 Pronounced Diversity in Electronic and Chemical Properties between the Catalytic Zinc Sites of Tumor Necrosis Factor-α-converting Enzyme and Matrix Metalloproteinases despite Their High Structural Similarity

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The metalloproteinase tumor necrosis factor-α-converting enzyme (TACE) is involved in the regulation of several key physiological and pathological processes. Therefore, potent and selective synthetic inhibitors are highly sought for the study of the physiological roles of TACE as well as for therapeutic purposes. Because of the high structural similarities between the active site of TACE and those of other related zinc endopeptidases such as disintegrin (ADAMs) and matrix metalloproteinases (MMPs), the design of such tailor-made inhibitors is not trivial. To obtain new insights into this problem, we have used a selective MMP inhibitor as a probe to examine the structural and kinetic effects occurring at the active site of TACE upon inhibition. Specifically, we used the selective MMP mechanism-based inhibitor SB-3CT to characterize the fine structural and electronic differences between the catalytic zinc ions within the active sites of TACE and MMP-2. We show that SB-3CT directly binds the metal ion of TACE as observed before with MMP-2. However, in contrast to MMP-2, the binding mode of SB-3CT to the catalytic zinc ion of TACE is different in the length of the Zn-S(SB-3CT) bond distance and the total effective charge of the catalytic zinc ion. In addition, SB-3CT inhibits TACE in a non-competitive fashion by inducing significant conformational changes in the structure. For MMP-2, SB-3CT behaved as a competitive inhibitor and no significant conformational changes were observed. An examination of the second shell amino acids surrounding the catalytic zinc ion of these enzymes indicated that the active site of TACE is more polar than that of MMP-2 and of other MMPs. On the basis of these results, we propose that although there is a seemingly high structural similarity between TACE and MMP-2, these enzymes are significantly diverse in the electronic and chemical properties within their active sites.

Tumor necrosis factor-α (TNF-α) is a key pro-inflammatory cytokine produced by activated monocytes and macrophages in response to a variety of exogenous and endogenous insults (1, 2). The overproduction of TNF-α strongly correlates with diverse pathologies such as human immunodeficiency virus infection (3), rheumatoid arthritis (4), septic shock (5), and insulin resistance (6). Precursor TNF-α is produced as a membrane-bound cytokine that is processed by the TNF-α-converting enzyme (TACE), a zinc-dependent metalloproteinase belonging to the ADAM (a disintegrin and metalloproteinase) family (ADAM-17) (7, 8).

The sequence alignment of TACE with members of the MMP family indicates substantial homology, particularly within the catalytic domain (35–44% identity). This high degree of conservation translates into substantial structural similarity. Fig. 1a shows the overlapped structures of the catalytic domains of TACE, MMP-2, and MMP-9. The core structures of these enzymes are highly similar, varying mostly within the peripheral loops. Specifically, the three-dimensional structural elements of TACE and MMP-2 surrounding the zinc-binding site are almost identical, showing a consensus helix followed by a loop that serves as a scaffold for the three His residues that coordinate the catalytic zinc ion (Fig. 1b). On the basis of this remarkable structural similarity, it is reasonable to assume that these enzymes must exhibit the similar mechanisms of catalysis and susceptibility to inhibitors. Yet, each protease is unique in terms of its specificity requirements for substrate cleavage and inhibition (9). This indicates that additional factors other than their active site structure must be critical for controlling both their catalytic properties and inhibition profiles.

