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Evidence for the negative cooperativity of the two active sites within bovine somatic angiotensin-converting enzyme

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Abstract The somatic isoform of angiotensin-converting enzyme (ACE) consists of two homologous domains (N- and C-domains), each bearing a catalytic site. We have used the two-domain ACE form and its individual domains to compare characteristics of different domains and to probe mutual functioning of the two active sites within a bovine ACE molecule. The substrate Cbz-Phe-His-Leu (*N*-carbobenzoxy-L-phenylalanyl-L-histidyl-L-leucine; from the panel of seven) was hydrolyzed faster by the N-domain, the substrates FA-Phe-Gly-Gly (*N*-(3-[2-furyl]acryloyl)-L-phenylalanyl-glycyl-glycine) and Hip-His-Leu (*N*-benzoyl-glycyl-L-histidyl-L-leucine) were hydrolyzed by both domains with equal rates, while other substrates were preferentially hydrolyzed by the C-domain. The inhibitor captopril ((2*S*)-1-(3-mercapto-2-methylpropionyl)-L-proline) bound to the N-domain more effectively than to the C-domain, whereas lisinopril ((*S*)-N^{α}-(1-carboxy-3-phenylpropyl)-L-lysyl-L-proline) bound to equal extent with all ACE forms. However, active site titration with lisinopril assayed by hydrolysis of FA-Phe-Gly-Gly revealed that 1 mol of inhibitor/mol of enzyme abolished the activity of either two-domain or single-domain ACE forms, indicating that a single active site functions in bovine somatic ACE. Neither of the k_{cat} values obtained for somatic enzyme was the sum of k_{cat} values for individual domains, but in every case the value of the catalytic constant of the hydrolysis of the substrate by the two-domain ACE represented the mean quantity of the values of the corresponding catalytic constants obtained for single-domain forms. The results indicate that the two active sites within bovine somatic ACE exhibit strong negative cooperativity.

Key words: Angiotensin-converting enzyme; N-domain; C-domain; Negative cooperativity

1. Introduction

Angiotensin-converting enzyme (ACE, peptidyl dipeptidase A, EC 3.4.15.1) is a type-I membrane-anchored glycoprotein, a member of the gluzincins, defined by a HExxH motif and a glutamic acid residue as the third zinc ligand [1]. This enzyme is essential for blood pressure control and water–electrolyte homeostasis through the renin–angiotensin–aldosterone system [2].

Two distinct ACE isoenzymes have been identified in mammalian tissues, the primary structure of which was determined by molecular cloning and sequencing of the complementary DNA [3,4]. The somatic form of ACE (1277 amino acids in human enzyme) consists of two homologous domains (N- and C-domains) within a single polypeptide chain, and each domain bears its own catalytic site [3]. The tertiary structure of somatic ACE is still unknown.

The other ACE isoenzyme is present exclusively in testes and is associated with mature germ cells [5]. Testicular ACE is identical to the C-terminal half of the somatic enzyme except for a short N-terminal sequence and thus contains only one catalytic site [4]. The crystal structure of testicular ACE was recently described [6].

The N-domain of ACE was also found in humans, and this ACE form is believed to be a result of limited proteolysis of the parent somatic form [7]. Several studies indicate that 'the bridge sequence' between the two domains can be cleaved in vitro. Native human ACE molecule was found to be susceptible to endoproteinase Asp-N producing both domains of the enzyme [8], while a variety of serine proteases were able to cleave partially denatured ACE [9–11]. The latter approach always results in the isolation of only the ACE N-domain [10,11], whereas the remaining part of the molecule undergoes denaturation and proteolytic digestion. Moreover, different heat stabilities of the domains allow selective inactivation of the C-domain and underlie the method of acquiring an active N-domain with inactive C-domain within a full-length ACE [12]. The possibilities described above suggest that the two domains within the somatic ACE molecule are rather separate and distinctive structures.

