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Design of an engineered ACE2 as a novel therapeutics against COVID-19

Zahra Payandeh, Mohammad Reza Rahbar, Abolfazl Jahangiri, Zahra Sadat Hashemi, Alireza Zakeri, Moslem Jafarisanif, Mohammad Javad Rasaeeg, Saeed Khalili

Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
Pharmaceutical Sciences Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
Applied Microbiology Research Center, Systems Biology and Poisonings Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran
ATMP Department, Breast Cancer Research Center, Motamed Cancer Institute, ACECR, Tehran, Iran
Department of Biology Sciences, Shahid Rajaee Teacher Training University, Tehran, Iran
Clinical Biochemistry, School of Medicine, Shahroud University of Medical Sciences, Shahroud, Iran
Department of Medical Biotechnology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

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Abstract
The interaction between the angiotensin-converting enzyme 2 (ACE2) and the receptor binding domain (RBD) of the spike protein from severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) plays a pivotal role in virus entry into the host cells. Since recombinant ACE2 protein has been suggested as an anti-SARS-CoV-2 therapeutic agent, this study was conducted to design an ACE2 protein with more desirable properties. In this regard, the amino acids with central roles in enzymatic activity of the ACE2 were substituted. Moreover, saturation mutagenesis at the interaction interface between the ACE2 and RBD was performed to increase their interaction affinity. The best mutations to increase the structural and thermal stability of the ACE2 were also selected based on B factors and mutation effects. The obtained resulted revealed that the Arg273Gln and Thr445Gly mutation have drastically reduced the binding affinity of the angiotensin-II into the active site of ACE2. The Thr27Arg mutation was determined to be the most potent mutation to increase the binding affinity. The Asp427Arg mutation was done to decrease the flexibility of the region with high B factor. The Pro451Met mutation along with the Gly448Trp mutation was predicted to increase the thermodynamic stability and thermostability of the ACE2. The designed therapeutic ACE2 would have no enzymatic activity while it could bear stronger interaction with Spike glycoprotein of the SARS-CoV-2. Moreover, decreased in vivo enzymatic degradation would be anticipated due to increased thermostability. This engineered ACE2 could be exploited as a novel therapeutic agent against COVID-19 after necessary evaluations.

1. Introduction

The SARS-CoV-2 is a non-segmented positive sense RNA virus which belongs to the genus Beta Coronavirus within the Coronaviridae family. Its 30 kilo base pairs genome encodes for multiple structural and non-structural proteins. Like SARS and the Middle East Respiratory Syndrome Coronavirus (MERS-CoV), it mainly causes fever, chronic fatigue, dry cough, shortness of breath, and severe respiratory distress (Cabeça et al., 2013; Corman et al., 2018; Enjuanes et al., 2006; Perlman and Netland, 2009; van Boheemen et al., 2012). The envelope-anchored trimeric spike (S) protein at the surface of the virus is considered to be the key protein for virus entry into the host cells. The ACE2 (a homologue of ACE) is reported to be the potent human receptor for SARS-CoV-2 (Othman et al., 2020). This receptor primarily plays physiological roles in the control of vasoconstriction and blood pressure. As a type I membrane protein, ACE2 is found to be expressed in lungs, heart, kidneys and intestine (Donoghue et al., 2000; Zhang et al., 2020; Zhao et al., 2020). It also has been shown that the cardiovascular diseases are associated with reduced ACE2 expression (Crackower et al., 2002). Given these properties, ACE2 has been served as a drug target for cardiovascular and kidney diseases. Although the RBD of the SARS-CoV-2 spike protein contains 6 amino acid substitutions compared to the RBD of the SARS spike protein, existence of highly conserved residues at this region corroborates the ACE2 as the receptor for SARS-CoV-2 entry (Boehm and Nabel, 2002; Donoghue et al., 2000; Ge et al., 2013). To determine whether the SARS-CoV-2 uses ACE2 as the cell entry receptor, Peng Zhou et al. conducted a virus infection study using HeLa cells
expressing ACE2 protein and cells without ACE2 expression. They have demonstrated that the SARS-CoV-2 can only infect ACE2-containing cells. They also have demonstrated that SARS-CoV-2 does not use other corona virus receptors including aminopeptidase N, dipetidyl and peptidase 4 (Wu et al., 2020).

