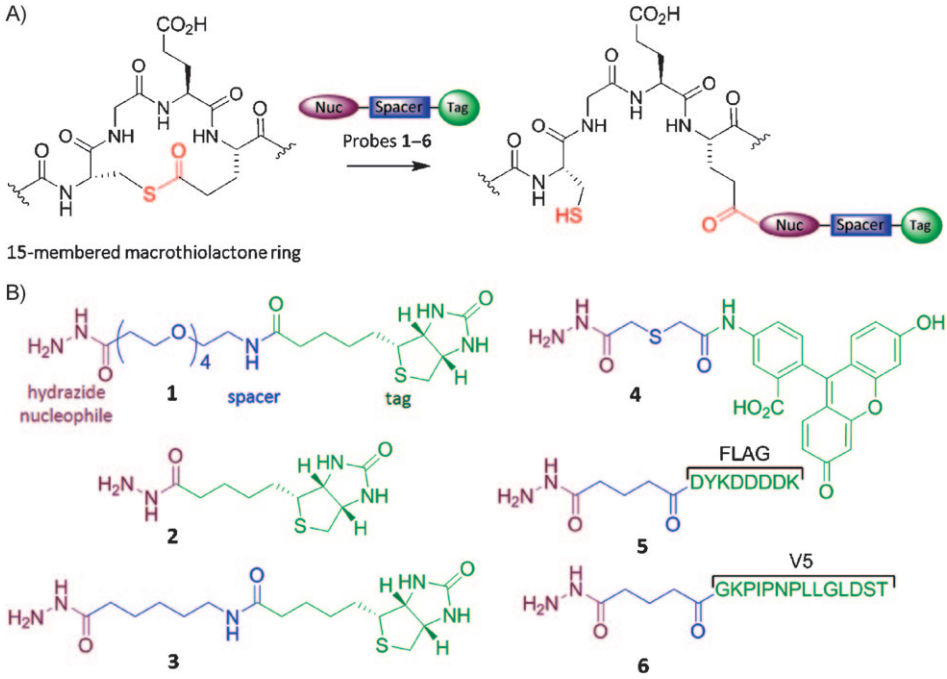
The complement proteins C3 and C4 and the pan-protease inhibitor α_2-macroglobulin (α_2M) are highly abundant serum proteins (C3: 1.2 g L^{-1}, C4: 0.2–0.4 g L^{-1}, α_2M: 1–2 g L^{-1}) that play a key role in host defense.[1–3] All these proteins (~1400–1800 residues; α_2M is a homotetramer) possess an internal 15-membered thiolactone ring formed by the joining of cysteinyl and glutamyl side chains in the conserved sequence CGEQ (Scheme 1 A).[3–5] Under normal physiological conditions, the thioester domains are buried within the protein core and shielded from solvent.[3,6,7] Upon activation, usually by proteolytic cleavage of the anaphylotoxin domain in C3 and C4 or the bait region of α_2M, the proteins undergo a conformational transition during which the thioester moves a considerable distance (~80 Å) to the surface, thus facilitating attack by foreign particle nucleophiles.[7,8] It is the reaction between this thioester and biologically relevant oxygen, nitrogen, or sulfur nucleophiles that facilitates covalent attachment of C3 and C4 to the surface of foreign particles or α_2M to proteases.[3,5,7]



Scheme 1. A) The putative reaction between a hydrazide probe and the 15-membered thiolactone ring of TEPs (thioester function in red). B) Molecules **1**–**6** used in this study: nucleophile (maroon), spacer (blue), functional tag (green).

Protection of the thioester bond before proteolytic activation is not absolute, and it has long been known that small nucleophiles such as water can induce a similar conformational rearrangement.[5,9] For C3, this slow hydrolysis ($t_{1/2}$ ~166 h) is thought to trigger activation of the alternative complement pathway in vivo.[9] In addition, small nucleophiles such as ammonia, hydroxylamine, hydrazine, putrescine, and methyl-, ethyl-, and isopropylamine have similarly been shown to access the thioester site, although the steric hindrance to this process is highlighted by the fact that isopropylamine reacts 750-fold slower than methylamine and *tert*-butylamine does not react at all.[9–11] Given that the reaction of thioester proteins (TEPs) with sterically hindered nucleophiles is slow,[5,10,11] it has not been demonstrated whether the intrinsic chemical reactivity of the thioester locus can be exploited to incorporate large functional molecules into TEPs in their native state. If the barriers to such an approach could be overcome, it would, for the first time, allow the synthesis of structurally well-defined TEP-derived conjugates with chemically engineered functionalities and provide a direct method to characterize the processing and interaction of these important immunoproteins in vivo.