The two catalytic sites within the somatic ACE molecule were long considered to function independently, as the activity of the wild-type enzyme was found to be equal to the sum of the activities of the N- and C-domains [13]. This observation contradicted the fact that titration of the two-domain human somatic ACE and the single-domain rabbit testicular ACE with competitive inhibitor lisinopril ((*S*)-N^{α}-(1-carboxy-3-phenylpropyl)-L-lysyl-L-proline) and FA-Phe-Gly-Gly (*N*-(3-[2-

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Abbreviations: ACE, angiotensin-converting enzyme; FA-Phe-Gly-Gly, *N*-(3-[2-furyl]acryloyl)-L-phenylalanyl-glycyl-glycine; FA-Phe-Ala-Ala, *N*-(3-[2-furyl]acryloyl)-L-phenylalanyl-L-alanyl-L-alanine; FA-Phe-Ala-Lys, *N*-(3-[2-furyl]acryloyl)-L-phenylalanyl-L-alanyl-L-lysine; FA-Phe-Ala-Pro, *N*-(3-[2-furyl]acryloyl)-L-phenylalanyl-L-alanyl-L-proline; FA-Phe-Phe-Arg, *N*-(3-[2-furyl]acryloyl)-L-phenylalanyl-L-phenylalanyl-L-arginine; Hip-His-Leu, *N*-benzoyl-glycyl-L-histidyl-L-leucine; Cbz-Phe-His-Leu, *N*-carbobenzoxy-L-phenylalanyl-L-histidyl-L-leucine; captopril, (2*S*)-1-(3-mercapto-2-methylpropionyl)-L-proline; lisinopril, (*S*)-N^{α}-(1-carboxy-3-phenylpropyl)-L-lysyl-L-proline

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furyl]acryloyl)-L-phenylalanyl-glycyl-glycine) as a substrate revealed only a single active site in both enzyme forms [14]. These results were explained by preferential binding of both substrate and inhibitor at the assay conditions on the C-domain only [14]. Further studies, however, revealed that the activity of somatic ACE often could not be represented by a sum of the activities of the two domains [11,15–17]. This observation led to the idea [8,17] that the two domains can no longer be considered as totally independent. The arrangement of the two domains within the somatic ACE molecule and the kinetic mechanism of the mutual functioning of the two active sites still defy description.

Here we present evidence that the two active sites within the bovine ACE molecule are absolutely dependent, which, in turn, implies tight proximity of the two domains within the somatic enzyme molecule.

2. Materials and methods

2.1. Enzyme purification

Bovine lung and bovine testis ACEs were purified by lisinopril affinity chromatography as described in [18]. The N-domain of ACE was obtained by limited proteolysis of the parent somatic ACE after partial denaturation of the enzyme in NH_4OH solution as described in [11]. All ACE preparations were proved to be homogeneous according to electrophoresis by the Laemmli method [19] in polyacrylamide gel in the presence of 0.1% SDS and β-mercaptoethanol. Proteins were stained with Coomassie brilliant blue G-250. Protein concentration was determined according to the modified Lowry method [20,21].

2.2. Inhibition of ACE activity

For all kinetic experiments, we chose nearly physiological conditions, namely, pH 7.5, 150 mM NaCl, 1 μM $ZnCl_2$. Assays with inhibitors were performed with Cbz-Phe-His-Leu (*N*-carbobenzoxy-L-phenylalanyl-L-histidyl-L-leucine) as a substrate. Because both captopril ((2*S*)-1-(3-mercapto-2-methylpropionyl)-L-proline) and lisinopril are competitive slow-tight binding inhibitors, the preincubation time required to produce equilibrium between enzyme and inhibitor was preliminarily determined [22]. Prior to the addition of substrate (100 μl), enzyme was preincubated with inhibitors for 3 h at 37°C in a total volume of 1.9 ml. All ACE forms were found to be stable during this period of time. The values of inhibition constants, K_i, were determined at five substrate concentrations within 0.5–3 K_m; the enzyme concentration in the reaction medium was equal to the K_i value determined in the preliminary experiment; the inhibitor concentration was varied from 0.5 to 10 $[E]_0$. Apparent ACE inhibition constants, K_i^{app}, were determined by linearization of the data in Henderson coordinates [23], $[I]_0/(1-v_i/v_0)$ versus v_0/v_i, where $[I]_0$ is the total inhibitor concentration and v_0 and v_i are the initial rates in the absence and in the presence of the inhibitor. The true values of the inhibition constants, K_i, were calculated by graphical extrapolation of K_i^{app} to $[S]_0 = 0$.

