# Enthalpy of captopril-angiotensin I-converting enzyme binding

Emilia Ortiz-Salmerón[a], Carmen Barón[a,b], Luis García-Fuentes[a,b,*]

[a] *Departamento de Química Física, Bioquímica y Química Inorgánica, Facultad de Ciencias Experimentales, Universidad de Almería, La Cañada de San Urbano, 04120 Almería, Spain*
[b] *Instituto de Biotecnologia de Granada, Granada, Spain*

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**Abstract High-sensitivity titration calorimetry is used to measure changes in enthalpy, heat capacity and protonation for the binding of captopril to the angiotensin I-converting enzyme (ACE; EC 3.4.15.1). The affinity of ACE to captopril is high and changes slightly with the pH, because the number of protons linked to binding is low. The determination of the enthalpy change at different pH values suggests that the protonated group in the captopril-ACE complex exhibits a heat protonation of approximately −30 kJ/mol. This value agrees with the protonation of an imidazole group. The residues which may become protonated in the complex could be two histidines existing in two active sites, which are joined to the amino acids coordinated to $Zn^{2+}$. Calorimetric measurements indicate that captopril binds to two sites in the monomer of ACE, this binding being enthalpically unfavorable and being dominated by a large positive entropy change. Thus, binding is favored by both electrostatic and hydrophobic interactions. The temperature dependence of the free energy of binding $\Delta G°$ is weak because of the enthalpy-entropy compensation caused by a large heat capacity change, $\Delta C_p = -4.3 \pm 0.1$ kJ/K/mol of monomeric ACE. The strong favorable binding entropy and the negative $\Delta C_p$ indicate both a large contribution to binding due to hydrophobic effects, which seem to originate from dehydration of the ligand-protein interface, and slight conformational changes in the vicinity of the active sites.**



*Key words:* Angiotensin I-converting enzyme; Captopril; Microcalorimetry; Binding

## 1. Introduction

Angiotensin I-converting enzyme (ACE; EC 3.4.15.1) plays an important role in blood pressure regulation. It is a dipeptidyl carboxypeptidase which converts angiotensin I into the potent vasopressor peptide angiotensin II and inactivates the vasodepressor peptide bradykinin [1]. ACE is an unusual zinc-metallopeptidase in that it is activated by chloride and lacks a narrow in vitro substrate specificity [2].

Inhibition of ACE is a widely used approach in the treatment of hypertension. The first available competitive inhibitors of ACE were the naturally occurring peptides in snake venom. Clinical studies using the nonapeptide teprotide, the most efficient of these snake venom peptides in vivo, demonstrated the potential of ACE inhibitors as antihypertensive drugs. A lot of highly potent inhibitors of ACE which can be taken orally have subsequently been developed during the past two decades [3,4].

The molecular cloning and sequencing of the complementary DNA for human ACE revealed that ACE (somatic isozyme of ACE) is a glycoprotein that consists of a single polypeptide chain containing two homologous domains called the N and C domains, each bearing a potential catalytic site [5–7]. ACE is found as a membrane-bound enzyme via its hydrophobic C-terminal segment and also as a circulating molecule in plasma [8]. The latter seems to be secreted from the former by the action of an unknown protease and shows enzymatic activity [9] indicating that the C-terminal region of the native enzyme is not needed for the catalytic activity [10–12]. In vitro, a soluble active form of it can be obtained from the lung enzyme by limited proteolytic cleavage [11,13].

The three-dimensional structure of ACE is still unknown, but the crystal structures of other zinc metalloproteases have been elucidated. Although carboxypeptidase A and ACE are quite distinct structurally, both enzymes are zinc-containing exopeptidases with some similar properties. These facts suggested that the nature of the catalytic process, and therefore of the active sites, was similar in both cases. Thus, taking into account the analogy among the catalytic sites of carboxypeptidase A, thermolysin and ACE, potent competitive inhibitors of ACE have been designed [4]. The first of these, captopril (D-[3-mercapto-2-methylpropanoyl]-L-proline), was designed with the help of a theoretical model of the active site of ACE, which was based on its presumed similarity to the known active site of carboxypeptidase and also with reference to the C-terminal sequences of the venom peptides which complete with substrates [5–17].

