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A Study of Chimeras Constructed with the Two Domains of Angiotensin I-Converting Enzyme

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ABSTRACT. Angiotensin I-converting enzyme (ACE) is composed of two highly similar domains called the N and C domains, which display some contrasting enzymatic properties. We constructed two ACE chimeras: chimera 1, comprised of the N domain containing the central 60 amino acid residues of the C domain, and chimera 2, comprised of the C domain containing the central 60 amino acid residues of the N domain. Chimeras 1 and 2 displayed K_m values for Hip-His-Leu and Z-Phe-His-Leu and k_{cat} ratios for these two substrates similar to that of the N and C domains, respectively. Thus, the short sequence exchanged between the two domains does not confer the specific properties of that domain for these two substrates but, rather, such specific properties must arise from the sequences surrounding the central region in each domain. BIOCHEM PHARMACOL 51;1: 11–14, 1996.

KEY WORDS. chimeras; monoclonal antibody

ACE§ (EC 3.4.15.1) is a zinc metallopeptidase primarily involved in the conversion of angiotensin I to the vasopressor peptide angiotensin II and in the inactivation of the vasodilatory peptide bradykinin [1]. The involvement of ACE in the metabolism of these two vasoactive peptides has led to the development of a large number of specific and highly potent ACE inhibitors, many of which are used as orally active drugs in the treatment of hypertension and congestive heart failure.

Mammalian ACE is composed of two highly similar domains, referred to as the N and C domains, each of which is catalytically active [2, 3], binds a zinc ion [4, 5], and a competitive ACE inhibitor [4, 6]. Previously, truncated ACE mutants were produced that contain only a single intact domain [3]. The two domains of ACE display some contrasting enzymatic properties: the C domain hydrolyzes Hip-His-Leu, angiotensin I, and substance P at a faster rate compared to the N domain [3, 7]; in contrast, the N domain hydrolyzes luteinising hormone-releasing hormone at a faster rate and at a specific amino-terminal cleavage site compared to the C domain [4, 7]. In addition, the two domains interact differently with a number of specific ACE inhibitors [6, 8] and display conformational differences as determined by immunological analyses [9]. The aim of the present study was to identify residues that may give rise to some of the observed differences between the two domains. We constructed two chimeras composed of the truncated fragments of either the N or C domain with the central 60 amino acid sequence exchanged between the two domains. This sequence was chosen for the exchange because it contains essential active site residues, notably the consensus sequence for zinc binding and the glutamate third zinc ligand. In addition, this sequence displays the highest level of homology between the two domains (78%). The enzymatic and immunological analyses of the expressed mutants are described.

MATERIALS AND METHODS

Site-Directed Mutagenesis

cDNAs encoding the N and C fragments of ACE were produced as described previously [3] except that the soluble form of the C fragment was produced with a stop codon located before the membrane-anchoring sequence [10]. Unique *Pvu*I and *Mlu*I sites were introduced into the cDNAs encoding the two fragments contained in an M13 vector by site-directed mutagenesis (Bio-Rad Laboratories) by a method based on that described by Kunkel [11]. The restriction sites are situated 5′ and 3′ to the sequence encoding the zinc-binding motif (HEXXH) and the glutamate zinc ligand [12] (Fig. 1). The introduced restriction sites produced silent mutations; their presence was confirmed by restriction mapping and DNA sequencing (Sequenase 2.0, U.S.B., OH); the absence of spurious mutations was also confirmed by sequencing. The cDNAs were subcloned into the expression vector pECE and chimera constructs were produced by digestion with *Pvu*I and *Mlu*I, purification and ligation of appropriate fragments (Fig. 1). The chimera constructs encode chimera 1 comprised of the N fragment with 13 amino acid changes derived from the C fragment and chimera 2 comprised of the C fragment with 13 amino acid changes derived from the N fragment. The 13 amino acids that differ between the two domains within the 60 amino acid sequence exchanged are shown in Fig. 2.

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§ Abbreviations: ACE, angiotensin I-converting enzyme; Hip, Hippuryl; Z, benzoyloxycarbonyl; MoAb, monoclonal antibody.
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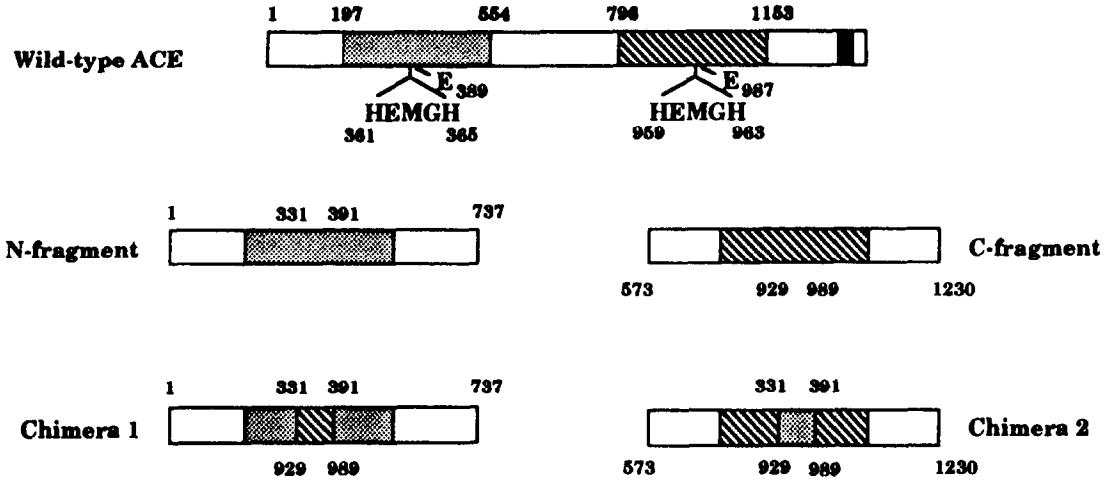

