Inhibition of the Tumor Necrosis Factor-α-converting Enzyme by Its Pro Domain

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Tumor necrosis factor-α-converting enzyme (TACE) is a disintegrin metalloproteinase that processes tumor necrosis factor and a host of other ectodomains. TACE is biosynthesized as a preproenzyme, and activation requires the removal of an inhibitory pro domain. Little is known about how the pro domain exerts inhibition for this class of enzymes. To study the inhibitory properties of the pro domain of TACE, we have expressed it in isolation from the rest of the protease. Here we show that the TACE pro domain (TACE Pro) is a stably folded protein that is able to inhibit this enzyme. TACE Pro inhibited the catalytic domain of TACE with an IC₅₀ of 70 nM. In contrast, this inhibitory potency decreased over 30-fold against a TACE form containing the catalytic plus disintegrin/cysteine-rich domains (IC₅₀ greater than 2 μM). The disintegrin/cysteine-rich region in isolation also decreases the interaction of TACE Pro with the catalytic domain. Surprisingly, we found that the cysteine switch motif located in TACE Pro was not essential for inhibition of the enzymatic activity of TACE; the pro domain variant C184A showed the same inhibitory potency against both TACE forms as wild type TACE Pro. X-ray absorption spectroscopy experiments indicate that binding of TACE Pro to the catalytic domain does include ligation of the catalytic zinc ion via the sulfer atom of its conserved Cys¹⁸⁴ residue. Moreover, the binding of TACE Pro to the catalytic zinc ion partially oxidizes the catalytic zinc ion of the enzyme. Despite this, the nature of the interaction between the pro and catalytic domains of TACE is not consistent with a simple competitive model of inhibition based on cysteine switch ligation of the zinc ion within the active site of TACE.

The tumor necrosis factor-α-converting enzyme (TACE or ADAM 17) is a zinc metalloproteinase that cleaves the precursors, membrane-bound form of tumor necrosis factor-α and other ectodomains (1–3). TACE is a member of a family of proteases known as the ADAMs (a disintegrin and metalloproteinase). ADAMs are multidomain proteins typically comprising a pro domain, metalloprotease domain, a disintegrin/cysteine-rich domain, transmembrane domain, and a cytoplasmic tail (4, 5). ADAMs are synthesized as latent precursors and later converted to the mature enzyme, lacking the pro domain, by furin or a furin-like enzyme in the secretory pathway (6, 7).

It has been shown previously that the pro domain of TACE seems to act as an inhibitor of this enzyme, because the activity of TACE was only recovered upon its removal (8). TACE Pro includes a cysteine switch box (PKVCGY¹³⁶), a feature present in most metzincins, including matrix metalloproteinases and ADAMs. It has been proposed that the pro domains of metzincins act as inhibitors of their catalytic domains through a mechanism that involves ligation of the cysteinyl thiol within the cysteine switch box to the zinc ion in the active site (9–11). The cysteine switch present in TACE Pro did appear to be important for inhibition of this enzyme, because thiol-modifying reagents such as 4-aminophenylmercuric acetate and octylthioglucoside promoted pro domain release from the catalytic domain and, therefore, enzyme activation (8).

TACE Pro also serves a second function: it is essential for the secretion of functional enzyme. In insect cells, a recombinant form of this enzyme lacking the pro domain failed to be secreted and was extensively degraded intracellularly (8). Similar results have been reported for other members of the ADAM family (12, 13). At first approximation, this is similar to the secreted bacterial serine proteases subtilisin and α-lytic protease, in which the pro domain accelerates the folding of the catalytic domain by several orders of magnitude, apparently by lowering the conformational energy barrier between the unfolded and native states (14–17).

Here we show that the isolated pro domain of TACE is a potent inhibitor of the catalytic domain of this enzyme. TACE Pro is a less effective inhibitor of a form of TACE comprising the catalytic plus disintegrin/cysteine-rich domains, suggesting a role for the disintegrin/cysteine-rich domain in prodomain removal and enzyme activation. We also show that the cysteine switch of TACE, although present in the zymogen form, is not required for the interaction between the pro and catalytic domains of TACE.

