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 peptides with high catalytic efficiency: angiotensin II (1–8) ($k_{cat}/K_m = 1.9 \times 10^6$ M^{-1} s^{-1}), apelin-13 ($k_{cat}/K_m = 2.1 \times 10^6$ M^{-1} s^{-1}), and dynorphin A 1–13 ($k_{cat}/K_m = 3.1 \times 10^6$ 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 ($k_{cat}/K_m = 1.3 \times 10^5$ M^{-1} s^{-1}), but it does not hydrolyze bradykinin. An alignment of the ACE2 peptide substrates reveals a consensus sequence of: Pro-X$_{(1–3 residues)}$-Pro-Hydrophobic, where hydrolysis occurs between proline and the hydrophobic amino acid.

Human angiotensin-converting enzyme-related carboxypeptidase (ACE2)[1] is a close homolog of human endothelial angiotensin I-converting enzyme (ACE, EC 3.4.15.1), with 42% protein sequence identity between the catalytic domains (for sequence alignment, see Ref. 1). ACE, a component of the renin-angiotensin system, is a zinc metalloprotease that catalyzes cleavage of the C-terminal dipeptide from Ang I to produce the potent vasopressor octapeptide Ang II (2). ACE-inhibiting drugs have an antihypertensive effect and substantially lower the long-term risk of death, heart attack, stroke, coronary revascularization, heart failure, and complications related to diabetes mellitus (for review, see Ref. 3). ACE also inactivates bradykinin by catalyzing the cleavage of the C-terminal dipeptide from the nonapeptide hormone (4), and ACE inhibitor-induced cough has been attributed to inhibition of bradykinin metabolism (5).

Like ACE, ACE2 is expressed in endothelial cells, although its expression is restricted to fewer tissues, which include the heart, kidney, and testis (1). ACE2 was identified as a zinc metalloprotease due to its canonical HE*XX*H sequence (amino acids 374–378) (1), its inhibition by EDTA (6), and its sequence identity with the catalytic residues of ACE (7). The ACE inhibitors captopril, lisinopril, and enalaprilat are not inhibitors of ACE2 (1, 6). The physiological and pathophysiological role of ACE2 is not yet clearly understood. To better understand the physiological role of ACE2, a detailed biochemical analysis of ACE2 substrate preference was undertaken.

We reported previously that secreted recombinant ACE2 expressed in Chinese hamster ovary cells catalyzes cleavage of the C-terminal residue of the biological peptides Ang I, des-Arg⁹-bradykinin, neurotensin 1–13, and kinetensin (1). Similarly, Tipnis *et al.* (6) reported that unpurified ACE2 expressed in Chinese hamster ovary cells catalyzes the hydrolysis of the C-terminal residue of Ang I and Ang II. Herein is the first report of characterization of the catalytic activity of purified ACE2. A sensitive fluorogenic substrate was developed and used to assess the dependence of ACE2 hydrolytic activity on pH and on the presence of monovalent anions. Also, ACE2 substrates were identified from screening biological peptides, and the kinetic constants were determined for hydrolysis of those peptides. The identified peptides are candidate ACE2 physiological substrates.

EXPERIMENTAL PROCEDURES

Materials—HPLC columns were purchased from the Waters Corp. (Milford, MA). Toyopearl columns were purchased from Tosoh Biosep (Montgomeryville, PA). The peptide Mca-APK(Dnp) was synthesized by Anaspec, Inc. (San Diego, CA). Biological peptides were purchased from Sigma-Aldrich Co., Bachem Bioscience (King of Prussia, PA), and American Peptide Co. (Sunnyvale, CA). Specifically, Ang I, Ang II, and dynorphin A 1–13 were purchased from Sigma-Aldrich Co. 7-Methoxycoumarin-4-yl)acetyl-YVADAPK(2,4-dinitrophenyl)-OH (M-2195), apelin-13, β-casomorphin, des-Arg⁹-bradykinin, Lys-des-Arg⁹-bradykinin, and neurotensin 1–8 were purchased from Bachem Bioscience.