In order to determine and quantify the forces driving association of captopril and ACE, we have studied the energetic of binding of the inhibitor to the enzyme as both a function of temperature and pH using isothermal titration calorimetry (ITC). The characterization of the binding was carried out over the pH range 6–8 in a series of buffer solutions with different heats of ionization, in order to have an estimate of the high-affinity equilibrium binding constant for the ACE/captopril interaction [18]. Those experiments allow us also to calculate the number of protons linked to ligand binding.

In the present study, the calorimetric titration of captopril to ACE was examined at four temperatures within the range 16–30°C. At pH 7, the enthalpy changes are positive within the range of temperature studied and decrease linearly with the temperature, given a large negative value of $\Delta C_p$. The formation of complex between captopril and the enzyme is entropically driven. The results obtained are discussed in terms of the possible interaction between the inhibitor and the enzyme.

*Corresponding author. Fax: (34) (50) 215008.
E-mail: igarcia@ualm.es

*Abbreviations:* ACE, angiotensin I-converting enzyme; Cacodylate, cacodylic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; Aces, 2-[(2-amino-2-oxoethyl)amino]ethanesulfonic acid; FAPGG, 2-furanacryloyl-L-phenylalanyl glycyl-glycine; ITC, isothermal titration calorimetry; Captopril, D-[3-mercapto-2-methylpropanoyl]-L-proline


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## 2. Materials and methods

### 2.1. Chemicals

Cacodylate, MES, Aces, Tris, HEPES and FAPGG were purchased from Sigma. $ZnCl_2$ was from Merck. Sephacryl S-300 HR was purchased from Pharmacia. Centripred 30 concentrators were from Amicon. All other chemicals were reagent grade of the highest purity available.

### 2.2. Enzyme

ACE was prepared from bovine lung by the method described by García-Fuentes et al. [13]. A soluble angiotensin I-converting enzyme form was obtained from purified membrane-bound enzyme, using trypsin treatment and separated by Sephacryl S-300 HR chromatography [13]. The trypsin solubilizes the protein by deletion of a fragment of 10 kDa, approximately. Thus, the molecular mass of the monomer was taken as 160 kDa [13]. The enzyme showed a single-band pattern in polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and showed no detectable trypsin activity.

Solutions of ACE were concentrated on Centriprep 30 concentrators and prepared by dialysis of the enzyme against several changes of 0.3 M NaCl, 50 μM $ZnCl_2$, 50 mM buffer A (cacodylate, MES and Aces at pH 6; cacodylate and HEPES at pH 7; HEPES and Tris at pH 8), and 4°C. Protein concentration was determined from absorbance measurements at 280 nm using the bovine-lung-enzyme absorbance coefficient $2.1\times10^5$ M/cm [19]. The activity of lung ACE was determined by the spectrophotometric method of Holmquist et al. [20] at 25°C, with 2-furanacryloyl-L-phenylalanylglycylglycine (FAPGG) as substrate. Reaction mixtures contained 100 μM FAPGG in 50 mM buffer A, 0.3 M NaCl, 10 μM $ZnCl_2$ and 5–12 nM ACE at pH 7.5. Absorbance measurements at 334 nm were carried out in a Beckman DU-7400 spectrophotometer with the cells maintained at 25°C. One unit of activity is defined as the amount of ACE that produces a $\Delta A_{334}$/min of 1.0. The specific activity of purified enzyme was 24–26 min/mg.

All solutions were degassed and clarified through a 0.45-μm Millipore filter immediately before use.

### 2.3. Calorimetric experiments

ITC experiments were performed in a calorimeter built in our laboratory, interfaced with a personal computer using an A/D converter board (Data Translation DT-2805) for automatic instrument control and data collection. The characteristics of this instrument are similar to other calorimeters, such as those described by McKinnon et al. [21] and Freire et al. [22]. A circulating water bath was used to help stabilize the experimental temperature. The titration experiments were carried out as described elsewhere [23,24]. The instrument was calibrated by (i) measuring the area under a test electrical heat pulse and comparing it with the heat input and (ii) measuring the heat of a standard chemical reaction. For the latter, the heat of protonation of glycine by HCl was measured [25]. The two calibration methods were in excellent agreement. Test runs were repeated periodically to ensure continued accuracy in the performance of the calorimeter. The calorimeter includes an electrical compensation circuit for a rapid return to base-line level after each injection. To measure the dilution heat of captopril in the absence of ACE, control experiments were performed in which the protein solution in the sample cell was replaced by the dialysate buffer. The dilution heats in the presence of the ACE-captopril complex were estimated from the enthalpies for injections of captopril measured after all ACE binding sites had been saturated. The dilution heats in the presence of complex obtained from the last peaks were used to correct the binding enthalpy for the heat of dilution.