MATERIALS AND METHODS

Plasmid Constructs—A plasmid for expression of the pro domain of TACE in Escherichia coli, lacking the signal peptide, was constructed using the vector pRSET B (Invitrogen). A DNA fragment encoding residues Asp²⁰² to Arg³¹¹ with an NdeI site at the 5′-end and a BamHI site at the 3′-end was generated by polymerase chain reaction amplifi-
cution using a previously reported pFastBac1 TACE plasmid as a template (8). The fragment was inserted at the BamHI and NdeI sites of pRSET B to obtain TACE Pro. TACE Pro C184A was made by cassette mutagenesis. The cassette was designed with EcoRV and Accl-compatible ends for insertion into TACE Pro and carried a Cys to Ala mutation at position 184. Additional constructs for expression of double alanine mutants in the TACE Pro cysteine switch region (PKVCYGLKDVD[98]) were made by the same cassette mutagenesis approach. The Cys to Ala mutation in position 184 and an additional alanine substitution at each individual position in the cysteine switch region.

Expression of Recombinant TACE Pro Proteins—E. coli BL21(DE3) electrocompetent cells were transformed with the corresponding TACE Pro plasmid and plated on LB plates containing 150 μg/ml ampicillin. After selection at 37°C, the clones were picked, grown overnight at 0.1 mm (2.92 mg/ml) and then with the same buffer containing 0.1% Triton X-100. Washed pellets were solubilized in 20 mM Tris-HCl, pH 8, containing 6 M Gdn-HCl and centrifuged at 26,000 × g for 30 min. The supernatant was applied to a 20-mL Ni²⁺-nitrilotriacetic acid column. The column was washed with 20 mM Tris-HCl, pH 8, 6 M Gdn-HCl and then with the same buffer containing 20 mM imidazole. The protein was eluted with 20 mM Tris-HCl, pH 8, 6 M Gdn-HCl, 300 mM imidazole. The eluate was dialyzed against water. Under these conditions, the protein formed a white precipitate that was then resuspended in 20 mM Tris-HCl, pH 8, 6 M Gdn-HCl to a concentration of 6 mg/ml. 50 μl of this protein solution were incubated with 950 μl of refolding buffer (FoldIt Screen Formulation 16, Hampton Research) with addition of 1 mM reduced glutathione, 0.1 M oxidized glutathione, and 3 mM lauryl maltoside for 4 h at 4°C with gentle stirring. After refolding, the solution was centrifuged at 10,000 × g for 10 min and filtered through a 0.22-μm filter. Finally, it was dialyzed against 250 ml of 20 mM Tris-HCl, pH 8, with 150 mM NaCl (buffer A). The final protein concentration obtained after this procedure was 4 μM (0.09–0.14 mg/ml). The proteins were purified as described previously (8). Logarithmically growing Trichoplusia ni cells were infected with baculovirus strains encoding R473 or R651 at a multiplicity of infection of 1. The cultures were harvested 48 h after infection, and the proteins were purified as described previously (8).

TACE Activity and Inhibition Assays—The inhibitory activity of TACE Pro was determined by an high performance liquid chromatography-based assay using the synthetic peptide dinitrophenyl-SPLAQAVRRSS-R-NH₂ as the substrate. TACE (50 ng) was incubated for 1 h at 37°C with a series of TACE Pro concentrations ranging from 0 to 6 μM in buffer A. The incubation took place for 20 min at 37°C with gentle shaking. The reaction was initiated by the addition of substrate at a final concentration of 20 μM. The reactions were incubated at 37°C for 30 min with gentle shaking, quenched by the addition of an equal volume of 1% heptafluorobutyric acid, and filtered using polyvinyl difluoride membrane filters. The quenched reaction mixtures were applied to a 150-mm C18 column (Vydac) and resolved using a gradient of 0.1% heptafluorobutyric acid in water and 0.1% heptafluorobutyric acid in acetonitrile. The ratio of product/substrate was obtained after averaging 5–10 independent XAS measurements for each sample. Each data set was aligned using the first inflection point of a reference zinc metal foil (9659 eV). Subsequently, the absorption coefficients for different samples were shifted in x-ray energy until their first inflection points were aligned at the same energy.