Baculovirus Expression and Purification of Soluble ACE2—An expression vector was generated encoding a secreted form of human ACE2 (1) (amino acids 1–740) in the pBac Pak9 vector (CLONTECH). Sf9 insect cells were infected at a multiplicity of infection of 0.1 with ACE2

[1] 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-YVADAPK(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; MES, 4-morpholineethanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid; bis-Tris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane.

baculovirus of titer 1.1×10^9 pfu/ml. A 10-liter fermentation run was carried out with SF9 cells grown to a density of 1.3×10^6 cells/ml in SF900II 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 $5000 \times g$ centrifugation, and the culture supernatant was collected, frozen, and stored at -80 °C.

The thawed supernatant was filtered (0.2-μm filter) and loaded onto a Toyopearl QAE anion exchanger column, and the column was washed with buffer A (25 mM Tris-HCl, pH 8.0). A 0–50% gradient elution was then performed with increasing buffer B (1.0 M NaCl and 25 mM Tris-HCl, pH 8.0) using a total of 5 column volumes. The ACE2-containing fractions, as detected by Coomassie Blue-stained SDS-PAGE, were pooled, and $(NH_4)_2SO_4$ 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 $(NH_4)_2SO_4$ and 25 mM Tris-HCl, pH 8.0) using 5 column volumes and then gradient-eluted with buffer A (0–100%). The ACE2-containing fractions, as detected by Coomassie Blue-stained SDS-PAGE, were pooled and dialyzed against buffer A at 4 °C overnight. The dialyzed ACE2 protein sample was sequentially loaded onto MonoQ column (Amersham Biosciences) and gradient-eluted with buffer B. The ACE2-containing fractions from the MonoQ column, as detected by Coomassie Blue-stained SDS-PAGE, were concentrated with a Centricon (Millipore Corp., Bedford, MA) concentrator, with a molecular mass cutoff of 30 kDa. The concentrated sample was loaded onto a TSK G3000SWxl size exclusion column and eluted with buffer A.

Determination of Dependence of ACE2 Activity on pH and Monovalent Anion Concentration—Mca-APK(Dnp) was dissolved in 100% Me_2SO and quantitated by measuring absorbance at 350 nm using an extinction coefficient of $15,000$ M^{-1} cm^{-1}. All reactions were performed in microtiter plates with a 100-μl total volume at ambient temperature. To each well, we added 75 μl of salt (NaCl, NaF, NaBr, or KCl), 5.0 μl of buffer (50 mM, final concentration), and 10 μl of Mca-APK(Dnp) (50 μM, final concentration), and the reaction was initiated by the addition of 10 μl of ACE2 (0.15 nM, final concentration). The buffers used in the pH dependence studies were sodium acetate, MES, bis-Tris propane, CHES, and CAPS, and the buffer used in the anion dependence studies was MES. The final Me_2SO concentration in the assay was 0.7%. The assay was monitored continuously by measuring the increase in fluorescence (excitation = 320 nm, emission = 405 nm) upon substrate hydrolysis using a Polarstar Galaxy fluorescence plate reader (BMG Lab Technologies, Durham, NC). Initial velocities were determined from the rate of fluorescence increase over the 15–60-min time course corresponding to ≤10% product formed. The pH was found to have no significant effect on product fluorescence across the range of pH 5 to pH 10 in the assay buffers.

Determination of ACE2 Hydrolysis of Biological Peptides—Reactions were performed in microtiter plates at ambient temperature. To each well, we added 5 μl of 1 mM peptide (50 μM, final concentration) and 45 μl of buffer (50 mM MES, 300 mM NaCl, 10 μM $ZnCl_2$, and 0.01% Brij-35 pH 6.5), and reaction was initiated by the addition of 50 μl of 100 nM ACE2 (50 nM, final concentration) or buffer (control). Reactions were performed at room temperature for 2 h and quenched with 20 μl of 0.5 M EDTA. Samples were then analyzed by MALDI-TOF mass spectrometry for detection of hydrolysis and determination of products formed. Mass spectrometry was performed on a Voyager Elite biospectrometry MALDI-TOF spectrometer (PerSeptive Biosystems, Framingham, MA) as described previously (1). The peptides that were found to be hydrolyzed by ACE2 were re-assayed under the same conditions in the presence of a high concentration of a potent, specific inhibitor of ACE2[2] to confirm specific cleavage by this protease.

