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Limited Proteolysis of Human Kidney Angiotensin-Converting Enzyme and Generation of Catalytically Active N- and C-Terminal Domains

Edward D. Sturrock,*,† Sergei M. Danilov,‡ and James F. Riordan*,[1]

**Center for Biochemical and Biophysical Sciences and Medicine, Harvard Medical School, 250 Longwood Avenue, Boston, Massachusetts 02115; †Medical Research Council Liver Research Centre, University of Cape Town Medical School, Observatory 7925, South Africa; and ‡Department of Anesthesiology and Pharmacology, University of Illinois College of Medicine, Chicago, Illinois 60612*

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The somatic form of angiotensin converting enzyme is a class I ectoenzyme that is bound to the surface of endothelial calls. It consists of two homologous, catalytic domains of approximately 600 residues each; a juxtamembrane "stalk" region; a transmembrane, hydrophobic sequence; and a 30 residue, C-terminal cytosolic domain. We have used limited proteolysis to probe the structural and functional properties of the enzyme. Endoproteinase Asp-N cleaves both the Thr^{615} - Asp^{616} and the Leu^{1219} - Asp^{1220} peptide bonds to generate the two catalytic domains which were isolated by a combination of immunoaffinity and lisinopril Sepharose affinity chromatography. The enzymatic characteristics of the N and C fragments were examined with angiotensin I, hippuryl-His-Leu, and luteinizing hormone-releasing hormone and indicate that both fragments contain catalytically active sites that retain their individual functional integrity. © 1997 Academic Press

Limited proteolysis has been used to elucidate the structure and function of numerous proteins (1, and references therein). For example endoproteinase Lys-C and trypsin digestions revealed that the C domain of CD45 contains a functional protein tyrosine phosphatase active site (2). More recently a combination of limited proteolysis and mass spectrometry has been used to determine the structural properties of DNA-binding proteins (3) and facilitated the crystal structure determination of the HIV-I Nef protein (4). The success of limited proteolysis depends on the principle that protection from proteolysis is conferred on regions of the protein that are located in a rigid structure, are buried, or are involved in protein-protein interactions. In contrast, regions that are solvent accessible and flexible will be less protected and therefore more susceptible to proteolytic cleavage.

The somatic form of angiotensin I-converting enzyme (ACE) is a ubiquitous ectozyme of 150-180 kDa that consists of two homologous domains (N and C domains) probably derived from gene duplication. Several studies have indicated that somatic ACE undergoes limited proteolysis to produce an 80-90 kDa fragment. This low molecular weight form of the enzyme was first observed during the purification of rabbit lung ACE (5). Iwata *et al.* showed that an enzymatically active 82 kDa fragment, containing the same N-terminal sequence as the native protein, could also be obtained from lung ACE after treatment with ammonium hydroxide (6). A variety of serine proteases were used to cleave denatured human plasma ACE to give a single 90 kDa fragment (7). However, in the absence of denaturation the enzyme resisted proteolysis. It is reasonable to infer from these results that these fragments correspond to one or both of the two domains and that they are linked by a short span of hydrophilic amino acid residues which is susceptible to cleavage by proteinases.

In all of the earlier limited proteolysis studies on ACE the cleavage sites were not identified and the fragments, if isolated, were only partially characterized. Thus the origins and functional significance of the proteolytic polypeptides were not established. We have found that endoproteinase Asp-N clips human kidney ACE between the N and C domains, as well as in the juxtamembrane stalk region, and generates two major fragments of 86 kDa and 81 kDa. The two fragments

[1] Address correspondence to this author at the Center for Biochemical and Biophysical Sciences and Medicine, Harvard Medical School, 250 Longwood Avenue, Boston, MA 02115. Fax: (617) 566-3137. E-mail: jriordan@warren.med.harvard.edu.

Abbreviations: ACE, angiotensin-converting enzyme; LH-RH, luteinizing hormone releasing hormone.

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were resolved by immunoaffinity chromatography and were characterized in terms of their hydrolysis of angiotensin I, hippuryl-His-Leu and LH-RH.

