

# Calorimetric analysis of lisinopril binding to angiotensin I-converting enzyme

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Received 18 November 1997

**Abstract** Isothermal titration microcalorimetry has been used to measure changes in enthalpy and heat capacity for binding of lisinopril to the angiotensin I-converting enzyme (ACE; EC 3.4.15.1) and to its apoenzyme at pH 7.5 over a temperature range of 15–30°C. Calorimetric measurements indicate that lisinopril binds to two sites in the monomer of both holo- and apo-ACE. Binding of lisinopril to both systems is enthalpically unfavorable and, thus, is dominated by a large positive entropy change. The enthalpy change of binding is strongly temperature-dependent for both holo- and apo-ACE, arising from a large heat capacity change of binding equal to  $-2.4 \pm 0.2$  kJ/K/(mol of monomeric holo-ACE) and to  $-1.9 \pm 0.2$  kJ/K/(mol of monomeric apo-ACE), respectively. The negative values of  $\Delta C_p$  for both systems are consistent with burial of a large non-polar surface area upon binding. Although the binding of lisinopril to holo- and apo-ACE is favored by entropy changes, this is more positive for the holoenzyme. Thus, the interaction between  $\text{Zn}^{2+}$  and lisinopril results in a higher affinity of the holoenzyme for this drug due to a more favorable entropic contribution.

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

## 1. Introduction

Angiotensin I-converting enzyme (ACE; EC 3.4.15.1) is a zinc metallopeptidase that plays an important role in blood pressure regulation. ACE catalyzes the hydrolysis of inactive angiotensin I to the potent vasoconstrictor angiotensin II, and converts the vasodilator bradykinin into an inactive peptide [1]. The involvement of ACE in the metabolism of these two vasoactive peptides has been responsible for the attention paid to it as a target for antihypertensive drugs. One of the compounds widely used in the treatment of hypertensive disease is lisinopril ( $N^\alpha$ -[(S)-1-carboxy-3-phenyl]-L-lysyl-L-proline), which is an inhibitor of ACE. The 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 [2–5]. ACE is found as a membrane-bound enzyme via its hydrophobic C-terminal segment and also as a circulating molecule in plasma [6]. The latter seems to be secreted from the former by the

action of an unknown protease and shows enzymatic activity [7], indicating that the C-terminal region of the native enzyme is not needed for the catalytic activity [8–10]. In vitro, a soluble active form of it can be obtained from the lung enzyme by limited proteolytic cleavage [9,11].

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 between the catalytic sites of the carboxypeptidase A, thermolysin and ACE, potent competitive inhibitors of ACE have been designed, one of them being lisinopril [12,13]. From a thermodynamic viewpoint drug design is seen primarily as the process of optimizing specific biomolecular interactions, which is achieved by the estimation of the difference between the Gibbs energy of a compound in water compared to that of the same compound bound to a specific protein receptor site. The Gibbs energy changes are linked to the enthalpy and entropy changes. Thus, the knowledge of the effect of specific interactions in the  $\Delta H$  and  $\Delta S^\circ$  values of binding will provide information, which in turn can be used in the drug design process. In order to understand the forces driving the association between lisinopril and ACE, we studied the energetics of binding of the inhibitor to the enzyme as a function of temperature using isothermal titration calorimetry (ITC). As far as we know, this is the first time that a calorimetric study has been carried out on ACE. Earlier studies have only been focused on measuring inhibition of ACE or binding affinities for different drugs [5,14–18], but a thermodynamic characterization of the binding of drugs to ACE has not been undertaken. In this work, we report on calorimetric titration of lisinopril to ACE and to its apoenzyme at four temperatures within the range 15–30°C. In both systems, the enthalpy changes are positive within the temperature range studied and decrease linearly with the temperature, given a large negative value of  $\Delta C_p$ . The formation of complexes between lisinopril and the holo- and apo-enzymes is entropically driven, although the thermodynamic parameters obtained are different. Those differences are discussed in terms of the possible interaction between the zinc cofactor and the inhibitor.

