

The Structure of Testis Angiotensin-Converting Enzyme in Complex with the C Domain-Specific Inhibitor RXPA380^{†,‡}

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ABSTRACT: Angiotensin I-converting enzyme (ACE) is central to the regulation of the renin–angiotensin system and is a key therapeutic target for combating hypertension and related cardiovascular diseases. Currently available drugs bind both active sites of its two homologous domains, although it is now understood that these domains function differently *in vivo*. The recently solved crystal structures of both domains (N and C) open the door to new domain-specific inhibitor design, taking advantage of the differences between these two large active sites. Here we present the first crystal structure at a resolution of 2.25 Å of testis ACE (identical to the C domain of somatic ACE) with the highly C-domain-specific phosphinic inhibitor, RXPA380. Testis ACE retains the same conformation as seen in previously determined inhibitor complexes, but the RXPA380 central backbone conformation is more similar to that seen for the inhibitor captopril than enalaprilat. The RXPA380 molecule occupies more subsites of the testis ACE active site than the previously determined inhibitors and possesses bulky moieties that extend into the S₂' and S₂ subsites. Thus the high affinity of RXPA380 for the testis ACE/somatic ACE C domain is explained by the interaction of these bulky moieties with residues unique to these domains, specifically Phe 391, Val 379, and Val 380, that are not found in the N domain. The characterization of the extended active site and the binding of a potent C-domain-selective inhibitor provide the first structural data for the design of truly domain-specific pharmacophores.

Angiotensin I-converting enzyme (ACE)¹ is a zinc metallopeptidase and a major drug target for cardiovascular disease. It has a central role in the renin–angiotensin system (RAS), regulating blood pressure, fluid and electrolyte homeostasis, and renal and vascular function, through its ability to produce angiotensin II from the precursor decapeptide angiotensin I and to inactivate bradykinin. As such, it is a key therapeutic target for the treatment of hypertension, congestive heart failure, myocardial infarction, renal failure, and diabetic nephropathy (1). Somatic ACE (sACE) is a monomeric, membrane-tethered protein, located on the plasma membrane of epithelial cells. It has two enzymatic domains, termed the N and C domains, and is heavily

glycosylated (2). A second form of ACE is found in the testis, termed testis ACE (tACE), and plays an important role in fertilization (3). This is also a membrane-bound protein but consists of only one enzymatic domain and a short heavily O-glycosylated N-terminal sequence (4).

Initial drug development for ACE was an early success for rational drug design, in the absence of the 3D structure for ACE (5). The simultaneous development of ACE inhibitors captopril and enalapril was based on the structures of carboxypeptidase A and thermolysin. These drugs have been hugely successful, even though the recent ACE crystal structure did not show significant structural similarity to either of these enzymes (6). Furthermore, it has become apparent since the advent of these drugs that sACE has the two homologous, but not identical, N and C enzymatic domains (7). These two domains both cleave angiotensin I and bradykinin but have different specificities for other substrates and inhibitors. The C domain is more efficient at cleaving the substrate Hip-His-Leu (HHL) (8) whereas the N domain has a preference for Ang1–7 (9) and Ac-Ser-Asp-Lys-Pro (AcSDKP) (10). They also differ in their glycosylation patterns and chloride activation (8, 11). These data suggest that the two domains might have different physiological roles. Indeed, the N domain contributes to the regulation of hematopoietic stem cell differentiation and proliferation through its hydrolysis of AcSDKP (10, 12), whereas the C domain is sufficient to maintain the regulation

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¹ Abbreviations: ACE, angiotensin-converting enzyme; RAS, renin–angiotensin system; tACE, testis ACE; sACE, somatic ACE; HHL, Hip-His-Leu; AcSDKP, Ac-Ser-Asp-Lys-Pro; CHO, Chinese hamster ovary; PEG, poly(ethylene glycol).

of the renin–angiotensin system (13). Therefore, it has been postulated that the use of domain-specific inhibitors may allow more precise control of ACE activities for specific therapeutic use and possibly reduce side effects such as cough and angioedema (14). Angioedema and cough are widely accepted to be caused by raised levels of bradykinin (15–17), and whereas angiotensin I cleavage is inhibited by blockage of either of the active sites, bradykinin can still be degraded if one of the active sites is unoccupied (18).

To further elucidate the significance of these two active sites, two new domain-specific inhibitors based on phosphinic peptides have been developed. All ACE inhibitors to date have been peptide analogues, usually with a sulfhydryl (captopril), carboxyl (lisinopril/enalaprilat), or ketone (keto-ACE) group for coordination of the zinc ion (19). Phosphinic peptides have been shown to have an advantage over the traditional inhibitors in terms of selectivity, due to the weaker coordinating power of the phosphate toward the zinc ion, allowing binding to be regulated by other determinants of the molecule (20). Initially, an N-domain-specific inhibitor was developed, RXP407, with a K_i for the N domain of 7 nM, 1000 times lower than for the C domain (21). Furthermore, RXP407 upregulated AcSDKP metabolism, increasing its plasma levels by 4–6-fold with no effect on blood pressure regulation (22). This was followed by RXPA380, a specific inhibitor for the C domain, which has a K_i of 3 nM for the C domain, 3000 times lower than for the N domain (18). In order to study the structural basis for this difference in domain selectivity between these inhibitors, we have crystallized tACE, which is identical to the C domain of sACE, with the RXPA380 inhibitor.