2.3. Kinetic characterization

Stoichiometric titration of ACE active molecules was performed with specific competitive inhibitor lisinopril as in [11]. The rates of catalytic hydrolysis of FA-containing substrates were monitored spectrophotometrically by the method of [24]. The substrates FA-Phe-Phe-Arg (*N*-(3-[2-furyl]acryloyl)-L-phenylalanyl-L-phenylalanyl-L-arginine), FA-Phe-Ala-Lys (*N*-(3-[2-furyl]acryloyl)-L-phenylalanyl-L-alanyl-L-lysine), Hip-His-Leu (*N*-benzoyl-glycyl-L-histidyl-L-leucine), and Cbz-Phe-His-Leu were initially dissolved in methanol; the final methanol concentration in the reaction medium was 5%. Hip-His-Leu and Cbz-Phe-His-Leu hydrolyses were followed fluorimetrically by the release of His-Leu, which was derivatized with *o*-phthalaldehyde [25]. Initial rates were measured during the first 5% of substrate hydrolysis. The kinetic constants K_m and k_{cat} were calculated by the Lineweaver–Burk analysis from at least three independent experiments. The standard deviations of the K_m and k_{cat} values were less than 10%.

3. Results

3.1. Inhibition of ACE activity

Cbz-Phe-His-Leu hydrolysis by the two-domain and single-domain ACE forms was inhibited in a dose-dependent manner by the specific competitive ACE inhibitors, lisinopril and captopril. The true values of the inhibition constants K_i are presented in Table 1. Previously [22], we have shown that the K_i values obtained for testicular and somatic bovine ACEs do not depend on the nature of the tripeptide substrate. The data of Table 1 indicate that captopril preferably binds to the N-domain of bovine ACE while lisinopril equally inhibits all ACE forms.

3.2. Active site titration

Under conditions of $[E]_0$, $[I]_0 \gg K_i$, the reaction

$$E + I \rightleftharpoons EI$$

is driven predominantly to the right, i.e. to the formation of the enzyme–inhibitor complex. The linear parts of the plots of residual activity versus $[I]_0$ extrapolated to the *x*-axis give intersection points representing moles of the inhibitor per mol of ACE required to abolish enzymatic activity [26]. We titrated the number of active sites in three ACEs with lisinopril, the inhibitor with equal inhibitory potency towards single-domain forms (Table 1). The results (Fig. 1) indicate that for the two-domain somatic ACE and for both single-domain ACE forms, binding of 1 mol of lisinopril per 1 mol ACE was sufficient to block FA-Phe-Gly-Gly-hydrolyzing activity, suggesting that all three bovine ACE forms reveal one active site per molecule. Moreover, inhibition with lisinopril of Cbz-Phe-His-Leu-hydrolyzing activity also demonstrated that only one equivalent of the inhibitor was necessary to abolish the activity of all ACE forms (data not shown).

3.3. Kinetic parameters of the hydrolysis of synthetic tripeptide substrates

Kinetic parameters of the hydrolysis of different tripeptide substrates by the three bovine ACE forms are presented in Table 2. Apparent K_m values for the hydrolysis of all substrates appeared to be similar for the three ACE forms, suggesting similar affinities of the two domains for these substrates. However, the comparison of k_{cat} values revealed the difference between ACE domains. Amongst the seven tripeptides surveyed, only Cbz-Phe-His-Leu was preferentially hydrolyzed by the N-domain, whereas FA-Phe-Ala-Ala (*N*-(3-[2-furyl]acryloyl)-L-phenylalanyl-L-alanyl-L-alanine), FA-Phe-Ala-Lys, FA-Phe-Ala-Pro (*N*-(3-[2-furyl]acryloyl)-L-phenylalanyl-L-alanyl-L-proline), and FA-Phe-Phe-Arg were hydrolyzed faster by testicular ACE (C-domain). Both single-domain forms exhibit equal activity towards the substrates Hip-His-Leu and FA-Phe-Gly-Gly.

Table 1
Inhibitor-binding properties of the three forms of bovine ACE

Inhibitor	Enzyme K_i (M)		
	Somatic ACE	N-domain	Testicular ACE
Lisinopril	$(1.8 \pm 0.2) \times 10^{-10}$	$(2.0 \pm 0.1) \times 10^{-10}$	$(1.2 \pm 0.2) \times 10^{-10}$
Captopril	$(6.0 \pm 0.5) \times 10^{-10}$	$(5.0 \pm 0.2) \times 10^{-10}$	$(9.1 \pm 0.9) \times 10^{-9}$

Conditions: 50 mM HEPES, pH 7.5, 150 mM NaCl, 1 μM $ZnCl_2$, 37°C. Substrate, Cbz-Phe-His-Leu.

Although both domains in somatic ACE are active, the activity of somatic ACE never represented the sum of the corresponding activities of the single-domain forms (Table 2). Moreover, the k_{cat} value for the hydrolysis of any substrate by the two-domain ACE was the mean quantity of the k_{cat} values for the hydrolysis of this substrate by the single-domain forms.

