TNF-α converting enzyme (TACE) is inhibited by TIMP-3

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Abstract TNF-α converting enzyme (TACE; ADAM-17) is a membrane-bound disintegrin metalloproteinase that processes the membrane-associated cytokine proTNF-α to a soluble form. Because of its putative involvement in inflammatory diseases, TACE represents a significant target for the design of specific synthetic inhibitors as therapeutic agents. In order to study its inhibition by tissue inhibitors of metalloproteinases (TIMPs) and synthetic inhibitors of metalloproteinases, the catalytic domain of mouse TACE (rTACE) was overexpressed as a soluble Ig fusion protein from NS0 cells. rTACE was found to be well inhibited by peptide hydroxamate inhibitors as well as by TIMP-3 but not by TIMP-1, -2 and -4. These results suggest that TIMP-3, unlike the other TIMPs, may be important in the modulation of pathological events in which TNF-α secretion is involved.

Key words: Metalloproteinase; Disintegrin metalloproteinase; Tissue inhibitor of metalloproteinases; Tumour necrosis factor; TNF-α converting enzyme

1. Introduction

Tumour necrosis factor-α (TNF-α) is a cytokine produced in response to infection or injury and identified as a major mediator of chronic autoimmune diseases such as rheumatoid arthritis and inflammatory pathologies such as sepsis [1]. ProTNF-α is initially produced as a membrane-bound form that is processed by TNF-α converting enzyme (TACE), a recently identified type I membrane-bound metalloproteinase [2,3]. TACE represents a potential target for the development of therapeutic agents for inflammation and related diseases [4]. TACE is a member of the ADAM (A disintegrin and metalloprotease) family of proteins which have been implicated in cell interaction events including cell fusion [5,6] and has been designated ADAM-17. TACE has a higher sequence identity with MADM (ADAM-10) than any other mammalian ADAMs [2]. MADM was originally implicated in myelin degradation [7], but was recently shown to be potentially capable of processing proTNF-α to its soluble form [8,9]. The Drosophila ADAM-10 orthologue, KUZ, can cleave the extracellular portion of the transmembrane receptor protein Notch which has been implicated in cell fate determination. Notch processing is probably a required step in the synthesis of a functional receptor [10].

The X-ray crystal structure of the catalytic domain of human TACE shows similarities with that of the snake venom metalloproteinase adamalysin II [11]. Snake venom metalloproteinases and ADAMs are included in the wider family of metalloproteinases, the metzincins [12], which also contains the matrix metalloproteinases (MMPs), involved in the degradation of extracellular matrix. MMP activities are regulated by a group of physiological inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) [13–15]. The question of the ability of these inhibitors to act on ADAMs has been raised [11,16]. However, TIMP-1 or -2 did not inhibit the processing of proTNF-α [17] or the shedding of the adhesion molecule L-selectin [18], excluding the participation of any of the MMPs characterised so far. More recent evidence indicates that shedding of receptors to cytokines such as TNF and IL-6 is inhibited by TIMP-3 [19,20].

One important issue is whether TACE is involved in the shedding of membrane proteins other than proTNF-α [21]. In common with as yet unidentified enzymes known as 'secretases' or 'sheddases', TACE is inhibited by broad spectrum synthetic MMP inhibitors and upregulated by phorbol esters [22]. In this report, we describe the expression and purification of the metalloproteinase domain of mouse TACE and the comparative study of its inhibition by TIMPs and synthetic inhibitors designed for MMPs.