EXPERIMENTAL PROCEDURES

Purification. A variant of human tACE (tACE Δ 36NJ) was expressed in CHO cells and purified to homogeneity using lisinopril–Sepharose affinity chromatography, as described previously (23). We refer to this variant throughout the manuscript as tACE. tACE activity was determined by measuring the hydrolysis of hippuryl-histidyl-leucine (Sigma) in a fluorometric assay (24). Protein concentration was determined by a Bradford protein assay (Bio-Rad protein microassay). Synthesis of RXPA380 [(2S)-2-[(2-[(1R)-1-[(benzyloxy)carbonyl]amino]-2-(phenylethyl)(hydroxyl)phosphinyl]cyclopentyl]carbonyl)amino]-3-(1H-indol-3-yl)propionic acid (Cbz-Phe Ψ [P(O)(OH)CH]Pro-Trp-OH)] was as described previously (25).

Crystallization and Data Collection. Equal volumes of tACE (6.4 mg/mL in 10 mM Hepes, pH 7.5) and RXPA380 (2 mM in water) were incubated on ice for 4 h. One microliter of this mixture was mixed with an equal volume of reservoir solution (13.5% PEG 4000, 0.12 M sodium malonate, 90 mM sodium acetate, pH 4.7, and 9 μ M zinc sulfate) and suspended above the well as a hanging drop at 16 °C. Crystals grew within a couple of days. A single crystal was cryocooled (100 K) using reservoir solution plus 25% (v/v) glycerol as a cryoprotectant. Diffraction data to 2.25 Å were collected on station X13 of DESY in Hamburg and a low-resolution dataset at BM14 of ESRF in Grenoble. The data were processed and scaled in HKL2000 (26) (see Table 1). The symmetry and systematic absences were consistent

Table 1: Crystallographic Data

| | |
|---|--------------|
| resolution range, Å | 20–2.25 |
| space group | $P2_12_12_1$ |
| no. of reflections measured | 278277 |
| no. of unique reflections | 22494 |
| $I/\sigma(I)$ (outer shell) ^a | 7.1 (1.5) |
| completeness (outer shell), ^a % | 72.2 (59.1) |
| R_{symm}^b (outer shell), ^a % | 10.3 (46.1) |
| R_{cryst}^c (last shell), % | 21.5 (31.9) |
| R_{free}^d (last shell), % | 26.2 (41.9) |
| average temperature factor, Å ² | |
| protein atoms | 22.4 |
| RXPA380 | 17.6 |
| solvent (no. of water molecules) | 15.8 (107) |
| deviation from ideal values (rms) | |
| bond lengths, Å | 0.008 |
| bond angles, deg | 1.2 |

^a Outer shell, 2.33–2.25 Å. ^b $R_{\text{symm}} = \sum_i \sum_j [|I_i(h) - \langle I(h) \rangle| / \sum_i \sum_j I_i(h)]$, where I_i is the i th measurement and $\langle I(h) \rangle$ is the weighted mean of all measurements of $I(h)$. ^c $R_{\text{cryst}} = \sum_h |F_o - F_c| / \sum_h F_o$, where F_o and F_c are the observed and calculated structure factor amplitudes of reflection h , respectively. ^d R_{free} is equal to R_{cryst} for a randomly selected 4% subset of reflections not used in the refinement.

with the spacegroup $P2_12_12_1$ (unit cell dimensions $a = 56.47$ Å, $b = 84.75$ Å, and $c = 133.51$ Å) with one molecule in the asymmetric unit. Data reduction was carried out using the CCP4 program TRUNCATE (27).

Structure Determination. The atomic coordinates of tACE were used as the starting model (PDB code 1O8A) (6). Refinement was carried out with Refmac (28), with 4% of the reflections kept aside for R_{free} calculation (29). Model building, including the addition of RXPA380 and water molecules, was done with Coot (30). Figures were produced with PYMOL (DeLano Scientific, San Carlos, CA).

RESULTS

Overall Structure. The tACE domain forms an ellipsoid with the active site buried in the center. In all structures determined so far it retains the same closed conformation associated with inhibitor binding (6, 31), although it has been proposed to flex in a manner similar to that observed for ACE2 to provide access to the active site (32, 33). Of the total construct, residues 37–627 and residues 40–623 are observed in the crystal structure. This includes an additional six C-terminal residues to those observed for the other native and ligand-bound tACE structures, but which were observed for the tACE-g13 glycosylation mutant structure (32). Interestingly, no sugars were observed at any of the six glycosylation sites, possibly because of the lower resolution of this structure.