To explore the fine kinetic and structural details within the active sites of TACE and MMP-2, we used the mechanism-based inhibitor, SB-3CT, designed to selectively inhibit metalloproteinases (MMP-2 and MMP-9) (10). SB-3CT (Fig. 2) consists of three segments: 1) a biphenyl moiety designed to interact with the P1′ subsite consisting of a large hydrophobic pocket; 2) a sulfone group intended to bind protein backbone amides (Leu193 and Ala192 in MMP-2); and 3) a threonine ring meant to coordinate the active site zinc ion and undergo ring opening by nucleophilic attack of the catalytic glutamate, thus exposing a sulfide ion for coordination to the catalytic zinc ion (10).
mode of binding of SB-3CT to MMP-2 was well characterized both structurally and kinetically by Kleifeld et al. (11) and Brown et al. (10). This inhibitor coordinates the zinc ion of MMP-2 in a monodentate fashion via the thirane sulfur. Such coordination activates the thirane for nucleophilic addition by the conserved active-site glutamate (Fig. 2), resulting in an irreversible inhibition of the protease. An interesting outcome of this inhibitory reaction is that both the structural and electronic conformations of the active site around the zinc ion resemble that of the zymogen (latent) form of MMP-2. This may explain the remarkable selectivity of SB-3CT for MMP-2 and MMP-9 (11).

Herein we show that SB-3CT constitutes a highly sensitive molecular probe for exploring the mechanistic features of the catalytic zinc sites of TACE and MMPs. To gain insight into the fine structure and electronics of the zinc-binding site of TACE in both the active and SB-3CT-inhibited forms, the zinc ion coordination shell was studied by x-ray absorption spectroscopy (XAS) and dynamic structural docking analysis. In addition, we examined the gross conformation of TACE by far-UV circular dichroism (CD) and the activity by steady-state kinetic analysis.

We found that SB-3CT is a micromolar inhibitor of TACE, substantially less potent than that for the inhibition of MMP-2 (10). Our EXAFS results suggest that SB-3CT binds the catalytic zinc ion of TACE via its thirane sulfur in a distorted tetra-coordinated sphere that is different from the case of the zinc-SB-3CT complex observed with MMP-2 in which the thirane ring is presumably opened-up, promoting the coordination of the thiolate to the catalytic zinc ion (11). We also show that, despite the high three-dimensional structural similarity among the active sites of TACE and MMPs, the TACE conformational structure around the catalytic zinc ion and the total effective charge of this metal ion differ from MMP-2. Consistent with this finding, the kinetics and geometry of binding of SB-3CT to TACE is different compared with MMP-2. Therefore, SB-3CT binds to the active site of TACE in a different mode from the one predicted for the precursor TNF-α substrate and hydroxamate-based competitive inhibitors (12).

A close examination of the second shell amino acid residues around the zinc ion in TACE and various MMPs revealed that the active site of TACE is significantly more polar than the active sites of most MMPs. This may account for the pronounced differences in electronic, structural, and kinetic behavior observed between the active sites of MMP-2 and TACE. Therefore, we propose that the fine active site structural, chemical, and electronic features of these proteinases should be taken into account when designing inhibitors, even in cases where high sequence and structural similarity are observed.

**EXPERIMENTAL PROCEDURES**

**Materials**

The catalytic domain of human TACE was expressed using a recombinant baculovirus expression system. This truncated was purified to homogeneity from the culture medium of infected Trichoplussia ni cells as described earlier (7, 13). The catalytic domain of MMP-2 (amino acids 110–467) was expressed in Escherichia coli in a pET-11a expression vector under T7 promoter BL-21 cells. The cells were induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 5 h. The cell pellet was resuspended in 50 mM Tris, pH 8.0, 0.5 mM EDTA, 50 mM NaCl, 5% glycerol, and 1% Triton X-100 at a 1:25 ratio of the buffer to the original culture volume. The suspension was centrifuged for 10 min at 15,000 rpm, and the pellet was dissolved in 50 mM Tris, pH 8.0, 0.5 mM EDTA, 50 mM NaCl, 5% glycerol, and 0.2% Sarkosyl followed by a 30-min incubation on ice. The supernatant fraction was loaded onto a 5-ml gelatin-Sepharose column (prepacked, Amersham Biosciences), preequilibrated, and washed with dialysis buffer (50 mM Tris, pH 8.0, 5 mM CaCl₂, 10 μM ZnCl₂, 0.02% Brij). The protein was eluted with 50 mM Tris, pH 8.0, 1 mM NaCl, 5 mM CaCl₂, 10 μM ZnCl₂, 0.02% Brij, and 15%
Mechanism-based Inhibition of TACE and MMP-2

The synthetic mechanism-based inhibitor SB-3CT was synthesized as described previously (10). The internally quenched fluorescent peptide QF-45 (Mca-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Arg-Lys(dinitrophenyl)-NH₂) was synthesized at the Weizmann Institute core facilities as described by Amour et al. (16).