## 2. Materials and methods

Angiotensin-converting enzyme was prepared from bovine lung by the method described by García-Fuentes et al. [11]. A soluble ACE form was obtained from purified membrane-bound enzyme, using

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**Abbreviations:** ACE, angiotensin I-converting enzyme; Cacodylate, cacodylic acid; FAPGG, 2-furanacryloyl-L-phenylalanyl glycyl-glycine; ITC, isothermal titration calorimetry; lisinopril,  $N^\alpha$ -[(S)-1-carboxy-3-phenyl]-L-lysyl-L-proline

trypsin treatment and size exclusion chromatography [11]. The trypsin solubilizes the protein by deletion of a fragment of approximately 10 kDa. Thus, the molecular mass of the monomer was taken as 160 kDa [11]. The purification yield was around 15–20 mg of apparently pure protein from 1 kg of bovine lung. 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  $\mu$ M Zn(AcO)<sub>2</sub>, 50 mM cacodylate at pH 7.5 and 4°C. Protein concentration was determined from absorbance measurements at 280 nm using the bovine lung enzyme absorbance coefficient  $2.1 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$  [19]. The activity of lung ACE was determined by the spectrophotometric method of Holmquist et al. [20] at 25°C, with 2-furanacryloyl-L-phenylalanyl glycyl-glycine (FAPGG) as substrate. Reaction mixtures contained 100 mM FAPGG in 50 mM HEPES, 0.3 M NaCl, 10  $\mu$ M Zn(AcO)<sub>2</sub> 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  $\Delta A_{334}/\text{min}$  of 1.0. The specific activity of purified enzyme was 24–26  $\text{min}^{-1} \text{ mg}^{-1}$ .

### 2.1. Preparation of apo-ACE

The zinc-free enzyme was prepared by dialysis of 30–84  $\mu$ M ACE against seven changes of a 100-fold volume excess of EDTA (10 mM) dissolved in 10 mM HEPES at pH 7.5 and 4°C for 1 week. The EDTA was removed by dialysis against three changes of 100-fold volume excess of 0.3 M NaCl, 50 mM cacodylate buffer solution at pH 7.5 and 4°C. The specific activity of the apoenzyme was less than 1% of the activity of the holoenzyme, and in the presence of Zn<sup>2+</sup> the activity is completely recovered.

All chemicals used were of the highest purity available and were purchased from either Merck or Sigma. Centriprep 30 concentrators were from Amicon. All solutions were degassed and clarified through a 0.45- $\mu$ m Millipore filter immediately before use.

### 2.2. Calorimetric experiments

ITC experiments were performed in a calorimeter built in our laboratory, interfaced to a microcomputer 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]. The titration experiments were carried out at pH 7.5 and 14.9°C, 19.9°C, 25°C and 29.9°C, as described elsewhere [23,24]. The protein concentrations of the solutions employed in the calorimetric experiments were in the range 34–90  $\mu$ M and 34–65  $\mu$ M for holo- and apo-ACE, respectively. All the appropriate corrections for dilution heats were applied. 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-lisinopril complex from calorimetric experiments showed less than 1% activity. Similarly, the pH values of the buffer, lisinopril, and protein solutions were checked at each temperature before and after the binding reaction.

### 2.3. Analysis of calorimetric titration data

The binding of a ligand,  $L$ , to two equal and independent sites on the protein molecule was assumed,  $K$  being its characteristic microscopic association constant,  $\Delta H$  the molar enthalpy change of binding and  $n_{H^+}$  the number of protons taken up by the protein-ligand complex. The heat released or absorbed for each ligand concentration is given by the equation [24]

$$Q = 2[P]V\Delta H \frac{K[L]}{1+K[L]} + 2[P]Vn_{H^+}\Delta H_i \frac{K[L]}{1+K[L]} \quad (1)$$

where  $[P]$  stands for the monomer protein concentration in the calorimeter cell,  $V$  for the reaction volume and  $\Delta H_i$  for the buffer ionization heat. The free ligand concentration after each injection must be calculated from these values according to

$$[L] = \left\{ \sqrt{(1-K[L]_T + 2K[P])^2 + 4K[L]_T} + K[L]_T - 2K[P] - 1 \right\} / 2K \quad (2)$$

where  $[L]_T$  is the total ligand concentration.

Eq. 1 involves three fitting parameters:  $\Delta H$ ,  $n_{H^+}$  and  $K$ . If the buffer ionization heat is close to zero, the last term in Eq. 1 can be neglected and, then, that equation has only two fitting parameters, the enthalpy change and the association constant. Once the convergence criteria are satisfied, the values of those parameters can be obtained for each temperature. A computer program for specific data analysis has been written including a function optimization based on the Levenberg-Marquardt algorithm [25]. Heat capacity changes of binding were obtained by measuring the enthalpy changes on association over a temperature range of 15–30°C.

