Novel mechanism of inhibition of human angiotensin-I-converting enzyme (ACE) by a highly specific phosphinic tripeptide

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INTRODUCTION

Membrane-bound zinc metallopeptidases such as ACE (angiotensin-I-converting enzyme, EC 3.4.15.1), NEP (neutral endopeptidase, also known as neprilysin, EC 3.4.24.11) and ECE-1 (endothelin-converting enzyme-1, EC 3.4.24.71) exert vasoconstrictive and vasodilatory activities [via Ang-II (angiotensin-II), and ET-1 (endothelin-1), and natriuretic peptides and BK (bradykinin) respectively] and are well-established targets for the treatment of cardiovascular disease [1–6]. Despite the success of ACE inhibitor therapy in the treatment of hypertensive patients, it has been established that chronic use of these inhibitors causes undesirable side effects, such as persistent dry cough, loss of taste and angiooedema, which are most likely due to elevated levels of BK [7,8]. Moreover, ACE inhibition can induce an increase in chymase activity in cardiac interstitial fluid, providing an alternative pathway for the formation of Ang-II [9]. Hence, blood pressure control remains suboptimal in a significant proportion of patients on this therapy, as well as those on other antihypertensive drug treatments [10]. This provides a compelling case for the development of new improved strategies for the treatment of patients with high blood pressure, a major risk factor for cardiovascular complications. To address this concern, potent vasopeptidase inhibitors able to target ACE and NEP were first developed [11,12], followed by dual NEP/ECE-1 inhibitors and ultimately triple inhibitors blocking ACE/NEP/ECE-1 simultaneously [13]. Even though the dual ACE/NEP inhibitor omapatrilat was more clinically efficacious than a single ACE inhibitor, the higher incidence of angiooedema observed in patients treated with omapatrilat halted the development of omapatrilat and raised concerns about the risk/benefit ratio of dual ACE/NEP inhibitors for therapeutic applications [14]. Although the incidence of angiooedema associated with triple ACE/NEP/ECE-1 inhibitor treatment is yet to be evaluated, it has been suggested that NEP inhibition (in the context of either dual or triple inhibitor treatment) might be responsible for the increase in adverse effects [13]. Indeed it has been established that inhibition of NEP results in increased levels of BK and ET-1. These data initiated the development of novel inhibitors that interact with the C-domain of ACE (C-ACE) and ECE-1 [15] (Figure 1). The discovery of the first potent and selective dual C-ACE/ECE-1 inhibitors revealed that the configuration of the P1′ residue was a key factor in the control of inhibitor selectivity (Figure 1). Instead of the classical S configuration in the P1′ position of the inhibitor (corresponding to an l amino acid, as observed in all ACE inhibitors reported to date) (compound FI in Figure 1), we discovered that an R configuration in compounds (compound FII in Figure 1) containing long and bulky P1′ side chains was well accommodated by ACE, as well as by ECE-1, but much less so by NEP (for details see [15]). Furthermore, in a spontaneous hypertensive rat model, an intravenous administration of a C-ACE/ECE-1 dual inhibitor (FII)
Table 1  Chemical structures of ACE/ECE-1 dual inhibitors FI and FII and their potency

| Inhibitor | ACE C-domain | ACE N-domain | NEP | ECE-1 |
|-----------|--------------|--------------|-----|-------|
| FI        | 0.41±0.03    | 180±25       | 172±24 | 275±60 |
| FII       | 0.65±0.03    | 150±20       | 6700±300 | 14±2  |

Figure 1  Chemical structures of ACE/ECE-1 dual inhibitors FI and FII and their potency

Compound FI adopts the S configuration and compound FII adopts the R configuration.

(10 mg/kg of body weight) lowered the mean arterial blood pressure by 24±2 mmHg (1 mmHg = 0.133 kPa) as compared with controls [15].

In humans there are two ACE isoforms: somatic ACE, which comprises two homologous enzymatic domains (N- and C- with ~60% amino acid sequence identity) [16] and testis ACE, which is a single domain protein identical with the C-domain of somatic ACE [17]. Although both cleave angiotensin-I, it has been shown that C-ACE is sufficient to maintain the regulation of blood pressure in vivo [18] and hence viewed as the dominant site of Ang-II generation. On the other hand the N-domain (N-ACE) contributes to the regulation of haemopoietic stem cell differentiation and proliferation through its hydrolysis of the anti-fibrotic haemoregulatory peptide AcSDKP (AZ-acetyl-seryl-aspartyl-lysyl-proline, a biological substrate of ACE) [19,20]. In addition, these domains have their own distinctive physiochemical properties, such as thermostability [21], resistance to proteolysis [22], chloride-ion dependence [23,24] and substrate preference [19,25,26]. Subtle differences in the crystal structures of the apo and bound forms of the two domains have been exploited for the development of domain-selective ACE inhibitors [27–33].