2. Materials and methods

2.1. Materials and general procedures

Fmoc amino acids were from Alexis Corporation (Nottingham, UK). Fmoc-Lys(Dnp)-OH was made as described [23]. HATU, HOAt and Fmoc-PAL-PEG-PS resin were from PerSeptive Biosystems (Hertford, UK). BB-94 was from British Biotech Pharmaceuticals Ltd (Oxford, UK). Ro-31-9790 was from Roche (Welwyn Garden City, UK). Protein A-Sepharose, soybean trypsin inhibitor and 7-methoxycoumarin-4-acetic acid were from Sigma (Poole, UK). Recombinant enterokinase (1.7 unit/μl, where 1 unit is defined as the amount of enzyme needed to cleave 50 μg of fusion protein in 16 h) was from Novagen (Cambridge Bioscience, Cambridge, UK). Recombinant human TIMP-1, -2 and -3 were expressed in NS0 mouse myeloma cells and purified as previously described [13,24]. Full-length mouse TIMP-4 was expressed from *E. coli* and refolded (manuscript in preparation).

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Abbreviations: DMSO, dimethylsulfoxide; Dnp, 2,4-dinitrophenyl; Fmoc, 9-fluorenylmethoxycarbonyl; HATU, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HOAt, 7-aza-1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; Mca, (7-methoxycoumarin-4-yl)acetyl; NS0, Non-secretor zero; PAL, peptide amide linker; PEG, poly(ethylene glycol); PS, polystyrene; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid

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Recombinant progelatinase-A (proMMP-2) was purified from conditioned medium from transfected mammalian cell lines and activated as described previously [24].

Unless indicated otherwise, all fluorimetric measurements were made with a LS50B Perkin-Elmer spectrofluorimeter (λ_{ex} = 328 nm, λ_{em} = 393 nm) equipped with thermostable cuvette holders (37°C) in 2.5 ml of buffer A (10 mM HEPES, pH 7.5, 0.02% NaN_3, 0.05% $Brij_{35}$) with constant amount of DMSO (2% v/v). Kinetic analyses were achieved with the software package GraFit 3.0 (Erithacus Software Ltd, Staines, Middlesex, UK).

HPLC analyses were done on a Perkin-Elmer Integral 4000 instrument equipped with a Vydac 218TP54 column maintained at 40°C. Solvents were: A, 0.1% TFA in water; B, 0.08% TFA in acetonitrile. A linear gradient from 5 to 95% B was run over 20 min at 1.25 ml/min and the eluate was monitored at 230 and 335 nm.

2.2. cDNA cloning of mouse TACE and expression of the catalytic domain

Poly A^+ RNA was separated by oligo-dT spin chromatography (Pharmacia) from total RNA isolated from NS0 cells (ECACC No. 85110503) by the method of Chomczynski and Sacchi [25]. It was converted into a cDNA library of approximately 10^6 individual clones in lambda ZAPII according to the protocol provided by the supplier (Stratagene, Amsterdam, Netherlands). The library was plated out and pooled into 10 aliquots which were each converted into a population of cDNAs in pBluescript (Stratagene). Murine TACE cDNA was amplified from one aliquot by PCR using the following oligonucleotide primers based on the human TACE sequence [2]: 5′-GAGAGATTCTAGAGCCACCATGAGGCAGTCTCTCCTATTCCTG-3′ and 5′-GAGAGAATTCTTAGCACTCTGTTTCTTTGCTGTC-3′.

Following TA subcloning in pMOSblue (Amersham) six independent clones containing the resulting 2.5 kb fragment were sequenced using an ABI 373A automated sequencer and a consensus sequence was established (Accession number AJ007365). It was used as a template together with the 5′ oligonucleotide described above and a second oligonucleotide (5′-GAGAGAGTCGACCACCTTGTTGCTGCGCTCCTGG-3′) to PCR amplify a DNA fragment encoding the pre pro catalytic domain. The resulting DNA fragment has a 3′ *Sal*1 site (underlined) that replaces the sequence in TACE that encodes amino acids C_{478} G_{479} found at the start of the disintegrin domain. It also has a 5′ *Xba*1 site upstream of the initiating methionine codon, ATG (also underlined). It was ligated into a version of pEE12 [26] such that it was joined at the *Sal*1 site to a sequence encoding the human IgG1 heavy chain constant region, hinge, CH1 and CH2 domains. A DNA adapter encoding an enterokinase cleavage site ([V]DDDDK) was then introduced at the *Sal*1 site. The vector was transfected into mouse NS0 myeloma cells and the most productive clones were identified by two site ELISA for the human IgG Fc domain [27] and then grown up in serum-free media as described [28]. The vector was also used to generate serum-free conditioned media following its transient expression in CHO-L761h cells as previously described [29].