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With this in mind, we studied the reaction between a panel of hydrazide probes **1**–**6** (Scheme 1B) and native state C3, C4, and α_2M. The key structural elements of **1**–**6** are a chemoselective hydrazide nucleophile, the presence of a spacer (of varying length and composition), and a functional molecular tag (biotin, fluorescein, or peptide).

As a method to follow the reaction of these probes with the TEPs, we made use of the fact that chemical- or heat-induced denaturation of TEPs induces an autolytic cleavage reaction.[12] Thus, proteins with an intact thioester undergo autolytic cleavage when denatured, but proteins in which the thioester has been disrupted, for example by water or other nucleophiles, will not (Figure S1 in the Supporting Information).[5,12]

The proteins C3, C4, and α_2M (3 μg) were incubated with probes **1**–**6** (0–25 mM) in phosphate-buffered saline (PBS; 10 mM phosphate, 140 mM NaCl, pH 7.4) at 37 °C for 24 h and then treated under autolysis conditions (Table S1, footnote [a] in the Supporting Information). For each hydrazide probe, the extent of thioester modification was qualitatively examined by comparing the decrease in autolytic fragment formation relative to the decrease induced by methylamine (50 mM; Table S1 and Figures S2–S5).[11,13,14] To confirm this modification, C3, C4, and α_2M were also incubated with hydrazides **1**–**6** at 5 mM, a concentration determined from the autolytic cleavage data to effect measurable protein adduct formation, and the labeling was analyzed by Western blot (probes **1**–**3, 5, 6**) or fluorescence detection (probe **4**). Gratifyingly, hydrazide probes **1**–**6** all modified the TEPs but to varying extents (Figures 1B and S6). Importantly, for all cases in which TEP modification was observed, only the thioester-containing α chains of C3 (113 kDa) and C4 (92 kDa), not the β chain of C3 (72 kDa) or the β and γ chain of C4 (72 and 32 kDa, respectively) were functionalized by the probes (Figure 1A and C).

Hydrazides **1** and **4** react with C3, C4, and α_2M to approximately the same extent, matching $MeNH_2$ (50 mM) reactivity at ~5–10 mM for C3 and C4 and ~25 mM for α_2M (Figures S2a and 3b). In contrast, probes **2** and **3** exhibit measurable modification of the TEPs, but even at 25 mM do not achieve the same extent of modification as $MeNH_2$ (50 mM; Figures S2b and 3a). The peptide-containing hydrazides **5** and **6** displayed only slight reaction with C3 and C4 and no measurable modification of α_2M at concentrations up to 25 mM (Figures 1C and S4). Interestingly, the reactivity of the hydrazide and linker component of probes **5** and **6** paralleled that of **5** and **6** in toto (as determined by autolytic cleavage inhibition). Thus, it did not modify α_2M, but showed better reaction with C3 and C4 (at >10 mM), suggesting that the molecular size and composition of the linker/spacer is the critical factor in determining successful TEP adduction (Figure S5a).

Seeking to gain a partial understanding of the kinetics underlying the hydrazide labeling, a pseudo-first-order kinetics study of the reaction of hydrazide probe **1** (5.0 mM) and C3 (8.6 μM) in PBS (pH 7.4) at 37 °C was undertaken. The results revealed that the conversion of C3 to form conjugate C3–**1** proceeds efficiently with a measured half-life ($t_{1/2}$) for loss of C3 of 5.8 h (and a rate constant for C3 modification by **1** of $k'_{hydrazid} = (3.36\times10^{-5})\,s^{-1}$; Figure S9). In addition, kinetics studies in which probe **4** concentrations were varied at a fixed C3 concentration revealed that adduct formation proceeds with a sigmoidal dependence on hydrazide concentration, representative of a classic receptor–ligand type process (Figure S10 and Methods section in the Supporting Information).

