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ACE2, the Receptor that Enables Infection by SARS-CoV-2: Biochemistry, Structure, Allostery and Evaluation of the Potential Development of ACE2 Modulators

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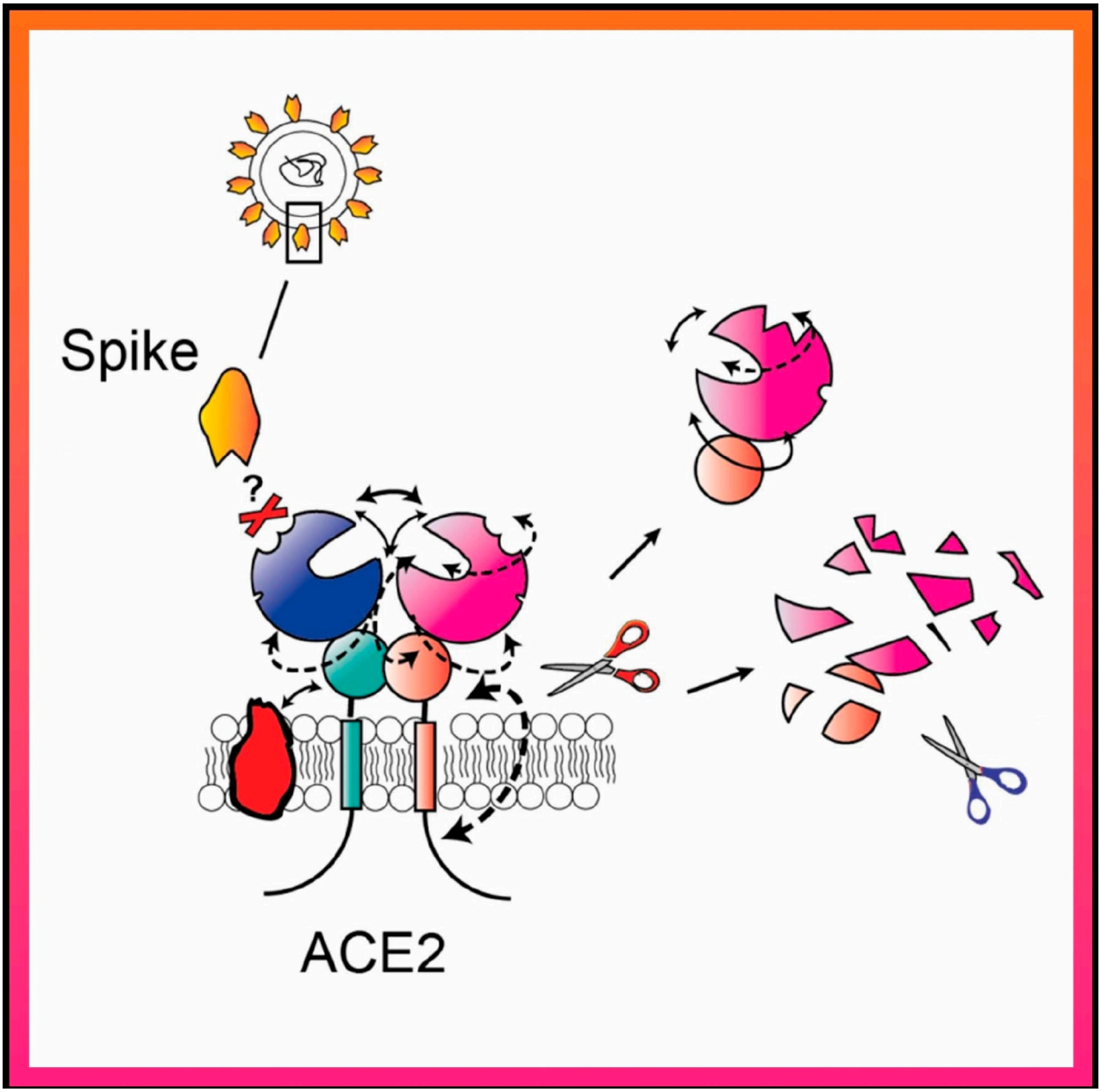
Dedicated to the victims of the COVID-19 pandemic.



Angiotensin converting enzyme 2 (ACE2) is the human receptor that interacts with the spike protein of coronaviruses, including the one that produced the 2020 coronavirus pandemic (COVID-19). Thus, ACE2 is a potential target for drugs that disrupt the interaction of human cells with SARS-CoV-2 to abolish infection. There is also interest in drugs that inhibit or activate ACE2, that is, for cardiovascular disorders or colitis. Compounds binding at alternative sites could allosterically affect the interaction with the spike protein. Herein, we review biochemical, chemical biology, and structural information on ACE2, including the recent cryoEM structures of full-length ACE2. We conclude that ACE2 is very dynamic and that allosteric drugs could be developed to target ACE2. At the time of the 2020 pandemic, we suggest that available ACE2 inhibitors or activators in advanced development should be tested for their ability to allosterically displace the interaction between ACE2 and the spike protein.

