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A calorimetric study of the binding of lisinopril, enalaprilat and captopril to angiotensin-converting enzyme

M. Andújar-Sánchez, A. Cámara-Artigas, V. Jara-Pérez*

Departamento Química Física, Bioquímica y Química Inorgánica, Universidad de Almería, Carretera Sacramento s/n, Almería 04120, Spain

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Abstract

The angiotensin I-converting enzyme (ACE; EC.3.4.15.1) is a dipeptidyl carboxypeptidase that plays a central role in blood pressure regulation. The somatic form of the enzyme is composed of two highly similar domains, usually referred to as N and C domains, each containing one active site. Nevertheless, a 1:1 stoichiometry for the binding of lisinopril, captopril or enalaprilat to somatic pig lung ACE is shown by isothermal titration calorimetry (ITC) and enzymatic assays. The binding of the three inhibitors at neutral pH is very tight and the enthalpy changes are positive, indicating that the binding is entropically driven. The origin of this thermodynamic signature is discussed under the new structural information available.

Keywords: Angiotensin I-converting enzyme; Inhibitors; Isothermal titration calorimetry; Enthalpy; Stoichiometry; Binding

1. Introduction

The angiotensin-converting enzyme (ACE; EC 3.4.15.1) is a type I membrane anchored dipeptidyl carboxypeptidase that plays an important role in blood pressure regulation, converting the inactive decapeptide angiotensin I to the potent vasopressor octapeptide angiotensin II by the removal of the C-terminal dipeptide His–Leu [1]. ACE control of blood pressure also takes place degrading the nonapeptide bradykinin, a blood vessel dilatator that reduces blood pressure [2]. ACE has been under extensive study for many years in order to develop antihypertensive drugs [3,4].

In mammals, there are two forms of ACE: a larger one, usually referred to as somatic ACE, which is composed of approximately 1300 amino acids and which is present in most tissues [3], and a smaller one referred to as testicular ACE (t-ACE) with only 730 amino acids and which is found exclusively in the testicles and appears to be involved in male fertility [5]. Somatic ACE is composed of two highly similar domains with an overall 60% homology in both nucleotide and amino acid sequence, known as N and C domains according to their position in the N-terminal or C-terminal half of the single chain protein [6,7]. On the other hand, testicular ACE contains a single domain that shows high identity to the C-terminal domain of the somatic ACE [8–10]. Both domains contain a canonical Zn-binding sequence motif, HExxH (His–Glu–x–x–His), and both domains have protease, dipeptidyl carboxypeptidase and endopeptidase activities [11]. In order to study the function of both catalytic sites, experiments with mutants that had one domain suppressed by deletion or one active site inactivated by point mutations have been performed [12]. These experiments have shown that the C-terminal domain is more important for blood pressure regulation. The C-terminal domain has a higher catalytic constant for angiotensin I and the nonphysiological substrate hippuryl–histidyl–leucine. The N-terminal domain shows a higher catalytic constant for other substrates such as the luteinizing-hormone releasing hormone and *N*-Ac-Ser-Asp-Lys-Proline (AcSDKP), which is a negative regulatory factor of hematopoietic stem cell differentiation and proliferation [13]. Therefore, the physiological functions of ACE are not limited to its cardiovascular role, and ACE may also be involved in hematopoietic stem cell regulation by constantly degrading AcSDKP [14]. The two domains of somatic ACE also show differences in stability to thermal denaturation.

* Corresponding author. Tel.: +34-950015623; fax: +34-950015008.
E-mail address: vjara@ual.es (V. Jara-Pérez).

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Differential scanning calorimetry of the enzyme revealed two distinct thermal transitions with melting points at 55.3 and 70.5 °C, which correspond to the denaturation of C- and N-domains, respectively [15]. Also, both domains show differing sensitivity to proteolysis, with the C-terminal domain more sensitive to degradation with proteases [16].

Recently, the crystal structures of the human testicular ACE (t-ACE) [17] and *Drosophila* AnCE (D-AnCE) [18] have been solved at 2.0 and 2.6 Å resolutions, respectively. D-AnCE contains a single ACE domain similar to that of t-ACE. Moreover, lisinopril-ACE structures have been solved for both sources, and the captopril-ACE structure is available from *Drosophila* AnCE. The knowledge of the interaction of these potent inhibitors of ACE at molecular level will provide useful information for the design of new drugs to combat high blood pressure. However, the design of new drugs can also be enhanced by thermodynamic data showing the nature of the binding [19]. Isothermal titration calorimetry (ITC) has been widely used to obtain thermodynamic information about the binding process in biological macromolecules [20], and it has been proposed as an important tool to understand the correlation between structure and thermodynamic parameters [21,22]. ITC studies have been conducted with bovine lung ACE [23,24] and the inhibitors lisinopril and captopril. In both cases, the enthalpy of binding is positive and the binding process is entropically driven. The basis of the unfavorable enthalpy change is unclear as both inhibitors show extensive polar interactions upon the binding to t-ACE and D-AnCE.