## 3. Results and discussion

### 3.1. Binding lisinopril to holo-ACE

Direct calorimetric measurements were performed in order to determine the enthalpy change for the binding of lisinopril inhibitor to holo-ACE. A typical thermogram for binding of lisinopril to monomeric ACE in cacodylate buffer at pH 7.5 and 25°C is shown in Fig. 1. Cacodylate buffer was chosen for its small ionization heat of approximately  $-1.25 \text{ kJ/mol}$  [26]. Thus, the thermal effects of possible buffer protonation or ionization must be almost negligible. However, the heat measured might include a possible contribution due to the heat induced by proton uptake or release from the ligand-protein complex. For the titration of ACE there are 12 consecutive equivalent 30  $\mu$ l injections (spaced at 4 min intervals) of a lisinopril solution into the enzyme solution. The positive sign of the measured heat indicates that the enthalpy change for each injection was positive and that the process of binding under these conditions was endothermic. Control experiments involved the same number of 30  $\mu$ l injections of inhibitor solution into the same buffer but without enzyme present. Control injections represent the dilution heat of lisinopril and the heat effects from non-chemical reaction sources,

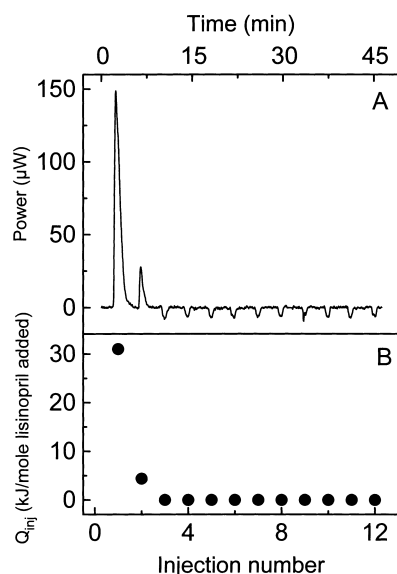


Fig. 1. Calorimetry titration of the binding of lisinopril to bovine lung ACE at pH 7.5 and 25°C. A: The programmed sequence consisted of 12 injections of 30  $\mu$ l each of 10 mM lisinopril stock solution. The lisinopril and buffer solution were injected into a sample cell containing 2.9 ml of 90.3  $\mu$ M monomeric ACE and a reference cell containing 2.9 ml of buffer (0.3 M NaCl, 50  $\mu$ M Zn(AcO)<sub>2</sub>, 50 mM cacodylate). The data in A are the differences between the sample cell and the reference cell. B: Integrated heat change for each injection per mol lisinopril injected after subtracting the control injection is plotted vs. injection number.

which have not been compensated with dual injection into the sample and reference cells. The negative deflections observed from the third injection to the end of the titration in Fig. 1A are very similar to the control injections and show that saturation has been reached in the first two injections. The area under each peak is the heat for each injection. The integrated heats after subtraction of the small heat of the control experiment are divided by the mol ligand injected, and the resulting values are shown vs. injection number in Fig. 1B. In the reaction cell, total enzyme and lisinopril concentrations were 89.3  $\mu\text{M}$  and 109.1  $\mu\text{M}$ , respectively, after the first injection. In these conditions, a site in the monomer of enzyme would be saturated if the association constant was higher than  $10^6 \text{ M}^{-1}$ . As it is well known that the affinity of ACE to lisinopril is very high [14,18], the result proves that lisinopril binds to the two potential active sites identified in the sequence of somatic ACE [5]. Also it agrees with the fact that lisinopril binds to both ileal fluid and rabbit testicle enzymes, which have analogous sequences to the N and C domains in the somatic enzyme [17], respectively. Because the reaction of ACE with lisinopril occurs with extremely high affinity, no attempt was made to obtain the equilibrium binding constants from the calorimetric titration data, since these constants are too large to be measured by titration microcalorimetry [27–29]. For the tight binding process, however, this technique allows precise measuring of the enthalpy change of binding. The total heat of the saturation process can be calculated from the summation of the heats involved in each injection. The value of  $\Delta H$  obtained at 25°C is shown in Table 1. As the enthalpy change is positive,  $\Delta S^\circ$  must also be positive and the binding process is entropy driven. The entropy change was calculated from the  $\Delta H$  obtained and using the value of  $\Delta G^\circ$  calculated from the microscopic binding constants [17]. The  $\Delta S^\circ$  value is also displayed in Table 1. 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.