METHODS

Enzyme purification. Human kidney ACE was purified to homogeneity by methods detailed elsewhere (8). Amino acid analysis was determined by 6-aminoquinolyl-*N*-hydroxy succinimidyl carbamate precolumn derivatization (9). Samples for sequencing were coupled to a DITC membrane and then subjected to N-terminal analysis on a Millipore Pro Sequencer.

Endoproteinase Asp-N digest. Purified kidney ACE (1.0 nmol) in 0.25 ml of 50 mM Hepes, pH 8.0 was digested with 2 μg endoproteinase Asp-N (Boehringer Mannheim) for 16 h at 37°C. The products were analyzed by SDS-PAGE and stored at −20°C.

Separation of proteolytic fragments. Phase separation of ACE in Triton X-114 was carried out as detailed elsewhere (10, 11). Monoclonal antibodies 9B9 and i2H5 that recognize epitopes from the N domain (12) were combined and coupled to protein G Sepharose 4B (Pharmacia) and crosslinked with dimethyl pimelimidate dihydrochloride (13). The bound material was eluted from the column as described (14) but with 250 mM triethylamine, pH 11.0, and fractions were collected in tubes containing 1M Tris.HCl, pH 6.8.

Kinetic characterization. Initial rates were measured during the first 5% of substrate hydrolysis. Angiotensin I and hippuryl-His-Leu hydrolyses were followed by the release of His-Leu which was derivatized with *o*-phthalaldehyde (15, 16). For LH-RH assays, quantitation of peptides LH-RH_{1-3} and LH-RH_{4-10} was performed by HPLC (17).

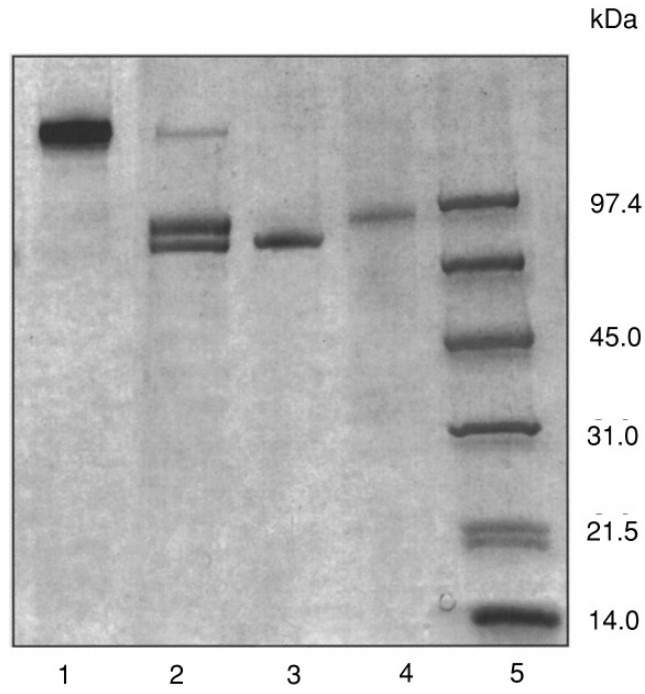

FIG. 1. SDS polyacrylamide gel electrophoresis of kidney ACE and the fragments produced after treatment with endoproteinase Asp-N on 4–20% gradient gel stained with Coomassie Brilliant Blue. *Lane 1,* kidney ACE; *lane2,* kidney ACE digested with endoproteinase Asp-N; *lane 3,* C-terminal fragment of kidney ACE; *lane 4,* N-terminal fragment of kidney ACE; *lane 5,* molecular mass markers.

RESULTS AND DISCUSSION

Endoproteinase Asp-N digestion of kidney ACE. Previously, limited digestion of somatic ACE has either yielded one dominant fragment or, as in the case of ammonium hydroxide treatment of rabbit ACE, an active 82 kDa fragment and a smaller 62 kDa fragment devoid of enzymatic activity. In our experiments proteolysis of native human kidney ACE with endoproteinase Asp-N gave two major bands on SDS-PAGE (Fig. 1, lane 2). The two bands were transferred to DITC membranes and subjected to N-terminal sequence analysis. The following sequences were obtained:

Upper band	(Leu) Asp Pro Gly Leu Gln Pro Gly Asn Phe Ser
Lower band	(Asp) Glu Ala Glu Ala Ser Lys Phe Val Glu Glu

These data correspond to residues 1-11 and 616-626, respectively, of human ACE and proved that the two fragments were derived from the N- and C- terminal halves of the of the intact protein. The cleavage site between the N and C domains could thus be assigned to Thr^{615}-Asp^{616}. Moreover, the similarity of the molecular weights suggested that the transmembrane and cytoplasmic domains had been cleaved from the C-terminal half.