Binding of RXPA380. RXPA380 (Figure 1A) is the longest inhibitor whose 3D structure in complex with tACE has been solved using X-ray crystallography. This phosphinic peptide not only has residues occupying the S_2' , S_1' , and S_1 subsites (Trp, Pro, and Phe, respectively) but also has a second Phe moiety extending into the S_2 subsite. RXPA380 binds the active site in an elongated conformation, with both phosphinyl oxygens coordinating the zinc (Figure 1B). The RXPA380 is positioned by eight direct hydrogen bonds with the protein, as calculated by HBPLUS (34), including three with the phosphinyl oxygens (Figure 2A) (Table 2). The N-terminus is positioned by an aromatic interaction with Phe 391 and a hydrogen bond between the carbonyl oxygen

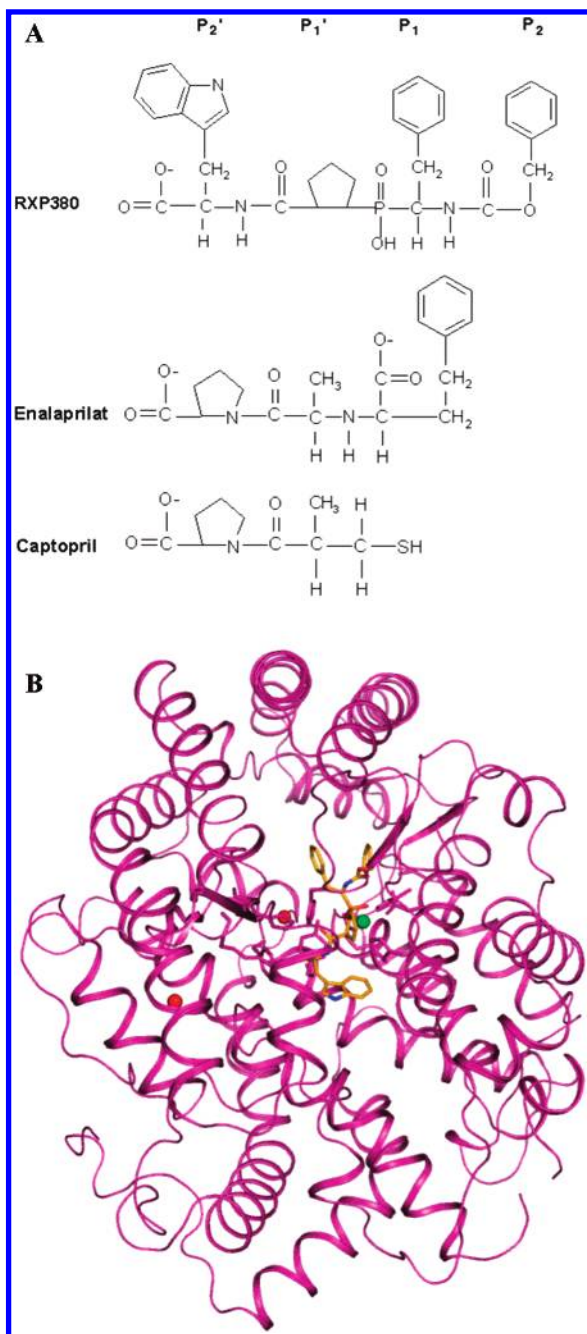


FIGURE 1: Testis ACE with RXPA380. (A) Chemical structures of RXPA380, enalaprilat, and captopril with the residue positions (P_2' , P_1' , P_1 , and P_2) labeled relative to the substrate-cleavage site. The inhibitors are in the same orientation as in Figure 2. (B) Cartoon of tACE (pink) showing RXPA380 (orange) bound in the active site. The zinc and chloride ions are shown as green and red spheres, respectively.

between the two Phe moieties and the backbone nitrogen of Ala 356. In contrast, the carbonyl oxygen on the C-terminal side makes two hydrogen bonds with His 353 and His 513. Finally, one oxygen of the C-terminal carboxylate is anchored by Lys 511 and Tyr 520, while the other oxygen makes a hydrogen bond with a water molecule.

Comparison with Other Known Inhibitors. Superposition of the RXPA380 structure with the previously determined structures of captopril, enalaprilat, and lisinopril shows a similar, but not identical, conformation to these peptide mimics (Figure 2B,C). The C-terminus is almost identically placed in all four complexes, interacting with Lys 511 and

Tyr 520. Despite the differences in the zinc coordination moieties (carboxyl in the case of enalaprilat and lisinopril, sulfhydryl for captopril, and phosphate for RXPA380) the inhibitor coordinating atoms are similarly positioned. The two phosphinyl oxygens of RXPA380 overlap almost precisely with those from the carboxylate groups of enalaprilat and lisinopril, with one ~ 2 Å away and the other ~ 2.5 Å away from the zinc. The P_1 Phe moieties of RXPA380, lisinopril, and enalaprilat also occupy the same space, although the difference in the backbone composition results in a slightly different angling of this substituent. Interestingly, the backbone of RXPA380 most closely follows the backbone conformation of captopril on the C-terminal side of the zinc-binding group. In the case of lisinopril and enalaprilat, the backbone bends away from the zinc to leave space to accommodate the longer carboxyl moiety next to the zinc (Figure 2C). RXPA380 makes eight hydrogen bonds with the protein, one more than enalaprilat and three more than captopril. Of these, however, four are conserved between all the inhibitors. These are the interactions of the C-terminal carboxyl and the adjacent carbonyl oxygen moieties with Lys 511 and Tyr 520 and with His 353 and His 513, respectively. In addition, the carboxylic acid is observed to bind a water molecule in all four inhibitor structures.