Determination of Kinetic Constants for ACE2 Hydrolysis of Peptides—Rates of substrate hydrolysis were determined by reversed phase chromatography using a capillary HPLC system (Agilent, Palo Alto, CA). Reactions were performed in 100 μl in microtiter plates at ambient temperature. Reactions were initiated by the addition of 50 μl of ACE2 (0.025–0.70 nM, final concentration) to 50 μl of peptide in assay buffer (50 mM MES, 300 mM NaCl, 10 μM $ZnCl_2$, and 0.01% Brij-35, pH 6.5). 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. Substrate concentrations ranged from 0.8–2000 μM, and hydrolysis was limited to ≤15% product formed (initial velocity conditions). Concentrations of the biological peptides were determined spectrophotometrically. Substrate and product peptides were resolved on a YMC ODS-A 1.0×50-mm

120A 5-μm column using a gradient of 10–45% B (A, water/0.1% trifluoroacetic acid (v/v); B, acetonitrile/0.1% trifluoroacetic acid (v/v)) and detected by absorbance at 215 nm. The peptide Mca-APK(Dnp) and its reaction product were resolved in the same manner using a gradient of 15–65% B and detected by absorbance at 350 nm. Injection volumes ranged from 0.5–20 μl. The extent of hydrolysis was determined from the areas of the substrate and product peaks (area of product peak/(area of product peak + area of substrate peak)) and converted to micromoles of product formed. Initial velocities (v) of substrate hydrolysis were calculated from the slopes of micromoles of product formed *versus* time. Initial velocities (v) were plotted *versus* substrate concentration and fit to the Michaelis-Menten equation ($v = V_{max}[S]/K_m + [S]$) using Grafit software (Erithacus Software Ltd., Surrey, United Kingdom). Turnover numbers (k_{cat}) were calculated from the equation $k_{cat} = V_{max}/[E]$, using a calculated ACE2 molecular mass of 85,314 Da and considering the enzyme sample to be essentially pure and fully active.

RESULTS

Recombinant soluble human ACE2, encoding amino acids 1–740 of the 805-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-YVADAPK(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-YVADAPK(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 was expressed in Sf9 insect cells and isolated to >98% purity, based on SDS-PAGE (Fig. 1*C*), 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 $K_m = 147 \pm 0.7$ μM, $k_{cat} = 114 \pm 0.7$ s^{-1}, and $k_{cat}/K_m = 7.7 \times 10^5$ M^{-1} s^{-1} ($n = 2$), as determined by an HPLC/UV 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 $ZnCl_2$ in the buffer (data not shown). ACE2 was stable for >6 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
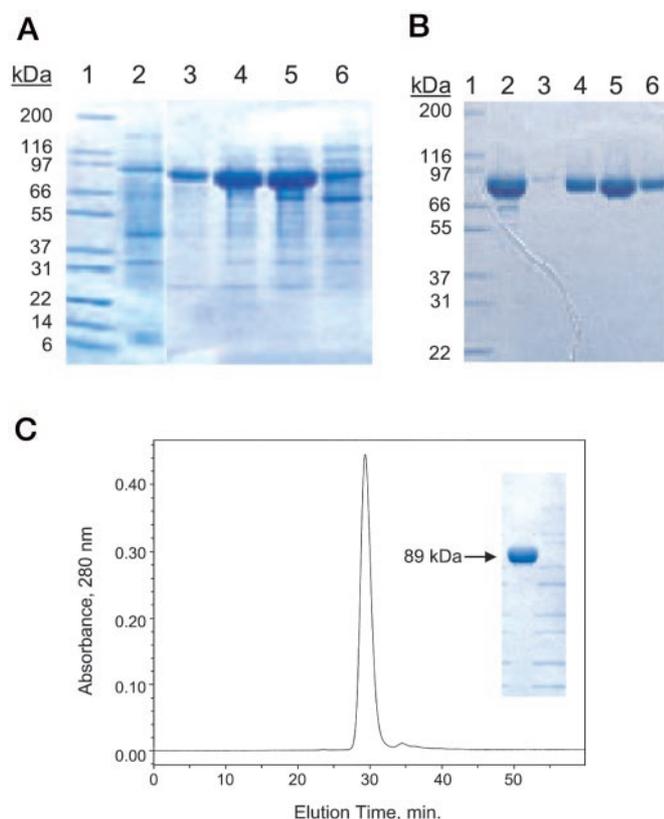