Given the crucial role of ACE2 and RBD interaction in the pathogenesis of SARS-CoV-2, inhibitory moieties to halt this interaction would prevent the virus from infecting the host cells. Targeting the interacting interfaces of ACE2 and RBD via antibodies or other therapeutic small molecules has already been practiced in numerous studies (Chen et al., 2017; Coleman et al., 2014; Der Sarkissian et al., 2008; Pang et al., 2020). Developing these kind of conventional therapeutics is inherently time consuming and demands recourses. An alternative approach to interfere with the interaction between ACE2 and RBD is the application of molecules which could compete with the cell membrane ACE2 to bind RBD. A recombiant ACE2 molecule which is designed to interact with RBD with higher affinity, without enzymatic activity and higher thermostability could be a suitable alternative.

Studies shown that specificity, binding affinity, thermostability and immunogenicity of therapeutic proteins can be altered by amino acid substitutions. Since several amino acids at the binding interface of the interacting molecules are responsible for binding affinity and immunogenicity, substitution of such key amino acids can significantly alter the affinity and immunogenicity (Clark et al., 2006; Xu et al., 2009). Such effective amino acids could be determined in vitro or using bioinformatics tools and replaced with more efficient amino acids. Bioinformatics approaches provide the opportunity to assess the effect of various sequence alterations on protein affinity, thermostability and enzymatic activity by reduced research costs and time (Payandeh et al., 2018; Payandeh et al., 2019; Sefid et al., 2019). Given the importance of SARS-CoV-2, its transmission and rapid spread to all countries around the world, it is important to develop new therapeutic approaches to deal with it. Since the ACE2 receptor plays the most important role in transmitting the virus into the pulmonary cells, the purpose of this study is to design a human ACE2 inhibitor that lacks its enzymatic activity and bears higher thermostability and higher affinity towards RBD.

2. Methods

2.1. Sequence and structure preparation

The protein sequences for RBD of the COVID-19 spike protein and the ACE2 protein were searched in UniProt (https://www.uniprot.org/) and NCBI (https://www.ncbi.nlm.nih.gov) databases. The 3D structures of these proteins along with their interacting complexes were obtained from PDB database. The latest structures with highest resolution were used for further analyses.

2.2. Deactivation of enzymatic activity

Since there were no resolved structures for ACE2 and angiotensin 2 (ANG2) complex, the structures of these molecules were docked together using CABSdock (http://biocomp.chem.uw.edu.pl/CABSdock) and ClusPro (https://cluspro.bu.edu/login.php) servers. The complex of ACE2 and ANG2 was used to find the most suitable point mutation candidates.

2.2.2. Network based protein analysis for functional and structural key residues

The residue interaction network of ACE2 and ANG2 was extracted from the tertiary structure of the complex of ACE2 and ANG2 by Residue Interaction Network Generator (Piovesan et al., 2016) as provided by http://protein.bio.unipd.it/. Strong and reliable interactions were generated by selecting the strict distance threshold. The network policy for identification of interacting pairs was based on calculating the distance between mass centers of two interacting residues. All types of interactions were considered in this analysis. The generated network further analyzed and visualized by Cytoscape 3.7.2 (Shannon et al., 2003). To investigate the key residues in correct folding and function of the protein, a centrality analysis was carried out by RINspector (Brysbaert et al., 2018) application of Cytoscape. The centrality analysis was based on the change of average shortest path length under removal of each residue (node in the network) as proposed by (del Sol et al., 2006) and embedded in RINspector. The shortest path length is the minimum number of edges that are required for connecting the first node to the second one in the network. The mean of these numbers, related to all possible pairs of residues in the network of interaction, is defined as average shortest path. The significance of average shortest path length is determined by the calculation of Z-score. The Z-scores greater than 2 are considered as relevant (Brysbaert et al., 2018).

2.2.3. Saturation mutagenesis to check the impact on binding affinity

The complex of ACE2 and ANG2 was fed as input for the mCSM-PII2 server (http://biosig.unimelb.edu.au/mcsm_pii2/) to analyze the effects of single-point mutations on protein–protein interactions. All possible mutations were performed for each mutation site to find the most deactivating mutation.