In order to gain structural insight into the dual ACE/ECE-1 inhibitor (FII) binding to ACE we have determined the crystal structure of FII in complex with C-ACE and N-ACE at high resolution. In the present paper we describe the novel and unexpected binding features of a highly specific and unusual dual inhibitor FII.

EXPERIMENTAL

Synthesis of the phosphinic tripeptide (FII) [(2S)-(3-[ hydroxyl(2-phenyl-[1R]-1-[(benzylxoy)carbonyl]-amino)ethyl]-phosphinyl]-2-[3-phenylisoxazol-5-ylmethyl]-1-oxo-propyl] amino)-3-(4-hydroxy-phenyl) propanoic acid) was performed as described by Jullien et al. [15]. Potency of inhibitors towards C-ACE and N-ACE, as well as potency towards NEP and ECE-1, were determined as described by Jullien et al. [15,34]. \( K_i \) values were determined at 25°C in 50 mM Hepes (pH 6.8), 200 mM NaCl, 10 \( \mu \)M ZnCl\_2 and 0.02 % Brij-35.

Protein purification and crystallization of ACE/ECE-1 dual inhibitor (FII) complex

C-ACE

A variant of human testis ACE (tACE\_A36-g13, underglycosylated protein) was purified to homogeneity from CHO (Chinese-hamster ovary) cells, as described previously [35]. The crystals of the C-ACE complex with the dual inhibitor were grown at 16°C by the hanging-drop vapour diffusion method. C-ACE protein (11.5 mg/ml in 50 mM Hepes, pH 7.5) was pre-incubated with FII (1 mM) on ice for 5 h before crystallization. The pre-incubated sample (2 \( \mu \)l) was mixed with the reservoir solution consisting of 13.5 % PEG [poly(ethylene glycol)] 4000, 50 mM sodium acetate (pH 4.7) and 10 \( \mu \)M ZnSO\_4, and suspended above the well. Diffraction quality co-crystals appeared after approximately 1 week.

N-ACE

The minimally glycosylated construct of the N-domain of somatic ACE, N-ACE389, was generated by site-directed mutagenesis, as described previously [33]. The recombinant protein was expressed in CHO cells and purified to homogeneity by lisinopril affinity chromatography. The crystals of N-ACE in complex with FII were grown at 16°C by the hanging-drop method. An initial hit was obtained in the presence of 0.06 M divalent cations, 0.1 M Tris/Bicine (pH 8.5) and 30 % PEG 550MME/PEG 20000, and was refined using the Hampton Research silver bullets additive screen. Briefly, the N-ACE protein (4 mg/ml) was pre-incubated with FII (at a molar ratio of 1:5) on ice before crystallization, 2 \( \mu \)l of pre-incubated sample was mixed with 1 \( \mu \)l of reservoir solution and 1 \( \mu \)l of G3 of silver bullet condition No 3 and suspended above the well. Diffraction quality crystals appeared after approximately 1 week.
RESULTS AND DISCUSSION

Overall structures

For the crystal structure determination an underglycosylated form of C-ACE was used that only had two of the potential seven N-linked glycans. Although it is possible that introducing mutations to disrupt glycosylation sites may affect the structure of a protein, previous work has shown that enzymatically deglycosylated C-ACE, as well as various glycosylation mutants of N- and C-ACE, have comparable enzymatic activities to the WT (wild-type) forms [33,35,46]. These findings imply that the crystal structures of minimally glycosylated N- and C-ACE are likely to be highly informative of the WT structures. Furthermore, aligning the X-ray structures of minimally glycosylated N- and C-ACE to the structures of their WT forms revealed RMSDs (root mean square deviations) for all atoms of 0.70 Å (1 Å = 0.1 nm) and 0.51 Å for the N- and C-ACE comparisons respectively. Thus the minimally glycosylated structures of the N- and C-domains are essentially identical with their glycosylated counterparts, and mutation of the glycan sequons does not seem to affect the structure or function of the enzyme.