FIG. 1. **Purification of soluble ACE2.** Purification of recombinant soluble ACE2 expressed in SF9 cells, as described under "Experimental Procedures." Samples were analyzed by gel electrophoresis with a 4–20% gradient of acrylamide under denatured/reduced conditions and stained with Coomassie Blue. *A*, fractions from the first chromatography step with QAE anion exchanger column. *Lane 1*, molecular mass markers; *lane 2*, sample loaded onto column; *lanes 3–6*, fractions from gradient elution. *B*, fractions from MonoQ column chromatography. *Lane 1*, molecular mass markers; *lane 2*, sample loaded onto column; *lanes 3–6*, fractions from gradient elution. *C*, elution profile from final purification step by size exclusion chromatography; absorbance detection at 220 nm. *Inset*, SDS-PAGE of pooled ACE2 sample from the final chromatography step. Molecular mass was determined by MALDI-TOF mass spectrometry to be 89.6 kDa.
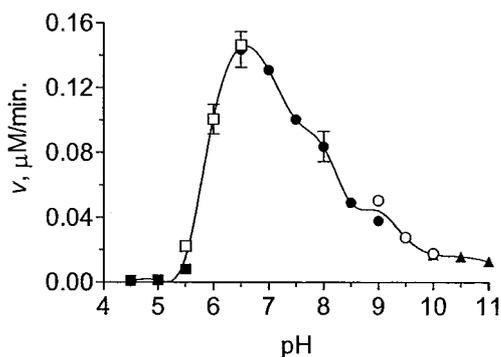

FIG. 2. **pH dependence of ACE2 proteolytic activity.** ACE2-catalyzed hydrolysis reactions were performed with 0.15 nM ACE2 and 50 μM Mca-APK(Dnp) in 1.0 M NaCl, 10 μM ZnCl$_2$, 0.01% Brij-35, and 50 mM buffer as described under "Experimental Procedures." Rates of hydrolysis of the internally quenched 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 ($\leq$10% hydrolysis) over 15–60 min. All values are an average (n = 2), and the S.D. is shown. ■, sodium acetate; □, MES; ●, bis-Tris propane; ○, CHES; ▲, CAPS.

but is not enhanced in the presence of bromide ion (Fig. 3). The catalytic activity is optimal in the presence of 1.0 M NaCl.

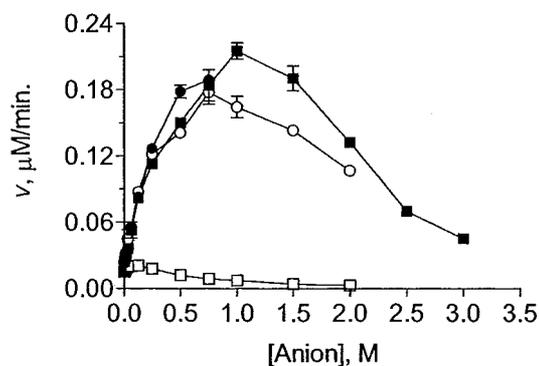The proteolytic activity of ACE2 was profiled against 126

FIG. 3. **Dependence of ACE2 proteolytic activity on anion concentration.** ACE2-catalyzed hydrolysis reactions were performed with 0.15 nM ACE2 and 50 μM Mca-APK(Dnp) in salt, 10 μM ZnCl$_2$, 0.01% Brij-35, and 50 mM MES, pH 6.5, as described under "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 ($\leq$10% hydrolysis) over 15–60 min. All values are an average (n = 2), and the S.D. is shown. ■, NaCl; □, NaBr; ●, NaF; ○, KCl.

