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Thiol ester role in correct folding and conformation of human α_2-macroglobulin

Properties of recombinant C949S variant

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Abstract

To determine the role of the thiol ester in the folding of human α_2-macroglobulin (α_2M) in the active conformation, we have characterized a recombinant variant of α_2M, C949S, expressed in baby hamster kidney cells, that lacks the thiol ester-forming cysteine. C949S α_2M behaves like methylamine-treated plasma α_2M, with correctly formed inter-subunit disulfide bridges, non-covalent association of covalent dimers to form tetramers, and exposure of the receptor binding domain, but an inability to inhibit proteinases, and inaccessibility of the bait regions to proteolysis We concluded that correct folding of monomers or their association to give tetrameric α_2M does not require a pre-formed thiol ester. Active α_2M may form in vivo by a two-step process involving initial folding to give a structure resembling that of C949S α_2M followed by thiol ester formation and a conformational change that gives the native active state.

Key words: α_2-Macroglobulin; Thiol ester; Site-directed mutagenesis; Baby hamster kidney cell; Human

1. Introduction

An unusual linkage, a thiol ester, is present in human α_2-macroglobulin, in α-macroglobulins from some other species, and in the complement proteins C3 and C4 [1]. All of these proteins are structurally related, though the complement proteins are 190 kDa monomers, whereas the α-macroglobulins are mostly dimers or tetramers. In human α_2-macroglobulin the thiol ester is formed between the side chains of cysteine-949 and glutamine three residues away at position 952. Limited sequence data on some of the other proteins show that the same relationship of only two intervening residues is maintained and that there are highly conserved residues between and immediately surrounding the cysteine and glutamine involved in the thiol ester linkage [2].

The thiol ester appears to serve two distinct functions. One is to provide a means of covalent binding to target proteins, either proteinases in the case of the α-macroglobulins, or cell surface proteins and immune complexes in the case of C3 and C4. The other is to mediate a large scale conformational rearrangement, which in the case of the dimeric or tetrameric α-macroglobulins is sufficient, even in the absence of covalent binding, to inhibit proteinases by engulfing them. On non-denaturing PAGE this is clearly seen as a change in electrophoretic mobility from a slow, active state to a fast, inactive state. An important consequence of the conformational change is to expose receptor binding sites, which mediate clearance in the case of α_2-macroglobulin-proteinase complexes.

There are two major questions with respect to the thiol ester in both human α_2-macroglobulin and in the other thiol ester-containing proteins. One is what role the thiol ester plays in the correct folding of the protein, and the other is what role it plays in maintaining the native conformation of the protein.

To investigate these questions for human α_2-macroglobulin and determine whether the thiol ester is needed for each of these roles, we have expressed a variant human α_2-macroglobulin, C949S, in which the cysteine that normally forms the thiol ester has been replaced by serine, thereby preventing the formation of a thiol ester linkage. We found that the absence of a thiol ester did not prevent the α_2-macroglobulin polypeptide from folding correctly, but that it did prevent adoption of the

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Abbreviations. α_2M, α_2-macroglobulin; BHK, baby hamster kidney; HNE, human neutrophil elastase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium.

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native conformation of the active inhibitor. Instead, the conformation was found to be indistinguishable (as judged by electrophoretic mobility) from that which occurs upon thiol ester cleavage of active wild-type α_2-macroglobulin by reaction with methylamine, the so-called 'fast-form'. These results suggest that the native conformation of human α_2-macroglobulin may be generated in vivo following complete folding of the protein into the fast-form conformation by closure of the thiol ester linkage. Closure of the thiol ester linkage, whether enzymatically mediated or spontaneously formed, could then change the conformation in a manner similar to the effect of cyanylation [3,4], to give a reversal of the more familiar slow-to-fast conformational change.

