**This is a pre- or post-print of an article published in
Chen, H.-L., Lünsdorf, H., Hecht, H.-J., Tsai, H.
Porcine pulmonary angiotensin I-converting enzyme-Biochemical
characterization and spatial arrangement of the N- and C-domains by three-
dimensional electron microscopic reconstruction
(2010) Micron, 41 (6), pp. 674-685.**

**Porcine pulmonary angiotensin I-converting enzyme–Biochemical
characterization and spatial arrangement of
the N- and C- domains by three-dimensional electron microscopy
reconstruction**

5

Hui-Ling Chen ^{a,1}, Heinrich Lünsdorf ^{b,1}, Hans-Jürgen Hecht ^{c,2}, and Hsin Tsai ^{d,*}

^aDevelopment Center for Biotechnology, Taipei County 221, Taiwan, ROC

10 ^b Department of Vaccinology and Applied Microbiology, Helmholtz–Center of Infection
Research,
38124 Braunschweig, Germany

^c Department of Structural Biology, Helmholtz–Center of Infection Research,
38124 Braunschweig, Germany

15 ^d Graduate Institute of Biotechnology, Chinese Culture University, Taipei 111, Taiwan, ROC

¹ Contributed equally to this work; ² in memoriam, who died in November 2009

* Corresponding author. Tel: +886 2 28623660; fax: +886 2 28623660

E-mail address: htsai@faculty.pccu.edu.tw

Abstract

The somatic angiotensin I-converting enzyme (sACE; peptidyl-dipeptidase A; EC 3.4.15.1) was isolated from pig lung and purified to homogeneity. The purified enzyme had a molecular mass of about 180 kDa. Upon proteolytic cleavage, two approximately 90 kDa fragments were obtained and identified by amino-terminal sequence analysis as the N- and C- domains of sACE. Both purified domains were shown to be catalytically active. A 2.3 nm low resolution model of sACE was obtained by three-dimensional electron microscopy reconstruction of negatively stained sACE particles, based on atomic X-ray data fitting. Our model shows for the first time the relative orientation of the sACE catalytically active domains and their spatial distance.

Keywords: Somatic angiotensin I-converting enzyme; Pig Lung; N-domain; C-domain; Electron microscopy; 3D reconstruction.

Abbreviations:

sACE, somatic angiotensin converting enzyme; tACE, testicular angiotensin converting enzyme; Con A, concanavalin A; DEAE, diethylaminoethyl; DIEA, diisopropylethylamine; DMF, *N,N*-dimethylformamide; DTT, dithiothreitol; EDTA, ethylene diamine tetraacetic acid; FA-Phe-Gly-Gly, L-furylacryloyl-L-phenylalanyl-glycylglycine; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; Hepes, *N*-(2-hydroxyethyl) piperazine-*N'*-(2-ethanesulfonic acid); HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; IEF, isoelectric focusing; MALDI-MS, matrix assisted laser desorption ionization mass spectrometry; pI, isoelectric point; PNGase F, peptide *N*-glycosidase F; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane

1. Introduction

Somatic angiotensin I-converting enzyme (sACE; EC 3.4.15.1) is a zinc metalloprotease that catalyzes the hydrolysis of carboxyl-terminal dipeptides from oligopeptide substrates, most notably the decapeptide angiotensin I and the nonapeptide bradykinin (Corvol et al., 1995).

5 sACE exists primarily as a cell-surface integral membrane glycoprotein with the bulk of its mass, including the catalytic sites, facing the extracellular milieu; therefore, it is an ectoenzyme (Coates, 2003). ACE has been purified from most mammalian tissues and two distinct isozymes have been identified. The larger form, Mr 150–180 kDa, is present throughout the body, with the highest abundance in the lung and kidney. This form is
10 generally referred to as endothelial (or somatic) ACE. A smaller ACE isozyme of Mr 90–100 kDa, is found exclusively in the testis and generally referred to as testicular (or germinal) ACE. The first full-length cDNA clone for ACE was constructed from the transcripts of ACE gene of human kidney (Soubrier et al., 1988). Human endothelial ACE consists of a single polypeptide chain composed of 1277 residues, of which 14 are cysteine residues. It contains 17 potential
15 N-glycosylation sites but no Ser/Thr rich region for O-glycosylation.

Molecular cloning of the cDNA of the sACE also demonstrated that the enzyme is composed of two homologous domains, referred to here as the N- and C-domains, which are supposed to result from gene duplication (Hubert et al., 1991; Riordan, 2003). The overall sequence similarity between the two domains is 60% in both nucleotide and amino acid sequences but
20 increases to 89% in a sequence of 40 amino acids in each domain containing essential active site residues. Therefore, each domain of sACE contains a catalytic site (Soubrier et al., 1988; Wei et al., 1991). There is no sequence similarity between the carboxyl- and amino-terminal extremities of the sACE molecule, and the central region which separates the two homologous domains, called the 'interdomain linker' (Corradi et al., 2006) is also unique.

25 Although the physiological importance of ACE has long been recognized, its structure and mechanism of action were not understood in detail. Recent advances in molecular biological techniques made it possible to grow crystals of the N- domain of sACE (Corradi et al., 2006), tACE (Natesh et al., 2003), and ACE2 (Towler et al., 2004), a C-domain homologue. However, a three-dimensional crystal structure of full-length somatic ACE is still lacking.

To investigate the structure–function relationship of sACE, we have purified full–length sACE from porcine lung, and its individual N– and C–domains by limited proteolysis. This enabled us to investigate the molecular and enzymatic properties of these proteins and to compare the properties of their native and individual domain forms. In addition, the electron microscopic reconstruction and structure fitting reveals for the first time the tertiary structure of intact sACE and the relative orientation of its N– and C–domains.

10

2. Experimental procedures

2.1. Materials

15 Fresh pig lungs were taken from the local slaughterhouse. PMSF (phenyl–methylsulfonyl fluoride), Triton X–100, Fractogel EMD DEAE–650(M), *p*–aminobenzoic acid and 6–aminocaproic acid were obtained from Merck. Epoxy–activated Sepharose 6B and Con A–Sepharose, PhastGel™ homogeneous 7.5, IEF 4–6.5 and IEF 3–9 were supplied by GE healthcare. 2–(1H–benzotriazol–1–yl)–1,1,3,3–tetramethyluronium hexafluorophosphate (HBTU) and 1–hydroxybenzo–triazole (HOBt) were from Applied Biosystems. Lisinopril (*N*²–[(*S*)–1–carboxy–3–phenylpropyl]–L–lysyl–L–proline) was a gift by Mr. R. Lüdecke from MSD Sharp & Dohme GmbH, Germany. FA–Phe–Gly–Gly (L–furylacryloyl–L–phenylalanyl–glycylglycine) and methyl– α –D–mannopyranoside were purchased from Sigma.

20 Endoproteinase Asp–N (EC 3.4.24.33), Endoglycosidase F (EC 3.2.1.96), N–glycosidase F (EC 3.5.1.52) and DIG glycan differentiation kit (containing SNA, MAA, blocking reagent, anti–digoxigenin–AP and NBT / X–phosphate solution) were purchased from Boehringer Mannheim GmbH. Molecular weight markers for electrophoresis were from Bio–Rad. All other chemicals were of reagent grade.

2.2. Extraction of membrane-bound ACE and ammonium sulfate fractionation

All procedures were carried out at 4°C. Pig lung tissue (0.9 kg) was cut into small pieces and suspended in 2 volumes of 1 mM PMSF, 20 mM Tris-HCl buffer, pH 7.8 (suspension buffer).