1. General Introduction

Angiotensin converting enzyme 2 (ACE2), first described with a role in the renin-angiotensin system,[1] is now considered a regulator of cardiovascular physiology, dietary amino acid homeostasis, innate immunity, and gut microbial ecology. It is also the receptor of the severe acute respiratory syndrome coronaviruses (SARS-CoVs), including SARS-CoV-2, responsible for the 2020 pandemic. A number of excellent reviews focusing on different aspects of ACE2 function and relevance in disease have been published.[2–6] An earlier review had a focus on biochemistry, including details of ACE2 glycosylation, substrate specificity, the requirement for pH and Cl^- for activity.[7] Here we review the structural and biochemical information on ACE2, analyse its dynamics and allosteric properties, and assess the possibility of identifying small compounds able to disrupt the interaction with SARS-CoV-2 allosterically.

1.1. ACE2

ACE2 is a transmembrane protein with an extracellular carboxypeptidase domain, located at the cell membrane in a variety of epithelial cells, including lung and airways, olfactory system, heart, kidneys, liver, pancreas and intestine.[2,8–9] ACE1 and ACE2 are coded by different genes and share a 40% amino acid sequence identity in the catalytic domain. Whereas ACE1 catalyses the formation of angiotensin II (angiotensin 1–8; DRVYIHPF) leading to vasoconstriction, increased blood pressure, cardiac hypertrophy and inflammation, among other effects, ACE2 processes angiotensin II to angiotensin 1–7 (DRVYIHP) and angiotensin I to angiotensin 1–9 (DRVYIHPFH), both enhancing vasodilatation and reducing blood pressure (Figure 1). Thus, ACE2 is protective in multiple cardiovascular diseases, such as hypertension and heart failure.[10–11] The difference at the active sites between ACE1 and ACE2 is such that ACE1 inhibitors (i.e., enalapril, lisinopril, captopril) do not crossreact with ACE2. ACE2 inhibitors have been developed, including MLN-4760 (also termed GL1001), DX-600, and 416F2.[12–14] Inhibition of ACE2 with MLN-4760 showed beneficial effects on a model of colitis in mice[15] and entered clinical trials for the treatment of ulcerative colitis (www.clinicaltrials.gov).

ACE2 is cleaved at the cell membrane by the ADAM17 protease (tumour necrosis α-convertase, TACE),[16] and by other proteases such as TMPRSS2, HAT and hepsin.[17–18] The cleavage by ADAM17, in a process termed shedding, releases catalytically active soluble forms of ACE2 into the circulation, with a still unclear physiological function.[19–20] Recombinant human ACE2 18–740 (rhACE2; i.e., GSK2586881/APN01) is being tested in clinical trials for diverse disorders including lung injury and pulmonary arterial hypertension.[21] GSK2586881/APN01 and B38-CAP, a bacterial-derived carboxypeptidase that cleaves both Ang I and Ang II to Ang 1–7,[22] are also in clinical trials for the treatment of SARS-CoV-2 infections.

ACE2 exerts indirect physiological functions on intestinal amino acid homeostasis, such as expression of antimicrobial

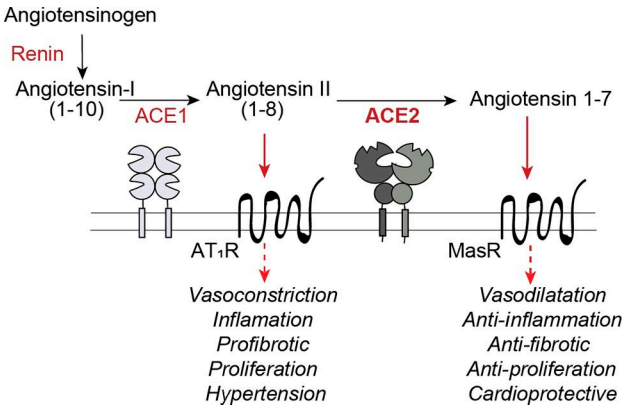


Figure 1. Simplified scheme of the role of ACE2 in the renin–angiotensin system. The cleaving of angiotensinogen by the enzyme renin results in the decapeptide angiotensin I (1–10). ACE1 cleaves angiotensin I to angiotensin II (1–8). Angiotensin II is a potent vasoconstrictor that binds to the type 1 angiotensin II receptor (AT_1R) to set off actions that result in higher blood pressure and inflammation. ACE2 cleaves angiotensin II to produce angiotensin 1–7, which binds to the Mas receptor (MasR) producing vasodilation and other cardioprotective actions. ACE2 is cleaved by ADAM17, which releases the active ACE2 protease catalytic domain to the circulation.