2.2.4. Exerting the mutations and assessing the effects on enzyme substrate interactions

The results of performed analyses along with the results of previous reports were considered to select the key amino acids which their mutation would terminate the enzymatic activity of the ACE2. The candidate mutations were exerted using the protein preparation tool from Molegro VIRTUAL DOCKER (v.6.0). The mutated amino acids and the neighboring amino acids were energy minimized to arrive at proper conformations. The Molegro VIRTUAL DOCKER was employed to determine the cavity sizes within the ACE2 structure before and after exertion of the mutations. The structures of the mutated ACE2 and ANG2 were docked again using CABSdock and ClusPro servers to analyze the consequences of the mutations.

2.3. Increasing ACE2 affinity toward RBD

2.3.1. ACE2 mutation analyses at its interface with RBD

The structure of ACE2 and RBD complex was extracted from the previously resolved protein complex under the PDB ID of 6M17. This complex was fed as the input for the mCSM-PII2 server (http://biosig.unimelb.edu.au/mcsm_pii2) to analyze the effects of single-point mutations on protein–protein interactions. The Saturation Mutagenesis tool of this server was employed to mutate all residues at the interaction interface to all 19 standard amino acids. The effect of each mutation is reported as predicted affinity change (ΔΔG affinity) (positive values correspond to affinity increase and vice versa). The LigPlus software was employed to draw the 2D plot of the interactions between the amino acids of ACE2 and RBD at their interaction interface.
2.3.2. Mutation of ACE2 to increase its RBD binding affinity

The mutations with highest possibility to increase the binding affinity between the ACE2 and RBD were exerted in the ACE2 structure by Molegro VIRTUAL DOCKER. The mutated amino acids and the neighboring amino acids were energy minimized to arrive at proper conformations. The effects of the mutation were also analyzed by LigPlus software by drawing the 2D plots of ACE2 and RBD interactions after each mutation.

2.4. ACE2 mutation to increase thermal stability

Average B factor for each residue was calculated by Chimera software for both 1R42 and 6M17. The results for 19 possible mutations at the residue with highest B factor were done by iStable2.0 software (http://ncblab.nchu.edu.tw/iStable2/result.php). The PoPMuSiC and HotMuSiC (https://soft.dezyme.com/) were respectively used for the prediction of protein thermodynamic stability changes upon single-site mutations and the prediction of protein thermostability changes upon single-site mutations. A systematic prediction was performed to have the prediction results for 19 mutations per ACE2 residue. The best mutations to enhance the thermal stability were exerted and optimized by Molegro VIRTUAL DOCKER.

2.5. Flexibility simulation

The CABSflex (http://biocomp.chem.uw.edu.pl/CABSflex2) server was employed for or fast simulation of protein structure flexibility. This tool generates protein dynamics at highly reduced (3 orders of magnitude) computational cost, although with some decrease in resolution. The ACE2 structure with 6 mutations was used as input for flexibility simulation.

2.6. Molecular dynamics (MD) analysis

The MD analysis was used to investigate the stability of the ACE2 protein following the exerted mutations. This method allows the protein atoms to move freely and find the best location based on the applied force-fields, while placed at a specific temperature and pressure conditions. If the mutated protein continues to maintain its structure and stability after the completion of the molecular dynamics process, the structure could be considered as stable. The GROMACS software version 4.6.5 and the GROMOS96-54 A7 force field were used to perform the MD simulation. The complex was put into a cube box and filled with water using the TIP3 model. If required, the system was neutralized by using Na+ or Cl– ions. Finally, the MD simulation was performed for 30 ns. The Root Mean Square Deviation (RMSD) and radius of gyration (Rg) was used to evaluate the stability of the mutated ACE2 protein.

2.7. Molecular docking analyses for mutant ACE2

The predicted mutations were exerted in the structure of the ACE2 from 1R42. The interaction orientation for mutant ACE2 and RBD was reanalyzed by molecular docking. The docking analysis was performed using HADDOCK server (https://wenmr.science.uu.nl/haddock2.4/) (Van Zundert et al., 2016). The results of the docking analyses were superimposed on the original ACE2 and RBD complex and the RMSD value was calculated using Chimera software (v.1.10.2).

2.8. Immunological analyses

The sequences of wild type and mutant ACE2 proteins were analyzed by Bepipred linear epitope prediction tool (http://tools.iedb.org/bcell/) of the IEDB database. The glycosylation sites of the wild type and mutant ACE2 proteins were analyzed by NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/) server.