Conserved Water Structure. Comparison of the active sites shows that much of the water structure is conserved following the binding of the inhibitors. The RXPA380 structure is at a lower resolution to the other structures, but of the waters modeled in the active site of this structure, all but one are conserved in the other structures. The water interacting with Lys 454 is not observed in the lisinopril and enalaprilat structures because it is replaced by a glycine molecule. These waters are scattered along the active site but clustered more toward the S_2 end of the active site (Figure 2D). The extra length provided by the N-terminal Phe extending into the S_2 subsite also displaces two waters conserved between the lisinopril and enalaprilat structures linked to the backbone nitrogen of Ala 356 (Figure 2C). Two similar waters are also seen in the captopril structure. The larger size of the tryptophan moiety at the S_2' subsite also displaces a water molecule conserved in the other three structures that interacts with Gln 281 (Figure 2C).

Comparison with the N Domain of Somatic ACE. RXPA380 was developed as a C domain-specific inhibitor for comparison of the N and C domain catalytic sites in sACE (18) with its K_i for the C domain 3000 times lower than that for the N domain. Thus, it is interesting that the tACE residues that form hydrogen bonds with RXPA380 are all conserved in the N domain, although the aromatic interaction with Phe 391 would be lost as this is replaced with Tyr 369. Superposition of the N domain (Figure 3) with this structure places the hydroxyl group of Tyr 369 within 2 Å of the P_2 Phe moiety, illustrating that the same orientation for RXPA380 would not be possible in the N domain. The combination of the lack of this interaction and the decrease of hydrophobicity in the S_2 subsite is likely to contribute to the C domain selectivity of RXPA380.

However, potency comparisons of RXPA380 with other phosphinic peptides suggest that the selectivity of RXPA380 for the C domain is due to its proline and tryptophan residues, in the P_1' and P_2' positions, respectively. The tryptophan moiety of RXPA380 is ~ 4.5 Å from two valines (Val 379