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 re-assayed in the same manner in the presence of a high concentration of a potent, specific ACE2 inhibitor[2] 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-

[3] Biologically active peptides not hydrolyzed by ACE2 (115 in total) are as follows: adrenocorticotrophic hormone 1–39 (human), adrenocorticotrophic hormone fragment 1–14, allatostatin I, allatostatin II, alytesin, amylin, β-amyloid peptide (1–28), angiotensin 1–9, angiotensin 1–7, angiotensin 1–5, antiflammin-1, antiflammin-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, dermorphin, dynorphin A 1–17, dynorphin B (porcine), eledoisin, endomorphin-1, endomorphin-2, α-endorphin, β-endorphin (human), β-neoendorphin, endothelin, Met-enkephalin, enterostatin (human), fibronectin adhesion-promoting peptide, fibronectin fragment 1371–1382, N-formyl-Met-Leu-Phe, galanin (human), galantide, gastric inhibitory polypeptide, gastrin I (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), isotocin, kemptide, (Trp^4)-kemptide, (Val^6, Ala^7)-kemptide, kinetensin (human), leptin (full-length), leptin fragment 22–56 (human), Leu-enkephalin, litorin, luteinizing hormone-releasing hormone, malantide, mast cell-degranulating peptide HR1, mastoparan (wasp), melanin-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, neuromedin N, neuropeptide FF (porcine), neuropeptide K, neuropeptide Y (human), neurotensin (1–13), nociceptin, nocistatin (bovine), orcokinin, orexin A, orexin B, oxytocin, pancreastatin 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), (Arg^8)-vasopressin, and (Arg^8)-vasotocin.

Table I

Hydrolysis of biologically active peptides by soluble human ACE2

Detection of ACE2 peptide hydrolysis and site of hydrolysis by MALDI-TOF MS. Site of hydrolysis is indicated by an arrow. Some analogs of peptide substrates not hydrolyzed are shown for sequence comparison. C, complete hydrolysis; P, partial hydrolysis; N, no hydrolysis; pE, pyroglutamyl.

Substrate	Sequence	Hydrolysis
Angiotensin I	DRVYIHPFH ↓ L	P
Angiotensin 1–9	DRVYIHPFH	N
Angiotensin II	DRVYIHP ↓ F	C
Angiotensin 1–7	DRVYIHP	N
Angiotensin 1–5	DRVYI	N
Apelin-13	QRPRLSHKGPMP ↓ F	C
Apelin-36 (C terminus shown)	. . .QRPRLSHKGPMP ↓ F	C
Bradykinin	RPPGFSPFR	N
des-Arg[9]-bradykinin	RPPGFSP ↓ F	C
Lys-des-Arg[9]-bradykinin	KRPPGFSP ↓ F	P
Bradykinin fragment 1–7	RPPGFSP	N
β-Casomorphin	YPFVEP ↓ I	C
Neocasomorphin	YPVEP ↓ I	C
Dynorphin A 1–13	YGGFLRRIRPKL ↓ K	C
Ghrelin (C terminus shown)	. . .ESKKPPAKLQP ↓ R	P
Neurotensin 1–8	pE-LYENKP ↓ R	P

drolytic activity is dependent on the C terminus sequence of the substrate, which is evident from the data with the angiotensin peptides. After 2 h, ACE2 hydrolyzes Ang I partially and Ang II completely, although there is no hydrolysis of angiotensin 1–9, angiotensin 1–7, and angiotensin 1–5, which possess the same N terminus. Similarly, ACE2 hydrolysis is specific for des-Arg[9] forms of 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 × 10^6 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-Arg[9]-bradykinin peptides, β-casomorphin, and neurotensin 1–8 with substantial catalytic efficiency (k_{cat}/K_m ≥ 1 × 10^5 M^{-1} s^{-1}).

DISCUSSION

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 6.5 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.

A characteristic of ACE that is unique among metalloproteases is its activation by monovalent anions, including Cl^-, Br^-, and Fl^- (10–12). ACE2 proteolytic activity is also activated by monovalent anions, with a 10-fold enhancement in the presence of 1.0 M NaCl. Fl^- 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[514], 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.

ACE2 Biological Peptide Substrates—The identification and kinetic characterization of ACE2 biological peptide substrates are useful in providing an initial understanding of the substrate specificity of the protease and the physiological role of ACE2. If ACE2 is a "converting enzyme," as ACE is, then its proteolytic activity either produces or degrades (or both) a peptide with biological activity. Therefore, an understanding of the biological activity of ACE2 substrates and products suggests putative physiological roles for ACE2.

