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Fluorescence Polarization Studies of Different Forms of Angiotensin-Converting Enzyme

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Abstract—The interaction of three forms of bovine angiotensin-converting enzyme (ACE) with the competitive peptide inhibitor lisinopril with a fluorescent label was studied by the fluorescence polarization technique. The dissociation constants K_d of the enzyme–inhibitor complexes in 50 mM Hepes-buffer, pH 7.5, containing 150 mM NaCl and 1 μM $ZnCl_2$ at 37°C were $(2.3 \pm 0.4)\cdot10^{-8}$, $(2.1 \pm 0.3)\cdot10^{-8}$, and $(2.1 \pm 0.2)\cdot10^{-8}$ M for two-domain somatic ACE, single-domain testicular ACE, and for the N-domain of the enzyme, respectively. The interaction of the enzyme with the inhibitor strongly depended on the presence of chloride in the medium, and the apparent dissociation constant of the ACE–chloride complex was $(1.3 \pm 0.2)\cdot10^{-3}$ M for the somatic enzyme. The dissociation kinetics of the complex of the inhibitor with somatic ACE did not fit the kinetics of a first-order reaction, but it was approximated by a model of simultaneous dissociation of two complexes with the dissociation rate constants (0.13 ± 0.01) sec^{-1} and (0.026 ± 0.001) sec^{-1} that were present at approximately equal initial concentrations. The dissociation kinetics of the single-domain ACE complexes with the inhibitor were apparently first-order, and the dissociation rate constants were similar: (0.055 ± 0.001) and (0.041 ± 0.001) sec^{-1} for the N-domain and for testicular ACE, respectively.

Key words: angiotensin-converting enzyme, N-domain, fluorescence polarization, inhibition

Angiotensin-converting enzyme (ACE, peptidyl dipeptidase A, EC 3.4.15.1) is physiologically significant. This enzyme plays a major role in the regulation of arterial blood pressure and water–salt balance [1, 2]. Inhibitors of ACE are successfully used in medicine in the treatment of hypertension and cardiovascular diseases [3, 4].

By molecular cloning of ACE from human endothelium (1306 amino acid residues), the somatic enzyme was shown to consist of two highly homologous domains (N- and C-), each domain bearing an active site [5, 6]. The testicular isoform of ACE is found in the germinal cells in the testes. This form consists of a single domain that is virtually identical to the C-domain of somatic ACE except for the N-terminal sequence of 67 residues [7]. The physiological role of testicular ACE is not yet clear. Some findings suggest that this enzyme should be involved in reproduction [8, 9]. A form of ACE corresponding to the single N-domain of somatic enzyme was found in surgical operations in the "ileal fluid" of patients [10]. This form of the enzyme seems to be a result of limited proteolysis *in vivo* of the somatic ACE form. The N-domain of ACE can also be obtained *in vitro* by treatment of the parent somatic enzyme (usually after slight denaturation) with such proteinases as trypsin, chymotrypsin, staphylococcus proteinase, endoproteinase Asp-N, etc. [11, 12].

The investigations of different mutant forms of human ACE [13-16] and of the forms which correspond to separate N- and C-domains of the enzyme [10, 17, 18] demonstrated that ACE catalytic sites vary significantly in the kinetic parameters of substrate hydrolysis and in inhibitor binding constants [13, 16, 19].

Few data for ACE from other sources are available to describe functions of the active sites of the different domains of the enzyme. Nevertheless, even these few findings [20, 21] show that the active sites located in the two domains of bovine ACE function somewhat differently than in human ACE. Thus, rates of Hip-His-Leu hydrolysis at both sites of bovine ACE are the same [20], whereas this substrate is hydrolyzed mainly at the C-domain site of human ACE [12]. In the case of Cbz-Phe-His-Leu as a substrate, the picture is different: this substrate is similarly hydrolyzed at two domains of human ACE, whereas the hydrolysis is faster at the N-domain of the bovine enzyme [20].

When inhibitory analysis is performed by conventional kinetic approaches, there is a question whether a substrate affects the resulting values of the inhibition kinetic constants K_i. The interpretation of findings can be

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simplified by elimination of the substrate from the system. There are some methodological approaches for this purpose: equilibrium dialysis, the use of immobilized inhibitors, fluorescence quenching during complex formation, resonance energy transfer from an enzyme fluorophore onto an inhibitor fluorophore, and fluorescence polarization technique.

