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Angiotensin-Converting Enzyme: Zinc- and Inhibitor-Binding Stoichiometries of the Somatic and Testis Isozymes†

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ABSTRACT: The blood pressure regulating somatic isozyme of angiotensin-converting enzyme (ACE) consists of two homologous, tandem domains each containing a putative metal-binding motif (HEXXH), while the testis isozyme consists of just a single domain that is identical with the C-terminal half of somatic ACE. Previous metal analyses of somatic ACE have indicated a zinc stoichiometry of 1 mol of Zn^{2+}/mol of ACE and inhibitor-binding studies have found 1 mol of inhibitor bound/mol of enzyme. These and other data have indicated that only one of the two domains of somatic ACE is catalytically active. We have repeated the metal and inhibitor-binding analyses of ACE from various sources and have determined protein concentration by quantitative amino acid analysis on the basis of accurate polypeptide molecular weights that are now available. We find that the somatic isozyme in fact contains 2 mol of Zn^{2+} and binds 2 mol of lisinopril (an ACE inhibitor) per mol of enzyme, whereas the testis isozyme contains 1 mol of Zn^{2+} and binds 1 mol of lisinopril. In the case of somatic ACE, the second equivalent of inhibitor binds to a second zinc-containing site as evidenced by the ability of a moderate excess of inhibitor to protect both zinc ions against dissociation. However, active site titration with lisinopril assayed by hydrolysis of furanacryloyl-Phe-Gly-Gly revealed that 1 mol of inhibitor/mol of enzyme abolished the activity of either isozyme, indicating that the principal angiotensin-converting site likely resides in the C-terminal (testicular) domain of somatic ACE and that binding of inhibitor to this site is stronger than to the second site. The second zinc- and inhibitor-binding site, instead, may be involved in a previously reported, unusual endoproteolytic cleavage of luteinizing hormone releasing hormone [Skidgel, R. A., & Erdös, E. G. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1025–1029], which we find is performed >300-fold faster by somatic compared to testis ACE. Taken together, these data indicate that the somatic enzyme may have an additional substrate specificity distinct from that for conventional substrates.

Angiotensin-converting enzyme (ACE;[1] peptidyl dipeptide hydrolase, EC 3.4.15.1) is a zinc-metallopeptidase that typically cleaves C-terminal dipeptides from oligopeptide substrates, as in its best-known substrate angiotensin I (Ehlers & Riordan, 1990). On the basis of numerous kinetic and chemical modification studies and on measured zinc stoichiometries of 1 mol of Zn^{2+}/mol of enzyme, ACE was inferred to contain a single carboxypeptidase A like active site [reviewed in Patchett and Cordes (1985); Ehlers & Riordan, 1990]. Indeed, the design of the current generation of potent, active site directed, zinc-coordinating inhibitors was based on this model (Ondetti et al., 1977; Cushman et al., 1977; Cushman & Ondetti, 1980; Patchett et al., 1980). These compounds are so effective that inhibition of the ACE-catalyzed conversion of angiotensin I to angiotensin II has become an important strategy in the treatment of hypertension and congestive cardiac failure (Gavras, 1990).

Recent results from molecular cloning have predicted that the somatic isozyme of ACE, the predominant isozyme that is widely distributed in mammalian tissues, consists of two homologous domains, each containing a putative metal-binding site of the type HEXXH (Soubrier et al., 1988), a motif that is common to many metallopeptidases exemplified by thermolysin but that differs from that present in carboxypeptidases (Vallee & Auld, 1990). There is also a unique testis isozyme of ACE that consists of and is essentially identical with the second, i.e., C-terminal domain of the somatic isozyme and thus contains only one of the two metal-binding motifs (Ehlers et al., 1989; Lattion et al., 1989; Kumar et al., 1989).

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[1] Abbreviations: ACE, angiotensin-converting enzyme; hTACE, recombinant human testis ACE; HK-ACE, ML-ACE, RL-ACE, and RT-ACE, human kidney, mouse lung, rabbit lung, and rabbit testis ACE, respectively; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Fa-FGG, 2-furanacryloyl-L-phenylalanylglycylglycine; Hepes, N-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; LH-RH, luteinizing hormone releasing hormone; Dnp-F, 1-fluoro-2,4-dinitrobenzene; Dnp, dinitrophenyl; OP, 1,10-phenanthroline; TFA, trifluoroacetic acid; buffer A, 50 mM Hepes, pH 7.5/0.3 M NaCl; HPLC, high-performance liquid chromatography.

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We (Ehlers et al, 1991) have confirmed the previous observation (El-Dorry et al., 1982) that the two ACE isozymes are kinetically identical with respect to typical substrates and chloride activation. Moreover, chemical modification of both isozymes with 1-fluoro-2,4-dinitrobenzene (Dnp-F) indicated that two critical residues, a tyrosine and a lysine, were present only in the C-terminal (testicular) domain of somatic ACE, as well as in testis ACE (Chen & Riordan, 1990). These results indicate that the somatic isozyme contains only one principal angiotensin-converting site that resides in the C-terminal domain.

Despite these data, the possibility remains that, as inferred from its primary structure, the somatic isozyme contains a second, zinc-binding active site, possibly with a novel activity. This would be of considerable interest in view of the widespread use of ACE inhibitors that may block a second as yet unidentified activity of the somatic enzyme unrelated to blood pressure regulation [for a recent review of the physiology of ACE see Ehlers and Riordan (1989)]. Hence, we undertook a careful reevaluation of the zinc- and inhibitor-binding stoichiometries of both isozymes as well as a search for an unconventional substrate(s) hydrolyzed by the somatic enzyme only. We have found that somatic ACE indeed contains a second zinc-containing, inhibitor-binding site that may account for a unique N-terminal endopeptidase activity.

Materials and Methods

ACE Purification. Human kidney ACE (HK-ACE), rabbit lung ACE (RL-ACE), rabbit testis ACE (RT-ACE), and mouse lung ACE (ML-ACE) were purified from Triton X-100 extracts of tissue homogenates by Sepharose-28–lisinopril affinity chromatography as detailed elsewhere (Pantoliano et al., 1984; Ehlers et al., 1986, 1989). Recombinant human testis ACE (hTACE) was purified from the conditioned media of Chinese hamster ovary cells stably transfected with the expression vector pLEN-ACEVII (Ehlers et al., 1991). Enzyme purity, assessed by SDS–PAGE and specific activity toward Fa-FGG under standard conditions (Bicknell et al., 1987), was judged to be >95%. In all cases, protein concentrations were determined by amino acid analysis using the Pico-tag method (Bidlingmeyer et al., 1984) as described (Strydom et al., 1986). Quantitation was based on inferred polypeptide molecular weights from cDNA sequence data (Soubrier et al., 1988; Bernstein et al, 1989; Kumar et al., 1989; Ehlers et al., 1989), rounded off to 147 000 for HK- and ML-ACE and 81 000 and 80 000 for RT-ACE and hTACE, respectively; the polypeptide of RL-ACE was assumed to be similar to that of HK- and ML-ACE.

