Protonation states of amino acid residues in metalloprotease complexed with its inhibitor: *ab initio* molecular simulations

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Abstract

The metalloprotease pseudolysin (PLN) secreted by the gram negative bacteria *Pseudomonas aeruginosa* degrades extracellular proteins to produce bacterial nutrition. Since PLN has a Zn ion around its inhibitor-binding site, the interactions between Zn and PLN residues as well as inhibitor can be significantly changed depending on the protonation states of the PLN residues around the inhibitor-binding site. To determine stable protonation states of these residues, we here considered some types of the protonation states for Glu and His residues existing around Zn and investigated the electronic states of the PLN+inhibitor complex, using *ab initio* molecular simulations. His223 was found to affect significantly on the PLN-inhibitor interactions.

Keywords

Metalloprotease; Bacterial virulence; Molecular simulation; Fragment molecular orbital; Protein-ligand interactions; Drug design; Inhibitor; Hydroxamate compound; Antimicrobial resistance.

1. Introduction

Antimicrobial agents inhibiting the growth of bacteria have been used widely in the treatment for infectious diseases [1]. However, the emergence of drug-resistant strains of bacteria and their spread pose a major threat to humans [2]. It is therefore necessary to develop novel potent antibiotics against bacterial infections with novel modes of action and a low risk of drug-resistance.

Pseudomonas aeruginosa (P. aeruginosa) is a pathogen for which new antibiotics are heavily needed. *P. aeruginosa* secrets the virulence factor pseudolysin (PLN) which is a proteolytic enzyme of the M4-family of metalloproteases [1]. The *P. aeruginosa* growth may be suppressed by inhibitors effectively binding the active site of PLN. In keeping with this notion, novel inhibitors against PLN have been developed as putative new antimicrobial agents [1, 2]. Since these agents do not attack the bacteria directly, there is a reduced risk for producing drug-resistant bacteria.

The structure of PLN contains a Zn and a Ca ion. The Ca ion contributes to stabilizing the PLN structure, while the Zn ion is necessary for the catalytic activity, and is located close to the cleavage site. The specificity of a protease is determined by the topology of the active site, which contains specific binding pockets for the substrate. Binding pockets that interact with amino acids N-terminal for the cleavage site in the substrate are called non-prime sites (S), while the pockets binding amino acids C-terminal for the cleave site is called primed sites (S′). The topology of the active site is not only important for the specificity and binding of substrates, but small molecule inhibitors also interact with the Zn and surrounding non-prime and prime sites. In PLN, the S1’ pocket includes, among others, the Leu132 and Val137 residues and preferentially recognizes aromatic and hydrophobic amino acids, while the S2’ pocket includes Asn112, Phe129, Ile186, Leu197 and Arg198 and recognize hydrophobic amino acids, and the S1 pocket composed of Tyr114, Trp115 and Tyr155 recognizes hydrophilic amino acids [1, 2].

In a recent study [3], a hydroxamic acid-based agent was developed as an potent inhibitor. Its inhibition effects against human matrix metalloproteases (MMPs) as well as PLN were investigated to reveal its specificity. In addition, using X-ray crystallography, the reason for this specific interaction was attributed to a distinct binding mode into the S1’ pocket. These results also pointed out the possibility for the hydroxamic acid-based agent to achieve selective PLN inhibition in front the MMPs. Therefore, it is expected that the agent and its structurally similar agents can be putative drugs against bacterial infections with a low risk of side effect as well as drug-resistance.

Since PLN has a Zn ion around its ligand-binding site, there is a possibility that the interactions between the Zn ion and some amino acid residues as well as ligand are

significantly changed depending on the protonation states of the residues around Zn. In our previous molecular simulations [4] for the complexes of vitamin D receptor (VDR) and its ligands having the same chemical structure with different chirality, we determined the stable protonation states of two histidine (His) residues, which contribute significantly to the binding between VDR and the ligand, using *ab initio* fragment molecular orbital (FMO) calculations. The results illustrated the possibility that the difference in the chirality of ligands can induce the change in protonation states of the His residues of VDR. This finding also provided an important warning that the protonation states of His residues existing near the ligand should be considered more precisely in the molecular simulations for investigating the specific interactions between protein and its ligand.