The last approach [22] is now widely used in studies of the interaction of antibodies with antigens [23, 24], but it also seems promising for studies of complex formation in other systems that consist of components of different molecular weights such as proteins and their low-molecular-weight ligands [25]. This approach is essentially based on the different mobility of the fluorophore incorporated in molecules with different weights. When a solution containing small and rapidly rotating ligand molecules with a fluorophore group is exposed to a plane-polarized light, the light emitted will be virtually completely depolarized because the excited molecules will change their orientation in space within their lifetime. On the contrary, in the case of a solution containing large molecules of a protein–ligand complex, the light emitted will retain its polarization. The determination of the vertical (I_{vert}) and horizontal (I_{hor}) components of the emitted light provides the fluorescence polarization value:

$$P = \frac{I_{vert} - I_{hor}}{I_{vert} + I_{hor}} \quad . \qquad (1)$$

For determination of the fluorescence polarization, the unit *mP*: 1 $mP = 1000 \cdot P$ is generally used.

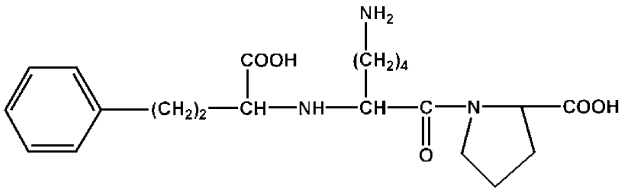
Using the fluorescence polarization technique, in the present work we compared the inhibition of three forms of bovine ACE (the somatic isoform, the testicular isoform, and the isolated N-domain) by a derivative of a competitive inhibitor of ACE, lisinopril supplemented with a fluorescent label.

MATERIALS AND METHODS

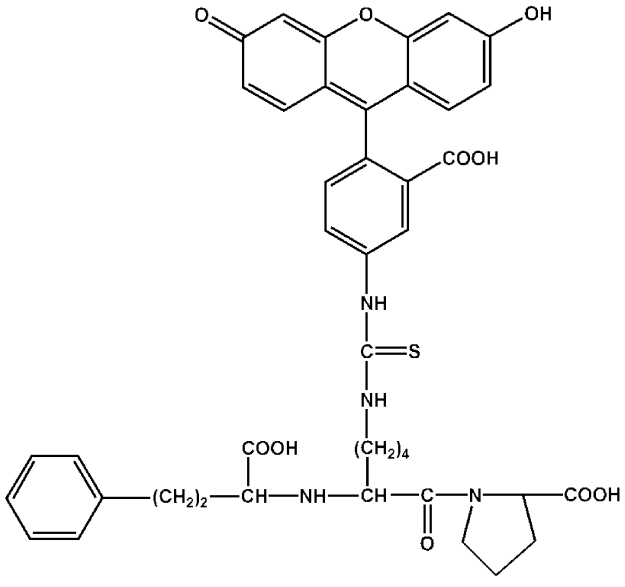
Reagents used. N-3-(2-furyl)-acryloyl-L-phenylalanyl-glycylglycine (FA-Phe-Gly-Gly), N-(S-1-carboxy-3-phenylpropyl)-L-lysyl-L-proline (lisinopril), N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid (HEPES), and fluorescein isothiocyanate (isomer I) were from Sigma (USA); other reagents were of chemical purity and analytical purity grades.

Lisinopril was modified with fluorescein isothiocyanate (F-lisinopril was prepared) as described earlier [26] as follows: 10 mg (~25 μmoles) lisinopril (Fig. 1) was dissolved in 0.5 ml of methanol, then 50 μl of triethylamine and 10 mg (~25 μmoles) fluorescein isothiocyanate were added, and the mixture was left for 24 h in the dark at room temperature. The resulting F-lisinopril (Fig. 1) was separated from the unreacted substances by TLC on a Silufol plate using chloroform–methanol (4 : 1). The substance from the band with $R_f = 0.3$ displayed both the lisinopril-specific activity to ACE and the ability for light absorption at the wavelength of 492 nm that is specific for fluorescein. The product was collected and dissolved in methanol. The repeated running of the substance of this band and subsequent development of the chromatogram with iodine or ninhydrin showed the absence of admixtures of both the initial fluorescein ($R_f = 0.5$) and of lisinopril ($R_f = 0$). The concentration of the resulting F-lisinopril was determined by the molar extinction coefficient in aqueous solution $\varepsilon_{492} = 8.78 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ (0.5 M carbonate buffer, pH 9.6) [27]. F-Lisinopril was stored in methanol solution at −20°C.