The C-ACE structure in complex with FII (one molecule per asymmetric unit) was refined to 1.97 Å resolution. No noticeable conformational change was observed in the structure upon inhibitor binding (RMSDs of 0.38 and 0.51 Å for Cα atoms and all atoms respectively). This is consistent with the previously determined structures of C-ACE inhibitor complexes [27,28,30–32]. The overall protein structure (Figure 2A) contains a Zn$^{2+}$ ion, two Cl$^{-}$ ions, two molecules of FII (for details see below), one Hepes molecule (from the crystallization medium), partially visible N-linked glycans at two potential glycosylation sites (Asn72 and Asn109) and 563 water molecules (Table 1).

The N-ACE structure in complex with FII (two molecules per asymmetric unit) was refined to 2.15 Å resolution. As in the case of previously determined N-ACE complexes [29,33], no noticeable conformational change was observed in the structure upon inhibitor binding (RMSDs of 0.56 and 0.70 Å for Cα atoms and all atoms respectively). This co-ordinate and parameter files for FII were generated using the PRODRG server [42]. Structure validation was conducted with the aid of programs PROCHECK [43] and MOLPROBITY [44]. There were no residues in the disallowed region of the Ramachandran plot. Figures 2 and 3 were drawn with PyMOL (http://www.pymol.org). Hydrogen bonds were verified with the program HBPLUS [45]. Details of crystallographic data are given in Table 1.

Figure 2 Overall topology of C-ACE and N-ACE molecules

(A) A cartoon representation of the C-ACE structure with bound dual-inhibitor molecules at sites A and B (FII-A and FII-B) and the observed N-glycosylated sugars (green sticks) at the N-glycosylation sites (Asn$^{72}$ and Asn$^{109}$). The bound inhibitor molecules at the two sites, FII-A and FII-B, are shown in stick representation, coloured in yellow and cyan respectively. The active-site Zn$^{2+}$ ion and the location of the two Cl$^{-}$ ions are shown in green and skyblue spheres respectively. (B) A cartoon representation of N-ACE in complex with the dual inhibitor. The bound dual inhibitor (FII) and the N-glycosylated sugars are shown as yellow and green sticks respectively. The Zn$^{2+}$ ion and the bound Cl$^{-}$ ion are also shown and coloured as in C-ACE.

Data collection and structure determination

Two datasets were collected on stations IO3 and IO4 at Diamond Light Source. No cryoprotectant was used to keep the crystal at constant temperature (100K) under the liquid nitrogen jet during data collection. Raw data images were indexed and scaled with the HKL2000 software package [36]. Data reduction was carried out using the CCP4 program TRUNCATE [37]. The structures were solved by molecular replacement using PHASER [38] with the aid of the native C-ACE (PDB code 1O86) [27] and N-ACE (PDB code 2C6F) [29] structures as search models. The resultant structures were refined for stereochemically restrained positional and temperature factors using REFMAC5 [39]. In total 5% of reflections were separated as the $R_{	ext{free}}$ set and used for cross validation [40]. Manual adjustments of the models were carried out using COOT [41]. Water molecules were added at positions where $F_o - F_c$ electron density peaks exceeded 3σ and potential hydrogen bonds could be made. On the basis of clear electron density interpretation, two FII molecules in the C-ACE complex structure and one FII molecule in the N-ACE structure were added and further refinement was carried out. The co-ordinate and parameter files for FII were generated using the PRODRG server [42]. Structure validation was conducted with the aid of programs PROCHECK [43] and MOLPROBITY [44]. There were no residues in the disallowed region of the Ramachandran plot. Figures 2 and 3 were drawn with PyMOL (http://www.pymol.org). Hydrogen bonds were verified with the program HBPLUS [45]. Details of crystallographic data are given in Table 1.
molecule of FII (for details see below) each monomer, five PEG molecules of different lengths (from the crystallization medium), partially visible N-linked glycans at three potential glycosylation sites (Asn^{45}, Asn^{416} and Asn^{480}) and a total of 663 water molecules (Table 1).