2. Materials and methods

2.1. Site-directed Mutagenesis of α_2M cDNA

The α_2M expression vector p1167 [5] was digested with the restriction endonucleases *Bsi*WI and *Xba*I to release a 2836-bp fragment containing the region covering the residues involved in thiol ester formation. This fragment was cloned into M13mp19 that had been modified to contain a new 24-bp polylinker (*Xho*I–*Bsi*WI–*Afl*II) between the *Sph*I and *Pst*I sites of the original polylinker region. An *Xho*I digest of the M13-α_2M fragment construct released a 965-bp α_2M cDNA fragment, which was subsequently subcloned into the *Xho*I site of an M13mp18 also modified to contain the new *Xho*I–*Bsi*WI–*Afl*II polylinker. Site-directed mutagenesis was performed on the single stranded M13mp18 construct containing the α_2M coding strand using the 21 base oligonucleotide 5′-CTG CTC TCC AGA GCC ATA GGG-3′, for which the underlined codon represents the cysteine → serine change at residue 949. Mutagenesis was carried out using the Amersham in vitro Mutagenesis System Version 2.1, which is based on phosphorothioate-modified DNA [6–9]. Greater than 80% positive plaques containing the desired G → C change at base 4310 (p1167 numbering system) were obtained, which was confirmed by sequencing of the single-stranded phage DNA. The 965-bp fragment containing the changed base at position 4310 was cloned back into M13mp19 at the *Xho*I site to regenerate the 2836-bp insert. Phage containing the insert in the correct orientation were determined by sequencing the single-stranded form. The 2836-bp insert was excised by digestion with *Bsi*WI and *Xba*I and ligated into p1167 that had been cut with the same restriction endonucleases and purified from the wild-type insert by electrophoresis on β-agarose. Sequencing was carried out on the plasmid to confirm the presence of the altered base as position 4310. This gave a new expression vector, p1167-C949S, coding for C949S variant α_2M.

2.2 Stable transfection of BHK cells

The procedure used for establishing stably transfected BHK cells expressing either wild-type or C949S variant α_2M was the same as we have used for stable expression of antithrombin [10], and a modification of the procedure described previously by Boel et al. [5]. BHK 21 cells were co-transfected with 20 μg p1167 (containing either wild-type or C949S variant coding sequences), 5 μg pRMH140, and 5 μg pSV2dhfr by calcium phosphate precipitation in T25 flasks for 5 h. After a 3-min glycerol shock, the cells were washed three times with phosphate-buffered saline and placed in DMEM plus serum. Transient expression of α_2M was detectable by radial immunodiffusion assay of the 48hr growth medium. The cells were split 1:5 into DMEM supplemented with 10% fetal bovine serum as well as 400 $\mu g \cdot ml^{-1}$ G418 and 0.4 μM methotrexate. Selection for stably transfected cells expressing the highest levels of α_2M was carried out over 4 weeks by gradually increasing the concentration of methotrexate to 10 μM while keeping the G418 concentration constant. The level of expression of the polyclonal culture after this period was 32 μg/10^6 cells/24 h for the C949S variant α_2M. For purification of large amounts of α_2M, cells were grown to confluence in roller bottles and cycled every 24 h between medium devoid of fetal bovine serum and drugs and medium supplemented with these items. Only medium from the serum-free cycles was harvested.

2.3. Isolation of α_2M

Plasma α_2M was isolated from outdated human plasma obtained from the Vanderbilt Blood Bank by chromatography on zinc chelate resin, Cibachron blue gel, and AcA22, as described previously [11]. Recombinant human α_2M, both wild-type and C949S variant proteins, were isolated from pooled serum-free growth medium by the protocol of Dangott and Cunningham [11], but with omission of the Cibachron blue and AcA22 chromatography steps, which were judged to be unnecessary for isolation of pure α_2M. The α_2M was judged to be homogeneous by SDS-PAGE. Concentrations of all forms of α_2M were determined spectrophotometrically using the extinction coefficient for the plasma protein of 564,000 $M^{-1} \cdot cm^{-1}$ [12].

2.4. Polyacrylamide gel electrophoresis

Nondenaturing PAGE was carried out on 5% acrylamide slab gels [13]. SDS-PAGE was carried out on 7.5% slab gels according to the procedure of Laemmli [14]. Unless otherwise indicated, all samples for SDS-PAGE were denatured and reduced for 45 min at 37°C in buffer containing 1% SDS and 0.8% dithiothreitol. 1 mM phenylmethylsulfonylfluoride was added to α_2M samples containing proteinase to prevent additional cleavage of the α_2M during denaturation. Where molecular weight standards were included, they consisted of myosin 200 kDa; β-galactosidase, 116.3 kDa; phosphorylase B, 97.4 kDa; serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa, trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa; and aprotinin, 6.5 kDa.