3. Results

3.1. Prepared sequences and structures

The sequences for RBD of the SARS-CoV-2 spike protein and the ACE2 protein were found under the NCBI accession numbers of QHD43416 and NP_001358344, respectively. The 3D structure for interacting complex of the RBD from spike protein and the ACE2 protein was stored under the PDB ID of 6M17. The ACE2 structure was obtained under the PDB ID of the 1R42. The extra protein chains and the non-protein moieties were removed from both 6M17 and 1R42 structures. The RBD of the SARS-CoV-2 spike protein was also extracted from the 6M17 as a separate structure for further analyses.

3.2. Deactivation of enzymatic activity

The molecular docking analyses between the ACE2 and ANG2 proteins indicated that the ANG2 could form a tight interaction with the ACE2 protein within the expected central cleavage of the enzyme. Correct deployment of the ANG2 within the ACE2 active site could be interpreted as the suitability of the employed docking method.

3.2.1. The 2D interaction plot

The 2D interaction plot (Fig. 1) of the ACE2 and ANG2 complex shows that a number of amino acids are involved in hydrophobic interactions and hydrogen bonds. Amongst the involved amino acids, the Arginine 273 and the Threonine 445 amino acids of the ACE2 molecule are involved with hydrogen bonds. Since hydrogen bonds are highly important for the stability of protein-peptide interaction, these amino acids should have significant roles in the stability of the ACE2 and ANG2 complex.

![Fig. 1. 2D plot of the ACE2 and ANG2 interaction (hydrogen bonds are indicated by dashed lines between the atoms involved, hydrophobic contacts are represented by an arc with spokes radiating towards the ligand atoms they contact, purple lines are either covalent bonds between protein and ligand, or “elastic” bonds within the ligand). The ANG2 is located at the center of the plot, while the ACE2 residues are around it. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)](image-url)
3.2.2. Network analyses of the complex

To determine the functional residues in the protein, a centrality analysis was performed on the residue interaction network of the ACE2-ANG2 complex. As expected, the residue interaction network is somehow a complex network (Fig. 2, left panel). To have a more clear view, the active pocket of the enzyme (ACE2), the interface residues between ACE2 (Chain A) and ANG2 were extracted from the whole network (Fig. 2, upper panel and Table 1). Moreover, the significant residues in the term of centrality were separated and illustrated in Fig. 2 lower panel. The Arginine 273 and the Thr39ine 445 of the ACE2 are among the amino acids attributed with high and significant Z-scores. Moreover, the Thr39ine 445 is shown to have a hydrogen bond with the Arginine 2 of the ANG2 molecule, while the Arginine 273 is shown to have a hydrogen bond with Aspartate 1 of the ANG2 molecule. The Arginine 2 of ANG2 is determined to be the central residue of ANG2. The increased level of Z-score indicates the increase in the average shortest path length upon node removal. When a significant Z-score assigned to a node (residue), it can be inferred that the related node may play an important role in the communication of the network. Therefore, the subjected residue may be an important structural or functional one. The illustration of active pocket is represented in protein structure to get a better insight about the native structure of the enzyme (Fig. 3).

3.2.3. Saturation mutagenesis for selected mutation sites

The Arginine 273 and the Thr39ine 445 of the ACE2 molecule were mutated to all 19 possible amino acids. The results of the

