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Chemical labelling of active serum thioester proteins for quantification

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ABSTRACT

The complement serum proteins C3 and C4 and the protease inhibitor α-2 macroglobulin are all members of the C3/α-2M thioester protein family, an evolutionarily ancient and conserved family that contains an intrachain thioester bond. The chemistry of the thioester bond is a key to the function of the thioester proteins. All these proteins function by covalently linking to their target by acyl transfer of the protein via the thioester moiety. We show that the signature thioester bond can be targeted with nucleophiles linked to a bioreporter molecule, site-specifically modifying the whole, intact thioester protein. Conditions were optimised to label selectively and efficiently pull-down unprocessed thioester-containing proteins from serum. We demonstrated pull-down of full-length C3, α-2M and C4 from sera in high salt, using a biotinylated nucleophile and streptavidin-coated resin, confirmed by MALDI-TOF MS identification of the gel bands. The potential for the development of a quantitative method for measuring active C3 in serum was investigated in patient sera pre and post operation. Quantifying active C3 in clinical assays using current methods is difficult. Methods based on antibody detection (e.g. nephelometry) do not distinguish between active C3 and inactive breakdown products. C3-specific haemolytic assays can be used, but these require use of relatively unstable reagents. The current work represents a promising robust, enzyme- and antibody-free chemical method for detecting active thioester proteins in blood, plasma or serum.

Introduction

The C3 and C4 thioester proteins of the complement pathway and the protease inhibitor α-2 macroglobulin (α-2M) form the first line of defence of the innate immune system against pathogens. Following limited proteolysis, C3 and C4 attach covalently to pathogen surfaces by acyl transfer via their thioester functionality and this irreversible attachment is central to pathogen recognition and clearance. α-2M interacts with exogenous proteases, and binds covalently to them, inhibiting their activity. In contrast to their protective effects, inappropriate or excessive activation of C3 is involved in many autoimmune, degenerative and inflammatory diseases. There is therefore great interest in techniques that can monitor the concentration of the active forms of these proteins as potential indicators of disease progression.

The site-specific labelling of proteins within a complex biological mixture like serum or plasma requires chemoselective reaction of the label with a unique site of a protein under near physiological conditions and in the presence of a vast array of other biomolecules. Some chemoselective reactions have been harnessed for activity based labelling, contributing significantly to the elucidation of numerous biological processes. Complement protein C3, as well as other proteins of the C3/α-2M thioester protein family all possess an eponymous thioester bond that bridges the cysteine and glutamine side chains of the conserved sequence GCGEQ to form the internal β-cysteinyl-γ-glutamyl, fifteen-membered thiolactone ring, a distinctive post-translational modification of this class of proteins that makes this family theoretically amenable to site-specific labelling (Fig. 1). In almost all cases of activity-based labelling, a protein activity is targeted by an electrophilic warhead (Heal et al. 2011), however for the C3/α-2M family this situation is reversed, as the thioester bond is itself the warhead and can be targeted with an appropriately reactive nucleophile. Thioester proteins are present in high concentrations in blood plasma as the broad-spectrum protease inhibitor α-2M (2.0 mg ml^{-1}) and as C3 (1.2 mg ml^{-1}) and C4 (~0.6 mg ml^{-1}) of the complement pathway.

Abbreviations: C3, complement component 3; C3(H_2O), C3 with hydrolysed thioester; C3(NH_2NH_2), hydrazine labelled C3; C3(bio), C3 labelled with biotin–PEG_4–hydrazide; C3(N), C3 labelled with nucleophile at thioester bond; fI, complement factor I; fH, complement factor H; EACA, epsilon amino caproic acid; α-2M, α-2 macroglobulin; PEG-plasma, plasma precipitated by PEG 3350; SA, streptavidin; HRP, horseradish peroxidase.

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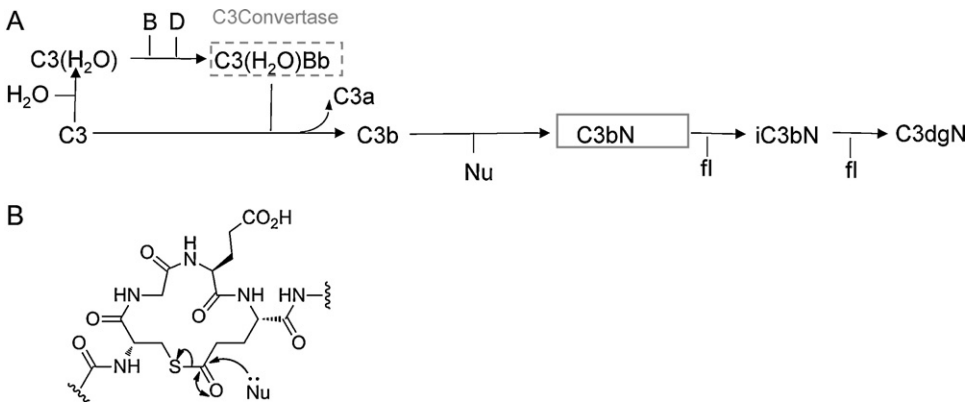

Fig. 1. Reactivity of the unactivated thioester of complement protein C3. (A) The tick over event of the alternative pathway. C3(H_2O) is formed by the slow hydrolysis of C3. Interaction between factor B and C3(H_2O) forms an active C3 convertase complex that can generate more C3b molecules by cleavage of the C3a domain from C3, forming a positive feedback loop. C3b has a metastable thioester group that can react rapidly with nucleophiles such as the hydroxyl groups on carbohydrates or amines on proteins. (B) A generalised reaction mechanism for the reaction of a nucleophile with the thioester of full-length C3 (tick over event). This gives a route for the modification of the whole C3 protein without activation. The fifteen-membered intramolecular thiolactone ring is the defining feature of the C3/α-2M thioester protein family, an evolutionary ancient family of proteins that all function by forming covalent linkage of the protein to a target via the thioester bond. The thioester moiety is formed by bridging the cysteine and glutamine side chains with a β-cysteinyl-γ-glutamyl bond.