Kinetic Studies

The enzymatic activity of TACE was measured at 37 °C by monitoring the degradation of the fluorescent peptide QF-45 at λmax = 340 nm and λem = 390 nm as described by Knight et al. (15). The standard assay mixture contained 50 mM Tricine buffer, pH 7.5, 100 mM NaCl, 10 mM CaCl₂, and 1 mM ZnCl₂. The steady-state kinetic data were processed and analyzed by linear and non-linear regression using Microsoft Excel, Magellan, and Origin 7.0. The initial velocities were calculated by using a linear fit followed by non-linear fit to the Michaelis-Menten equation as shown in Equation 1 (17),

\[
\frac{1}{v} = \frac{1}{V_{\text{max}}} + \frac{K_m}{V_{\text{max}}} S
\]  

where \( V_i \) is the initial velocity, \( V_{\text{max}} \) is the maximum velocity, \( S \) is the substrate concentration, and \( K_m \) is the Michaelis-Menten constant.

The inhibition mode was determined by analyzing the linear regression of Lineweaver-Burk plots of the kinetic data (1/V versus 1/S). The \( K_i \) values were calculated using Equation 2 (17).

\[
\frac{1}{V_{\text{max app}}} = \frac{1}{V_{\text{max}}} + \frac{1}{V_{\text{max}}} (1 + \frac{I}{K_i})
\]

Circular Dichroism (CD)

CD measurements were performed employing an Aviv 202 spectropolarimeter using a 1-mm path length quartz cuvette at 25 °C. Wave-length scans were done in the 190–260-nm far-ultraviolet range. The CD spectra of TACE were recorded at a concentration of 0.1 mg/ml in 150 mM NaCl, 20 mM Tris buffer, pH 8, at 2 s/nm. The data sets were averaged and then normalized to the base line at 250 nm.

Inductively Coupled Plasma Atomic Emission Spectroscopy

The metal content of TACE samples was measured using an inductively coupled plasma atomic emission spectrometer (Spectroflame, Spectro, Kleve, Germany). Prior to measurement, the samples were digested with nitric acid and the volume was adjusted to 6 ml (final concentration 10%) per sample. The zinc content was determined relative to a zinc reference solution. The results indicated a stoichiometry of 0.9 zinc ion/protein molecule as determined in two independent measurements. These results are in agreement with the crystal structure of the catalytic domain of TACE (12), indicating one zinc ion/protein molecule.

XAS Studies

Sample Preparation—The enzyme was concentrated by ultrafiltration using Vivaspin 6-ml units (10-kDa cutoff, Vivascience AG, Hanover, Germany) to a final concentration of 0.1 mM (2.92 mg/ml). Samples were loaded into copper sample holders (10 × 5 × 0.5 mm) precoated with Mylar tape 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.

Data Collection—XAS data collection was performed at the National Synchrotron Light Source (NSLS), Brookhaven National Laboratory (beamline X2B). 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. To calibrate the beam energy, the transmission signal from a zinc foil was measured with a reference ion chamber simultaneously with fluorescence. Several scans of each sample were collected for a total of 1 × 10⁶ counts across the edge. 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.