Previous studies on the chemical modification of these proteins maintained that accessibility of the thioester was limited by the steric hindrance and the overall size of the attacking nucleophile.[10,11] Our results indicate a more complex system in which access depends on the specific protein (C3 and C4 were generally more readily modified than α_2M), and the composition of both the spacer and the tag being adducted. In-depth structural effects of linker and tag on TEP modification are currently being studied in further detail.

The enzymatic processing of the C3 and C4 conjugates formed after reaction with hydrazides **1** and **4**–**6** was then studied by using the complement regulatory protease Factor I in the presence of Factor H or soluble complement receptor type 1 (sCR1 or CD35; Figures S7 and 1B and D). Western blot and fluorescent analyses revealed these conjugates were enzymatically processed as if they were physiologically reacted immunoproteins, albeit slower, suggesting that such TEP conjugates may find use in vivo.

The decreased rate of enzymatic processing was highlighted by the fact that the C3–α73 conjugates of all probes studied were not completely digested after 12 h incubation with Factor I. In addition, conjugates C3–**4** and C4–**4** showed resistance to cleavage; C3–**4** exhibited only slight processing, and C4–**4** required twice the concentration of Factor I/sCR1 to achieve complete processing (Figure S8).

The tag-dependent nature of this processing is highlighted by the fact that C4–**6** was not only slowly processed, but also displayed partial cleavage fragments which correspond to each of the potential Factor I cleavage sites on C4 (Figures S7 and 1B and D). Overall, this slow processing is consistent with previous work, which showed that the alternative pathway activators, C3–H_2O and cell-bound C3b, are resistant to processing by regulatory enzymes and also alludes to a possible role that endogenous nucleophiles might play in activating the alternative pathway.[15,16]

ABPP is a functional proteomics tool that uses active-site-directed chemical probes to characterize enzymes in native biological samples.[17] Having shown that the biotin–hydrazide probe **1** specifically labels the thioester domain of all the TEPs studied, we wondered whether probe **1** could react and 'profile' TEP processing within the complex biological milieu of the plasma proteome. Thus, human plasma (citrated) was incubated with biotin–hydrazide probe **1** (5 mM) for 12 h at 37 °C, and biotinylated proteins were captured on neutravidin–agarose beads. The sequestered proteins were then resolved by Western blot SDS-PAGE under reducing conditions, with both streptavidin (to detect biotinylated peptide fragments) and anti-TEP polyclonal antibodies (to detect all chain fragments of C3, C4, and α_2M; Figure 2). These analyses revealed that α_2M was sequestered from plasma, as were C3 (α and β chains), and C4 (α, β, and γ chains). In addition, there was evidence of considerable proteolytic processing of C3–**1** and C4–**1** in plasma, fur-