The concentration of the solutions employed in the calorimetric experiments were in the range 20–50 μM of ACE. The thermal effect of the protein dilution was negligible in all cases. The activity of the enzyme was routinely checked just before and after the calorimetric experiment. The ACE-captopril complex from calorimetric experiment showed less than 1% activity.

Similarly, the pH values of the buffers, captopril, and protein solutions were controlled at each temperature before and after the binding reaction.

### 2.4. Relationship between protonation and pH dependence of ligand-binding affinity

The number of protons linked to a ligand-binding reaction of a macromolecule, $n_{H^+}$, can be determined indirectly from the measured values of the ligand-binding equilibrium constant, $K$, vs. pH, according to the relationship

$$\frac{\partial \log K}{\partial \mathrm{pH}} = n_{H^+} \quad (1)$$

where temperature, pressure, and the thermodynamic activities of all solution components other than the protons are held constant. Carrying out integration of Eq. 1 between two pH values leads to

$$\log K^{\mathrm{pH}_2} = \log K^{\mathrm{pH}_1} - \int_{\mathrm{pH}_1}^{\mathrm{pH}_2} n_{H^+}\, d\mathrm{pH} \quad (2)$$

This equation indicates that we can determine the ligand binding equilibrium at a given pH by two independent measurements, represented by the two terms on the right-hand side of Eq. 2: (i) determination of the binding equilibrium constant under convenient pH conditions, and (ii) integrated $n_{H^+}$ vs. pH data.

## 3. Results and discussion

### 3.1. FA-Phe-Gly-Gly hydrolysis: buffer and pH dependence

The variation of $\log(v_o/[E])$ for FAPGG hydrolysis as a function of pH is shown over the pH range from 6 to 9 (Fig. 1) at different buffer solutions. Throughout this range, the hydrolysis of substrate follows first-order kinetics. The assays were carried out in 0.3 M chloride, which is the optimal concentration where the initial velocity is maximal. The pH-rate profile exhibits an optimum centered at pH 7.3 and falls off when the pH is different from this value. This profile is characterized by the ionization of two groups on the free enzyme. Thus, for the buffers examined and within the pH range studied, there is no effect of buffer concentration in the rate of FAPGG hydrolysis.

### 3.2. Stoichiometry and affinity of ACE-captopril binding

Fig. 2 shows a sample data set for the calorimetric titration of monomeric ACE with captopril in buffer HEPES at pH 7 and 16°C. For the titration of ACE there are 9 consecutive equivalent 15-μl injections (spaced at 4-min intervals) of a captopril solution into the enzyme solution. The positive sign of the measured heat indicates that the enthalpy change

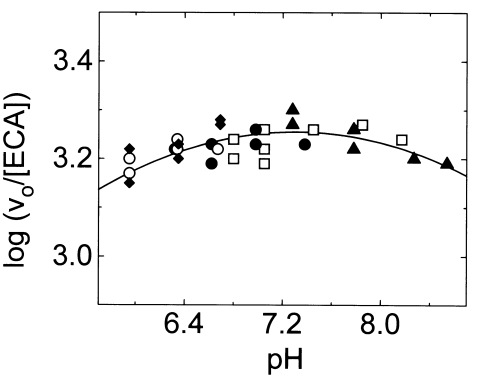


Fig. 1. pH dependence of the bovine lung angiotensin I-converting enzyme catalyzed hydrolysis of 100 μM of FAPGG determined at 25°C in 0.3 M sodium chloride in either 50 mM HEPES (□), Aces (●), Tris (▲), MES (○) or cacodylate (♦) buffers. The solid line describes a theoretical curve for the pH dependence of a diprotic system with apparent p$K$ values of 6.4 and 8.2.

