Residues affecting the chloride regulation and substrate selectivity of the angiotensin-converting enzymes (ACE and ACE2) identified by site-directed mutagenesis

Christopher A. Rushworth, Jodie L. Guy and Anthony J. Turner

Institute of Molecular and Cellular Biology, Faculty of Biological Sciences, University of Leeds, UK

Angiotensin-converting enzyme (ACE) and its homologue angiotensin-converting enzyme 2 (ACE2) are critical counter-regulatory enzymes of the renin–angiotensin system, and have been implicated in cardiac function, renal disease, diabetes, atherosclerosis and acute lung injury. Both ACE and ACE2 have catalytic activity that is chloride sensitive and is caused by the presence of the CL1 and CL2 chloride-binding sites in ACE and the CL1 site in ACE2. The chloride regulation of activity is also substrate dependent. Site-directed mutagenesis was employed to elucidate which of the CL1 and CL2 site residues are responsible for chloride sensitivity. The CL1 site residues Arg186, Trp279 and Arg489 of testicular ACE and the equivalent ACE2 residues Arg169, Trp271 and Lys481 were found to be critical to chloride sensitivity. Arg522 of testicular ACE was also confirmed to be vital to the chloride regulation mediated by the CL2 site. In addition, Arg514 of ACE2 was identified as a residue critical to substrate selectivity, with the R514Q mutant, relative to the wild-type, possessing a fourfold greater selectivity for the formation of the vasodilator angiotensin-(1–7) from the vasoconstrictor angiotensin II. The enhancement of angiotensin II cleavage by R514Q ACE2 was a result of a 2.5-fold increase in \( V_{\text{max}} \) compared with the wild-type. Inhibition of ACE2 was also found to be chloride sensitive, as for testicular ACE, with residues Arg169 and Arg514 of ACE2 identified as influencing the potency of the ACE2-specific inhibitor MLN-4760. Consequently, important insights into the chloride sensitivity, substrate selectivity and inhibition of testicular ACE and ACE2 were elucidated.

Abbreviations
Abz, o-aminobenzoic acid; ACE, angiotensin-converting enzyme; Dnp, 2,4-dinitrophenyl; Mca, (7-methoxycoumarin-4-yl)acetyl; RAS, renin–angiotensin system; sACE, somatic ACE; tACE, testicular ACE.
Chloride regulation of ACE and ACE2

C. A. Rushworth et al.

FEBS Journal 275 (2008) 6033–6042 © 2008 The Authors Journal compilation © 2008 FEBS

has two homologous domains (N- and C-domains), and each of these contains an active site. tACE only possesses a single catalytic domain, which corresponds to the C-domain of sACE.

The requirement of chloride ions for the hydrolysis of angiotensin I by ACE has long been recognized [15], and ACE2 activity is also regulated by chloride ions [16], with the chloride regulation of both of these enzymes being substrate dependent. The cleavage of angiotensin I by ACE is activated by chloride ions, whereas bradykinin cleavage is maximal at a concentration of 20 mM, with increases in chloride concentration above this value producing an inhibitory effect on activity [17–19]. The presence of chloride also increases the hydrolysis of angiotensin I by ACE2, but inhibits cleavage of the vasoconstrictor angiotensin II [20]. It has been proposed that chloride binding induces subtle changes in the conformation of the active site, which either facilitate or hinder substrate binding [21]. Both sACE and ACE2 have high levels of expression in the kidney, where extracellular chloride ion levels fluctuate. Consequently, chloride regulation of ACE and ACE2 could serve as a regulatory mechanism to maintain a physiologically appropriate balance of activities.

The crystal structure of tACE shows that two chloride-binding sites are present [22]. The first site (CL1) is located some distance away from the zinc ion of the active site (20.7 Å), whereas the second site (CL2) is considerably closer, being 10.4 Å from the zinc ion. The N-domain of sACE has been shown to possess a CL2 site only, and so the enzyme has three chloride-binding sites in total [23]. The CL2 chloride site is absent in ACE2 as a result of substitution of the tACE residues Pro407 and Pro519 with Glu398 and Ser511, and the resulting projection of their side-chains into the location of this site. Consequently, ACE2 only binds a chloride ion at one CL1 site [24].

Previous mutagenesis studies of sACE have shown that the CL2 site residue Arg1098 is essential for chloride sensitivity, with this residue being conserved as Arg522 in tACE and Arg514 in ACE2 [25]. An Arg514 to Glu mutation did not result in the loss of ACE2 chloride ion sensitivity when the synthetic peptide Mca-Dnp (7-methoxycoumarin-4-yl)acetyl; Dnp, 2,4-dinitrophenyl) served as the substrate [26]. Hence, it would appear that the CL1 site must be solely responsible for the chloride sensitivity of ACE2, whereas, in tACE, the phenomenon is a result of the combined effects of the CL1 and CL2 sites.

