Hydrolysis of Biological Peptides by Human Angiotensin-converting Enzyme-related Carboxypeptidase*

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Human angiotensin-converting enzyme-related carboxypeptidase (ACE2) is a zinc metalloprotease whose closest homolog is angiotensin I-converting enzyme. To begin to elucidate the physiological role of ACE2, ACE2 was purified, and its catalytic activity was characterized. ACE2 proteolytic activity has a pH optimum of 6.5 and is enhanced by monovalent anions, which is consistent with the activity of ACE. ACE2 activity is increased ~10-fold by Cl− and F− but is unaffected by Br−. ACE2 was screened for hydrolytic activity against a panel of 126 biological peptides, using liquid chromatography-mass spectrometry detection. Eleven of the peptides were hydrolyzed by ACE2, and in each case, the proteolytic activity resulted in removal of the C-terminal residue only. ACE2 hydrolyzes three of the high catalytic activity: angiotensin II (1–8) (kcat/Km = 1.9 × 105 M−1 s−1), apelin-13 (kcat/Km = 2.1 × 106 M−1 s−1), and dynorphin A 1–13 (kcat/Km = 3.1 × 106 M−1 s−1). The ACE2 catalytic efficiency is 400-fold higher with angiotensin II (1–8) as a substrate than with angiotensin I (1–10). ACE2 also efficiently hydrolyzes des-Arg9-bradykinin (kcat/Km = 1.3 × 105 M−1 s−1), but it does not hydrolyze bradykinin. An alignment of the ACE2 peptide substrates reveals a consensus sequence of: Pro-X1–2 residue-Pro-Hydrophobic, where hydrolysis occurs between proline and the hydrophobic amino acid.

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The abbreviations used are: ACE2, angiotensin-converting enzyme-related carboxypeptidase; ACE, angiotensin I-converting enzyme; Ang I, angiotensin I (1–10); Ang II, angiotensin II (1–8); Mca-APK(Dnp), (7-methoxycoumarin-4-yl)acetyl-Ala-Pro-Lys(2,4-dinitrophenyl)-OH; Mca-YVADPK(Dnp), (7-methoxycoumarin-4-yl)acetyl-Tyr-Val-Ala-Asp-Ala-Pro-Lys(2,4-dinitrophenyl)-OH; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; HPLC, high pressure liquid chromatography; HES, 1,3-bis[tris(hydroxymethyl)methylamino]propane.

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baculovirus of titer 1.1 × 10^10 pfu/ml. A 10-liter fermentation run was carried out with SF9 cells grown to a density of 1.3 × 10^7 cells/ml in SF9001 serum-free medium (Invitrogen), 18 mM l-glutamine, and 1× antibiotic-antimycotic (from 100X stock; Invitrogen) at 27 °C. At 96 h after infection, cells were pelleted at 5000 × g centrifugation, and the culture was then filtered, concentrated, dialyzed against 0.15–0.20 M NaCl, and stored at -50 °C until use. The thawed supernatant was filtered (0.2-μm filter) and loaded onto a Toyopearl QAE anion exchange column, and the column was washed with buffer A (25 mM Tris-HCl, pH 8.0) after all of the culture had been collected. The column was equilibrated with buffer A (25 mM Tris-HCl, pH 8.0) at ambient temperature. To each sample was added buffer A (100 μl) and buffer B (10 μl of 1.5 M NaCl and 25 mM Tris-HCl, pH 8.0) such that a total volume of 0.5 ml was used. The ACE2-containing fractions were detected by Coomassie Blue-stained SDS-PAGE, were pooled, and (NH₄)₂SO₄ was added to a final concentration of 1.0 M. The sample was then loaded onto a Toyopearl Phenyl column. After loading, the column was washed with buffer C (1.0 M (NH₄)₂SO₄ and 25 mM NaCl, pH 6.5). However, ACE2 maintains substantial catalytic activity at pH 5.0 and has optimal activity at pH 8.0. Reactions were performed at room temperature for 0, 15, 22.5, or 30 min and quenched by the addition of 10 μl of 0.5 M EDTA. Samples were then analyzed by MALDI-TOF mass spectrometry for detection of ACE2 and its product peptides by MALDI-TOF mass spectrometry to identify a suitable fluorescent substrate for initial enzyme characterization. The caspase-1 substrate Mca-TVAADPK(Dnp) was found to be hydrolyzed by ACE2, as measured by a time-dependent increase in fluorescence (excitation = 320 nm, emission = 405 nm). Analysis of the reaction products by MALDI-TOF mass spectrometry indicated hydrolysis of the Pro-Lys(2,4-dinitrophenyl) peptide bond. A truncated peptide with more efficient intramolecular fluorescence quenching, Mca-APK(Dnp), was synthesized and assayed as an ACE2 substrate with the goal of improving the fluorescence signal of the assay. Complete hydrolysis of 40 μM Mca-APK(Dnp) resulted in a 300-fold fluorescence increase over background, whereas complete hydrolysis of the same concentration of Mca-TVADPK(Dnp) resulted in a 21-fold increase over background. The Mca-APK(Dnp) substrate is hydrolyzed by ACE2 and was used for characterization of the enzyme activity.

