Volume 308, number 3, 280–282 FEBS 11474 August 1992

Spontaneous thioester bond formation in α_2-macroglobulin, C3 and C4

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Recieved 2 July 1992

Purified α_2-macroglobulin and complement proteins C3 and C4 were treated with ammonia to break their intramolecular thioester bonds and reform the original free cysteinyl and glutamyl side chains. When this reaction was performed at low temperature a conformational intermediate was trapped which lacked a thioester, but which could refold to the native structure and spontaneously reform the thioester and full biological function. The findings suggest that these proteins may undergo spontaneous post-translational self-modification forming the thioesters without involvement of enzymes or high energy metabolites such as ATP.

α-Macroglobulins; Protease inhibitor; Complement 3; Complement 4; Post-translational protein modification

1. INTRODUCTION

Formation of a thioester bond between the side chains of the amino acids cysteine and glutamine in a single polypeptide is rare, but occurs in three human plasma proteins involved in complement and coagulation [1–6]. This bond results from a post-translational modification which joins a cysteinyl -SH to a glutamyl residue with expulsion of ammonia.

Thioester-containing proteins fall into two functional classes. α_2-Macroglobulin (α_2M) is a proteinase inhibitor with broad specificity. Proteases are first attracted to a 'bait region' of α_2M then are both physically trapped in a molecular cage and covalently linked to the inhibitor via the thioester site (reviewed in [7]). The complement proteins C3 and C4 are homologous [8] to α_2M, but utilize the thioester to covalently attach themselves to pathogens and other particles on which the complement system is activating [9,10]. Covalent attachment is largely nonspecific allowing attachment to sugars or proteins on the surface of any organism.

A recent report described thioester reformation in C3 following treatment with ammonia [11]. This is now shown to be a general phenomenon shared by C4 and α_2M suggesting that all three proteins possess an active site [12] which functions intramolecularly to condense the Cys and Gln side chains.

2. MATERIALS AND METHODS

C3 [13,14], C4 [15] and α_2M [6] were purified from human plasma as previously described. Trypsin was purchased from Sigma and radioiodinated to approximately 1 μCi/μg using Iodogen (Pierce).

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Thioester-dependent function of α_2M was assayed by measuring its ability to couple covalently to trypsin. Samples were mixed with 10 μM $HgCl_2$ to block further thioester reformation and a 3-fold molar excess of [^{125}I]trypsin in PBS pH 7.4 containing 0.1% gelatin was added. After incubation for 5 min at 25°C covalent attachment was assessed by gel chromatography through S300HR (0.6×30 cm) after denaturation in 4 vols. of 9 M guanidine. The amount of native C3 reformed was determined by HPLC on a Mono S column (Pharmacia) or by hemolytic function as previously described [11]. Where necessary ammonia was rapidly removed by centrifugal desalting through Sephadex G-25. Thioester reformation in C4 was determined at 37°C after 500-fold dilution into complement assay buffer (0.1% gelatin, 5 mM sodium barbital, 145 mM NaCl, 10 μM $HgCl_2$, pH 7.4). Thioester-dependent hemolytic activity was assessed by measuring lysis of antibody-coated sheep erythrocytes incubated in C4-depleted human serum [16].

3. RESULTS

Thioester-containing proteins were treated with ammonia to reform the original Cys and Gln residues present immediately after translation. The reaction with ammonia at low temperature (Fig. 1) allowed the accumulation of a conformational intermediate which in the case of C3 can be isolated by cation exchange HPLC at a pH near the pI of C3 (peak 2, Fig. 2). This intermediate has been shown to lack an intact thioester [11]. The ability of this intermediate to refold during incubation at higher temperatures (25°C) to the native structure and reform the thioester bond is illustrated in Fig. 3. Fig. 3 also shows the spontaneous recovery of thioester-dependent functions by α_2M and C4. In each case the native protein containing the thioester was purified from human plasma, incubated with ammonium chloride at pH 8.4 between 0°C and 5°C and the ammonia was then rapidly removed or diluted. During incubation in the absence of ammonia C3 regained more than 70% of its activity over 2 h. α_2M which had been 90% inactivated regained 27% of its ability to covalently attach