The rough atomic background was then removed with the AUTOBK program of the UWXAFS data analysis package, developed at the University of Washington at Seattle (18). For background removal, the energy shift, E₀, was chosen as the edge shift of the processed data for each sample and used as the origin of the photoelectron energy. The R space region for minimizing the signal below the first shell was chosen between 1.0 and 1.2 Å. Upon background removal, the useful k range (energy range used in fitting procedure, in wave units) in the resultant k₁-weighted (k) was between 0 and 10.0 Å⁻¹. Model data for fitting procedures were constructed by extracting the structural zinc site coordinates (in a radius of 4 Å from the zinc) of gelatinase A (Protein Data Bank code 1CK7) and stromelysin-1 (Protein Data Bank code 1SLM). The theoretical photoelectron scattering amplitudes and phase shifts were calculated for each zinc ligand (path), using the computer code FEFF7 (19, 20). The total theoretical signal (k) was constructed by adding the most important partial k values that contributed to the R range of interest. The theoretical XAFS signal was fitted to the experimental data using the nonlinear least squares method, implemented in the program FEFFIT 2.98. The fit was performed with four independent procedures on both theoretical and experimental data. The experimental data and theoretical values were weighted by k and multiplied by a Hanning window function in Fourier transforms.

RESULTS

The Pro Domain of TACE Can Be Expressed in Isolation—It has been shown previously that the pro domain of TACE displays inhibitory activity against this enzyme (8). To characterize the role of TACE Pro as an enzyme inhibitor, we attempted its expression in E. coli.

TACE Pro was expressed at high levels and accumulated as inclusion bodies. The purification protocol involved a nickel chelate chromatography step under denaturing conditions followed by refolding via dialysis. Refolding of TACE Pro was the limiting step in obtaining large amounts of the protein, because
of its tendency to aggregate at concentrations over 8 μM. Nevertheless, the final yield for this refolding step was typically 31–48%. After refolding, TACE Pro was over 95% pure judging by reducing SDS-PAGE analysis. The protein partitioned between two forms: a free monomer and a disulfide-linked dimer (Fig. 1). The free monomer constituted 40% of the purified protein, as determined by densitometric analysis of gels run at different total protein concentrations.

TACE Pro appeared to be folded according to both our fluorescence and circular dichroism spectroscopy data. The intrinsic tryptophan fluorescence emission of TACE Pro after excitation at 280 nm showed a maximum at 325 nm, suggesting that its two tryptophan residues (Trp$^{111}$ and Trp$^{153}$) were in an environment of low polarity (Fig. 2A). For comparison, when the protein was subjected to chemical denaturation with 6 M Gdn-HCl, the fluorescence emission maximum shifted to the red, indicating the exposure of the tryptophans to bulk solvent in the unfolded polypeptide (Fig. 2A). The circular dichroism spectrum of TACE Pro showed a minimum between 208 and 230 nm, which revealed the presence of significant secondary structure in the protein (Fig. 2B). Upon chemical denaturation with Gdn-HCl, the CD ellipticity in this region was lost (Fig. 2B).

To determine whether the pro domain of TACE is stably folded in isolation, we studied its unfolding under equilibrium conditions by monitoring the change in 1) the center of mass of the fluorescence emission and 2) the circular dichroism ellipticity versus denaturant concentration (Fig. 3). Both of these probes reported a single transition between the folded and unfolded states, characteristic of a cooperative, thermodynamically stable native state. The midpoint of this transition was observed at 1 M Gdn-HCl.