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peptides, and on the gut microbiome, by stabilizing the amino acid transporter B°AT1 with a chaperone-like mechanism.[5,23] In animal models, the expression of ACE2 is upregulated by inhibitors of ACE1 and AT_1R blockers, but the results have not been replicated in humans.[24] ACE2 expression is enhanced by interferon IFNα in human airway epithelial cells.[25]

1.2. ACE2 and the mechanism of SARS-CoV/CoV-2 infection

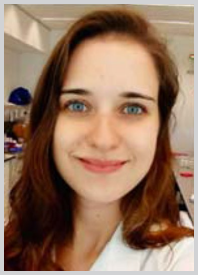
SARS-CoV-2's mechanism of infection is similar to that of SARS-CoV-1 (formerly known as SARS-CoV). The infection is mediated by a viral protein termed the spike, or S-protein, and ACE2 using a type I mechanism of fusion with target cells.[6,26–34] Although CD209 L (L-SIGN) has been described as a co-receptor of SARS-CoV,[35] the sole transfection of ACE2 into SARS-CoV-refractory cell lines confers susceptibility to infection.[36] The cleavage of ACE2 is linked to the entry of SARS-CoV-1 into cells.[17,18,37] TMPRSS2 also cleaves the spike protein and primes it to allow viral entry into the cells.[32] Inhibition of TMPRSS2 by the clinically approved drug camostat mesylate inhibits viral infection.[32,38] Therefore, cells throughout the body that express both proteins could be target of the coronavirus infection.[9]

Altogether, there is plenty of evidence to sustain that ACE2 is the key receptor that enables infection of human cells by coronaviruses infecting humans like SARS-CoV-1,[6,39] HCoV-NL63[40] and SARS-CoV-2.[32] Therefore, pharmacological targeting of human ACE2 could lead to decreased interaction with the spike protein from SARS-CoVs, inhibiting infectivity and becoming a means for the treatment of infected patients. Targeting human ACE2 instead of aiming the viral spike protein directly has the benefit of being a more widely applicable treatment in the event of future strains or mutations of SARS-CoVs.

It is established that the spike protein from SARS-CoVs attaches to ACE2 at a site different from the ACE2 peptidase active site.[8] Thus, it is not expected that ACE2 inhibitors that bind at the active site of ACE2 could directly compete with the spike protein for the same interaction site. On the other hand, if ACE2 were allosteric, a drug binding at the active site, or to an alternative pocket, could affect the interaction of the spike protein at the distant site allosterically and could be a possible approach for the treatment of COVID-19 patients.

1.3. Allostery

Allostery is a central mechanism that mediates cellular regulation. Allostery involves the structural and dynamic communication between at least two sites on a protein, site A and site B, in such a way that the occupancy of site A, will affect the conformation of site B, determining a physiological



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Prof. Dr. Dr. Albrecht Piiper, originally from Göttingen, studied medicine and biology. He has a broad field of interests and expertise, ranging from in vitro to clinical research. Since 2008 he has been at the Clinic of Internal Medicine I. Current interests range from improving the tumour delivery of anti-cancer drugs, via liver diseases to the identification of biomarkers.

Dr. Ricardo M. Biondi received his PhD at the University of Buenos Aires, then spent most of his scientific career in France, Scotland and Germany. Over the last 20 years, he has led detailed studies of the mechanisms of allosteric regulation and allosteric drug development to AGC kinases. Since 2016, he has headed a research group at IBioBA-CONICET, Buenos Aires, while also being a visiting scientist in the Clinic of Internal Medicine I at Frankfurt University Hospital.

response.[41–42] As allostery is bidirectional, the occupancy of site B can also affect the conformation of site A.

Numerous allosteric drugs have been approved, the oldest being benzodiazepines.[43–44] Over the last two decades, rational allosteric drugs have been developed to the G protein-coupled receptor family[45] including maraviroc and cinacalcet. Allosteric drugs such as nevirapine[46] and sofosbuvir[47] have also provided treatment options for human immunodeficiency virus and human hepatitis C virus infections. When compared to drugs binding at orthosteric sites, allosteric inhibitors can add the benefit of higher selectivity. Interestingly, as could be the case for ACE2, drugs directed to the orthosteric/active sites of enzymes can also produce effects on distant sites and allosterically enhance or disrupt interactions.[48]

Studies on allostery and drug development to allosteric proteins show that the allosteric effect is often not visible from the analysis of crystal structures. Molecular dynamic studies can predict if a small compound binding at a first site can induce rigidity or mobility at a second distant site; however, rigidity or mobility at a particular site does not directly relate to increase or decreased binding of interacting proteins.[49]

Specific studies on ACE2 dynamics and allostery have not been performed, although molecular dynamics simulations are presently being carried out.[50–51] In the absence of specifically designed experimental studies, we can deduce dynamics and allostery from biochemical, chemical biology and structural work published over the years.

2. Deduced Dynamics and Allostery from Biochemical and Structural Studies on ACE2

In the following paragraphs we summarize biochemical and structural knowledge on ACE2 that can provide information about the possible existence of allostery and participation in an allosteric network.