In the present study, we report the ITC results on the binding of the three potent inhibitors to somatic ACE from pig lung. We analyze these results taking into account the new structural information provided by the t-ACE and D-AnCE structures to explain the thermodynamic behavior of the binding process.

2. Experimental setup

2.1. Materials

Concanavalin A-Sepharose and epoxy-activated Sepharose 6B were purchased from Amersham Biosciences. Lisinopril, captopril, HEPES, cacodylate, MES, ACES, Tris and PIPES were from Sigma. Enalaprilat was a gift from Merck. All other chemicals were of reagent grade and obtained locally. Lisinopril was coupled to epoxy-activated Sepharose 6B as described elsewhere [25].

Somatic ACE was purified from pig lung using two-step affinity chromatography with Concanavalin A-Sepharose followed by Lisinopril Sepharose 6B as described previously [26]. The purity of the protein was confirmed by SDS/PAGE. ACE concentrations were determined using a molar absorption coefficient of 2.18×10^5 M^{-1} cm^{-1} at 280 nm determined by the Gill and Von Hippel method [27] as described by Andujar et al. [26].

2.2. Enzyme activity studies

ACE activity was assayed using furanacryloyl-L-phenylalanylglycylglycine (FAFGG) as substrate [28]. The assays were performed in 50 mM HEPES, 0.3 M NaCl and 10 μM $ZnCl_2$ pH 7.5, at 25 °C. A total of 200 μl of 0.5 mM FAFGG was diluted in 770 μl of buffer, and the reaction was initiated by adding 30 μl of enzyme solution containing 1–3 μg of protein. Absorbance decrease at 334 nm was monitored and initial velocities were determined. One unit of activity is defined as the amount of ACE that produces a unit decrease in absorbance at 334 nm in 1 min. Purified somatic ACE from pig lung had a specific activity of 35–37 U/mg.

In order to study the stoichiometry of the inhibition, activity assays were performed under conditions of inhibitor and enzyme concentrations much higher than the binding constant. As ACE inhibition is strongly dependent on the presence of chloride ions [12], two different NaCl concentrations (20 and 300 mM NaCl) were used in the assays. Aliquots of the enzyme containing 1–3 μg of protein were incubated with different amounts of the inhibitors for 2 h before enzyme activity assay in order to ensure the complex formation. The partition ratio of the inhibitor/enzyme association was determined by plotting the fractional activity (velocity of the inhibited enzyme reaction/velocity of the uninhibited enzyme reaction) versus the ratio of inhibitor/enzyme ([I]/[E]). Linear regression analyses were performed to determine the intercept between the two straight lines that yields the stoichiometry of the inhibition.

2.3. Isothermal titration calorimetry

Isothermal calorimetric titrations were performed using an MCS Microcalorimeter from Microcal (Northampton, MA, USA) [29]. The reference cell was filled with water, and the instrument was calibrated using standard electrical pulses. A circulating water bath was used to stabilize the temperature. The instrument was allowed to equilibrate overnight. All solutions were thoroughly degassed by stirring under vacuum before use. Solutions of ACE were titrated with 10 identical 10-μl injections at 6-min intervals. The injection syringe, on which a paddle is mounted, stirred the solutions at 300 rpm, ensuring immediate mixing. Concentrations of ACE used for the titrations were in the range from 3.5 to 10.2 μM, while concentrations of the inhibitors were 0.33–0.51 mM for lisinopril, 0.15–0.46 mM for captopril and 0.15–0.54 mM for enalaprilat.

The peaks of the thermograms were integrated using an ORIGIN 5 software (Microcal) supplied with the instrument. In the titration experiment, total heat measured is the contribution of the intrinsic binding heat of the ligand to the protein together with the ligand dilution heat and the protonation heat. Dilution experiments were performed by

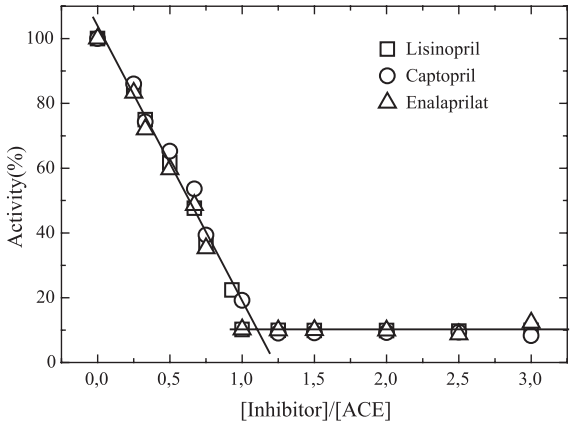

Fig. 1. Titration of active sites in pig lung angiotensin-converting enzyme using lisinopril, captopril and enalaprilat as competitive inhibitors. The samples were incubated with the inhibitor 2 h at 25 °C in 50 mM HEPES, 0.3 M NaCl and 10 μM $ZnCl_2$. Substrate used for activity measure was FAFGG.

identical injections of ACE inhibitor into the cell containing buffer alone. The thermal effect of protein dilution was negligible in all cases. The protonation heat arises from the fact that the binding of a ligand to a protein is usually accompanied by proton release or uptake. To determine the number of protons involved in the binding process, ITC experiments were carried out using three different buffers with different ionization enthalpies (HEPES, cacodylate and ACES) in the presence of 300 mM NaCl and 0.05 mM Zinc Acetate. The data were analyzed using the following equation:

$$\Delta H_{obs} = \Delta H_b + n\Delta H_{ioniz}$$

where n is the number of protons exchanged with the buffer media to form the complex, and ΔH_{ioniz} is the ionization enthalpy of the buffer.