Somatic ACE from bovine lungs was prepared by extraction of the enzyme with buffer in the presence of Triton X-100 (Ferak, Germany) with subsequent affinity chromatography on lisinopril-agarose as described in [28].



Lisinopril



F-Lisinopril

Fig. 1. Structures of lisinopril and of F-lisinopril.

Testicular ACE from bovine testes was prepared as described in [29]: the enzyme was extracted with buffer in the presence of Triton X-100 and subjected to cascade affinity chromatography on phenyl-silochrome and lisinopril-agarose.

The N-domain of somatic ACE was prepared by limited proteolysis of the somatic enzyme form with trypsin as described in [20] with subsequent separation of the ACE N-domain and the initial somatic enzyme by gel chromatography on Sephadex G-200 superfine (Pharmacia, Sweden).

Homogeneity of the three forms of ACE was tested by polyacrylamide gel electrophoresis in the presence of 0.1% SDS and β-mercaptoethanol using the method of Laemmli [30] and by determination of the concentrations of the enzyme active molecules by stoichiometric titration with the competitive ACE inhibitor lisinopril as described in [31] with FA-Phe-Gly-Gly as a substrate.

Constants of ACE inhibition by lisinopril and F-lisinopril and also the enzyme concentration were determined by the decrease in the activity on varying the inhibitor concentration at different concentrations of the substrate. Reaction mixture (0.9 ml) of the inhibitor (10^{-9}-10^{-7} M) and 10^{-8} M ACE in 50 mM Hepes-buffer, pH 7.5, containing 150 mM NaCl and 1 μM $ZnCl_2$ was incubated for 30 min at 37°C in a spectrophotometric cuvette. Then 100 μl of the substrate FA-Phe-Gly-Gly was added at varied concentration (4-10 mM), the reaction mixture was stirred, and the rates of changes in the absorption at wavelengths 335, 340, and 345 nm were recorded with a Shimadzu UV-265FW spectrophotometer (Japan). The reaction rate was calculated using differential molar extinction coefficients $\varepsilon_{(s\text{-}p)}$ = 1900, 1120, and 575 $M^{-1}\cdot cm^{-1}$, respectively. The data were processed by plotting the dependences in the coordinates of Dixon [32] and Henderson [33].

Dissociation constants of ACE–F-lisinopril complexes were determined by two approaches.

1. The complete curve of the labeled inhibitor titration with the enzyme was obtained (Fig. 2). To do this, solutions with a fixed concentration of F-lisinopril (10^{-8} M) and varied (10^{-10}-$5\cdot10^{-7}$ M) concentrations of the enzyme in 50 mM Hepes-buffer, pH 7.5, containing 150 mM NaCl and 1 μM $ZnCl_2$ were incubated for 30 min at 37°C to reach equilibrium. Then the fluorescence polarization was determined with a TDX Analyzer (ABBOT, USA) or with a BEACON 2000 (Panvera, USA). Concentrations of the enzyme–inhibitor complex and of the free enzyme and free inhibitor were calculated from the fluorescence polarization values for each experimental point using the following relations [22]:

$$P_m = \frac{P_b \cdot C_b + P_f \cdot C_f}{C_b + C_f}, \quad (2)$$

$$C_f = I_0 - C_b, \quad (3)$$

$$C_f(E) = E_0 - C_b, \quad (4)$$

$$C_b = \left(\frac{P_m - P_f}{P_b - P_f}\right) \cdot I_0, \quad (5)$$

where P_m are the fluorescence polarization values determined at different concentrations of the enzyme in the reaction mixture; P_f is the fluorescence polarization value for the free inhibitor (which corresponds to the lower plateau value in the complete titration curve); P_b is the polarization of the enzyme–inhibitor complex (the upper plateau value in the titration curve); I_o and E_o are the total concentrations of the inhibitor and of the enzyme, respectively; C_f and $C_f(E)$ are the equilibrium concentrations of the free inhibitor and enzyme; C_b is the concentration of the enzyme–inhibitor complex. The processing of C_b and $C_f(E)$ values in the $C_b/C_f(E)$ versus C_b coordinates (Scatchard's coordinates) provides the dissociation constant of the ACE–lisinopril complex.