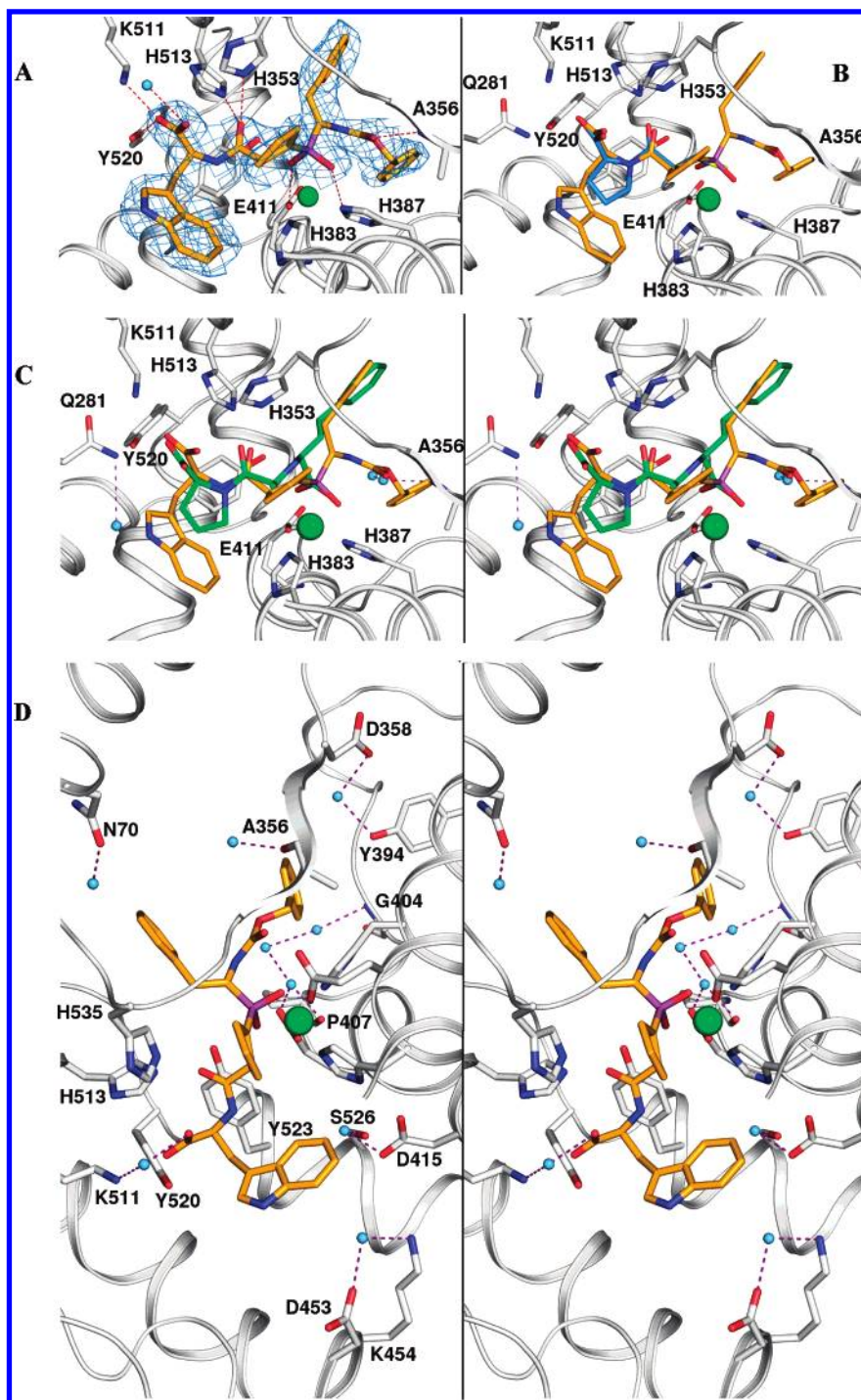


FIGURE 2: A close-up view of the tACE active site showing RXPA380 bound (orange). The zinc ion is shown in green, and (D) shows the water molecules from the RXPA380 complex in light blue. Atoms are colored as follows: gray for carbon, red for oxygen, blue for nitrogen, and purple for sulfur. (A) Omit map density (1σ , blue) for the inhibitor. Residues forming hydrogen bonds with the inhibitor are shown as sticks with the hydrogen bonds represented as dashed red lines. A water molecule bound to the inhibitor is shown as a light blue sphere. (B) The inhibitor captopril (blue) is superimposed on RXPA380. The sulfur atom can just be seen in yellow behind the purple phosphorus atom of RXPA380. (C) Stereoview of enalaprilat (green) superimposed on RXPA380. Three waters that were observed in the lisinopril and enalaprilat complex structures, but are displaced in the RXPA380 structure, are shown as light blue spheres with hydrogen bonds illustrated as purple dashed lines. (D) Stereoview of waters in the RXPA380 complex active site. The view is rotated 90° from the previous figures. Water-bound residues are shown as sticks and the waters as light blue spheres. Hydrogen bonds are illustrated as purple dashed lines.

and Val 380 in tACE) lining the S_2' subsite, making van der Waals interactions with them likely. The change of these residues to the polar Ser 357 and Thr 358 in the N domain suggests that it is the decrease in hydrophobicity in this area that makes the binding of RXPA380 to the N domain less efficient.

It was also suggested that the NH of the indole ring of the P_2' moiety may form a hydrogen bond with a nearby aspartate (Asp 453 in tACE) and that this interaction may be lost with the exchange of this residue for glutamate in the N domain (25). However, Asp 453 is 5.5 Å away from the tryptophan ring and is not close enough to make this

Table 2: Comparison of the Hydrogen Bonds between tACE and Inhibitors RXPA380, Captopril, and Enalaprilat

| tACE | | RXPA 380 | | captopril | | enalaprilat | |
|---------|------|----------|--------------|-----------|--------------|-------------|--------------|
| residue | atom | atom | distance (Å) | atom | distance (Å) | atom | distance (Å) |
| H353 | NE2 | O5 | 2.9 | O1 | 2.5 | O1 | 2.7 |
| A354 | O | | | | | N1 | 3.0 |
| A356 | N | O2 | 2.7 | | | | |
| H383 | NE2 | O3 | 3.3 | | | | |
| E384 | OE2 | | | S1 | 3.1 | O2 | 2.7 |
| H387 | NE2 | O4 | 3.0 | | | | |
| K511 | NZ | O6 | 2.6 | O2 | 2.7 | O4 | 2.8 |
| H513 | NE2 | O5 | 2.9 | O1 | 2.7 | O1 | 3.1 |
| Y520 | OH | O6 | 2.6 | O2 | 2.7 | O4 | 2.6 |
| Y523 | OH | O3 | 2.7 | | | O3 | 2.8 |
| W82 | O | OXT | 3.0 | O3 | 2.6 | O5 | 2.7 |

interaction (Figure 3). Comparison with the N domain shows that the equivalent glutamate is also over 4 Å away.

