

# Activation of ADAM 12 protease by copper

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**Abstract** Conversion of latent proteases to the active form occurs by various mechanisms characteristic for different protease families. Here we report that the disintegrin metalloprotease ADAM 12-S is activated by Cu(II). Copper activation is distinct from the cysteine switch component of latency: elimination of the ADAM 12 cysteine switch by a point mutation in the propeptide had no effect on copper activation, whereas mutation of an unpaired cysteine residue in the catalytic domain resulted in a mutant form of ADAM 12-S that was insensitive to copper. This suggests a multi-step activation mechanism for ADAM 12 involving both furin cleavage and copper binding. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** ADAM; Disintegrin metalloprotease; Copper; Protease activation

## 1. Introduction

ADAMs (A disintegrin and metalloprotease) are a family of cell surface or secreted proteins that are related to snake venom metalloproteases and matrix metalloproteases (MMPs). ADAMs function in a wide variety of physiological processes, by virtue of either their cell adhesive activity or their proteolytic activity [1–4]. ADAM 12 has been implicated in myogenesis and tumorigenesis [5–7]. Human ADAM 12 is expressed in two forms, a membrane-anchored form (ADAM 12-L) typical for the ADAM family, and a shorter form (ADAM 12-S) that is secreted as a soluble protein [6]. ADAM 12-S appears to be one of the proteases responsible for cleavage of IGFBP-3 in blood during pregnancy [8,9].

Protease function in vivo can be regulated at several levels. A common means of modulating function is by delaying activation of the protease until it reaches its target site. Both ADAM proteases and MMPs are synthesized as zymogens, in which the N-terminal prodomain represses proteolytic activity by means of a cysteine switch. This latency mechanism involves an unpaired cysteine residue in the prodomain that directly coordinates the zinc ion at the catalytic site [10,11].

Activation of most ADAM proteases is initiated in the *trans*-Golgi network, when furin cleaves the propeptide from the protease [4,12]. Activation of proMMPs occurs in the extracellular environment in a stepwise manner, where the first step is cleavage of the propeptide by an exogenous protease at a specific site, followed by complete removal of the propeptide either by autocleavage or by intermolecular proteolysis [13]. In vitro, proMMP activation can be initiated by oxidizing or thiol-modifying reagents, and it is possible that this mechanism also functions in vivo. Such reagents function by modifying the unpaired cysteine residue in the propeptide, thereby inactivating the cysteine switch [14]. However, in some cases these oxidizing or thiol-modifying reagents activate proMMPs by an unknown mechanism that is cysteine switch-independent [15,16].

In this study, using purified recombinant ADAM 12-S protease, we have found that Cu(II) can activate ADAM 12-S by a cysteine switch-independent mechanism. Our results suggest that activation of ADAM 12 and possibly other ADAM proteases is a multistep process involving more than just furin cleavage of the propeptide. These data may also provide new insights into the complicated activation mechanism of MMPs.

## 2. Materials and methods

### 2.1. Reagents

All chemicals were purchased from Sigma. Chloride salts of divalent transition metal ions were used. Purified human  $\alpha_2$ -macroglobulin ( $\alpha_2$ M) was kindly provided by Claus Oxvig (University of Aarhus, Denmark).

### 2.2. Preparation of ADAM 12-S protein

Recombinant human ADAM 12-S protein (GenBank accession number AF023477) was purified as previously described [9]. The C179S mutation as well as the furin site mutation allowing expression of the ADAM 12-S proform have been published previously [12]. The C273A mutation was made by site-directed mutagenesis on ADAM 12-S cDNA using the GeneEditor site-directed mutagenesis system from Promega. Mutant ADAM 12 cDNAs were cloned in the pCEP4 expression vector (Invitrogen), yielding plasmids p1573 (C179S), p1572 (furin site mutation), and p1630 (C273A). They were transfected into 293-EBNA cells, and the mutant proteins were purified as for wild-type ADAM 12-S [9].