2. In some experiments, the method described in [34] was used. This method can be used in the case of failure to obtain the upper plateau of the titration curve to calculate the position of this plateau based on the following premises.

From (2) the following relation holds:

$$\frac{C_b}{C_f} = \frac{P_m - P_f}{P_b - P_m}. \quad (6)$$

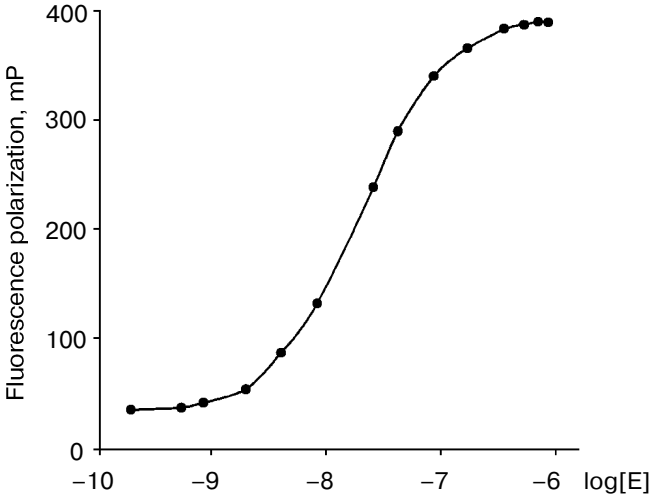


Fig. 2. Complete curve of F-lisinopril titration with ACE. Conditions: 50 mM Hepes-buffer, pH 7.5, 150 mM NaCl, 1 μM $ZnCl_2$, 37°C.

From the Scatchard equation obtain:

$$\frac{C_b}{C_f} = \frac{C_{b\,max}}{K_d} - \frac{C_b}{K_d}, \quad (7)$$

where K_d is the dissociation constant; $C_{b\,max}$ is the maximum quantity of the enzyme that can be involved in the complex formation. In the case of one-site interactions, $C_{b\,max}$ is equal to the concentration of the inhibitor labeled.

At $C_b \to 0$ obtain:

$$\frac{C_b}{C_f} = \frac{C_{b\,max}}{K_a}, \quad (8)$$

let $P_m \to P'$, then, taking into account (6), obtain:

$$P' = P_b - \frac{K_a \cdot (P' - P_f)}{K_a \cdot C_{b\,max}} \cdot \quad (9)$$

This approach that provides a significant decrease in the enzyme expenditure during the experiments is performed essentially as follows: for certain fixed concentrations of the inhibitor in the range of 10^{-9}-$2\cdot10^{-8}$ M, the fluorescence polarization P_m was measured at certain concentrations of the enzyme which were the same for each concentration of the inhibitor (the enzyme concentrations were varied from $5\cdot10^{-9}$ to $5\cdot10^{-8}$ M). Processing the findings in coordinates of P_m/P_f versus I_o resulted in a set of straight lines. The intercepts cut by these lines on the Y-axis determined the limiting values P' for each concentration of the enzyme chosen. The further processing of the findings in coordinates P' versus $(P' - 1)/[E]_o$ provides from the intercept on the Y-axis the enzyme–inhibitor polarization value normalized to the free inhibitor polarization P_b/P_f. As we have the fluorescence polarization of the free inhibitor P_f obtained experimentally, it is easy to obtain from the P_b/P_f value the fluorescence polarization P_b of the enzyme–inhibitor complex. Then, as in the first case, the value of K_d for the ACE–F-lisinopril complex was obtained by processing the data in the Scatchard's coordinates.