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 removed with the AUTOBK program of the University of Washington XAFS data analysis package developed at the University of Washington (Seattle, WA) (18). The same energy, \( E_0 = 9659 \text{ eV} \), was chosen for background removal as the origin of the photoelectron energy. For signal minimization below the first shell, the R-space region was chosen between 0.6 and 1.2 Å. The useful k-range in the resultant \( k^2 \)-weighted (\( k \)) spectra was between 0 and 10.0 Å⁻¹ upon background removal. The model data for fitting procedures were constructed by extracting the structural zinc site coordinates (in a radius of 4 Å from the zinc) of MMP-2 (Protein Data Bank (PDB) code 1CK7), stromelysin-1 (PDB code 1SLM), and CatS 10 from the Cambridge small molecule data bank. The theoretical photoelectron-scattering amplitudes and phase shifts for each zinc ligand (path) were calculated using the computer code FEFFIT (19, 20). The total theoretical signal (\( k \)) was constructed by adding the most important partial (\( h \)) values that contributed to the \( r \)-range of interest.

The theoretical XAFS signal was fitted to the experimental data using the non-linear least squares method implemented in the program FEFFIT 2.96 (18) in R-space by Fourier transformation of both theoretical and experimental data. Both data were weighted by \( h \) and multiplied by a Hanning window function in Fourier transforms.

Molecular Modeling—The crystallographic three-dimensional structure of TACE (PDB code 1BKC) was used to model SB-3CT bound to the active site of this protease. Initial structures for the conformers were generated from a systematic conformation search using SPARTAN (21) and then optimized by the means of density functional theory using the Gaussian 98 (22) program package. The B3LYP (23, 24) exchange-correlation functional was used in conjunction with the cc-pVDZ (25) basis set where a high exponent “d” function was added to sulfur as recommended by Martin (26). Low energy conformers of SB-3CT were used for docking into the catalytic site of TACE, employing the FlexAid program (27).

RESULTS AND DISCUSSION

The high degree of similarity between the active sites of MMPs and TACE prompted us to use SB-3CT as a molecular tool to probe structural and electronic features of the active site of TACE involved in interactions with this inhibitor. Based on results discussed below, it seems that SB-3CT interacts with the active sites of TACE and MMP-2 in entirely distinct ways despite the high sequence and structural similarity of their catalytic domains.

SB-3CT Is a Non-competitive Inhibitor of TACE—The
steady-state kinetics of TACE substrate cleavage was studied by monitoring the degradation of the model substrate QF-45 (15, 16). SB-3CT was found to inhibit TACE in the low micromolar range with a $K_i$ value of 3.7 nM, which is worse approximately by two orders of magnitude compared with the inhibition of MMP-2 by the same inhibitor. Surprisingly, we found the inhibition mode of TACE by SB-3CT to be non-competitive (Fig. 3); this compound was previously found to be a competitive inhibitor of MMP-2 (10). This observation was surprising because the active sites of the two enzymes are very similar. Whereas we could readily anticipate a somewhat worse dissociation constant for inhibition of TACE by SB-3CT compared with the case of inhibition of MMP-2, the fact that the modes of inhibition were entirely distinct (non-competitive versus competitive) was counter-intuitive based on the structural considerations. The difference in inhibition mechanisms suggested that SB-3CT does not bind to TACE via the substrate binding pocket at the hydrophobic cleft proposed by Maskos et al. (12). Moreover, the affinity of SB-3CT for MMP-2 and MMP-9 is substantially higher than that for TACE as indicated by comparison of $K_i$ values (14 nM for MMP-2 and 600 nM for MMP-9) (10). These differences in binding affinity probably reflect differential modes of interaction of SB-3CT to TACE versus MMP-2/MMP-9.

XAS Studies—The active site structures surrounding the catalytic zinc ion of the recombinant catalytic domain of human TACE in its active and inhibited forms were studied by XAS. XAS corresponds to modulations in the x-ray absorption coefficient, $\mu(E)$, around the x-ray absorption edge of a given atom. This is a valuable spectroscopic technique for elucidating the local structure of a variety of metal-binding sites in metalloproteins (28). The XAS spectra are divided into two parts: 1) the x-ray absorption near edge spectra and 2) the EXAFS regions. X-ray absorption near edge spectra include information regarding the effective charge of the metal ion and its geometry. Complementary to this finding, an analysis of the EXAFS region provides information on the local structure around the analyzed metal ion including average bond distances, mean square variation in distance, metal coordination number, and ligand type.