Experiments identical to those described above were also carried out at 14.9°C, 19.9°C and 29.9°C. In these cases saturation was also allowed on the second injection (data not shown), and the binding enthalpy change was positive. As the temperature increased,  $\Delta H$  became less endothermic and its contribution to the Gibbs energy change of binding was less unfavorable. Fig. 2 shows a linear dependence of binding enthalpy change on the temperature, and a constant heat capacity change was obtained from the slope of a linear regression analysis of  $\Delta H$  vs.  $T$  data (Table 1). As can be seen in Fig. 2, the enthalpy change decreased quickly with the temperature due to a large negative heat capacity. The binding process is accompanied by a large positive entropy change, which also depends strongly on temperature, while  $\Delta G^\circ$  will change less than  $\Delta H$  and  $\Delta S^\circ$  with temperature because of the enthal-

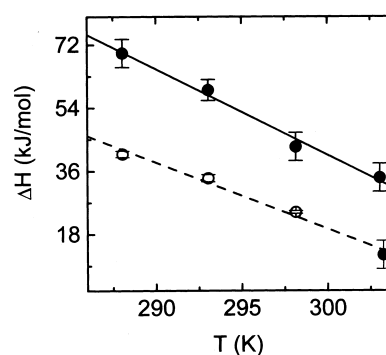


Fig. 2. Temperature dependence of the enthalpy change for the binding of lisinopril to bovine lung holo- and apo-ACE at pH 7.5. ●: average of two independent titrations; ○: obtained from the fitting of the experimental data to Eq. 1 (see text). The solid line is the linear least-squares fit of the data for the lisinopril-holo-ACE system, while the dashed lines correspond to the lisinopril-apo-ACE complex. The heat capacity changes, given by the slope of the plots of  $\Delta H$  vs.  $T$ , are shown in Table 1.

py-entropy compensation. This behavior is usual in many ligand-protein interactions [24,30–32], because the binding of a ligand to a protein is frequently accompanied by a large  $\Delta C_p$ .

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 lisinopril-ACE complex has an electrostatic interaction between the zinc ion and some group from lisinopril. 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 [12,13]. In the spatial structure of these two related enzymes [33–35], 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 [2]. Monzingo and Matthews [35] crystallized some inhibitors bound to thermolysin and found that the carboxylate of their phenylalanine is bound to the  $\text{Zn}^{2+}$ . Moreover, in the active sites of carboxypeptidase A [33] and thermolysin [35] there is an arginine, which binds the C-terminal carboxylate of the substrate. Similar interactions are possible between a carboxylate group of lisinopril and zinc ion of ACE and between an arginine residue of ACE and the carboxy terminus of lisinopril. X-ray analysis also showed a hydrogen bond between the inhibitors and the thermolysin [35], and this interaction has also been suggested to exist in the inhibitor-ACE association [12]. However, the obtained thermodynamic parameter values seem to indicate that hydrogen bonds do not play an important role in the binding of lisinopril to ACE. On the other hand, the

Table 1  
Apparent thermodynamic parameters for the binding of lisinopril to the monomer of bovine lung ACE at pH 7.5 and 25°C

ACE	$\Delta G^\circ$ (kJ/mol)	$\Delta H$ (kJ/mol)	$\Delta S^\circ$ (J/K/mol)	$\Delta C_p^\circ$ (kJ/K/mol)
Holoenzyme <sup>a</sup>	$-123.1 \pm 0.7^c$	$42.9 \pm 4.0$	$557 \pm 5$	$-2.4 \pm 0.2$
Apoenzyme <sup>b</sup>	$-49.8 \pm 0.4$	$24.3 \pm 0.4$	$248 \pm 2$	$-1.9 \pm 0.2$

<sup>a</sup>The errors of the parameters are calculated as standard deviations of two experiments at each temperature.

<sup>b</sup>The uncertainties are standard errors in fitting of the curves.

<sup>c</sup>This value has been calculated from Deddish et al. [17].