The tissue was homogenized in a Waring blender by six 30 Sec bursts at top-speed. Triton X-

5 100 was added to the homogenate at a final concentration of 0.2 % (w/v). The suspension was stirred for 90 min, followed by centrifugation at 9000 x g for 30 min. The pellets were resuspended in 300 ml of suspension buffer and re-extracted with 0.2 % (w/v) Triton X-100. The combined ACE-containing supernatant was kept on an ice-bath, and further purified by ammonium sulfate fractionation. Solid ammonium sulfate was added slowly to a final
10 concentration of 30 % (w/v). After stirring for 1 hour, the mixture was centrifuged at 10,000 x g for 20 min and the supernatant was collected. Solid ammonium sulfate was added to the supernatant to a final concentration of 70 % (w/v), stirred for 1 hour and centrifuged as described above. The precipitates were collected and redissolved in 20 mM Tris-HCl buffer (pH 7.8) and dialysed three-times against 24 volumes of the same Tris-HCl buffer for 24 hour
15 at 4 °C.

2.3. DEAE-Fractogel chromatography

The enzyme was further purified by column chromatography on a Fractogel EMD DEAE-650(M) column (5.0cm x 20cm) equilibrated with 20 mM Tris-HCl buffer (pH 7.8). The
20 dialyzed solution was centrifuged at 14,000 rpm to remove the insoluble particles and then loaded onto the DEAE column at a flow rate of 3 ml/min. The column was washed with 6 column volumes (total 2400 ml) of 20 mM Tris-HCl, pH 7.8, and the enzyme was eluted over a 3000 ml 0-0.25 M NaCl linear gradient in 20 mM Tris-HCl, pH 7.8. Active fractions were pooled.

25

2.4. Con A-Sepharose chromatography

The pooled ACE fractions from the DEAE column were applied to a 2.6 cm x 15 cm Con A-Sepharose column. The flow rate was 1 ml/min. The column was washed with 5 column volumes (400ml) of buffer A (20 mM Tris-HCl, 0.2M NaCl, pH 7.4), and the enzyme was eluted

with 10 column volume (800 ml) of buffer B (20 mM Tris-HCl, 100 mM methyl- α -D-mannopyranoside, pH7.4) in 20 ml fractions. Active fractions were collected and pooled for further purification.

5 2.5. Preparation of Sepharose-lisinopril affinity resin

Preparation of lisinopril-coupled Sepharose was modified from Hooper and Turner (1987).

384 mg of *p*-aminobenzoic acid (2.8 mmol) were dissolved in 12 ml of DMF and 0.5 ml of DIEA, and mixed with 4 g of epoxy-activated Sepharose 6B suspended in water. The coupling reaction was performed in a glass reactor by gently shaking for 15 hours at room

10 temperature. After filtering and washing the matrix with DMF and H₂O, residual epoxy groups were blocked with 20 ml of 1M ethanolamine for at least 1 h. Following this, the matrix was washed with H₂O and finally with DMF extensively.

Next, the modified Sepharose was activated for 2.5 hours with 1.1 g of HBTU (2.8 mmol) and 378 mg of HOBT (2.8 mmol) which were dissolved in 12 ml of DMF and 0.2 ml of DIEA,

15 respectively. The solution was filtered, and the second coupling reaction was performed by adding 551 mg of 6-aminocaproic acid (4.2 mmol) to 12 ml of 0.1 M Na₂CO₃ (pH 11.8) solution for 24 hours at room temperature. After the coupling was completed, the gel was filtered and washed again with H₂O and finally DMF.

The filtered and extensively washed matrix was activated with HBTU/HOBT as described

20 above. At last 486 mg of lisinopril (1.2 mmol) was coupled to the extended matrix arm in 12 ml of 0.1 M K₂CO₃ (pH 11.8) for 24 hours at room temperature. Unreacted groups were blocked with 1M glycine (pH 10.0), and the resulting Sepharose-lisinopril gel was washed extensively with H₂O, followed by 0.5 M NaCl, 0.1 M Tris-HCl buffer (pH 8.5), sodium acetate buffer (pH 4.5) containing 0.5 M NaCl, and finally equilibration buffer (20 mM Tris-HCl, pH
25 7.2, 0.3 M NaCl, 100 μ M ZnCl₂) prior to usage.

The coupling of *p*-aminobenzoic acid and 6-aminocaproic acid was monitored by UV_{254 nm} absorption. When caproic acid is coupled to the epoxy-activated Sepharose an absorption peak is observed at approx. 292 nm. The coupling of lisinopril to the affinity resin can be monitored by a decrease in the lisinopril content of the coupling solution as measured by

reverse-phase HPLC. The concentration of covalently bound lisinopril in the affinity gel was 1–2 $\mu\text{mol/ml}$ of gel as determined by reverse-phase HPLC analysis of the coupling solution.

2.6. sACE purification by Sepharose–lisinopril affinity chromatography

5 The Sepharose–lisinopril affinity gel preparation was packed into a column (6 cm x 1.6 cm) and equilibrated with 20 mM Tris–HCl (pH 7.2) buffer, containing 300 mM NaCl, and 100 μM ZnCl_2 . Pooled fractions from the Con A–Sepharose were loaded onto the column at a flow rate of 0.6 ml/min. sACE was eluted with 50 mM $\text{Na}_2\text{B}_4\text{O}_7$ (pH 9.5) buffer and collected in 2 ml fractions. Each tube was prefilled with 0.2 ml of 1 M Tris–HCl (pH 7.2) and 3 M NaCl buffer in
10 order to balance the pH of eluents.

2.7 Enzyme assay

sACE activity measurements were made using a spectrophotometer (Beckman Model DU640) by continuously monitoring the absorbance at 340 nm. The standard assay volume is 1 ml and
15 consists of 0.5 mM FA–Phe–Gly–Gly in 50 mM Hepes buffer (pH 7.5) and 0.3 M NaCl (Holmquist et al., 1979). The reaction was performed at 37 °C and the initial rate was determined by absorbance at 340 nm. The data of $\Delta A/\text{min}$ were converted into U/ml according to the following formula: $\text{U/ml} = (\Delta A/\text{min}) \div (\Delta\epsilon \times 1\text{ cm}) \times (V_t / V_s)$, where V_t is the final volume of reaction mixture and V_s is the volume of enzyme sample used. $\Delta\epsilon$ is the
20 differential molar extinction coefficient which is $0.977\text{ mM}^{-1} \times \text{cm}^{-1}$. One unit of ACE activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of FA–Phe/min at 37 °C. The specific activity was expressed as units/mg of protein. The protein concentration was determined by using the Bio–Rad protein assay kit using bovine serum albumin as a standard.

25 2.8. Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoreses (SDS–PAGE) were performed on Pharmacia PhastGel Homogeneous 7.5. Isoelectric focusing (IEF) was on Pharmacia PhastGel IEF 4–6.5. All electrophoresis were carried out on the separation and control unit and the protein bands were detected by sensitive silver staining on the development unit of

PhastSystem (Pharmacia LKB Biotechnology; now GE Health Care). The molecular mass and the isoelectric point (pI) values were calibrated, using the high molecular weight marker and IEF standard marker from Bio-Rad.

5 **2.9. Protein sequence determination**

The N-terminal sequences of proteins were determined by automated Edman degradation in an Applied Biosystems model 473 protein sequencer. Protein bands separated by SDS-PAGE were transferred onto PVDF membrane by Western blotting. The membranes or the intact protein solutions were loaded on the sequencer for automated analysis.

10

2.10. Limited proteolysis with endoproteinase Asp-N

Purified pig lung ACE (1.6 mg) in 0.25 ml of 50 mM Hepes (pH 8.0) buffer was digested with 2 µg of endoproteinase Asp-N for 18 hours at 37 °C. The extent of digestion was monitored by SDS-PAGE.