At present, the essential residues for CL1 site-mediated regulation of tACE and ACE2 activities are unknown. In addition, although Arg1098 has been identified as essential to chloride regulation of the CL2 site of sACE, the roles of the equivalent tACE and ACE2 residues have not been investigated previously. In this study, candidate residues potentially involved in chloride binding at the CL1 and CL2 sites of ACE and ACE2 were changed by site-directed mutagenesis, and the effects on chloride sensitivity, substrate selectivity and inhibitor potency were observed.

Results

PCR mutagenesis of CL1 and CL2 site residues of tACE and ACE2

The residues surrounding the chloride ion at the CL1 and CL2 sites of tACE and ACE2 are shown in Fig. 1. In tACE, the residues that coordinate the chloride ion at the CL1 site are Arg186, Trp485 and Arg489, which are conserved as Arg169, Trp477 and Lys481 in ACE2 [22,24]. All of these residues can influence chloride binding and therefore the chloride sensitivity of enzyme activity. Trp271 of ACE2 and the equivalent Trp279 tACE residue are also implicated in CL1 site-mediated chloride sensitivity, as they are in close proximity to the CL1 chloride ion, with Trp271 of ACE2 also lying two residues upstream of Arg273, which is known to be critical for substrate binding [26].

The CL2 site chloride in tACE is bound by Tyr224, a water molecule and Arg522, with Tyr204 and Arg514 being the corresponding ACE2 residues [20,22]. Arg1098 in the C-domain of sACE has previously been shown by an R1098Q mutant to serve a vital role in chloride dependence [25], and is the equivalent residue to Arg514 of ACE2 and Arg522 of tACE.

PCR mutagenesis was employed with tACE and ACE2 on the CL1 and CL2 site residues described above, which are listed in Table 1, in order to investigate their roles in chloride sensitivity. R186QR489QR522Q tACE and R169QR481QR514Q ACE2 triple mutants were also constructed to determine whether a synergistic effect on chloride sensitivity occurred.

Expression of wild-type and mutant forms of tACE and ACE2

Stable expression of tACE and ACE2 mutants was established in HEK293 cells, and was shown to be comparable with that of the wild-type forms. All of the mutant proteins also migrated on SDS-PAGE with the same apparent Mr as the wild-type enzymes (Fig. 2). Consequently, any differences observed in the activity levels of the enzyme variants are not attributable to significant alterations in protein expression.
Altered enzymatic activity of the mutant forms of tACE and ACE2

With angiotensin I as the substrate, and at 100 mM NaCl, the physiological concentration of chloride ions in human plasma [27], all of the mutants had, to varying degrees, a level of activity less than that of the wild-type. Of the tACE mutants, those which contained a CL2 site mutation had the lowest levels of activity, with R522Q and R186QR489QR522Q tACE possessing 21.7% and 16.3% relative activity, respectively. In contrast with the tACE equivalent variants, of the ACE2 mutants, R169Q had the lowest relative activity (5.2%) (Table 2).

The rate of angiotensin II cleavage was also recorded at 100 mM NaCl for all of the ACE2 variants (Table 3), as this is the physiological substrate of the enzyme [28]. Surprisingly, the CL2 site mutant R514Q and the triple mutant R169QK481QR514Q showed enhanced levels of angiotensin II cleavage compared with the wild-type at the physiological concentration of chloride, possessing 179.3% and 204.4% relative activity, respectively. The results strongly suggest that Arg514 of ACE2 contributes to the substrate selectivity of the enzyme, particularly as R514Q has a 35-fold higher level of activity.

### Table 1. tACE and ACE2 residues subjected to site-directed mutagenesis.

| Equivalent residue | Chloride-binding site | Role of residue                                                                 |
|--------------------|-----------------------|---------------------------------------------------------------------------------|
| tACE               | ACE2                  |                                                                                  |
| R186               | R169                  | CL1                               | Coordinates Cl\(^{-}\) by ionic interaction                                     |
| W279               | W271                  | CL1                               | Close proximity to both the Cl\(^{-}\) and residue R273 in ACE2, a residue critical to substrate binding |
| R489               | K481                  | CL1                               | Coordinates Cl\(^{-}\) by ionic interaction                                     |
| R522               | R514                  | CL2                               | Coordinates Cl\(^{-}\) by ionic interaction                                     |

Fig. 1. Chloride-binding sites of tACE (orange) and ACE2 (red): (A) CL1 binding site; (B) CL2 binding site. Residue numbering for tACE is first. The chloride ion is shown in green and is a fixed position relative to both TACE and ACE2 residues. Residues subjected to site-directed mutagenesis are shown in bold. Cl\(^{-}\) coordinating residues are shown in italic. Cl\(^{-}\) is unable to be bound by ACE2 at the CL2 site as a result of the side-chains of Glu398 and Ser511 projecting into this region.

Fig. 2. Expression of wild-type and mutant variants of tACE and ACE2. Aliquots containing 10 μg of total protein obtained from transfected HEK293 cells were separated by SDS-PAGE (10% polyacrylamide gel). Detection of TACE (A) and ACE2 (B) was visualized by immunoblotting using specific human polyclonal antibodies.