**TACE Pro Is an Inhibitor of TACE**—We proceeded to test whether refolded TACE Pro could act as an inhibitor of TACE *in vitro*. We assayed the inhibitory activity of TACE Pro against two different TACE forms: 1) the catalytic domain of TACE alone and 2) the catalytic and disintegrin/cysteine-rich domains of TACE. TACE Pro proved to be a very potent inhibitor of the catalytic domain (IC$^{50}$ = 70 nM; Fig. 4A). Interestingly, this inhibitory potency dropped over 30-fold against the form containing the catalytic and disintegrin/cysteine-rich domains (IC$^{50}$ higher than 2 μM; Fig. 4A). The inhibition curve for the mature ectodomain of TACE could not be completed because TACE Pro aggregated at concentrations around 8 μM. The disintegrin/cysteine-rich domain seems to affect the ability of the pro domain to stably bind the catalytic domain. To test whether the disintegrin/cysteine-rich domain of TACE promotes dissociation of TACE Pro from the catalytic domain, we preincubated inactive pro-catalytic domain complexes with increasing amounts of disintegrin/cysteine-rich domain and then assayed for enzymatic activity. As shown in Fig. 5, even a short preincubation time (5 min) resulted in significant recovery of the catalytic activity of TACE (for example, at 10 μM of disintegrin/cysteine-rich domain, 38% of activity was observed relative to a control preparation of enzyme devoid of the pro domain; in contrast, only 3.5% of activity was observed with no addition of the disintegrin/cysteine-rich domain). Larger increases in activity were observed after 2 h of preincubation (Fig. 5; 58% activity at 10 μM disintegrin/cysteine-rich domain, versus 3.4% in its absence). Therefore, this domain of TACE appears to decrease the affinity of the pro domain for the catalytic domain. The low levels of spontaneous activation in the absence of the disintegrin/cysteine-rich domain are in

![Fig. 1. SDS-PAGE of purified TACE Pro.](http://www.jbc.org/)

![Fig. 2. Fluorescence and circular dichroism spectra of TACE Pro and TACE Pro C184A.](http://www.jbc.org/)

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*Fig. 1.* SDS-PAGE of purified TACE Pro. After refolding, TACE Pro was partitioned between a monomeric and a disulfide bond dimeric form. Lane 1, markers; lane 2, monomeric TACE Pro under reducing conditions; lane 3, monomeric and dimeric TACE Pro under nonreducing conditions.

*Fig. 2.* Fluorescence and circular dichroism spectra of TACE Pro and TACE Pro C184A. Native spectra were taken in 20 mM Tris-HCl, pH 8, 150 mM NaCl. Denatured spectra were taken in the same buffer plus 6 M Gdn-HCl. A, fluorescence spectra. ○, native TACE Pro; ○, native TACE Pro C184A; ■, denatured TACE Pro; □, denatured TACE Pro C184A. The maximum observed at 370 nm corresponds to material that aggregated during the course of the experiment. B, CD spectra. The symbols are the same as for A. (deg/cm^2/dmol) × 10^-3.
agreement with previous observations showing that the catalytic domain activation of TACE was only promoted efficiently upon the addition of hydrophobic thiol-modifying reagents (8). The inhibitory activity of TACE Pro could potentially be attributed to chelation of the zinc ion in the active site of the enzyme by the C-terminal His6 purification tag in TACE Pro. We addressed this possibility by using a TACE Pro variant that lacked that purification tail. This pro variant proved to have similar inhibitor profiles when tested against both forms of TACE (data not shown).

An Intact Cysteine Switch Is Not Required for Inhibition—The pro domain of TACE contains a conserved cysteine residue at position 184 in the context of a cysteine switch consensus motif. This residue was expected to mediate inhibition via coordination to the zinc ion in the active site, as proposed in the cysteine switch model (9–11). To study the importance of the cysteine switch motif in the inhibition of TACE, we generated a variant of TACE Pro carrying a cysteine to alanine substitution at position 184 (TACE Pro C184A). The mutant protein was expressed in E. coli and purified using the same protocol as for TACE Pro. TACE Pro C184A purified entirely as a monomer (data not shown) and had similar solution properties as its wild type counterpart. The fluorescence emission and CD spectra of TACE Pro C184A were basically indistinguishable from those of the wild type protein (Fig. 2). TACE Pro C184A also had similar equilibrium denaturation profiles to those of TACE Pro (Fig. 3).