Separation of the N and C domains. Both of the fragments partitioned into the detergent poor phase on treatment with Triton X-114 preventing resolution by phase separation. Based on earlier work where membrane anchored ACE partitioned into the detergent-rich phase (10) this finding confirmed the absence of the hydrophobic, transmembrane segment (and *pari passu* the cytosolic domain) due to the presence of a second cleavage site in the extracellular stalk region.

Owing to the similar physicochemical properties of the two fragments little success was achieved in isolating the individual polypeptides by ion exchange, hydrophobic interaction and gel filtration chromatography. Instead, an immunoaffinity column was generated with monoclonal antibodies recognizing epitopes from the antigenic region of the N domain of ACE, coupled to protein G Sepharose. The N-terminal fragment bound to the column whereas the C-terminal fragment emerged in the void volume. The former was eluted with 250mM trimethylamine, pH 11.0, and the latter was further purified using a lisinopril Sepharose affinity column (8). The two fragments ran as single bands on SDS-PAGE at Mr = 81 and 86 kDa (Fig. 1, lanes 3 and 4) corresponding to the C and N domains, respectively.

TABLE 1

Amino Acid Composition of N and C Fragments of Human Kidney ACE

Amino acid	N Fragment	C Fragment
Asx	60.2 (58)	62.8 (61)
Glx	70.1 (69)	79.6 (73)
Ser	31.3 (35)	32.1 (36)
Gly	34.3 (31)	34.6 (33)
His	19.9 (18)	20.3 (20)
Arg	30.6 (32)	27.3 (28)
Thr	28.9 (29)	30.2 (31)
Ala	49.8 (52)	46.5 (45)
Pro	33.1 (34)	32.3 (35)
Tyr	24.8 (28)	22.9 (26)
Val	27.3 (28)	27.0 (28)
Met	11.5 (12)	16.5 (17)
Ile	19.9 (19)	24.9 (25)
Leu	65.3 (63)	63.7 (64)
Phe	27.6 (29)	25.5 (26)
Lys	25.5 (23)	31.8 (30)

Note. The numbers in parentheses are based on the amino acid sequence of human somatic ACE (23) and correspond to residues 1–615, N fragment, and 616–1219, C fragment. The amino acid composition of the latter suggests that Asp-N cleavage in the juxtamembrane stalk region of ACE occurs between Leu^{1219} and Asp^{1220}, resulting in the loss of residues 1220–1277.

The similarity between these two homologous domains is demonstrated by their amino acid composition (Table 1). When compared to the C fragment the composition of the N fragment is notable for significantly lower amounts of Met, Ile and Lys (20 to 30%). The composition of the C fragment suggests that the second site of cleavage is between Leu^{1219} and Asp^{1220} which is in the stalk region, eight residues from the start of the transmembrane region (Fig. 2).

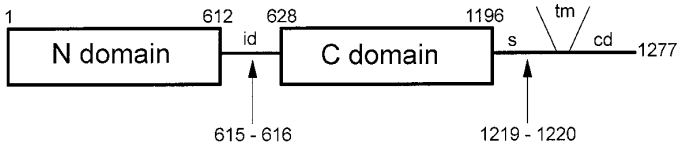

FIG. 2. Schematic structure of ACE (not to scale). The N domain extends from residue 1 to ~ residue 612. The interdomain (id) region extends from ~ residue 612 to ~ residue 628 and encompasses residues 615 and 616, the site of Asp-N cleavage. The C domain extends from ~ residue 628 to ~ residue 1196. Asp-N also cleaves ACE in the juxtamembrane stalk region (s), likely between residues 1219 and 1220 based on the amino acid analysis of the C domain (Table 1). This removes the remaining 8 stalk residues, the 22 residue transmembrane domain (tm) and the 28-residue cytosolic domain (cd). The exact length of the C domain and the juxtamembrane stalk region are not known with certainty but are estimated from the effects of mutagenesis on the rate of proteolytic release of membrane-bound ACE from chinese hamster ovary cells (26) and more recent results from this laboratory (M.R.W. Ehlers, personal communication). The lack of Asp-N cleavage at Asp^{1210} likely reflects the presence of Pro at position 1209.