Enzyme activity was checked before and after the calorimetric experiment. After titration, the ACE-inhibitor complex showed less than 1% activity in all the experiments. The pH values of the buffers, inhibitors and protein solutions were controlled before and after titration.

2.4. Structure superposition and ligand–protein contacts

Coordinates from the Protein Data Bank (PDB) for human testicular ACE (1o8a), human testicular ACE-lisinopril complex (1o86), *Drosophila* AnCE (1j38), *Drosoph-*

Table 1
Binding enthalpies for lisinopril, captopril and enalaprilat

Inhibitor	Temperature (°C)	pH	Buffer	n	ΔH_T (kJ/mol)	ΔH_b[a] (kJ/mol)
Lisinopril	25	7	HEPES	1.06 ± 0.08	49.17 ± 1.89	26.90 ± 1.70
			Aces		60.19 ± 1.95	
			Cacodylate		24.82 ± 1.63	
	20		HEPES	1.05 ± 0.06	54.70 ± 1.25	32.90 ± 1.20
			Aces		66.02 ± 1.36	
			Cacodylate		31.25 ± 1.18	
	16		HEPES	1.08 ± 0.11	70.52 ± 1.36	48.30 ± 1.50
			Aces		82.48 ± 1.45	
			Cacodylate		46.95 ± 1.63	
Captopril	25	7	HEPES	0.42 ± 0.03	30.92 ± 1.20	22.10 ± 1.00
			Aces		35.29 ± 1.30	
			Cacodylate		21.28 ± 0.90	
	20		HEPES	0.40 ± 0.04	42.10 ± 1.65	33.80 ± 1.60
			Aces		46.42 ± 1.60	
			Cacodylate		33.17 ± 1.55	
	16		HEPES	0.38 ± 0.04	47.52 ± 1.25	39.70 ± 1.20
			Aces		51.73 ± 1.36	
			Cacodylate		39.23 ± 1.15	
Enalaprilat	25	7	HEPES	1.02 ± 0.07	44.23 ± 0.52	22.80 ± 0.60
			Aces		54.84 ± 0.75	
			Cacodylate		20.80 ± 0.63	
	20		HEPES	1.01 ± 0.10	53.77 ± 0.32	32.80 ± 0.50
			Aces		64.66 ± 0.85	
			Cacodylate		31.21 ± 0.22	
	16		HEPES	1.01 ± 0.09	67.88 ± 1.36	47.10 ± 1.50
			Aces		79.07 ± 1.56	
			Cacodylate		45.84 ± 1.52	
	16	6	Mes	1.83 ± 0.14	54.10 ± 1.36	25.95 ± 1.79
			Aces		83.61 ± 1.95	
	16	8	Tris	0.89 ± 0.08	92.75 ± 2.06	49.95 ± 1.74
			HEPES		68.22 ± 1.25	

[a] These values were obtained considering the ionization values for the buffers reported for HEPES (ΔH_{ion} = 21.01kJ/mol, $\Delta C_{p,ion}$ = 48.74J/Kmol), Cacodylate (ΔH_{ion} = − 1.96kJ/mol, $\Delta C_{p,ion}$ = − 78.49J/Kmol), Aces (ΔH_{ion} = 31.41kJ/mol, $\Delta C_{p,ion}$ = − 26.64J/Kmol), Mes (ΔH_{ion} = 15.53kJ/mol, $\Delta C_{p,ion}$ = 16.37J/Kmol), Tris (ΔH_{ion} = 47.44kJ/mol, $\Delta C_{p,ion}$ = − 72.78 J/Kmol).

ila AnCE-lisinopril complex (1j36) and *Drosophila* AnCE-captopril complex (1j36) were used. In order to determine changes in the structures as a result of the inhibitor binding, superposition and RMS deviations of the structures were performed using the CCP4 program LSQKAB [30]. Contacts between inhibitor and residues in the active site were calculated with the program CONTACT from CCP4 suite [31].

3. Results

3.1. Active sites titration

Active sites titration of pig lung ACE with lisinopril, enalaprilat and captopril under low and high chloride ion concentrations are shown at Fig. 1. Plots of residual activities expressed as percentage of activity in the absence of inhibitor versus the ratio inhibitor concentration over enzyme concentration [I]/[ACE] result in two linear portions. When the first linear portion is extrapolated to the *x*-axis, the intersection point takes place at an inhibitor/enzyme molar ratio of approximately 1. Thus, only 1 mol of inhibitor is required to abolish ACE FAFGG hydrolyzing activity.