Novel inhibitor-binding mode

C-ACE–FII complex

Two bound inhibitor molecules were identified unambiguously in the C-ACE active site in close proximity to one another,
Table 1 Crystallographic statistics for the ACE/ECE-1 dual inhibitor FII complex

| Statistic                  | C-ACE   | N-ACE   |
|----------------------------|---------|---------|
| Resolution (Å)             | 1.97    | 2.15    |
| Space group                | P2(1)2(1) | P1     |
| Number of molecules/ASU    | 1       | 2       |
| Cell dimensions            | a:b:c   | 58.7, 86.1, 134.8 Å | 89.1, 64.4, 75.8 Å |
| α,β,γ                       | 90, 90, 90° | 78.8, 76.4, 82.9° |
| Total number of observations| 526714  | 718177  |
| Number of unique reflections| 49449  | 84896   |
| Completeness (%)           | 97.8 (93.4) | 97.0 (95.0) |
| F(obs)/F(expected)         | 15.6 (3.3) | 14.1 (2.4) |
| Rmerge, Rwork**            | 7.4 (31.4) | 7.3 (48.1) |
| Rmerge, Rwork‡             | 19.8/23.2 | 21.9/25.5 |
| Deviation from ideality    | 0.007   | 0.008   |
| Bond lengths (Å)           | 1.204   | 1.16    |
| Bond angles (°)            | 20.8    | 33.7/93.2 |
| B-factor analysis (Å²)     | 29.2    | 33.1    |
| Protein atoms (chain A/B)  | 17.4    | 32.3/34.1 |
| Solvent atoms              | 14.5    | 25.1/23.6 |
| Inhibitor atoms            | 47.4    | 58.6/71.5 |
| Glycosylated sugars        | 64      | 64      |
| Heps atoms                 | 14.5    | 23.8/31.6 |
| CI⁻ ions (2) (chain A/B)   | 57.0/55.8 | 57.0/55.8 |
| PEG atoms (chain A/B)      | 0.007   | 0.008   |

* Rmerge = Σ||I(h)−<I(h)>||/ΣI(h), where I(h) is the measured intensity and <I(h)> is the weighted mean of all the measurements of I(h).
†Rwork = Σ||Fo−Fc||/ΣFo, where Fo and Fc are observed and calculated structure factor amplitudes of reflection h respectively.
‡Rwork is equal to Rmerge for a randomly selected 5% subset of reflections.

by inspection of the Fourier difference electron density map (Figures 3A and 3C). One molecule (hereafter called FII-A, the primary site) occupies the expected S1, S2, S1′ and S2′ subsites of the C-ACE active site, whereas the unexpected second molecule (hereafter called FII-B, the secondary site) forms intermolecular aromatic (pi–pi) interactions with FII-A through a ‘hand-shake’ conformation and extends towards the non-primed region of the active site involving some 46 contacts (Figure 3D).

FII-A binding

The phosphoryl zinc-binding group of FII-A interacts with the catalytic Zn²⁺ ion through its two oxygen atoms (distances of 2.04 Å and 2.74 Å respectively). However, the accommodation of the unusual P1′ side chain with the R configuration of FII-A in the active site of C-ACE has resulted in a significantly different positioning of the inhibitor. This can be seen by comparing the superimposition of the binding modes of lisinopril (a well-known clinically used ACE inhibitor with Kᵢ values of 44 and 2.4 nM for N-ACE and C-ACE of human somatic ACE respectively [23,27]), RXPA380 [a C-ACE-specific phosphinic inhibitor with a Kᵢ value of 3 nM (2500 nM for N-ACE)] [30,47] and FII-A (Figure 3F).

Despite different chemical structures and the zinc-binding groups, the backbone of lisinopril and RXPA380 inhibitors, as well as the orientation of their respective side chains, superimpose well [30] (Figure 3F). In contrast, comparing the binding mode of FII-A to either that of lisinopril or RXPA380 reveals a different picture. There is a marked difference in the positioning of FII-A in the C-ACE active site and, more specifically, the positioning of its phosphoryl zinc-binding group and the orientation of the inhibitor molecule at the P₁, P₁′ and P₂′ positions. In the P₁′ position, the nitrogen atom of the isoxazole ring is held by a water-mediated interaction with Asp³¹⁵. The oxygen atom of the isoxazole ring appears to make a direct interaction with the active-site residue His⁸³⁶. Surprisingly, the bulky side chain of FII at the P₁′ position points in an unusual orientation and extends towards the S₁′ pocket. This particular orientation is probably stabilized by hydrophobic interactions provided by Val³⁸⁰ and the P₁′ aromatic ring of the inhibitor molecule (Figure 3F), but it is also rendered possible by the unique topology of the S₁′ and S₂′ subsites of ACE, which form a single and very large cavity, sufficiently wide enough to accept the bulky P₁′ side chain in the S₁ or R configuration (Figure 3G). In the S₁′ configuration, the P₁′ side chain would probably assume an orientation similar to that observed for the lysine residue in lisinopril. In addition to these points, several polar and van der Waals interactions observed between FII and ACE, as compared with more ‘classical ACE inhibitors’ (see below), are probable reasons why inhibitors FII and FII exhibit similar potency towards ACE.

