Inhibition of the TNFα Converting Enzyme (TACE) by its Pro Domain

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Running title: TACE inhibition by its pro domain
Abstract

TACE is a disintegrin metalloproteinase that processes tumor necrosis factor and a host of other ectodomains. TACE is biosynthesized as a zymogen 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. In order 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 TACE Pro is a stably folded protein that is able to inhibit this enzyme. TACE Pro inhibited the catalytic domain of TACE with an IC\textsubscript{50} of 70nM. In contrast, this inhibitory potency decreased over 30-fold against a TACE form containing the catalytic plus disintegrin/cysteine-rich domains (IC\textsubscript{50} greater that 2\muM). 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 TACE’s enzymatic activity: the pro domain variant C184A showed the same inhibitory potency against both TACE forms as wild type TACE Pro. X-ray absorption spectroscopy (XAS) experiments indicate that binding of TACE Pro to the catalytic domain does include ligation of the catalytic zinc ion via the sulfur 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. In spite of 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 TACE’s active site.
Introduction

The tumor necrosis factor-alpha (TNF-$\alpha$)\(^1\) converting enzyme (TACE or ADAM 17) is a zinc metalloproteinase that cleaves the precursor, membrane bound form of TNF-$\alpha$ 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, metalloproteinase domain, 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, since TACE’s activity was only recovered upon its removal (8). TACE Pro includes a cysteine-switch box (PKVCGY186), 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 cysteiny1 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, since 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 a first approximation, this is similar to the secreted bacterial serine proteases subtilisin and

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\(^1\) Abbreviations: TNF-$\alpha$, tumor necrosis factor-alpha; TACE, TNF-$\alpha$ converting enzyme; ADAM, a disintegrin and metalloproteinase; TACE Pro, TACE’s pro domain; LB, Luria-Bertani broth; IPTG, isopropyl $\beta$-thiogalactoside; Gdn-HCl, guanidinium hydrochloride; Ni-NTA, Nickel$^{2+}$-nitrilotriacetic acid; Sf9, Spodoptera frugiperda cells; T.ni Trichoplussia ni cells; HFBA, heptaflurobutyric acid; PVDF, polyvynilene difluoride; HPLC, high performance liquid chromatography; CD, circular dichroism; XAS, X-ray absorption spectroscopy; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; XANES, X-ray Absorption Near Edge Spectra; EXAFS, Extended X-ray
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 pro domain removal and enzyme activation. We also show that TACE’s cysteine-switch, although present in the zymogen form, is not required for the interaction between TACE’s pro and catalytic domains.

Materials and Methods

**Plasmid constructs** – A plasmid for expression of the pro domain of TACE in *E. coli*, lacking the signal peptide, was constructed using the vector pRSET B (Invitrogen). A DNA fragment encoding residues Asp22 to Arg214 with an NdeI site at the 5'-end and BamHI site at the 3'-end was generated by polymerase chain reaction amplification 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 AccI 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 TACE Pro’s cysteine-switch region (PKVCGYKLDVD190) were made by the same cassette mutagenesis approach. They carried a 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 overnight incubation at 37 °C, cells were resuspended in 1L of LB with 150 µg/mL ampicillin. Cells were grown at Absorption Fine Structure; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases.
37 ºC, induced with 1 mM isopropyl thiogalactoside (IPTG) at an OD of 0.6 and harvested 3 hours post induction.

Purification and refolding of wild type and mutant TACE Pro – Cell pellets were washed once with 20 mM Tris-HCl, pH 8 and twice with this 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 x g for 30 minutes. The supernatant was applied to a 20 mL Ni-NTA column. The column was washed with 20 mM Tris-HCl pH 8, 6 M Gdn-HCl and then with this 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 which 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 mM oxidized glutathione and 3 mM lauryl maltoside for four hours at 4 ºC with gentle stirring. After refolding, the solution was centrifuged at 10,000 x g for 10 minutes 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 4-6 µM (0.09-0.14 mg/mL).