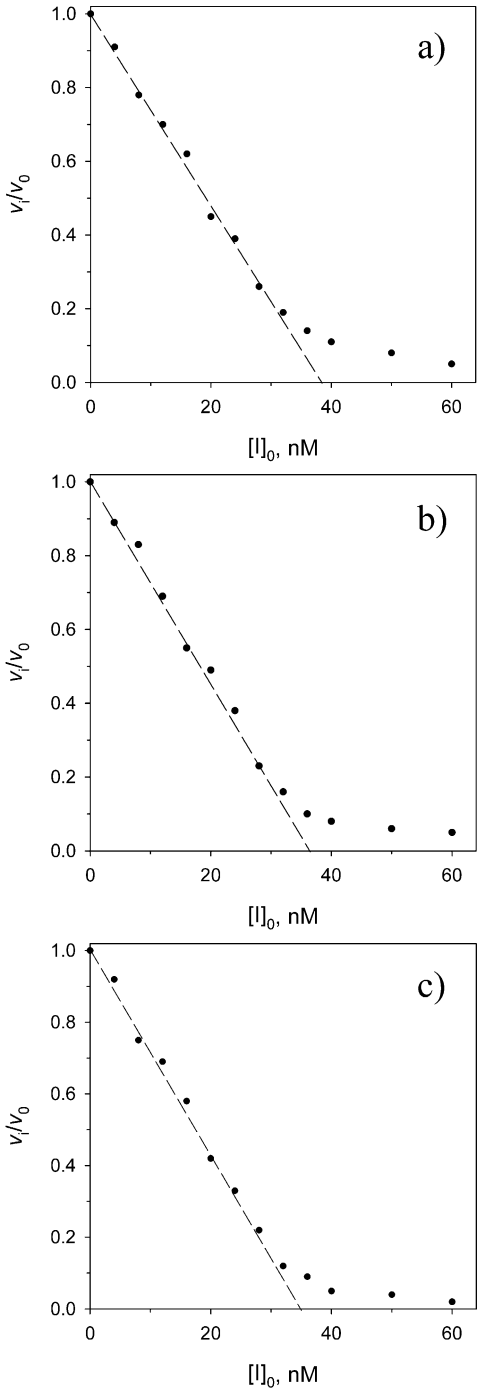

Fig. 1. Stoichiometric titration of active sites in ACE preparations with lisinopril. a: Somatic ACE; b: N-domain; c: testicular ACE (C-domain). Conditions: ACE solution (39 nM) in 50 mM HEPES-buffer, pH 7.5, containing 150 mM NaCl, 1 μM $ZnCl_2$, was incubated with 0–2.5 equiv. of lisinopril at 25°C for 30 min in 980 μl. Residual enzyme activities were then determined by adding 20 μl of 5 mM FA-Phe-Gly-Gly in the same buffer and measuring the initial rates of hydrolysis.

Table 2
Kinetic parameters for substrate hydrolysis by the three bovine ACE forms

	Somatic ACE	N-domain	Testicular ACE
FA-Phe-Gly-Gly			
k_{cat} (s^{-1})	280 ± 16[a]	279 ± 10[a]	260 ± 14
K_m (mM)	0.70 ± 0.06[a]	1.40 ± 0.02[a]	0.50 ± 0.02
Hip-His-Leu			
k_{cat} (s^{-1})	12 ± 1[a]	12 ± 1[a]	12 ± 1
K_m (mM)	0.90 ± 0.10[a]	0.50 ± 0.05[a]	0.60 ± 0.03
Cbz-Phe-His-Leu			
k_{cat} (s^{-1})	58 ± 5[a]	122 ± 10[a]	19 ± 2
K_m (mM)	0.25 ± 0.02[a]	0.15 ± 0.02[a]	0.13 ± 0.02
FA-Phe-Ala-Ala			
k_{cat} (s^{-1})	108 ± 11[a]	35 ± 3[a]	178 ± 15
K_m (mM)	0.05 ± 0.01[a]	0.05 ± 0.02[a]	0.05 ± 0.01
FA-Phe-Ala-Lys			
k_{cat} (s^{-1})	54 ± 2	35 ± 3	85 ± 7
K_m (mM)	0.15 ± 0.02	0.14 ± 0.01	0.17 ± 0.02
FA-Phe-Ala-Pro			
k_{cat} (s^{-1})	30 ± 3	8 ± 1	45 ± 1
K_m (mM)	0.008 ± 0.003	0.008 ± 0.003	0.005 ± 0.002
FA-Phe-Phe-Arg			
k_{cat} (s^{-1})	60 ± 5[a]	45 ± 4[a]	76 ± 8
K_m (mM)	0.05 ± 0.01[a]	0.05 ± 0.01[a]	0.12 ± 0.02

Conditions: 0.05 M HEPES, pH 7.5, 0.15 M NaCl, 1 μM $ZnCl_2$, 25°C.
[a]From [11].