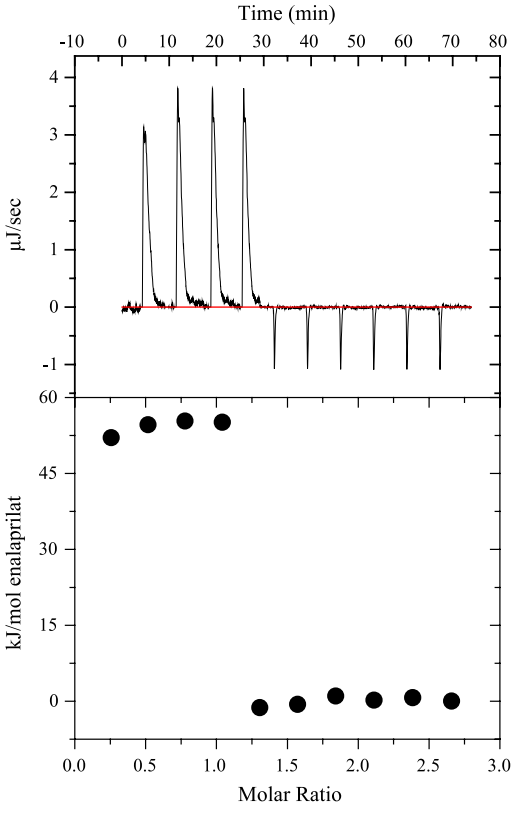

Fig. 2. Calorimetric titration of the binding of enalaprilat to pig lung angiotensin-converting enzyme in 50 mM HEPES, 0.3 M NaCl and 50 µM $Zn(CH_3COO)_2$ at pH 7 and 16 °C. The experiment consisted of 10 injections of 10 µl each of a 0.25-mM stock solution of enalaprilat. Enalaprilat was injected into a sample cell (volume = 1.38 ml) containing 7.06 µM of pig lung angiotensin-converting enzyme. Injections were at 6-min intervals. Similar experiments were performed with lisinopril and captopril.

Table 2
ΔC_p, ΔASA_{ap}, ΔASA_{pol} values for lisinopril, captopril and enalaprilat at pH 7

Inhibitor	ΔC_p (kJ/Kmol)	ΔASA_{ap} (Å^2)	ΔASA_{pol} (Å^2)
Lisinopril	− 2.4 ± 0.2	− 1795.3	− 912.2
Captopril	− 2.1 ± 0.2	− 1550.8	− 792.2
Enalaprilat	− 2.4 ± 0.2	− 1843.1	− 949.0

3.2. Isothermal titration calorimetry

ITC experiments were conducted at pH 7 and different temperatures to determine the thermodynamic parameters of the binding of the three potent ACE inhibitors lisinopril, captopril and enalaprilat (Table 1). A typical ITC profile for the binding of enalaprilat to pig lung ACE at pH 7 is shown in Fig. 2A. Fig. 2B includes a plot of the total heat evolved per mole of the inhibitor versus the relation inhibitor concentration over enzyme concentration. It shows that saturation of the enzyme corresponds to a stoichiometry of 1 mol of enalaprilat per mole of the enzyme. The same results were obtained for lisinopril and captopril.

As the binding process is very tight, ITC allows precise measuring of the enthalpy change of binding but not the binding constant. Binding enthalpies for the three inhibitors are shown in Table 1. ΔH_b decreases linearly with increasing temperature, and from the slope of the graphical representation of ΔH_b versus temperature, the values of heat capacity changes ΔC_p were determined. As Table 2 shows, the ΔC_p for the binding of lisinopril, captopril and enalaprilat to pig lung ACE are negative and large. Heat capacity changes involved in protein–ligand binding arise from changes in the degree of surface hydration in the free and complex molecules, and to a lesser extent, from changes in molecular vibrations [32,33]. In the association process of a ligand to a protein, a substantial fraction of polar and nonpolar surface is buried, and some semiempirical methods have been developed to calculate the molecular surface buried in the complex. The application of Murphy's approach [32] to the experimentally determined values (Table 2) indicates that the surface area buried on complex formation comprises 66% nonpolar surface and 34% polar surface for the three inhibitors.

4. Discussion

Our results show that the binding of one molecule of the inhibitor to one of the active sites prevents the hydrolysis of substrate from the second active site. The stoichiometry of the binding obtained by means of the activity inhibition experiments agrees with that obtained from the ITC experiments. This result is in agreement too with Binevski et al [34] that obtained a 1:1 stoichiometry for the binding of lisinopril to bovine ACE and suggests that both sites of bovine ACE

are active, but that binding of the substrate or lisinopril to one of the active sites prevents binding to the other site.