Expression and purification of TACE truncates R473 and R651 – The construction of TACE truncates R473 and R651 and production of baculovirus in Sf9 (Spodoptera frugiperda) for protein production in insect cells have been described previously (8). Logarithmically growing T.ni (Trichoplusia ni) cells were infected with baculovirus strains encoding R473 of R651 at a multiplicity of infection of 1. Cultures were harvested 48 hours post-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 HPLC-based assay using the synthetic peptide Dnp-SPLAQAVRSSSR-NH$_2$ as the substrate. TACE (R473 and R651), at a final concentration of 1 nM, was
incubated with a series of TACE Pro concentrations ranging from 0 to 6 µM in buffer A. The incubation took place for 20 minutes at 37 °C with gentle shaking. The reaction was initiated by addition of substrate at a final concentration of 20 µM. Reactions were incubated at 37 °C for 30 minutes with gentle shaking, quenched by addition of an equal volume of 1% heptafluorobutyric acid (HFBA) and filtered using polyvinylidifluoride (PVDF) membrane filters. Quenched reaction mixtures were applied to a 150 mm C18 column (Vydac) and resolved using a gradient of 0.1% HFBA in water and 0.1% HFBA in acetonitrile. The ratio of product/substrate was obtained by integration of the absorbance of the resolved peaks at 350 nm.

Circular dichroism spectroscopy – Circular dichroism (CD) spectra were recorded in an AVIV 62DS spectropolarimeter. Protein stocks were maintained at a concentration of 180 µM in buffer A containing 6 M Gdn-HCl. Those stocks were rapidly diluted sixty-fold in buffer A to reach a final concentration of 3 µM of refolded protein. Spectra were taken between 208-260 nm using a 1 cm-path fused quartz cuvette. Scans were done in triplicate.

For denaturation studies, the protein stocks were diluted into buffer A containing Gdn-HCl concentrations ranging from 0 to 7.5 M. The ellipticity at 222 nm was recorded for four independent protein dilutions at each Gdn-HCl concentration.

Fluorescence spectroscopy – Fluorescence emission scans were obtained using an AVIV ATF-105 spectrofluorometer. Samples were prepared in the same way as for CD spectroscopy. Protein samples were excited at 280 nm and the intrinsic tryptophane emission spectra were recorded from 300 to 400 nm using a 3 mm-path cuvette. The photomultiplier tube was pre-set to 50% of the maximum output at a fixed excitation wavelength of 280 nm and emission wavelength of 350 nm.

For denaturation studies, the protein stocks were also diluted in buffer A containing a range of Gdn-HCl concentrations from 0 to 7.5 M. The emission spectra were recorded from 300 to 400 nm and the center of mass of each spectrum (\( n_c \)) was calculated using the equation:

\[
\bar{\bar{c}} = \frac{\sum F_i n_i}{\sum F_i}
\]
where $F_i$ is the fluorescence emitted at wavenumber $n$.

**Sample preparation for X-ray absorption spectroscopy studies** – TACE’s catalytic domain was concentrated by ultra filtration using vivaspin 6 mL units (Vivascience AG, Hannover, 10 kDa cutoff), to a final concentration of 0.1 mM (2.92 mg/mL). Samples were loaded into copper sample holders (10 x 5 x 0.5 mm) pre-covered with Mylar tape which is transparent to X-rays, immediately followed by freezing in liquid nitrogen. Frozen samples were then mounted inside a Diplex closed cycle helium cryostat and their temperature was maintained at 30 K to minimize thermal disorder in the XAS data.

**XAS data collection** – Data collection was performed at the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratory, beam line X9B. The spectra were recorded at the zinc K-edge in fluorescence geometry at low temperature (30 K). The beam energy was defined using a flat Si (111) monochromator crystal. The incident beam intensity $I_0$ was recorded using an ionization chamber. The fluorescence intensity was recorded using a 13-element germanium detector. The transmission signal from a zinc foil was measured with a reference ion chamber simultaneously with fluorescence to calibrate the beam energy position throughout all measurements. 5-6 scans of each sample were collected. The samples were checked for burning marks after each scan, and the beam position on the sample was changed before each scan to minimize radiation damage. The enzyme integrity was checked before and after exposure to X-rays. In these experiments, TACE was found to be intact and fully active.

**XAS Data Processing and Analysis** - The average zinc K-edge absorption coefficient $\mu(E)$, was obtained after averaging 5-13 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 smooth atomic background was then removed with the AUTOBK program of the UWXAFS data analysis package, developed at the University of Washington, Seattle (18). For background removal, the energy shift, $E_0$, 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 0.6 and 1.2 Å. Upon background removal, the useful k-range in the resultant k2-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 (PDB) code 1CK7) and Stromelysin-1 (PDB 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 (18) in R-space, by Fourier transforming of both theoretical and experimental data. 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). In order 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, due to its tendency to aggregate at concentrations over 8µM. 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 (Figure 1). The free monomer constituted 40% of the purified protein, as determined by densitometric analysis of gels ran 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 (Trp111 and Trp153) were in an environment of low polarity (Figure 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 (Figure 2A). TACE Pro’s circular dichroism spectrum showed a minimum between 208 and 230 nm, which revealed the presence of significant secondary structure in the protein (Figure 2B). Upon chemical denaturation with Gdn-HCl, the CD ellipticity in this region was lost (Figure 2B).