### Altered enzymatic activity of the mutant forms of tACE and ACE2

With angiotensin I as the substrate, and at 100 mM NaCl, the physiological concentration of chloride ions in human plasma [27], all of the mutants had, to varying degrees, a level of activity less than that of the wild-type. Of the tACE mutants, those which contained a CL2 site mutation had the lowest levels of activity, with R522Q and R186QR489QR522Q tACE possessing 21.7% and 16.3% relative activity, respectively. In contrast with the tACE equivalent variants, of the ACE2 mutants, R169Q had the lowest relative activity (5.2%) (Table 2).

The rate of angiotensin II cleavage was also recorded at 100 mM NaCl for all of the ACE2 variants (Table 3), as this is the physiological substrate of the enzyme [28]. Surprisingly, the CL2 site mutant R514Q and the triple mutant R169QK481QR514Q showed enhanced levels of angiotensin II cleavage compared with the wild-type at the physiological concentration of chloride, possessing 179.3% and 204.4% relative activity, respectively. The results strongly suggest that Arg514 of ACE2 contributes to the substrate selectivity of the enzyme, particularly as R514Q has a 35-fold higher level of activity.
for angiotensin II hydrolysis than for angiotensin I, whereas, for the wild-type, a difference of only 10-fold was observed (Tables 2 and 3).

**Substrate specificity of wild-type tACE and ACE2 chloride sensitivity**

Following the removal of chloride ions by extensive dialysis, the effect of increasing chloride concentration on the activities of wild-type tACE and ACE2 was observed (Fig. 3). As reported previously, angiotensin I cleavage by tACE and ACE2 is activated by chloride ions [18,20]. The activation profiles differ, however, between the two enzymes. tACE activity continues to increase as [NaCl] is increased up to 1 mM (Fig. 3A), whereas maximal activity is obtained at approximately 500 mM NaCl for ACE2 (Fig. 3B). The degree of chloride activation of angiotensin I hydrolysis is greater for tACE than ACE2, with an 8.1-fold increase in the level of activity recorded at 500 mM NaCl compared with 0 mM for tACE, whereas only a 3.9-fold increase in ACE2 activity occurs. The effect of increasing chloride concentration on the rate of ACE2 cleavage of angiotensin II is distinct from, and more complex than, that of angiotensin I (Fig. 3C). A twofold increase in activity is observed as [NaCl] is increased from 0 to 100 mM, but any further increase in chloride concentration produces an inhibitory effect on activity, before a plateau is reached at 500 mM NaCl. Consequently, the level of activity at 500 mM NaCl is 1.6-fold less than that in the absence of NaCl and 3.2-fold less than that at 100 mM NaCl.

**Effects of CL1 and CL2 site mutations on the chloride sensitivity of tACE and ACE2**

In order to observe the effects of the various mutations on the chloride sensitivity of tACE and ACE2, the fold differences between the activity at 0 and 500 mM NaCl with angiotensin I and 0 and 100 mM NaCl with angiotensin II were recorded (Fig. 4). These concentrations were chosen as they allowed the elucidation of the maximal level of chloride activation for wild-type ACE2. Intriguingly, W279A tACE showed an inhibition of angiotensin I cleavage induced by 500 mM NaCl, with the corresponding fold difference in activity being 0.6, compared with the 8.1-fold difference recorded for the wild-type (Fig. 4A). The R186Q CL1 site and R522Q CL2 site mutations also showed a pronounced effect, with no significant chloride sensitivity observed with either variant. The R186QW279AR522Q mutant behaved in a similar manner to these two mutants, as it also lacked chloride sensitivity.

Wild-type ACE2 showed a 3.9-fold increase in angiotensin I cleavage at 500 mM NaCl, but the K481Q and R514Q variants possessed significantly lower levels of chloride activation with this substrate, exhibiting 1.9-fold and 1.6-fold increases in activity, respectively (Fig. 4B). R169QW279AR522Q ACE2 showed a 1.5-fold increase in activity, and the chloride sensitivity of this enzyme was very similar to that of the R514Q variant. In addition, W271A ACE2 was found to lack any significant chloride sensitivity with this substrate, and R169Q ACE2 did not show any activity in the absence of chloride ions.

When angiotensin II served as the substrate, prominent differences from wild-type ACE2 were observed for several of the variants, with R169Q, W271A and R514Q

| tACE variant | v (nmol-min⁻¹-mg⁻¹) | Relative activity (%) | ACE2 variant | v (nmol-min⁻¹-mg⁻¹) | Relative activity (%) |
|--------------|---------------------|-----------------------|--------------|---------------------|-----------------------|
| Wild-type    | 16.67 ± 0.58        | 100.0                 | Wild-type    | 1.98 ± 0.12        | 100.0                 |
| R186Q        | 7.14 ± 0.09         | 42.8                  | R169Q        | 0.10 ± 0.01        | 5.2                   |
| W279A        | 3.79 ± 0.10         | 22.7                  | W271A        | 0.10 ± 0.05        | 5.3                   |
| R489Q        | 7.13 ± 0.34         | 42.8                  | K481Q        | 0.42 ± 0.05        | 21.0                  |
| R522Q        | 3.62 ± 0.09         | 21.7                  | R514Q        | 1.03 ± 0.05        | 52.0                  |
| R169QR489QR522Q | 2.72 ± 0.22 | 16.3                  | R169QR481QR514Q | 1.05 ± 0.03 | 53.2                  |