FIG. 1. Schematic diagram of the primary structures of wild-type ACE, truncated ACE fragments, and chimeras 1 and 2. Wild-type ACE (ACE) contains the C-terminal transmembrane domain (right black box) and the N and C domains: the amino acid sequence homology between these domains reaches 67.7% between residues 197–554 (dotted box) and 796–1153 (box with slanted shading). The zinc-binding motif "HEMGH" and the glutamate third zinc ligand is shown under each domain (human ACE numbering). *Pvu*I and *Mlu*I restriction sites were introduced into the cDNAs encoding the N and C fragments as described under Materials and Methods at positions corresponding to positions 331 (*Pvu*I) and 391 (*Mlu*I) in the N fragment and positions 929 (*Pvu*I) and 989 (*Mlu*I) in the C fragment. The *Pvu*I and *Mlu*I restriction sites, unique to the N and C fragments, were used to construct cDNAs encoding ACE chimeras 1 and 2.

Enzymatic Characterization

COS-7 cells were cultured and transfected as described previously [13]. The serum-free culture medium was collected and concentrated by Amicon filtration. ACE activity was determined using Hip-His-Leu (5 mM final concentration) and Z-Phe-His-Leu (0.5 mM final concentration). The release of His-Leu was measured fluorimetrically (detection limit 0.02% substrate hydrolysis) as described previously [14, 15]. Enzyme activities were calculated from the first 5% substrate hydrolysis and the specificity of the reaction was confirmed by the specific ACE inhibitor captopril (10 μM).

Plate Immunoprecipitation Assay and Inhibition of ACE Activity by Monoclonal Antibodies

The binding of ACE mutants to monoclonal antibodies MoAbs) was quantified by a plate immunoprecipitation assay described previously [9]. The inhibitory potencies of MoAbs i2H5 and 3A5 (specific inhibitors of the N domain active site) were determined for the hydrolysis of Z-Phe-His-Leu by the ACE mutants as described previously [9].

RESULTS

Enzymatic Characterization

The ratio of hydrolysis of Z-Phe-His-Leu/Hip-His-Leu can distinguish the activities of the N and C domains: Z-Phe-His-Leu is hydrolyzed at an equal rate by both domains [9], whereas Hip-His-Leu is hydrolyzed at a faster rate by the C domain active site [3]. Wild-type ACE, the N and C fragments, and chimeras 1 and 2 displayed similar K_m values for Hip-His-Leu; however, some variation was observed in the K_m values for the substrate Z-Phe-His-Leu, although these values were in the same order of magnitude. Catalytic activities were characterized by the ratio of hydrolysis of Z-Phe-His-Leu/Hip-His-Leu using the same quantities of the same enzyme sample for each substrate hydrolysis. In this case, the ratio of V_{max} becomes equal to the k_{cat} ratio of Z-Phe-His-Leu/Hip-His-Leu hydrolysis (k_{cat} ratio). The k_{cat} ratio of chimeras 1 and 2 were the same compared to the N- and C-fragments, respectively (Table 1).

Immunological Characterization and Inhibition by Monoclonal Antibodies

Chimera 1 displayed low, but significant, levels of cross-reactivity to MoAbs i2H5 and 6A12 (3–4% binding compared to the N fragment) (Fig. 3). Chimera 2 and the C fragment did not bind any of the MoAbs. The inhibition of the N and C fragments and chimeras 1 and 2 by MoAbs 3A5 and i2H5 was investigated using Z-Phe-His-Leu as substrate. The N fragment activity was inhibited by both antibodies, with i2H5 exhibit-

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N-fragment: 331-------Arg---------Arg-ThrMetAspGln-SerThr
C-fragment: 929-------Gly---------Thr-AsnLeuGluAsp-ValVal

            Val---------TyrLeu-------Ser--Arg----------391
            Ala---------PheMet-------Ala--Glu----------989
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FIG. 2. Alignment of the amino acid sequences encoded between the *Pvu*I and *Mlu*I sites in the N and C fragments of ACE. The sequences between the *Pvu*I and *Mlu*I restriction sites of the cDNAs encoding the N and C fragments encode 60 amino acids. These 60 amino acids are situated from residues 331–391 and 929–989 for the N and C fragments, respectively. Conserved residues between the N and C fragments are represented by dashes.

