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Role of Two Chloride-Binding Sites in Functioning of Testicular Angiotensin-Converting Enzyme

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Abstract—Modeling the structure of the C-domain of bovine angiotensin-converting enzyme revealed two putative chloride-binding sites. The kinetic parameters, K_m and k_{cat}, of hydrolysis of the substrate Cbz-Phe-His-Leu catalyzed by the testicular (C-domain) enzyme were determined over a wide range of chloride concentrations. Chloride anions were found to be enzyme activators at relatively low concentrations, but they inhibit enzymatic activity at high concentrations. A general scheme for the effect of chloride anions on activity of the C-domain of bovine angiotensin-converting enzyme accounting for binding the "activating" and "inhibiting" anions is suggested.

Key words: angiotensin-converting enzyme, testicular enzyme, C-domain, chloride

Angiotensin-converting enzyme (ACE, peptidyl dipeptidase A, EC 3.4.15.1) is metallopeptidase of the gluzincin group. Two ACE isoforms are synthesized in mammalian cells. The somatic form of the enzyme is found in almost all tissues and consists of two homologous N- and C-domains; each of them contains a Zn^{2+}-dependent catalytic site [1]. The testicular ACE isoform is synthesized only in testis and corresponds to the C-domain of somatic ACE (excluding the N-terminal sequence of 67 amino acids); thus, it has only one active site [2]. The two isoforms are encoded by the same gene but differ in transcription initiation point [3].

Activation of the enzyme by anions, especially chloride, is a unique feature of ACE. When describing a newly isolated ACE or studying new substrates, the degree of the enzyme activation by chloride is usually one of the parameters characterizing its catalytic activity. However, the mechanism of the effect of anions on this enzyme is still unclear, although there is a lot of information on ACE activation by anions. The reason is that the dependence of ACE activity on concentration of anions changes when using various substrates and inhibitors [4-6]. Based on the data obtained by Riordan and coworkers in the mid-80s, tripeptide ACE substrates were formally divided into three classes depending on the value of the apparent activation constant by chloride anion (K'_A) and on the apparent activation mechanism [5-7]. However, this approach did not rationalize all the peculiarities of ACE activation by chloride, and also at that time it was not yet known that somatic ACE consists of two homologous domains and thus has two active sites [1].

Now it is known that two active sites of ACE hydrolyze the same set of the substrates but with different efficiencies [8, 9]. Both domains are activated by chloride anions, but the N-domain is less chloride-dependent than the C-domain [8, 9]. Besides, the pathway of the substrate hydrolysis may be also determined by the concentration of chloride [9]. So, it is obvious that the observed activation of ACE by chloride is a result of a combination of various factors.

The spatial structure of the C-domain of human ACE and its complexes with three inhibitors was recently determined by X-ray analysis [10, 11]. As found, in the single-domain form of the human enzyme there are two chloride-binding sites separated by 20 Å from each other. The first chloride-binding site is 21 Å distant and the second 10 Å distant from Zn^{2+} of the active site [10]. According to the structural data on the C-domain of ACE, the microenvironment of these two chloride anions is as follows [12]: the first chloride anion (Cl^1, far from Zn^{2+} of the active site) is bound with two Arg residues and surrounded with a hydrophobic envelope of four Trp residues. The second chloride anion (Cl^2, close to Zn^{2+} of the active site) is bound with an Arg residue, which is crucial for manifestation of chloride dependence of ACE, as shown earlier [13]. Tyrosine and some

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other hydrophobic residues are also in the environment of Cl^2.

So, even in the single-domain form of human ACE there are two chloride-binding sites. Thus, it is necessary to study the activation mechanism of the single-domain forms of the enzyme before studying the anion-mediated activation mechanism of the somatic two-domain ACE.

In this work we present the data on binding of two chloride anions with the C-domain of bovine ACE (82.5% homology with the C-domain of human ACE) and show that binding of the second chloride can result not in activation, but in inhibition of the enzyme.

MATERIALS AND METHODS

Reagents. In this work we used N^{α}-3-(2-furyl)-acryloyl-L-phenylalanyl-glycyl-glycine (FA-Phe-Gly-Gly), N-(S-1-carboxy-3-phenylpropyl)-L-lysyl-L-proline (lisinopril), and N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (Hepes) of SigmaUltra grade from Sigma (USA); histidyl-L-leucine (His-Leu) from Serva (Germany); N^{α}-carbobenzoxy-L-phenylalanyl-L-histidyl-L-leucine (Cbz-Phe-His-Leu) from Bachem (USA); and potassium chloride of extra pure grade from Reakhim (Russia).