| ACE2 variant | v (nmol-min⁻¹-mg⁻¹) | Relative activity (%) |
|--------------|---------------------|-----------------------|
| Wild-type    | 20.30 ± 0.40        | 100.0                 |
| R169Q        | 0.22 ± 0.01         | 1.1                   |
| W271A        | 0.19 ± 0.01         | 0.9                   |
| K481Q        | 14.58 ± 0.77        | 71.8                  |
| R514Q        | 36.41 ± 1.49        | 179.3                 |
| R169QR481QR514Q | 40.19 ± 1.45 | 204.4                 |
ACE2 all lacking a significant level of chloride sensitivity (Fig. 4C). The R514Q mutant has already been identified to possess a greater level of activity than the wild-type with this substrate at 100 mM NaCl (Table 3), but even more pronounced is the illustration in Fig. 4C that, in the absence of chloride ions, R514Q ACE2 has a 3.2-fold greater level of activity than the wild-type. As with the substrate angiotensin I, the chloride sensitivity of the R169QK481QR514Q variant with angiotensin II was very similar to that of R514Q ACE2.

**Kinetic parameters of angiotensin II cleavage by wild-type and R514Q ACE2**

The R514Q ACE2 variant showed an elevated level of angiotensin II cleavage and a decreased level of angiotensin I cleavage compared with the wild-type at physiological NaCl concentration (Tables 2 and 3). To further investigate the altered substrate selectivity of this variant, the kinetic parameters $K_m$ and $V_{max}$ of wild-type and R514Q ACE2 cleavage of angiotensin II at 100 mM NaCl were elucidated (Table 4). It was found that there was no significant difference in the $K_m$ values of these two variants, but $V_{max}$ was 2.5-fold higher for R514Q. The catalytic efficiency ($V_{max}/K_m$) of R514Q ACE2 was also 2.8-fold greater than that of the wild-type under these conditions. These data indicate that the R514Q mutation increases angiotensin II hydrolysis by enhancing the maximal level of activity, but not by altering the substrate-binding affinity.

**Effects of CL1 and CL2 site mutations on the chloride sensitivity of tACE and ACE2 inhibition**

It has been shown previously that the potency of N- and C-domain ACE inhibition by captopril, lisinopril and enalaprilat is enhanced as [NaCl] is increased [29]. However, there has been no previous report on the chloride sensitivity of the potency of the ACE2-specific inhibitor MLN-4760. Hence, dose–response curves were obtained for the inhibition of the ACE2 variants by MLN-4760, and the inhibition of the tACE variants by captopril, in the absence and presence of NaCl (500 mM). The IC$_{50}$ values derived from these dose–response curves showed that the inhibition of wild-type ACE2 was sensitive to chloride concentration, as observed for wild-type tACE (Fig. 5). The MLN-4760 IC$_{50}$ value recorded at 500 mM NaCl was 10-fold lower than that in the absence of chloride ions for wild-type ACE2, and the captopril IC$_{50}$ value with wild-type tACE was decreased 3.3-fold at 500 mM NaCl. The chloride sensitivity of inhibitor potency was lacking in R522Q tACE (Fig. 5A), which is in agreement with the absence of chloride sensitivity of angiotensin I cleavage by this variant (Fig. 4A). The R169Q ACE2 variant had an IC$_{50}$ value 21-fold and 9-fold greater than that recorded for the wild-type in the wild-type with this substrate at 100 mM NaCl (Table 3), but even more pronounced is the illustration in Fig. 4C that, in the absence of chloride ions, R514Q ACE2 has a 3.2-fold greater level of activity than the wild-type. As with the substrate angiotensin I, the chloride sensitivity of the R169QK481QR514Q variant with angiotensin II was very similar to that of R514Q ACE2.
absence and presence of 500 mM NaCl, respectively (Fig. 5A). Similarly, the R514Q ACE2 variant had an IC₅₀ value 27-fold and 50-fold greater than that of the wild-type in the absence and presence of 500 mM NaCl, respectively. It is therefore clear that these two mutations reduce the potency of MLN-4760.

**Discussion**

The findings of this study provide further support for the physiological relevance of the chloride sensitivity of ACE and ACE2 activity. It has been confirmed that an increase in [Cl⁻] above 100 mM, which is the physiological concentration in human plasma [27], increases angiotensin I and decreases angiotensin II cleavage by ACE2 and increases angiotensin I cleavage by ACE. This would have the effect of increasing the localized concentration of the vasoconstrictor angiotensin II. A decrease in [Cl⁻] would lead to the opposite scenario of a reduced localized angiotensin II concentration. The high levels of ACE and ACE2 in the kidney expose these enzymes to fluctuations in [Cl⁻] which do not occur in the plasma. Therefore, in vivo Cl⁻ sensitivity may serve to regulate the localized concentration of angiotensin peptides, particularly in the kidney, thus acting as a homeostatic regulatory mechanism.