2.1. Biochemical studies

ACE2 consists of a catalytic domain (protease catalytic domain, PD) and a collectrin-like domain (CLD) that includes a neck domain, a single transmembrane sequence and a cytoplasmic 43 amino acid tail (Figure 2A, B).

The enzymatic activity of the PD can be inhibited by compounds or polypeptides binding to the substrate-binding site, that is, MLN-4760. MLN-4760 does not affect the interaction of ACE2 with the spike protein of the SARS-CoV-1 in immunoprecipitation experiments nor does it affect the coronavirus infection of cells in culture.[52] MLN-4760's potency is improved by increased chloride concentrations and mutagenesis analysis showed that Arg169 and Arg514 were critical to mediate the chloride-dependent increase in the potency of MLN-4760.[53] Interestingly, Arg169 of ACE2 is approximately at a 16 Å distance from MLN-4760 binding site and so it is unlikely to directly affect inhibitor binding, but rather to allosterically affect the active site. Supporting this hypothesis, chloride was also shown to modulate substrate selectivity of ACE2.[54]

Is the ACE2 active site linked to other distant allosteric sites? Although mutations of ACE2 at the active site render ACE2 inactive, the mutant protein still interacts with the spike protein and mediates infection, thus indicating that the catalytic activity of ACE2 is not required for SARS-CoV-1 interaction and infection.[28] The effect of spike protein S1 domain on ACE2 enzymatic activity was tested using purified recombinant ACE2 and a peptide substrate that fluoresces upon cleavage (Mca-YVADAPK(Dnp)-OH substrate). Li et al. found that the spike protein does not affect the *in vitro* activity of ACE2[52] – although the data are not shown in the manuscript. This result provides evidence that at least some interactions at the spike-binding site may not affect the catalytic activity of ACE2. The finding suggests that if there was an endogenous protein binding to the spike-binding site of ACE2, or a treatment with exogenous receptor binding domain (RBD) from the spike protein itself, it could block the interaction of human cells with SARS-CoVs without affecting ACE2 catalytic activity and its physiological function in the regulation of the renin–angiotensin system.

2.2. Structural and structure-based studies on the protease catalytic domain (PD)

There are over 20 crystal and cryoEM structures of the PD deposited in the Protein Data Bank. Most structures of the isolated PD show the catalytic domain with the active site in an "open" conformation (i.e., PDB IDs: 1R4 L, 6 M17, 6 M18, 6LZG). A closed conformation is observed only in complex with MLN-4760 (PBD 1R42;[55] Figure 2C, D). There are also structures in an "intermediate" conformation, all of them in complex with the RBD of spike from SARS-CoV-1 or a chimera of SARS-CoV-1 and 2 (i.e., PDB IDs: 6ACK, 2AJF, 6VW1). Open and closed conformations vary at the active site and at the "back", along the hinge region (shown as schemes in Figure 2C).

Activators of ACE2 could be beneficial as drugs for the treatment of diabetic nephropathy, heart failure, or hypertension.[56] The structures of the isolated protease catalytic domain in open and closed conformations were used to screen *in silico* for compounds that bind at sites different from the active site. In a first study, Hernandez Prada et al. identified two small compounds (XNT and resorcinolnaphthalein) binding at the hinge region (Figure 2B, C) that in an *in vitro* ACE2 activity assay (Mca-YVADAPK(Dnp)-OH substrate) activated ACE2 1.8 and 2.2 fold, respectively, with EC_{50} values (concentration to achieve 50% enhancement of activity) of approximately 20 μM.[57] Using the same approach, diverse FDA-approved drugs were identified as low-μM binders, and the compound diminazene was described to activate ACE2 ($EC_{50} = 8$ μM), by modifying both K_m and V_{max} (Mca-YVADAPK(Dnp)-OH substrate; Figure 2E, H).[58] Together, these studies suggest that there is an allosteric communication between the hinge region and the active site.

There is also evidence for possible allostery between the active site and the spike protein binding site. Huentelman et al.