Bovine testicular ACE (C-domain) was obtained by a procedure including extraction of the enzyme by 50 mM phosphate buffer, pH 7.5, containing 150 mM KCl, 1 μM $ZnCl_2$, and 0.5% Triton X-100 (Dia-M, Russia) and cascade affinity chromatography [14]. Electrophoresis of ACE preparation was performed in 7.5% polyacrylamide gel in the presence of 0.1% SDS and β-mercaptoethanol according to Laemmli [15]. Protein was stained by Coomassie Brilliant Blue G250.

The concentration of the active ACE in the preparation was determined by stoichiometric titration [16] with specific competitive inhibitor lisinopril in 50 mM Hepes, pH 7.5, containing 150 mM KCl and 1 μM $ZnCl_2$ at 25°C. On establishment of the equilibrium between free enzyme and the enzyme–inhibitor complex, FA-Phe-Gly-Gly solution was added to the reaction mixture and the residual activity of the enzyme was determined spectrophotometrically [17].

Kinetics of Cbz-Phe-His-Leu hydrolysis catalyzed by ACE in the presence of chloride at various concentrations was studied fluorometrically; the reaction product His-Leu was determined with *o*-phthalaldehyde [18]. Experiments were performed in 50 mM Hepes, pH 7.5, containing 1 μM $Zn(CH_3COO)_2$ and 0-700 mM KCl. All buffers were prepared using deionized water from a MilliQ (Millipore, USA).

Data obtained in at least three independent experiments were analyzed in $[S]/v - [S]$ coordinates, where [S] is the substrate concentration and v is the rate of enzymatic hydrolysis. Parameters of the scheme of action of chloride anions in the reactions catalyzed by ACE were calculated using Sigma Plot and Microsoft Excel programs.

ACE was desalted by gel chromatography on Sephadex G-25 coarse using microcolumns from Filtration Column, J. T. Baker, 6 ml in volume. The carrier was washed several times with deionized water and excess water was removed by centrifugation at 3000 rpm for 15 min using a VEB MLW centrifuge (Germany). Then the enzyme sample was applied onto the carriers and centrifuged again for 10 min. Desalting was monitored conductometrically. Activity of the enzyme solution was tested via the initial rate of FA-Phe-Gly-Gly hydrolysis in 50 mM Hepes, pH 7.5, containing 150 mM KCl and 1 μM $ZnCl_2$.

The effect of ionic strength on ACE activity was studied by comparison of the enzyme activity in Cbz-Phe-His-Leu hydrolysis in 50 mM Hepes, pH 7.5, containing 150 mM KCl; 600 mM KCl; 150 mM KCl and 450 mM KNO_3; 150 mM KCl and 150 mM Na_2SO_4. All solutions contained 1 mM $Zn(CH_3COO)_2$. The ionic strength of the first solution was 0.15 M and that of the others was 0.6 M.

Amino acid sequences of the C-domain of human ACE [1] and the C-domain of bovine ACE [19] were aligned using the Clustal-X program [20] and BLOSSUM30 homology matrix.

The structure of the C-domain of bovine ACE was modeled based on the alignment of amino acid sequences and on the structure of the C-domain of human ACE (PDB: 108 A) using the Biopolymer modulus of Sybyl 6.9 package [21]. On mutation of non-homologous amino acid residues and insertion of the corresponding loops in the template (PDB: 108 A), molecular geometry of the C-domain of bovine ACE was completely optimized using a Tripos force field.

RESULTS AND DISCUSSION

Modeling of the structure of the C-domain of bovine ACE. Detection of putative chloride-binding sites. To detect the putative chloride-binding sites in the C-domain of bovine ACE, we first compared the amino acid sequences of the C-domains of human and bovine ACE (Fig. 1). It appeared that these sequences are 82.5% identical and that all the amino acids present in the microenvironment of chloride anions in the C-domain of human ACE are also present in the C-domain sequence of bovine ACE (Table 1).

We constructed a spatial model of the C-domain of bovine ACE using a spatial structure of the C-domain of human ACE as a matrix [10]. According to this model, both putative chloride-binding sites identical to the binding sites of human ACE are present in the structure of the C-domain of bovine ACE.