Metal Analysis. In general, precautions against adventitious zinc ion contamination were taken as described (Falchuk et al., 1988). Buffers extracted with dithizone (Holmquist, 1988) and water purified by a Milli-Q reagent water system (Waters) contained $<2 \times 10^{-8}$ M Zn^{2+}. Plasticware was soaked overnight in 20% HNO_3 and rinsed extensively with ultrapure water prior to use. Enzyme samples were prepared for metal analysis by washing in Centricon 30 microconcentrators (Amicon) as follows. Centricon components, except the filter-bearing unit, were soaked in 20% HNO_3 overnight and rinsed. The membranes were then washed, by repeated centrifugation according to the manufacturer's recommendations, with 4 × 2 mL of 10 mM HCl, followed by 2 × 2 mL of water, and 2 × 2 mL of metal-free 5 mM Hepes, pH 7.5, or until the filtrate zinc concentration was identical with that of the buffer ($<2 \times 10^{-8}$ M). Enzyme samples were then added to the Centricons, generally 0.5–1.0 mL of affinity column eluates that contained $\sim 1 \times 10^{-6}$ M ACE. The enzyme samples were alternately concentrated to ~40 μL and diluted to 2 mL with metal-free 5 mM Hepes, pH 7.5, until the filtrate contained $<5 \times 10^{-8}$ M zinc. At this point, the enzyme concentrates (~40 μL containing $\sim 2 \times 10^{-5}$ M ACE) were diluted 100–400-fold in 0.2% HNO_3 (Ultrex), and zinc concentrations were determined by graphite furnace atomic absorption spectroscopy on a Perkin-Elmer Model 5000 atomic absorption spectrophotometer (Falchuk et al, 1988). All zinc determinations were performed at two dilutions (falling within a standard curve), each in duplicate. Duplicate samples of the enzyme concentrates as well as of the optimal dilutions were submitted directly for amino acid analysis for protein quantitation.

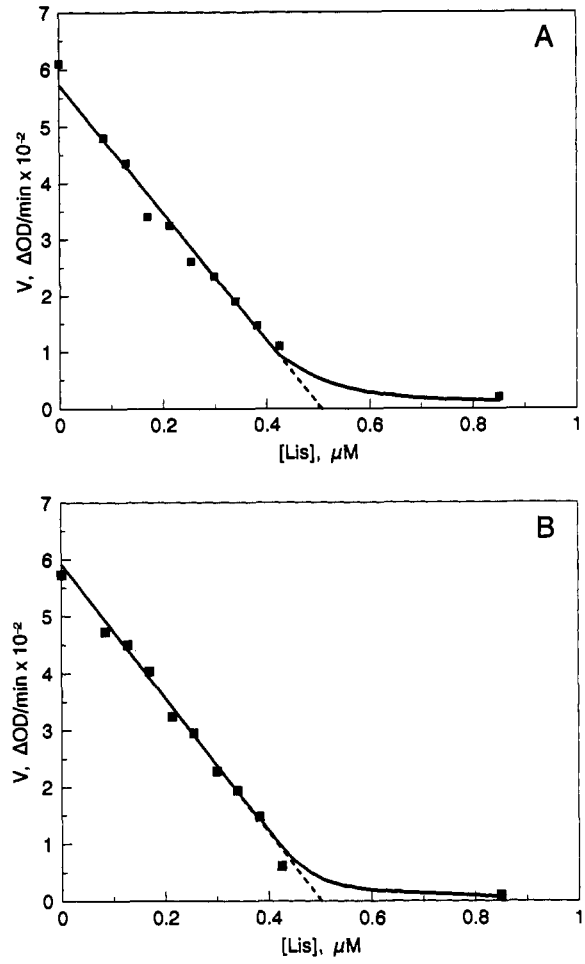

FIGURE 1: Titration of active sites in the somatic and testis isozymes of ACE by binding of the competitive inhibitor lisinopril. (A) 0.47×10^{-6} M human kidney ACE (somatic isozyme) and (B) 0.45×10^{-6} M rabbit testis ACE (testis isozyme) were incubated with 0.2–2.0 equiv of lisinopril ($K_i \leq 2.5 \times 10^{-11}$ M) at room temperature for 30 min in 50 mM Hepes, pH 7.5/0.3 M NaCl, in a volume of 20 μL. Residual enzyme activities were then determined by addition of 990 μL of Fa-FGG (0.15 mM) in the same buffer to 10 μL of the incubates and measuring initial rates during the first minute of hydrolysis. Enzyme and lisinopril concentrations were estimated by amino acid analysis. Data were plotted as residual activity v vs lisinopril concentration ([Lis]). Intersection of the extrapolated linear portion of the curve (broken line) with the x axis gives the concentration of bound lisinopril.

Lisinopril-Binding Studies. The number of inhibitor-binding sites per molecule of somatic and testis ACE was estimated by binding of lisinopril [(*S*)-N^{α}-(1-carboxy-3-phenylpropyl]-L-lysyl-L-proline, a gift from A. A. Patchett, Merck). Lisinopril, a tight-binding, active site directed ACE inhibitor, can be expected to bind stoichiometrically to enzyme under conditions where $[E] \gg K_i$; these conditions are easily achieved as the K_i for lisinopril is $\sim 2.5 \times 10^{-11}$ M (Ehlers et

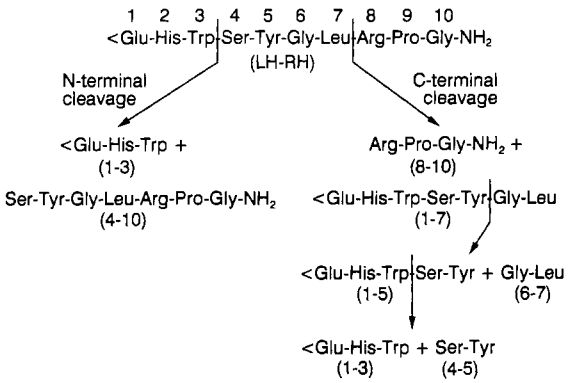

FIGURE 2: Cleavage pathways for the ACE-catalyzed hydrolysis of LH-RH [modified after Skidgel and Erdös (1985)]. Note that fragment 4–10 is resistant to further degradation (Table V) and is unique to the N-terminal pathway. Fragment 1–3 is an endproduct of both pathways.

al., 1991). Inhibitor-binding stoichiometries were determined by active site titration and equilibrium binding as detailed in Figure 1 and Table III, respectively. In both cases enzyme at ($\sim$0.2–0.5) $\times$ 10^{-6} M was incubated with 0.2–8.0 equiv of lisinopril in buffer A at room temperature for 30–60 min. Under these conditions maximal inhibition was achieved by 30 min of incubation and $t_{1/2}$ (half-life of dissociation) of the lisinopril–ACE complex is 105 min (Bull et al., 1985a). The enzyme and lisinopril stock solutions were quantitated by amino acid analysis (acid hydrolysis of lisinopril releases proline).