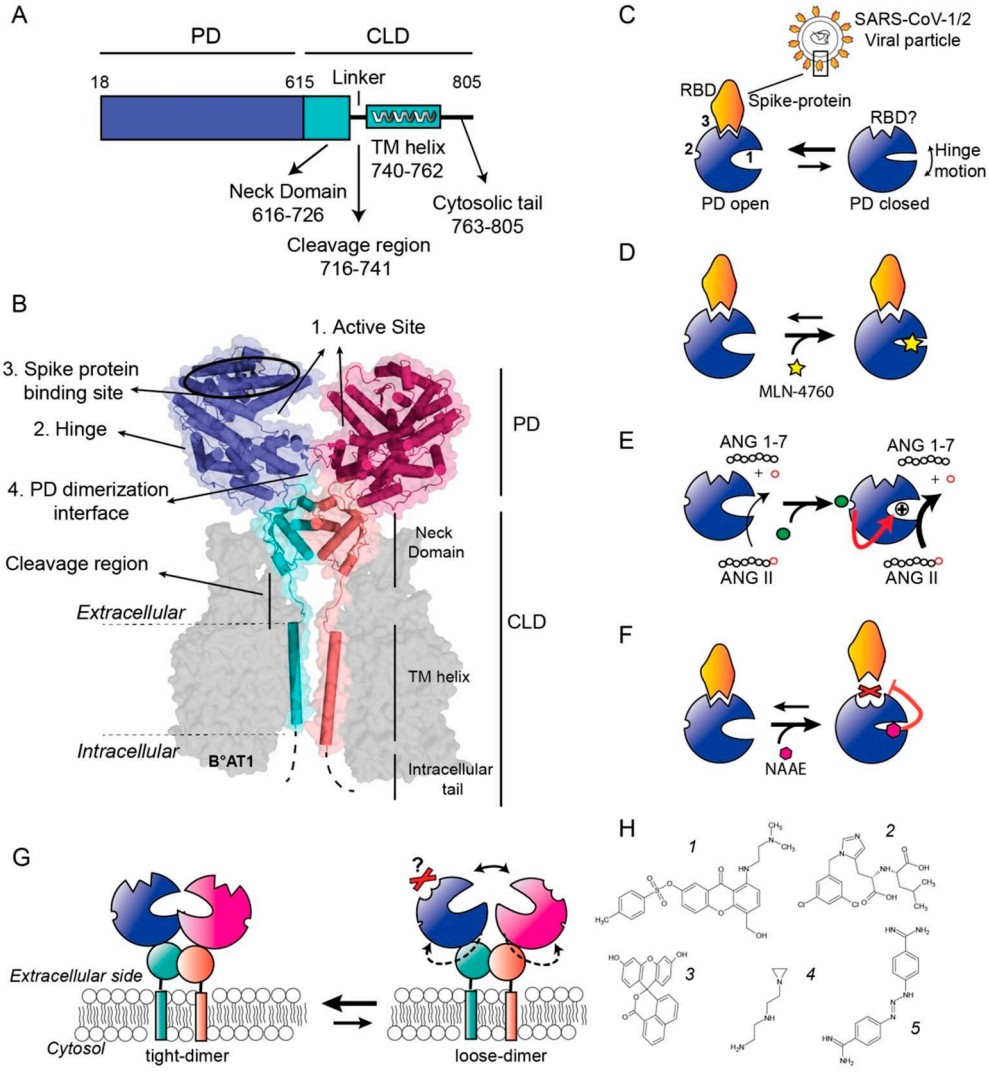


Figure 2. Structure and conformations of ACE2. A) Scheme of motifs and domains of ACE2. ACE2 has an N-terminal protease catalytic domain PD (blue) and a C-terminal collectrin-like-domain CLD (cyan). The first 17 amino acids correspond to the signal peptide that is cleaved during the maturation of the protein (not shown). The CLD consists of an extracellular neck domain, a linker, a single transmembrane (TM) helix and an intracellular tail of 43 amino acids. The sites of cleavage by proteases that release soluble ACE2 to the circulation are indicated. B) Structure of full-length ACE2 (tight dimer) in complex with B°AT1 (PDB ID: 6 M17). ACE2 is represented as cylindrical helices and loops with surface; the surface of B°AT1 is presented in grey. For simplification, the RBDs present in this structure are not shown. The monomers of ACE2 are coloured in blue and pink (following the colours of A). The different regions of ACE2 and the four key regulatory sites (1: active site, 2: hinge, 3: claw-like or spike (RBD)-binding site, 4: PD dimerization interface) are indicated. C) Schematic representation of the open–close hinge movement of the PD. The active site of the PD can adopt an open, intermediate (not shown) or closed conformation. The hinge pocket is disassembled in the closed structure. D) MLN-4760 binds at the active site, stabilizes the closed structure and does not affect the interaction with spike. E) Small compounds designed to bind at the hinge region, i.e., diminazene, increase the activity of ACE2. F) Small compounds designed to bind at the active site in the closed structure of PD (NAAE) displace interaction with spike protein. G) Schematic representation of the structure of full-length ACE2 dimers in two conformations identified by cryoEM in complex with B°AT1. In the absence of the spike protein RBD, the two conformations are found in a 3:1 proportion. The tight dimer (left) is a scheme representing the structure shown in B. In both dimer conformations, the neck domains form tight interactions. In the loose dimer (right), the PDs rotate with respect to the neck domain and the PD–PD interaction is lost. In the presence of spike protein RBD, only the tight dimer ACE2 structure is present. In the loose dimer, the conformation of the spike protein binding site in the PD appears modified (detailed in Figure 3). H) Chemical structures: 1: XNT, 2: MLN-4760, 3: resorcinolnaphthalein, 4: NAAE, 5: diminazene. The mechanisms of action for XNT, resorcinolnaphthalein, NAAE and diminazene (E) and (F) are deduced from biochemical work and not validated structurally.