15

2.11. Electron microscopy and 3D reconstruction

Purified sACE was diluted in 20 mM Tris-HCl buffer (pH 7.8) to a final protein concentration of 70 µg/ml. The sample was adsorbed onto a carbon-foil (Valentine et al., 1968) and examined by negative-staining electron microscopy as described in detail by Winkler et al. (1997). The in focus, high contrast imaging was done with the 'in-column' energy-filtered transmission electron microscope (EF-TEM; Zeiss CEM 902, Oberkochen, Germany). Electron spectroscopic imaging (ESI) was done at $U_{04,5}$ -edge at 115 eV at medium dose exposures. For 3D reconstruction images at Scherzer focus were taken near low-dose exposure settings at 50000x magnification in the elastic bright-field mode (30 µm objective aperture; 200 µm
20 condenser aperture; energy-slit width: 15 eV; emission current: 1 µA; acceleration voltage: 80 kV). Images were collected on AgfaScientia plate film and developed to full strength with Kodak D-76.

25

Digitization of negatives, which were checked for drift and astigmatism by optical diffraction, was done using an Agfa Duoscan T2500 at 2000 lpi-setting, corresponding to a 0.278 nm

final sampling size at the probe level. A total of 3918 particles was sampled using the 'boxer' routine of EMAN (Ludtke et al., 1999) from 3x3 median-filtered images with a box size set to 72 pixels. 3D reconstruction was done using the EMAN software suite with a symmetry-setting of C1 after rough classification of the original data set (running the EMAN command
5 line 'refine 2d.py start.hed' iteratively to get some unbiased 2D class views) and selecting 10 representative classes of typical particle projections. Finally, 12 'refinement' iterations were run to reach FSC convergence.

The 'Ix_mapman' program (Kleywegt and Jones, 1996) was used to convert the EMAN voxel data into the CCP4-format for 3D-modeling using 'Chimera' (version 1, build 2470) (Pettersen
10 et al., 2004) and manually fitting of atomic coordinates into the electron microscopic volume densities. Voxel-slice viewing was done with VMD (version 1.8.6.) program (Humphrey et al., 1996). Atomic coordinates used for structure fitting of the C-(ACE2; PDB entry code 1R42; Towler et al., 2004) and N-domains (human sACE; PDB entry code 2C6F; Corradi et al., 2006) were obtained from the Protein Data Bank (Research Collaboratory for Structural
15 Bioinformatics, Rutgers University, New Brunswick, NJ). CoreIDRAW Graphics Suite 13 was used for general image processing and presentation.

20

3. Results

3.1. sACE preparation from porcine lung

sACE was purified as in Materials and Methods described, the purified enzyme was concentrated to 14 mg/ml and stored at -20 °C. The purity of the enzyme preparation was
25 determined by SDS-polyacrylamide gel electrophoresis (PAGE; 7,5 % gel concentration). On silver staining, only a single band of 170 kDa was observed. This indicates that the enzyme has been purified to homogeneity. A summary of the purification procedures and results are presented in Table 1. The final yield was about 14 mg of homogeneous sACE from 0.9 kg of fresh pig lung tissue. The overall purification was 875 fold with a final 45% recovery of

activity. The specific activity of the sACE preparation is about 219 units/mg protein, which is considerably higher than that previously reported in the literature (Andujar-Sánchez et al., 2003).

5 3.2. ACE N- and C-domain isolation

3.2.1. Properties of the sACE

The effect of pH and buffer nature on the hydrolysis of FA-Phe-Gly-Gly was studied over a pH range from pH 5.5 to 9.5 (Mes from pH 5.5 to 6.5; Hepes from pH 7.0 to 8.5, and sodium borate from pH 9.0 to 10.0). Hydrolysis of FA-Phe-Gly-Gly (5×10^{-4} M) followed first-order
10 kinetics over this pH range. The optimum pH is at 7.5 as determined from the pH-rate profile which fell off markedly when the pH was below 7.0 (fig.1; dashed line). All measurements were carried out in the presence of 0.3 M sodium chloride.

The decrease in activity below pH 7.0 was partly due to dissociation of zinc ions from the enzyme. Under acidic pH conditions, the dissociation of metal ions could be overcome by
15 addition of zinc (10^{-4} M) to the assay mixture. This resulted in an increase of sACE activity, whereas in the pH range above 7.5, zinc suppressed the activity slightly. Thus, in the presence of 10^{-4} M of zinc, the activity versus pH profile showed that the pH optimum could be shifted from 7.5 to 6.5 (fig.1, solid line).

When the enzyme was incubated at 37 °C for 24 h up to several days in a mixed buffer (such
20 as acetate, phosphate, Tris and borate) with pH values ranging from 4.0 to 11.0, it was found that the enzyme was relatively stable over a pH range from 7.0 to 8.0, and more than 90 % of activity could be retained after this treatment.

The K_m and k_{cat} values of the enzyme were obtained from Lineweaver-Burk plots, and were determined to be 7.8×10^{-4} M and $5.27 \times 10^4 \text{ min}^{-1}$, respectively, when FA-Phe-Gly-Gly was
25 used as the substrate. The catalytic efficiency (k_{cat}/K_m) of sACE was calculated to be $67.6 \times 10^6 \text{ M}^{-1} \cdot \text{min}^{-1}$ as shown in Table 2. This value is in good agreement with data obtained by Bünning et al. (1983). Table 3 presents the K_i values for captopril and BPP_{9a}, as determined by the graphical method of Dixon et al. (1979). The molecular mass of the enzyme was determined to be 170 kDa by SDS-PAGE and 175 kDa by MALDI mass spectrometry, which is

in good agreement with reported data (Andujar-Sánchez et al., 2003).

3.2.2. N-terminal sequencing of sACE

5 The N-terminal amino acid sequence analysis of the purified porcine lung sACE revealed the following sequence: Leu-Asp-Ser-Ala-Leu-Gln-Pro-Gly-Asp-Phe-Thr-Ala-Asp-Glu-Ala-Gly-Ala-Glu-Asp-Phe-Ala-Gln-Ser-Phe-Asp-Ser-Ser-X-Glu-Gln-Val-Leu-Phe-Gln. It is 76% identical to that of human sACE (Soubrier et al., 1988).

3.2.3. Purification of the N- and C-domains

10 The proteolytic products of sACE obtained from endoproteinase Asp-N digestion were separated and purified by Sepharose-lisinopril affinity chromatography. The affinity column was equilibrated with 20 mM Tris-HCl (pH7.2), 0.3 M NaCl, and 100 μ M ZnCl₂. After washing with the same buffer to remove unbound proteins, peak I was eluted with 20 mM Mes-NaOH (pH 6.0) containing 10 μ M ZnCl₂ (buffer B, fig. 2). Peak II was then eluted with 20 mM Hepes-
15 NaOH (pH 6.0) containing 10 μ M ZnCl₂ (buffer C, fig. 2). Both peak fractions were analysed by amino acid sequencing for protein identification (see below).

3.2.4. N-terminal sequence of the N- and C-domains

The intact sACE protein was subjected to limited proteolysis with endoproteinase Asp-N,
20 leading to the presence of two major protein bands in the SDS-PAGE (fig. 3, lane 3). The molecular mass of these two protein fragments was estimated to be 90 kDa and 80 kDa. The bands were transferred to PVDF membranes and analyzed by N-terminal sequencing. The results are shown in Table 4. The sequence data correspond to residues 1-24 and 616-640 of human sACE (Soubrier et al., 1988) and suggest that they were derived from the N- and C-
25 domains, respectively. Peak I (fig. 2) which was eluted with 20mM Mes-NaOH (pH 6.0) and 10 μ M ZnCl₂ represents the 90 kDa N-domain (fig.3; lane4). Peak II (fig. 2) which was eluted with 20mM Hepes-NaOH (pH 6.0) and 10 μ M ZnCl₂ corresponds to the 80 kDa C-domain (fig.3; lane 5).