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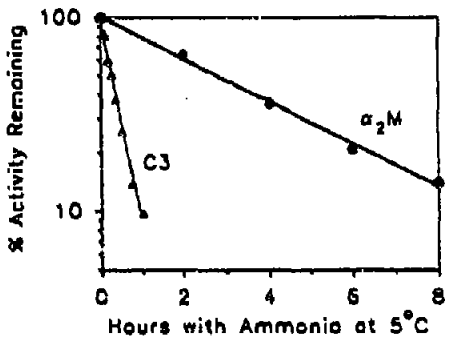

Fig. 1. Rates of inactivation of thioester-dependent functions of C3 and α_2M incubated with 100 mM NH_4Cl at 5°C at pH 8.4 (70 mM bicine, 70 mM NaCl).

to trypsin. α_2M inactivated only 50% by brief exposure (1 h at 5°C) to a higher concentration of ammonia (200 mM) was able to regain 80% of the inactivated thioester function (not shown). C4 exhibited the poorest recovery increasing from 5% active to only 7% active after 2 h at 37°C. The relative inefficiency of reformation by C4 may be due to the fact that this protein is a three chain molecule [16] having undergone two proteolytic post-translational modifications. The presence of the SH blocking agent mercury chloride (10μM) prevented the recovery of functional activity of all three proteins, but did not affect preexisting activity.

Higher temperatures were required to reform significant levels of functional C4 and α_2M than to reform C3. As Fig. 4 illustrates little or no α_2M function was recovered at 25°C, while this was the optimal temperature for C3.

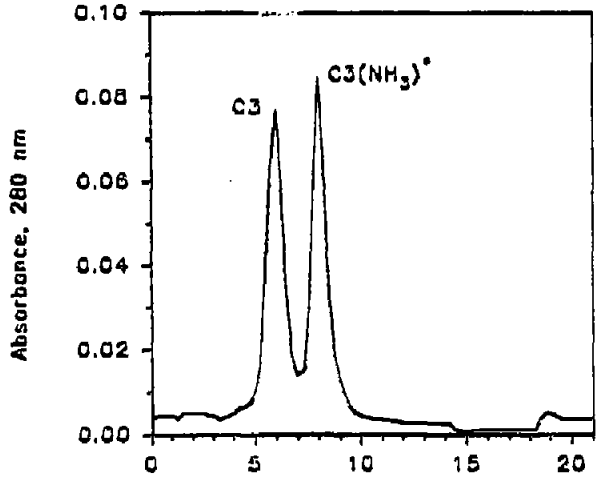

Fig. 2. Isolation by cation exchange HPLC of a conformational intermediate ($C3(NH_3)^*$) trapped during inactivation of C3 with ammonia at 5°C (30 min as in Fig. 1). Chromatography was performed on a Mono S column at pH 6 as previously described [11].