Determination of the apparent dissociation constant of the ACE–Cl^- complex. To obtain the complete titration curve on the binding of chloride with ACE, the concentrations of the enzyme and of F-lisinopril were maintained constant at $3\cdot10^{-7}$ and 10^{-8} M, respectively, and the concentration of NaCl was varied in the range from 0 to 0.3 M. The reaction mixture supplemented with all components was incubated in 50 mM Hepes-buffer, pH 7.5, containing 1 μM $ZnCl_2$ for 30 min at 37°C, and then the fluorescence polarization was determined. The data were processed, and the apparent K_{Cl} was determined by plotting the $C_b/[Cl^-]$ dependences on C_b, where C_b was the concentration of the ACE–Cl^-–F-lisinopril complex calculated by formula (5).

Dissociation kinetics of the ACE–F-lisinopril complexes were followed by changes with time in the fluorescence polarization on the displacement of F-lisinopril from its complex with the enzyme (with the somatic or testicular enzyme or with the N-domain) by excess lisinopril. The typical experiment was performed as follows: 1 ml of 10^{-8} M F-lisinopril and $(1\text{-}5)\cdot10^{-7}$ M ACE in 50 mM Hepes-buffer, pH 7.5, containing 150 mM NaCl and 1 μM $ZnCl_2$ were incubated for 30 min at 37°C, and then the fluorescence polarization of the resulting complex was determined. Afterwards, 10 μl of lisinopril in the same buffer was added to the final concentration of 10^{-4} M, the cuvette was shaken rapidly, and changes in the fluorescence polarization with time were determined using the BEACON 2000. From the resulting values of the fluorescence polarization, the concentrations of the free and bound inhibitors were calculated for every time point as described above. The calculations and data processing by nonlinear regression methods and the estimation of errors were performed with the Sigma Plot program (SPSS Inc., USA).

RESULTS AND DISCUSSION

The most widely used inhibitors of ACE have no intrinsic fluorophores. Therefore, a fluorescent label was incorporated into the competitive peptide inhibitor lisinopril. Lisinopril is one of the most effective inhibitors of ACE, the inhibition constant for the human enzyme being $3.9\cdot10^{-10}$ M [35]. The inhibition constants determined by us with lisinopril for three forms of bovine ACE were $(1.8 \pm 0.2)\cdot10^{-10}$, $(7.4 \pm 0.8)\cdot10^{-11}$, and $(2.0 \pm 0.1)\cdot10^{-10}$ M for somatic ACE, testicular ACE, and the N-domain, respectively. Thus, unlike human ACE, which is characterized by the most efficient lisinopril inhibition of the active site on the C-domain [35], both active sites of bovine ACE are inhibited by lisinopril similarly.

The amino group of lisinopril is accessible for modification. We used fluorescein isothiocyanate for labeling. The structures of the resulting labeled inhibitor and of the initial lisinopril are presented in Fig. 1.

To determine by fluorescence polarization the dissociation constant (K_d) of the enzyme–inhibitor complex ACE–F-lisinopril, the complete curve of inhibitor titration with the enzyme was plotted. A typical curve in semilogarithmic coordinates is presented in Fig. 2. However, it is known that both the ACE activity and the efficiency of its inhibition are strongly dependent on the chloride concentration in the reaction medium. This is exemplified in Fig. 3 by titration curves of the somatic enzyme with F-

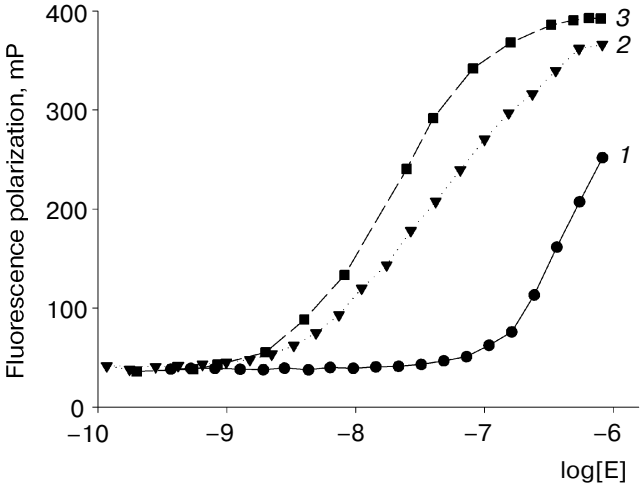


Fig. 3. Titration curves of labeled lisinopril with the bovine somatic enzyme in the absence of chloride (*1*) and at 0.05 M (*2*) and 0.3 M (*3*) NaCl. Conditions: 50 mM Hepes-buffer, pH 7.5, 1 μM $ZnCl_2$, 37°C.