performed an *in silico* screening based on the closed conformation of ACE2 to identify new compounds binding to the active site and described that the molecule NAAE (*N*-(2-aminoethyl)-1 aziridine-ethanamine) inhibits ACE2 activity (IC_{50} 50 μM).[59] Treatment with this molecule inhibited SARS-CoV-1 membrane fusion. The finding indicates an allosteric communication between the active site of ACE2 and its site of interaction with spike protein from SARS-CoV-1 (shown schematically in Figure 2F), suggesting that drugs may be developed to bind at the active site and disrupt the interaction with the SARS-CoV-2 spike protein.[59]

2.3. Structural studies on full-length ACE2

The spike protein is a trimer. Interestingly, recent work shows that a monomeric form of the PD (ACE2 18–640) binds efficiently to the isolated RBD from spike, but does not significantly bind full-length spike trimers, while the PD (18-640)-Fc dimer can bind full-length spike trimers with reduced on-rate but also reduced off-rate.[60] CryoEM studies with SARS-CoV virions show that it binds to three soluble ACE2-Fc molecules.[61] After interaction with ACE2, the spike trimer undergoes conformational changes that promote membrane fusion. It is not yet known whether ACE2 must also undergo conformational changes to enable infection. The structure of full-length ACE2 was recently elucidated by cryoEM in a complex with the amino acid transporter B°AT1[8] (Figure 2B). It shows that ACE2 is a dimer, as previously described biochemically.[61,62] Dimers provide additional possibilities for cooperative allosteric effects, although these have not been described in ACE2. In the full-length solved structure, B°AT1 supports the formation of dimers by stabilizing the neck domain and the transmembrane helix of ACE2. This is in agreement with previous work showing that ACE2 constructs comprising the extracellular regions, neck domain and PD, are dimers. Dimers of ACE2 comprising PD fused to the Fc domain of antibodies, have also been employed in research.[61–62]

The cryoEM solved structure of full-length ACE2 revealed the existence of two types of dimers: the authors describe them as "closed dimer" and "open dimer".[8] To avoid confusion with the "open" and "closed" conformations of the PD, we will refer to them as tight dimer and loose dimer, respectively (shown schematically in Figure 2G). In the tight-dimer conformation, the dimerization interface consists of a strong interaction between the CLD neck domain and a second interaction between the two PDs. Interestingly, when the authors investigated the structure of full-length ACE2 in the presence of the RBD by cryoEM, they identified only the ACE2 tight-dimer in complex with RBD (in the presence of 10 mM leucine). In the loose-dimer conformation, there is a rotation between the CLD and the PD (Figures 2G and 3A), which breaks the dimer interaction between the PDs and separates both domains about 25 Å. As a result, the PDs do not interact, while the dimer still remains stable, mediated by the interface within the neck domain. More notably, the cryoEM structure of the full-length ACE2 reveals that the PD can be stabilized in a new structural "twisted" conformation (Figures 2G and 3B). In this new conformation, the PD claw-like surface shows changes which include the shifting of residues involved in the interaction with the RBD,[8,63] most notably at the α1 helix of ACE2, the main point of interaction with the viral protein (Figures 2G and 3C).

3. Analysis on ACE2 Dynamics and Allostery

The structural information of ACE2 obtained by crystallography and cryoEM provide snapshots depicting conformations that ACE2 can stably achieve. Taken together with the biochemical studies, we propose a cartoon model of ACE2 with various key sites that appear dynamic (Figure 2C–F). We identify four key sites on the PD, the carboxypeptidase active site (site 1, Figure 2B, C), the hinge pocket at the back of the active site (site 2, Figure 2B, C) and the claw-like surface that interacts with the RBD within the S1 domain of the viral spike protein (site 3, Figure 2B, C). In addition, based on the cryoEM full-length structure, we must also consider the existence of a dimerization interface (site 4, Figure 2B, F). How are these sites related to each other? The proteolytic domain can be observed in "open", "closed" and "intermediate" conformations (Figure 2C). These conformations refer to the "opening" of the active site, where the hinge region participates in the movement. The only structure in "closed" conformation corresponds to the crystal bound to the ACE2 inhibitor MLN-4760 (Figure 2D). The finding that compounds binding at the hinge region, site 2, can modulate the protease activity, site 1, provides evidence that the two sites are allosterically connected (Figure 2E). While MLN-4760 binding to the active site does not affect the binding of the spike protein to ACE2, NAAE designed to bind at the active site in the closed conformation disrupted interaction with the spike protein (Figure 2F). Therefore, we must also consider that a subset of compounds binding at ACE2 active site (site 1) could affect site 3 and enhance or inhibit interaction with the spike protein. The finding of the twisted conformation of the PD in the full-length loose dimer shows a novel stable conformation of the PD (Figure 2G). In addition, the twisted conformation has a modified claw-like surface, which, if stabilized, could impair efficient binding to RBD from the spike protein at the claw-like site (site 3) and inhibit interaction with SARS-CoVs.