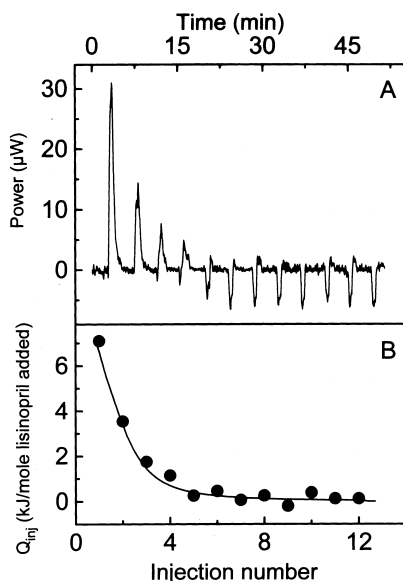


Fig. 3. Isothermal titration of the binding of lisinopril to bovine lung apo-ACE at pH 7.5 and 25°C. A: Data for 12 injections of 30  $\mu$ l each of a 8.0 mM lisinopril solution into a sample cell containing 2.9 ml of 54.3  $\mu$ M apoenzyme. B: Integrated data per mol drug injected after subtraction of control vs. injection number. The solid curve is the theoretical one for the parameter values shown in Table 1. Buffer conditions: 0.3 M NaCl and 50 mM cacodylate.

interaction between apolar groups from lisinopril 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. The aromatic groups of lisinopril may be accommodated in a hydrophobic binding pocket similar to that observed in the three-dimensional structure of carboxypeptidase A and thermolysin. If a significant apolar surface area is buried at the interaction interface, the complex formation is accompanied by negative changes in the heat capacity of the system [36–41]. As Table 1 shows, the  $\Delta C_p$  for the binding of lisinopril to holo-ACE is negative and large. Murphy and Freire [42] and Spolar and Record [43] 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 little to  $\Delta C_p$ . Thus, the enthalpy and heat capacity values provide an estimation of solvent accessibility changes during the binding [42,44], the values of which are still unknown since the spatial structure of ACE has not yet been determined. If we assume that the contribution to  $\Delta H$  and  $\Delta C_p$  of possible protonation or ionization of groups of the protein and/or ligand during binding is almost negligible, the application of Murphy's approach [42] to the experimentally determined values (Table 1) indicates that the surface area buried on complex formation comprises 70% non-polar surface (approximately 1720  $\text{\AA}^2$ ) and 30% polar surface (approximately 775  $\text{\AA}^2$ ).

### 3.2. Binding lisinopril to apo-ACE

Fig. 3 shows data from the calorimetric titration of 2.9 ml of 54.3  $\mu$ M monomeric apo-ACE in the sample cell at 25°C with a 8 mM solution of lisinopril, both in the same buffer (0.3 M NaCl, 50 mM cacodylate at pH 7.5). The sample data represent 12 injections of 30  $\mu$ l each spaced at 4 min intervals. The first four injections are all endothermic, while the last

seven are exothermic and very similar to the control experiment, which indicates that saturation has been reached. The integrated heats after subtraction of the small heats of the control experiment are shown as the enthalpy per mol ligand injected vs. injection number in Fig. 3B. For this system the shape of a calorimetric titration curve is sensitive to both the enthalpy change and the association constant for the binding ligand to protein. If the curve in Fig. 3B is compared to that in Fig. 1B it is evident that the holoenzyme has a higher affinity for lisinopril than the apoenzyme. The calorimetric titrations were also carried out at other temperatures between 14.9 and 19.9°C and are shown in Fig. 4 as curves of cumulated changes of enthalpies. Therefore, any point, at a given ligand concentration, represents the total heat obtained summing all previous heats and divided by the mol monomeric protein. In order to calculate more accurately the heat capacity change value, the enthalpy change of the binding of lisinopril to the apoenzyme was also determined at 30°C.