2.3. Purification of rTACE

Conditioned medium was mixed with an equal volume (25 ml) of buffer A and batch-bound for 16 h at 4°C to 0.5 ml of Protein A-Sepharose previously equilibrated with buffer A. The resin was washed with 50 ml of buffer A containing 0.5 M NaCl prior to addition of 2.5 ml of buffer A containing 50 mM NaCl, 2 mM $CaCl_2$ and 10 units of recombinant enterokinase. The slurry was agitated for 16 h at room temperature and then for a further hour in presence of 100 μg/ml of soybean trypsin inhibitor to inactivate the enterokinase. The supernatant was removed from the resin and the eluted protein analysed by SDS-PAGE (10% polyacrylamide) under reducing conditions and by N-terminal sequencing.

N-terminal sequence analysis was obtained as follows: 5 μg of purified TACE was analysed by SDS-PAGE (4–20% polyacrylamide gradient gel, NOVEX), and the protein was electrophoretically transferred to a PVDF membrane (Immobilon P, Millipore). The membrane was stained with Ponceau Red, washed extensively with distilled water, and the band corresponding to TACE was excised for N-terminal sequencing using an Applied Biosystems 470 microsequencer.

2.4. Synthesis of QF-45 and QF-48

The quenched fluorescent substrates Mca-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Ser-Arg-Lys(Dnp)-NH_2 (QF-45) and Mca-Ser-Ser-Met-Ala-Gln-Thr-Leu-Thr-Leu-Arg-Ser-Ser-Ser-Lys(Dnp)-NH_2 (QF-48) are based on the TACE-sensitive sequences in human and mouse TNF-α, respectively. QF-45 and QF-48 were synthesised as C-terminal amides on Fmoc-PAL-PEG-PS resin in a PerSeptive Biosystems 9050 Plus PepSynthesiser. Briefly, Fmoc amino acids (4 eq.) were activated with HATU (4 eq.) in the presence of diisopropylethylamine (8 eq.) [30]. HOAt (4 eq.) was added when coupling Gln. Fmoc deprotection was with a mixture of 2% (v/v) piperidine and 2% (v/v) 1,8-diazabicyclo[5,4,0]undec-7-ene. For the coupling of 7-methoxycoumarin-4-acetic acid (4 eq.), the resin was gently shaken with HATU (4 eq.) in the minimum volume of dimethylformamide. A second portion of HATU was added after 4 h and the reaction was allowed to proceed to completion overnight. Peptides were released by treatment with TFA/water/triisopropylsilane (92.5:5:2.5, by vol.) for 2 h at 21°C, applied to a column of Vydac 218TPB1520 and eluted with a gradient of 5 to 50% acetonitrile in 0.1% TFA. Fractions containing homogeneous product were identified by analytical HPLC, pooled and freeze-dried. The identity of the purified peptides was confirmed by matrix-assisted laser desorption-ionisation mass spectrometry.

2.5. Enzyme assays and identification of cleavage products

Initial velocities (V_i) corresponding to the rTACE-catalysed hydrolyses of QF-48 (0.2 to 0.8 μM) and QF-45 (0.2 to 2 μM) were recorded at concentrations of substrate well below the Michaelis constant ($S \ll K_M$). Under these conditions, catalytic efficiencies (k_{cat}/K_M) were derived by linear regression of the simplified Michaelis equation: $V_i = (k_{cat}/K_M) \times E_o \times S$. The enzyme concentration E_o was determined as described below. The effect of pH was observed from pH 6.0 to 11.0 in buffer 20 mM MES, 20 mM Tris, 20 mM CAPS. The influence of ionic strength over enzyme activity was examined by adding sodium chloride (0 to 0.5 M) to buffer A. The requirement for metal ions was examined after 1 h incubation in presence of 1 mM of EDTA followed by addition of 2 mM $CaCl_2$, $ZnCl_2$ or $MgCl_2$.