### 2.3. Protease assays

Assays were carried out in 50 mM PIPES (pH 7.0), 50 mM NaCl, 2 mM CaCl<sub>2</sub>, 0.5 mg/ml CHAPS at 37°C for 2 h. ADAM 12-S was included at 0.1  $\mu$ M, based on an extinction coefficient at 280 nm of 67.8 mM<sup>-1</sup>.  $\alpha_2$ M was included at a concentration of 0.5 mg/ml. Reactions were terminated by boiling in reducing SDS sample buffer and analyzed by SDS-PAGE on 6% Tris-glycine gels (Novex), followed by Western blotting using a polyclonal antibody against the ADAM 12 cysteine-rich domain [17]. Detection was performed with a horseradish peroxidase-conjugated second antibody and the chemiluminescence SuperSignal West Pico reagent from Pierce.

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**Abbreviations:** ADAM, A disintegrin and metalloprotease;  $\alpha_2$ M,  $\alpha_2$ -macroglobulin; MMP, matrix metalloprotease; PAGE, polyacrylamide gel electrophoresis

#### 2.4. Gel filtration chromatography

8  $\mu$ g purified ADAM 12-S protein was applied to a Superose 12 HR column, using the ÄKTA Basic FPLC system (Amersham Pharmacia Biotech) at a flow rate of 0.5 ml/min. The column buffer was 20 mM Tris (pH 7.5), 200 mM NaCl, 1 mg/ml CHAPS. Absorbance at 280 nm was used to monitor the eluant, and peak fractions were analyzed by SDS-PAGE on a 10% Tris-glycine gel (Novex), followed by immunoblotting using antibodies against both the cysteine-rich domain and the propeptide of ADAM 12.

### 3. Results and discussion

We examined the requirement of ADAM 12-S protease for biologically relevant transition metal ions, making use of the  $\alpha$ 2M cross-linking reaction. In previous studies we used conditioned medium from transfected COS cells as a source of ADAM 12-S, while in this study we used purified ADAM 12-S [11,12]. There was no detectable protease activity in the absence of transition metal ions (Fig. 1A). Both Zn(II) and Cu(II) were strong activators of ADAM 12-S protease, while the other metal ions had no effect. In the presence of 50  $\mu$ M Zn(II) or 10  $\mu$ M Cu(II), most of the ADAM 12-S was converted to high molecular weight cross-linked products, showing that the majority of the ADAM 12-S molecules were proteolytically active.

These reactions contain two proteins, hence it is possible that Zn(II) or Cu(II) stimulated activity by binding to either  $\alpha$ 2M or ADAM 12-S. Another assay for ADAM 12-S protease is the autocleavage of the ADAM 12-S proform that occurs upon chemical or genetic inactivation of the cysteine switch [11]. This assay has the advantage that the only protein present is ADAM 12-S. Fig. 1B shows that the only transition metal ion capable of activating the ADAM 12-S proform was Cu(II). Autocleavage was detectable at 1  $\mu$ M Cu(II) and was