The local structure of the catalytic zinc ion of TACE and the TACE-SB-3CT complex was characterized by XAS. Specifically, the XAS data from zinc k-edge of the active and inhibited forms of TACE were collected and analyzed. Fig. 4a shows the x-ray absorption near edge spectra of active TACE and the TACE-SB-3CT complex. The edge energy position of the TACE-SB-3CT complex is shifted to a lower energy by $\Delta E \approx 0.5$ eV relative to active TACE (Fig. 4b). Although difficult to quantify, such
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The uncertainties for the fits are given for the distance parameters. The symbols F and V stands for “fixed” and “varied,” respectively, and indicate how the respective parameter was treated in the fit. R is distance of atoms from the catalytic zinc ion in Å, \( \sigma^2 \) is the Debye-Waller factor.

| Fit number | \( \chi^2 \) | Path | \( \Delta E_0 \) | R | \( \sigma^2 \) |
|------------|-------------|-----|----------------|---|------------|
| **Active TACE** | | | | | |
| Fit 1 | 44.0 | Zn-N/O × 3.47 ± 1.1 [V] | 5.3 [F] | 2.03 ± 0.01 | 6.5E-03 [V] |
| Fit 2 | 180 | Zn-N/O × 2 [F] | 6.0 [F] | 2.07 ± 0.02 | 1.0E-05 [V] |
| Fit 3 | 3.13 | Zn-N/O × 3 [F] | 7.2 [F] | 3.06 ± 0.07 | 9.2E-03 [V] |
| Fit 4 | 31 | Zn-N/O × 3 [F] | 7.2 [F] | 3.11 ± 0.01 | 5.6E-03 [V] |

| **TACE-SB3CT complex** | | | | | |
| Fit 1 | 0.36 | Zn-N/O × 3 [F] | 6.0 [F] | 2.05 ± 0.01 | 1.8 E-03 [V] |
| Fit 2 | 1.03 | Zn-N/O × 3 [F] | 6.0 [F] | 2.09 ± 0.01 | 3.1 E-02 [V] |
| Fit 3 | 16.9 | Zn-N/O × 3 [F] | 6.0 [F] | 2.02 ± 0.01 | 1.0E-06 [V] |
| Fit 4 | 3.7 | Zn-N/O × 3 [F] | 6.0 [F] | 2.09 ± 0.01 | 1.8E-03 [V] |

- Estimated error for \( \sigma^2 \) did not rise over 10% where \( \sigma^2 \) was varied.

shifts to a lower energy indicate a partial reduction in the total effective charge of the catalytic zinc ion within the active site of TACE upon SB-3CT binding. In contrast, the zinc k-edge position of the MMP-2-SB-3CT complex overlaps well with the edge of active MMP-2, resulting in no significant change in the total charge of the metal ion upon binding to the inhibitor. In contrast, the pronounced spectral changes in the post-edge features of MMP-2 versus MMP-2-SB-3CT (11) were not detected between active TACE and TACE-SB-3CT (Fig. 4a).

Differences in the total effective charge of the catalytic zinc ion between the SB-3CT-inhibited complexes of TACE and MMP-2 suggest that the microenvironments surrounding the catalytic zinc ions of these two enzymes are not equivalent (29, 30). To obtain a detailed picture of the zinc ligands and their relative distances, we analyzed the EXAFS spectra of both the active and inhibitor-bound forms of TACE. The EXAFS curve-fitting analysis was preformed using previously reported procedures developed by our group (31, 32). The normalized background subtracted x-ray absorption data were fitted to theoretical phase shifts and amplitudes. Theoretical models of the proposed structural and catalytic zinc sites were constructed from the crystal structure of pro-MMP-2 (33) and the catalytic domain of TACE (12) (see “Experimental Procedures”). Zinc sites were fitted to the Zn-N, Zn-O, Zn-S, and Zn-C theoretical phases and amplitudes using different combinations of varied and constraint parameters. In addition, different initial values for distances, Debye-Waller factors, and \( \Delta E_0 \) shifts were applied during the fitting procedure. The \( \Delta E_0 \) shifts and the reduction factor \( S_0 \) were varied for each fit and fixed at their optimal values. These fitting results are listed in Table I and shown graphically in Fig. 4, c–d.