The quenched fluorescent substrates QF-45 (10 μM) and QF-48 (5 μM) were incubated for 16 h at 37°C with 100 nM rTACE. An initial HPLC analysis with 10 μl of each digest showed that the cleavage products were well separated. 300 μl of each digestion sample was then fractionated by HPLC and the eluate was collected at 0.5 min intervals. Fractions containing the products were dried in vacuo over P_2O_5 and submitted for mass spectrometry by Dr L.C. Packman (Cambridge Centre for Molecular Recognition). No hydrolysis of QF-45 and QF-48 was obtained in control assays where rTACE was excluded.

2.6. Inhibition procedures

Reaction mixtures between rTACE (~1 nM) and inhibitors were pre-incubated 2 h at 37°C with TIMP-1 (0 to 152 nM), TIMP-2 (0 to 128 nM), TIMP-4 (0 to 115 nM), CT-2256 (0 to 25 nM), BB-94 (0 to 25 nM), Ro-31-9790 (0 to 250 nM), CT-420 (0 to 1 μM), CT-548 (0 to 24 μM) and CT-1746 (0 to 1 μM). The steady-state velocities V_s were measured with QF-45 (1 μM). Apparent inhibition constants K_i' (equal to $K_i(1+S/K_M)$) and E_o were estimated by least-squares fit to Eq. 1 for competitive tight-binding inhibition [31]:

$$V_s = (V_o/2E_o) \times \left\{E_o - I_o - K_i' + [(E_o - I_o - K_i')^2 + 4E_oK_i']^{1/2}\right\}, \quad (1)$$

where I_o and V_o are total concentration of inhibitor and velocity in absence of inhibitor, respectively. The same method was used for the inhibition of rTACE by TIMP-3 (0 to 0.77 nM) and of MMP-2 (~0.1 nM) by TIMP-1, -3 and -4 (0 to 90 pM), except the pre-incubation periods were extended to 16 h. MMP-2 activity was followed at 25°C with 1 μM of the fluorogenic peptide Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (QF-24) in 100 mM Tris (pH 7.5), 150 mM NaCl, 10 mM $CaCl_2$, 0.05% $Brij_{35}$ and 0.02% NaN_3.

The association rate constant (k_{on}) of rTACE with TIMP-3 was estimated as previously published [24]. The dissociation rate constant (k_{off}) was determined by initially incubating rTACE and TIMP-3 at an equimolar concentration of 100 nM and diluting 0.5 to 2.5 μl of the preformed enzyme-inhibitor complex into 2.5 ml of assay buffer containing 1 μM QF-45. Recovery of enzyme activity was followed for 5 h. Estimates of k_{off} have been calculated as previously published [24]. The method employed for determining an overall dissociation constant (K_D) for MMP-2 and TIMP-2 is described elsewhere [32].

3. Results and discussion

3.1. Amino acid sequence of murine TACE

Comparison of the predicted amino acid sequence of murine and human TACE reveals a high degree of sequence conservation (92.6% identity). When compared with other mammalian ADAMs, both versions of TACE are clearly more closely related to MADM than to other members of this family [2]. This is particularly apparent within the catalytic domain, where TACE and MADM share a conserved arrangement of six cysteine residues that differs significantly from the other family members. This may have relevance to the catalytic properties of the two enzymes, as both are reported to cleave proTNF-α [8,9]. Currently it is not clear whether one or both enzymes play this important role in vivo. Similarly it is not known whether TACE shares the ability of MADM to cleave Notch.