| Wild-type | Res-number | Mutant | ΔG<sub>ppi2</sub>-prediction (Kcal/mol) | Affinity | Wild-type | Res-number | Mutant | ΔG<sub>ppi2</sub>-prediction (Kcal/mol) | Affinity |
|-----------|------------|-------|--------------------------------------|----------|-----------|------------|-------|--------------------------------------|----------|
| ARG       | 273        | GLY   | 1.548                                | Decreasing| THR       | 445        | GLY   | 1.344                                | Decreasing|
| ARG       | 273        | MET   | 1.51                                | Decreasing| THR       | 445        | PRO   | 1.238                                | Decreasing|
| ARG       | 273        | ALA   | 1.51                                | Decreasing| THR       | 445        | ALA   | 0.854                                | Decreasing|
| ARG       | 273        | CYS   | 1.464                                | Decreasing| THR       | 445        | CYS   | 0.709                                | Decreasing|
| ARG       | 273        | PRO   | 1.414                                | Decreasing| THR       | 445        | MET   | 0.701                                | Decreasing|
| ARG       | 273        | VAL   | 1.362                                | Decreasing| THR       | 445        | VAL   | 0.65                                | Decreasing|
| ARG       | 273        | LEU   | 1.229                                | Decreasing| THR       | 445        | LEU   | 0.448                                | Decreasing|
| ARG       | 273        | ILE   | 1.205                                | Decreasing| THR       | 445        | ILE   | 0.431                                | Decreasing|
| ARG       | 273        | THR   | 1.153                                | Decreasing| THR       | 445        | PHE   | 0.36                                | Decreasing|
| ARG       | 273        | ASN   | 1.123                                | Decreasing| THR       | 445        | TRP   | 0.176                                | Decreasing|
| ARG       | 273        | SER   | 1.073                                | Decreasing| THR       | 445        | LYS   | 0.105                                | Decreasing|
| ARG       | 273        | ASP   | 1.045                                | Decreasing| THR       | 445        | GLN   | 0.002                                | Decreasing|
| ARG       | 273        | HIS   | 1.019                                | Decreasing| THR       | 445        | TYR   | 0.023                                | Increasing|
| ARG       | 273        | LYS   | 0.889                                | Decreasing| THR       | 445        | HIS   | 0.11                                | Increasing|
| ARG       | 273        | PHE   | 0.754                                | Decreasing| THR       | 445        | ARG   | 0.13                                | Increasing|
| ARG       | 273        | TYR   | 0.752                                | Decreasing| THR       | 445        | SER   | 0.341                                | Increasing|
| ARG       | 273        | TRP   | 0.594                                | Decreasing| THR       | 445        | ASN   | 0.394                                | Increasing|
| ARG       | 273        | GLU   | 0.449                                | Decreasing| THR       | 445        | GLU   | 2.02                                | Increasing|
| ARG       | 273        | GLN   | 1.724                                | Increasing| THR       | 445        | ASP   | 2.331                                | Increasing|
mutagenesis analyses for both selected amino acids have indicated that the mutation to Glycine is the best mutation to decrease the binding affinity between the ACE2 and the ANG2 molecules (Table 1). Given the previously reported experimental evidences, mutating the Arginine 273 residue to Glutamine would drastically change the active site interactions with the ANG2 protein. Therefore, we preferred to mutate the Arginine 273 residue to Glutamine and the Threonine 445 residue to Glycine. Choosing the Glutamine and the Glycine residues to substitute the original amino acids would prevent drastic structural changes due to their similar sizes to the original residues.

3.2.4. Mutation effects on ACE2 and ANG2 interaction

Reanalysis of the molecular docking between the mutated ACE2 and ANG2 molecules indicates that following the exerted mutations the ANG2 molecule loses its ability to make a stable interaction with the active site of the mutated enzyme. The cavity search within the structures of ACE2 molecule before and after mutations shows that the volume of central cavity of the ACE2 (active site) is not drastically affected from the performed mutations (4764.67 Å³ before mutations and 4819.46 Å³ after mutations). The mutant ACE2 interacts with the ANG2 in a significantly different orientation (Fig. 4). These results indicate that the performed mutations would most likely terminate the enzymatic activity of the ACE2 by removing highly stabilizing bonds which were provided by original amino acids at positions 273 and 445.

3.3. Increasing the binding affinity

3.3.1. Determination of suitable mutations across the ACE2 and RBD interface

The results of the Saturation Mutagenesis analyses were obtained and sorted according to their effects to increase the binding affinity between ACE2 and RBD. The best possible mutations...
are listed in Table 2. The mCSM-PPI2 models the effects of variations on the inter-residue non-covalent interaction network using graph kernels, evolutionary information, complex network metrics and energetic terms to better assess the molecular mechanism of the mutation. The employed graph-based structural signatures called mCSM has been shown to be an accurate and high-throughput approach to predict the impact of mutations on protein structure and function, and was one of the first methods capable of assessing the impact of mutations on protein interaction binding affinity (Rodrigues et al., 2019). The important amino acids which are involved in the interaction between ACE2 and RBD were also plotted by LigPlus software. The amino acids which are involved in hydrogen bonds or hydrophobic interactions are depicted in Fig. 5.

3.3.2. Exerting the mutations at ACE2 and RBD interface
Analyses of various single or multiple mutations were performed using their 2D interaction plots. The mutations which were able to conserve the original interacting amino acids at the ACE2 and RBD interface and increasing the number of involved amino acids were considered as suitable mutations. The obtained results demonstrated that the threonine 27 to Arginine mutation is the only mutation which met the selection criteria (Fig. 6). Obeying these criteria would guarantee the preservation of the ACE2 and RBD interactions in their original orientation.