The EXAFS curve-fitting data analysis of active TACE shows that the zinc ion is coordinated tetrahedrally with three Zn-His bond distances at 2.06 ± 0.01 Å, one Zn-N/O bond distance at 1.89 ± 0.01 Å, three Zn-C bond distances at 2.85 ± 0.01 Å, and three Zn-C bond distances at 3.11 ± 0.01 Å (Fig 3). The attempts to fit the first coordination shell of the active enzyme with only three Zn-N/O or additional Zn-S contributions resulted in unstable fits exhibiting high \( \chi^2 \) values. The first coordination shell zinc-ligand bond distances of active TACE are in agreement with the x-ray crystal structure (12) showing three Zn-N contributions from 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 (11).

The curve-fitting analysis of the TACE-SB-3CT complex demonstrates the binding of SB-3CT to the catalytic metal ion (Table I). Specifically, the zinc ion in TACE-SB-3CT exhibited one Zn-S(SB-3CT) at 2.35 ± 0.01 Å, three Zn-N(His) ligands at 2.05 ± 0.01 Å, three Zn-C at 2.94 ± 0.01 Å, and three Zn-C at 3.15 ± 0.09 Å (Fig 1). The rather long Zn-S(SB-3CT) bond distance indicates that the catalytic zinc ion of TACE in the TACE-SB-3CT complex is bound to the thirane sulfur and not the thiolate ion, which would have been generated by the reaction between the conserved active site glutamate residue and the thirane ring, as documented for MMP-2. This obser-
vation underscores further the different binding modes of SB-3CT to TACE and to MMP-2. Our fitting results do not exclude outright the possibility that the zinc ion in the inhibited enzyme would form a pentavalent complex with an additional Zn-O/N contribution at \(1.73 \pm 0.01\) Å (Table I, fit 2). This fit was obtained by fixing the Zn-S distance at 2.27 Å and refining the coordination number. However, the additional Zn-N/O distance is relatively short and therefore cannot be justified. Refinement of this bond distance resulted in a larger value but higher \(R^2\) values (Table I, fit 3). Attempts to exclude the Zn-S contribution from the first coordination shell of the zinc ion resulted in unstable fits and higher \(R^2\) values (Table I, fit 4).

The first coordination shell of the zinc ion of TACE appears to be slightly distorted relative to the one observed for the MMP-2-SB-3CT complex (11). This is because of the elongation of the Zn-S(SB-3CT) bond distances in TACE-SB-3CT (2.35 ± 0.01 Å) versus MMP-2-SB-3CT (2.22 ± 0.02 Å). Based on this finding, we propose that, in the case of MMP-2, SB-3CT is bound via the thiolate coordination to the catalytic zinc ion (shorter bond distance), whereas in the case of TACE, the thiirane sulfur coordinates the catalytic zinc ion (longer bond distance). This finding suggests that the opening of the thiirane ring by the nucleophilic attack of the catalytic glutamate residue, a key feature in the design of SB-3CT, does not take place in the case of TACE.

Molecular Modeling—To obtain further insights into the mode of interaction of SB-3CT with TACE, we performed molecular modeling studies using a novel dynamic docking procedure (27). Unlike rigid body-docking procedures, the dynamic docking algorithm that we employed utilizes a global search procedure based on genetic algorithms (34) and surface complementarity (35). Using this strategy, the modeling of the interaction of SB-3CT with TACE and MMP-2 by this approach is not biased by any previous knowledge.