4. Discussion

The discovery that ACE possesses two active sites was made more than a decade ago [3]; however, the mechanism of mutual functioning of the two active sites within the somatic ACE molecule is still unclear. Experiments with recombinant human ACE and various mutant forms with a single active site in the hydrolysis of Hip-His-Leu, angiotensin I, and bradykinin seemed to vindicate the hypothesis that the two active sites in full-length ACE function totally independently [13,15]. Recently, this hypothesis has received valuable support by findings of selective inhibitors able to differentiate ACE active sites [27–29]. The phosphinic peptide RXP 407 totally blocked the N-domain in vitro, but efficient hydrolysis of angiotensin I by the C-domain still occurred under these conditions [27]. This inhibitor also affected the metabolism of the negative regulator of hematopoiesis AcSDKP in mice without notable effect on angiotensin I metabolism [28]. Bradykinin-potentiating peptides selectively inhibiting the C-domain were described recently as well [29]. However, some uncertainty concerning the independence of ACE active sites still remains. The experiments with substance P and LH-RH as substrates did not comply with the scheme for full independence of the domains [15]. The data on the hydrolysis of angiotensin I and FA-Phe-Gly-Gly allowed the authors of [8] to conclude that the two domains may not function independently, and a thorough study of the hydrolysis of bradykinin-related peptides by recombinant wild-type ACE and its full-length mutants revealed that the two domains of ACE do not operate independently but may cooperate or coordinate [17].

In these studies we used, besides purified bovine somatic and testicular ACEs, purified bovine individual N-domain obtained by limited proteolysis of the parent somatic form.

Bovine somatic ACE has 84% overall homology in amino acid sequence with the human somatic enzyme [30]. The homology between the N-domains of bovine and human ACEs is 89%, while the homology between the C-domains of these enzymes is 82%.

Numerous previous studies of the recombinant variants of human ACE, testicular rabbit ACE (highly homologous to the C-domain of human ACE), and individual N-domain of human enzyme obtained from ileal fluid or by limited proteolysis of the somatic form demonstrated that, as a whole, the enzymatic properties of naturally occurring and recombinant individual domains are similar [7,10] and coincide with the properties of full-length recombinant ACEs with critical mutation in one of two domains [13,15,31]. Thus, the usage of different ACE forms allows us to compare catalytic properties of the individual domains of the enzyme and, furthermore, to assess the mutual functioning of the two active sites within the somatic ACE molecule.

We showed similar affinities of the two bovine ACE domains to the tripeptide substrates and to lisinopril at 150 mM NaCl (Tables 1 and 2). The latter observation is in contrast with earlier data for human recombinant enzyme [31], for which lisinopril was found to inhibit preferably the C-domain, but coincides with more recent data for the same enzyme [32]. The potency of the inhibitor captopril was 10 times more pronounced towards the bovine N-domain (Table 1). For comparison, captopril inhibited only slightly better the activity of the N-domain of human ACE with Hip-His-Leu as a substrate at 300 mM NaCl and inhibited much better the activity of the N-domain at low Cl^- concentration (20 mM) [31], while this inhibitor was reported to be 16 times more efficient for blocking the activity of the human N-domain, than the C-domain, with N-Ac-Ser-Asp-Lys-Pro as a substrate at 50 mM NaCl [16].

Two active sites of bovine ACE demonstrate equal catalytic constants in the hydrolysis of the substrates Hip-His-Leu and FA-Phe-Gly-Gly. The substrate Cbz-Phe-His-Leu is hydrolyzed faster on the N-domain, while other tripeptide substrates are hydrolyzed faster on the C-domain (Table 2). These data do not correspond to those for human ACE, for which Hip-His-Leu is preferentially hydrolyzed on the C-domain [13], while Cbz-Phe-His-Leu is hydrolyzed on both domains with equal rates [33]. Thus, despite overall protein homology, bovine and human ACEs exhibit species substrate specificity.