Surprisingly, the introduction of this cysteine to alanine mutation had only a modest effect in the inhibitory potency of TACE Pro. The IC50 values obtained for TACE Pro C184A were 40 nM against the catalytic domain and higher than 2 μM against the mature ectodomain (catalytic plus disintegrin/cysteine-rich domains). This suggests that the cysteine residue cannot determine by itself the interaction between the pro and catalytic domains of TACE. Other residues within the cysteine switch region (PKVCGYLKVD190) do not appear to be critical for the pro-catalytic domain interaction either, because alanine substitutions at each individual position of the switch region, in the context of TACE Pro C184A, affected the inhibitory ability of TACE Pro modestly, within 2-fold of the IC50 observed with wild type TACE Pro (Table I). Additional sequence elements within the pro domain different from the cysteine switch region, yet to be identified, must mediate the pro-catalytic domain interaction.

![Fig. 3. Chemical denaturation of TACE Pro and TACE Pro C184A with Gdn-HCl. Protein denaturation was performed in 20 mM Tris-HCl, pH 8, 150 mM NaCl and the indicated concentrations of Gdn-HCl. A, denaturation followed by changes in ellipticity at 222 nm. B, TACE Pro; C, TACE Pro C184A. B, denaturation followed by changes in intrinsic fluorescence after excitation at 280 nm. D, TACE Pro; E, TACE Pro C184A.](image_url)

![Fig. 4. Inhibition of TACE R473 and R651 with TACE Pro. A range of different concentrations of TACE Pro was incubated with TACE R473 (catalytic domain, ○) or R651 (catalytic plus disintegrin/cysteine-rich domains, ○) prior to incubation with substrate, as described under “Materials and Methods.” A, inhibitor profiles obtained with TACE Pro. B, inhibitor profiles obtained with TACE Pro C184A. The data are expressed as percentages of the TACE activity observed in the absence of inhibitor with either R473 or R651.](image_url)
Conformational Changes within the Active Site of the TACE Zymogen Probed by XAS

We wanted to address whether binding of TACE Pro to the catalytic domain of this enzyme changes the conformation and electronic state of the coordination shells surrounding the catalytic zinc ion. For this, we used x-ray absorption spectroscopy. XAS refers to modulations in x-ray absorption coefficient, \( \mu(E) \), around an x-ray absorption edge of a given atom. Fig. 6A shows the XANES spectra of active and latent (bound to the pro domain) forms of TACE. The edge energy position of the pro-catalytic complex is shifted to higher energy by \( 0.45 \) eV in comparison with active TACE (catalytic domain alone). A shift in edge position is often an indication of structural modification at the metal site, such as different ligation \((21)\). Specifically, this shift reflects a change in the total effective charge of the zinc ion in TACE, which is partially oxidized upon binding of the pro domain. The post-edge spectra exhibit slight modifications of the peak intensities around 9680 and 9737 eV, indicating mild conformational changes within the environment of the catalytic zinc ion of TACE.

To study the structure of the zinc site in the enzyme, we performed EXAFS curve fitting analysis of TACE in its latent and active states. The analysis was conducted by fitting the theoretical phase shifts and amplitudes extracted from the crystal structure of pro-MMP-2 \((22)\) and the catalytic domain of TACE to the corresponding raw data. The fitting parameters are listed in Table II.