The positive value for the number of protons exchanged (Table 1) indicates that protein-inhibitor complex takes up protons upon binding. Moreover, some protein residues must show an increase in the pK_a value in order to become protonated upon binding. Several ionizable groups are present at the active site of ACE: His513 and His 353 at t-ACE (His497 and His337 at D-AnCE) are located close to the carbonyl group of lisinopril and captopril (Fig. 3), and they may be responsible for the proton uptake. In addition, mutation of the His1089 residue of human somatic ACE, corresponding to the His513 residue at t-ACE (His497 residue of D-AnCE), prevents binding of captopril and lisinopril to the enzyme [35].

Nevertheless, lisinopril and enalaprilat show a higher number of protons exchanged than captopril. This difference could be explained in the dissimilar interactions established between protein residues and inhibitors. So far, no structure is available for pig lung ACE but structures for t-ACE and D-AnCE have been recently reported [17,18] and residues directly involved in the Zinc and inhibitor binding are completely conserved in the known ACE sequences [18]. Fig. 3 shows contacts between lisinopril and t-ACE protein residues and captopril and D-AnCE protein residues. Captopril coordinates to Zinc ion through its thiol group, while lisinopril and enalaprilat are coordinated through a carboxylate group. At pH 7, lisinopril and enalaprilat carboxylate groups will be unprotonated whereas the captopril thiol group, with a pK_a = 9.8 [36], would be protonated. In order to interact with the zinc atom, the thiol group must lose its proton, so the difference in the number of protons exchanged between captopril and lisinopril or enalaprilat would arise from this ionizable group.

The three inhibitors show a positive enthalpy for the binding process at pH 7 and over the range of temperatures studied, so the binding process is entropically driven. When the dominant driving force for binding is a large positive entropy change, this originates primarily from a large positive solvation entropy due to the burial of a large hydrophobic surface upon binding and a small loss of conformational entropy due to the little flexibility of inhibitors preshaped to the geometry of the binding site [37]. ACE-inhibitor binding has a negative ΔC_p, so a reduction of solvent accessibility to nonpolar surfaces accompanies protein–ligand binding. In spite of the size differences between lisinopril, captopril and enalaprilat, the same percentage of polar and apolar surface area is buried upon binding, being the apolar surface value almost twice than for polar surface. These changes are bigger than those expected for a rigid body binding, and contributions other than the change in polar–apolar surface area upon binding must be considered. To date, all ACE structures available are homologous to the carboxyl-terminal domain of the somatic ACE, and superposition of the free inhibitor ACE structure with lisinopril/captopril-ACE complex structures doesn't show major