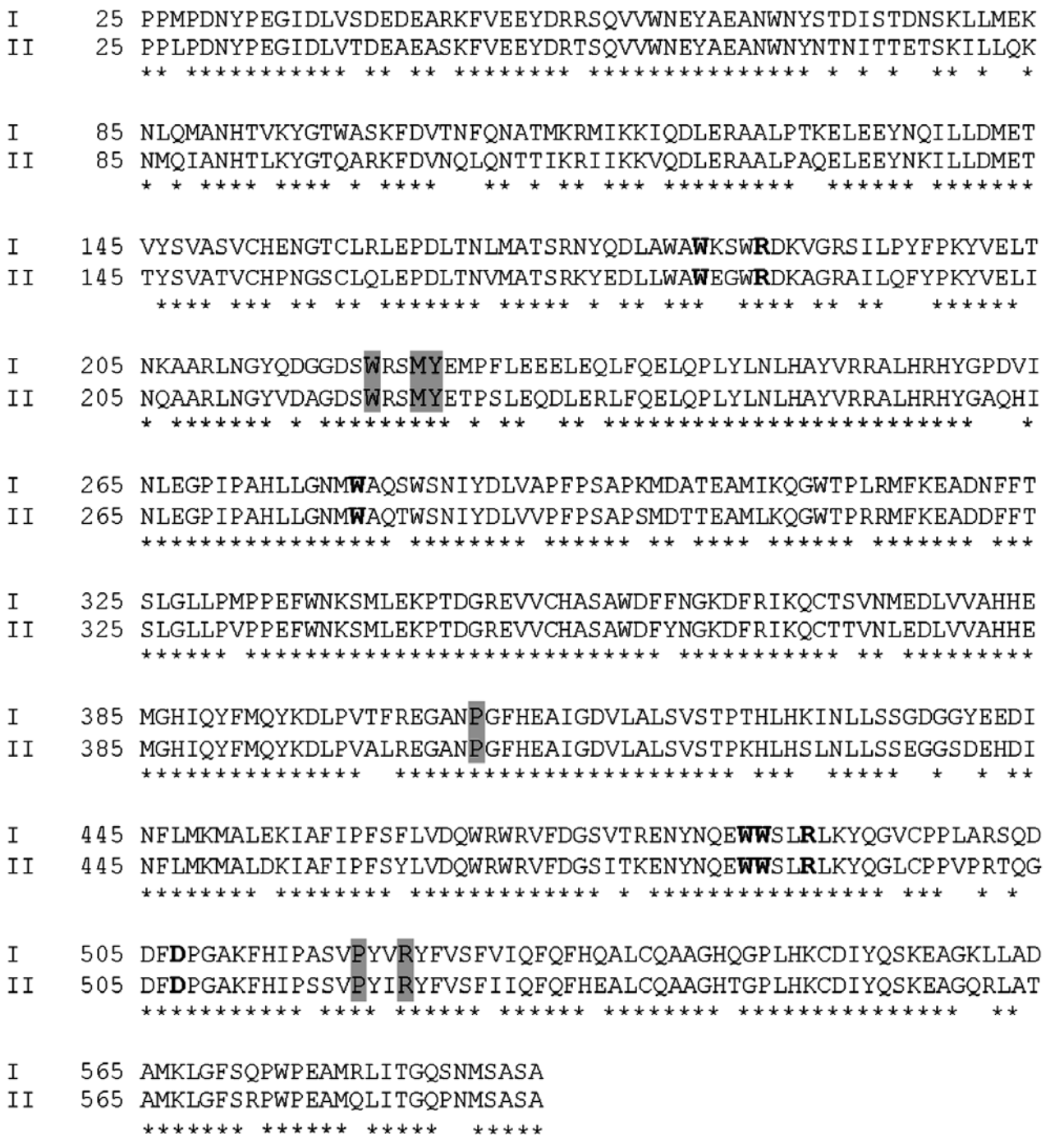

Fig. 1. Comparison of the amino acid sequences of the C-domains of bovine (I) and human ACE (II). Amino acids composing the binding site of the near chloride anion (Cl^2) are highlighted in gray and those composing the binding site of the far chloride anion (Cl^1) are highlighted in bold. The conservative amino acids are marked by asterisks.