2.5. Assays

Trypsin trapping assays were carried out as described previously [15] by measuring residual trypsin enzymatic activity following addition of an excess of soybean trypsin inhibitor to complex any non-α_2M-trapped trypsin. Trypsin activity was measured spectrophotometrically using the chromogenic substrate *N*-tosyl-L-arginine methyl ester.

2.6. Materials

Reagents, enzymes, and oligonucleotides were obtained as follows. Low melting point agarose, maximum efficiency DH5αF′ IQ competent *E. coli*, DMEM, and G418 from Gibco/BRL. Sequenase version 2.0 DNA sequencing kit from United States Biochemical Corporation. M13mp18 (RF), M13mp19 (RF), and *Bsi*WI from Boehringer Mannheim. *Xba*I, *Pst*I, *Sph*I, *Xho*I, β-agarase 1, T4 DNA ligase, and T4 polynucleotide kinase from New England Biolabs. Plasmid purification kits from Qiagen. Oligonucleotide-directed in vitro mutagenesis system kit version 2.1 from Amersham. [α-^{32}P]dATP and [γ-^{32}P]ATP from New England Nuclear. Radial Immunodiffusion plates for human α_2M from The Binding Site, Inc. Methotrexate ((+)-amethopterin), phenylmethsulfonylfluoride, and *N*-tosyl-L-arginine methyl ester from Sigma. Trysin from Cooper Biomedical. Human neutrophil elastase from Athens Research and Technology, Inc. Fetal bovine serum, premium grade from Bioproducts for Science, Inc.

3. Results

3.1. Structural properties of C949S variant α_2M

To determine whether the mutation of cysteine-949 to serine had affected the ability of the polypeptide to fold in the correct manner, to form the correct inter-subunit disulfide bonds, and to oligomerize to form a tetramer, the structural properties of the recombinant C949S variant of α_2M were determined by several methods. The immunoreactivity of the protein towards anti-human α_2M polyclonal antibody was examined in a radial immunodiffusion plate assay. The C949S α_2M was immunoreactive and gave a degree of interaction consistent with the total amount of α_2M present (estimated inde-

pendently from intensity on SDS-PAGE). This indicated that the protein had folded sufficiently well to be secreted into the medium and possessed some or all of the epitopes present in wild-type or plasma α_2M.

On a polyacrylamide gel run under non-denaturing conditions the C949S variant α_2M had the same mobility (Fig. 1, lane 7) as wild-type or plasma α_2M that had been treated with trypsin or HNE (Fig. 1, lanes 2,3,5 and 6), but different mobility from native wild-type or plasma forms (Fig. 1, lanes 1 and 4). Reaction of C949S with either trypsin or HNE did not change its mobility (Fig. 1, lanes 8 and 9) consistent with it already being in the fast-form conformation. Since the mobility under non-denaturing conditions depends on the tertiary as well as the primary structure of a protein, this indicated that the overall structure resembled that of fast form α_2M both in the folding of the monomer and the association to form tetrameric α_2M.

Upon treatment of C949S variant with 1% SDS, the protein changed mobility on a non-denaturing gel from that of tetrameric α_2M (Fig. 2, lanes 1–5) to the same position as the 360-kDa disulfide-linked half molecules of plasma and wild-type α_2M on a polyacrylamide gel (Fig. 2, lanes 6–10). Following reduction, the variant gave a single band at the same position as the 180-kDa monomer position (Fig. 3, lanes 1,4, and 7). These results indicated that the correct disulfides had been formed between monomers to give covalently linked dimers and that no thiol ester was present in the molecule. Heating the C949S sample at 90°C for 5 min prior to running on SDS-PAGE did not result in the heat fragmentation that is thought to result from intramolecular attack of the amide nitrogen of Gln 952 on the carbonyl of the thiol ester (Fig. 4A, lane 10), consistent with the absence of a thiol ester.