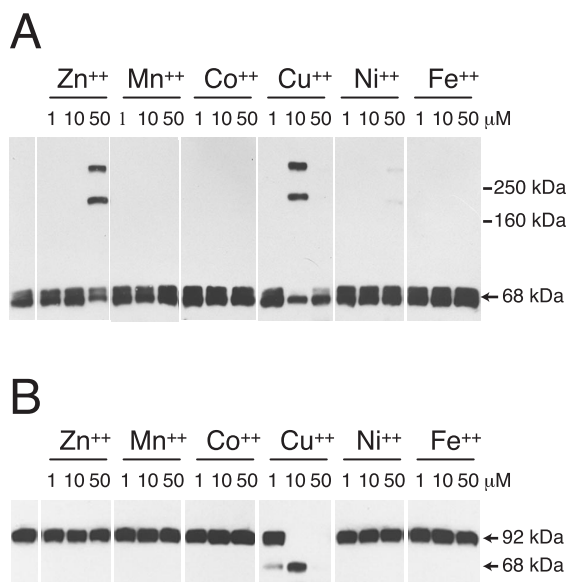


Fig. 1. A:  $\alpha$ 2M crosslinking assay on ADAM 12-S protease, carried out either in the absence of transition metal ions (first lane) or with individual ions at the indicated concentration. Cleavage of the  $\alpha$ 2M bait region results in conversion of 68 kDa ADAM 12 protease to high molecular weight cross-linked products. B: Autocleavage of ADAM 12-S proform. ADAM 12-S protein containing a mutation to prevent cleavage of the propeptide by furin was purified and incubated either in the absence of transition metal ions (first lane) or with individual ions at the indicated concentration.

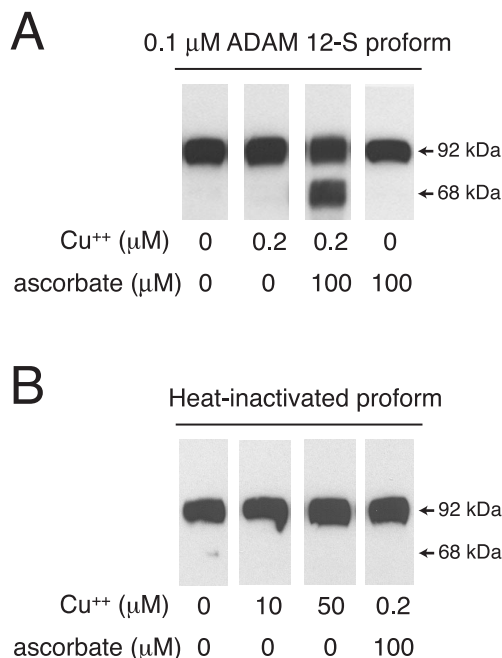


Fig. 2. Autocleavage of ADAM 12-S proform induced by metal-catalyzed oxidation. A: The proform was incubated with Cu(II) and ascorbate at the indicated concentrations. Conversion of the 92 kDa proform to a 68 kDa form was detected by immunoblotting. B: The proform was incubated at 70°C for 15 min to destroy its catalytic activity, prior to incubation with Cu(II) and ascorbate.

complete at 10  $\mu$ M Cu(II). Western blotting with domain-specific antibodies showed that cleavage occurred near the junction of the prodomain and the catalytic domain (data not shown). Higher concentrations of Cu(II) resulted in further degradation of the protease.

Copper and other transition metal ions are capable of oxidizing proteins at amino acid residues such as His or Cys [18]. Such metal-catalyzed oxidation can be highly specific. In the presence of a reducing agent (e.g. ascorbate), Cu(II) can bind to and oxidize a single amino acid residue in a protein, resulting in dramatic changes in protein structure [19–21]. We tested for activation of ADAM 12-S protease by metal-catalyzed oxidation using the ascorbate/Cu(II)/O<sub>2</sub> system [21,22]. ADAM 12-S proform was incubated with 0.2  $\mu$ M Cu(II), at a ratio of two copper ions per molecule of ADAM 12-S. At this concentration of copper, there was no detectable autoproteolysis (Fig. 2A). Addition of 100  $\mu$ M ascorbate resulted in activation of ADAM 12-S protease. Ascorbate alone had no effect. When the enzymatic activity of the proform was destroyed by heat treatment prior to incubation with copper or copper/ascorbate, no cleavage was seen (Fig. 2B). Conversion of 92 kDa ADAM 12-S to a 68 kDa form was therefore not due to direct cleavage of peptide bonds by copper, but rather autocatalysis of the activated proform.