C3 is a 190 kDa protein consisting of a 113 kDa α-chain, containing the thioester bond, and a 75 kDa β-chain linked by a single disulphide bond. In recent years, the crystal structures (Fredslund et al. 2006; Janssen et al. 2005, 2006) of intact complement C3 and its activation product C3b (Fig. 1) have revealed the two end points of the structural transition that takes place when the C3 molecule is activated and cleaved: upon going from C3 to C3b, the C3 domain containing the thioester (TED domain) undergoes a large rearrangement, while the domains surrounding the anaphylotoxin domain (ANA) also change orientation and expose surfaces for interaction with Factor B (fB), Factor I (fI) and its cofactors such as fH. Although it has not received the attention of C3, α-2M is an important serum protein that acts as a broad range protease inhibitor by physically trapping proteases and preventing them reaching substrate (Salvesen and Barrett 1980). α-2M has a 'bait' region with high substrate activity for a wide range of proteases. Analogous to the C3a or ANA domain in C3, cleavage of this bait region causes a dramatic conformational change. Related thioester containing proteins are represented across metazoans (Dodds and Law 1998) and include the thioester protein (Tep) of *Drosophila* (Baxter et al. 2007).

The intact thioester proteins behave as typical protein thioesters with an estimated half-life of hydrolysis of the thioester of intact C3 (~160 h) within the range of a peptide thioester (Davies and Sim 1981). Characteristically for thioesters they are much more susceptible to aminolysis than hydrolysis (Yang and Drueckhammer 2001) and this observation has been used in many studies to incorporate low molecular weight nucleophiles (Law and Dodds 1997). Furthermore, the slow hydrolysis of intact C3 that constitutes the 'tick over' event of the alternative pathway to form C3(H_2O) shows that the thioester bond is in contact with solvent. C3(H_2O) can form a convertase C3(H_2O)Bb (Fig. 1) like its truncated relative C3b and is a substrate for fI, and although no structure has yet been reported for C3(H_2O) it is assumed to behave similarly to C3b (Bexborn et al. 2008). Cleavage (in this case hydrolysis) of the thioester bond is enough to cause transition to a C3b-like structure. This pathway would therefore seem an ideal route to incorporate nucleophiles attached to a bioreporter (as suggested in Figs. 1 and 2A). The thioester bond is a very rare post-translational modification for an extracellular protein and reactive to nucleophiles so that it can be chemoselectively derivatized. Nevertheless chemical approaches to labelling and regulating complement have been largely neglected. When activated by limited proteolysis the thioester becomes extremely reactive with a very short serum lifetime, and will react rapidly with many nucleophiles (Sim and Law 1985; Sim et al. 1981). Conjugation of C3b to ovalbumin and other targets (Cretin et al. 2007; Villiers et al. 1999) has been achieved by adding trypsin to C3 in an excess of the conjugating agent to take advantage of this activation, although coupling efficiency is low. Radiolabelled methylamine has been incorporated into serum fractions of many organisms to screen for the presence of thioester proteins across metazoans (Dodds et al. 1998). Recently, an ingenious method has been used to label C3 efficiently with larger molecules, C3 is treated with methylamine, and as the thioester is aminolysed the cysteine side-chain becomes transiently available for derivatization. This promising approach has been used to conjugate C3 to harness its immunological properties (Mitchell et al. 2008). We have previously demonstrated, that purified C3 thioester can be directly reacted via the thioester bond with much larger biomolecules than were used before. However it was still difficult to measure the efficiency of the reaction, estimated by indirect methods such as ability to perform the autocleavage reaction (Cole et al. 2009). Attempts at pull-down directly from sera gave complex product mixtures from post-derivatization processing. We considered that it would be worthwhile to reinvestigate serum pull-down of full-length derivatized protein with the knowledge that high salt inhibits formation of C3(N)fHfI (Soames and Sim 1997). This approach would yield efficient labelling by pull-down of the intact, derivatized material, directly from serum.

Our aim is to develop a detection reagent that reacts with thioester proteins in serum, without need to purify the protein. An activity-based approach using a nucleophile attached to a reporter molecule would potentially be able to extract and detect the active thioester proteins present (Heal et al. 2011). This chemical approach would accelerate and simplify complement detection by obviating the need for expensive antibodies and dedicated machinery. More importantly this technique will allow us to directly quantify active complement C3 or C4 in serum. The pivotal role of complement in the immune response is reflected in the number of medical conditions correlated with abnormal complement activation or consumption (Morgan and Harris 2003; Nilsson et al. 2011). Thioester labelling would also be useful for screening and comparison of thioester proteins from a wide variety of metazoans, but most importantly for direct longitudinal studies of active C3, C4 levels in different diseases.