To determine whether TACE’s pro domain 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 at 222 nm versus denaturant concentration (Figure 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 1M 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 TACE Pro’s inhibitory activity against two different TACE forms: 1) TACE’s catalytic domain alone and 2) TACE’s catalytic plus disintegrin/cysteine-rich domains. TACE Pro proved to be a very potent inhibitor of the catalytic domain (IC$_{50}$ = 70 nM, Figure 4A). Interestingly, this inhibitory potency dropped over thirty fold against the form containing the catalytic and disintegrin/cysteine-rich domains (IC$_{50}$ higher that 2 µM, Figure 4A). The inhibition curve for the mature ectodomain of TACE could not be completed because TACE Pro aggregated at concentrations around 8µM. The disintegrin/cysteine rich domain seems to affect the ability of the pro domain to stably bind the catalytic domain. In order to test whether TACE’s disintegrin/cysteine rich domain promotes dissociation of TACE Pro from the catalytic domain, we pre-incubated inactive pro-catalytic domain complexes
with increasing amounts of disintegrin/cysteine-rich domain and then assayed for enzymatic activity. As shown in Figure 5, even a short pre-incubation time (5 min) resulted in significant recovery of TACE’s catalytic activity (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 hours of pre-incubation (Figure 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 agreement with previous observations showing that TACE’s catalytic domain activation was only promoted efficiently upon addition of hydrophobic thiol-modifying reagents (8).

TACE Pro’s inhibitory activity could potentially be attributed to chelation of the zinc ion in the active site of the enzyme by the C-terminal His-6 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). In order 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 (Figure 2A and 2B). TACE Pro
C184A also had similar equilibrium denaturation profiles to those of TACE Pro (Figure 3).

Surprisingly, the introduction of this cysteine to alanine mutation had only a modest effect in the inhibitory potency of TACE Pro. The IC\textsubscript{50} 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, since 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 two-fold of the IC\textsubscript{50} observed with wild-type TACE Pro (Table 1). Additional sequence elements within the pro domain different from the cysteine-switch region, yet to be identified, must mediate the pro-catalytic domain interaction.

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). XAS refers to modulations in x-ray absorption coefficient, \( \mu(E) \), around an x-ray absorption edge of a given atom. Figure 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 to 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 eV and 9737 eV, indicating mild conformational changes within the environment of TACE’s catalytic zinc ion.

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 (23) and fitted to the experimental XAS data of active and latent TACE respectively. Figure 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 2. The zinc sites in the various forms of the enzyme were fitted to the Zn-N, Zn-O, Zn-S, and Zn-C paths using different combinations of variable and constraint parameters.

Stable and reproducible fits of pro-TACE (Table 2) were consistent with a tetrahedral coordination of the zinc ion with three Zn-N (His) at 2.06±0.03 Å and one Zn-S(Cys) at 2.27±0.01 Å in the first coordination shell, and seven Zn-C contributions (three Zn-C 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 Zn-N/O or additional Zn-O contributions resulted in unstable fits and relatively high chi-square 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 Zn-N(His) bond distances at 2.06 ± 0.01 Å, one Zn-N/O bond distance at 1.89 ± 0.01 Å, 3Zn-C bond distances at 2.85 ± 0.01 Å and 3Zn-C bond distances at 3.11 ± 0.01 Å. Attempts to fit the first coordination shell of the active enzyme with only 3Zn-N/O or additional Zn-S contributions resulted with unstable fits and high chi-square values. The first coordination shell Zn-ligand bond distances of active TACE are in agreement with the x-ray crystal structure (23), showing three Zn-N contributions of the histidine residues chelating the catalytic zinc ion. Similarly, the catalytic zinc ion of MMP-2 is coordinated by three Zn-N(His) at 1.97 ± 0.02 Å, and one Zn-N/O (presumably Zn-O 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 pro domain-mediated
inhibition: ADAM 9 (25), ADAM 10 (12), ADAM 12 (13), ADAM 15 (26), ADAM 17 (TACE, 8, 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 are essential for secretion of functional enzyme, since 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 subtilysin BPN and [I]-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 ADAMs’ 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 has 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 ADAM’s pro domains, because of the low homology across the family within this region and the fact that ADAM’s pro domains are substantially larger than those of MMPs.