The C-terminal tyrosine side chain at the P₁′ position of the inhibitor is held by hydrophobic interactions mediated through aromatic residues Phe⁵²⁷ and Phe⁵⁷³, an orientation slightly different to that adopted by the tryptophan side chain of RXPA380 (Figure 3F). Furthermore, the hydroxy group of the tyrosine moiety of the inhibitor is anchored via water-mediated hydrogen bonds with Asp³¹⁵, His³⁸³ and Lys³⁸⁴. The phenyl side chain at the P₁ position is held by hydrophobic interactions provided by the side chains of Phe⁵¹² and Val⁵¹⁸. Finally, the phenyl group at the P₂ position of the dual inhibitor makes aromatic contact with Phe³⁸⁰, as observed for RXPA380 [30]. Despite this unusual binding mode, FII-A is involved in strong interactions with the protein atoms: 16 hydrogen bonds, which include seven water-mediated hydrogen bonds, as calculated by HBPLUS (Figure 3A and Table 2); and several atoms of FII-A are in close proximity to ten protein side chain (Ser³⁵⁵, Glu⁷⁷⁶, Val⁷⁷⁹, Val⁸⁰¹, Phe⁹¹⁰, His¹⁴⁰, Phe³⁵⁷, Phe¹³², Val¹⁵⁸ and Phe³⁵²), most of them making extensive van der Waals contacts. This network of interactions provides an excellent structural basis for the sub-nanomolar affinity of this dual inhibitor towards ACE.

FII-B binding

For the first time, an inhibitor molecule is observed at a secondary binding site (FII-B) in the ACE structure. The inhibitor is held by nine water-mediated hydrogen bonds and two direct interactions with Asn⁵⁰⁸ and Arg⁴⁷³, as calculated by HBPLUS (Table 2). In addition, Glu¹⁴³, Glu¹⁴⁶ and Arg¹⁴² also mediate direct interactions with the inhibitor (Figure 3C). The isoxazole phenyl of FII-B makes strong aromatic (pi–pi) stacking interactions with the amino benzyol group of inhibitor FII-A at the primary site (Figure 3D). The P₁ phenyl group of the dual inhibitor participates in an aromatic interaction with Trp³¹⁰ of the protein molecule. The isoxazole phenyl group of FII-B makes an aromatic interaction with Trp³ⁱ⁰ and the tyrosine group at the P₁′ position of FII-B appears to make a hydrophobic interaction with Tyr⁵². The phosphinic oxygen atoms appear to make direct interactions with Glu¹³² and Glu⁴⁰⁳, and a weak interaction with Lys¹¹⁸. In the native C-ACE structure, and in all previously studied C-ACE inhibitor-bound complexes [27,28,30,31], this binding site of FII-B is occupied by a string of bound water molecules.

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N-ACE–FII complex

One bound inhibitor molecule per monomer of FII was found in the primary site (FII-A, occupying all four substrate binding sites: S2, S1, S1′ and S2′) and was identified unambiguously in the active site of N-ACE (Figures 3B and 3E). No secondary FII-binding site was observed. As in the case of the C-ACE–FII complex (Figure 3B and 3E), the binding pocket retains the full complement of conserved hydrogen-bond interactions in the N-ACE active site (described above), the binding pocket is held by hydrophobic interactions mediated through His388 and of conserved hydrogen-bond interactions in the N-ACE active site (Figure 3I). These observations provide a structural basis for the lower specificity of FII for N-ACE as compared with C-ACE.