Recombinant soluble human ACE2, encoding amino acids 1–740 of the 808-amino acid full-length enzyme and deleting the C-terminal transmembrane domain, was expressed in Chinese hamster ovary cells and isolated to ≥90% purity by SDS-PAGE (as described previously, Ref. 1). This ACE2 sample was used to screen a number of commercially available intramolecularly quenched fluorescent peptides to identify a suitable fluorescent substrate for initial enzyme characterization. The caspase-1 substrate Mca-TVADPK(Dnp) was found to be hydrolyzed by ACE2, as measured by a time-dependent increase in fluorescence (excitation = 320 nm, emission = 405 nm). Analysis of the reaction products by MALDI-TOF mass spectrometry indicated hydrolysis of the Pro-Lys(2,4-dinitrophenyl) peptide bond.

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Recombinant soluble human ACE2 was expressed in Sf9 insect cells and isolated to ≥98% purity, based on SDS-PAGE (Fig. 1C), by a four-step chromatography protocol. The purified protein sample was confirmed to be ACE2 by peptide mapping of trypsin-digested protein, analyzed by MALDI-TOF mass spectrometry (data not shown). The molecular mass of the purified ACE2 (89.6 kDa, as determined by MALDI-TOF mass spectrometry) is greater than that predicted from the peptide sequence (85.314 kDa). The higher molecular mass is likely to be due to glycosylation, as has been reported for ACE2 (6). The ACE2 sample efficiently hydrolyzes the fluorogenic peptide Mca-APK(Dnp), with kinetic constants of $k_{cat} = 147 ± 7.7 \text{ M}^{-1} \text{s}^{-1}$ and $K_{m} = 7.7 \times 10^{-5} \text{ M}$, determined by an HPLC/UVC detection-based assay, as described under “Experimental Procedures.” The activity of ACE2, which was identified as a zinc metalloprotease, was found to be stabilized by the presence of 10 μM ZnCl₂ in the buffer (data not shown). ACE2 was stable for >2 h at room temperature in assay buffer. This ACE2 sample from Sf9 insect cells was then used for all subsequent studies described.

The dependence of ACE2 proteolytic activity on pH and monovalent anion concentration was determined as described under “Experimental Procedures.” ACE2 activity has a strong pH dependence under acidic conditions (Fig. 2), such that the enzyme is almost inactive at pH 5.0 and has optimal activity at pH 6.5. However, ACE2 maintains substantial catalytic activity under basic conditions (pH 7–9). ACE2 proteolytic activity is greatly enhanced by high concentrations of chloride or fluoride.

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2. E. Callewood, N. Dales, A. Gould, B. Guan, T. Ocain, and M. Patane, unpublished results.
biological peptides at the pH optimum for the enzyme and in the presence of Cl⁻ (pH 6.5, 0.3 M NaCl). The extent and site of peptide hydrolysis after a 2-h incubation with 50 nM ACE2 were analyzed by MALDI-TOF mass spectrometry. Peptides that were found to be hydrolyzed by ACE2 were then reassayed in the same manner in the presence of a high concentration of a potent, specific ACE2 inhibitor² to confirm that proteolysis was catalyzed by ACE2. Eleven peptides are hydrolyzed by ACE2 and are shown in Table I. There were 115 peptides that were not hydrolyzed by ACE2.³ In all cases, ACE2 exhibits only carboxypeptidase activity. The ACE2 hy-

"Experimental Procedures." Rates of hydrolysis of the internally quenched fluorescent peptide Mca-APK(Dnp) were determined by measuring the slope of increase in fluorescence (excitation = 320 nm, emission = 405 nm) under initial velocity conditions (≤10% hydrolysis) over 15–60 min. All values are an average (n = 2), and the S.D. is shown. ■, NaCl; □, NaBr; ●, NaF; ○, KCl.