3.2. *Functional properties of C949S variant α_2M*

To determine whether the fast electrophoretic mobility of the C949S variant α_2M on non-denaturing PAGE indicated that the variant had also lost the ability to interact with and inhibit proteinases, we examined the interaction of the protein with trypsin and human neutrophil elastase (HNE) and compared the behavior with that of wild-type recombinant and plasma α_2M. With both trypsin and HNE the bait region of plasma α_2M

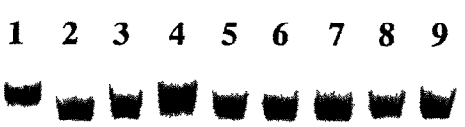

Fig. 1. Demonstration of fast-form mobility for C949S α_2M on non-denaturing polyacrylamide gel. Lanes 1–3, human plasma α_2M; lanes 4–6, wild-type recombinant α_2M; lanes 7–9, C949S variant recombinant α_2M. For each protein the first lane is unreacted, the second after reaction with trypsin, and the third after reaction with HNE.

Fig. 2. SDS-induced dissociation of α_2M species into disulfide-linked half molecules on a 5% polyacrylamide gel run under non-denaturing non-reduced conditions. Lane 1, native plasma α_2M; lane 2, methylamine-treated plasma α_2M; lane 3, wild-type recombinant α_2M; lane 4, methylamine-treated recombinant α_2M; lane 5, C949S variant recombinant α_2M. Lanes 6–10, are the same samples as in lanes 1–5, respectively, but after incubation in 1% SDS prior to loading.

was completely cleaved to give the characteristic bands on SDS-PAGE at about 90 kDa, corresponding to cleaved monomer as well as fainter high molecular weight bands near the top of the gel corresponding to monomer fragments covalently cross-linked to the proteinase via ε-lysyl-γ-glutamyl bonds (Fig. 3, lanes 1–3) (the cross linked bands are either absent or of greatly reduced intensity in the HNE sample, probably as a result of the absence of lysine residues in HNE). Almost identical behavior was found with recombinant wild-type α_2M (Fig. 3, lanes 4–6). The C949S variant, however, was only slightly susceptible to bait region cleavage by either trypsin or elastase (Fig. 3, lanes 7–9).

To determine if the limited proteolysis of the bait region of the C949S variant simply reflected a much slower rate of reaction with trypsin compared with plasma or wild-type α_2M, the variant was incubated with 2.2 eq. trypsin for different periods of time before inactivation of the proteinase. For comparison, methylamine-treated plasma α_2M was similarly incubated with trypsin. SDS-PAGE of the C949S reaction mixtures showed that a small fraction of the bait regions is accessible to trypsin

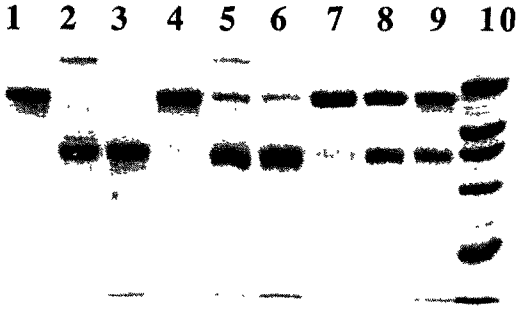

Fig. 3. Ability of proteinases to react with plasma, wild-type recombinant, and C949S recombinant α_2Ms from appearance of cleavage and cross-linkage products on reduced SDS-PAGE. Lanes 1–3, plasma α_2M; lanes 4–6, recombinant wild-type α_2M; lanes 7–9, C949S recombinant variant α_2M. lane 10, high molecular weight markers, as described in section 2.4, from myosin to carbonic anhydrase. For each protein the first lane contains unreacted α_2M, the second α_2M reacted with 2.2 eq. trypsin, and the third lane α_2M reacted with 2.2 eq. HNE.