3.4. Mutations to increase thermal stability
Five ACE2 residues with highest average B factors are listed in Table 3. The aspartate residue at position 427 has been among top 5 residues with high B factor. The region with highest B factor is depicted in Fig. 7. These high B factor regions are more flexible regions which could be affected by protease activity. The results of mutation analyses have shown that the mutation form aspartate 427 to arginine would increase the thermal stability of the ACE2. The Proline 451 to methionine mutation (PoPMuSiC prediction) and the glycine 448 to tryptophan mutation (HotMuSiC prediction) were predicted to increase the thermodynamic stability and thermostability of the ACE2. These mutations were exerted and optimized using Molegro VIRTUAL DOCKER.

3.5. Protein flexibility simulation
Analyzing the RMSF plot of the perfumed simulation indicated that the flexibility of mutated amino acids during the simulation is not high (Fig. 8a). This means that there are no large-scale con-

Table 2
Mutation candidates to increase the affinity. The mutations with highest capability to increase the binding affinity between the ACE2 and RBD are listed in the table.

| Wild-type | Res-number | Mutant | Mscm-ppi2-prediction (ΔAG Binding) Kcal/mol | Affinity |
|-----------|------------|--------|---------------------------------------------|---------|
| THR       | 27         | ARG    | 1.371                                       | Increasing |
| LYS       | 31         | GLU    | 1.311                                       | Increasing |
| ASP       | 38         | VAL    | 1.27                                        | Increasing |
| THR       | 27         | GLU    | 1.26                                        | Increasing |
| PHE       | 28         | TRP    | 1.152                                       | Increasing |

Fig. 5. The amino acids involved in ACE2 and RBD interaction. The structural visualization of the interactions across the interface is depicted in part (a). The 2D interaction plot of the interactions across the interface is depicted in part (b). The horizontal dashed line represents the interface. The amino acids located above the line belong to RBD and the amino acids located below the line belong to ACE2 (hydrogen bonds are indicated by dashed lines between the atoms involved, hydrophobic contacts are represented by an arc with spokes radiating towards the other atoms they contact).
formational transitions of protein system at the mutations sites. The superimposed structures of 10 protein models during the simulation has also indicated the low structural fluctuation (Fig. 8b)

3.6. The stability of mutated ACE2 through MD analysis

The RMSD diagram of the mutated was plotted as a function of time. The diagram shows the deviation of the protein structure at different simulation times compared to the pre-simulation structure. The results showed that the enzyme reached stability after 10 ns. The fluctuations of the RMSD graph should be due to the amino acid changes. However, at the end of the simulation time the system reached stability (Fig. 9a). The Rg is another important factor for protein stability analysis. The lower the Rg, the more folded and stable is the protein. As shown, the radius of gyration of the mutant protein decreased during the simulation, which means that its stability is increased during the simulation (Fig. 9b).

3.7. Molecular docking of mutant ACE2 and RBD

The results of the molecular docking analyses indicated that the mutant ACE2 is capable of interacting with RBD with proper orientation (Fig. 10). This interaction orientation could include all original interactions across the ACE2 and RBD interface. The low RMSD (0.8 Å) between the original and the mutated ACE2 proteins indicates that the exerted mutation does not make a drastic changes in

| Table 3 |
The top 5 positions with highest average B factors for 6M17 and 1R42 structures (the B factors are normalized to the scale of 0 to 100 Å²).

| 6M17 | Normalized B factor | 1R42 | Normalized B factor |
|------|---------------------|------|---------------------|
| 427  | 100                 | 86   | 100                 |
| 426  | 99                  | 338  | 99                  |
| 425  | 99                  | 427  | 99                  |
| 428  | 95                  | 85   | 98                  |
| 429  | 95                  | 339  | 96                  |
the protein structure. These results indicate that the mutant ACE2 could compete with the cell membrane ACE2 to bind to the RBD of the Spike protein.

3.8. Immunological analyses

The immunological analyses indicated that the mutant ACE2 does not contain any newly formed epitopes as the consequence of the exerted mutations. Moreover, none of the glycosylation sites were omitted or added to the glycosylation pattern of the ACE2 protein following the mutations. The 55, 92, 105, 324, 424 and 548 positions were predicted to be the glycosylation sites at both wild type and mutant ACE2 protein. The intact pattern of the glycosylation would help the mutant ACE2 to be considered as a self-protein upon clinical administration.