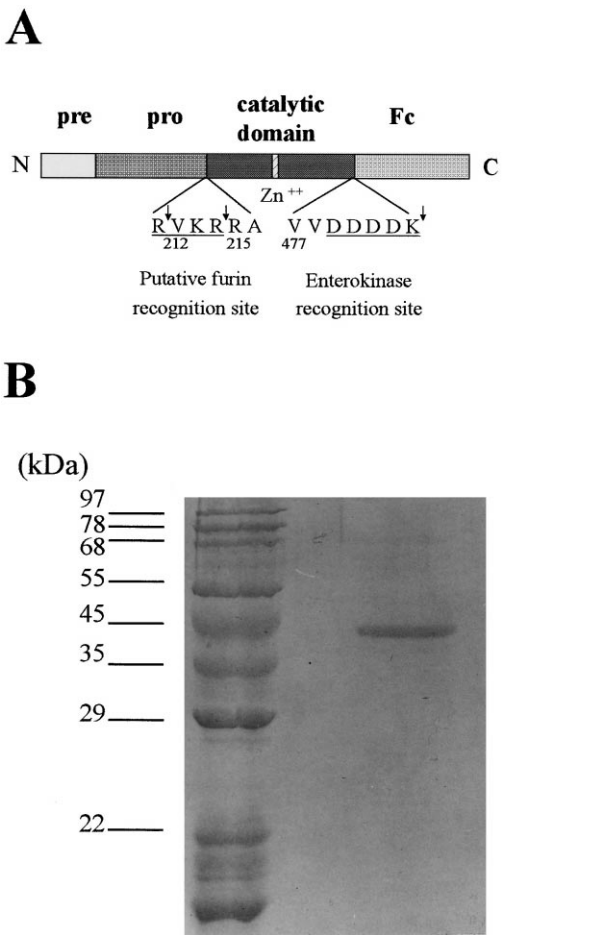

Fig. 1. A: Schematic representation of the TACE pre pro catalytic domain expressed as a Fc fusion. The arrowheads to the left indicate the propeptide processing sites as determined by N-terminal sequencing. The arrowhead to the right indicates the anticipated cleavage that occurs after treatment with enterokinase. B: Coomassie stained SDS-PAGE of purified rTACE. The fusion protein could be bound through the Fc portion to Protein A-Sepharose and free rTACE eluted by enterokinase cleavage, yielding a protein band at an apparent molecular weight of 43 kDa. N-terminal sequencing of this protein band showed that it corresponded to the catalytic domain of rTACE lacking the pro-domain.

3.2. Purification of rTACE

Purified recombinant TACE catalytic domain was analysed by SDS-PAGE, and N-terminal sequencing. rTACE was released from the Fc fusion through cleavage at an engineered enterokinase site at the C-terminus of the catalytic domain (Fig. 1A). The resulting protein had an apparent molecular weight of 43 kDa as analysed by SDS-PAGE and Coomassie staining (Fig. 1B). N-terminal sequencing of the purified material identified two species sequences present in equal amounts, VKRRAEPN and RAEPNPLK, with the N-terminal residues being Val-212 and Arg-215 respectively. N-terminal sequencing of human TACE, expressed in CHO cells [11] revealed processing at the same two sites, indicating that the dual cleavage sites may be an authentic part of the activation pathway, presumably involving a furin-like enzyme [2,3].

In conclusion the recombinant TACE purified and used in this study, has an apparent molecular weight (43 kDa) and N-terminal sequence consistent with it being the bona fide catalytic domain of the enzyme (Fig. 1).