Conclusion

The S1′ pocket of ACE can accommodate a variety of P1′ residues including alanine, histidine, phenylalanine and lysine with its more extended conformation. However, our structural data did not reveal any direct interactions between the bulky bicyclic P1′ group of FII-A and the S1′ pocket but, unexpectedly, this side chain was oriented more towards the S2′ pocket, rationalizing the accommodation of a bulky P1′ group in the R configuration by the active site. It should be noted that an S configuration of the P1′ residue was reported to be an absolute requirement in all clinical ACE inhibitors [15]. As shown in the present study, the accommodation of an R configuration not only concerns the orientation of the phenyl-isoxazole side chain, but also implies a striking shift in positioning of FII-A in the C-ACE and N-ACE active site, compared with the binding modes of other known ACE inhibitors. Despite this particular binding mode, FII-A has been shown to be engaged in multiple polar and non-polar interactions, an observation that expands the current ‘dogma’ regarding the chemical space defined by the ACE active sites.

In addition, the unexpected observation of the binding of the dual inhibitor at the secondary site of C-ACE, FII-B, (involving displacement of conserved water molecules), provides further leads to be explored with regard to subtle modifications of the chemical space at the active site. It also demonstrates the active-site plasticity in accommodating potent inhibitors. Thus the present study for the first time has provided a wealth of fundamental knowledge about ACE/ECE-1 dual-inhibitor (FII) specificity which will no doubt prove useful in understanding the selectivity profiles of different vasoactive hormones.

Table 2 Comparison of hydrogen-bond interactions with the dual inhibitor FII in C-ACE and N-ACE complexes

| Domain | Site | Inhibitor atoms | Interacting protein atoms | Distance (Å) |
|--------|------|-----------------|--------------------------|-------------|
| C-ACE  | Primary (FII-A) | O | G281NE2 | 3.02 |
|        | OAC  | H353NE2 | 2.61 |
|        | OAB  | A336H | 2.67 |
|        | OAG  | H383NE2 | 3.15 |
|        | OAG  | H387NE2 | 3.06 |
|        | O    | K511NZ | 2.69 |
|        | OAC  | H513NE2 | 2.83 |
|        | O    | Y520OH | 2.59 |
|        | OAD  | Y530H | 2.59 |
|        | NBI  | Wat | 3.10 |
|        | OXT  | Wat | 2.69 |
|        | O    | Wat | 3.45 |
|        | OH   | Wat | 2.80 |
|        | OH   | Wat | 2.76 |
|        | OMB  | Wat | 3.36 |
|        | NBG  | Wat | 2.81 |
|        | OH   | Wat | 2.86 |
|        | O    | Wat | 2.54 |
|        | OH   | Wat | 2.72 |
|        | OMB  | Wat | 3.22 |
|        | NBG  | Wat | 2.66 |
|        | O    | G259NE2 | 3.00 |
|        | OAC  | H351NE2 | 2.64 |
|        | OAB  | A334N | 2.76 |
|        | NBG  | T358DG1 | 3.45 |
|        | OAG  | H365NE2 | 2.90 |
|        | O    | K489NZ | 2.62 |
|        | OAC  | H491NE2 | 2.98 |
|        | O    | Y490OH | 2.58 |
|        | OAD  | Y501OH | 2.53 |
|        | N   | Y501OH | 2.53 |
|        | NBI  | Wat | 2.99 |
|        | OXT  | Wat | 2.67 |
|        | OH   | Wat | 2.44 |

*Additional contacts identified for the second molecule in the asymmetric unit at the primary-binding site.

However, a structure-based alignment of residues from the two complexes revealed some significant differences in residues at the periphery of this primary site (Figure 3H).

A comparison of the N-ACE active site with the FII-B-binding site of C-ACE clearly showed the presence of several key residues occluding the binding of FII. Specifically, Leu132, Ser135, Val16, Trp365, and Arg381 in the N-ACE active site are replaced by Trp59, Tyr62, Ala63, Met221, and Glu401 in the C-ACE respectively (Figure 3I). The S1′ pocket of C-ACE is occupied by a larger side chain, compared with N-ACE, which may explain the lower specificity of FII for N-ACE as compared with C-ACE.

AUTHOR CONTRIBUTION

Sylva Schwager and Colin Anthony performed the protein production and purification. Bertrand Czarny and Fabrice Beau synthesized phosphinic peptide inhibitors and performed biochemical assays. Mohd Akif performed the crystallization experiments. Mohd Akif and Ravi Acharya conducted the structure determination. Mohd Akif, Vincent Dive, Edward Sturrock and Ravi Acharya designed the research, analysed the data and wrote the manuscript. All authors helped with editing the manuscript before submission.

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