In order 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 (M. A. Leesnitzer, unpublished work). The in vitro refolded TACE Pro inhibits the catalytic domain in this same range (IC$_{50}$=70nM). 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 (figure 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 coworkers found that an unrelated proteinaceous inhibitor of TACE, TIMP-3, also exhibited decreased affinity against the ectodomain form of the enzyme relative to the isolated catalytic domain (31, 32).

The pro domain of TACE contains a consensus cysteine-switch box, found both in the MMP and ADAM families. It has been proposed that the pro domain of metzincins exert their inhibitory role through ligation of the cysteiny1 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 ADAM9, ADAM10 and ADAM12. 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 due to protein misfolding. In the case of ADAM12, 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 TACE’s inhibition 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-aminophenylmercuric acetate and octyl thiogluicoside (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.

In order to address whether Cys184 actually ligated TACE’s catalytic zinc ion, 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: the X-ray Absorption Near Edge Spectra (XANES) and the Extended X-ray Absorption Fine Structure (EXAFS). XANES provide 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 TACE’s pro-catalytic complex possesses a stable Zn-S bond at the active site. However, our biochemical evidence shows that the binding of TACE Pro’s Cys184 to the catalytic zinc site in TACE is energetically neutral. Differently from the MMPs, the Zn-S (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 (Solomon et al, accompanying report).

The above results pose an obvious question: if TACE’s cysteine switch is physically present in the zymogen form, but is not needed for enzyme inhibition, what is then 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 maturated 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 degradation (Jennifer D. Leonard, unpublished work). 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 Cys184 thiol coordination to the catalytic zinc ion by XAS methods (Figure 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.

Acknowledgements
We thank J. David Becherer for advice and for generously providing the original TACE clone used for vector construction and Jennifer Leonard for help with vector design, construction and for sharing reagents. We also thank Walter Englander for allowing us access to a spectropolarimeter and Mark Bickett for help with data analysis. Supported by grant AR-45949 from NIH/NIAMS. I.S. is supported by the Israeli Science Foundation project 3771.

References
Table 1. Half-inhibitory concentrations (IC$_{50}$) for a series of alanine mutants scanning the cysteine switch region of TACE, in the presence of a C184A mutation. Each protein was assayed against the catalytic domain of TACE as described in the Materials and Methods section. Data are the average of two independent determinations (experimental errors within 15%) and are expressed as the nanomolar concentration of the respective pro domain double mutant.

<table>
<thead>
<tr>
<th>TACE Pro mutant</th>
<th>IC$_{50}$ (nM)</th>
</tr>
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<tbody>
<tr>
<td>P181A</td>
<td>48.55</td>
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<tr>
<td>K182A</td>
<td>72.58</td>
</tr>
<tr>
<td>V183A</td>
<td>99.71</td>
</tr>
<tr>
<td>G185A</td>
<td>131.7</td>
</tr>
<tr>
<td>Y186A</td>
<td>70.98</td>
</tr>
<tr>
<td>L187A</td>
<td>57.32</td>
</tr>
<tr>
<td>K188A</td>
<td>87.98</td>
</tr>
<tr>
<td>V189A</td>
<td>69.24</td>
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<tr>
<td>D190A</td>
<td>81.87</td>
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Table 2: EXAFS fit results