Through the formation of tACE and ACE2 mutants, several CL1 site residues critical to the chloride sensitivity of activity were identified here for the first time. Arg186 and Arg489 of tACE, and the equivalent ACE2 residues Arg169 and Lys481, coordinate the chloride ion at this site by ionic interactions [22]. The removal of this interaction by mutagenesis abolishes or greatly reduces the level of chloride sensitivity. Trp271 of ACE2 is located in close proximity to the chloride ion at the CL1 site (4.88 Å) and lies two residues upstream of Arg273, which is known to be critical for substrate binding [26]. By mutagenesis, Trp271 and the tACE equivalent
Trp279 have also been identified as residues critical to the chloride sensitivity of the two enzymes. The CL1 site is unlikely to influence zinc binding as it is 20.7 Å from this ion in tACE [22]. However, it has been suggested to be important in stabilizing complex formation for the residues in subdomain II involved directly in substrate binding [20].

The CL2 site of ACE has previously been suggested to contain the mechanistically binding chloride ion, which, when bound by the enzyme, breaks the salt bridge between Arg522 and Asp465 of C-domain sACE and facilitates the movement of Tyr523 towards the active site [30]. It was found in this study that the tACE CL2 site mutant R522Q possesses no significant chloride sensitivity, as predicted from a previous study of the equivalent R1098Q sACE mutant [25]. Arg522 (Arg1098) is consequently critical to the chloride dependence of both tACE and sACE. The X-ray crystal structure of tACE reveals that Arg522, in combination with Tyr224 and a water molecule, binds the chloride ion at the CL2 site [22]. The ionic interaction of this residue with the chloride ion is therefore essential to the chloride sensitivity mediated by the CL2 site. Although the CL2 site chloride is absent in ACE2 [24], the R514Q variant affects the chloride sensitivity of the enzyme, with the observed increase in activity being twofold less than that of the wild-type with angiotensin I and absent with angiotensin II. In ACE2, it would thus appear that this mutation is able to transmit long-range effects on the chloride sensitivity of the enzyme induced by the CL1 site. The chloride sensitivity of the tACE and ACE2 triple mutants was consistently found to be similar to that of R522Q tACE and R514Q ACE2, respectively. This suggests that it is R522/R514 that has the greatest influence on the chloride regulation of activity, and that the CL2 site of ACE does indeed contain the mechanistically binding chloride ion.

The ACE2 CL2 site residue Arg514 strongly influences substrate selectivity. The removal of the positive charge of this residue by the creation of an R514Q mutant produces an enzyme that, compared with the wild-type, shows twofold greater activity with the substrate angiotensin II, but twofold less activity with angiotensin I at physiological [NaCl]. The crystal structure of ACE2 reveals that the topology and chemical environment of the S1 subsite is dictated by four residues (Tyr510, Arg514, Phe504 and Thr347), which are expected to restrict the size of substrate P1 side-chains [24]. Therefore, the R514Q mutation most probably alters the environment of the S1 subsite to make it more favourable for angiotensin II hydrolysis, but less favourable for angiotensin I hydrolysis. The kinetic data confirm that this variant has a threefold greater catalytic efficiency than the wild-type with angiotensin II, and this is not a result of an alteration in substrate-binding affinity but of an increase in $V_{max}$. The R514Q ACE2 variant or variants with enhanced activity towards angiotensin II have potential therapeutic value in acute lung injury, as mice suffering from this condition have previously shown markedly improved disease following the injection of recombinant ACE2 into the abdomen [11].

In this study, the potency of both tACE and ACE2 inhibitors was discovered to be chloride sensitive, with high [Cl\textsuperscript{-}] increasing inhibitor potency. This is believed to occur as a result of the facilitation of inhibitor binding by the conformational changes induced by chloride...
binding. Intriguingly, in contrast with these findings, the potency of ACE inhibitors is known to be increased in rats subjected to low-salt diets [31]. It is therefore apparent that there are additional salt-sensitive mechanisms in vivo that influence ACE inhibitor potency, and that these override the chloride binding-induced alteration in ACE inhibitor potency observed here. The ACE2 residues Arg169 and Arg514 have been identified as critical to the potency of MLN-4760, as their mutation drastically reduces the IC<sub>50</sub> values recorded in comparison with the wild-type. Arg514 of ACE2 is located next to the S<sub>1</sub> subsite [24], and so the R514Q mutation is hypothesized to have altered the environment of this region to one that is less accommodating to MLN-4760 binding. Arg169 of ACE2 is approximately 16 Å from the dichlorobenzyl group of MLN-4760 [24] and so is unlikely to directly hinder inhibitor binding. The most probable explanation is that the R169Q mutation transmits a conformational change to the active site over a long distance, which makes the environment for MLN-4760 binding less favourable.