3.2.5. Kinetic parameters of the N- and C-domains

During the course of proteolytic digestion with endoproteinase Asp-N, the activity of the Asp-N treated sACE was unaltered with respect to the time of digestion as shown in fig. 4. This indicated that, after proteolysis, both domains are as active as native sACE. The purified N- and C-domains have similar K_m values for the tested substrate, FA-Phe-Gly-Gly. However, the kinetic data (Table 5) indicated that the catalytic efficiency (K_{cat}/K_m) of the isolated C-domain is about two-fold higher than that of the native sACE. These results are compatible with the notion that the catalytic activity of the C-domain is negatively regulated by its N-domain counterpart in native sACE (Binevski et al., 2003; Woodman et al., 2005; Skirgello et al., 2005).

3.3. Electron microscopy analysis

3.3.1. Structural studies of porcine lung sACE and its isolated N- and C-domains.

The isolated sACE molecules, as analyzed by electron microscopy, showed that the enzyme preparation is pure and homogeneous (fig. 5 a and b). The molecules have a characteristic bilobed shape (fig. 5 a and b; circles) as seen from either the global elastic bright-field (fig.5a) or the inelastic darkfield view (reversed contrast at the uranium edge $O_{4,5} = 115$ eV; fig.5b). The overall dimensions of sACE range from 11.7 nm to 19.2 nm in length, with a mean length of 15.3 nm ($N = 129$) and a mean width of 7.4 nm ($N = 154$) within the range from 4.2 nm to 10.3 nm. Both domains, which appear similar in size, differ in their substructural, tertiary organization, as revealed from details in their projection features. While one domain shows an overall triangular projection contour, the other appears as an orthogonal rectangular projection (fig. 5-c). Furthermore, both domains are connected by a distinct filigree hinge-region (fig. 5c; black/white arrowheads), also termed 'interdomain-linker'. In the inelastic darkfield view at the Gaussian focus both domains show a distinct spatial arrangement of centers of mass. The triangular projections (fig.5c) often appear to be composed of three (fig. 5c; solid triangles) or four protein masses arranged at the vertices of a tetrahedron (fig. 5c; arrows). These triangular projection contours are characteristic for the

N-domains, which were isolated from lung sACE after mild proteolysis (fig.5d). Conversely, the compact orthogonal projection, representing a substructure of two parallel elongated centers of mass, was mainly observed for the isolated C-domain (fig. 5e). Obviously mild proteolysis and purification of these domains did not affect the overall tertiary structure. Thus the apparent different conformational states of the isolated N- and C-domains are similar to those in the native enzyme.

3.3.2. 3D-image reconstruction of sACE.

Negatively stained sACE molecules, analysed by electron microscopy and reconstructed by single-particle analysis with EMAN from digitized negatives – free from drift and astigmatism – lead to a 3D model of the native molecule with 2.3 nm low resolution. A total of 3918 particle projections was boxed at a 72 x 72 pixel box size (fig. 6a) and was analyzed using C1 symmetry. Exposure of negatives at medium dose and focusing near Scherzer focus resulted in noisy micrographs which were 3x3 median-filtered prior to particle selection. Reference-free 2D refinement (EMAN subprogram 'refine 2d.py') was done to get characteristic means of noiseless particle projections of the sACE molecules. Ten typical views (fig. 6b) were chosen and the 'startany' routine was run, resulting in a total of 236 classes. The presence of tertiary substructure and their connection by a faint bridge could be verified (fig. 6b), which was already visible from inspection at the individual molecule level (fig. 5c).

A total of 16 refinement iterations was calculated, and convergence was reached after seven refinement cycles. All 3D-models of the corresponding iterations were checked for homogeneity of class distribution in Euler-space. Finally the 3D electron microscopic density of the 11th iteration was chosen for final modeling since it showed the most uniform class distribution in Euler-space (fig. 6d). Resolution was checked by Fourier-Shell-correlation (FSC) at a threshold of 0.5σ , which proved 2.31 nm for the corresponding 3D-model. A collection of 18 out of 236 class views (fig. 6e) shows top views and different side views as averages of the corresponding ACE projections. Based on visual inspection of all sections of the matrix view, looking for the onset and disappearance of protein densities along the z-axis, 55 layers were found to be occupied by protein density, leading to a molecule height of

15.3 nm for sACE (data not shown). This length is consistent with direct length measurements from survey view micrographs as shown above (fig. 5a and b).

3D-model refinement was done using Chimera over a volume range from - 4.28 to 9.04. The final level was set to 2.45 and a surface smoothing was applied with three iterations and 0.5

factor-setting, followed by Gaussian filter setting the width (2 sdev) to 5 at step 1. This led to the final 3D-model and supports the notion that sACE is an asymmetric molecule. Four main aspects can be discerned by rotation of the model around its z-axis, termed 'front view', 'left side view', 'backside view' and 'right side view' (fig. 7a). Both N- and C-domains are linked by a single oligopeptide segment, distinctly visible as a filigree spacer that represents the

'interdomain-linker'. This spacer is made up from assumed 15 amino acids and is prone to proteolysis (Sturrock et al., 1997). The linker keeps both domains at a distance of about 2.0-2.6 nm. Nevertheless, even at the given low resolution, both domains show individual dissimilarities, which can be recognized mainly as (1) surface mass deficient areas termed 'central holes' (fig. 7a; arrows, ch-N, ch-C). (2) A distinct protuberance is visible mainly at the lower domain (fig. 7a; arrowhead) and belongs to the subdomain I (SD I; see Towler et al., (2004) for nomenclature) residual. (3) Both C- and N-domains show intradomain cleft-like mass deficiencies, which indicate distinct tertiary structure details.

By slice viewing of the electron microscopic density model (fig.7b) the protein mass distribution and the molecule's interior organization could be revealed. Here, eight slices,

indicated by the black vertical or horizontal bars of the 3D-model icons, represent the main section 'on-views' of sACE. It is obvious from views #2, 3, 5, 7 and 8 (fig.7b) that the interior of both domains show bright-shining voids, surrounded by protein densities colored blue.

These voids obviously are not arranged symmetrically to each other and also their shape is dissimilar to one another. Section on-view #6 shows characteristic features of the molecule as

already observed from individual negatively stained particles (fig. 5c-e), i.e. N-domain projections appear triangular in contour and the C-domain shows orthogonal mass projections. Furthermore, the molecule seen from its 'right side view' (fig.7a) has been cut mainly through the ventral part of the C-domain (fig.7b; #4), and its corresponding 'slice-view' distinctly reveals a 'cone-like' protuberance (fig.7b; #4, arrow), which represents a

prominent feature of the sACE molecule, as it is indicated in fig. 7a by arrowheads. This difference in shape of the internal voids of both domains is further emphasized by their appearance as Z-slices (fig. 7c), which show central circular dark void areas in the N-domain's interior, which is distinctly different from the inner C-domain, characterized by dark elongated void contours, framed yellow and pile up for roughly 14 nm along the z-axis, and thus form a cleft-like interior space, distinctly larger than the N-domain's equivalent.

3.3.3. Molecular fitting of atomic models for the N- and C-domain into the 3D electron microscopic densities of porcine lung sACE.