### Table I

| TACE Pro mutant | IC\(_{50}\) (nM) |
|-----------------|----------------|
| P181A           | 48.55          |
| K182A           | 72.58          |
| V183A           | 99.71          |
| G185A           | 131.7          |
| Y186A           | 70.98          |
| L187A           | 57.32          |
| K188A           | 87.98          |
| V189A           | 69.24          |
| D190A           | 81.87          |
TACE (23) and fitted to the experimental XAS data of active and latent TACE, respectively. Fig. 6B shows the Fourier transform presentations of best fitting results for the EXAFS analysis of the various structures. The fitting parameters and the quality of the fits are listed in Table II. The zinc sites in the various forms of the enzyme were fitted to the zinc-nitrogen, zinc-oxygen, zinc-sulfur, and zinc-carbon paths using different combinations of variable and constraint parameters.

Stable and reproducible fits of TACE Pro (Table II) were consistent with a tetrahedral coordination of the zinc ion with three zinc-nitrogen (His) paths at 2.06 ± 0.03 Å and one zinc-sulfur (Cys) path at 2.27 ± 0.01 Å in the first coordination shell, and seven zinc-carbon contributions (three zinc-carbon paths at 2.79 ± 0.02 Å and four at 3.07 ± 0.02 Å) in the second coordination shell. Attempts to fit the first coordination shell of the active enzyme with four zinc-nitrogen/oxygen or additional zinc-oxygen contributions resulted in unstable fits and relatively high $\chi^2$ (the sum of the residuals squared. It represents robustness of the fit) values. The zinc ligand distances derived from our EXAFS analysis for the pro enzyme are in agreement with the crystal structure of pro-MMP2 (22) and previous data of pro-MMP-2 EXAFS analysis (24).

The EXAFS curve fitting data analysis of active TACE shows that the zinc ion is tetrahedrally coordinated with three zinc-nitrogen (His) bond distances at 2.06 ± 0.01 Å, one zinc-nitrogen/oxygen bond distance at 1.89 ± 0.01 Å, three zinc-carbon bond distances at 2.85 ± 0.01 Å, and two zinc-carbon bond distances at 3.11 ± 0.01 Å. Attempts to fit the first coordination shell of the active enzyme with only three zinc-nitrogen/oxygen contributions or additional zinc-sulfur contributions resulted with unstable fits and high $\chi^2$ values. The first coordination shell zinc-ligand bond distances of active TACE are in agreement with the x-ray crystal structure (23), showing three zinc-nitrogen contributions of the histidine residues chelating the catalytic zinc ion. Similarly, the catalytic zinc ion of MMP-2 is coordinated by three zinc-nitrogen (His) at 1.97 ± 0.02 Å and one zinc-nitrogen/oxygen (presumably zinc-oxygen contribution from water) at 2.01 ± 0.05 Å (24).

### DISCUSSION

The role of the pro domains of the ADAM family of metalloproteinases in the regulation of the activity of these enzymes is not fully understood yet. Only a few ADAMs have been characterized in terms of their secretion and prodomain-mediated inhibition: ADAM 9 (25), ADAM 10 (12), ADAM 12 (13), ADAM 15 (26), ADAM 17 (TACE, Refs. 8 and 27 and this report), ADAM 19 (7), and ADAM 28 (28). A recent report suggested that ADAM 33 may differ from other ADAMs; its pro domain appears not to inhibit catalytic activity (29). It has been reported that the pro domain of ADAMs is essential for secretion of functional enzyme, because variants lacking this domain were rapidly degraded intracellularly (8, 12, 25, 27). One possibility is that the pro domains of ADAMs may work in an analogous way to the ones of the bacterial serine proteases subtilisin BPN and α-lytic protease. For those enzymes, the pro domain is needed to catalyze protein folding by effectively decreasing the conformational energy barrier between the folded and unfolded states (folding under kinetic control) (14–17). On the other hand, the role of ADAM pro domains may be restricted to aid in the trafficking of these enzymes through the secretory pathway by acting as intramolecular chaperones without a direct role in catalysis of folding. In an analogous manner to several molecular chaperones, these pro domains may actually arrest the folding of the catalytic domain, instead of promoting it. Under this alternate interpretation, the final assembly of ADAM proteinases into their native, functional conformations occurs only after pro domain dissociation. At this point, there is no evidence to distinguish between these two possibilities for any ADAM. Pro domains have also been shown to be inhibitors of the catalytic domain on the basis that the enzyme only becomes fully active after removal of its pro domain (8, 12, 25). As mentioned above, ADAM 33 seems to be an exception to this rule, although definitive data to support this claim have not been provided yet (29). This pro domain removal can be either autocatalytic or mediated by a furin-like enzyme, depending on each individual ADAM family member (8, 12, 25–30). There is very limited structural information on
the pro domains of ADAMs. Although extensive research has been done on the pro domains of MMPs, it is difficult to use that structural information to model the pro domains of ADAM, because of the low homology across the family within this region and the fact that the pro domains of ADAM are substantially larger than those of MMPs.