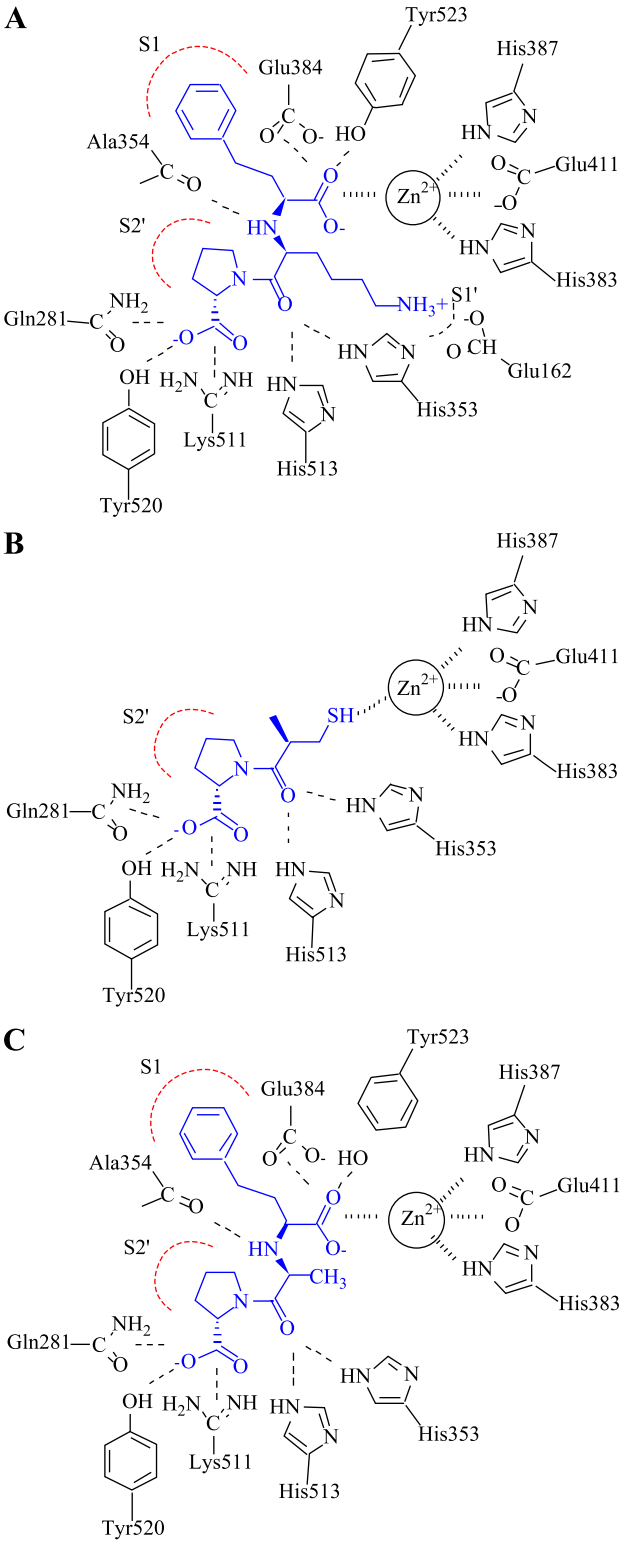

Fig. 3. Contacts between residues in the active site of ACE structures and lisinopril and captopril. (A) Contacts between ACE and lisinopril, (B) captopril and (C) enalaprilat. Amino acids at the active site show the t-ACE sequence's number. Captopril contacts at t-ACE were modelled from the D-AnCE-captopril structure. Enalaprilat was modelled either from t-ACE or D-AnCE-lisinopril structures.

changes (RMS displacement 0.152 Å, maximum displacement 1.138 Å), but molecular modelling of the amino-terminal domain [38] and some structural features (for example, the distal position of the chloride binding sites from the active site) indicate the possibility of conformational changes [17,18].

The three inhibitors reported in this study have a pseudo-peptide structure with a proline residue at the position P′ 2 (Fig. 3) that confers rigidity to the molecule. This proline residue is also present in the natural substrate of ACE, angiotensin I, and it has been suggested that the preference of proline at position P′ 2 of the peptide may reside in its rigid conformation that allows the carboxy terminus to be placed in a favorable alignment for the interaction with a positively charged amino acid of the active site of the enzyme [39]. In t-ACE structure, Lys511, Gln281 and Tyr520 (Lys495, Gln265 and Tyr504 at D-AnCE) are forming salt bridge and hydrogen bond interactions with the proline carboxy terminus of the inhibitor. It has been proven that these interactions play a central role in the binding of peptidic substrates to ACE2, a novel human zinc metalloprotease homologous to ACE [40] that is unable to bind ACE inhibitors, such as lisinopril, captopril and enalaprilat. As can be seen at Fig. 3, lisinopril shows several additional polar interactions as compared to captopril: Glu162–Lysil moiety, Glu384 and Tyr523 with the carboxyl of lisinopril, which coordinates the Zinc ion. The first is lost too in enalaprilat but the Glu384 and Tyr523 interactions are expected to be conserved in the enalaprilat-ACE complex. These enthalpic favorable interactions could play a role in the specificity of the binding process, but their contribution to the energetics of the binding is overcome by the hydrophobic contribution to the binding as shown by the positive value of the enthalpies that is approximately the same for the three inhibitors. This thermodynamic behavior, high binding constants values but positive enthalpies, has been previously reported for the HIV-1 protease binding to most of its inhibitors currently in clinical use and to its natural substrate [41,42] and has been attributed to the predominantly hydrophobic nature of the binding.

ACE is a metalloproteinase that contains zinc at the active site, and it is the prime example of mechanism-based design of inhibitors taking into account the role of the metal at the catalysis [43]. In t-ACE structure (PDB code 1o8a), zinc is coordinated with an acetate ion while in D-AnCE structure (PDB code 1j38), it is coordinated to two water molecules. An entropy gain can be expected when these ions or water molecules are released from the zinc site upon inhibitor binding. In lisinopril and enalaprilat, the carboxylic group is involved in zinc chelation. These two carboxylic oxygens make direct coordination to zinc with penta-coordinated geometry that appears to mimic the nucleophilic water and the carboxyl oxygen of the substrate in the tetrahedral intermediates during enzymatic reaction [18]. Furthermore, an increase in ligand field stabilization energy is expected when these inhibitors are bound to ACE by means of the relaxation of a low symmetry centre to one of high symmetry [44], and this factor may play an important role in the exceptionally tight binding of these inhibitors [45]. It has been proven that the removal of the zinc atom from ACE results in a huge decrease of its affinity for enalaprilat and other ACE inhibitors [12].

5. Conclusions

The results presented here show a tight binding entropically driven of lisinopril, enalaprilat and captopril to pig lung ACE. Comparing the three inhibitor structures, an important role of the proline moiety at the binding process is proposed. No conformational changes can be discarded from the values of the heat capacity changes and the changes of accessible surface areas calculated from them. Moreover, the stoichiometry value of 1:1 for the complete inhibition of the enzyme indicates that both active sites would work in a cooperative manner.