Direct inspection of negatively stained sACE molecules and the corresponding 3D electron microscopic density, suggest that the enzyme's conformational state is stable and even withstands mild proteolysis followed by affinity-chromatographic purification. This led us to examine the density model more closely by fitting high resolution atomic coordinates of the N-domain of human sACE into the assumed N-domain moiety, and the atomic coordinates of ACE2 into the analogous C-domain part (fig. 8). The fitting procedure was done semi-automatically using the Chimera 'Fit map into model' routine. Since the resolution of the model is 2.31 nm, only a few distinct markers could be used to align overall atomic model features. The C-domain as the basal part of the sACE-model (fig. 8a and c) showed at least three characteristic aspects, which are consistent with those of the corresponding atomic model. These are first the central hole (fig. 8a; ch-C), which offers a deep view into the interior void of the C-domain, as is recognized from volume slice-views (fig. 7b; #1, 2, 3). Second, the 'cone-like protuberance' (Fig. 7a, and 8a, c; solid arrowheads), and third, the neck-like 'interdomain-linker'. The atomic model did fit and docked rather well to the first two features in such a way that (1) the 'substrate cleft' in its substrate-unbound state (fig. 8b; sc (open)) aligned with the 'central hole' and (2) the SD I of the C-domain (fig. 8b) with the 'cone-like' protuberance. This 'two-point-alignment' is straight forward and leads to acceptable fitting of high and low resolution data. From this alignment it is obvious that the position of the 'interdomain-linker' of the atomic model (fig. 8d; red-colored C-terminus of

the N-domain) is different from the electron microscopic density model.

Conversely, fitting the N-domain atomic structure, here in its 'closed substrate-bound' state, into the corresponding EM density was not straightforward. The split 'interdomain-linker' end, i.e. the C-terminus of the N-domain (Fig. 8b and d; Asp612), was oriented relative to its corresponding counterpart (Fig. 8b and d; Ser19) and the residual part of the atomic model was rotated and shifted to optimally fit with the rudimentary 'central hole' (Fig. 8a and c; ch-N) into the electron microscopic density model. The absence of a prominent cone-like protuberance (fig. 8a and c; massive arrowheads) and the missing of a pronounced cleft-like 'central hole' within the N-domain of the sACE obviously is indicative for the 'closed substrate-cleft' conformation.

4. Discussion

4.1. Isolation and biochemical characterization of sACE from porcine lung.

It has been reported that sACE from human kidney can be split into two active domains by limited proteolysis using endoproteinase Asp-N. Here, we have obtained similar results with purified sACE from porcine lung. Due to the similar physicochemical properties of the two sACE domains, it has proven very difficult to isolate the individual domains using standard chromatography techniques. Sturrock et al., (1997) therefore, generated an immunoaffinity column with monoclonal antibodies recognizing specific epitopes, which were used for separation of the N-domain of sACE.

Although the N- and C-domains of sACE appear to be very similar, subtle differences exist between them. For example, ileal ACE, the naturally occurring active N-domain, has been found to poorly bind to a Sepharose-lisinopril affinity column with eluting buffer containing only 0.3M NaCl; however, it bound well in the presence of a higher salt (Deddish et al., 1994). Ileal ACE bound to a Sepharose-lisinopril column in 10 mM Hepes (pH 8.0) containing 0.8 M NaCl and 10 μ M ZnSO₄ and could be eluted with 10 mM Hepes (pH 6.0) containing 10 μ M ZnSO₄. In another work the importance of pH on the dissociation of lisinopril and sACE

was pointed out (Bull et al., 1985). Here we used the Sepharose–lisinopril affinity column to separate the rather similar N– and C–domains of sACE taking advantage of the subtle difference that exists between the two domain forms. Native sACE as well as both N– and C–domains can bind to Sepharose–lisinopril in buffer consisting of 20 mM Tris–HCl (pH 7.2), 0.3 M NaCl and 100 μ M ZnCl₂. Surprisingly, only the N–domain was eluted from column when washed with 20 mM Mes–NaOH (pH 6.0) containing 10 μ M ZnCl₂. The C–domain was eluted by changing the nature of the buffer to Hepes and could be eluted in 20 mM Hepes (pH 6.0) and 10 μ M ZnCl₂. Finally, the remaining bound protein could be eluted with 50 mM Na₂B₄O₇ (pH 9.5) buffer, which was identified as native sACE. It is quite remarkable that the intact sACE, the N– and the C– domain molecules all can be separated by Sepharose–lisinopril affinity column chromatography simply by changing the buffer system.

4.2. Native structure of the sACE.

Though low in resolution, the presented TEM–structure of negatively stained sACE and that of its isolated N– and C–domains provide close–up views of the main conformational state of the native molecule. To our knowledge this is the first detailed molecular structure analysis of sACE from porcine lung and complements earlier dynamic light and neutron scattering data of Baudin et al. (1988), which showed the porcine lung sACE to have a radius of gyration of 44.5 Å. The authors assumed the molecule to be rod–shaped, measuring 4–6 nm in diameter and 13.5 nm in length. These values are close to our data, i.e. 15.3 nm in length and 7.4 nm in width.

Based on our data it is possible to describe three distinct substructures of sACE, which correspond to (1) the 'hinge–region' or 'interdomain–linker', (2) the two–centered, orthogonally shaped moiety, and (3) the monocentered, triangular contoured moiety (see Fig. 5). Both N– and C– domains were isolated after mild proteolysis of the purified sACE, followed by affinity chromatography, and were confirmed by N–terminal sequencing. The different molecular shapes can be assigned to either different conformations or differences in shape of both domains. Remarkably, neither mild proteolysis nor affinity chromatography abolished enzymatic activity, indicating that the conformation of the individual domains represent

stable conformers, which is also true for intact sACE. This is consistent with a 'two-mass' conformation, with the orthogonal moiety representing the C-domain in its substrate-unbound, 'open-cleft' state, while the triangular moiety representing the N-domain with the substrate-cleft 'closed' (see Fig. 5b-e). From X-ray crystallographic studies of inhibitor-bound and unbound ACE2, which is a homologue of the sACE's C-domain, both these distinct conformational states were confirmed. It was shown, that SD I in the 'open-cleft' state moves about 1.5 nm towards the SD II subdomain upon binding of one molecule of lisinopril, which fixes the ACE2 domain in the 'closed-cleft' state (Towler et al., 2004). This relative movement of the SD I subdomain was verified and described in detail with glycosylation mutants of the germinal tACE as the ACE2-homologue (Watermeyer et al., 2006). Naverova et al. (2008) were able to create monoclonal antibodies (mAbs) directed to conformational epitopes on the C-domain surface of human ACE. Two of these showed some anticatalytic activities to the C-domain, though significantly lower than mAbs directed to the N-domain (Danilov et al., 2007; Skirgello et al., 2006). According to the resulting epitope binding maps Naperova et al. (2008) constructed a putative 3D-arrangement of N- and C-domains, based on corresponding X-ray data, and thus simulated a model of the somatic ACE molecule. This model, however, shows both domains in contact to each other and the interdomain-linker in the position of the central axis of the molecule, contradicting our data of clearly separated and distant domains and a lateral, off-axis position of the linker.

In the present 3D reconstruction of the electron microscopic densities a significant structural similarity to the 'open' conformational state could be established, referred to as the 'central hole' of the C-domain (Fig. 7A and 8a, c), which represents the large void space of the C-domain's interior (Fig. 7b and c). The 'central hole', next to the origin of the neck-like 'interdomain-linker', forms the entrance to the interior void-pocket which represents the open substrate cleft. The 'open-state' of the C-domain's cleft is outlined by the intense binding and/or accumulation of the cationic uranyl stain molecules, since the inside of the cleft is aligned with acidic amino acids (Towler et al., 2004). This is in congruence with the appearance and dimensions of the substrate-unbound 'open-cleft' conformation, which was found to be characteristic for the active form of human sACE and ACE2 in the presence of

chlorine (Ehlers and Kirsch, 1988). Similarly prominent deep clefts have been observed from neurolysin (Brown et al., 2001) and a carboxypeptidase from the hyperthermophilic archaeon *Pyrococcus furiosus* (Arndt et al., 2002). Both enzymes are functionally homologous to ACE and belong to the metalloprotease (zincin) superfamily (Sturrock et al., 2004).