DISCUSSION

The currently available ACE inhibitors are well-established therapeutics for high blood pressure and related diseases but do not bind preferentially to either of the two domains of somatic ACE. The binding strength for these inhibitors is largely due to their ability to coordinate the zinc ion strongly through sulfhydryl, carboxylate, or keto groups. Also, a calorimetric study has shown that the binding of captopril, lisinopril, and enalaprilat is entropically driven (35), which may be the reason that inhibitors lacking the rigidity of the prolyl moiety have reduced binding affinity (36). In contrast, the phosphate moiety is a weaker coordinator of the zinc ion, and this enhances the potential for selective inhibitors whose binding strength is aided by their other substituents (20). RXPA380 is the first example of a phosphinic peptide that is truly selective for the C domain. To understand this selectivity further and to probe the mechanism of this preferred binding affinity, we have determined the crystal structure of tACE, which is equivalent to the C domain of sACE, complexed with RXPA380.

The structure reveals that the binding of RXPA380 to the C domain involves more direct interactions than the previously determined ACE inhibitor structures. This can be partly ascribed to the larger size of the inhibitor and may also be required to compensate for the weaker coordination of the zinc by the phosphate. Interestingly, the specificity of RXPA380 for the C domain does not appear to depend on hydrogen bonding to the protein, as all of these residues are conserved in the N domain, but on the shape and polarity of the active site. The exchange of three hydrophobic residues (Phe 391, Val 379, and Val 380 in tACE) for polar residues in the N domain (Tyr 369, Ser 357, and Thr 358, respectively) results in the reduced affinity for the N domain mediated by the P₂ Phe and P₂' Trp moieties of RXPA380. The question remains, however, whether other more drug-like inhibitors can be designed to take further advantage of differences between the two active sites. One obvious difference between the sites is the presence of a positive charge at the base of the S₁' pocket in the N domain. This positive charge presumably contributes to the fact that lisinopril, with its lysyl moiety at the P₁' position, is 20 times more selective for the C domain than the N domain (8). However, as enalaprilat, which lacks the lysyl group, also

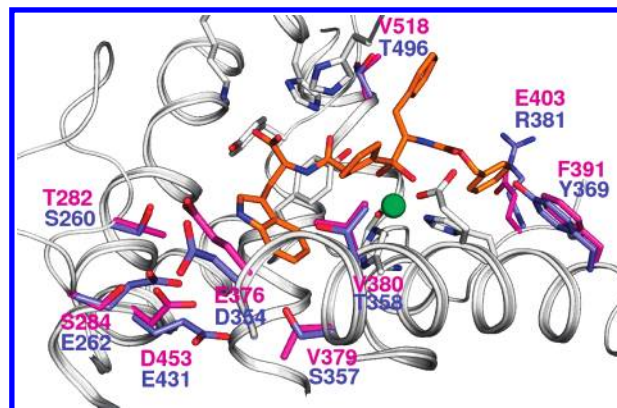


FIGURE 3: Superposition of the tACE active site with the N domain. Residues unique to tACE/C domain are shown as pink sticks, and the equivalent residues from the N domain are superimposed as blue sticks. The label colors match the residue colors, pink and blue for tACE and the N domain, respectively.

has less affinity for the N domain, the contribution of this moiety is hard to quantify.

More relevant, perhaps, is the increased hydrophobicity of the S₂' pocket of the C domain, where it has been noted that bulky P₂' residues, such as tryptophan, confer C domain selectivity (36, 37). Although the Trp moiety utilizes this space, there is still more space in this subsite for a larger hydrophobic moiety extending toward Asp 453 (tACE numbering). This extra space in the C domain, compared to the N domain, is due to the larger Glu 431 in the N domain. The other key difference, at the nonprime end of the binding site, is the charge of the S₂ pocket. The incorporation of the bulky phenyl group at the P₂ position contributes to the selectivity of RXPA380 for the C domain due to the presumed repulsion of this moiety by the larger Tyr 369 in the N domain. However, the addition of a positive charge to the P₂ group may increase selectivity by ionic interactions with this pocket in the C domain (Glu 403, tACE numbering) and, similarly, repel the positive charge in the N domain (Arg 381) (Figure 3). Indeed, the carboxylate group at this position in the N domain-specific inhibitor RXP407 is known to be one of the determinants of its specificity.

Another consideration, given the evidence for entropic binding of captopril, lisinopril, and enalaprilat to the active site, is the ability of new inhibitors to displace the water in the active site. With a clearer understanding of the conserved water structure of the active site, inhibitors could be designed to displace extra waters at known sites. For example, a more bulky P₂' residue may displace some of the conserved waters in the extensive S₂' subsite, whereas an additional positive charge on the P₂ residue may favor interactions with Asp 358 at the expense of another conserved water (Figure 2D).

The necessity for drugs to meet many criteria such as suitable solubility and bioavailability makes it hard to predict which inhibitors will make good drugs in vivo. An effective drug development strategy, where there are existing drugs for a specific target, is the subtle modification of known inhibitors in order to maintain drug-like properties. Thus, it has been shown that even minor changes in ACE inhibitor structures can lead to 1 or 2 orders of magnitude difference in inhibition of N/C domain selectivity in vitro (36, 37). The future of domain-specific ACE inhibitors lies in finding those compounds that best utilize the chemical differences between

the domains while maintaining strong inhibition and drug-like properties.