³ Biologically active peptides not hydrolyzed by ACE2 (115 in total) are as follows: adrenocorticotropic hormone 1–39 (human), adrenocorticotropic hormone fragment 1–14, allatostatin I, allatostatin II, alytesin, amylin, β-amyloid peptide (1–28), angiotensin 1–8, angiotensin 1–7, angiotensin 1–5, antifilamin-1, antifilamin-2, atrial natriuretic peptide, bombesin, bradykinin, bradykinin fragment 1–7, brain injury-derived neurotrophic peptide brain natriuretic peptide-32 (porcine), caerulein, calcitonin (human), α-calcitonin gene-related peptide (human), cholecystokinin-8 sulfated, corticotropin-releasing factor (human), CSH 103, derrnorphin, dynorphin A 1–17, dynorphin B (porcine), eldoisine, endomorphin-1, endomorphin-2, α-endorphin, β-endorphin (human), β-neocendorphin, endothelin, Met-enkephalin, enterostatin (human), fibroenitin adhesion-promoting peptide, fibroenitin fragment 1371–1382, N-formyl-Met-Leu-Phe, galalin (human), galantide, gastric inhibitory polypeptide, gastrin 1 (human), Arg-Arg-gastrin fragment 22–30 (human), gastrin-releasing peptide (human), glucagon, glucagon-like peptide 1 (7–37), glucagon-like peptide 1 fragment 7–36, glucagon-like peptide 2 guanylin (rat), histidyl-proline (cyclized form), inhibin β-subunit fragment 67–94 (human), isocitocin, kemptide, (Trp⁶)-kemptide, (Val¹, Ala⁷)-kemptide, kineotens (human), leuotonin (full-length), leuotonin fragment 22–56 (human), Leu-enkephalin, litorin, luteinizing hormone-releasing hormone, malantide, mast cell-degranulating peptide HK1, mastoparan (wasp), melacin-concentrating hormone (human), α-melanocyte-stimulating hormone, β-melanocyte-stimulating hormone (human), γ-melanocyte-stimulating hormone, morphiceptin, motilin (porcine), myelin basic protein fragment 4–14 (bovine), neurogranin fragment 28–43, α-neurokinin, neurokinin A, neurokinin B, neuromedin B, neuromedin C, neuromedin K, neuropeptide FF (porcine), neuropeptide K, neuropeptide Y (human), neuropeptide TI, nociceptin, nocistatin (bovine), oreokinin, orexin A, orexin B, oxytocin, pancreaticastatin fragment 37–52, pancreatic polypeptide, parathyroid hormone 1–34 (human), peptide histidine methionine-27 (human), peptide T, peptide YY (human), pituitary adenylate cyclase-activating peptide 1–27, protein kinase C substrate, ranatensin, RGDS, sauvagine (frog), secretin (human), small, cardioactive peptide A, somatostatin-14, somatostatin-28, substance P, thyrotropin-releasing hormone, tyrosine protein kinase substrate, urocortin, urodilatin, valosin (porcine), vasoactive, intestinal peptide (human), (Arg⁶)-vasopressin, and (Arg⁶)-vasotocin.
ACE2 Biological Peptide Substrates

| Substrate Sequence | Hydrolysis |
|--------------------|------------|
| Angiotensin I       | DRVYIHFPFH | L | P |
| Angiotensin 1–9     | DRVYIHFPFH | N |
| Angiotensin II      | DRVYIHFPFH | F |
| Angiotensin 1–7     | DRVYIHFPFH | C |
| Angiotensin 1–5     | DRVYIHFPFH | N |
| Apelin-13           | QRPRLSHKGFP | F |
| Apelin-36 (C terminus shown) | QRPRLSHKGFP | F |
| Bradykinin          | RPFPFSFR   | N |
| des-Arg⁹-bradykinin | RPFPFSFR   | F |
| Lys-des-Arg⁹-bradykinin | RPFPFSFR | F |
| Bradykinin fragment 1–7 | RPFPFSFR | N |
| β-Casomorphin       | YPFYVEP    | I |
| Neocasomorphin      | YPFYVEP    | C |
| Dynorphin A 1–13    | YGGFLRIRRFLK | K |
| Ghrelin (C terminus shown) | ESSKKPKLQ | P |
| Neurotensin 1–8     | pE-LYENKP  | P |

**Comparison of ACE2 and ACE Catalytic Activity**—ACE2 has been purified to homogeneity, and its activity was characterized for comparison with its closest homolog, ACE. The catalytic activity of human soluble ACE2 was found to have a pH optimum of 8.5 in the presence of 1.0 M NaCl. There is a sharp pH dependence under acidic conditions, such that ACE2 is almost inactive at pH 5.0 but has substantial catalytic activity at pH 9.0. A similar pH dependence has been reported for the catalytic activity of rabbit lung ACE in the presence of 1.0 M NaCl (8). In this report, the pH dependence of ACE activity was determined to be dependent on the anion concentration, and the same may be true for ACE2.

ACE2 proteolytic activity is specific for des-Arg⁹-bradykinin, although it cleaves neither bradykinin nor bradykinin fragment 1–7.