Different conformers of SB-3CT were modeled by molecular mechanics procedures followed by the density functional theory calculation using the B3LYP module of the Gaussian 98 software package (22) (data not shown). SB-3CT conformers were calculated with the thiirane ring intact. The best conformers where chosen based on the free energy values (Fig. 5a). The selected conformers (Fig. 5b) were docked dynamically into TACE and MMP-2. The different models obtained from the described docking procedures were evaluated for chemical and structural relevance by examining the types of atomic interaction and steric clashes. Fig. 5, c–d, shows the predicted interactions of SB-3CT with MMP-2 and TACE. As expected from previous studies, the biphenyl moiety of SB-3CT directly inter-
acts with the P1’ hydrophobic pocket of MMP-2; thus, the
inhibitor spans the hydrophobic pocket within the substrate-
binding cleft (11). The specific topography of the substrate-
binding hydrophobic cleft of MMP-2 is conserved remarkably in
binding cleft (11). The specific topography of the substrate-
inhibitor spans the hydrophobic pocket within the substrate-
binding pocket and while the catalytic zinc ion is coordinated by
the thiorane sulfur moiety. This is a surprising result because of
the high sequence and structural homology between the cata-
lytic domains of MMP-2 and TACE. Our model of the TACE-SB-
3CT complex suggests a binding site for SB-3CT almost enti-
tirely non-overlapping with the proposed substrate-binding
cleft, consistent with the non-competitive kinetic behavior of
SB-3CT with TACE.

**CD Measurements of TACE and TACE Complexes**—Our ki-
netic, XAS, and modeling studies suggest that SB-3CT binds
the catalytic zinc ion via an alternative binding site. Non-
competitive compounds binding outside of the active site often
exert their inhibitory functions through the induction of con-
formational changes that render the target enzyme inactive. To
examine whether this is the case for TACE, we performed
far-UV CD-spectroscopic studies. We analyzed the spectra of
the free, active, and catalytic domains of TACE and MMP-2,
comparing them to the ones obtained for the inhibited
TACE-SB-3CT and MMP-2-SB-3CT complexes. Far-UV CD
spectroscopy is useful in determining the secondary structure
content of polypeptides (38).

Far-UV CD analysis of TACE revealed a dramatic conforma-
tional change upon the addition of SB-3CT (Fig. 6a), an event

that would take place after the complex between SB-3CT and
the enzyme (Fig. 5d) has formed. A loss of ~15–20% in the
eellipticity within the 208–228-nm region of the spectrum cor-
responding to changes in the α-helix or β-sheet content was
evident. This significant change in the secondary structure for
TACE suggests that SB-3CT may act as a negative allosteric
regulator of TACE, binding at a site different from the sub-
strate-binding cleft and inducing a conformational change
in this enzyme. In contrast, no significant conformational changes
were detected between the free and SB-3CT-complexed forms
of MMP-2 (Fig. 6b).

In this work, we have presented a detailed investigation of
the microenvironment surrounding the catalytic zinc ion of
TACE using structural, kinetic, and spectroscopic analyses.
The active site of TACE shares high sequence and structural
similarity to that of the enzymes of the MMP family, specifi-
cally to MMP-2 (Fig. 1a). Despite this finding, we have demonstrated
that the behavior of these two enzymes differs mark-
edly upon binding to the mechanism-based inhibitor, SB-3CT.
Our results reveal that there is a pronounced distinction be-
tween the catalytic zinc sites of TACE and MMPs despite their
high structural similarity within the active sites. A close ex-
amination of the second shell amino acid residues around the
zinc ions of TACE and MMPs (Fig. 7a) reveals the basis of the
observed distinction among the various members of this family.
It appears that while the peptide backbone structure is highly
conserved among the different enzymes, the electrochemical
potential induced by the active site residues may be quite
different. For example, the second shell residues surrounding
the zinc ion residues in TACE are Thr404, Glu406, Gly408,
Asn410, Asp416, and Glu414 versus Ala502, Glu404, Gly406, Ala408,
Glu412, and Ser414 for MMP-2. Therefore, the microenviron-
ment surrounding the catalytic zinc ion of the TACE active site
is significantly more polar than that of MMP-2. Fig. 7a shows
that the catalytic zinc site of TACE is also more polar than the
catalytic sites of MMP-1, MMP-3, MMP-7, and MMP-9. Elec-
trostatic surface analysis of the catalytic site of TACE versus
MMP-2 shows that the catalytic zinc environment of the TACE
is more polar compared with that of MMP-2 (Fig. 7, b–c). The
role of metal-ligand second shell residues has been analyzed by
ab initio theoretical calculations and documented in previous
studies. In a related study, Colletta and co-workers (37) inves-
tigate the kinetic behavior of MMP-2, MMP-8, and MMP-9 as a
function of the protonation state of the active site residues.
Significant changes in kinetic parameters were evident not
only between these homologous enzymes at the same pH but
also in the same enzyme at different protonation states (37).
Cross et al. (38) use ab initio calculations to show that the
protonation states of the catalytic glutamate of TACE (Glu406)
and inhibitors are important factors in understanding the in-
hibitory properties of hydroxamic and carboxylic acid com-
ounds. They computed pK_a values using a molecular model
of TACE to predict the mechanism of inhibitor binding to the
catalytic enzyme site. It consisted of a lowering of the pK_a of
the bound ligand and the raising of the pK_a of the active site
Glu406. They showed that the inhibitor-induced protonation of
Glu406, pre- or post-binding, was crucial for determining both
the binding mode and inhibitory potency (38).