Assays of Oligopeptide Hydrolysis. Bombesin, eledoisin, litorin, ranatensin, and luteininzing hormone releasing hormone (LH-RH; Gn-RH, gonadotropin-releasing hormone) were from Bachem; des-Arg^9-bradykinin, bradykinin fragment 1–5, substance P, substance P fragment 9–11, LH-RH fragment 4-10, des-Gly^{10}-LH-RH ethylamide, and endothelin were from Sigma. All peptides were dissolved in water or 0.1 M acetic acid. Peptide hydrolysates were analyzed by high-performance liquid chromatography (HPLC) on an Altex Ultrasphere-IP C_{18} column (Beckman) developed with a 30-min linear gradient of 0.1% TFA to 80% CH_3CN/0.1% TFA, and absorbance was monitored at 214 nm. Hydrolysis products were identified by comparison of retention times to those of authentic standards or by amino acid analysis of eluted peaks; product analyses were not undertaken for every substrate.

LH-RH Kinetics. Under conditions where <10% substrate was hydrolyzed, initial rates at [S] = (0.38–5.0) $\times$ 10^{-4} M were determined from product formation [fragments 1–3 and 4–10, Figure 2] quantitated by comparing integrated peak areas to the area of a known standard. Apparent kinetic constants for the overall hydrolysis and for N-terminal cleavage alone of LH-RH were derived from Lineweaver–Burk plots of initial rates of formation of fragments 1–3 and 4–10, respectively (see below under Results for a description of LH-RH cleavage pathways). LH-RH-binding constants were also determined from estimates of the K_i for inhibition of Fa-FGG hydrolysis, measured at [Fa-FGG] = 0.05 and 0.1 mM in the presence of (0.37–3.7) $\times$ 10^{-4} M LH-RH; K_i values were derived from Dixon plots of initial rates.

RESULTS

Zinc Stoichiometry. Zn analyses were performed on three representatives of the somatic isozyme, HK-, RL-, and ML-ACE, and two testis isozymes, RT-ACE and hTACE. Calculated Zn stoichiometries, summarized in Table I and derived from two to five separate preparations for each enzyme, clearly and reproducibly show that the somatic isozyme of ACE contains 2 mol of Zn/mol while the testis isozyme contains 1 mol of Zn/mol.

Table I: Zinc Stoichiometries of the Somatic and Testis Isozymes of ACE[a]

	mol of Zn^{2+}/mol of ACE					
	prepn 1	prepn 2	prepn 3	prepn 4	prepn 5	average
			Somatic Isozymes[b]			
HK	1.9	2.3	2.2	2.0	1.8	2.0
RL	1.7	2.1	2.4	1.7		2.0
ML			2.2	1.9		2.1
			Testis Isozymes[c]			
RT	0.77	1.1	1.0	0.92		0.95
hT		1.0	0.99	0.91		0.97

[a] ACE samples were washed in microconcentrators with metal-free 5 mM Hepes, pH 7.5, until the filtrates contained <5 $\times$ 10^{-8} M Zn^{2+}. The ACE samples were then concentrated to $\sim$2 $\times$ 10^{-5} M, diluted 100–400-fold in 0.2% metal-free HNO_3, and analyzed for zinc by graphite furnace atomic absorption spectroscopy. Each preparation was analyzed in duplicate at two optimal dilutions, and samples were simultaneously taken for direct quantitation of protein concentration by amino acid analysis. [b] Somatic isozymes: HK, RL, and ML, human kidney, rabbit lung, and mouse lung ACE, respectively. [c] Testis isozymes: RT, rabbit testis ACE; hT, human recombinant testis ACE expressed in CHO cells stably transfected with pLEN-ACEVII (Ehlers et al., 1991).

Table II: Zinc Stoichiometry of the ACE–Lisinopril Complex in the Presence of OP or Acid[a]

	mol of Zn^{2+}/mol of ACE				
	OP-treated ACE[b]				
	prepn 1	prepn 2	prepn 3	pH 5.5[c]	control[d]
			Somatic Isozymes		
HK	2.01	2.01	2.0	2.1	0.38
RL	2.02	1.72	1.9	2.2	0.49
ML	2.2	2.0	1.84	2.1	
			Testis Isozymes		
RT	1.04	1.03	1.03	1.3	0.04
hT	1.18	0.94	1.04	1.2	0.03

[a] Enzyme [(0.16–1.6) $\times$ 10^{-6} M], rendered free of adventitious Zn^{2+} as described in Table I, was incubated with 2 μM lisinopril in buffer A (50 mM Hepes, pH 7.5/0.3 M NaCl) for 30 min at room temperature and then washed repeatedly in a Centricon with the same solution. The enzyme was then treated in one of the following ways. [b] The enzyme was washed with 2 $\times$ 1 mL buffer A containing 0.1 mM OP and 2 μM lisinopril for $\sim$60 min, followed by 2 $\times$ 2 mL buffer A with 2 μM lisininopril and no OP (the latter caused matrix interference effects during Zn analyses). [c] The enzyme was incubated overnight at room temperature in 20 mM Mes, pH 5.5/0.3 M NaCl, containing 2 μM lisinopril, and then washed with 5 $\times$ 2 mL of the same buffer. ACE samples were concentrated and analyzed for Zn^{2+} and protein concentrations as detailed in Table I. [d] Controls were washed with 0.1 mM OP in buffer A without lisinopril; treatment with pH 5.5 alone was not tested. Isozymes are as described in Table I.