Data from screening 126 biological peptides indicate that ACE2 is a carboxypeptidase, distinguishing it from its closest homolog, ACE, which is a C-terminal dipeptidyl-peptidase. This result is consistent with our earlier report on ACE2 catalytic activity. Our previous results, however, showed hydrolysis of the C-terminal residue of neurotensin 1–13 and kinetensin by ACE2 expressed in Chinese hamster ovary cells (1). In the current studies, neither peptide was confirmed as an ACE2 substrate. The difference in the two results may be due to the difference in the degree of purity of ACE2 used in the studies (>98% in the current study). Additionally, in the current study, proteolysis was confirmed to be catalyzed by ACE2 by demonstrating inhibition of proteolysis with a specific ACE2 inhibitor.

A P5-P1′ alignment of the seven peptide substrates that ACE2 efficiently hydrolyzes (k_{cat}/K_m > 1 × 10^5 M^{-1} s^{-1}) indicates a consensus sequence of $Pro-X_{(1-3 \text{ residues})}$-Pro-Hydrophobic, with hydrolysis between proline and the hydrophobic amino acid. The proline in the P1 site is the most conserved residue in the ACE2 substrates, present in six of the seven peptides. Proline is also in the P3 or P5 position for five of the seven substrates. Five of the seven substrates have a hydrophobic amino acid in the P1′ position. ACE2 will also hydrolyze a basic residue in P1′, as exemplified by dynorphin A and neurotensin 1–8. The ACE2 peptide substrates consist primarily of basic and hydrophobic residues. A more detailed understanding of the requirement for each substrate residue in binding and catalysis will require determination of ACE2 catalytic properties on peptides with systematic changes in substrate sequence.

Of the peptide components of the renin-angiotensin system, ACE2 only hydrolyzes Ang II with high catalytic efficiency (k_{cat}/K_m ≥ 1 × 10^5 M^{-1} s^{-1}). The other peptides, Ang I, angiotensin 1–9, angiotensin 1–7, and angiotensin 1–5, are poorly hydrolyzed or not hydrolyzed at all by ACE2. The biochemical evidence therefore indicates that ACE and ACE2 may have complementary functions. ACE proteolysis generates Ang II, and ACE2 proteolysis degrades it, although there is no evidence that the observed ACE2 activity *in vitro* is representative of the physiological activity of the enzyme. Ang II is a potent vasoconstrictor that promotes vascular hypertrophy (14). The *in vitro* ACE2-catalyzed hydrolysis of Ang II produces Ang 1–7, whose vasodilator and antihypertensive effects are counter to those of Ang II (15, 16). Therefore, if the physiological role of ACE2 is conversion of Ang II to Ang 1–7, it would be expected that ACE2 plays a role in vasodilation. There is evidence, however, that the *in vivo* hydrolysis of Ang II to produce Ang 1–7 is catalyzed by prolyl-endopeptidase (EC 3.4.24.26), neprilysin (EC 3.4.24.11), and metalloendopeptidase (EC 3.4.24.15)
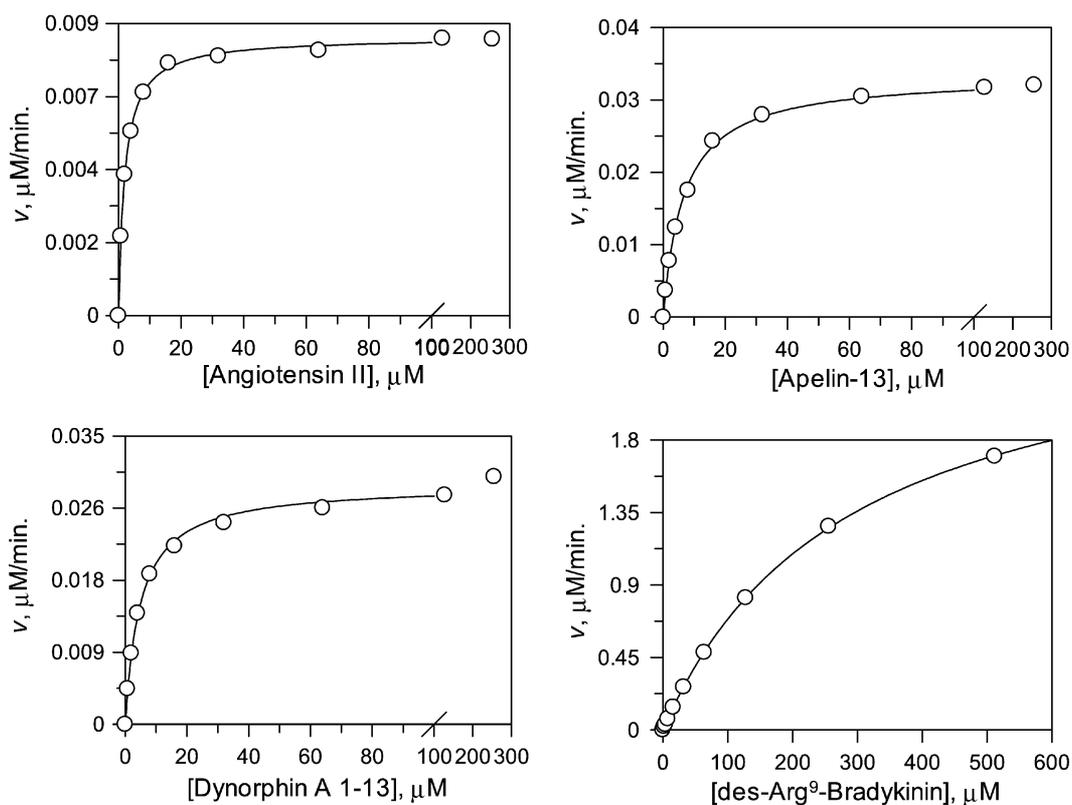
ACE2 Biological Peptide Substrates