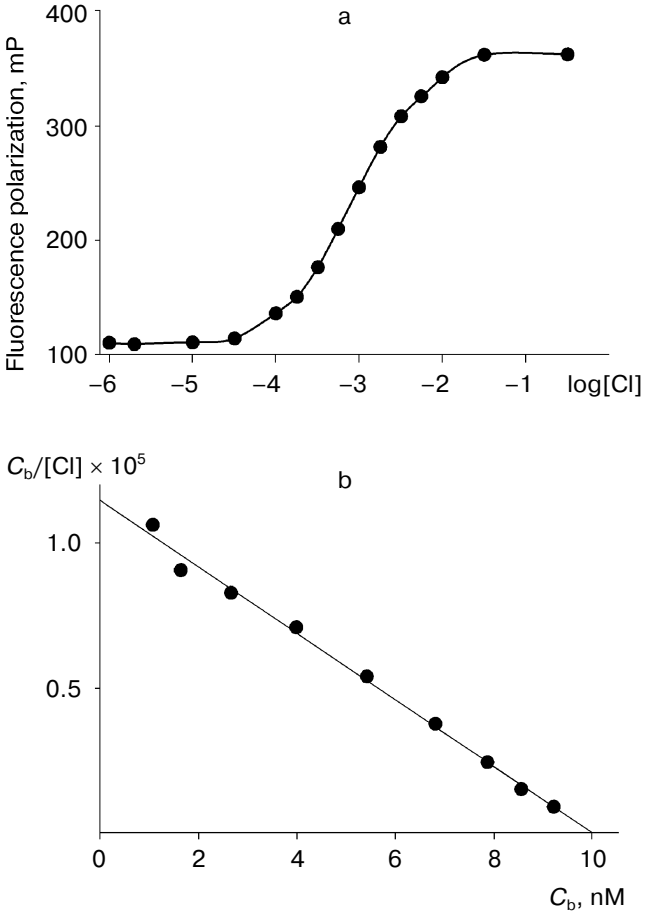


Fig. 4. Determination of the apparent constant K_d of the ACE–chloride complex. a) The general appearance of the complete titration curve with sodium chloride of the bovine somatic enzyme ($3 \cdot 10^{-7}$ M) and F-lisinopril (10^{-8} M) mixture; b) processing of this curve in Scatchard coordinates. Conditions: 50 mM Hepes-buffer, pH 7.5, 1 μM $ZnCl_2$, 37°C.

lisinopril at varied concentrations of chloride. Thus, the fluorescence polarization allowed us to calculate the apparent dissociation constant for the enzyme–chloride complex. The value of this constant was obtained from the titration curve with chloride at constant concentrations of the enzyme and of the inhibitor (Fig. 4a). The data processing in Scatchard's coordinates (Fig. 4b) gave the K_{Cl} value of $(1.3 \pm 0.2) \cdot 10^{-3}$ M.

However, the dissociation constant K_{Cl} of the chloride complex with the enzyme determined from the experimental kinetics is, in fact, an apparent constant and depends on both the substrate hydrolyzed and the inhibitor. Thus, for ACE from rabbit lungs this constant varied in the range $(3\text{-}150) \cdot 10^{-3}$ M on the hydrolysis of different substrates [36]. The K_{Cl} value of $2 \cdot 10^{-3}$ M calculated in [37], which was determined by the effect of chloride on the binding of dansyl derivatives of different inhibitors to ACE from rabbit lungs (i.e., in the absence of substrate), was close to the lowest value of the apparent dissociation constant for the enzyme–chloride complex found for the rabbit enzyme and also close to the K_{Cl} value found by us for ACE from bovine lungs.