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REFERENCES

- Wong, J., Patel, R. A., and Kowey, P. R. (2004) The clinical use of angiotensin-converting enzyme inhibitors, *Prog. Cardiovasc. Dis.* 47, 116–130.
- Soubrier, F., Alhenc-Gelas, F., Hubert, C., Allegrini, J., John, M., Tregear, G., and Corvol, P. (1988) Two putative active centers in human angiotensin I-converting enzyme revealed by molecular cloning, *Proc. Natl. Acad. Sci. U.S.A.* 85, 9386–9390.
- Hagaman, J. R., Moyer, J. S., Bachman, E. S., Sibony, M., Magyar, P. L., Welch, J. E., Smithies, O., Krege, J. H., and O'Brien, D. A. (1998) Angiotensin-converting enzyme and male fertility, *Proc. Natl. Acad. Sci. U.S.A.* 95, 2552–2557.
- Ehlers, M. R. W., Fox, E. A., Strydom, D. J., and Riordan, J. F. (1989) Molecular cloning of human testicular angiotensin-converting enzyme: the testis isozyme is identical to the C-terminal half of endothelial angiotensin-converting enzyme, *Proc. Natl. Acad. Sci. U.S.A.* 86, 7741–7745.
- Acharya, K. R., Sturrock, E. D., Riordan, J. F., and Ehlers, M. R. W. (2003) Ace revisited: A new target for structure-based drug design, *Nat. Rev. Drug Discovery* 2, 891–902.
- Natesh, R., Schwager, S. L. U., Sturrock, E. D., and Acharya, K. R. (2003) Crystal structure of the human angiotensin-converting enzyme-lisinopril complex, *Nature* 421, 551–554.
- Turner, A. J., and Hooper, N. M. (2002) The angiotensin-converting enzyme gene family: genomics and pharmacology, *Trends Pharmacol. Sci.* 23, 177–183.
- Wei, L., Clauser, E., Alhenc-Gelas, F., and Corvol, P. (1992) The two homologous domains of human angiotensin I-converting enzyme interact differently with competitive inhibitors, *J. Biol. Chem.* 267, 13389–13405.
- Deddish, P. A., Marcic, B., Jackman, H. L., Wang, H. Z., Skidgel, R. A., and Erdos, E. G. (1998) N-domain-specific substrate and C-domain inhibitors of angiotensin-converting enzyme: angiotensin-(1–7) and keto-ACE, *Hypertension* 31, 912–917.
- Rousseau, A., Michaud, A., Chauvet, M. T., Lenfant, M., and Corvol, P. (1995) The hemoregulatory peptide N-acetyl-Ser-Asp-Lys-Pro is a natural and specific substrate of the N-terminal active site of human angiotensin-converting enzyme, *J. Biol. Chem.* 270, 3656–3661.
- Jaspard, E., Wei, L., and Alhenc-Gelas, F. (1993) Differences in the properties and enzymatic specificities of the two active sites of the two active sites of angiotensin I-converting enzyme (kininase II). Studies with bradykinin and other natural peptides, *J. Biol. Chem.* 268, 9496–9503.
- Azizi, M., Rousseau, A., Ezan, E., Guyene, T. T., Michelet, S., Grognet, J. M., Lenfant, M., Corvol, P., and Menard, J. (1996) Acute angiotensin-converting enzyme inhibition increases the plasma level of the natural stem cell regulator N-acetyl-seryl-aspartyl-lysyl-proline, *J. Clin. Invest.* 97, 839–844.
- Fuchs, S., Xiao, H. D., Cole, J. M., Adams, J. W., Frenzel, K., Michaud, A., Zhao, H., Keshelava, G., Capecchi, M. R., Corvol, P., and Bernstein, K. E. (2004) Role of the N-terminal catalytic domain of ACE investigated by targeted inactivation in mice, *J. Biol. Chem.* 279, 15946–15953.
- Dickstein, K., and Kjekshus, J. (2002) Effects of losartan and captopril on mortality and morbidity in high-risk patients after acute myocardial infarction: the OPTIMAAL randomised trial, *Lancet* 360, 752–760.
- Nussberger, J., Cugno, M., Amstutz, C., Cicardi, M., Pellacani, A., and Agostoni, A. (1998) Plasma bradykinin in angio-oedema, *Lancet* 351, 1693–1697.
- Beltrami, L., Zingale, L. C., Carugo, S., and Cicardi, M. (2006) Angiotensin-converting enzyme inhibitor-related angioedema: how to deal with it, *Expert Opin. Drug Saf.* 5, 643–649.
- Ehlers, M. R. (2006) Safety issues associated with the use of angiotensin-converting enzyme inhibitors, *Expert Opin. Drug Saf.* 5, 739–740.
- Georgiadis, D., Beau, F., Czarny, B., Cotton, J., Yiotakis, A., and Dive, V. (2003) Roles of the two active sites of somatic angiotensin-converting enzyme in the cleavage of angiotensin I and bradykinin: insights from selective inhibitors, *Circ. Res.* 93, 148–154.