Acknowledgements

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References

[1] L.T. Skeggs, J.R. Kahn, N.P. Shumway, Preparation and function of the hypertension converting enzyme, J. Exp. Med. 103 (1956) 295–299.
[2] M.R. Ehlers, J.F. Riordan, Angiotensin-converting enzyme: new concepts concerning its biological role, Biochemistry 28 (1989) 5311–5318.
[3] A.A. Patchett, E.H. Cordes, The design and properties of *N*-carboxyalkyldipeptide inhibitors of angiotensin-converting enzyme, Adv. Enzymol. Relat. Areas Mol. Biol. 57 (1985) 1–84.
[4] M.A. Ondetti, From peptides to peptidases: a chronicle of drug discovery, Annu. Rev. Pharmacol. 34 (1994) 1–16.
[5] J.R. Hagaman, J.S. Moyer, E.S. Bachman, M. Sibony, P.L. Magyar, J.E. Welch, O. Smithies, J.H. Krege, D.A. O'Brien, Angiotensin-converting enzyme and male fertility, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 2552–2557.
[6] F. Soubrier, F. Alhenc-Gelas, C. Hubert, J. Allegrini, M. John, G. Tregear, P. Corvol, Two putative active centers in human angiotensin I-converting enzyme revealed by molecular cloning, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 9386–9390.
[7] P. Corvol, T.A. Williams, in: A.J. Barrett, N.D. Rawlings, J.F. Woessner (Eds.), Handbook of Proteolytic Enzymes, Academic Press, New York, 1998, pp. 1066–1076.
[8] R.S. Kumar, J. Kusari, S.N. Roy, R.L. Soffer, G.C. Sen, Structure of testicular angiotensin-converting enzyme. A segmental mosaic enzyme, J. Biol. Chem. 264 (1989) 16754–16758.
[9] A.L. Lattion, F. Soubrier, J. Allegrini, C. Hubert, P. Corvol, F. Alhenc-Gelas, The testicular transcript of the angiotensin I-converting enzyme encodes for the ancestral, non-duplicated form of the enzyme, FEBS Lett. 252 (1989) 99–104.