Determination of kinetic parameters of Cbz-Phe-His-Leu hydrolysis catalyzed by testicular bovine ACE at various KCl concentrations. To prove the presence of two chloride-binding sites in the C-domain of bovine ACE, we studied the effect of chloride on the activity of testicular enzyme in hydrolysis of Cbz-Phe-His-Leu tripeptide substrate. Kinetic parameters of hydrolysis, K_m and k_{cat}, were determined at various KCl concentrations (from 0 to 700 mM).

As shown in Fig. 2, the value of K_m decreases with increase in KCl concentration. The data indicate that Cl^- binding to ACE results in tighter binding of the substrate

Table 1. Amino acid residues composing the binding sites of chloride anions (Cl^1 and Cl^2) in the C-domains of bovine and human ACE

Cl^1	Cl^2
Arg 186	Tyr 224
Arg 489	Arg 522
Trp 182	Pro 519
Trp 486	Pro 407
Trp 485	Met 223
Trp 279	Trp 220
Asp 507	

Note: Numeration of the testicular ACE is used in this table.

to enzyme, which is consistent with the literature [22]. As for k_{cat}, at first it increases with increasing KCl concentration, reaches a maximum at about 300 mM KCl, and then it begins to decrease (Fig. 2). Thus, at relatively low concentrations chloride anions activate the C-domain of bovine ACE, but at high concentrations (that is, occupying both binding sites) chloride inhibits the enzyme activity.

It is known that the activating effect of chloride is specific and is not governed by the ionic strength of the solution. Anions of various chemical natures vary in their activating effect on ACE, chloride being the most efficient activator [6]. To find whether it is possible to consider ACE inhibition in the presence of high KCl concentrations as a specific action of chloride, we performed two series of experiments. First, we showed that replacement of K^+ for Na^+ did not influence the enzyme activity. Second, our comparisons of the effect of anions of various chemical natures on the activity of testicular ACE at the same ionic strength of solution showed that the effect depends on the nature of the anion: nitrate inhibited ACE activity more efficiently than chloride, whereas sulfate activated the enzyme (Table 2). Thus, at both low and high salt concentrations, the effect of anions on ACE activity is governed by the chemical nature of the anion rather than the ionic strength of the solution.

So, the results indicate the presence of at least two chloride-binding sites in the C-domain of bovine ACE: binding of chloride to one of the sites causes activation of the enzyme, whereas chloride binding to the second site results in inhibition of the enzymatic activity. For comparison, we performed an analogous experiment studying the effect of chloride on the activity of human testicular ACE in Cbz-Phe-His-Leu hydrolysis. It appeared that dependencies of the rate of enzymatic hydrolysis on KCl concentration are similar for human and bovine ACE, that is, chloride anions at the relatively low concentrations are activators, but at high concentrations they inhibit ACE activity (data not shown).

Scheme of the action of chloride in the reactions catalyzed by the C-domain of bovine ACE. Based on dependencies of $K_{m\ app}$ and $k_{cat\ app}$ on KCl concentration, we suggested a general scheme of the action of chloride in reactions catalyzed by the C-domain of bovine ACE (Fig. 3). According to this scheme, the values of $K_{m\ app}$ and $k_{cat\ app}$ can be defined as follows:

$$k_{cat\ app} = k_{cat}\frac{\gamma + [KCl]/(\alpha K_{dis,A}) + (\omega[KCl])/(\beta K_{dis,I}) + (\delta[KCl]^2)/(\alpha\beta K_{dis,A}K_{dis,I})}{1 + [KCl]/(\alpha K_{dis,A}) + [KCl]/(\beta K_{dis,I}) + [KCl]^2/(\alpha\beta K_{dis,A}K_{dis,I})},$$

$$K_{m\ app} = K_{m,0}\frac{1 + [KCl]/K_{dis,A}}{1 + [KCl]/(\alpha K_{dis,A}) + [KCl]/(\beta K_{dis,I}) + [KCl]^2/(\alpha\beta K_{dis,A}K_{dis,I})}.$$

The experimental results are most adequately described by the scheme in which ES, Cl^1ES, and