Then the dissociation constants K_d of the enzyme–inhibitor complex ACE–F-lisinopril were determined at 150 mM NaCl. On plotting the complete titration curve, the fluorescence polarization value of the free inhibitor (P_f) was 36 ± 2 mP. The dependence of the fluorescence polarization on the complex weight is a curve with saturation, and the complex weight on this saturation depends on the lifetime of the excited fluorophore. For fluorescein, such saturation had to occur at molecular weight of the complex above 80 kD [38]. In our case, we exceeded this limit for all forms of ACE; this is why the fluorescence polarization value for the enzyme–inhibitor complex (P_b) was 390 ± 10 mP regardless of the enzyme form.

The processing of points in the ascending part of the titration curves in Scatchard's coordinates (Fig. 5) resulted in values of K_d for the enzyme–inhibitor complex as follows: $(2.3 \pm 0.4) \cdot 10^{-8}$, $(2.1 \pm 0.3) \cdot 10^{-8}$, and $(2.1 \pm 0.2) \cdot 10^{-8}$ M for two-domain somatic ACE, single-domain testicular ACE, and for the N-domain of the enzyme, respectively. The inhibition constant of bovine somatic ACE by F-lisinopril determined by suppression of hydrolysis of FA-Phe-Gly-Gly was $(1.8 \pm 0.3) \cdot 10^{-8}$ M, i.e., the resulting value of K_d was in agreement with the corresponding value of K_i within the error limits. This finding demonstrates the equivalence of results obtained by these two approaches. The comparison of these constants for unmodified lisinopril and for F-lisinopril suggests that the introduction of the voluminous label should cause steric hindrances for penetration of the inhibitor to the active site of the enzyme resulting in a two-order-of-magnitude deterioration of the inhibition constant.

Note, that although the somatic enzyme molecule has two active sites, the concentration determined by stoichiometric titration with inhibitor never exceeded the

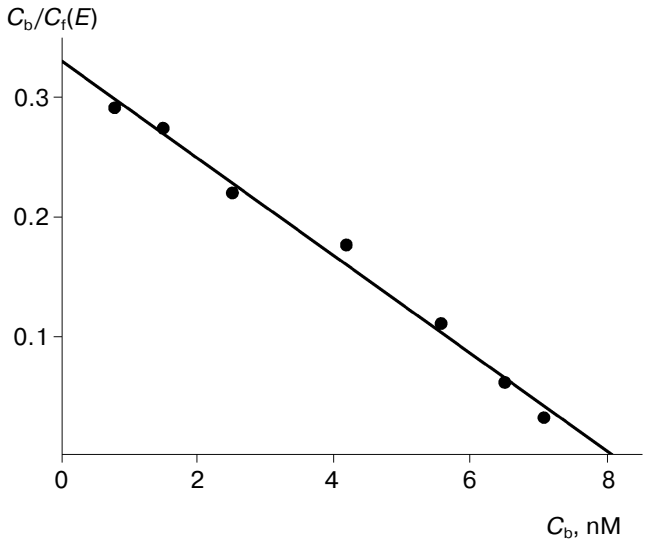


Fig. 5. Processing of the complete titration curve of the labeled inhibitor with bovine somatic ACE in Scatchard's coordinates.

protein concentration of the enzyme [20, 31]. Thus, during the titration with lisinopril the behavior of the full-sized ACE was the same as the behavior of the enzyme with only one active site. We have shown that under conditions of stoichiometric titration [33] with lisinopril and also under conditions of non-stoichiometric titration [33] with F-lisinopril only one site of the somatic enzyme was titrated.

The fluorescence polarization method allows us to follow the formation or dissociation of complexes with time. The ACE–F-lisinopril complex formation was rapid and virtually complete within the dead-time of the device, which was 12 sec in our case.

Kinetics of the significantly slower dissociation of the enzyme–inhibitor complex was followed during the displacement of F-lisinopril from the equilibrium complex by a large excess of unlabeled lisinopril. The typical time dependence of the complex concentration after addition of the unlabeled inhibitor is shown in Fig. 6.