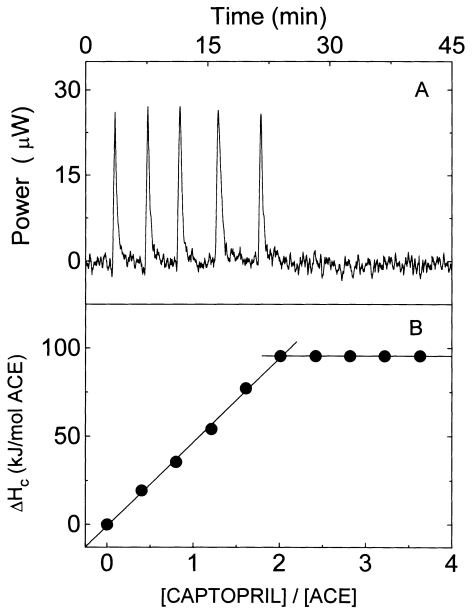


Fig. 2. Calorimetry titration of the binding of captopril to the bovine lung angiotensin I-converting enzyme at pH 7 and 16°C. A: The programmed sequence consisted of 9 injections of 15 μl each of 1.2 mM captopril stock solution. The captopril and buffer solution were injected into a sample cell containing 2.9 ml of 15.4 μM monomeric ACE and a reference cell containing 2.9 ml of buffer (0.3 M NaCl, 50 μM $ZnCl_2$, 50 mM HEPES). The data shown in A are the differences between the sample and reference cells, and in B the cumulative enthalpy, $\Delta H_c$, as a function of molar ratio of captopril to ACE monomer.

for each injection was positive and that the process of binding under these conditions was endothermic. Control experiments were made as described in Section 2 and represent the dilution heat of captopril and the heat effects from non-chemical reaction sources, which have not been compensated with dual injection into the sample and reference cells. The fact that no signals are observed from the sixth injection to the end of the titration in Fig. 2A shows that saturation has been reached in the first five injections. Fig. 2B shows how the total heat increases linearly with the ligand concentration until saturation is reached at a stoichiometry of 2 mol of captopril per mol of ACE monomeric. The heat absorbed in the first five injections of captopril is seen to be identical, indicative of tight binding. This result agrees with previous works, which have demonstrated that captopril binding to ACE is tight [26–28]. At pH 7 the intrinsic binding constant estimated by using a two site independent and non-interacting model is $>10^8$ $M^{-1}$, which is the upper limit of detectability for the instrument. Identical experiments to that described above were carried out at pH 6 and 8 in different buffer solutions. The enthalpy changes obtained are given in Table 1. As we will describe below, these experiments allow the estimation of the number of protons linked to the complex formation. These values are too small (Table 1) to decrease the affinity of captopril to monomeric ACE with a change of pH, and thus it is impossible to calculate the first term on the right-hand side of Eq. 2. The second term can be calculated using the values given in Table 1. If the same number of protons taken up by each site in the monomer of ACE is assumed, the result indicates that the equilibrium constant changes less than an order of magnitude with a variation of pH between 6 and 8. Even if the number of protons taken up by each site were different, the variation in the equilibrium constant would also be small. For this reason, in other buffer solutions and pH values, no attempt has been made to obtain equilibrium binding constant from the calorimetric titration data, since the high affinity of ACE-captopril association precludes the determination of affinity by calorimetric titration as described by Wiseman et al. [29]. Experiments were designed to facilitate the accurate measurement of the enthalpy and stoichiometry of ACE-captopril binding. The results obtained prove that captopril, as lisinopril [30], binds to the two potential active sites identified in the sequence of somatic ACE [7], with an association constant higher than $10^8$ $M^{-1}$, which changes little over a pH range of 6–8.

### 3.3. Measurements of protons linked to ligand binding

As has been said above, a series of titration calorimetry binding measurements were made for captopril to ACE as a function of pH in the presence of two or three different buffer solutions, which are given in Table 1.

If the binding reaction involves a change in protonation, the observed heats by titration calorimetry are apparent enthalpies, which include contributions from the binding enthalpy and ionization enthalpy of the buffer in proportion to uptake or release of ligand-linked protons. The measured $\Delta H_T$ is the sum of the reaction binding enthalpy, $\Delta H_b$, which is independent of the buffer, and a term proportional to the change of ionization of the buffer

$$\Delta H_T = \Delta H_b + n_{H^+}\Delta H_{ion} \quad (3)$$

where $n_{H^+}$ is the number of protons taken up by the complex during the binding reaction and $\Delta H_{ion}$ the ionization heat of the buffer. The binding enthalpy and number of linked protons were calculated from $\Delta H_T$ obtained for each buffer at constant pH by Eq. 3. These data are also listed in Table 1. As the binding enthalpy change, $\Delta H_b$, is positive, $\Delta S°$ must also be positive and the binding process is entropy driven over the pH range of 6–8. Therefore, the interaction of this drug with ACE is strongly endothermic in nature. This behavior is similar to that showed by the interaction lisinopril/ACE [30].