3.3. Activity of rTACE

rTACE cleaved peptide substrates covering the cleavage sites of human and mouse proTNF-α (QF-45 and QF-48). HPLC analysis showed that both substrates were cleaved at a single site. The molecular masses of the end products were consistent with rTACE-catalysed hydrolysis occurring at the following peptide bonds (-↓-): -Leu-Thr-↓-Leu-Arg- (QF-48) and -Gln-Ala-↓-Val-Arg- (QF-45). These results revealed that rTACE cleaved QF-48 and QF-45 at the processing sites of mouse and human proTNF-α, respectively. The selective cleavage of the second Thr-Leu bond in QF-48 emphasised the importance of the residues surrounding the cleaved bond in determining substrate specificity. The catalytic efficiencies (k_{cat}/K_M) for the rTACE-catalysed hydrolysis of QF-45 and QF-48 obtained at 37°C by fluorimetry had values of $1.3(\pm 0.1)\times 10^5$ and $3.5(\pm 0.4)\times 10^3$ M^{-1} s^{-1}, respectively. Thus the human TNF-α based peptide was hydrolysed 37 times more rapidly by the catalytic domain of mouse TACE than the mouse TNF-α based peptide. TNF-α converting activity, partially purified from a human monocytic cell line (THP-1), processed human proTNF-α, but did not cleave mouse proTNF-α [33]. These data suggest that peptides based on mouse proTNF-α are poor in vitro substrates of both human and murine TACE. Interestingly, the fluorogenic peptide QF-24, which is cleaved by most MMPs between Gly and Leu, is also a mediocre rTACE substrate. The calculated catalytic efficiency for rTACE-catalysed hydrolysis of QF-24 ($k_{cat}/K_M = 1.2(\pm 0.1)\times 10^3$ M^{-1} s^{-1} at 37°C) is 500-fold lower than that reported for MMP-2 [34].

rTACE activity was inhibited by EDTA, but inhibition could be reversed by incubation with Zn^{2+}. Other metal ions tested (Ca^{2+} and Mg^{2+}) did not reactivate the enzyme, providing evidence for the specificity of the Zn^{2+} for the catalytic activity of rTACE. Activity of MADM had previously been reported to be higher at pH values between 9 and 10 [8]. Similarly, rTACE was active within a pH range of 6 to 10.5 (data not shown), with the enzymatic activity increasing 7-fold from pH 6.5 to 10 (its optimum pH). At pH 10.5, rTACE was still active, but less stable ($t_{1/2} \sim 10$ min). The presence of 0.5 M NaCl had no notable effect on rTACE activity.