TABLE 1. Enzymatic characterization of chimeras 1 and 2

Enzyme	K_m mM Z-Phe-His-Leu	K_m mM Hip-His-Leu	k_{cat}/k_{cat} Z-Phe-His-Leu/Hip-His-Leu
Wild-type	0.15	1.6	0.95 ± 0.05
N-fragment	0.93	2.5	4.50 ± 0.35
Chimera 1	0.37	1.5	4.50 ± 0.42
C-fragment	0.20	1.5	0.67 ± 0.07
Chimera 2	0.20	1.6	0.67 ± 0.09

Results are the mean of 3–4 independent determinations. Assays were performed in the presence of 300 mM chloride at pH 8.3. The k_{cat}/k_{cat} ratio is quoted ± SEM for the calculated values of V_{max} (Z-Phe-His-Leu)/V_{max} (Hip-His-Leu), which is equivalent to k_{cat} (Z-Phe-His-Leu)/k_{cat} (Hip-His-Leu). Values of K_m and V_{max} were derived from Lineweaver-Burk plots.

ing a greater inhibitory potency compared to 3A5. The C fragment and chimeras 1 and 2 were not inhibited by either i2H5 or 3A5.

DISCUSSION

The construction of chimeric proteins can potentially map regions of a protein to a specific function. In the present study, we have used the truncated mutants of ACE, of either the N or C domain [3], to produce two ACE chimeras. Enzymatic characterization of recombinant mutants indicated that chimeras 1 and 2 compared to the N and C fragments, respectively, displayed similar enzymatic characteristics for the ratio of Z-Phe-His-Leu/Hip-His-Leu hydrolysis. In addition, chimeras 1 and 2 did not display any significant modification of the K_m for these substrates compared to the N and C fragments, indicating that substrate-binding subsites were conserved by the 60 amino acid transition. It would appear that the 13 amino acids within the 60 amino acid sequence that differ between the two domains are not responsible for conferring the distinct enzymatic properties of the domains for the hydrolysis of Z-Phe-His-Leu and Hip-His-Leu.

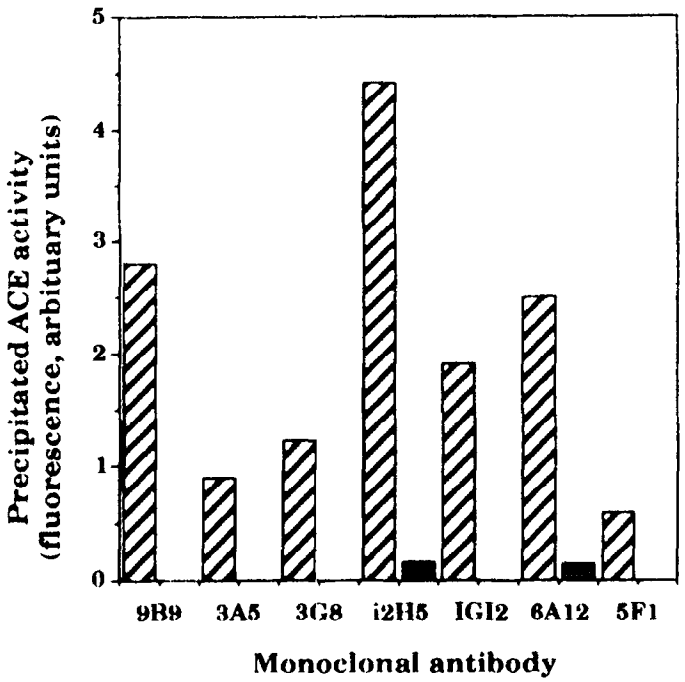

FIG. 3. ACE activities of the N fragment and chimera 1 precipitated by monoclonal antibodies. No detectable MoAb-precipitated ACE activity was observed for the C fragment and chimera 2. Immunoprecipitated ACE activity of the N fragment and chimera 1 was measured as described under Materials and Methods using a panel of 7 monoclonal antibodies isolated and characterised previously. ▩N fragment; ▨Chimera 1.

Three arginine residues (amino acid positions 339, 349, and 381) have been substituted for different residues in chimera 1 compared to the N fragment. In the proposed active site model of ACE, as in carboxypeptidases, an arginine residue is considered to be important to position the substrate reacting with the free carboxy-terminus of the peptide substrate to allow hydrolysis of the penultimate peptide bond. The present data indicate that this function is not served by Arg 339, 349, or 381 in the N domain of ACE.

The binding of a series of anti-ACE monoclonal antibodies to chimeras 1 and 2 was investigated and compared to the N and C fragments. An absence of binding was observed for chimera 2 and the C fragment. For the C fragment, this observation is consistent with previous results [9]. In contrast, the N fragment displayed high levels of binding to the panel of monoclonal antibodies and chimera 1 (composed primarily of the N fragment) displayed 3–4% of antibody binding compared to the N fragment. The series of monoclonal antibodies used in this study, with the exception of 5F1, recognize conformational epitopes. If the conformation of chimera 1 has been altered compared to the N fragment, such an alteration is nonetheless tolerated for enzymatic activity.

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