**Experimental procedures**

**Construction of human tACE and ACE2 variants**

The peptides angiotensin I and angiotensin II were obtained from Bachem International (St Helens, UK). The fluorogenic peptides Mca-APK(Dnp) and Abz-FRK(Dnp) (Abz, α-aminobenzoic acid) were obtained from BIOMOL International (Exeter, UK). PIRE5-neo tACE cDNA (containing nucleotides 126–2219) was provided by E. T. Parkin (University of Leeds, UK) and pCI-neo ACE2 cDNA (containing nucleotides 104–2323) was provided by J. L. Guy (University of Leeds, UK).

**Site-directed mutagenesis**

Mutagenic PCRs were carried out in 0.2 mL Eppendorf tubes with 50 µL reaction volumes as described previously [26]. Plasmid DNA was prepared from a single colony and fully sequenced to ensure the presence of the desired point mutations and the absence of unintended mutations.

**Stable transfection of tACE and ACE2 variants in HEK293 cells**

HEK293 cells were cultured under an atmosphere of 5% CO<sub>2</sub> at 37 °C in DMEM, and grown to approximately 60% confluence in a Petri dish. Immediately prior to transfection with 5 µg of plasmid DNA, the cell monolayer was washed twice with NaCl/P<sub>e</sub>. GeneJuice transfection reagent was used at a ratio of DNA to reagent of 1 : 3 (w/v). This was added to the Petri dish with 2.5 mL of DMEM and incubated for 16 h before the addition of supplemented DMEM. At 72 h after transfection, the cells were passaged and allowed to grow in supplemented medium containing antibiotic G418 (1 mg/mL<sup>-1</sup>). The cells were subjected to repeated rounds of selection with G418 until they reached 80% confluence, when they were passaged and allowed to continue to grow in selection medium. To collect the soluble secreted ACE2 protein, the cells were incubated with 5 mL of OptiMEM for 24 h before harvesting. These samples were concentrated using Centricon (Millipore, Billerica, MA, USA) 10 kDa cut-off filter units. Full-length tACE protein was obtained following medium removal by washing the cells three times with NaCl/P<sub>e</sub> and then scraping off with 1.5 mL of NaCl/P<sub>e</sub>. For the chloride activation assays, the samples were exchanged into 50 mM HEPEs/KOH, pH 7.4, using Centricon 10 kDa cut-off filter units.

**One-step RT-PCR**

Total RNA was isolated from cells using an RNeasy Mini Kit (Valencia, CA, USA), according to the manufacturer’s guidelines. RT-PCR was carried out using a Titanium<sup>™</sup> one-step RT-PCR kit (BD Biosciences, San Jose, CA, USA), according to the manufacturer’s guidelines. The following PCR profile was used: one cycle (50 °C for 1 h); one cycle (94 °C for 5 min); 30 cycles (94 °C for 30 s, 65 °C for 30 s, 68 °C for 1 min); one cycle (68 °C for 2 min). Amplicons were sequenced to confirm the integrity of the product, and this process was carried out for each of the mutant variants.

**Protein determination**

Protein concentrations were determined using the bicinchoninic acid assay with bovine serum albumin as standard [32].

**SDS-PAGE**

Protein samples were prepared in 2 × gel loading buffer (Sigma, Poole, UK) and heated to 100 °C for 5 min. The samples were separated by SDS-PAGE using the method described by Laemmli [33] with 10% polyacrylamide running gels and 6% polyacrylamide stacking gels. Broad-range pre-stained protein standards were run alongside the samples.

**Immunoelectrophoretic analysis**

The proteins were electrophoretically transferred to a poly(vinylidene difluoride) membrane from the polyacrylamide gels. The membrane was saturated with NaCl/Tris (10 mM Tris/HCl, pH 7.4, 150 mM NaCl) containing 5% (w/v) nonfat milk for 1 h. For tACE detection, the mem-

---

C. A. Rushworth et al.
brane was incubated overnight at 4 °C with mouse monoclonal anti-human ACE ectodomain IgG (1 : 100) obtained from R and D Systems Europe Ltd (Abingdon, UK) in 3% (w/v) bovine serum albumin in NaCl/Tris containing 0.1% (v/v) Tween 20 (TBST). After rinsing with TBST, the membrane was washed three times in TBST for 10 min at room temperature. The membrane was then incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-goat IgG (1 : 2000) obtained from Sigma.

The TBST washes were repeated before visualization of the immunoreactive proteins by chemiluminescence using an ECL kit. An identical method for ACE2 detection was employed, except that the primary antibody was goat polyclonal anti-human ACE2 ectodomain IgG (1 : 100) obtained from R and D Systems, and the secondary antibody was horseradish peroxidase-conjugated anti-goat IgG (1 : 5000) obtained from Sigma.