- 5 The reconstructed N-domain of the porcine sACE does not show this prominent central hole with a large interior void. Instead, only a small, pocket-like indentation in register with the 'central hole' at the periphery could be observed. It is assumed that the N-domain has its cleft closed, thus reflecting the 'substrate/inhibitor-bound state' (Fig. 8a and c). The indentation is structurally similar to the interior void, which can be seen from volume-slices of the voxel
10 cube (fig. 7b,c).

These voids thus reflect the domains' interior state at the tertiary structure level. The quality and potency of the preparation method to come down to this level of resolution was shown to outline features down to the secondary structure level by cryo-negative stain electron microscopy of chaperonins (De Carlo et al., 2008) and as is shown simple negative stain is
15 potent to outline the 'interdomain-linker' as a single polypeptide chain. Our 3D-EM reconstruction of sACE reveals an asymmetric molecule (Fig. 7a).

Some assumptions of the relative orientation of the substrate clefts of the sACE molecule and the corresponding orientation of the N- and C-domains relative to the neck-like 'interdomain-linker' can be made in relation to the long axis of the molecule. The plane of the C-domain's
20 cleft appears inclined relative to the long axis by roughly 60° (Fig. 8D; dashed line). In contrast, the N-domain has its cleft oriented nearly normal to the molecule's long axis, and relative to the long axis both clefts – and thus both domains – are twisted about 90° to each other.

Based on our data three main points regarding the spatial arrangement of both sACE domains
25 are noticeable. (i) The voids within both domains are different in volume and represent the active site or substrate-binding cleft. (ii) Along the molecule's central axis both these clefts appear inclined to about 60° to each other. Thus, the substrate entry site of both domains are oriented differently to the bulk solvent. (iii) The N- and C-domains are not in contact with each other but instead are separated by an 'interdomain-linker' of 2.0–2.5 nm in distance.

Our 3D-model obtained by semi-automatic fitting atomic coordinates into the electron microscopic densities suggests that subdomain SD I of the C-domain is closest to the N-domain's SD II vertex. This domain arrangement keeps the 'flexible loops' (Fig.8b) at intermediate distance to each other. This means that the flexible C-domain's SD I is distant to the corresponding, flexible N-domain's SD I and comes close to the N-domain's SD II helix H27 (residues 568–588) and helix H19 (residues 417–432) (Corradi et al., 2006). Additionally, based on the actual atomic fitting the 'flexible loop', comprising residues 128–136 of the human somatic N-domain, does not appear proximal or in even contact with the 'interdomain-linker', contradicting the model view of the human sACE (Corradi et al., 2006).

Although human as well as bovine sACE display negative cooperativity between the N- and C-domains' active sites upon binding of diverse peptides or inhibitors (Binevski et al., 2003; Woodman et al., 2005; Skirgello et al., 2005), the enzymic activities are not linked to the glycosylation state. Deglycosylation of sACE from porcine lung did not markedly affect the biochemistry or structural properties of the enzyme (Baudin et al., 1997). However, this has been disputed by Watermeyer et al. (2006), who found that interactions of glycan chains at N72 and N109 in tACE stabilized the $\alpha 1$ and $\alpha 2$ helices of the lid.

Concerning the degree of spatial freedom both domains receive their flexibility by the 'interdomain-linker', whose N-terminal portion (residues 602–612) has been well defined (Corradi et al., 2006). At full stretch it may span a length of 3.98 nm based on a peptide length of 0.362 nm (Corey, Pauling, 1953). This length is long enough to bridge the interdomain distance of 2.0–2.5 nm observed in the electron microscopic density model. From the hinge end at the N-domains's C-terminus Asp⁶¹² to the N-terminus Asp⁶¹⁶ of the C-domain (Sturrock et al., 1997) (cf. the 80 kDa protein, Table 4) the tripeptide Leu-Val-Thr (residues 613–615 of human sACE, Soubrier et al., 1988) has to be added to the 'interdomain-linker' of the intact molecule, thus giving it an overall length of 15 amino acids.

The data presented here provide new insight into the biochemical characteristics of the sACE. Isolating the N- and C-domains combined with electron microscopy studies, provided for the first time detailed tertiary structure information for both domains. The 3D reconstruction of

sACE obtained at 2.3 nm resolution allowed interactive fitting of the atomic coordinates for the N- and the C-domain determined by X-ray crystallography into the electron microscopic density. The focus for alignment of both atomic models with their open (C-domain) and closed (N-domain) 'substrate clefts' was set to the orientation of their electron microscopic counterparts, i.e. the interior voids of both domains.

Here, we have presented for the first time the conformational state of isolated porcine lung sACE and addressed the spatial relationship of the N- and C-domain's active sites at the tertiary structure level. Future studies will focus on electron microscopy studies of substrate- and inhibitor-bound complexes, which should provide key insight into how the conformational states will differ during catalysis.

Acknowledgements

Hui-Ling Chen was recipient of a fellowship from the NSC-DAAD (National Science Council, Republic of China/ Deutscher Akademischer Austauschdienst, Germany) 'Sandwich' Program. We thank Mrs. Rita Getzlaff for protein sequencing; and Dr. Francis T. F. Tsai, Baylor College of Medicine, for critical reading of this manuscript.

References

- Andujar-Sánchez, M., Cámara-Artigas, V., Jara-Pérez, V., 2003. Purification of angiotensin I converting enzyme from pig lung using concanavalin-A Sepharose chromatography. *J. Chromatogr. B* 783, 247-252.
- Arndt, J.W., Hao, B., Ramakrishnan, V., Chen, T., Chan, S.I., Chan, M.K., 2002. Crystal structure of a novel carboxypeptidase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *Structure* 10, 215-224.
- Baudin, B., Timmins, P.A., Drouet, L., Legrand, Y., Baumann, F.Ch. 1988. Molecular weight and

shape of angiotensin-I converting enzyme. A neutron scattering study. *Biochem. Biophys. Res. Commun.* 154, 1144–1150.

5 Baudin, B., Alves, N., Pilon, A., Bénétteau-Burnat, B., Giboudeau, J. 1997. Structural and biological roles of glycosylations in pulmonary angiotensin I-converting enzyme. *Glycobiology* 7, 565–570.

10 Binevski, P.V., Sizova, E.A., Pozdnev, V.F., Kost, O.A., 2003. Evidence for the negative cooperativity of the two active sites within bovine somatic angiotensin converting enzyme. *FEBS Lett.* 550, 84–88.

15 Brown, C.K., Madauss, K., Lian, W., Beck, M.R., Tolbert, W.D., Rodgers, D.W., 2001. Structure of neurolysin reveals a deep channel that limits substrate access. *Proc. Nat. Acad. Sci. U.S.A.* 98, 3127–3132.

Bull, H.G., Thornberry, N.A., Cordes, E.H., 1985. Purification of angiotensin-converting enzyme from rabbit lung and human plasma by affinity chromatography. *J. Biol. Chem.* 260, 2963–2972.

20 Bünning, P., Holmquist, B., Riordan, J. F., 1983. Substrate specificity and kinetic characteristics of angiotensin converting enzyme. *Biochemistry* 22, 103–110.

25 Checler, F., Vincent, J.P., Kitabgi, P., 1983. Degradation of neurotensin by rat brain synaptic membranes: involvement of a thermolysin-like metalloendopeptidase (enkephalinase), angiotensin-converting enzyme, and other unidentified peptidases. *J. Neurochem.* 41, 375–384.

Cheung, H.S., Cushman, D.W., 1973. Inhibition of homogeneous angiotensin-converting

enzyme of rabbit lung by synthetic venom peptides of *Bothrops jararaca*. Biochim. Biophys. Acta 293, 451–463.