Table 1
Captopril binding heats of ACE in different buffers and at different pH conditions to yield the binding enthalpies and linked protons

| pH | Buffer | $\Delta H_{ion}$ (kJ/mol) | $\Delta H_T$ (kJ/mol) | $\Delta H_b$ (kJ/mol) | Linked protons (per mol ACE) |
|---|---|---|---|---|---|
| 6 | Cacodylate | −1.25 | 80.17 | 81.10 | 0.54 |
| 6 | Cacodylate | −1.25 | 81.03 | 81.10 | 0.54 |
| 6 | MES | 15.38 | 88.30 | 81.10 | 0.54 |
| 6 | Aces | 31.5 | 99.00 | 81.10 | 0.54 |
| 7 | Cacodylate | −1.25 | 85.00 | 85.60 | 0.48 |
| 7 | HEPES | 20.57 | 95.56 | 85.60 | 0.48 |
| 8 | Tris | 48.09 | 94.82 | 98.94 | −0.08 |
| 8 | HEPES | 20.57 | 97.18 | 98.94 | −0.08 |

As can be observed in Table 1, the protons are taken up at pH 6 and 7 and are practically zero at pH 8. The protonation changes for captopril-ACE binding can be viewed as arising from a shift in the p$K$ of one or more groups on complex formation. Alterations in the protonation state of certain residues in the vicinity of the captopril binding site may explain the variation of number of protons at three pHs examined. Notice in Eq. 3 that while the binding enthalpy was corrected for the enthalpy of buffer ionization, $\Delta H_b$ retains contributions from the ionization enthalpies of groups on the protein, the ligand or both. Thus, $\Delta H_b$ can be the sum of a term that does not depend on the ionization of groups, and corresponds to the intrinsic binding enthalpy, and a term proportional to the change of ionization of ACE and/or captopril. At this point, it is interesting to consider that probably there are His residues in both active sites of N and C domains. Soubrier et al. [31] indicated that both His-360 and -388 in domain N, and both His-958 and -986 in domain C, are joined to the amino acid residues coordinated with $Zn^{2+}$ (His-361, His-365, Glu-389, His-959, His-963 and Glu-987). Considering the possible p$K$ value of this residue in the protein and its ionization enthalpy change, a shift in its p$K$ value can explain the uptake of protons obtained at the pH range studied. Since the number of protons taken is practically zero at pH 8, the intrinsic binding enthalpy would be approximately 98.94 kJ/mol ACE. There are several reasons that can explain these changes in p$K$ values. For instance, a variation in the micropolarity of the environment surrounding the side chains of certain active site residues as a result of captopril binding is a possibility. Alternatively, a protonated form could be stabilized by forming a hydrogen bond with a neighboring group. The contribution of protonation to $\Delta H_b$ suggests also that groups with low ionization heat like Glu or Asp practically do not take up protons upon binding. Since the $\Delta H_b$ changes with the pH within the range studied, a corresponding change in the $n_{H^+}$ with temperature given by the following relation [32,33] will take place

$$\left(\frac{\partial \Delta H_b}{\partial \mathrm{pH}}\right)_T = -2.3RT^2\left(\frac{\partial n_{H^+}}{\partial T}\right)_{\mathrm{pH}} \quad (4)$$

### 3.4. Heat capacity change $\Delta C_p$ and temperature dependence of the thermodynamic parameters

As a function of temperature between 16 and 30°C, we analyzed the interaction between captopril and ACE in cacodylate buffer and pH 7. The reaction heat observed, corrected by the buffer ionization heat (Table 1), $\Delta H_b$, depends linearly on temperature in the range 16–30°C. $\Delta C_p = -4.3 \pm 0.1$ kJ/K/mol was obtained from the slope of $\Delta H_b$ vs. temperature in Fig. 3. A rather high negative $\Delta C_p$ value is normal in binding studies [24,30,34,35], and is a distinctive feature of site-specific binding [36–38].