We considered the possibility that copper activation of ADAM 12 protease involves the cysteine switch. Cys179 in the ADAM 12 prodomain is an unpaired cysteine residue which represses proteolytic activity by coordinating the zinc ion at the active site of the catalytic domain [11]. The ADAM 12 propeptide remains bound to the protease after cleavage by furin [9], therefore the cysteine switch is potentially still functional in furin-cleaved ADAM 12-S. Cu(II) could conceivably disengage the cysteine switch by binding to or oxidizing

Cys179, similar to the way in which ADAM 12 or MMP proforms can be activated by alkylation of the free cysteine in the propeptide [11,13]. A mutant form of ADAM 12-S was prepared in which Cys179 was changed to Ser. The C179S mutant behaved identically to wild-type ADAM 12-S; it was activated by both Cu(II) and Zn(II) (Fig. 3). We conclude that the cysteine switch and copper activation are two separate components of the ADAM 12 latency/activation mechanism.

The sequence of the ADAM 12 prodomain and catalytic domain was examined for amino acid residues likely to constitute a copper binding site; cysteine and histidine are good candidates based on comparison to known copper binding proteins [23,24]. There is a cysteine residue in the ADAM 12 catalytic domain that is predicted to be unpaired, based on homology to other ADAM proteases [2]. We hypothesized that this residue, Cys273, together with histidine residues in the catalytic domain and/or propeptide, binds a copper ion, leading to a change in conformation of ADAM 12-S, and subsequent activation. ADAM 12-S protein with Cys273 mutated to Ala was purified as tested in the  $\alpha$ 2M cross-linking assay. This ADAM 12-S mutant was not activated by Cu(II) (Fig. 3). This is a specific effect rather than simple misfolding of the protease due to the mutation, because the C273A mutant does have  $\alpha$ 2M cross-linking activity in the presence of Zn(II).

These data, together with previously published results on the ADAM 12 cysteine switch [11], suggest a multi-step activation mechanism for ADAM 12-S protease. Newly synthesized ADAM 12-S in the endoplasmic reticulum is latent, due to coordination of the zinc ion at the active site by Cys179 in the prodomain. As it passes through the *trans*-Golgi network, ADAM 12-S is cleaved at the junction between the prodomain

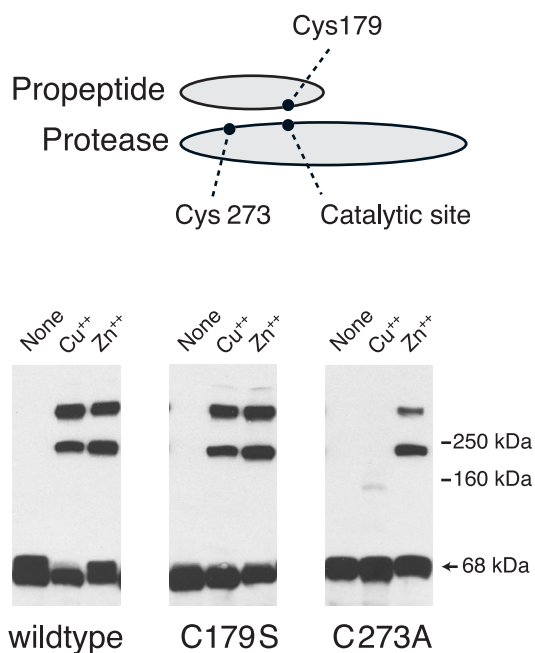


Fig. 3.  $\alpha$ 2M crosslinking assay on ADAM 12-S protease, comparing copper activation of wild-type ADAM 12-S to ADAM 12-S containing a point mutation in the cysteine switch (C179S) or a point mutation in an unpaired cysteine residue in the catalytic domain (C273A). Reactions were carried out either with no transition metal ions, with 10  $\mu$ M CuCl<sub>2</sub>, or with 50  $\mu$ M ZnCl<sub>2</sub>.