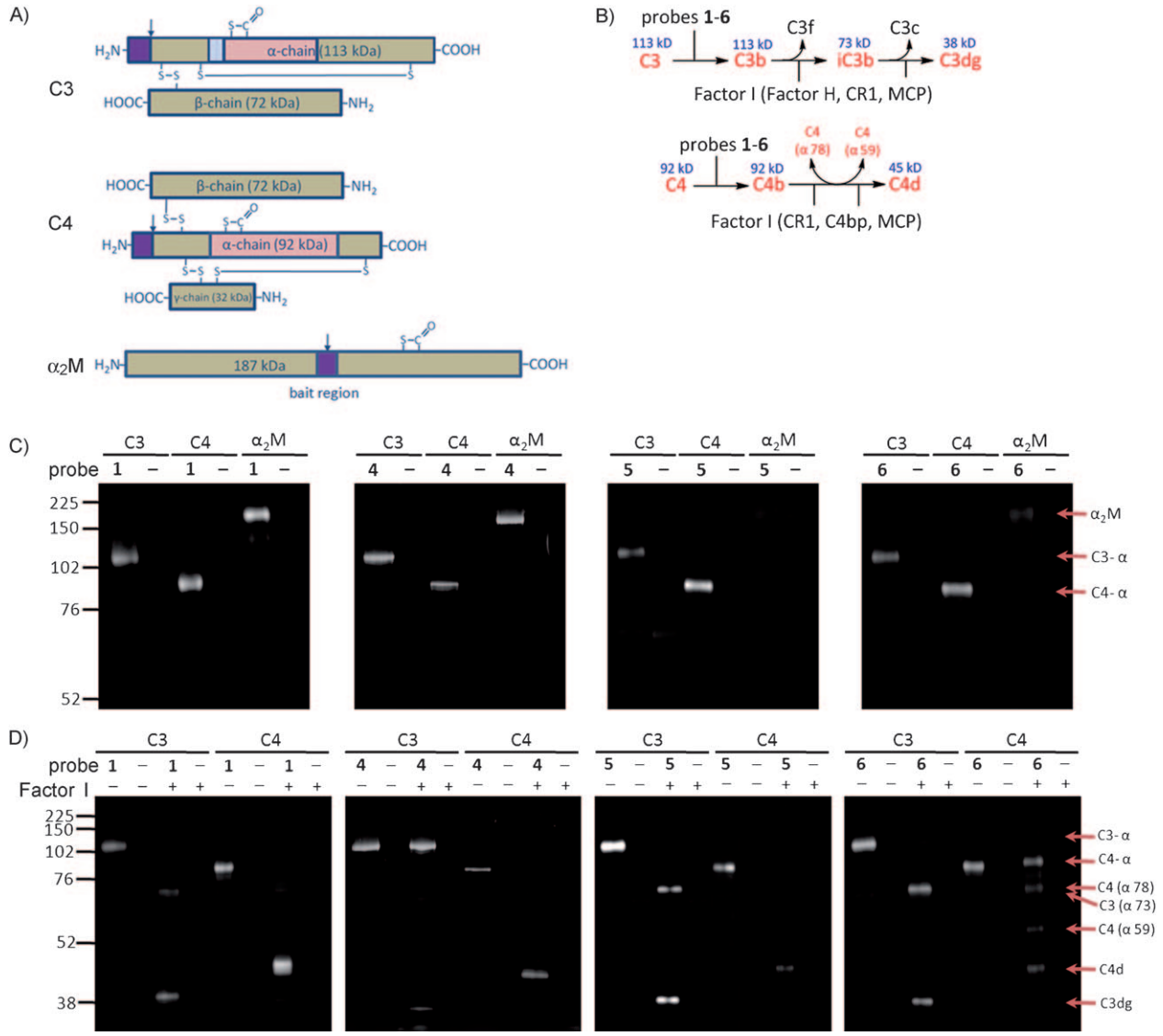

Figure 1. Analysis of TEP modification. A) Primary chain representation of TEPs C3, C4, and α_2M; anaphylatoxin domain or bait region (purple), and the thioester domain (red). Note: α_2M is a homotetramer of ~748 kDa, and each monomer possesses a thioester region. B) Observed in vitro enzymatic processing of probe-reacted C3 and C4 (i.e., with anaphylatoxin domain intact). Molecular weights for the thioester domain fragments under reducing conditions are given in blue. C) Western blot SDS-PAGE or fluorescent analyses of TEPs incubated with **1**, **4**, **5**, or **6** (detected with either streptavidin–HRP conjugate, fluorescence, anti-FLAG mAb, or anti-V5 mAb, respectively). Lanes that are labeled with a probe number (**1**, **4**, **5**, or **6**) represent TEP (3 μg) incubation with probe (5 mM) in PBS (pH 7.4) at 37 °C for 36 h and were then dialyzed. Lanes headed with – represent incubation of TEP under identical buffer conditions, but in the absence of probe. Visible bands are the C3 α chain (113 kDa), C4 α chain (92 kDa), and α_2M monomer (187 kDa). D) Western blot analysis of Factor I processing of C3 conjugates with **1**, **4**, **5**, or **6** and C4 conjugates with **1**, **4**, **5**, or **6**. After incubation of the TEP and probe as described in Figure 2C, C3–(**1**, **4**, **5**, or **6**) and C4–(**1**, **4**, **5**, or **6**; 3 μg; 1.4 μM) were incubated for 12 h at 37 °C in the presence or absence of Factor H and Factor I (0.5 μg of each) for C3 conjugates and sCR1 and Factor I (0.5 μg of each) for C4 conjugates. C4–**4** was cleaved with sCR1 (1 μg) and Factor I for the same time period. Labeled fragments were detected by Western blot SDS-PAGE as described in Figure 2C.