Table 2
Apparent thermodynamic parameters for the binding of captopril to the monomer of bovine lung angiotensin I-converting enzyme at pH 7

| Temperature (°C) | $\Delta H_b$ (kJ/mol) | $\Delta G°$ (kJ/mol) | $\Delta S°$ (J/K/mol) |
|---|---|---|---|
| 30.0 | 25.20 | −115.8 | 460 |
| 24.8 | 47.69 | −113.0 | 540 |
| 19.8 | 70.84 | −108.6 | 610 |
| 16.0 | 85.60 | −107.8 | 700 |

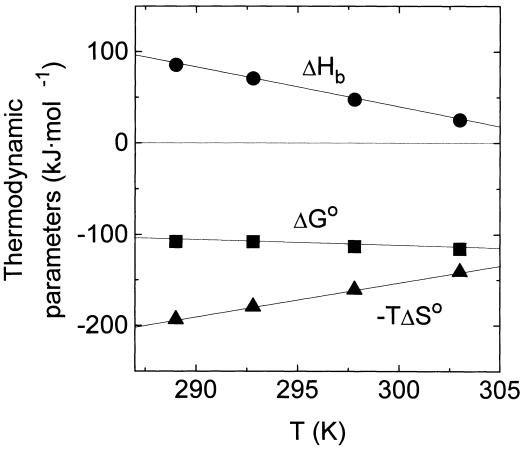


Fig. 3. Temperature dependence of the thermodynamic parameters for the binding of captopril bovine lung angiotensin I-converting enzyme at pH 7. The buffer used was 50 mM cacodylate, 0.3 M NaCl, and 50 μM $ZnCl_2$. The parameters have been corrected for effects of buffer ionization. The heat capacity change associated with the binding of captopril to ACE was determined by linear regression analysis as the slope of the plot of $\Delta H_b$ vs. temperature. Values of $\Delta G°(T)$ have been calculated using the equilibrium constants from Deddis et al. [28] and the van 't Hoff equation. The values of $-T\Delta S°(T)$ are calculated from $-T\Delta S°(T) = \Delta G°(T) - \Delta H_b(T)$.

The entropy change was calculated from the $\Delta H_b$ obtained and using the value of $\Delta G°$ calculated from the microscopic binding constants [28]. The $\Delta S°$ value is also displayed in Table 2. The standard state is that of 1 mol/l. The calculation of thermodynamic functions implies the usual approximation of setting standard enthalpies equal to the observed ones.

For all temperatures at which measurements have been made, the binding enthalpy contributes non-favorably to the free energy of binding. As temperature increases, the binding enthalpy becomes less endothermic (more favorable). As can be seen in Fig. 3, the enthalpy change decreases quickly with temperature due to a large negative heat capacity. The binding process is accompanied by a large positive entropy change, which depends also strongly on temperature, while $\Delta G°$ changes little with temperature because of the enthalpy-entropy compensation. This behavior has been found in many ligand-protein interactions [23,24,30,39–41].

The positive enthalpy change and large positive entropy change of binding upon complex formation can be justified by electrostatic and hydrophobic interactions. It is probable that the captopril-ACE complex has an electrostatic interaction between the zinc ion and the some group from captopril. Electrostatic interactions correlate well with some postulated interactions in designed inhibitors on the assumption that ACE has similar active sites to thermolysin and carboxypeptidase A [16,17]. In the spatial structure of these two related enzymes [42–44], two histidines, one glutamate and one water molecule coordinate the zinc ion. Although the three-dimensional structure of ACE has not been yet determined, the sequence is known and corresponding residues of His and Glu are found in the N and C domains [31]. Monzingo and Matthews [44] crystallized some inhibitors bound to thermolysin and found that the carboxylate of their phenylalanine is bound to the $Zn^{2+}$. Moreover, in the active site of carboxypeptidase A [45] and thermolysin [44] there is an arginine which binds the C-terminal carboxylate of the substrate. Similar interactions are possible between a sulfhydryl group of