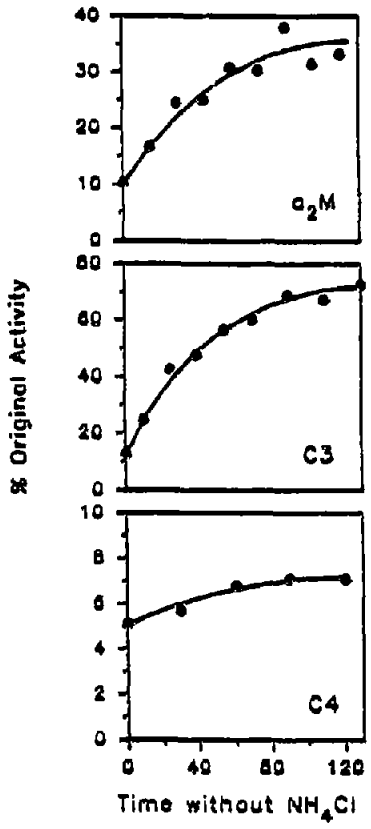

Fig. 3. Reformation of thioester-dependent functions of α_2M, C3 and C4 after treatment with and removal of ammonia. α_2M (8.4 mg/ml) was incubated for 15 h at 0°C with 0.1 M NH_4Cl in 70 mM bicine, 70 mM NaCl at pH 8.4. Ammonia was removed and the protein transferred to 20 mM sodium phosphate, 140 mM NaCl pH 7.4 by centrifugal desalting through Sephadex G-25 at 4°C. Thioester reformation was initiated by placing the sample at 37°C at zero time (min). Samples were removed, and assayed for thioester-dependent attachment to [^{125}I]trypsin. C3 at 3 mg/ml was treated with 50 mM NH_4Cl in 50 mM bicine, 60 mM NaCl pH 8.4 for 40 min at 25°C. Ammonia was removed by centrifugal desalting in 50 mM bicine, 60 mM NaCl pH 8.4 at 4°C. Thioester reformation was initiated by placing the sample at 25°C at zero time. Samples were removed and the amount of native C3 reformed was determined by HPLC as previously described [11]. C4 (0.55 mg/ml) was treated for 24 h at 0°C with 0.2 M NH_4Cl in 50 mM bicine, 70 mM NaCl pH 8.4. Thioester reformation was followed at 37°C by measuring thioester-dependent hemolytic activity. No thioester-dependent activity was reformed by any of the three proteins if 10 μM $HgCl_2$ was added at zero time.

4. DISCUSSION

Speculation on the mechanism of thioester formation has focused on similarities with the thioester of transglutaminases [11,17,18]. These bonds arise between Cys and Gln residues on separate proteins and the reaction proceeds without high energy metabolites [19]. Several reports, however, have suggested that cofactors are required for intramolecular thioester formation [20] and in one case that a C3-specific cofactor might be necessary [21].

As illustrated in Fig. 3, all three human plasma pro-

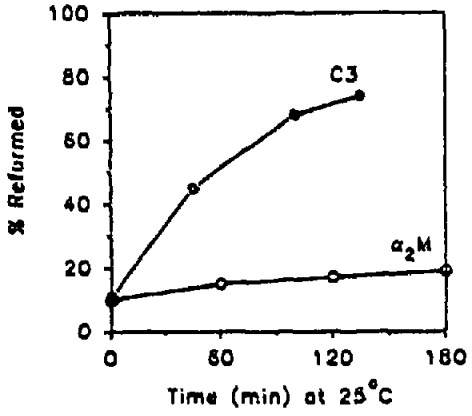

Fig. 4. Temperature dependence of thioester reformation illustrated by poor recovery of α_2M at the optimal temperature of C3 reformation (25°C).

teins are capable of spontaneously regaining their thioester-dependent functions after inactivation by ammonia. In each case reformation requires an unblocked -SH group suggesting that the process involves the single thioester-derived Cys and is similar to reformation in C3 [11]. C3 has been shown to undergo a stepwise conformational change following scission of the thioester by nucleophiles [11,22–24]. One of these intermediates, designated $C3(NH_3)^*$ when ammonia was the nucleophile, was trapped at low temperature and separated by HPLC from native C3 and other conformational forms. No comparable separation method has been found for the putative intermediates of α_2M or C4. Isolated $C3(NH_3)^*$ was shown to be capable of efficient thioester reformation and structural refolding to the native form [11]. Conformers arising subsequent to $C3(NH_3)^*$ in the unfolding pathway were not able to reform the thioester. The data suggests that α_2M also passes through a relatively stable intermediate form before unfolding to a stable form. Rapid conversion to the intermediate with minimal time for unfolding to the final conformation allowed the demonstration of a thioester reformation efficiency of 80% for α_2M, similar to that for C3. Thermodynamic measurements on C3 indicated that the Gibbs free energy of thioester formation and protein refolding was +5.2 kcal/mol and that refolding to the native form was kinetically favored over further unfolding to a stable conformation lacking a thioester bond [11]. Present data suggest similar kinetics and thermodynamics characterize α_2 M, but that reformation is hindered in C4.

These findings suggest that the thioester bond forms during folding of the proteins and requires only the energy derived from packing of the polypeptide chain. This post-translational self modification is stable until proteolysis initiates a large conformational change and activates the thioester site permitting nonspecific covalent attachment of the proteins to their targets via amide or ester bonds.

Acknowledgements: I thank Shirley A. Puckett and Kerry L. Wadey-Pangburn for technical assistance and the National Institutes of Health for support.

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