4. Discussion

There is still no definitive drug or effective vaccine to fight against COVID-19. Due to its pivotal role in SARS-CoV-2 infection, the interaction between ACE2 and RBD could be targeted for drug or vaccine development (Prompetchara et al., 2020). Vaccines are considered to be one of the most cost-effective healthcare tools in the prevention of infectious diseases. As expected, various strategies have been adapted to develop effective vaccines against RBD of SARS-CoV-2 (Chen et al., 2020a). This is while alternative
competitive molecules to interrupt this interaction could be as effective (Molina et al., 2020). ACE2 could be the alternative target for drug or vaccine design efforts against SARS-CoV-2. This enzyme is expressed in a variety of tissues with profound physiological roles (Der Sarkissian et al., 2008; Rentzsch et al., 2008; Tipnis et al., 2000; Wong et al., 2007). Therefore, ACE2 targeted treatments bear the risk to have negative effects on its inherent physiological roles. Given these circumstances, a recombinant ACE2 molecule which lacks its enzymatic activity with higher thermostability and affinity towards RBD would compensate for the challenges of targeting the cell membrane ACE2. In this regard, Changhui et al. have recently reported a recombinant ACE2-Ig with potential applications for diagnosis, prophylaxis, and treatment of COVID-19 (Lei et al., 2020). Moreover, Montiel et al. have demonstrated that recombinant ACE2 can significantly block early stages of SARS-CoV-2 infections (Monteil et al.). However, their study does not focus on enhancing the binding affinity and thermostability of the ACE2. It should be noted that recombinant ACE2 has already been tested in phase 1 and phase 2 clinical trials and its administration is reported to be well tolerated by healthy human subjects (Haschke et al., 2013; Khan et al., 2017).

Two mutations were performed at the active site of the ACE2 enzyme according the results of network analysis, 2D interaction plot and previously reported studies. One of the mutations at the active site of the ACE2 protein has already been shown to abolish the enzymatic activity of this protein via site directed mutagenesis (Goy et al., 2005). This study has corroborated the accuracy of mutation candidates introduced by the employed approach. Although the saturation mutation results proposed the Arginine 273 to Glycine mutation, we have preferred the experimentally approved Arginine to Glutamine mutation. It seems that this mutation could omit the hydrogen bond between Arginine 273 and Aspartate 1 of the ANG2 molecule, while the active site cavity would be occupied by the bulky side chain of the Glutamine. The Threonine 445 to Glycine mutation does the same regarding the hydrogen bond omission and preservation of the occupied volume by an equivalent sidechain. Replacing the selected residues with amino acids of similar size would limit the structural changes to a minimum. These mutations had been shown to have a significant effect on binding orientation and affinity of the ANG2 against the active site of the ACE2. The cavity size of the ACE2 active site has slightly become larger after the introduction of the mutations. This increase in cavity volume seems expected due to the slightly smaller size of the replaced amino acids. These changes would inhibit the ACE2 enzyme to perform its carboxypeptidase function due to the loss of key enzyme and substrate interactions and the lack of proper lock between enzyme and substrate in a correct orientation. Reduced enzymatic activity of ACE2 would bring about reduced physiological consequences upon its prescription.

Aside from reduced enzymatic activity, the engineered ACE2 needs to have higher affinity toward the RBD in comparison to the membrane attached ACE2 of the target cells. This property would give the engineered ACE2 to have a winning chance to interact with RBD and compete with membrane attached ACE2. Since the ACE2 and RBD molecules are complexed in a certain orientation (Chen et al., 2020b; Yan et al., 2020), the mutations to enhance the binding affinity should preserve this orientation. Our results indicate that the threonine 27 to Arginine mutation does not have a drastic effect on the interaction orientation and the interacting amino acids. However, new amino acids with extra hydrogen bonds and hydrophobic interactions are engaged at the interaction interface. These results could be construed as stronger interactions between ACE2 and RBD with similar interaction orientation following the threonine 27 to Arginine mutation.