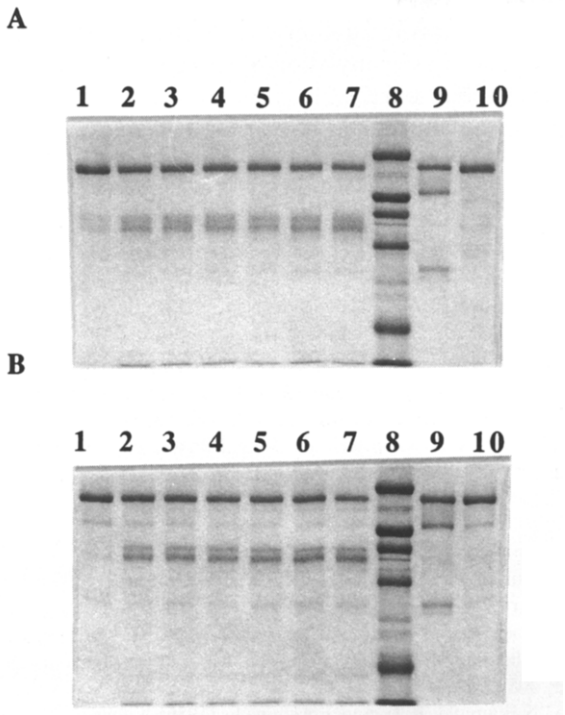

Fig. 4. Inaccessibility of the bait region to proteolysis by trypsin in methylamine-treated plasma α_2M and C949S recombinant variant α_2M demonstrated by SDS-PAGE of incubations of the α_2Ms with trypsin for different times. (A) C949S variant recombinant α_2M. (B) Methylamine-treated plasma α_2M. Lanes 1 through 7 represent incubations with trypsin for 0, 5, 10, 15, 30, 60, and 120 min. Lane 8 contains broad range molecular weight standards as described in section 2.4. Lane 9 in each panel contains heat-treated native α_2M and shows the characteristic heat fragmentation bands at about 120 and 60 kDa. Lane 10 in panel A contains heat-treated C949S α_2M, and lane 10 in panel B contains heat treated methylamine-treated plasma α_2M. Lane 10A shows no heat fragmentation band and lane 10b shows only a trace, presumably resulting from the presence of low levels of intact thiol ester prior to heating.

and is cleaved rapidly within the first few minutes (Fig. 4A, lanes 1 and 2). Most of the bait regions are not accessible to trypsin and remain uncleaved even after incubation with trypsin for 2 h (Fig. 4A, lanes 2–7). The ability of trypsin to cleave a small fraction of the bait regions was not, however, restricted to the C949S variant, since methylamine-treated plasma α_2M, which is also in the fast-form conformation, showed a similar pattern of limited cleavage (Fig. 4B, lanes 1–7).

A trypsin trapping assay was performed on the C949S variant α_2M to determine if the limited bait region cleaved seen above corresponded to a small amount of trypsin binding. One sample of the variant was incubated with 2.2 eq. trypsin for 5 min and another for 1 h. No trypsin activity was found associated with the α_2M. In contrast, plasma α_2M under the same conditions gave a value of 1.6 mol trypsin trapped/mol α_2M.

The final comparison between C949S α_2M and methylamine-treated plasma α_2M was in the behavior towards papain. Following reaction of plasma α_2M with either proteinase or methylamine, the receptor recognition domain is exposed and can be cleaved specifically by reaction with papain [16]. Native α_2M does not show this specific cleavage. A comparison of the papain-digestion patterns of native plasma α_2M, methylamine-treated plasma α_2M, and C949S α_2M, showed that the latter behaved like methylamine-treated α_2M, being able to release a fragment that ran on SDS-PAGE at the same positions as purified receptor binding domain (Fig. 5), whereas no such fragment release was observed with the native protein (Fig. 5, lane 2). Two bands are normally observed for the receptor recognition domain corresponding to non-glycosylated and glycosylated forms (Fig. 5, lane 7) [16,17]. In plasma α_2M the glycosylated form dominates (Fig. 5, lanes 4 and 7), whereas in the recombinant protein the non-glycosylated form dominates (Fig. 5, lane 6).