It called our attention that the association of the cytoplasmic tail of ACE2 with a ubiquitous calcium-binding protein, calmodulin, reduces the cleavage and release of its extracellular peptidase domain.[64–65] One simple explanation could be that the interaction at the cytoplasmic domain induces a direct stabilizing effect on the extracellular region of ACE2, avoiding the exposure of the cleavage site or inhibiting the interaction with the protease. Although other mechanisms could also explain the finding, it is tempting to speculate that there is an allosteric communication between the cytoplasmic tail of ACE2 and the extracellular domain that could physiologically signal in both directions. Independently of the mechanism, pharmacologically affecting the calmodulin binding to the intracellular tail would be expected to modulate the stability of the extracellular domain.

Another hint of allostery between distinct sites on ACE2 comes from the apparent contradiction that while ACE2 inhibitors appear to be beneficial for colitis, the knock-out of ACE2 produces colitis.[5] ACE2 ameliorates colitis indirectly by stabilizing the neutral amino acid transporter B°AT1 by a mechanism that does not require ACE2 catalytic activity.[66] One possible explanation for this contradiction is that the ACE2 inhibitors binding at the active site, like MLN-4760, could stabilize ACE2, which will also stabilize B°AT1 allosterically. As B°AT1 interacts with the neck domain and the transmembrane helix of ACE2, a direct effect by MLN-4760 could imply an allosteric communication between the active site of the PD and

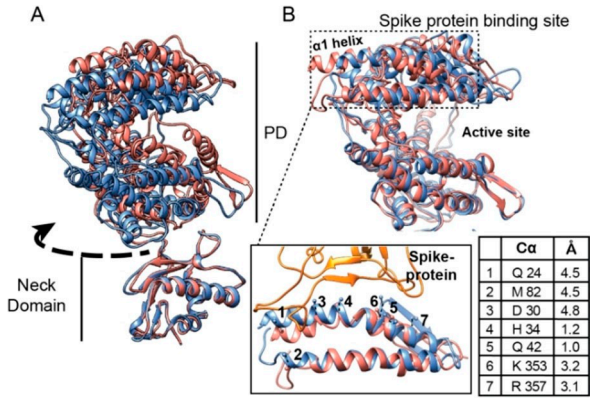
the CLD of ACE2. Following on the known interaction between B°AT1 and ACE2 it was also recently suggested that the B°AT1 inhibitor nimesulide, an approved drug in some countries, could potentially affect the interaction with the spike protein, allosterically.[67]

3.1. The strengths and limitations of the analysis

The main limitation is that the studies on ACE2 were not designed to investigate its dynamics and allostery. However, a strength is that there is consistent information suggesting that ACE2 is dynamic and possibly populated in equilibrium between different conformations depicted schematically in Figure 2. The *in vitro* biochemical characterization of small-molecule "activators" comprises an important experimental evidence of allostery. However, Haber et al. indicated that, in their hands, the claimed small-molecule "activators" did not affect the *in vitro* activity of ACE2 (using Mca-APK-Dnp as a substrate).[68] It is possible that the results of both groups of researchers may be correct, but that the conditions of the assay by Haber et al. did not reveal an increase in activity. There are different reasons for such discrepancy. For example, an allosteric activator can act by affecting the K_m; in such case, the concentration of substrate used could mask the activating effect. Therefore, the characteristics of the ACE2 substrate used should be well characterized before a study on a possible allosteric effect is investigated using catalytic activity as a readout. At any rate, the compounds claimed to activate ACE2 *in vitro* did produce the desired effect *in vivo*. Noteworthy, except for MLN-4760, a confirmation that the experimental compounds indeed bind at the expected sites is missing.

We here broadly assume that the understandings obtained with SARS-CoV-1 would turn out to be indistinguishable from SARS-CoV-2. Although several important aspects have been found to be identical, the specific studies on the interactions of ACE2 with the spike proteins may vary, as the RBD of the SARS-CoV-2 spike protein has additional residues participating in the interaction and higher affinity to ACE2.[63] Studies on ACE2 identified that it is phosphorylated at residue 680, and that this phosphorylation stabilizes the protein avoiding degradation.[69] It is not known if the phosphorylation could affect any of the characteristics of the protein described biochemically, by crystallography or by cryoEM. Also we should keep in mind that many studies on ACE2 have employed ACE2 PD–Fc dimers, which might not mimic the physiological dynamics of ACE2 dimers.