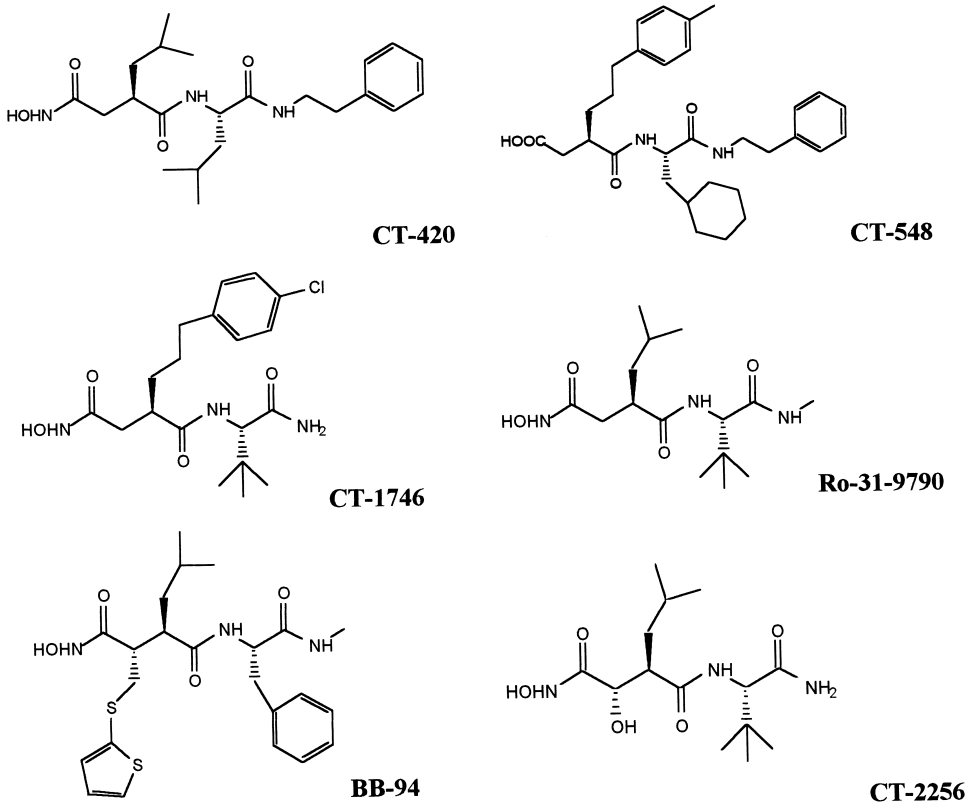

Fig. 2. Structures of the metalloproteinase inhibitors tested on rTACE.

3.4. Inhibition of rTACE by MMP inhibitors

The design of several MMP inhibitors has been based on the structural requirements of the active site of MMPs for its substrates [35]. The nature of the residue occupying the P_1' position at the cleavage site is a major determinant in MMP activity and selectivity. This residue is either leucyl or isoleucyl in collagen cleaved by collagenase-1 (MMP-1). Consequently, several inhibitors such as CT-420, Ro-31-9790, BB-94 and CT-2256 (Fig. 2) have a leucyl-type side chain (isobutyl) in a position likely to interact with the S_1' specificity pocket of collagenases or other MMPs [35]. These inhibitors are usually broad spectrum MMP inhibitors and their hydroxamate derivatives can also inhibit the shedding of several membrane proteins [22] as well as TACE [3]. The K_i values reported for the inhibition of TACE by BB-94, GW9471 and TAPI were respectively 4.5, 5.7 and 8.1 nM [3]. In our studies, compounds with an isobutyl side chain in the P_1' position were likewise efficient inhibitors of rTACE, particularly BB-94, the most potent inhibitor tested (Table 1). The value found here for BB-94 differs slightly from that reported by Moss et al. [3].

Table 1
Inhibitory activities of metalloproteinase inhibitors towards rTACE and MMP-1, -2 and -3

Inhibitor	rTACE	MMP-1	MMP-2	MMP-3
CT-420	85	7.6	2.85	45
CT-548	N.I.	44 700	21	842
CT-1746	126	122	0.04	10.9
Ro-31-9790	21	4.2	1.8	119
BB-94	0.54	1.5	0.26	2
CT-2256	4.8	4.7	10	34.2

Apparent K_i (nM); MMP-1, collagenase-1; MMP-2, gelatinase-A; MMP-3, stromelysin-1.

Extension of the P_1' hydrocarbon chain, as in CT-1746 and CT-548 (Fig. 2), is well tolerated by MMP-2 but less so by MMP-1 and rTACE (Table 1, [35]). The lack of inhibition of rTACE by CT-548 may also be due to the presence of a less efficient zinc binding group (carboxylate instead of hydroxamate; Table 1). To further study TACE inhibition by hydroxamate inhibitors, it would be interesting to try similar compounds bearing an isopropyl group in the P_1' position, thereby mimicking the valyl residue of human proTNF-α at this position.

3.5. Inhibition of rTACE by TIMPs

The activities of the natural MMP inhibitors, the TIMPs, against rTACE were assessed using QF-45. TIMP-3 was a good inhibitor of rTACE, whereas TIMP-2 and TIMP-4 were relatively weak inhibitors and TIMP-1 had no effect (Table 1). The inhibition of rTACE by TIMP-3 was characteristic of slow-binding inhibition following a simple bimolecular association [31]. The estimated second-order association rate constant k_{on} was $1.013(\pm 0.007)\times 10^6$ M^{-1} s^{-1} and the first-order dissociation rate constant k_{off} was $2.01(\pm 0.02)\times 10^{-4}$

Table 2
Inhibition of MMP-2 and rTACE by TIMPs[a]

TIMP	MMP-2	rTACE
-1	<3	N.I.
-2	0.0006[b]	130 000[c]
-3	<2	182
-4	<4	100 000[c]

[a]Apparent K_i (pM) except where indicated; [b]K_D (pM); [c]IC25 (pM); N.I., no inhibition.