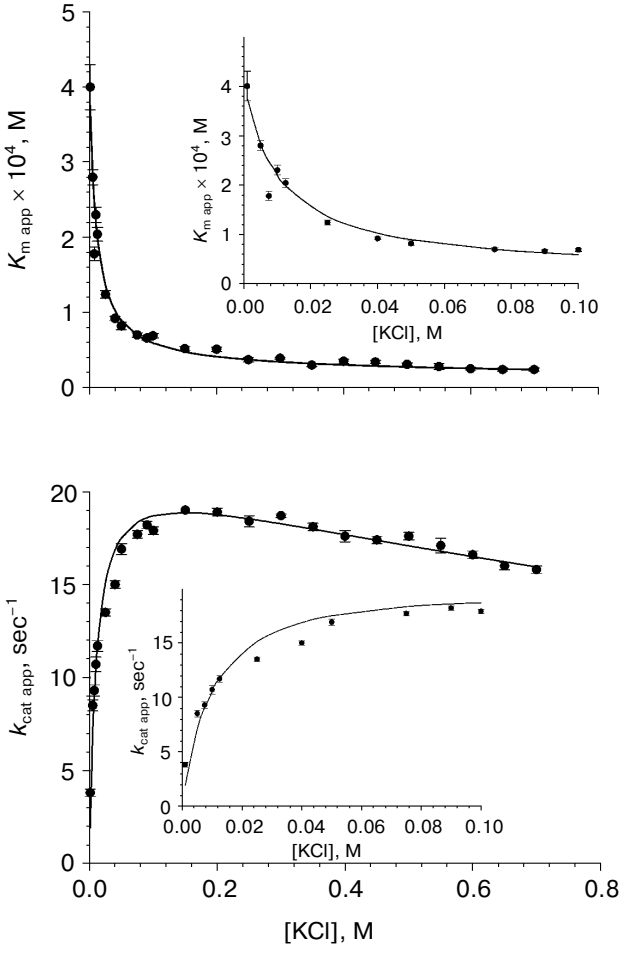

Fig. 2. Kinetic parameters of Cbz-Phe-His-Leu hydrolysis catalyzed by testicular bovine ACE versus concentration of chloride. Theoretical dependencies are presented by the curves. Data for low KCl concentrations are presented in the insets.

Table 2. Relative activity (*a*) of testicular ACE in Cbz-Phe-His-Leu hydrolysis in solutions of various salts

Salt	Ionic strength, M	*a*, %
0.15 M KCl	0.15	100
0.6 M KCl	0.6	88
0.15 M KCl + 0.45 M KNO_3	0.6	64
0.15 M KCl + 0.15 M Na_2SO_4	0.6	116

Note: Conditions: 50 mM Hepes, pH 7.5, 1 μM $Zn(CH_3COO)_2$, [S] = 0.5 mM, 25°C.

Table 3. Parameters of the scheme of action of chloride in Cbz-Phe-His-Leu hydrolysis catalyzed by testicular bovine ACE

Parameter	Value
$K_{m,0}$, mM	0.4 ± 0.1
$K_{dis,A}$, mM	160 ± 80
α	0.064 ± 0.036
k_{cat}, sec^{-1}	21.5 ± 1
$K_{dis,I}$, M	>10
$\beta K_{dis,I}$, M	2.1 ± 0.5

Cl^1ECl^2S complexes are inactive ($\gamma = \omega = \delta = 0$) and only ECl^2S complex with only one chloride-binding site occupied is active (Fig. 3). Using the abovementioned equations, we calculated the dissociation constants for the ACE complexes with the substrate and chloride, k_{cat} and α coefficient (Table 3). The α value appeared to be significantly less than 1 (Table 3); this means that binding of the activating chloride (designated as Cl^2 in the scheme) to the C-domain of ACE results in the tighter binding of the substrate, this corresponding with the data on K_m decrease with increase in concentration of chloride [22]. The calculated dissociation constant of the complex of activating chloride anion with the testicular ACE ($K_{dis,A}$) is 0.16 M, whereas the dissociation constant of the complex of inhibiting chloride anion (designated as Cl^1 in the scheme) with the enzyme ($K_{dis,I}$) exceeds 10 M and cannot be determined. However, substrate binding significantly increased affinity of the inhibiting chloride anion to the enzyme (Table 3, $\beta K_{dis,I}$). It should be noted that α and β coefficients used in the scheme (Table 3) characterize the enhanced affinity of the activating and inhibiting chloride anions to the enzyme on the substrate binding as well as enhanced affinity of the substrate to the testicular ACE on binding of the corresponding anions.