Recombinant ACE2 has already been used for severe acute lung injury, acute ANG2-induced hypertension, ANG2-induced heart hypertrophy, cardiac dysfunction, adverse myocardial remodeling, renal oxidative stress, inflammation, and fibrosis (Chen et al., 2016; Gu et al., 2016; Huentelman et al., 2005; Imai et al., 2005; Zhong et al., 2011; Zou et al., 2014). However, according to the pharmacokinetic studies the half-life of recombinant ACE2 is only hours in humans and mice due to its fast clearance rates (Haschke et al.; Wysocki et al., 2010). Resistance against proteolytic degradation is one of the most important factors which could determine the protein half-life. Prior studies involving L-asparaginase, β-galactosidase, λ-repressor, Clostridium difficile toxin A-specific antibodies, and Bacillus subtilis lipase have shown that increasing a protein’s thermostability may also increase its resistance to protease digestion (Ahmad et al., 2012; Daniel et al., 1982b; Hussack et al., 2011; Parsell and Sauer, 1989). Thermal and structural stability of proteins are important determinants of proteolytic stability (Akasako et al., 1995; Daniel et al., 1982a; Parsell and Sauer, 1989). Proteins are resistant against proteolytic degradation in their native conformation. However, they are highly susceptible to proteases after unfolding. The accessible and flexible loop regions of proteins could be attacked by unspecific proteases even in the native conformation of the highly compact and thermostable proteins (Hubbard, 1998; Sarath et al., 1989). A number of strategies have been harnessed to extend the low half-life of therapeutic proteins. Amino acid manipulation, post translational modification, bio-conjunction and carrier mediated delivery are the main categories of technologies reported for half-life extension (Zaman et al., 2019). In order to increase the thermostability of the ACE2 protein we have used the amino acid manipulation strategy. The first approach involved the mutation of amino acids with highest B factors (aspartate 427 to arginine). The diffusion of atomic electron densities with respect to their equilibrium positions due to thermal motion and positional disorder is represented by B factor. Thus, B factor shows the extent of mobility for individual amino acids. Replacing an amino acid with high B factor would increase the protein rigidity (Reetz et al., 2006; Xie et al., 2014). On the other hands, two other mutations were performed to increase the thermodynamic stability and thermostability of the ACE2. The mutations were selected at the regions which are far from the RDB binding site to avoid any destructive effects on the binding affinity. The obtained MD results have also corroborated the stability of the mutated ACE2 structure. We believe that increased thermodynamic stability and thermostability of the ACE2 would enhance the protein half-life. It should be noted that ACE2 amino acid manipulation could be accompanied with other half-life extension strategies like bio-conjugation.

Protein engineering is a powerful tool to design therapeutic proteins with desired properties. Bioinformatics tools are contemporary widely used for protein engineering (Khalili, 2017a,b). These tools could save a lot of time and effort in the case of diseases like COVID-19 which time is essential. Using these tools an engineered ACE2 protein has been designed which is expected to bind RBD with higher affinity while it is more thermostable and lacks enzymatic activity. Since ACE2 is reported to be the main RBD binding receptor, its application as an inhibitory agent provides an evolutionary advantage over other conventionally employed therapeutics. Moreover, using the extracellular domain of ACE2 reduces the possible adverse effects and immunological responses against this therapeutic molecule. In vivo an in vivo examination of the engineered protein would unveil its true potentials.

CRediT authorship contribution statement

Zahra Payandeh: Methodology. Validation. Formal analysis. Investigation. Resources. Writing - review & editing. Visualization.
Mohammad Reza Rahbar: Methodology, Validation, Formal analysis, Investigation, Resources, Writing - review & editing, Visualization. Abofiz Ajanghiri: Methodology, Validation, Formal analysis, Investigation, Resources, Writing - review & editing, Visualization. Zahra Sadat Hashemi: Methodology, Validation, Formal analysis, Investigation, Resources, Writing - review & editing, Visualization. Alineza Zakeri: Methodology, Validation, Formal analysis, Investigation, Resources, Writing - review & editing, Visualization. Moslem Jafarzani: Methodology, Validation, Formal analysis, Investigation, Resources, Writing - review & editing, Visualization. Mohammad Javad Rasaei: Methodology, Validation, Formal analysis, Investigation, Resources, Writing - review & editing, Visualization. Saeed Khalili: Methodology, Validation, Formal analysis, Investigation, Resources, Writing - review & editing, Visualization. Mohammad Javad Rasaei: Methodology, Validation, Formal analysis, Investigation, Resources, Writing - review & editing, Visualization. Saeed Khalili: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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