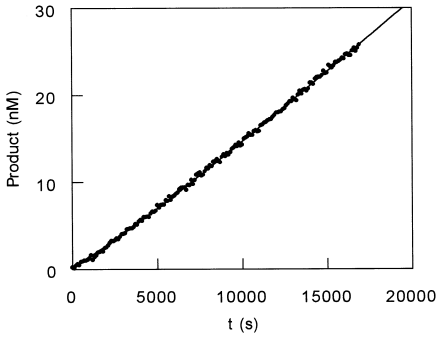

Fig. 3. Dissociation of rTACE-TIMP-3 complex. Preformed complex was diluted to a final concentration of 0.04 nM into QF-45 substrate solution (1 μM). Recovery of enzyme activity was monitored by product fluorescence ($\lambda_{ex} = 328$ nm, $\lambda_{em} = 393$ nm). Data were fitted to Equation 5 as described in [24] to give a dissociation rate constant $k_{off} = 2.0 \times 10^{-4}$ s^{-1}.

s^{-1} (Fig. 3). Combined, these parameters give a dissociation constant (K_d) of the EI complex of 198 pM. This value was in good agreement with the apparent inhibition constant: $K_i' = 182(\pm 76)$ pM (Table 2). The high standard error probably resulted from the effect of prolonged pre-incubation on enzyme stability [13].

The K_i' values for the inhibition of MMP-2 by TIMP-1, -3 and -4 were considerably lower (Table 2). Because $[E]_o \sim K_i'$ cannot be fulfilled, the estimates for K_i' are less accurate, with standard errors above 50%. For comparative purposes only, estimates of K_i' plus their standard error are shown in Table 2 as upper limits. TIMP-3 is at least two orders of magnitude more inhibitory towards MMP-2 than rTACE. However, the inhibition effect of TIMP-3 on rTACE is specific, whereas MMP-2 is tightly inhibited by all four TIMPs. MMP-2 and TIMPs interact not only through their N-terminal domains, but also through their C-terminal domains which contribute to the overall stability of their complex [24]. This is reflected by the very low dissociation constant of the MMP-2-TIMP-2 complex determined in our laboratory: $K_d = 6 \times 10^{-16}$ M [32]. However, for matrilysin (MMP-7), an MMP that naturally lacks a C-terminal domain, the inhibition by TIMP-1 ($K_i' = 370$ pM [36]), is comparable to that of rTACE by TIMP-3. The contribution of the disintegrin and cysteine-rich domains to the inhibition of TACE by TIMP-3 has yet to be determined.

Structural similarities between the active sites of stromelysin-1 (MMP-3) and the snake venom metalloproteinase, adamalysin II, had previously led Bode and co-workers to propose that TIMPs may be inhibitors of members of the ADAM family [16]. This was substantiated by more recent work on the crystal structure of the TACE catalytic domain [11]. From our studies we can conclude that only TIMP-3 is likely to be a physiologically relevant inhibitor of TACE. These data agree with the observations that cell shedding of the TNF receptor [19], the IL-6 receptor [20] and TNF-α and L-selectin (manuscript in preparation) are modulated by TIMP-3 but not by TIMP-1 or -2. It will be of interest to determine whether other ADAMs are inhibited by the TIMPs. MADM is a prime candidate for such studies, because of its relatively high sequence identity with TACE compared with other ADAMs [2] and its proTNF-α converting activity [8,9].

Structural studies of the interactions between TACE and TIMP-3 by X-ray crystallography or NMR spectroscopy may provide valuable information for the design of inhibitors with potential pharmacological applications or TIMP variants that selectively inhibit TACE, or specific MMPs.

TIMP-3 has the potential to be a physiologically relevant regulator of ectodomain shedding activities of cells due to its location within the extracellular matrix. Recent studies have suggested that TIMP-3 has apoptotic activities against some cell types which may, in part, be due to the inhibition of the relevant receptor shedding events [19,37].

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