4. Discussion

We have shown by a number of criteria that the C949S variant of α_2M, despite being unable to form a thiol ester, could fold into a form that was indistinguishable from that of methylamine-treated plasma α_2M. Thus C949S α_2M showed 'fast-form' electrophoretic mobility under non-denaturing conditions and possessed no proteinase inhibitory capacity. The latter was judged not to be due to incorrect folding, however, since the bait regions showed the same resistance to tryptic cleavage as

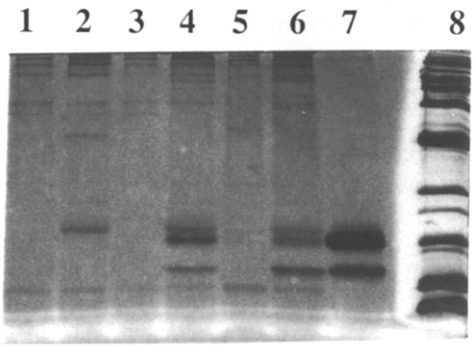

Fig. 5. Release of receptor recognition domain from C949S and methylamine-treated plasma α_2Ms, but not from native α_2M upon reaction with papain. Silver stained 12% SDS polyacrylamide gel run under non-reducing conditions. Lanes 1 and 2, native plasma α_2M; lanes 3 and 4, methylamine-treated plasma α_2M; lanes 5 and 6, C949S α_2M; lane 7, purified receptor recognition domain. Lane 8 contains molecular weight markers as described in section 2.4. Lanes 1,3 and 5 are samples unreacted with papain; lanes 2,4 and 6 are α_2M samples incubated with papain for 5 h. The conditions of the incubation were 0.4 mg/ml α_2M, and a ratio of α_2M to papain of 48:1 (w/w). Note that the receptor recognition domain occurs as two bands. Both have the same polypeptide structure but differ in glycosylation. The higher M.W. band carries one carbohydrate chain, whereas the lower band carries none. It appears that the relative proportions of glycosylated and non-glycosylated domain differ between the plasma protein and the recombinant BHK-derived protein, with more glycosylated form for the former and more non-glycosylated form for the latter.

methylamine-treated α_2M. Denatured or incorrectly folded α_2M would be expected to have a number of potential tryptic cleavage sites accessible to proteinase. The protein also appeared to have the same tetrameric form as both native and methylamine-treated plasma α_2Ms, being composed of a non-covalently associated pair of disulfide-linked dimers. In addition, the variant could also release the receptor recognition domain upon cleavage with papain. This cleavage is specific for the fast-form methylamine-treated structure of α_2M.

These results show that the thiol ester is not required for folding of the individual domains of α_2M, for correct disulfide-bond formation both within and between monomers, and for the correct association of the disulfide-linked half molecules to give the tetramer. However, the behavior of the variant as a non-inhibitory fast-form of α_2M, rather than an active slow-form species, again demonstrates the close relationship between the presence of an intact thiol ester and the maintenance of the active conformation. This relationship is a subtle one, which may not involve a large energy difference between the two states. Thus cyanylation or iodination of the free SH group in fast-form plasma α_2M can reverse the slow-to-fast conformational change even though the thiol ester is not re-formed [4]. In bovine α_2M, cleavage of the thiol ester alone by methylamine-treatment is not sufficient to cause the slow-to-fast conformational change [11]. Instead both thiol ester cleavage and bait region cleavage must occur for the conformational change to occur. In chicken ovostatin there is no thiol ester, but a proteinase-induced slow-to-fast conformational change still occurs [18]. Also, it has been shown for C3 that the thiol ester can spontaneously reform after cleavage by either ammonia or methylamine, with a ΔG^0 for the process of $+5.2$ kcal $\cdot$ mol^{-1}, corresponding to a K_d for the formation of slow form C3 and ammonia of about 150 μM [19]. With appropriately low concentrations of NH_3 at physiological pH, this could readily result in formation of the thiol ester and a conformational change to the slow-form, active species in C3 and also, presumably, in α_2M.

These results suggest a folding pathway for active α_2M that does not require the thiol ester to give the correct domain folding or monomer-monomer interactions, but only to provide the small energy change necessary to move the equilibrium in favor of the active conformation. This shift in equilibrium can be provided by other means, such as cyanylation of the cysteine, cross-linking with cis-platinum [20,21], or other undefined interactions such as must occur in bovine α_2M or ovostatin.

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