TABLE 2

Kinetic Parameters for Substrate Hydrolysis by ACE and Its C and N Fragments

	Angiotensin I		Hippuryl-His-Leu		LH-RH	
	K_M (μM)	k_{cat} (sec^{-1})	K_M (μM)	k_{cat} (sec^{-1})	K_M (μM)	k_{cat} (sec^{-1})
Kidney ACE	23[a]	32.6[b]	1900[c]	400	97[d]	1.32[d]
N Fragment	70	10	2600	46	138	0.72
C fragment	82	36	1387	315	63	0.08

[a] Overall hydrolysis as measured by sum of $LH\text{-}RH_{1\text{-}3}$ and $LH\text{-}RH_{4\text{–}10}$.
[b] From (24).
[c] From (18).
[d] From (25).

Secondary structure predictions. The Chou-Fasman technique (15) was used to predict the secondary structure of the protein in the vicinity of the cleavage site between the N and C domains. Asp^{616} is located in a hydrophobic region of the polypeptide that displays a high surface probability and a high degree of flexibilty (data not shown). Interestingly, the short span of residues encompassed by these peaks concurs with the N-terminal squence analyses, in which no minor sequences were observed. Thus it appears that negligible cleavage occurred at any of the 65 other aspartic acid residues in the protein, and especially not at the neighboring residues, Asp^{612} and Asp^{628}. This suggests that the stretch of residues linking the two domains, and susceptible to proteolysis, may only be 10-15 residues in length.

Kinetic characterization of the N and C domains. The enzymatic activities of the N and C fragments were measured using three substrates with different specificities for the N and C domains. The k_{cat} values for the hydrolysis of angiotensin I and hippuryl-His-Leu by the N fragment are 28% and 21% respectively of that of the C fragment. However, the K_m values of the two domains are similar for the hydrolysis of angiotensin I (see Table 2). This is in agreement with earlier work that suggested the two domains have similar affinities for these substrates although differing in their rates of hydrolysis (16). In contrast to these data, hydrolysis of the blocked decapeptide LH-RH by the N fragment was about an order of magnitude faster than that of the C fragment, confirming the concept that the N domain is predominantly responsible for the endopeptidase activity (17,18). These data demonstrate unequivocally that the two fragments from Asp-N cleavage of kidney ACE are derived from the N and C terminal halves, and that both contain active sites that retain their functional integrity.

To our knowledge this is the first successful attempt

at separating and purifying to homogeneity the two domains of native, tissue-derived ACE, with retention of enzymatic activity. A recent study showed that a naturally-occurring fragment of human ACE corresponding to the N-terminal domain and extending to between residues 443 and 559 is present in ileal fluid (19). This fragment was less active toward bradykinin but more active toward LH-RH than testicular ACE, i.e. the C-terminal domain (20). Our results provide independent verification of these data and those obtained with recombinant N and C domains of somatic ACE in which it was first shown that each of the domains is catalytically active (16,17). Both domains have been shown to exhibit similar catalytic activities toward the physiological substrates angiotensin I and bradykinin (17), but the two active sites display different patterns of chloride activation as well as different inhibitor binding properties (21). Moreover, the N domain preferentially hydrolyses substrates such as LH-RH (17,18) and the hemoregulatory tetrapeptide N-acetyl-Ser-Asp-Lys-Pro (22).

Our data reaffirm the previous conclusion, based on a comparison of the activities of the somatic and testis ACE isozymes, that although both domains in somatic ACE are active, the intact somatic enzyme does not have a higher k_{cat} for conventional substrates (e.g., angiotensin I and furanacryloyl-Phe-Gly-Gly) than the isolated C domain, implying that with respect to their substrates the two domains do not function independently. An understanding of the precise arrangement of the two domains and the nature of the interaction between their respective active sites awaits the determination of the three-dimensional crystal structure. For this and related structure-function studies, the production of active N and C domains from the native enzyme by limited proteolysis should provide a useful additional investigative tool.

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