5 Coates, D., 2003. Molecules in focus. The angiotensin converting enzyme (ACE). Int. J. Biochem. Cell. Biol. 35, 769–773.

Corey, R.B., Pauling, L., 1953. Fundamental dimensions of polypeptide chains. Proc. R. Soc. (Lond.) B141, 10–20.

10 Corradi, H.R., Schwager, S.L.U., Nchinda, A.T., Sturrock, E.D., Acharya, K.R., 2006. Crystal structure of the N domain of human somatic angiotensin I-converting enzyme provides a structural basis for domain-specific inhibitor design. J. Mol. Biol. 357, 964–974.

15 Corvol, P., Williams, T.A., Soubrier, F., 1995. Peptidyl dipeptidase A: angiotensin I-converting enzyme. Methods Enzymol. 248, 283–305.

Cushman, D.W., Cheung, H.S., Sabo, E.F., Rubin, B., Ondetti, M.A., 1979. Development of specific inhibitors of angiotensin I converting enzyme (kininase II). Federation Proc. 38, 2778–2782.

20 Danilov, S.M., Watermeyer, J., Balyasnikova, I.V., Gordon, K., Kugaevskaya, E.V., Elisseeva, Y.E., Albrecht, R.F. II., Sturrock, E.D. 2007. Fine epitope mapping of mAb 5F1 reveals anticatalytic activity. Biochemistry 46, 9019–9031.

25 De Carlo, S., Boisset, N., Hoenger, A., 2008. High-resolution single-particle 3D analysis on GroEL prepared by cryo-negative staining. Micron. Doi:10.1016/j.micron.2007.11.003

- Deddish, P.A., Wang, J., Michel, B., Morris, P.W., Davidson, N.O., Skidgel, R.A., Davidson, N.O.,
1994. Naturally occurring active N-domain of human angiotensin I-converting enzyme.
Proc. Natl. Acad. Sci. U.S.A. 91, 7807–7811.
- 5 Dixon, M., Webb, E.C., Thorne, C.J.R., Tipton, K.F., 1979. *Enzymes*. third ed. Longman Group
Ltd., London.
- Ehlers, M.R.W., Kirsch, R.E., 1988. Catalysis of angiotensin I hydrolysis by human
angiotensin-converting enzyme: effect of chloride and pH. Biochemistry 27, 5538–
10 5544.
- Holmquist, B., Bünning, P., Riordan, J.F., 1979. A continuous spectrophotometric assay for
angiotensin converting enzyme. Anal. Biochem. 95, 540–548.
- 15 Hooper, N.M., Turner, A.J., 1987. Isolation of two differentially glycosylated forms of
peptidyl-dipeptidase A (angiotensin converting enzyme) from pig brain: a re-
evaluation of their role in neuropeptide metabolism. Biochem. J. 24, 625–633.
- Hubert, C., Houot, A.M., Corvol, P., Soubrier, F., 1991. Structure of the angiotensin I-
20 converting enzyme gene. Two alternate promoters correspond to evolutionary steps of
a duplicated gene. J. Biol. Chem. 266, 15377–15383.
- Humphrey, W., Dalke, A., Schulten, K., 1996. VMD – visual molecular dynamics. J. Mol. Graph.
14, 33–38.
- 25 Kleywegt, G.J., Jones, T.A., 1996. xDIMPAN and xDIDATAMAN–programs for
reformatting, analysis and manipulation of biomacromolecular electron-density maps

and reflection data sets. *Acta Crystallogr.* D52, 826–828.

- Ludtke, S.J., Baldwin, P.R., Chiu, W. 1999. EMAN: semiautomated software for high-resolution single-particle reconstructions. *J. Struct. Biol.* 128, 82–97.
- 5 Naperova, I.A., Balyasnikova, I.V., Schwartz, D.E., Watermeyer, J., Sturrock, E.D., Kost, O.A., Danilov, S.M. 2008. Mapping of conformational mAb epitopes to the C domain of human angiotensin I-converting enzyme. *J. Proteome Res.* 7, 3396–3411.
- 10 Natesh, R., Schwager, S.L.U., Sturrock, E.D., Acharya, K.R., 2003. Crystal structure of the human angiotensin-converting enzyme-lisinopril complex. *Nature* 421, 551–554.
- Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C., Ferrin, T.E., 2004. UCSF chimera – a visualization system for exploratory research and
- 15 analysis. *J. Comput. Chem.* 25, 1605–1612.
- Riordan, J.F., 2003. Angiotensin I-converting enzyme and its relatives. *Genome Biol.* 4, 225.
- Skirgello, O.E., Binevski, P.V., Pozdnev, V.F., Kost, O.A. 2005. Kinetic probes for inter-domain
- 20 co-operation in human somatic angiotensin-converting enzyme. *Biochem. J.* 391, 641–647.
- Skirgello, O.E., Balyasnikova, I.V., Binevski, P.V., Sun, Z.-L., Baskin, I.I., Palyulin, V.A., Nesterovitch, A.B., Albrecht, R.F. II., Kost, O.A., Danilov, S.M. 2006. Inhibitory
- 25 antibodies to human angiotensin-converting enzyme: fine epitope mapping and mechanism of action. *Biochemistry* 45, 4831–4847.
- Soubrier, F., Alhenc-Gelas, F., Hubert, C., Allegrini, J., John, M., Tregear, G., Corvol, P., 1988.

Two putative active centers in human angiotensin I converting enzyme revealed by molecular cloning. Proc. Natl. Acad. Sci. U.S.A. 85, 9386–9390.

5 Strittmatter, S.M., Snyder, S.H., 1985. Characterization of angiotensin converting enzyme by [3H]captopril binding. Mol. Pharmacol. 29, 142–148.

Sturrock, E.D., Danilov, S.M., Riordan, J.F. 1997. Limited proteolysis of human kidney angiotensin-converting enzyme and generation of catalytically active N- and C-terminal domains. Biochem. Biophys. Res. Commun. 236, 16–19.

10

Sturrock, E.D., Natesh, R., van Rooyen, J.M., Acharya, K.R., 2004. Structure of angiotensin I-converting enzyme. Cell. Mol. Life Sci. 61, 2677–2686.

15

Towler, P., Staker, B., Prasad, S.G., Menon, S., Tang, J., Parsons, T., Ryan, D., Fisher, M., Williams, D., Dales, N.A., Patane, M.A., Pantoliano, M.W., 2004. ACE2 X-ray structures reveal a large hinge-bending motion important for inhibitor binding and catalysis. J. Biol. Chem. 279, 17996–18007.

20

Valentine, R.C., Shapiro, B.M., Stadtman, E.R., 1968. Regulation of glutamine synthetase; electron microscopy of the enzyme from *Escherichia coli*. Biochemistry 7, 3266–3273.

25

Watermeyer, J.M., Sewell, T., Schwager, S.L., Natesh, R., Corradi, H.R., Acharya, K.R., Sturrock, E.D., 2006. Structure of testis glycosylation mutants and evidence for conserved domain movement. Biochemistry 45, 12654–12663.

Wei. L., Alhenc-Gelas, F., Corvol, P., Clauser, E., 1991. The two homologous domains of the human angiotensin I-converting enzyme are both catalytically active. J. Biol. Chem.

266, 9002–9008.

Winkler, J., Lünsdorf, H., Jokusch, B.M., 1997 Energy-filtered electron microscopy reveals that talin is a highly flexible protein composed of a series of globular domains. Eur. J. Biochem. 243, 430–436.

5

Woodman, Z.L., Schwager, S.L.U., Redelinghuys, P., Carmona, A.K., Ehlers, M.R.W., Sturrock, E.D., 2005. The N domain of somatic angiotensin-converting enzyme negatively regulates ectodomain shedding and catalytic activity. Biochem. J. 389, 739–744.