Eight of the ACE2 peptide substrates identified were further characterized by determining the kinetic constants for their hydrolysis catalyzed by ACE2 using an HPLC separation/UV detection-based assay. Representative Michaelis-Menten plots of the data for four of the peptides are shown in Fig. 4, and the kinetic constants are summarized in Table II. ACE2 cleaves three biological peptide substrates with high catalytic efficiency: Ang II, apelin-13, and dynorphin 13. For all three substrates, the $K_m$ value is $<10$ μM, and the $k_{cat}$/$K_m$ value is $>1 \times 10^5$ M$^{-1}$ s$^{-1}$. By comparison, ACE2 hydrolyzes Ang I with a lower turnover number ($k_{cat}$ = 0.035 s$^{-1}$). ACE2 also cleaves des-Arg⁹-bradykinin peptides, β-casomorphin, and neurotensin 1–8 with substantial catalytic efficiency ($k_{cat}$/$K_m$ $\geq 1 \times 10^5$ M$^{-1}$ s$^{-1}$).

**Discussion**

The characteristic of ACE that is unique among metalloproteases is its activation by monovalent anions, including Cl$^-$, Br$^-$, and FI$^-$. ACE2 proteolytic activity is also activated by monovalent anions, with a 10-fold enhancement in the presence of 1.0 M NaCl. FI$^-$ enhances ACE2 activity to a similar degree, whereas Br$^-$ has no effect on activity. Recently, Husain and co-workers (12) determined that a residue conserved in all known ACE sequences, Arg$^{1098}$ in the C domain of the somatic form of human ACE, is critical for Cl$^-$ activation of the enzyme.

This residue is conserved in human and rat ACE2 (Arg$^{1098}$, alignment shown previously, Ref. 1) and may play a role in the observed ACE2 activation by Cl$^-$. Any observed differences in the characteristics of monovalent anion activation of ACE and ACE2 cannot be directly attributed to differences in the enzymes because other conditions, such as the substrate assayed, are known to affect ACE anion activation (8). It has been suggested that ACE activity may be regulated physiologically by Cl$^-$ concentration (13), and therefore ACE2 may be regulated in a similar manner.
in a tissue-specific manner. Studies to examine the in vivo effects of ACE2 inhibition on Ang II serum levels and on blood pressure will help us to understand the physiological role of the carboxypeptidase.

ACE2 hydrolyzes the hormone apelin-13 with high catalytic efficiency and cleaves apelin-36, whose C-terminal 13 amino acids are identical to those of apelin-13. These two forms of apelin were recently identified as endogenous ligands for the human APJ receptor (17, 18), which is a homolog of the angiotensin receptor AT1. Intravenous injection of apelin-13 in rat was found to decrease blood pressure (19), although a different group reported that the peptide is a potent vasoconstrictor (20).

It was also reported that intraperitoneal injection of apelin-13 in rat increases water intake (19).

Dynorphin A 1–13, identified as a good ACE2 substrate in vitro, is an endogenous opioid neuropeptide with antinociceptive effects (21). Dynorphin 1–12, the product of the ACE2 reaction, possesses 50- to 230-fold weaker binding affinity to the \( \kappa \)-opioid receptor than does dynorphin A 1–13 (22). Thus, this is an example in which the substrate and product of the ACE2-catalyzed hydrolysis in vitro have been reported to have different pharmacological effects in vivo.

The proteolysis of des-Arg\(^9\)-bradykinin by ACE2 in vitro may be relevant because of the physiological role of ACE in bradykinin metabolism. ACE has been demonstrated to be one of the primary proteases responsible for the hydrolysis of bradykinin and, to a lesser extent, des-Arg\(^9\)-bradykinin (4). Bradykinin and des-Arg\(^9\)-bradykinin possess different pharmacological properties; the former binds selectively to B2 receptors, and the latter binds selectively to B1 receptors. ACE2 hydrolyzes des-Arg\(^9\)-bradykinin but does not hydrolyze other forms of bradykinin. Whereas the turnover number for ACE2 hydrolysis of des-Arg\(^9\)-bradykinin (\( k_{\text{cat}} = 64 \text{ s}^{-1} \)) is comparable to the turnover number reported for ACE hydrolysis of bradykinin (\( k_{\text{cat}} = 11 \text{ s}^{-1} \); Ref. 23), the ACE2 \( k_{\text{cat}} \) value is 1600-fold higher than that of ACE (286 versus 0.18 \( \mu \text{M} \)).

Neurotensin 1–8 is a fragment of the known active form of the neuropeptide (for reviews, see Refs. 24 and 25) and is itself
not known to be biologically active. The biologically active forms, neurotensin-13 and neuromedin (9, 24), are not hydrolyzed by ACE2.

Although the biological peptides Ang II, apelin-13, dynorphin A 1–13, and des-Arg9-bradykinin are good ACE2 substrates in vitro, such evidence is only suggestive that they may be physiological substrates of ACE2. The measurement of changes in their in vivo levels in wild-type versus ACE-2 knockout animals or upon administration of an ACE2 inhibitor is needed to further understand the biological role of ACE2.

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