A possibly important finding emerges from the recently described loose dimers of ACE2. The loose-dimer structure is a stable structure in equilibrium with the tight dimers that bind the RBD. The "twist" rotation observed in the PD on the full-length loose dimer leads to the breaking of the α1 helix comprising residues that directly participate in the interaction with the spike protein (Q24, D30, K31, H34,Y41, Q42; Figure 3C). Although the structure of this dimeric conformation was solved to low resolution (4.5 Å), the modifications at the claw-like site are important and, if stabilized, could preclude high affinity



	Cα	Å
1	Q 24	4.5
2	M 82	4.5
3	D 30	4.8
4	H 34	1.2
5	Q 42	1.0
6	K 353	3.2
7	R 357	3.1

Figure 3. The rotated and twisted conformations of the full-length ACE2 loose dimer. The images are obtained by alignment of the tight (blue) and loose (pink) dimers. A) Rotation of the PD in relation to the CLD. The rotation is shown upon alignment of the CLD. B) The structure of the PD in the rotated-twisted loose dimer. The rotated PD is modified at the RBD-binding site. The image is produced by alignment of PD. The top region, which interacts with the RBD of the spike protein, undergoes changes, particularly in the α1 helix. The zoom depicts the RBD binding site of ACE2 upon aligning the last C-terminal portion of the α1 helix. In the loose dimer, some of the helix α1 residues that interact with the spike protein RBD move about 4.5 Å (measuring from the Cα). The table indicates the relative movement between the tight and loose dimers of Cα of relevant residues that interact with the spike protein RBD.

binding to the viral spike protein. However, if the α1 helix was flexible, the conformation could also enhance the interaction to the spike protein. The cryoEM twisted structure with disrupted RBD binding site shows that the PD active site is not significantly different from other open structures. This could indeed indicate that the modifications at the RBD binding site may not affect the active site. However, further dedicated investigations are needed to understand the ACE2 dynamic system in detail to support a complete analysis of the potential of development of allosteric drugs.

4. Summary and Outlook

Allostery is a central widespread mechanism in all life forms, once defined as "the second secret of life". In summary, ACE2 appears as a highly dynamic protein, for which allostery has been demonstrated to different degrees between the active site (site 1), the hinge region (site 2), the claw-like/spike protein binding site (site 3), and the Cl^- biding site. In addition, there are potential allosteric communications between the active site and the stability of the B°AT1, and between the intracellular tail and the cleavage of the extracellular domain releasing PD to the circulation. The twisted catalytic domain of ACE2 observed in the full-length cryoEM structure hints at the existence of structural communication between parts of the full-length protein and the claw-like/spike binding site, which may be exploited by drugs to allosterically inhibit interaction with the spike protein.

From the drug discovery perspective, the dynamic features of ACE2 and the knowledge accumulated throughout the years

support the possibility that ACE2 conformation and function could be modulated by allosteric drugs. On the one hand, we suggest that further studies should confirm the allosteric nature of ACE2, the role in physiology and the potential for exploiting the allosteric properties for drug discovery. On the other hand, the small compounds identified in chemical biology or drug-discovery projects will help to define more clearly the allosteric features of ACE2 and the possible exploitation for therapies. The screening of a small library of compounds using an AlphaLISA-based interaction assay between ACE2 and the RBD domain of the spike protein identified small compounds that displace the interaction.[70] Such kinds of assays can identify small compounds that bind to ACE2 with different mechanisms of action to displace the interaction with spike. Notoriously, one validated "hit" compound that displaced the interaction *in vitro* was the enalapril (IC_{50} 7.5 μM), a prodrug approved for the treatment of hypertension that is converted by de-esterification to enaprilat,[70] which is a potent ACE1 inhibitor. Together with the discussions above, the finding further highlights the possibility that enalapril, at the high concentrations used, might crossreact with ACE2, binding at the active site and allosterically displacing the interaction with the coronavirus spike protein as depicted in Figure 2F. The identified approved drugs are active *in vitro* at concentrations too high for use as antivirals, but could help to pave the way for future anti-SARS-CoV-2 drugs.

Given that ACE2 appears as a highly dynamic protein with a complex allosteric network between key sites, the development of compounds interacting at one site should ideally be tested for their effects on the different distant sites. For example, an activator compound that enhances ACE2 catalytic activity could have an effect on its ability to affect the interaction with the spike protein, the stability of B°AT1, the ability to be cleaved by ADAM17 or the intracellular interaction with calmodulin.

Still, a major question is whether high affinity small compounds and drugs will be able to bind distinct sites on ACE2 to modulate its activity and its interaction with SARS-CoVs. Additional remaining questions related to ACE2 dynamics abound. Does ACE2 dynamic equilibrium between conformations have a physiological function? Is there an endogenous ligand that binds to the spike protein binding site on ACE2? Does the interaction of the spike protein with ACE2 allosterically affect any other conformation-dependent function of ACE2, that is, B°AT1 stability, shedding, or intracellular signalling? Does SARS-CoVs infection require ACE2 conformational changes and dynamics? Do ACE2 polymorphisms[71] affect ACE2 dynamics and infection by SARS-CoVs?

Finally, in relation to the current COVID-19 pandemic we encourage the evaluation of all available drugs and advanced compounds targeting ACE2 for their abilities to allosterically inhibit the interaction with the spike protein and to inhibit the infectivity by SARS-CoV-2.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: ACE2 · allostery · coronavirus · drug development · protein dynamics

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