To confirm the presence of a second zinc ion in the somatic isozyme and to explore its functional significance, we incubated various species of ACE with a moderate excess of the tight-binding, active site directed, metal-coordinating inhibitor lisinopril. Lisinopril and the related compound enalaprilat bind to both isozymes of ACE with a $K_i \simeq 5 \times 10^{-11}$ M (Shapiro & Riordan, 1984b; Ehlers et al., 1991). It has previously been shown by magnetic circular dichroism that inhibitor binding to cobalt-substituted ACE converts a low-symmetry metal ligand environment to a tetrahedral geometry of high symmetry and that there is a direct interaction of inhibitor with the metal (Bicknell et al., 1987). It is likely, therefore, that the inhibitor "locks" the metal into the active site and thereby prevents loss of metal in the presence of agents that usually promote its dissociation, such as metal chelators or acidic buffers (Kleemann et al., 1986). Consequently, resistance of

Table III: Lisinopril-Binding Stoichiometries of the Somatic and Testis Isozymes of ACE[a]

	molar quantities (pmol)			mol of lisinopril bound/mol of ACE
	protein	lisinopril added	lisinopril eluted	
		Somatic Isozymes		
HK	90	220 (2.44)	108	1.24
	90	368 (4.11)	203	1.85
RL	106	485 (4.58)	292	1.82
	103	808 (7.84)	585	2.17
		Testis Isozymes		
RT	88	265 (3.01)	180	0.97
	101	397 (3.93)	281	1.15
hT	211	500 (2.34)	274	1.07
	211	750 (3.55)	524	1.07

[a] Enzyme ($\sim 0.2 \times 10^{-6}$ M) was incubated with 2–8 equiv of lisinopril (equivalents added given in parentheses) in 50 mM Hepes, pH 7.5/0.3 M NaCl at room temperature for 1 h and concentrated in a Centricon (~30-min centrifugation), and protein and unbound lisinopril were quantitated in the retentates and filtrates, respectively, by amino acid analysis, in triplicate; the data are from a single experiment. No allowance was made for the unbound lisinopril that remained in the retentate, as it represented <10% of the total. Isozymes are as in Table I.

both metal ions in the somatic ACE–inhibitor complex to removal by chelator or acid would not only confirm the Zn stoichiometry of 2 mol of Zn/mol of protein but would also establish that the second zinc is a component of a second site similar enough to the first site[2] to allow inhibitor binding.

As summarized in Table II, the results of the experiment are consistent with the proposed model. The ACE–lisinopril complex resisted dissociation of 2 zinc ions in the somatic isozyme and 1 zinc ion in the testis isozyme when these were treated with either 0.1 mM OP or a pH 5.5 buffer [zinc dissociates rapidly from ACE at pH < 7.0 (Bünning et al., 1983; Kleemann et al., 1985), and maximal lisinopril binding occurs at pH 5.5–6.5 (Bull et al, 1985a)]. In contrast, when the enzymes were treated with OP alone in the absence of lisinopril, the zinc was removed. Of interest is that with the OP treatment alone, the somatic isozymes retained ~20% of their zinc while the testis isozymes retained <4% (Table II).

Lisinopril-Binding Studies. Additional data regarding the number of lisinopril-binding sites in the two isozymes were obtained from direct binding experiments. Enzyme was equilibrated with 2–8 equiv of lisinopril, and unbound inhibitor was removed by concentration of the enzyme in a microconcentrator. Quantitation of the enzyme and unbound inhibitor concentrations revealed that the somatic isozyme binds 2 mol of lisinopril/mol of protein while the testis isozyme binds 1 mol/mol (Table III). However, it appears that the addition of ≥4 equiv of lisinopril was required under these conditions to achieve occupancy of both sites in somatic ACE (Table III). This may mean that the affinity of the second site for lisinopril is lower than that of the first site.

Active Site Titration. Under conditions of [E], [I] $\gg K_i$, the reaction

$$E + I \rightleftarrows EI \rightleftarrows EI^*$$

is driven predominantly to the right, especially in the presence of 0.3 M Cl^-, which promotes isomerization of EI to a more tightly bound complex, EI* (Shapiro & Riordan, 1984b; Bull et al., 1985a). We therefore titrated the number of active sites in ACE under these conditions and found that plots of residual activity v vs [I] contained linear portions that could be extrapolated to the x axis to give intersection points representing moles of lisinopril per moles of ACE required to abolish activity (Figure 1).

The results, summarized in Table IV, indicate that for both the somatic isozymes (HK- and RL-ACE) and testis isozymes (RT-ACE and hTACE), binding of 1 mol of lisinopril/mol of ACE was sufficient to abolish Fa-FGG-hydrolyzing activity, suggesting that both isozymes contain 1 angiotensin-converting site/molecule. Moreover, these data imply that, while somatic ACE appears to contain two lisinopril-binding sites, the angiotensin-converting site presumably residing in the C-terminal or "testicular" domain binds lisinopril more tightly. Thus, addition of 1 equiv of lisinopril leads to occupancy of the C-terminal active site first and its inhibition, and only upon addition of further equivalents of lisinopril is the second, N-terminal site occupied (Table III) This experiment was not attempted with ML-ACE because it has not been kinetically characterized and preliminary studies indicated that its specific activity toward Fa-FGG was ~50% lower, and the K_i for lisinopril ~10^2-fold higher, than for the other enzymes.

Hydrolysis of Atypical Substrates by ACE Isozymes. In light of data suggesting the presence of a potential second active site in somatic ACE, we surveyed a number of oligopeptides that would be considered atypical ACE substrates. If a substrate(s) could be identified that was hydrolyzed by the somatic isozyme but not by the testis isozyme, this might signal an activity unique to the second site of somatic ACE. This study was conducted with HK-ACE and hTACE, isozyme preparations that are highly purified and known to be free of isozymic contamination (Ehlers et al., 1989, 1991).

Of the 10 oligopeptides surveyed, only LH-RH and its derivative des-Gly^{10}-LH-RH ethylamide were preferentially hydrolyzed by the somatic isozyme (Table V). Indeed, this is the first instance where such a difference between the two isozymes has been observed. As described by Skidgel and Erdös (1985), LH-RH is hydrolyzed by somatic ACE by one of two cleavage pathways (Figure 2). The rate of N-terminal cleavage could be quantitated by measurement of the rate of fragment 4–10 formation, since this fragment resists further hydrolysis (Table V). The overall rate of LH-RH hydrolysis was measured by quantitation of fragment 1–3 formation, which is an endproduct of both cleavage pathways (Figure 2). In the presence of 0.3 M Cl^- the overall rate of LH-RH hydrolysis by somatic ACE was 13-fold faster than by testis ACE, but the rate of N-terminal cleavage was >300-fold faster; the rates of C-terminal cleavage differed by <2-fold (Table VI). Analysis of the respective chromatograms indicated that 87% of the hydrolysis of LH-RH in 0.3 M Cl^- by the somatic enzyme occurred via the N-terminal pathway, whereas only 4% of hydrolysis by the testis enzyme was by this pathway (Figure 3). The amino-terminal activity had an absolute dependence on chloride and became undetectable in the absence of chloride (Table VI), while the carboxy-terminal pathway was reduced 11-fold for both isozymes. Indeed, without chloride the hydrolysis patterns of the two isozymes became indistinguishable and the ratio of the overall rates of LH-RH hydrolysis by the somatic and testis isozymes, respectively, fell from 13:1 to 1.7:1 (Table VI). The hydrolysis of LH-RH by either isozyme was inhibited >99% by 40 μM lisinopril (Table VI). Kinetic constants for LH-RH hydrolysis are summarized in Table VII. Apparent K_m values for the

[2] Throughout this paper, the active site presumed to be located in the C-terminal domain of somatic ACE, the domain that is identical in sequence with the polypeptide from residue 37 to the C-terminus of testis ACE (Figure 4), is referred to as the "principal" or "first" site. A potential active site that may be located in the N-terminal domain of somatic ACE only is referred to as the "second" site.