Kinetic data interpreted from the structural viewpoint. We agree with suggestions [12] that Cl^2-binding site near the active site of the C-domain of ACE is responsible for the activating effect of chloride. According to these suggestions, in the absence of chloride, Arg522(1098) (number without parentheses corresponds to the testicular ACE, number in parentheses corresponds to the somatic human ACE) forms a salt bridge with Asp465(1041), whereas the neighbor residue Tyr523(1099) is far from the catalytic center and is not able to participate in stabilization of the enzyme–substrate complex. In the presence of chloride anions, this salt bridge is destroyed due to formation of the ionic bond between chloride and Arg522(1098), whereas Tyr523(1099) moves to the catalytic center [12]. This is in accord with the earlier reported data [13] that Arg1098 in the C-domain of somatic ACE is crucial for the activating effect of chloride. Thus, chloride plays a role of ionic "switch".

The second (far from the active site) chloride-binding site (Cl^1) is also considered as an activating one [12]. However, our kinetic data allow consideration of this site as one causing inhibition of enzymatic activity. In this case, the enhanced binding of the inhibiting chloride after binding of substrate to the enzyme (Table 3) can be elucidated as follows. In a free enzyme the positively charged side group Lys511(1087) forms a salt bridge with Asp507(1083), thus blocking the Cl^1-binding site (Fig. 4a). In the enzyme–substrate complex this salt bridge is destroyed and the Lys side chain turns towards the active site of enzyme and participates in stabilization of carboxyl group of a substrate (Fig. 4b). Asp507(1083) also turns from the Cl^1-binding site to Arg501(1077) and forms a strong ionic bond with the latter. Such a "switching" of Lys511(1087) side chain from Cl^1-binding site to the catalytic center of the enzyme should result in the enhanced

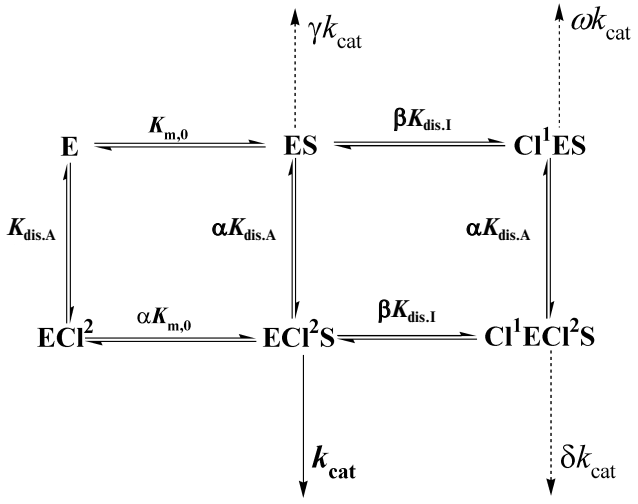

Fig. 3. General scheme of action of chloride in the reaction catalyzed by the C-domain of bovine ACE.