To study the determinants of the interaction between the pro domain and the catalytic domain of TACE, we expressed TACE Pro in E. coli. We found TACE Pro to have a thermodynamically stable structure, according to both our fluorescence and circular dichroism spectroscopy data. Denaturation experiments using both spectroscopic probes showed that TACE Pro has reversible, two-state unfolding behavior under equilibrium conditions upon dilution in solutions containing the chemical denaturant Gdn-HCl. TACE Pro showed an unfolding transition at a relatively low Gdn-HCl concentration (1 M) suggesting that, although it can behave as an independent folding unit, its thermodynamic stabilization may depend on its association with the catalytic domain.

TACE Pro refolded in vitro proved to be a very potent inhibitor of its catalytic domain. It was previously reported that although a TACE pro-catalytic domain complex is basically inactive, the activity of the enzyme was recovered upon dilution of the protein complex (8). Recovery of activity by dilution occurs in the nanomolar range. The in vitro refolded TACE Pro inhibits the catalytic domain in this same range (IC₅₀ = 70 nM). Therefore, this domain, after expression and purification in an isolated form, resembles the function of the naturally occurring pro domain. The inhibitory potency of TACE Pro was much weaker against the complete TACE ectodomain (catalytic plus disintegrin/cysteine-rich domains). This indicates that the disintegrin/cysteine-rich domain may play a role in displacing the pro domain of TACE from the catalytic domain upon processing by furin or a furin-like enzyme. We found that the disintegrin/cysteine-rich domain can remove the pro domain from the pro-catalytic complex in trans (Fig. 5). It is possible that part of the disintegrin/cysteine-rich domain of TACE may be positioned next to the substrate-binding cleft. Therefore, it may sterically hinder the interaction of the pro domain with this surface of the catalytic domain. In fact, Murphy and co-workers (31, 32) have shown that an unrelated proteinaceous inhibitor of TACE, tissue inhibitor of metalloproteinases-3 (TIMP-3), also exhibits decreased affinity against the catalytic domain of TACE Pro (11). Since the original proposal of the cysteine switch model (9), there have been conflicting reports on the importance of the cysteine and other residues within the pro domain in the folding and inhibitory roles of such domain in MMPs (9–11, 33).

The pro domain of TACE contains a consensus cysteine switch box found in both the MMP and ADAM families. It has been proposed that the pro domain of metzincins exert their inhibitory role through ligation of the cysteinyl thiol to the zinc ion in the active site of these enzymes (9–11). Since the original proposal of the cysteine switch model (9), there have been conflicting reports on the importance of the cysteine and other residues within the pro domain in the folding and inhibitory roles of such domain in MMPs (9–11, 33). In the case of MMP3, at least two residues in addition to the cysteine within the switch box are important for maintaining the latency of the enzyme (34). Within the ADAM family, the role of this motif has been studied in ADAM 9, ADAM 10, and ADAM 12. The cysteine switch has been shown to be a key element in the pro-catalytic interaction (12, 13, 25). For ADAM 9 and ADAM 10, a Cys to Ala mutation in the switch box prevented the production of functional enzyme, probably because of protein misfolding. In the case of ADAM 12, the cysteine residue does not seem to be required for secretion, but a mutation to alanine renders the pro domain incapable of inhibiting the activity of the catalytic domain. In this report, we demonstrate that an intact cysteine switch is not required for inhibition of TACE by its pro domain. A TACE Pro variant carrying a Cys to Ala mutation at position 184 proved to have similar solution properties and inhibitory potency to those of its wild type counterpart. Other residues adjacent to this central cysteine residue in the switch box do not seem to be required for the pro-catalytic domain interaction either. This result is not in contradiction with a previous publication showing that 4-aminoephynylmercuric acetate and octyl thioglucoside (two thiol-modifying reagents) are able to dissociate the pro-catalytic complex (8). As it has been reported previously for the MMPs, these reagents may disrupt the pro-catalytic domain interaction in a manner that does not involve thiol modification of the cysteine residue.