Table IV: Active Site Titration of the Somatic and Testis Isozymes of ACE with Lisinopril[a]

	experiment 1			experiment 2		
	[E] ($\times 10^{-6}$ M)	[Lis][b] ($\times 10^{-6}$ M)	mol of Lis/mol of ACE	[E] ($\times 10^{-6}$ M)	[Lis][b] ($\times 10^{-6}$ M)	mol of Lis/mol of ACE
			Somatic Isozymes			
HK	0.47	0.5	1.1	0.52[c]	0.50[c]	0.96[c]
RL	0.56	0.6	1.2	0.55	0.50	0.91
			Testis Isozymes			
RT	0.45	0.5	1.1			
hT	0.43	0.46	1.1			

[a] ACE and lisinopril were incubated under conditions where [E], [I] $\gg K_i$, residual activities were determined by addition of Fa-FGG, and curves of v vs [I] were plotted, as described in legend to Figure 1. Each curve consisted of 11 data points determined in duplicate. [b] [Lis], concentration of lisinopril at the intersection of linear extrapolation with the x axis (Figure 1). [c] Incubated in the presence of 10^{-5} M $ZnCl_2$. Isozymes are as in Table I.

Table V: Hydrolysis of Atypical Substrates by the Somatic and Testis Isozymes of ACE[a]

substrate	sequence	extent of hydrolysis (%) somatic	extent of hydrolysis (%) testis	hydrolysis time (min)
des-Arg^9-Bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe	98	98	30
Litorin	<Glu-Gln-Trp-Ala-Val-Gly-His-Phe-Met-NH_2	99.7	99	60
Ranatensin	<Glu-Val-Pro-Gln-Trp-Ala-Val-Gly-His-Phe-Met-NH_2	97	95	60
Substance P	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2	83	95	60
Bombesin	<Glu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2	6	6.5	120
Eledoisin	<Glu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH_2	0	0	60
Endothelin		0	0	150
LH-RH	<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2	37	2.9	90
des-Gly^{10}-LH-RH-NHEt	<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-NHEt	45	0.6	60
LH-RH (4-10)	Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2	0	0	180

[a] Enzyme, 2×10^{-8} M HK-ACE (somatic) or hTACE (testis), was incubated with 0.1 mM substrate in 50 mM Hepes, pH 7.5/0.3 M NaCl, in a total volume of 100 μL at 37 °C for 30–180 min. Hydrolysates were quenched in 0.5% TFA, and 30 μL was injected onto a C_{18} reversed-phase HPLC column developed with a 30-min linear gradient of 0.1% TFA to 80% CH_3CN/0.1% TFA. The extent of substrate hydrolysis was estimated by comparison of the integrated peak area of residual substrate with that of an unreacted control. Note that the HPLC product patterns for the two isozymes were identical for each of the substrates tested, except for LH-RH (Figure 3) and des-Gly^{10}-LH-RH ethylamide.

Table VI: Hydrolysis of LH-RH by ACE Isozymes: Differential Product Formation and Effect of Chloride and Lisinopril[a]

LH-RH hydrolysis conditions	rate of product formation[b] % of maximum[c] somatic ACE overall	somatic ACE C-term.	somatic ACE N-term.	testis ACE overall	testis ACE C-term.	testis ACE N-term.	ratio of somatic to testis ACE overall	ratio of somatic to testis ACE C-term.	ratio of somatic to testis ACE N-term.
standard	100	13	87	7.7	7.42	0.28	13	1.8	311
0 Cl^-	1.2	1.2	0	0.7	0.7	0	1.7	1.7	nd
lisinopril[d]	<1	0	0	0	0	0	nd	nd	nd

[a] Enzyme was incubated with substrate and hydrolysates were analyzed by HPLC as described in Table V. [b] Products analyzed were LH-RH fragments 1–3 (<Glu-His-Trp) and 4–10 (Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2), which reflect the rates of overall and N-terminal cleavage of LH-RH, respectively (Figure 2). The rate of C-terminal cleavage was deduced from the overall and N-terminal rates. [c] Values are given as percentages of the overall rate of hydrolysis of LH-RH by somatic ACE, which was determined from the rate of formation of fragment 1–3 under standard conditions (50 mM Hepes, pH 7.5/0.3 M NaCl) and designated maximal (=100). [d] [Lisinopril] = 40 μM; nd = not determined.

overall hydrolysis of LH-RH and for the N-terminal cleavage alone were virtually identical for both isozymes and in good agreement with the K_i values for inhibition by LH-RH of Fa-FGG hydrolysis.

Dependence of LH-RH Hydrolysis on ACE–Lisinopril Stoichiometry. As 1 equiv of lisinopril was sufficient to abolish Fa-FGG hydrolysis by the somatic enzyme and as this was likely due to preferential binding of inhibitor to the C-terminal active site (see under Active Site Titration above), we studied the effect of increasing amounts of lisinopril on LH-RH hydrolysis by somatic ACE. If the putative second active site was solely responsible for the N-terminal tripeptidyl endopeptidase activity, then addition of 1 equiv of lisinopril should leave this activity intact. However, this was not the case. Addition of 1 equiv of lisinopril to the somatic enzyme led to a 20-fold (95%) fall in Fa-FGG hydrolysis and a 5-fold (80%) decrease in LH-RH hydrolysis (Table VIII). Even 0.625 equiv decreased LH-RH hydrolysis 1.7-fold (40%). Of note is that with the addition of substoichiometric amounts of inhibitor, the decrease in LH-RH hydrolysis was 2–3 times less than the decrease in Fa-FGG hydrolysis (Table VIII).