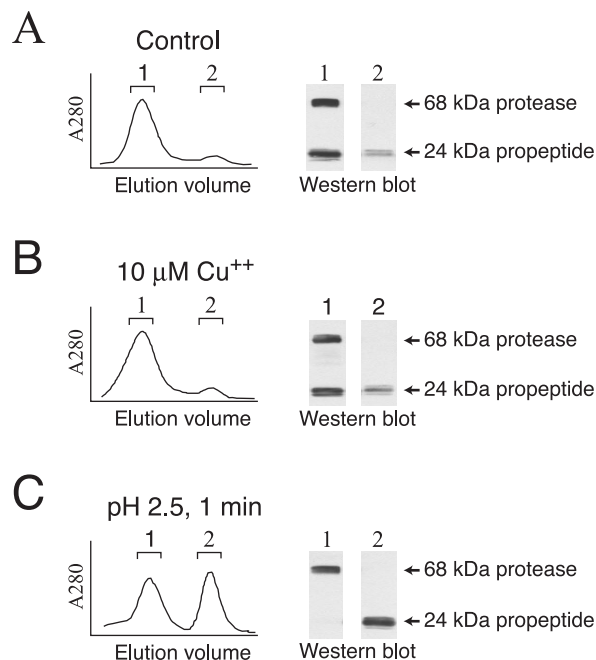


Fig. 4. Gel filtration chromatography on wild-type ADAM 12-S protein. A: Control. B: In the presence of 10  $\mu$ M CuCl<sub>2</sub>. C: Treated with pH 2.5 for 1 min prior to chromatography. Immunoblotting of the peak fractions using antibodies against both the propeptide and the protease showed that peak 1 contained the propeptide/protease complex, and peak 2 contained free propeptide. 10  $\mu$ M CuCl<sub>2</sub> had no effect, while low pH dissociated the propeptide/protease complex.

and the catalytic domain, but the propeptide remains bound to the protease. Furin-cleaved ADAM 12-S is markedly more active than the ADAM 12-S proform when assayed using either  $\alpha$ 2M [11,12] or IGFBP-3 as a substrate (F. Loechel, unpublished data), but an additional step involving binding of Cu(II) appears to be required for full activity of ADAM 12-S protease. This second activation step does not involve the cysteine switch (Fig. 3), nor does it seem to involve dissociation of the propeptide from the protease. The propeptide remains bound to the protease after furin cleavage, as evidenced by the fact that the two polypeptides migrate as a complex in gel filtration chromatography (Fig. 4A). A trace of free propeptide is also detectable. The protease/propeptide complex remained intact when chromatography was performed in the presence of 10  $\mu$ M CuCl<sub>2</sub> (Fig. 4B). In contrast, the propeptide could be dissociated from the protease by a brief incubation at pH 2.5 (Fig. 4C).

We favor a model in which copper binding, rather than dissociating the propeptide, results in oxidation of one of the amino acid residues in the binding site, and thereby changes the conformation of ADAM 12-S so that it is fully active. In vivo, such a two-step activation mechanism would allow for tighter control of ADAM 12 protease activity in blood or in the extracellular space. Copper is sequestered by proteins such as ceruloplasmin, and there is thought to be little free copper available [25]. Transfer of a copper ion to ADAM 12 protease in vivo could occur either directly from a copper chaperone, similar to what has been demonstrated for copper charging of superoxide dismutase [23], or by transient

increases in free copper concentration as a result of oxidation of proteolysis of copper-sequestering proteins.

Is copper likely to be involved in the activation of other proteases? Among ADAM proteases, ADAM 13 and ADAM 19 are good candidates, because they contain an unpaired cysteine residue in the catalytic domain at the same position as Cys273 of ADAM 12 [26]. Copper activation of ADAM 12 protease may have a parallel in the MMP family of zinc metalloproteases. It is known that MMPs, in addition to the zinc ion at the catalytic site, have an additional binding site for a 'structural' zinc in the catalytic domain [1]. Zinc occupancy of this site in a given MMP is dependent on the presence of other domains, as well as on the method of purification [27,28]. It has been proposed that the second zinc ion is part of an additional activation step, separate from the MMP cysteine switch [29]. It would be interesting to test whether this zinc binding site is actually a copper binding site that plays a role in MMP activation *in vivo*.

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