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<tr>
<th>Fit #</th>
<th>$\chi^2$</th>
<th>Path</th>
<th>$\Delta E_0$</th>
<th>$R$ [Å]</th>
<th>$\chi^2$ [Å]*</th>
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<td></td>
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<td>Active TACE</td>
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<td>Fit 1.</td>
<td>44.0</td>
<td>ZnN/O x 3.47±1.1 [V]</td>
<td>5.3 [F]</td>
<td>2.03±0.01</td>
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<td>Fit 2.</td>
<td>180</td>
<td>Zn-N/O x 2 [F]</td>
<td>6.0 [F]</td>
<td>2.07±0.02</td>
<td>1.0E-05 [V]</td>
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<td></td>
<td></td>
<td>Zn-N/O x 1 [F]</td>
<td>6.0 [F]</td>
<td>1.92±0.05</td>
<td>1E-06 [V]</td>
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<tr>
<td></td>
<td></td>
<td>Zn-C x 3 [F]</td>
<td>7.2 [F]</td>
<td>2.82±0.07</td>
<td>1.3E-02 [V]</td>
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<td></td>
<td>Zn-C x 3 [F]</td>
<td>7.2 [F]</td>
<td>3.06±0.07</td>
<td>9.2E-03 [V]</td>
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<tr>
<td>Fit 3.</td>
<td>3.13</td>
<td>Zn-N/O x 3 [F]</td>
<td>5.8 [F]</td>
<td>2.06±0.01</td>
<td>2.0E-03 [V]</td>
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<td></td>
<td>Zn-N/O x 1 [F]</td>
<td>5.8 [F]</td>
<td>1.89±0.01</td>
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<td>Zn-C x 3 [F]</td>
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<td>Zn-C x 3 [F]</td>
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<td>Zn-C x 3 [F]</td>
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<td>Zn-C x 4 [F]</td>
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<tr>
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<td>Zn-N/O x 1 [F]</td>
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* Estimated error for $\chi^2$ did not rise over 10%, where $\chi^2$ was varied (see Materials and Methods).
Figure legends

Figure 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; lane2, monomeric TACE Pro under reducing conditions; lane 3, monomeric and dimeric TACE Pro under non-reducing conditions.

Figure 2. **Fluorescence and circular dichroism spectra of TACE Pro and TACE Pro C184A.** Native spectra were taken in 20mM Tris-HCl, pH 8, 150mM NaCl. Denatured spectra were taken in the same buffer plus 6M Gdn-HCl. (A) Fluorescence spectra: native TACE Pro (closed circles), native TACE Pro C184A (open circles), denatured TACE Pro (closed squares), denatured TACE Pro C184A (open squares). The maximum observed at 370 nm corresponds to material that aggregated during the course of the experiment. (B) CD spectra. Symbols are the same as in A. [Q]: (deg - cm²/dmole) x 10⁻³.

Figure 3. **Chemical denaturation of TACE Pro and TACE Pro C184A with Gdn-HCl.** Protein denaturation was performed in 20mM Tris-HCl, pH 8, 150mM NaCl and the indicated concentrations of Gdn-HCl. (A) Denaturation followed by changes in ellipticity at 222 nm: TACE Pro (closed circles), TACE Pro C184A (open circles). (B) Denaturation followed by changes in intrinsic fluorescence after excitation at 280 nm: TACE Pro (closed squares), TACE Pro C184A (open squares).

Figure 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, closed circles) or R651 (catalytic plus disintegrin/cysteine-rich domains, open circles) prior to incubation with substrate, as described in Materials and Methods. (A) Inhibitor profiles obtained with TACE Pro. (B) Inhibitor profiles obtained with TACE Pro C184A. Data are expressed as percentages of the TACE activity observed in the absence of inhibitor with either R473 or R651.

Figure 5. **TACE activation following incubation with the disintegrin/cysteine-rich domain.** 100 nM samples of inactive TACE pro-catalytic domain complexes were pre-incubated for 5 minutes (open circles) or 4 hours (closed circles) with isolated TACE disintegrin/cysteine-rich domain in 20 mM Tris-HCl, pH 8, 150 mM NaCl. After incubation, samples were diluted and assayed for activity as described in the materials and methods section. Activity expressed as percent of control incubations lacking TACE Pro.

Figure 6. (A) **Zinc k-edge spectra of active, and latent forms of TACE.** Normalized raw XAS data in the zinc K-edge region of active (solid) and latent complex (symbol). A shift of ~0.45 eV in edge position to higher energy is observed upon the binding of the pro domain to active TACE. Moderate changes in XANES features (see arrows) are observed upon TACE inhibition. (B) **EXAFS fitting results for active and latent forms of TACE.** 1. TACE; 2. pro-TACE. The results are presented in the R-space and back transformed k-space of the experimental data (-) to simulated theoretical zinc ligand contributions (o). The fitting parameters are listed in Table 2.
Figure 1
Figure 2.

A

B
Figure 3.

A

B

![Graph A](image)

![Graph B](image)
Figure 4.

A

B
Figure 5.

[Graph showing TACE activity (% maximal activity) against TACE disintegrin/cysteine rich (M)]
Figure 6

A
Inhibition of the TNFα converting enzyme (TACE) by its Pro domain
Patricia E. Gonzales, Ariel Solomon, Ann B. Miller, M. Anthony Leesnitzer, Irit Sagi and Marcos E. Milla

J. Biol. Chem. published online April 20, 2004

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