LH–RH Hydrolysis by Dnp-Modified ACE. Chemical modification of HK-ACE and hTACE with Dnp-F under the conditions described (Chen & Riordan, 1990) resulted in

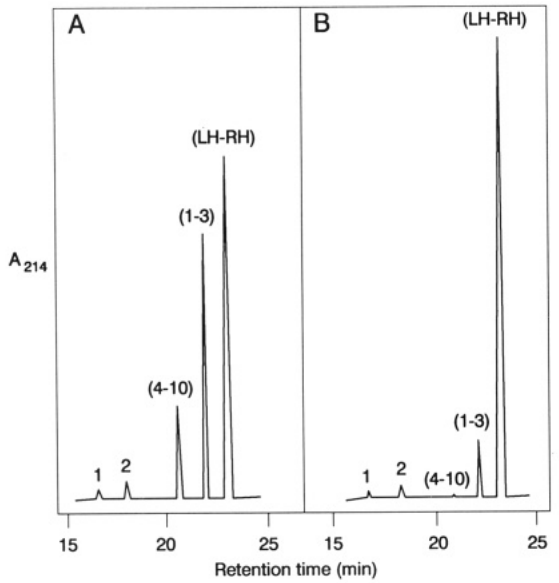

FIGURE 3: HPLC chromatograms (schematic) of the products of hydrolysis of LH-RH by the somatic (A) and testis (B) isozymes of ACE. LH-RH was hydrolyzed by equimolar amounts of either isozyme, and the products were analyzed by HPLC as described in Table V. Fragments 1–3 and 4–10 were identified by amino acid analysis and by comparison to authentic standards. Peaks 1 and 2 were not identified but are assumed to be identical with the similarly eluting LH-RH cleavage products described by Skidgel and Erdös (1985).

Table VII: Hydrolysis of LH-RH by ACE Isozymes: Kinetic Constants[a]

isozyme	k'_{cat} (min^{-1}) overall	k'_{cat} (min^{-1}) N-term.	K'_m ($\times 10^{-4}$ M) overall	K'_m ($\times 10^{-4}$ M) N-term.	K_i ($\times 10^{-4}$ M)
somatic	79	69	0.97	1.06	1.2
testis	6.4	<0.2[b]	0.92		1.2

[a] Initial rates were measured in 50 mM Hepes, pH 7.5/0.3 M NaCl, with HK-ACE (somatic) and hTACE (testis). Apparent K_m and k_{cat} values were determined from Lineweaver–Burk plots of rates of formation of LH-RH fragments 1–3 and 4–10, reflecting rates of overall and N-terminal cleavage, respectively, at [S] = (0.38–5.0) $\times 10^{-4}$ M. K_i values were determined from Dixon plots of the inhibition of Fa-FGG (0.05 and 0.1 mM) hydrolysis by (0.36–3.7) $\times 10^{-4}$ M LH-RH. [b] Estimate based on ratio of somatic to testis isozymes for rate of 4–10 product formation (see Table VI).

>98% inhibition of the initial Fa-FGG-hydrolyzing activity for both isozymes and >95% inhibition of LH-RH hydrolysis by testis ACE. However, 20% of the initial LH-RH-hydrolyzing activity was retained by the somatic isozyme.

Table VIII: Dependence of LH-RH Hydrolysis on ACE–Lisinopril Stoichiometry[a]

equiv of lisinopril added	substrate hydrolysis (% of control) Fa-FGG	LH-RH product (1–3)	LH-RH product (4–10)
	Somatic ACE		
0	100	100	100
0.625	20.7	59	56
0.75	16.6	37	38
0.875	13.6	26	27
1.0	4.6	16	18
2.0	3.5	4.3	5.1
4.0	2.1	1.8	<1
	Testis ACE		
0	100	100	
1.0	5.7	9.2	
2.0	3.3	3.4	
4.0	<1	<1	

[a] Somatic (HK-ACE) and testis (hTACE) isozymes, 0.2 $\times 10^{-6}$ M, were incubated with 0.625–4 equiv of lisinopril in 50 mM Hepes, pH 7.5/0.3 M NaCl, in a volume of 40 μL at room temperature for 1 h. Fa-FGG-hydrolyzing activity was then assayed as described in Figure 1. LH-RH hydrolysis was assayed by 20-min incubations at 37 °C of 10 μL of enzyme–lisinopril incubates added to 40 μL of the same buffer containing 0.1 mM LH-RH; hydrolysates were analyzed by HPLC as described in Table V. Values are percentages of rates in absence of lisinopril (control).

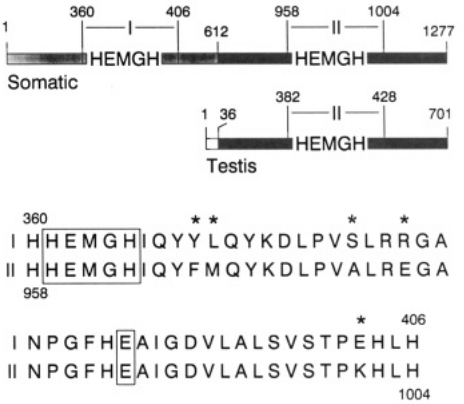

FIGURE 4: Schematic representation of somatic and testis ACE. The solid bars indicate 100% sequence identity between residues 612–1277 and 36–701 of the somatic and testis isozymes, respectively; the open bar represents sequence unique to the testis isozyme, and the shaded bar denotes homology between the N- and C-terminal domains of somatic ACE. The proposed metal-binding motifs (HEMGH) and third zinc ligands (E) (boxed) are shown together with flanking sequences as regions I and II from the N- and C-terminal domains of somatic ACE, respectively. I and II are 89% identical. Nonidentical residues are indicated by asterisks.

Discussion

The recent molecular cloning of the somatic and testis ACE isozymes from various sources provided a number of insights, most importantly that the somatic enzyme comprises two homologous domains that apparently arose by gene duplication, with each domain containing a putative metal-binding site (Soubrier et al., 1988; Bernstein et al., 1989). The testis isozyme consists of only a single domain that corresponds to the C-terminal half of the somatic enzyme (Ehlers et al., 1989; Lattion et al., 1989; Kumar et al., 1989). Of particular interest is that the two thermolysin-like metal-binding motifs (HEMGH) of the somatic enzyme are located in 47-residue sequences that are 89% identical (Figure 4). Both sequences contain a second conserved glutamate, 24 residues C-terminal to the HEMGH site (Figure 4), that may constitute a third zinc ligand (Vallee & Auld, 1990). The inferred structure for the somatic isozyme was inconsistent with Zn and inhibitor-binding stoichiometries for somatic ACE of 1 mol/mol reported by a number of laboratories, including our own (Das & Soffer, 1975; Fernley, 1977; Conroy et al., 1978; Bünning & Riordan, 1981, 1985; Bull et al., 1985a,b; Strittmatter & Snyder, 1986; Cumin et al,. 1989). However, review of these data indicates that these stoichiometries were based on ACE protein quantitations that may have been inaccurate owing to difficulties in the estimation of the molecular weight and molar absorptivity (Chen & Riordan, 1990; Skoglof et al., 1990).