FIG. 4. **Michaelis plots for ACE2 hydrolysis of biological peptides.** Initial velocity data for ACE2 hydrolysis of Ang II, apelin-13, dynorphin A 1–13, and des-Arg[9]-bradykinin were determined as described under "Experimental Procedures." Data were fit to the Michaelis-Menten equation ($v = V_{max}[S]/K_m + [S]$). Turnover numbers (k_{cat}) were calculated from V_{max} values ($k_{cat} = V_{max}/[E]$, using a molecular mass of 85,314 Da for ACE2). K_m and k_{cat} values are summarized in Table II.

TABLE II

Kinetic constants for hydrolysis of biological peptides by soluble human ACE2

Determinations were performed as described under "Experimental Procedures." All determinations are $n = 2$. K_m and k_{cat} values are reported ± S.E. pE, pyroglutamyl.

Substrate	Sequence	K_m	k_{cat}	K_{cat}/K_m
		(μM)	*(s⁻¹)*	*M⁻¹ s⁻¹*
Angiotensin I	DRVYIHPFH ↓ L	6.9 ± 0.50	0.034 ± 0.001	4.9×10^3
Angiotensin II	DRVYIHP ↓ F	2.0 ± 0.02	3.5 ± 0.1	1.8×10^6
Apelin-13	QRPRLSHKGPMP ↓ F	6.8 ± 0.30	13 ± 0.1	2.0×10^6
des-Arg[9]-bradykinin	RPPGFSP ↓ F	290 ± 0.7	62 ± 2.0	2.2×10^5
Lys-des-Arg[9]-bradykinin	KRPPGFSP ↓ F	130 ± 1.7	26 ± 3.0	2.0×10^5
β-Casomorphin	YPFVEP ↓ I	31 ± 0.6	6.8 ± 0.07	2.2×10^5
Dynorphin A 1–13	YGGFLRRIRPKL ↓ K	5.5 ± 0.8	16 ± 0.5	2.9×10^6
Neurotensin 1–8	pE-LYENKP ↓ R	300 ± 9	57 ± 12	1.9×10^5

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 κ-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 ($k_{cat} = 64$ s⁻¹) is comparable to the turnover number reported for ACE hydrolysis of bradykinin ($k_{cat} = 11$ s⁻¹; Ref. 23), the ACE2 K_m value is 1600-fold higher than that of ACE (286 *versus* 0.18 μM).

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-Arg[9]-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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