Figure legends:

Fig. 1. pH optimum of ACE activity. pH dependence of the ACE-catalyzed hydrolysis of FA-Phe-Gly-Gly (0.5mM) was determined at 37°C in 300mM NaCl containing none (dashed line) or 0.1mM zinc ion (solid line) in either 50mM Mes (pH 5.5–6.5), Hepes (pH 7.0–8.5) or borate (pH 9.0–10.0) buffer.

Fig. 2. Sepharose-lisinopril affinity chromatography for purification of N- and C-domains. About 1.6 mg of purified sACE was digested with 2 µg of endoproteinase Asp-N in 50 mM Hepes (pH 8.0) buffer at 37 °C for 18 hours. The proteolytic products were then purified by a Sepharose-lisinopril column, which was equilibrated with 20 mM Tris-HCl (pH 7.2), 0.3 M NaCl, 100 µM ZnCl₂. The N-domain of ACE (Peak I) was eluted first with buffer B: 20 mM Mes-NaOH (pH 6.0), 10 µM ZnCl₂. The C-domain (Peak II) was then obtained by elution with buffer C: 20 mM Hepes-NaOH (pH 6.0), 10 µM ZnCl₂.

Fig. 3. SDS-PAGE analysis of separation of N- and C-domains. The fractions of Peaks I and II (fig.2), were examined by 7.5% SDS-PAGE. Lanes 4 and 5 were Peaks I and II, respectively. Lane 2 was the pure sACE, and lane 3 was the pure sACE being digested with endoproteinase Asp-N. Lane 1 were high molecular mass markers, composed of myosin (200.0 kDa), *E. coli* β-galactosidase (116.3 kDa), rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa) and hen egg white ovalbumin (45.0 kDa).

Fig. 4. The time course of proteolytic digestion of sACE. About 120 µg of purified sACE was digested with 0.1 µg of endoproteinase Asp-N in Hepes (pH 8.0) at 37 °C for 20 hours. The ACE activity was measured at time intervals of 0h, 1h, 3h, 5h and 20 h (—●—). The control experiment was performed under the same condition except for the omission of endoproteinase Asp-N (---x---).

Fig. 5. Survey views of negatively stained porcine lung somatic ACE. (a) Elastic bright-field

view (zero-loss image) of purified molecules. (b) Corresponding ESI-view (U_{045} edge; 115 eV; same imaging mode for b, c, and e), which shows the protein molecules in reversed contrast. Encircled particles are identical in (a) and (b; scale valid for a). (c) Selected sACE molecules with distinct tertiary features. Black-white arrowhead: hinge or 'interdomain-linker', double
5 arrow: orthogonal, two-centered protein mass, solid triangle: triangular-shaped protein mass, arrow: four-centers of protein masses, tetrahedrally arranged. (d) Isolated N-domains (selected views). (e) Isolated C-domains (selected views). Bar: 20 nm, c, e; e valid for d.

Fig. 6: Single-particle analysis of negatively stained sACE molecules. (a) Gallery of boxed
10 particles, seen as zero-loss images. (b) Gallery of prefiltered views after an initial 2D refinement, classified as unbiased references for EMAN-image reconstruction. Distinct side view projections of two-domain molecules (0,1,2,3,5,7,8,9) and on-top views of mono- and/or bi-domain molecules (= 4,6) are shown. (c) Resolution of the electron microscopic density model, set at FSC 0.5 σ after 11 convergent refinements. (d) Class distribution in
15 Euler-space. (e) 18 representative views of totally 236 class averages.

Fig. 7: Electron microscopic density model of the somatic ACE. (a) 'En face' aspects of the sACE-model, rotated by 90° and indicated correspondingly. ch-N: central hole of the N-domain, ch-C: central hole of the C-domain, arrowheads indicate a cone-like protuberance.
20 (b) Divers volume-slices parallel or normal to the molecule's rotation (Z) axis. Protein densities are colored blue and voids inside the molecule are bright. Slice positions (black line) are shown relatively to the molecules from 1 to 8. (c) Gallery of volume-slices along the z-axis. Slices #7 to #33 from top to bottom encompass represent the N-domain, framed green; #34 to #61 represent the C-domain, framed red. Slices framed yellow, show sections with a
25 prominent elongated void, which pile up to form the interior cleft, about 14 nm in height, these slices are flanked up and down the z-axis by smaller circular voids, recognized as black central dots. Adequately, the N-domain slices only show central spot-like voids. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 8: Fitting of high-resolution X-ray atomic data into the low-resolution electron microscopic 3D reconstruction. (a) Backside-view of the semi-transparent model, showing the molecule's topography and inside orientation of the N-domain atomic data (human sACE-N-domain; PDB-code: 2C6F) and of the C-domain atomic data (human ACE2; PDB-code: 1R42). (b) Same view as in (a), showing the helix-sheet atomic models. (c) and (d) Corresponding aspects of the molecule, seen from the 'right side'. The N-domain is shown in green, the C-domain is colored red. ch-C: central hole of the C-domain; ch-N: central hole of the N-domain; arrowheads indicate the prominent protuberance; sc: substrate cleft; N-term: amino-terminus; C-term: carboxy-terminus; individual amino acids are indicated; SD: subdomain, H19, H27: helices, according to the nomenclature of Corradi et al. (2006). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 1. Summary of the purification of angiotensin I-converting enzyme from porcine lung

	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Recovery (%)
Crude extract	2300	26,85	6726	0.25	1	100
Ammonium sulfate precipitation	600	10,27	4918	0.48	1.9	73.1
Fractogel-DEAE	1460	2022	3900	1.93	7.7	58.0
ConA-Sepharose	710	149	3193	21.43	85.7	47.5
Sepharose-lisinopril	29	13.8	3017	218.62	874.5	44.9

Table 2. Comparison of kinetic parameters of ACE from different sources

	Porcine lung ACE	Rabbit lung ACE
k_{cat} (min^{-1})	52.700	19.000
K_m (M)	7.8×10^{-4}	3.0×10^{-4}
k_{cat}/K_m ($\text{M}^{-1} \cdot \text{min}^{-1}$)	67.4×10^6	63×10^6
Reference	This work	Bünning et al. (1983)

Table 3. Inhibition of porcine lung angiotensin I-converting enzyme

Inhibitor	K_i (nM)		
	This work	Literature	Reference
Captopril (SQ14,225)	1.7	1.7	Cushman et al. (1979)
		5.7	Checler et al. (1983)
		4.0	Strittmatter and Snyder (1985)
BPP _{9a} (SQ20,881)	124.7	130	Cushman et al. (1979)
		100	Cheung and Cushman (1973)

Table 4. The N-terminal sequences of porcine lung sACE and protein fragments obtained from endoproteinase Asp-N proteolysis^a

Fragment	Amino acid sequence
Intact pig lung ACE	L D S A L Q P G D F T A D E A G A E D F A Q S F
The 90kDa protein	L D S A L Q P G D F T A D E A G A E D F A Q S F
The 80kDa protein	D E A E A S K F V E E Y D R Q V V L N E Y A E

5 ^aOne-letter amino acid notation is used.

10

Table 5. Kinetic parameters of purified N- and C-domains of porcine lung ACE

Enzyme	K_m (mM)	k_{cat} (min ⁻¹)	k_{cat}/K_m (M ⁻¹ · min ⁻¹)
Native ACE	0.78	5.3×10^4	67.9×10^6
N-domain	0.58	1.1×10^4	19.0×10^6
C-domain	0.40	4.5×10^4	112.5×10^6

figure 1

figure 2

figure 3

figure 4

figure 5

figure 6

figure 7

figure 8