ACE and ACE2 activity assays

Activity assays were carried out in 50 mM HEPES buffer, pH 7.4, containing the stated concentrations of NaCl (final volume, 100 μL). The specific activity was determined by pre-incubation of 1 μg of protein with either 1 μM captopril (an ACE-specific inhibitor) [34] or 100 nM MLN-4760 (an ACE2-specific inhibitor) [35] for 20 min before the addition of 100 μM angiotensin I or angiotensin II. Reactions were carried out at 37 °C for 2 h and terminated by heating at 100 °C for 5 min. These conditions ensured that product formation was linear with respect to time and amount of protein. An aliquot of 80 μL of the assay solution was applied to a C18 reverse-phase HPLC column (5 μm particle size, 250 × 4.5 mm internal diameter; Phenomenex, Cheshire, UK) with a UV detector set at 214 nm. All separations were carried out at room temperature at a flow rate of 1.5 mL·min⁻¹. Mobile phase A consisted of 0.02% (v/v) trifluoroacetic acid in water, and mobile phase B consisted of 0.016% (v/v) trifluoroacetic acid in acetonitrile. A linear gradient of 11% B to 100% B over 15 min, with 5 min at final conditions and 8 min re-equilibration, was used. The elution positions of the products were determined using pure synthetic standards. \(K_m\) and \(V_{max}\) values were determined as described above, except that six concentrations of angiotensin I and II ranging between 50 and 400 μM were incubated with the various forms of ACE2 and tACE. The enzyme concentration was adjusted to ensure that < 15% of the substrate was consumed at the lowest substrate concentration, guaranteeing that product formation was linear with respect to time over the duration of the assay. \(K_m\) and \(V_{max}\) values were calculated by linear regression using the equation: \(v = V_{max} \times [S]/(K_m + [S])\). The IC₅₀ values for MLN-4760 inhibition of the ACE2 variants were determined as described previously [36]. The IC₅₀ values for captopril inhibition of the tACE variants were determined in a similar manner, except that Abz-FRK(Dnp) served as the substrate.

Acknowledgements

We thank the Biotechnology and Biological Sciences Research Council for financial support and Professor Nigel Hooper (University of Leeds, UK) for helpful advice and comments. JLG was in receipt of a British Heart Foundation Junior Research Fellowship.

References

1 Inagami T (1994) The renin–angiotensin system. Essays Biochem 28, 147–164.
2 Corvol P, Williams TA & Soubrier F (1995) Peptidyl dipeptidase A: angiotensin I-converting enzyme. Methods Enzymol 248, 283–305.
3 Tipnis SR, Hooper NM, Hyde R, Karran E, Christie G & Turner AJ (2000) A human homolog of angiotensin-converting enzyme. Cloning and functional expression as a captopril-insensitive carboxypeptidase. J Biol Chem 275, 33238–33243.
4 Zisman LS, Keller RS, Weaver B, Lin Q, Speth R, Bristow MR & Canver CC (2003) Increased angiotensin-(1–7)-forming activity in failing human heart ventricles. Evidence for up-regulation of the angiotensin-converting enzyme homologue ACE2. Circulation 108, 1707–1712.
5 Ishiyama Y, Gallagher PE, Averill DB, Tallant EA, Brosnihan KB & Ferrario CM (2004) Upregulation of angiotensin-converting enzyme 2 after myocardial infarction by blockade of angiotensin II receptors. Hypertension 43, 970–976.
6 Lely AT, Hamming I, van Goor H & Nvis GJ (2004) Renal ACE2 expression in human kidney disease. J Pathol 204, 587–593.
7 Tikellis C, Johnston CI, Forbes JM, Burns WC, Burrell LM, Risvanis J & Cooper ME (2003) Characterization of renal angiotensin-converting enzyme 2 in diabetic nephropathy. Hypertension 41, 392–397.
8 Wong DW, Oudit GY, Reich H, Kassiri Z, Zhou J, Liu QC, Backx PH, Penninger JM, Herzenberg AM & Scholowe JW (2007) Loss of angiotensin-converting enzyme-2 (Ace2) accelerates diabetic kidney injury. Am J Pathol 171, 438–451.
9 Candido R, Jandeleit-Dahm KA, Cao Z, Nестеров SP, Burns WC, Twigg SM & Dille RJ (2002) Prevention of accelerated atherosclerosis by angiotensin-converting enzyme inhibition in diabetic apolipoprotein E-deficient mice. Circulation 106, 246–253.
10 Yi CE, Ba L, Zhang L, Ho DD & Chen Z (2005) Immunolocalization of ACE2 and AT2 receptors in rabbit atherosclerotic plaques. J Histochim Cytochem 2, 73–82.
11 Imai Y, Kuba K, Rao S, Huan Y, Guo F, Guan B, Yang P, Sarao R, Wada T, Leong-Poi H et al. (2005) Angiotensin-converting enzyme 2 protects from severe acute lung failure. Nature 436, 112–116.
Chloride regulation of ACE and ACE2

C. A. Rushworth et al.

12 Li W, Moore MJ, Vasilieva N, Sui J, Wong SK, Berne MA, Somasundaran M, Sullivan JL, Luzuriaga K, Greenough TC et al. (2003) Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. Nature 426, 450–454.

13 Hubert C, Houot A-M, Corvol P & Soubrier F (1991) Structure of the angiotensin I-converting enzyme gene: two alternative promoters correspond to evolutionary steps of a duplicated gene. J Biol Chem 266, 15377–15383.