ther supporting the notion that TEP–large-molecule conjugates would be susceptible to the normal proteolytic processing in a biological setting (Figure 2). The Western blot results of the neutravidin affinity capture were corroborated using on-bead trypsin digest of the neutravidin bead and subsequent proteomic analysis of the peptide fragments detected (see the Supporting Information). Interestingly, in addition to C3, C4, and α_2M being detected by the proteomic analysis, Factor H was also detected, suggesting a possible coprecipitation of Factor H complexed with the hydrazide **1**-treated C3.

In summary, we have shown for the first time that the thioester proteins C3, C4, and α_2M can be functionalized with large molecules by attack on the thioester when the proteins are in the native state. The conjugates thus formed are structurally well defined and behave in the same way as activated TEPs, binding to and being processed by downstream cofactors (Factor H), receptors (sCR1), and enzymes (Factor I). At present, we are studying the scope and consequences of this phenomenon, but the ability to modify these central proteins of the immune system with large nucleophiles possessing

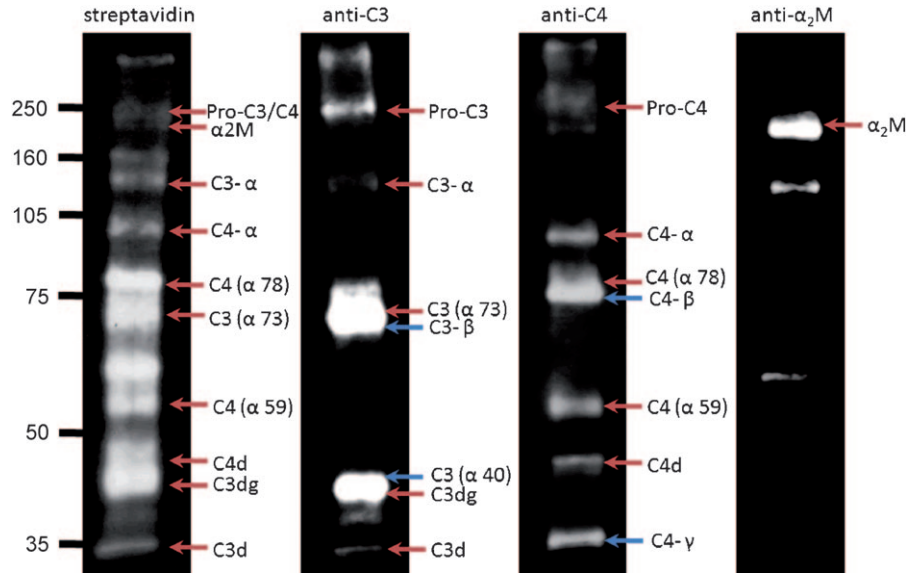

Figure 2. ABPP of human plasma (100 μL) incubated for 12 h at 37 °C with biotin–hydrazide **1** (5 mM). The SDS-PAGE gels were probed with streptavidin–HRP and polyclonal anti-C3, -C4 and -α_2M antibodies. In the streptavidin Western blot, only biotinylated peptide fragments were assigned that correspond to known TEPs or fragments thereof that are known to contain the thioester. Red arrows on the polyclonal antibody blots correspond to bands that should contain the thioester and also appear on the streptavidin–HRP blot. Blue arrows correspond to fragments of the proteins known not to contain the thioester and therefore should not appear on the streptavidin blot. The assignment of bands to specific fragments in the gels should be used as a guide rather than a definitive analysis due to the complexity of the samples and the potential for cross-reactivity and domain specificity of the polyclonal antibody samples.

complex and broad-ranging chemical functionality has significant biological implications and applications in the fields of immunodiagnostics, immunotherapy, and vaccine design.

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