The findings for somatic ACE could not be described by the first-order curve (Fig. 6, curve *1*). Nonlinear regression methods provided the simplest equation that adequately described the findings:

$$[EI]_t = a_1[EI]_0 \cdot e^{-k1t} + a_2[EI]_0 \cdot e^{-k2t}, \qquad (10)$$

where $[EI]_0$ is the initial concentration of the enzyme–inhibitor complex and $[EI]_t$ is the concentration of the enzyme–inhibitor complex at time t. This equation describes the model of parallel dissociation of two complexes with rate constants k_1 and k_2, whereas the parameters a_1 and a_2 show the contribution of each component.

Here the k_1 and k_2 values are 0.13 ± 0.01 and 0.026 ± 0.001 sec^{-1}, respectively, whereas a_1 and a_2 are 0.43 and 0.57, respectively, i.e., with similar concentrations of the complexes the rate constants of their dissociation differ fivefold.

The dissociation kinetics of the enzyme–inhibitor complexes for single-domain ACE forms were significantly different from the dissociation kinetics for the two-domain form (Fig. 6, curves *2* and *3*). The findings are linearized in the first-order coordinates and are excellently approximated by the dissociation model for a single complex with the rate constants of 0.055 ± 0.001 and 0.041 ± 0.001 sec^{-1} for the N-domain and for testicular ACE, respectively.

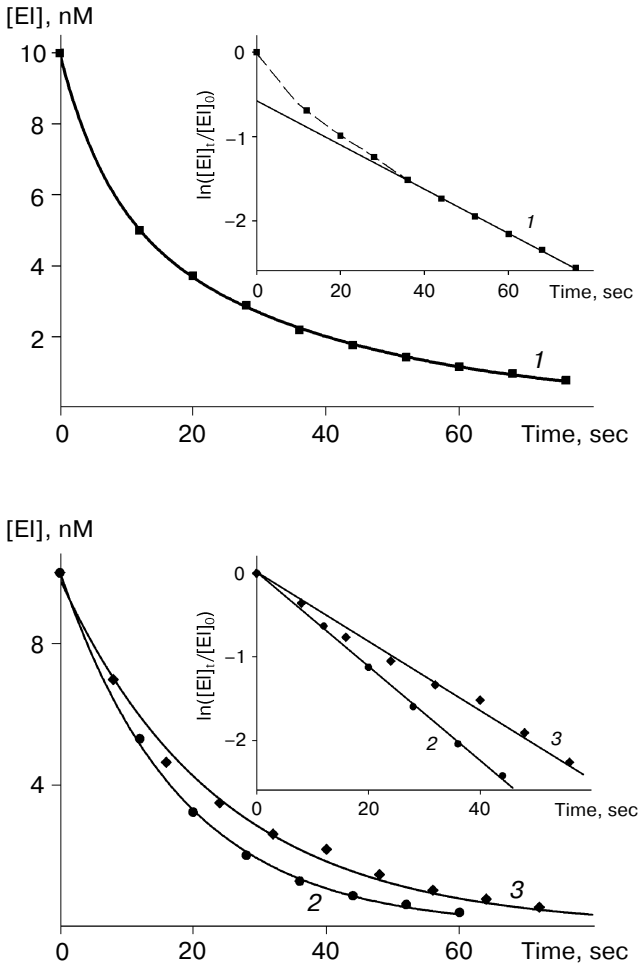


Fig. 6. Dissociation kinetics of the enzyme–inhibitor complex in the case of: the somatic enzyme (*1*), the N-domain (*2*), and the testicular enzyme (*3*). The points correspond to experimental data; the lines were calculated theoretically. The inserts present the data processing in first-order coordinates. Conditions: 50 mM Hepes-buffer, pH 7.5, 150 mM NaCl, 1 μM $ZnCl_2$, 37°C.

Thus, two populations of complexes found for the somatic enzyme seem to be the inhibitor complexes with different domains of the protein. Because the ratio of these two forms in the equilibrium complex is 1 : 1, it is suggested that in the case of somatic bovine ACE the inhibitor binding to either of the enzyme sites should be virtually equally probable. This is supported by substantial coincidence of the dissociation constants for complexes formed by the N- and C-domains of ACE. And the lack of coincidence of the dissociation rate constants of the enzyme–inhibitor complexes found for the single-domain forms of ACE with the dissociation rate constants found for the complex with the two-domain form suggests that the two catalytic sites of the full-size somatic enzyme should display some mutual influence.

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