Molecular biology studies of human somatic ACE predict the existence of two functional active sites in the protein monomer [2,45] and some ACE inhibitors bound to both sites [5,15–18]. Although the sequence of ACE reveals a high degree of internal homology between the N and C domains [2], the affinity of both sites for several inhibitors seems to be slightly different [5,16,17]. The equilibrium constants of lisinopril to holo-ACE are not equal but are of the same order of magnitude [5,17]. We tested different binding models and the analyses of the data in Fig. 4 are consistent with a stoichiometry of two equal and non-interacting sites in the apoenzyme for the binding of lisinopril. However, a model of two independent sets of binding sites in the monomer (two enthalpy changes and two apparent association constants) makes it possible to fit the experimental binding data, both equilibrium constants being very close to the one obtained, which is  $(2.3 \pm 0.2) \times 10^4 \text{ M}^{-1}$  at 25°C. Thus, our data allow us to suggest that lisinopril shows close affinity for both sites in the N and C domains and to determine the  $\Delta H$  for the binding of lisinopril to monomeric apo-ACE with fairly good precision. The results of the fitting of the experimental data for the lisinopril-apo-ACE complex, using the model to two equal and independent sites (see Section 2) are shown in Fig. 4 and the thermodynamic parameters at 25°C are summarized in

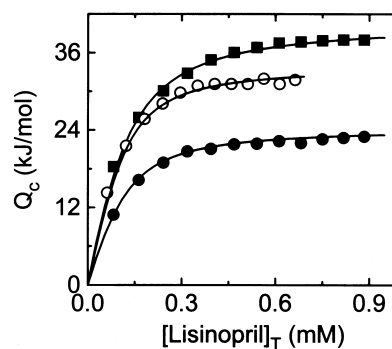


Fig. 4. Thermal titration of bovine lung apo-ACE at pH 7.5. The monomeric protein concentration was 34–65  $\mu$ M in 0.3 M NaCl and 50 mM cacodylate. The points correspond to the total cumulative heat effect in the titration process plotted as a function of the total lisinopril concentration at 14.9°C (■), 19.9°C (○) and 25°C (●). The solid lines are the theoretical ones corresponding to Eq. 1 divided by mol monomeric protein and obtained from the values shown in Table 1.

Table 1. The heat capacity change was calculated as described before for the ligand binding to the holoenzyme (Fig. 2). In this case, a large negative  $\Delta C_p$  was also obtained (Table 1). Thus, the enthalpy and entropy changes of lisinopril-apo-ACE complex formation depend strongly on temperature in the range 15–30°C, while  $\Delta G^\circ$  changes little with temperature because of enthalpy-entropy compensation. The large negative  $\Delta C_p$  suggests the possibility of a decrease in the exposure of hydrophobic groups from the ligand and the protein to water upon binding. The association lisinopril-apoenzyme is clearly governed by an entropic contribution at all temperatures examined. If the thermodynamic parameter values for the binding of the inhibitor to holo- and apo-ACE are compared, we can observe that the affinity of the ligand to the holoenzyme is higher, since the value of  $K$  is approximately five orders of magnitude higher. This higher affinity is due to a more favorable entropy change, which can be explained by electrostatic interactions between  $\text{Zn}^{2+}$  and some negative groups of lisinopril. These interactions seem to be accompanied by a positive  $\Delta H$ . However, an electrostatic interaction usually makes positive contributions to  $\Delta C_p$  and our results show a heat capacity more negative for the holoenzyme than for the apoenzyme, which seems to suggest light changes at the neighborhood of the sites in the apoenzyme with respect to the holoenzyme, which affect their interactions with the lisinopril.

The results presented in this study show that lisinopril binds to two sites in the somatic ACE monomer. The Gibbs energy change for both holo- and apo-ACE complex formation is entropy-controlled and although the enthalpy and entropy changes exhibit strong temperature dependence, arising from a significant negative heat capacity change, 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 lisinopril to holo-ACE is provided by electrostatic interactions and hydrophobic effects related to buried apolar surface with liberation of water molecules from both the protein and the drug, showing that the hydrophobic character of the groups in the drug is an important factor in the binding of an inhibitor to ACE. The comparison between the thermodynamic parameters for the binding of lisinopril to the holo- and the apo-enzyme shows that the interaction between the zinc ion and some group of lisinopril increases the affinity of holoenzyme for the inhibitor due to a more favorable entropic contribution.

**Acknowledgements:** We would like to thank Dr. O. López-Mayorga for the excellent technical assistance and the Biocalorimetry Center (NIH) of The Johns Hopkins University for their technical help with the construction of the titration calorimeter. This research was supported by Grants PB93-0731 and PB93-1163 from the Ministerio de Educación y Cultura, Spain. R.T.S. was supported by fellowships from the F.P.I (AP93), Ministerio de Educación y Cultura, Spain.

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