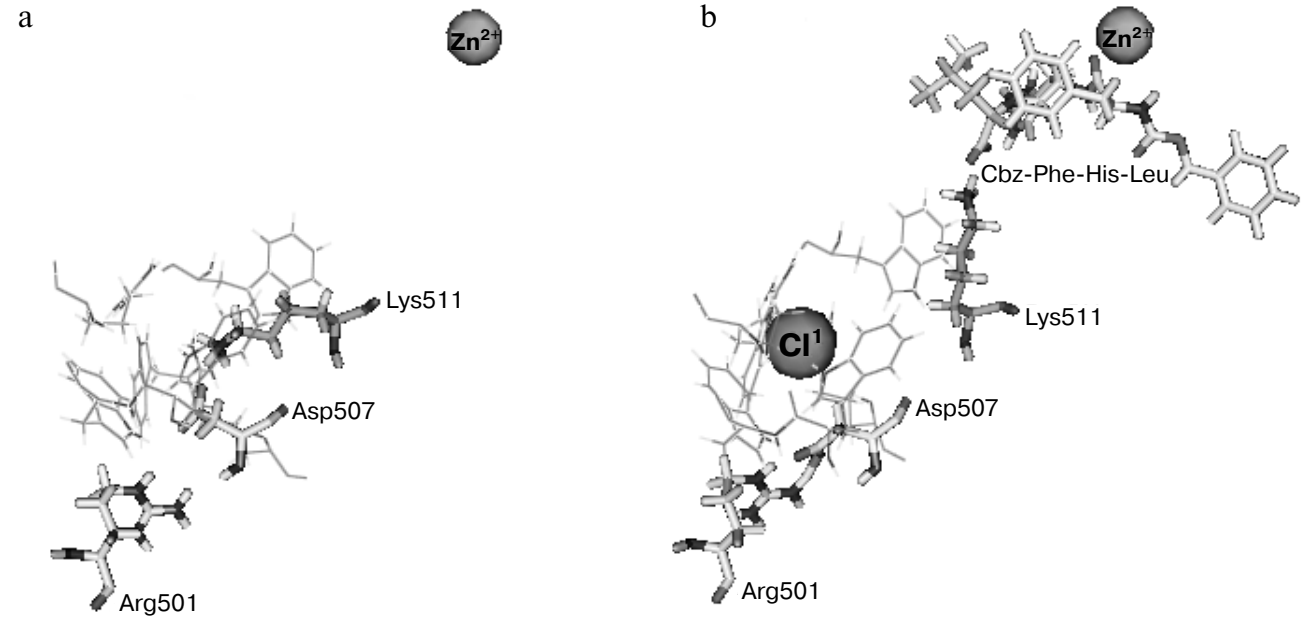

Fig. 4. Positions of Lys511 and Asp507 amino acid residues in free enzyme (a) and in the enzyme–substrate complex containing the inhibiting chloride anion (b).

binding of chloride to the enzyme–substrate complex. Structural justification of the origin of inhibitory action of chloride on the enzyme activity obviously demands further studies.

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REFERENCES

1. Soubrier, F., Alhenc-Gelas, F., Hubert, C., Allegrini, J., John, M., Tregear, G., and Corvol, P. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 9386-9390.
2. Ehlers, M. R. W., Fox, E. A., Strydom, D. J., and Riordan, J. F. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 7741-7745.
3. Hubert, C., Houot, A. M., Corvol, P., and Soubrier, F. (1991) *J. Biol. Chem.*, **266**, 15377-15383.
4. Shapiro, R., and Riordan, J. F. (1984) *Biochemistry*, **23**, 5234-5240.
5. Shapiro, R., and Riordan, J. F. (1983) *Biochemistry*, **22**, 5315-5321.
6. Bunning, P., and Riordan, J. F. (1983) *Biochemistry*, **22**, 110-116.
7. Shapiro, R., Holmquist, B., and Riordan, J. F. (1983) *Biochemistry*, **22**, 3850-3857.
8. Wei, L., Alhenc-Gelas, F., Corvol, P., and Clauser, E. (1991) *J. Biol. Chem.*, **266**, 9002-9008.
9. Jaspard, E., Wei, L., and Alhenc-Gelas, F. (1993) *J. Biol. Chem.*, **268**, 9496-9503.
10. Natesh, R., Schwager, S. L. U., Sturrock, E., and Acharya, K. R. (2003) *Nature*, **21**, 551-554.
11. Natesh, R., Schwager, S. L. U., Evans, H. B., Sturrock, E., and Acharya, K. R. (2004) *Biochemistry*, **43**, 8718-8724.
12. Tzakos, A. G., Galanis, A. S., Spyroulias, G. A., Cordopatis, P., Manessi-Zoupa, E., and Gerothanassis, I. P. (2003) *Prot. Eng.*, **16**, 993-1003.
13. Liu, X., Fernandez, M., Wouters, M. A., Heyberger, S., and Husain, A. (2001) *J. Biol. Chem.*, **276**, 33518-33525.
14. Kost, O. A., Grinshtein, S. V., Nikol'skaya, I. I., Shevchenko, A. A., and Binevski, P. V. (1997) *Biochemistry (Moscow)*, **62**, 321-328.
15. Laemmli, U. K. (1970) *Nature*, **227**, 668-672.
16. Binevski, P. V., Nikol'skaya, I. I., Pozdnev, V. F., and Kost, O. A. (2000) *Biochemistry (Moscow)*, **65**, 651-658.
17. Holmquist, B., and Bunning, P. (1979) *Analyt. Biochem.*, **95**, 540-549.
18. Conroy, J. M., and Lai, C. I. (1978) *Analyt. Biochem.*, **87**, 556-561.
19. Shai, S. Y., Fishel, R. S., Martin, B. M., Berk, B. C., and Bernstein, K. B. (1992) *Circ. Res.*, **70**, 1274-1281.
20. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. J. (1997) *Nucleic Acids Res.*, **25**, 4876-4882.
21. Sybyl 6.6. Tripos Inc.1699 South Hanley Road, St. Louis, MO 63144, http://www.tripos.com.
22. Harper, J. W., Shapiro, R., and Riordan, J. F. (1987) *Biochemistry*, **26**, 1284-1288.