[10] M.R.W. Ehlers, E.A. Fox, D.J. Strydom, J.F. Riordan, The testis isozyme is identical to the C-terminal half of endothelial angiotensin-converting enzyme, Proc. Natl. Acad. Sci. U. S. A. 86 (1989) 7741–7745.
[11] L. Wei, F. Alhenc-Gelas, P. Corvol, E. Clauser, The two homologous domains of human angiotensin I-converting enzyme are both catalytically active, J. Biol. Chem. 266 (1991) 9002–9008.
[12] L. Wei, E. Clauser, F. Alhenc-Gelas, P. Corvol, The two homologous domains of human angiotensin I-converting enzyme interact differently with competitive inhibitors, J. Biol. Chem. 267 (19) (1992) 13398–13405.
[13] M. Lenfant, J. Wdzieczak-Bakala, E. Guittet, J.-C. Prome, D. Sotty, E. Frindel, Inhibitor of hematopoietic pluripotent stem cell proliferation: purification and determination of its structure, Proc. Natl. Acad. Sci. U. S. A. 86 (1989) 779–782.
[14] M. Azizi, A. Rousseau, E. Ezan, T.-T. Guyene, S. Michelet, J.M. Grognet, M. Lenfant, P. Corvol, J. Menard, 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 (1996) 839–844.
[15] S. Voronov, N. Zueva, V. Orlov, A. Urutyunyan, O. Kost, Temperature-induced selective death of the C-domain within angiotensin-converting enzyme molecule, FEBS Lett. 522 (2002) 77–82.
[16] E.D. Sturrock, S.M. Danilov, J.F. Riordan, Limited proteolysis of human kidney angiotensin-converting enzyme and generation of catalytically active N - and C-terminal domains, Biochem. Biophys. Res. Commun. 236 (1997) 16–19.
[17] R. Natesh, S.L.U. Schwager, E.D. Sturrock, K.R. Acharya, Crystal structure of the human angiotensin-converting enzyme-lisinopril complex, Nature 421 (2003) 551–554.
[18] H.M. Kim, D.R. Shin, O.J. Yoo, H. Lee, J.O. Lee, Crystal structure of *Drosophila* angiotensin I-converting enzyme bound to captopril and lisinopril, FEBS Lett. 538 (2003) 65–70.
[19] P.C. Weber, F.R. Salemme, Applications of calorimetric methods to drug discovery and the study of protein interactions, Curr. Opin. Struck. Biol. 13 (2003) 115–121.
[20] S. Leavitt, E. Freire, Direct measurement of protein binding energetics by isothermal titration calorimetry, Curr. Opin. Struck. Biol. 11 (2001) 560–566.
[21] J.E. Ladbury, Isothermal titration calorimetry: application to structure-based drug design, Thermochim. Acta 380 (2001) 209–215.
[22] A. Velazquez-Campoy, I. Luque, E. Freire, The application of thermodynamic methods in drug design, Thermochim. Acta 380 (2001) 217–227.
[23] E. Ortiz-Salmerón, C. Barón, L. García-Fuentes, Enthalpy of captopril-angiotensin I-converting enzyme binding, FEBS Lett. 435 (1998) 219–224.
[24] R. Téllez-Sanz, L. García-Fuentes, C. Barón, Calorimetric analysis of lisinopril binding to angiotensin I-converting enzyme, FEBS Lett. 423 (1998) 75–80.
[25] H.G. Bull, N.A. Thornberry, E.H. Cordes, Purification of angiotensin-converting enzyme from rabbit lung and human plasma by affinity chromatography, J. Biol. Chem. 260 (1985) 2963–2972.
[26] M. Andújar-Sánchez, A. Camara-Artigas, V. Jara Perez, Purification of angiotensin I converting enzyme from pig lung using concanavalin-A sepharose chromatography, J. Chromatogr., B, Biomed. Sci. Appl. 783 (2003) 247–252.
[27] S.C. Gill, P.H. von Hippel, Calculation of protein extinction coefficients from amino acid sequence data, Anal. Biochem. 182 (1989) 319–326.
[28] B. Holmquist, P. Bünning, J.F. Riordan, A continuous spectrophotometric assay for angiotensin converting enzyme, Anal. Biochem. 95 (1979) 540–548.
[29] T. Wiseman, S. Williston, J.F. Brandts, L.-N. Lin, Rapid measurement of binding constants and heats of binding using a new titration calorimeter, Anal. Biochem. 179 (1989) 131–137.
[30] W. Kabsch, A solution for the best rotation to relate two sets of vectors, Acta Crystallogr., A 32 (1976) 922–923.
[31] Collaborative Computational Project, Number 4, The *CCP*4 suite: programs for protein crystallography, Acta Crystallogr., D Biol. Crystallogr. 50 (1994) 760–763.
[32] K.P. Murphy, E. Freire, Thermodynamics of structural stability and cooperative folding behavior in proteins, Adv. Protein Chem. 43 (1992) 313–361.
[33] R.S. Spolar, J.R. Livingstone, M.T. Record Jr., Use of liquid hydrocarbon and amide transfer data to estimate contributions to thermodynamic functions of protein folding from the removal of nonpolar and polar surface from water, Biochemistry 31 (16) (1992) 3947–3955.
[34] P.V. Binevski, E.A. Sizova, V.F. Pozdnev, O.A. Kost, Evidence for the negative cooperativity of the two active sites within bovine somatic angiotensin-converting enzyme, FEBS Lett. 550 (2003) 84–88.
[35] M. Fernandez, X. Liu, M.A. Wouters, S. Heyberger, A. Husain, Angiotensin I-converting enzyme transition state stabilization by HIS1089: evidence for a catalytic mechanism distinct from other gluzincin metalloproteinases, J. Biol. Chem. 276 (2001) 4998–5004.
[36] G.K. McEvoy (Ed.), Captopril, AHFS-96 Drug Information, American Society of Health-System Pharmacists, Bethesda, MD, 1996.
[37] A. Velazquez-Campoy, I. Luque, M.J. Todd, M. Milutinovich, Y. Kiso, E. Freire, Thermodynamic dissection of the binding energetics of KNI-272, a potent HIV-1 protease inhibitor, Protein Sci. 9 (2000) 1801–1809.
[38] J.H. Fernandez, M.A.F. Hayashi, A.C.M. Camargo, G. Neshich, Structural basis of the lisinopril-binding specificity in N- and C-domains of human somatic ACE, Biochem. Biophys. Res. Commun. 308 (2003) 219–226.
[39] A. Michaud, M.T. Chauvet, P. Corvol, N-domain selectivity of angiotensin I-converting enzyme as assessed by structure–function studies of its highly selective substrate, *N*-acetyl-seryl-aspartyl-lysyl-proline, Biochem. Pharmacol. 57 (1999) 611–618.
[40] J.L. Guy, R.M. Jackson, K.R. Acharya, E.D. Sturrock, N.M. Hooper, A.J. Turner, Angiotensin-converting enzyme-2 (ACE2): comparative modeling of the active site, specificity requirements, and chloride dependence, Biochemistry 18 (2003) 13185–13192.
[41] A. Velazquez-Campoy, M.J. Todd, E. Freire, HIV-1 protease inhibitors: enthalpic versus entropic optimization of the binding affinity, Biochemistry 39 (2000) 2201–2207.
[42] I. Luque, E. Freire, Structure-based prediction of binding affinities and molecular design of peptide ligands, Methods Enzymol. 295 (1998) 100–127.
[43] D. Leung, G. Abbenante, D.P. Fairlie, Protease inhibitors: current status and future prospects, J. Med. Chem. 43 (2000) 305–341.
[44] B.L. Vallee, R.J. Williams, Metalloenzymes: the entatic nature of their active sites, Proc. Natl. Acad. Sci. U. S. A. 59 (2) (1968) 498–505.
[45] R. Bicknell, B. Holmquist, F.S. Lee, M.T. Martin, J.F. Riordan, Electronic spectroscopy of cobalt angiotensin converting enzyme and its inhibitor complexes, Biochemistry 26 (23) (1987) 7291–7297.