Active site titration with lisinopril assayed by hydrolysis of FA-Phe-Gly-Gly revealed that 1 mol of inhibitor/mol of enzyme abolished the activity of either two-domain or single-domain bovine ACE forms, indicating that only one active site functions in somatic ACE. These data are in agreement with the previous observation that both somatic human ACE and testicular rabbit ACE [14] exhibited one active site per molecule upon titration with lisinopril. These results were explained before by preferential binding of both inhibitor and substrate on the C-domain of the somatic enzyme [14]. However, both active sites in bovine ACE possess equal affinity to lisinopril and possess equal catalytic properties with respect to the hydrolysis of FA-Phe-Gly-Gly (Tables 1 and 2), suggesting that either one of the active sites is 'silent' in bovine ACE or binding of the inhibitor to one active site prevents hydrolysis of the substrate at another site. The existence of a 'silent' active site is highly unlikely as bovine ACE is highly homologous to the human enzyme, which has two functional active sites. Moreover, in a previous fluorescence polarization study [34] of different bovine ACE forms we showed the presence of two populations of complexes between somatic enzyme and fluorescent-labeled lisinopril in conditions of a significant lack of the inhibitor; that was attributed to the formation of complexes of labeled lisinopril with different ACE domains.

The analysis of kinetic parameters of the hydrolysis of the substrates by different ACE forms confirms the existence of a strong negative cooperativity between the two bovine ACE domains. If the values of K_m were almost similar for all ACE forms, the values of k_{cat} markedly differed, and that allows assessing the mutual functioning of the two active sites within the somatic enzyme. The k_{cat} value of the hydrolysis of definite substrate by somatic enzyme by no means either represented the sum of corresponding k_{cat} values for individual domains or was higher than the value obtained for the more active domain. Moreover, the k_{cat} value for the two-domain ACE was always the average quantity of the k_{cat} values for single-domain forms.

There are three simple schemes for the mutual functioning of the two active sites within a single enzyme:

A. Active sites within a two-domain enzyme function as two separate independent enzymes. Then, the value of the catalytic constant, k_{cat}, for the whole enzyme will be represented by the sum of the k_{cat} values for individual sites.

B. Only one active site of the two functions at any moment. Then, the kinetic constants for the whole enzyme are derived from combination of kinetic constants for individual sites:

$$k_{cat} = (k_{cat,1}/K_{m,1} + k_{cat,2}/K_{m,2})/(K_{m,1} + K_{m,2})$$

$$K_m = K_{m,1} \times K_{m,2}/(K_{m,1} + K_{m,2})$$

C. The enzyme can bind another substrate molecule on the second active site when the first site is already occupied, but the resulting complex SES is non-productive. Therefore, the Lineweaver–Burk plot can be expected to decline at high substrate concentration due to enzyme inhibition, and the maximum rate of enzymatic hydrolysis should be observed at substrate concentration $[S] = (K_{m,1} \times K_{m,2})^{1/2}$.

In the present case, variant A is immediately eliminated as we have already noted. Variant C should be eliminated as well, as any declining from the Lineweaver–Burk plot for FA-Phe-Phe-Arg that we have observed elsewhere [22] was marked for both somatic and single-domain forms. The description of mechanism B can be further simplified, as the K_m values of the hydrolysis of any substrate by single-domain ACE forms appeared to be virtually identical. Hence, the k_{cat} value for the whole enzyme can be represented as a mean quantity of the values of k_{cat} for individual sites:

$$k_{cat} = (k_{cat,1} + k_{cat,2})/2$$

Our results demonstrate that the values of k_{cat} of the hydrolysis of tripeptide substrates by three bovine ACE forms perfectly coincide with mechanism B. Thus, while both sites in bovine two-domain ACE are active, random binding of the substrate molecule to one of the active sites prohibits or dramatically decreases binding of another substrate molecule to the second site.

Titration of a single active site within two-domain ACE

with inhibitor lisinopril and FA-Phe-Gly-Gly (or Cbz-Phe-His-Leu) as a substrate is also easily explained by mechanism B. In this case, the inhibitor randomly binds to any active site in somatic ACE, but the second active site fails to bind the substrate and, therefore, to reveal enzymatic activity.

The results demonstrate strong negative cooperativity between the two active sites within bovine somatic ACE, which suggests tight proximity of the two domains within the ACE molecule. However, a full understanding of the precise arrangement of the domains and the nature of the interactions between domains waits for the definition of the three-dimensional structure of the somatic enzyme.

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