14 Donoghue M, Hsieh F, Baronas E, Godbout K, Gosselin M, Stagliano N, Donovan M, Woolf B, Robison K, Jeyaseelan R et al. (2000) A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1–9. Circ Res 87, E1–E9.

15 Skeggs LT, Kahn JR & Shumway NP (1956) The preparation and function of the hypertensin-converting enzyme. J Exp Med 103, 295–299.

16 Vickers C, Hales P, Kaushik V, Dick L, Gavin J, Tang J, Godbout K, Parsons T, Baronas E, Hsieh F et al. (2002) Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. J Biol Chem 277, 14838–14843.

17 Bunning P & Riordan JF (1983) Activation of angiotensin converting enzyme by monovalent anions. Biochemistry 22, 110–116.

18 Shapiro R, Holmquist B & Riordan JF (1983) Anion activation of angiotensin converting enzyme: dependence on nature of substrate. Biochemistry 22, 3850–3857.

19 Dorer F, Ryan JW & Stewart JM (1974) Hydrolysis of bradykinin and its higher homologues by angiotensin-converting enzyme. Biochem J 141, 915–917.

20 Guy JL, Jackson RM, Acharya KR, Sturrock ED, Hooper NM & Turner AJ (2003) Angiotensin-converting enzyme-2 (ACE2): comparative modeling of the active site, specificity requirements, and chloride dependence. Biochemistry 42, 13185–13192.

21 Ehlers MR & Riordan JF (1990) Angiotensin-converting enzyme: biochemistry and molecular biology. In Hypertension: Pathophysiology, Diagnosis and Management (Laragh JH & Brenner BM, eds), pp. 1217–1231. Raven Press, New York, NY.

22 Natesh R, Schwager SL, Sturrock ED & Acharya KR (2003) Crystal structure of the human angiotensin-converting enzyme–lisinopril complex. Nature 421, 551–554.

23 Corradi HR, Schwager SL, Nchinda AT, Sturrock ED & Acharya KR (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.

24 Towler P, Staker B, Prasad SG, Menon S, Tang J, Parsons T, Ryan D, Fisher M, Williams D, Dales NA et al. (2004) ACE2 X-ray structure reveals a large hinge-bending motion important for inhibitor binding and catalysis. J Biol Chem 279, 17996–18007.

25 Liu X, Fernandez M, Wouters MA, Heyberger S & Husain A (2001) Arg1098 is critical for the chloride dependence of human angiotensin I-converting enzyme C-domain catalytic activity. J Biol Chem 276, 33518–33525.

26 Guy JL, Jackson RM, Jensen HA, Hooper NM & Turner AJ (2005) Identification of critical active-site residues in angiotensin-converting enzyme-2 (ACE2) by site-directed mutagenesis. FEBS J 272, 3512–3520.

27 Reinalter SC, Jeck N, Brichausen C, Watzer B, Nüising RM, Seyberth HW & Könhoff M (2002) Role of cyclooxygenase-2 in hyperprostaglandin E syndrome/antenatal Bartter syndrome. Kidney Int 62, 253–260.

28 Rice GI, Thomas DA, Grant PJ, Turner AJ & Hooper NM (2004) Evaluation of angiotensin converting enzyme (ACE), its homologue ACE2 and neprilysin in angiotensin peptide metabolism. Biochem J 383, 45–51.

29 Natesh R, Schwager DR, Evans HR, Sturrock ED & Acharya KR (2004) Structural details on the binding of antihypertensive drugs captopril and enalaprilat to human testicular angiotensin I-converting enzyme. Biochemistry 43, 8718–8724.

30 Tzakos AJ, Galanis AS, Spyroulias GA, Cordopatis P, Manessi-Zoupa E & Geroulanos IS (2003) Structure and function discrimination of the N- and C-catalytic domains of human angiotensin-converting enzyme: implications for Cl– activation and peptide hydrolysis mechanisms. Protein Eng 16, 993–1003.

31 Hamming I, van Goor H, Turner AJ, Rushworth CA, Michaud AA, Corvol P & Navis G (2008) Differential regulation of renal angiotensin-converting enzyme (ACE) and ACE2 during ACE inhibition and dietary sodium restriction in healthy rats. Exp Physiol 93, 631–638.

32 Smith PK, Krohn RJ, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ & Klenk DC (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150, 76–85.

33 Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

34 Cushman DW, Cheung HS, Sabo EF, Robin B & Ondetti MA (1979) Development of specific inhibitors of angiotensin I converting enzyme (kininase II). Fed Proc 38, 2778–2782.

35 Dales NA, Gould AE, Brown JA, Calderwood EF, Guan B, Minor CA, Gavin JM, Hales P, Kaushik VK, Stewart M et al. (2002) Substrate-based design of the first class of angiotensin-converting enzyme-related carboxypeptidase (ACE2) inhibitors. J Am Chem Soc 124, 11852–11853.

36 Rela M, Rushworth CA, Guy JL, Turner AJ, Langer T & Jackson RM (2006) Structure-based pharmacophore design and virtual screening for novel angiotensin converting enzyme 2 inhibitors. J Chem Inf Model 46, 708–716.