- Redelinghuys, P., Nchinda, A. T., and Sturrock, E. D. (2005) Development of domain-selective angiotensin I-converting enzyme inhibitors, *Ann. N.Y. Acad. Sci.* 1056, 160–175.
- Dive, V., Georgiadis, D., Matziari, M., Makaritis, A., Beau, F., Cuniassé, P., and Yiotakis, A. (2004) Phosphinic peptides as zinc metalloproteinase inhibitors, *Cell Mol. Life Sci.* 61, 2010–2019.
- Dive, V., Cotton, J., Yiotakis, A., Michaud, A., Vassiliou, S., Jiracek, J., Vazeux, G., Chauvet, M. T., Cuniassé, P., and Corvol, P. (1999) RXP 407, a phosphinic peptide, is a potent inhibitor of angiotensin I converting enzyme able to differentiate between its two active sites, *Proc. Natl. Acad. Sci. U.S.A.* 96, 4330–4335.
- Junot, C., Gonzales, M. F., Ezan, E., Cotton, J., Vazeux, G., Michaud, A., Azizi, M., Vassiliou, S., Yiotakis, A., Corvol, P., and Dive, V. (2001) RXP 407, a selective inhibitor of the N-domain of angiotensin I-converting enzyme, blocks in vivo the degradation of hemoregulatory peptide acetyl-Ser-Asp-Lys-Pro with no effect on angiotensin I hydrolysis, *J. Pharmacol. Exp. Ther.* 297, 606–611.
- Gordon, K., Redelinghuys, P., Schwager, S. L. U., Ehlers, M. R. W., Papageogiou, A. C., Natesh, R., Acharya, K. R., and Sturrock, E. D. (2003) Deglycosylation, processing and crystallization of human testis angiotensin-converting enzyme, *Biochem. J.* 371, 437–442.
- Friedland, J., and Silverstein, E. (1976) A sensitive fluorimetric assay for serum angiotensin-converting enzyme, *Am. J. Clin. Pathol.* 66, 416–424.
- Georgiadis, D., Cuniassé, P., Cotton, J., Yiotakis, A., and Dive, V. (2004) Structural determinants of RXP380, a potent and highly selective inhibitor of angiotensin-converting enzyme C-domain, *Biochemistry* 43, 8048–8054.
- Otwinowski, Z., and Minor, W. (1997) Processing of X-ray diffraction data collected in oscillation mode, *Methods Enzymol.* 276, 307–326.
- CCP4 (1994) The CCP4 suite: Programs for protein crystallography, *Acta Crystallogr. D* 50, 760–763.
- Murshudov, G. N. (1997) Refinement of macromolecular structures by the maximum-likelihood method, *Acta Crystallogr. D* 53, 240–255.
- Brünger, A. T. (1992) Free R value: a novel statistical quantity for assessing the accuracy of crystal structures, *Nature* 355, 472–475.
- Emsley, P., and Cowtan, K. (2004) Coot: Model building tools for molecular graphics, *Acta Crystallogr.* 60, 2126–2132.
- Natesh, R., Schwager, S. L. U., Evans, H. R., Sturrock, E. D., and Acharya, K. R. (2004) Structural details on the binding of antihypertensive drugs captopril and enalaprilat to human testicular angiotensin I-converting enzyme, *Biochemistry* 43, 8718–8724.
- Watermeyer, J. M., Sewell, B. T., Schwager, S. L., Natesh, R., Corradi, H. R., Acharya, K. R., and Sturrock, E. D. (2006) Structure of testis ACE glycosylation mutants and evidence for conserved domain movement, *Biochemistry* 45, 12654–12663.
- Towler, P., Staker, B., Prasad, S. G., Menon, S., Tang, J., Parsons, T., Ryan, D., Fisher, M., Williams, D., Dales, N. A., Patane, M. A., and Pantoliano, M. W. (2004) ACE2 structures reveal a large hinge-bending motion important for inhibitor binding and catalysis, *J. Biol. Chem.* 279, 17996–18007.
- McDonald, I. K., and Thornton, J. M. (1994) Satisfying hydrogen bonding potential in proteins, *J. Mol. Biol.* 238, 777–793.
- Andújar-Sánchez, M., Cámara-Artigas, A., and Jara-Pérez, V. (2004) A calorimetric study of the binding of lisinopril, enalaprilat and captopril to angiotensin-converting enzyme, *Biophys. Chem.* 111, 183–189.
- Nchinda, A. T., Chibale, K., Redelinghuys, P., and Sturrock, E. D. (2006) Synthesis and molecular modeling of a lisinopril-tryptophan analogue inhibitor of angiotensin I-converting enzyme, *Bioorg. Med. Chem. Lett.* 16, 4616–4619.
- Nchinda, A. T., Chibale, K., Redelinghuys, P., and Sturrock, E. D. (2006) Synthesis of novel keto-ACE analogues as domain-selective angiotensin I-converting enzyme inhibitors, *Bioorg. Med. Chem. Lett.* 16, 4612–4615.