These problems have now been overcome by quantitative amino acid analysis based on molecular weights inferred from cloned cDNAs. Further, in the case of the zinc analyses, we did not follow the standard practice of extensively dialyzing enzyme preparations against large volumes of metal-free buffer prior to measurement, since a comparatively weak K_D of 10^{-8}–10^{-9} M (Bull et al., 1985b; Kleemann et al., 1986) could lead to partial dissociation of zinc. Instead, adventitious zinc

was rapidly removed by washing enzyme preparations in metal-free microconcentrators with continuous monitoring of the zinc content of the filtrates; at no time were enzyme solutions diluted below 0.1×10^{-6} M. This allowed us to reproducibly determine that the zinc content of the somatic isozyme of ACE is 2 mol of Zn^{2+}/mol of enzyme, while that of testis isozyme is 1 mol of Zn^{2+}/mol (Table I). Moreover, 2 mol of zinc/mol of somatic ACE and 1 mol of zinc/mol of testis ACE were protected by the inhibitor lisinopril against dissociation in 0.1 mM OP or at pH 5.5 (Table II).

The results presented in Table II also suggest that the second zinc in the somatic enzyme is part of a site that can bind the tight-binding inhibitor lisinopril. The latter finding is surprising: although the metal-binding HEMGH motifs and C-terminal flanking sequences are highly conserved in the two domains of somatic ACE (Soubrier et al., 1988) (Figure 4), two specific active site residues, tyrosine and lysine, are only found in the C-terminal domain (Chen & Riordan, 1990); of these, the lysine is thought to be involved in chloride binding, which in turn, modulates substrate binding (Shapiro & Riordan, 1983). However, the lisinoprol-binding stoichiometry of 2 mol of lisinopril/mol of somatic enzyme was confirmed by a direct equilibrium binding study (Table III), although the second site may have a lower affinity, requiring the presence of >4 equiv of inhibitor to achieve full occupancy (Table III). Similar data were obtained previously for the stoichiometry of CA-Phe-Gly binding to somatic ACE from rabbit lung (Shapiro & Riordan, 1984a).

Differential affinity, in which lisinopril binds to the principal angiotensin-converting (presumably C-terminal) site first before binding to the second site, was also suggested by a kinetic active site titration experiment (see Figure 1 and Table IV). From this study it was clear that 1 equiv of lisinopril was sufficient to abolish activity toward Fa-FGG for both isozymes. [We have assumed that hydrolysis of Fa-FGG and angiotensin I occur at the same active site since no differences were noted for these substrates with either isozyme (Ehlers et al., 1991).] A similar result was recently obtained by Skoglof et al. (1990), who titrated rabbit lung ACE with enalaprilat and ramiprilat and determined an ACE–inhibitor stoichiometry of 1:1 from steady-state inhibition kinetics with Fa-FGG as substrate. However, their study, like ours, only demonstrates that 1 equiv of inhibitor is necessary and sufficient to block hydrolysis of Fa-FGG and related (angiotensin I like) substrates, and it does not rule out the presence of a second, non-Fa-FGG-hydrolyzing active site. Moreover, these data imply that the inhibitor-binding affinity for the second site is weaker than for the principal site. Taken together, these studies support the prior impression that in somatic ACE a single active site is responsible for the angiotensin-converting activity and that with conventional substrates no significant differences between the isozymes could be detected (El-Dorry et al., 1982; Ehlers et al., 1991; J. W. Harper, R. Shapiro, and J.F.R., unpublished results).

However, we now find that the atypical substrates LH-RH and des-Gly^{10}-LH-RH ethylamide are hydolyzed at significantly faster rates by the somatic isozyme (Table V). Moreover, the unusual N-terminal endopeptidase activity (Figure 2) is virtually unique to the somatic isozyme, with the testis enzyme showing only 0.3% of the activity of somatic ACE (Table VI). This N-terminal tripeptidyl endopeptidase activity was absolutely chloride dependent, and in the absence of added chloride the somatic and testis isozymes became almost indistinguishable in that both utilized only the C-terminal cleavage pathway of LH-RH hydrolysis (Table VI). The chloride dependence of N- and C-terminal cleavages of LH-RH is consistent with the nature of the cleavage sites (Figure 2), which can be classified as class I and II substrate sites, respectively [see Shapiro et al. (1983) for a classification of ACE substrates according to their chloride dependence]. The presence of two primary (initial) cleavage sites and additional secondary sites (Figure 2) complicates a kinetic analysis of the hydrolysis of LH-RH. If the secondary cleavages are discounted during the initial hydrolysis period, then the hydrolysis of LH-RH can be viewed as a two-substrate reaction that is susceptible to kinetic analysis if the rates of formation of products unique to the respective cleavages can be measured (Dixon & Webb, 1979). However, whereas the rate of formation of fragment 4–10, corresponding to the N-terminal cleavage (Figure 2), was easily quantitated, the peak area of fragment 8–10 could not be realiably integrated and therefore the rate of C-terminal cleavage could not be determined. Hence, we determined apparent constants for the overall reaction from the rate of fragment 1–3 formation and approximate constants for the N-terminal cleavage only (Table VII). In addition, we estimated the K_i for the inhibition of Fa-FGG hydrolysis by LH-RH, which presumably reflects the dissociation constant for LH-RH binding to the principal active site in both isozymes. Significantly, these various binding constants were virtually identical with one another for both isozymes (Table VII). Although these data are subject to the reservations noted above, this may mean that LH-RH binds to the same active site in each isozyme and that the difference between the isozymes in the hydrolysis of LH-RH cannot be accounted for by binding of this peptide to a putative second active site unique to the somatic enzyme.

The addition of 1 equiv of lisinopril to somatic ACE led to a 20-fold decrease in Fa-FGG hydrolysis and a 5-fold decrease in LH-RH hydrolysis (Table VIII), making it unlikely that hydrolysis of this substrate was occurring solely at the second site. This result was supported by the effects of chemically modifying the isozymes with Dnp-F under conditions previously shown to inhibit Fa-FGG hydrolysis >95% and to label one essential tyrosine and one essential lysine only in the C-terminal or testicular half of somatic ACE (Chen & Riordan, 1990): this modification also produced a 5-fold decrease in the N-terminal tripeptidyl endopeptidase activity of somatic ACE. These data can be interpreted to mean that in somatic ACE there is a second site or region within the second domain that cooperates with the principal or "classical" active site for the N-terminal tripeptidyl endoproteolytic cleavage of LH-RH. Thus, LH-RH binds to the principal active site, as inferred from the binding constants that are identical for the two isozymes (Table VII), but cleavage involves participation of or is influenced by the second domain. Alternatively, the differential effects of either lisinopril binding or chemical modification on the inhibition of LH-RH and Fa-FGG hydrolysis by somatic ACE may merely reflect substrate-dependent effects unrelated to a putative second active site. It is well known that the catalytic mechanism of ACE is complex and that the results of chloride activation, inhibition, and chemical modification are all substrate-dependent (Shapiro et al., 1983; Shapiro & Riordan, 1983, 1984a). Of course, some of this previously observed complexity (all relevant kinetic studies were preformed with the somatic enzyme from rabbit lung) may have its basis in the presence in somatic ACE of a duplicated domain that contains a potential second active site.