We have shown in this paper that despite high structural
similarity, variation in the polarity of the active site residues of
the catalytic zinc-binding site in MMPs and related enzymes
dictates their different electronic states. This stems from subtle
differences in distances, effective charge of the catalytic metal
ion, and orientation of the active site His imidazole rings to-
ward the zinc ion as suggested in the structure alignment in
Fig. 1a and our XAS results. A detailed understanding of the

![Fig. 6](image-url)
It is a common belief that the high structural similarity within the active sites of TACE, ADAMs, and MMPs is a serious obstacle in developing selective inhibitors for these related enzymes. Indeed, the cross-inhibitory properties of a number of known inhibitors for these enzymes has been proposed as the root cause of some of the recent clinical trials with MMP inhibitors (39, 40). This indeed has been a challenge. However, our findings reported herein argue that the seemingly high structural similarity may not be the full story in the prediction of the catalytic behavior of these enzymes or their predisposition toward inhibitors. That SB-3CT would serve as a non-competitive inhibitor for TACE was unanticipated based on solely structural considerations. However, a closer examination of the binding modes of the compound to the active site of TACE based on dynamic considerations shed light on the electronic state of the active site of TACE, polarity, chemistry, and fine structure will be of great utility in understanding the chemical basis of inhibition of both physiological and synthetic TACE inhibitors.

Mechanism-based Inhibition of TACE and MMP-2

**FIG. 7.** *a,* schematic representation of the number of polar or charged residues in the second coordination shell of the catalytic zinc ion of TACE and analogous catalytic sites of MMPs (PDB codes are provided in parentheses). The corresponding amino acid residues are as follows: TACE (Thr404, Glu406, Gln402, Gly404, Ala406, Asp410, Asp416, and Glu414); MMP-1 (Ala200, Glu202, Gly204, Ser206, Phe210, and Ser212); MMP-2 (Ala402, Glu404, Gly406, Ala408, Glu412, and Ser414); MMP-3 (Ala200, Glu202, Gly204, Ser206, Phe210, and Ser212); MMP-7 (Thr217, Glu219, Gly221, Ser223, Ser227, and Ser229); MMP-9 (Ala406, Gln402, Gly404, Ala406, Asp410, and Ser414). *b,* electric potential (Connolly drawing) of the catalytic site in TACE (PDB code 1BKC). The relative electrostatic scale from red to blue (on the right) indicates negative to positive charge, respectively. The zinc ion is designated as orange ball. *c,* electric potential (Connolly drawing, presented in stereo) of the catalytic site of MMP-2 (PDB code 1CK7). The relative electrostatic scale is the same as in b. The catalytic zinc ion is designated as orange ball. The catalytic zinc environment in TACE as observed in panel b is significantly more polar than in MMP-2.

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