To address whether Cys184 actually ligated the catalytic zinc ion of TACE, we performed XAS analysis of both the free and Pro-bound catalytic domain species. XAS is a valuable spectroscopic technique for elucidating the local structure of a variety of metal-binding sites in metalloproteins (35). The successful application of XAS to study electronic and local structures of the catalytic zinc site in the related MMPs systems has been demonstrated (24). XAS spectra are divided into two regions: XANES and EXAFS. XANES provides information about the effective charge of the metal ion and its geometry. Complementary to this, analysis of the EXAFS region provides the local structure around the analyzed metal ion, which includes average bond distances, mean square variation in distance, metal coordination number, and ligand type. Interestingly, our EXAFS results show that the binding of TACE Pro to the catalytic domain does involve ligation of the catalytic zinc ion in the enzyme via the sulfur ion of the conserved Cys184 amino acid residue. This indicates that the steady state pro-catalytic complex of TACE possesses a stable zinc-sulfur bond at the active site. However, our biochemical evidence shows that the binding of Cys184 of TACE Pro to the catalytic zinc site in TACE is energetically neutral. Differently from the MMPs, the zinc-sulfur (Cys) bond in TACE may be extremely susceptible to chemical attack by either adjacent polar residues within the active site or solvent molecules. Consistent with this hypothesis, the XANES features of active free catalytic domain and inactive pro-catalytic domain complex are different from the ones observed for MMP-2 and pro-MMP-2. This suggests that although the basic metal-ligand coordination is conserved, the chemical potential of the catalytic zinc site in TACE may be different relative to the MMPs. Such chemical diversity has been documented for TACE in comparative XAS studies with MMP-2 (36).

The above results pose an obvious question: if the cysteine switch of TACE is physically present in the zymogen form but is not needed for enzyme inhibition, what then is its function? Our own studies point at a role in preventing intracellular proteolysis of the TACE zymogen. An intact cysteine switch is not essential for the efficient biosynthesis and maturation of the TACE forms used in this study (catalytic domain and catalytic plus disintegrin/cysteine-rich domain). Mature, functional catalytic domain and TACE ectodomain were secreted from insect cells infected with baculoviral strains encoding the C184A variants of both enzyme forms. Furthermore, full-length TACE C184A was also biosynthesized and matured similarly to its wild type counterpart in these cells. However, the only difference we could detect is very telling; significant intracellular degradation of the zymogen forms was observed when Cys184 was missing, suggesting that a cysteine switch in the “closed” position may be required for protecting TACE from intracellular degra-

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2 M. A. Leesnitzer, unpublished observation.
dation. This may explain both the relatively high degree of conservation of this motif throughout the ADAM family of proteinases and our direct confirmation of the Cys\textsuperscript{184} thiol coordination to the catalytic zinc ion by XAS methods (Fig. 6).

In summary, here we report on the biophysical properties and inhibitory activity of the pro domain of TACE. We demonstrate that this domain can act as an inhibitor of TACE in trans, being more potent against the catalytic domain than against the whole ectodomain. Our results question the application of a classical cysteine switch mechanism for maintenance of the zymogen state by TACE Pro. The importance of other regions outside the cysteine switch box of TACE in maintaining the latency of the enzyme is currently under investigation.

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Inhibition of the Tumor Necrosis Factor-α-converting Enzyme by Its Pro Domain
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