It is difficult to assess the physiological relevance of ACE in the metabolism of LH-RH. As a membrane hydrolase,

ACE is ideally placed in the hypothalamus and pituitary and in the kidney and testis (epididymal and interstitial cells contain the somatic isozyme) to act on central and peripheral LH-RH (Skidgel & Erdös, 1985), but the k_{cat} and k_{cat}/K_m are 28-fold and 124-fold lower, respectively, than those for angiotensin I (Ehlers et al, 1991). Further, there is evidence that the primary LH-RH-metabolizing enzyme is endopeptidase 24.15 (Lasdun & Orlowski, 1990; Molineaux et al., 1988). Nevertheless, somatic ACE is the only documented enzyme capable of producing LH-RH fragment 1–3, a major metabolite of LH-RH at all in vivo sites where its degradation has been studied (Skidgel & Erdös, 1985, and references cited therein; Molineaux et al., 1988; Yokosawa et al., 1987), and specific inhibitors of ACE block the formation of fragment 1–3 (Yokosawa et al., 1987; Molineaux et al., 1988). Thus, somatic ACE may have a specialized function in generating fragment (1–3).

We were unable to infer what the determinants of the N-terminal tripeptidyl endopeptidase activity were. The N-terminal pyroglutamic acid is apparently important (Skidgel & Erdös, 1985), but in our survey other peptides with pyroglutamyl N-termini and amidated C-terminal were hydrolyzed equally, if at all, by both isozymes (Table V), presumably by an initial C-terminal tripeptidyl endopeptidase attack that appears to be characteristic for amidated peptides (Ehlers & Riordan, 1990). Only des-Gly^{10}-LH-RH ethylamide was also preferentially hydrolyzed by somatic ACE (Table V), indicating that the C-terminal residue and size of the blocking groups were not critical. Although we did not characterize this substrate further, fragment 1–3 was a major product of its hydrolysis (not shown).

In conclusion, the data presented in this report strongly suggest the existence of two zinc- and inhibitor-binding sites in somatic ACE. Only one of these sites, presumably the site in the C-terminal domain that is identical with that found in the testis enzyme, is necessary for the typical angiotensin-converting activity. The second site may participate in the unusual N-terminal tripeptidyl endopeptidase cleavage of LH-RH that is virtually unique to the somatic isozyme. The presence of, potentially, two active sites in a single polypeptide is unusual, but not unprecedented. At least two other brush border hydrolases are known that consist of two homologous domains and contain two similar but distinct active sites that apparently evolved from duplication of an ancestral gene: the prosucrase–isomaltase complex (Hunziker et al., 1986) and lactase–phlorizin hydrolase (Mantei et al., 1988). Similarly, the S6 kinase from *Xenopus* oocytes and the leukocyte common antigen (CD45) now thought to be a protein tyrosine phosphatase both consist of two homologous, tandem domains that may contain two similar but distinct catalytic centers (Jones et al., 1988; Charbonneau et al., 1988; Tonks et al., 1988). In view of the widespread clinical use of ACE inhibitors such as lisinopril that can bind to two sites in the somatic enzyme, it is of some importance to determine the characteristics of the second site and the function it subserves.

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Registry No. ACE, 9015-82-1; LH-RH, 9034-40-6; Zn^{2+}, 7440-66-6; lisinopril, 76547-98-3.

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Identification of Cysteine-319 as the Target Amino Acid of 8-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 5′-Triphosphate in Bovine Liver Glutamate Dehydrogenase†

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ABSTRACT: The affinity label 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-triphosphate (8-BDB-TA-5′-TP) has been shown to react with bovine liver glutamate dehydrogenase in the region of the GTP-dependent NADH inhibitory site with incorporation of about 1 mol of reagent/mol of subunit [Ozturk, D. H., Safer, D., & Colman, R. F. (1990) *Biochemistry 29*, 7112-7118]. The modified enzyme was shown to contain only 5 free sulfhydryl groups upon 5,5′-dithiobis(2-nitrobenzoate) titration as compared with 6 in the unmodified enzyme. In the unmodified enzyme digested with trypsin, 6 cysteinyl peptides were detected by high-performance liquid chromatography upon treatment with iodo[^{3}H]acetic acid. In contrast, only 5 (carboxymethyl)cysteinyl peptides were detected in 8-BDB-TA-5′-TP-modified enzyme. When carboxymethylated modified and unmodified enzymes were digested with thermolysin, 6 peptide sequences containing (carboxymethyl)cysteine were obtained in the unmodified enzyme, but only 5 were observed in the modified enzyme. The (carboxymethyl)cysteine which was absent in the modified enzyme was determined to be Cys-319, leading to the conclusion that 8-BDB-TA-5′-TP reacts with Cys-319, thereby preventing it from subsequent reaction with radioactive iodoacetate. It was previously reported that 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate (6-BDB-TA-5′-DP) modifies Cys-319 in this enzyme [Batra, S. P., & Colman, R. F. (1986) *Biochemistry 25*, 3508-3515]. Evidence is here presented that the reaction product is different for the two nucleotide analogues: while Cys-319 reacts at the methylene group of 6-BDB-TA-5′-DP by displacement of bromide, the same cysteine appears to attack the carbonyl group of 8-BDB-TA-5′-TP, leading to a thiohemiacetal adduct.

Bovine liver glutamate dehydrogenase [L-glutamate:NAD(P)$^+$ oxidoreductase (deaminating), EC 1.4.1.3] is an allosteric enzyme with multiple binding sites for purine nucleotides. The enzyme is inhibited by GTP and high concentrations of NADH, while ADP functions as an allosteric activator (Colman, 1990). The enzyme is composed of six identical subunits in its active form, which binds 2 mol of GTP/mol of subunit in the presence of NADH, but only 1 mol of GTP/mol of subunit in the absence of reduced coenzyme (Pal & Colman, 1979). Furthermore, the allosteric activator ADP,

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