captopril and zinc ion of ACE and between an arginine residue of ACE and the carboxyl terminal of captopril. On the other hand the heterocyclic ring of the prolyl residue of captopril may bind to a hydrophobic pocket of the enzyme. If significant apolar surface area is buried at the interaction interface, the complex formation is accompanied by negative heat-capacity changes of the system [45–49]. Murphy and Freire [50] and Spolar and Record [36] have suggested that the $\Delta C_p$ may be described as a phenomenon in hydration terms, pointing out that changes in vibrational modes apparently contribute litte to $\Delta C_p$. Similarly, Connelly has shown that the heat capacity of ligand binding can be approximated by contributions arising from dehydration of solvent exposed groups [52–54]. Thus, the enthalpy and heat capacity values provide an estimation of solvent accessibility changes during the binding [50,51], the values of which are still unknown since the spatial structure of ACE has still not been determined. Murphy's approach [50] can be applied to the intrinsic binding thermodynamic parameters, which do not retain the contribution due to protonation of groups in the complex. The intrinsic binding enthalpy change has already been determined (98.94 kJ/mol) at 16°C and the contribution of protonation of groups to heat capacity change can be corrected assuming that the His residue is the protonated group. The results of Murphy's approach indicate that the surface area buried on complex formation comprises 67% non-polar surface (approximately 3310 Å$^2$) and 33% polar surface (approximately 1650 Å$^2$). The amount of non-polar surface involved appeared too large to be accounted for in 'rigid body' association [36]. Hence our results suggest that slight conformational changes in the vicinity of the active sites were also coupled to binding. That could justify the accessible surface area values calculated.

### 3.5. Nature of the favorable entropy of binding

The positive entropy change can be explained by a strong hydrophobic effect. $\Delta S$ may be described as [36] $\Delta S° = \Delta S_{hydr} + \Delta S_{trans} + \Delta S_{specific}$ where $\Delta S_{hydr}$ is the contribution by the hydrophobic effect. $\Delta S_{trans}$ accounts for the reduction in the overall rotational and translational degrees of freedom, as well as the immobilization of amino acid side chains at the complex interface. $\Delta S_{specific}$ describes system-specific contributions such as reduction of main chain mobility and entropic contributions from polar interactions. $\Delta S_{hydr}$ can be estimated from $\Delta S_{hydr} = 1.35\ \Delta C_p\ \ln(T/386)$ where $\Delta C_p$ (in J/mol K) is the measured heat capacity change, $T$ the absolute temperature and 386 the reference temperature at which the entropy of transfer of non-polar liquids to water vanishes. For captopril-ACE complex at 25°C we obtain $\Delta S_{hydr} = 1.51$ kJ/K/mol. From $\Delta S° = 0.54$ kJ/K/mol, we calculated that $\Delta S_{trans} + \Delta S_{specific} = -0.97$ kJ/K/mol. Because of a lack of structural information on the captopril-ACE complex, we cannot provide information about the nature of $\Delta S_{trans}$ and $\Delta S_{specific}$ which together oppose binding. However, for a number of bimolecular association reactions, $\Delta S_{trans}$ has been thought to contribute $-0.21$ kJ/K/mol of rotational and translation entropy [36]. Hence, the remaining entropic loss of $-0.76$ kJ/K/mol must be contributed by the loss in the conformational restrictions of captopril and ACE. The latter is a significant contribution of the total entropic changes, and its magnitude is 50% lower than the favorable entropic contribution due to the hydrophobic effect. Hence, unlike the other cases of protein-ligand interaction, this binding of captopril to ACE cannot be taken as the 'rigid body' interaction [36]. The interaction between apolar groups of captopril and ACE requires the dehydration of both the protein and the drug and there is an entropic gain from the transfer of interfacial water into the bulk solvent. Assuming that $\Delta C_p$ value is due principally to the hydrophobic effect [34] and that the decrease in heat capacity per mol of water lost is, on average, 24 J/mol/K [55], one can calculate that about 175–180 water molecules are released.

The results of the study presented here can be summarized as: (i) the affinity of ACE to captopril is high and changes slightly with the pH. The binding is accompanied by a proton uptake which can be attributed to an increase in the p$K$ of one or more groups of the drug and/or enzyme in the complex. Although the nature of the protonated group remains ambiguous, the present results suggest that two histidines existing in both active sites of N and C domains which are not coordinated to $Zn^{2+}$ may become protonated in the complex. (ii) Captopril binds to two sites in the somatic ACE monomer. This binding is entropically driven, indicating a strong contribution from hydrophobic effects due to the release of water molecules when captopril and ACE associate. (iii) The observed heat capacity change is negative and the binding process remains dominated by entropy throughout the physiological temperature range. Thus, the thermodynamic parameter values suggest that the driving force for the binding of captopril to ACE is provided by electrostatic interactions and hydrophobic effects. Using published correlations between the heat capacity change and the burial of non-polar surface area, we are able to estimate the surface area that is buried in the captopril-ACE complex. These values suggest that the binding of captopril to ACE produces slight conformational changes in the vicinity of the active sites.

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