In the present study, we considered different protonation states for the glutamine acid (Glu) and His residues located around the Zn ion in PLN and elucidated which protonation state is more stable, using *ab initio* FMO calculations. In addition, specific interactions between PLN residues as well as Zn and the PLN inhibitor were investigated at an electronic level, using the *ab initio* FMO method. The results elucidated the reason for strong binding of the inhibitor to the S1' pocket of PLN. The present results provide useful information for developing novel antibacterial agents, highly potent and selective for PLN.

2. Details of *ab initio* molecular simulations

2.1. Construction of initial structure for PLN+inhibitor complex

In the present study, we employed a hydroxamate inhibitor shown in Figure 1a, which has significant inhibitory effect against PLN: Ki value is 12.3 μM [3]. Since the hydroxamic acids in general have quite high pKa values (8-9), we considered that most inhibitor molecules are protonated at physiological pH and the protonated state of the inhbitor was used in the calculations. The initial 3D-structure of PLN+inhibitor was obtained from the protein data bank (PDB ID: 6FZX) [3]. PLN has seven His residues, and their protonation states were assigned based on the pKa value predicted by the PROPKA3.0 program [5, 6]. His residues, which have a pKa value higher than 6 and are located on the surface of PLN, were assigned Hip^+ protonation, while His residues with a lower pKa value and located inside the PLN structure were assigned Hie or Hid protonation. The chemical structures of these protonation states are shown in Figure S1a of Supporting information. Since the determination of Hie or Hid depends significantly on the structure of the residues existing around the His residue, we assigned the protonation states by considering the steric hinderance around the His residue. The assigned protonation states of the His residues in PLN are listed in Table 1. His77 was

assigned as Hie, because the distance between the N atom of the ε-site of His77 and the O atom of Ile25 backbone is 2.8 Å and a hydrogen bond can be formed between them by introducing a H atom to the ε-site of His77, as shown in Figure S2 of Supporting information. On the other hand, H atom cannot be introduced to the N atom at the δ-site of His77, because the Thr39 has a OH group at the end of side-chain. His140 and His144 have Hid protonation in order to coordinate with the Zn ion at the ε-site N atom of their imidazole rings, although His140 has a high pKa value of 7.58. His224 has Hid protonation, because the distance between the N atom at the ε-site of His224 and the O atom of a crystal water molecule is short (2.7 Å) and the H atom of the water molecule is likely to contribute to the hydrogen between His224 and the water molecule in the PDB structure [3]. It is noted that His223 has lower pKa value of 5.51 and no steric hindrance to the surrounding PLN residues, indicating that His223 can have both Hid and Hie protonation states. We therefore considered both protonation states for His223 and determined which of them is more stable based on the total energies evaluated by *ab initio* FMO calculations.

In addition, we here considered three types of protonation states for Glu141, because this residue is located near the inhibitor and the interaction between Glu141 and the inhibitor is likely to be significantly affected by the change in the protonation state of Glu141. In the previous molecular simulations [7, 8] of the metalloprotease thermolysin, whose structure is very similar to the PLN structure, a proton was added to the carbonyl group of Glu141 side-chain, in order to neutralize the change of Glu141 and reproduce the structure of thermolysin obtained by the experiment. We here considered in details the protonation state of Glu141 and determined its most reliable protonation state, using molecular mechanics (MM) method to optimize the structure. In fact, we considered three types of protonation states (Glu, Glh-1 and Glh-2) for Glu141, as shown in Figure S1b of Supporting information. The Glu state is a non-protonated state with negative charge, while Glh-1 and Glh-2 are states with one or the other oxygen atom of the carbonyl group of Glu141 side-chain protonated. Therefore, electrostatic interactions between Zn ion and Glu141 as well as His223 are likely to be affected significantly by the change in their protonation states.

In the present simulations, we totaly considered the six types of combinations of the protonation states for His223 (Hid and Hie) and Glu141 (Glu, Glh-1 and Glh-2) in PLN and determined the most preferable state by the MM optimizations and *ab initio* FMO calculations.

In addition, PLN has two disulfide bonds between the cysteine (Cys) residues. Indeed, the sulfur atoms of Cys30 and Cys58 lose the hydrogen atom to form a disulfide

bond, while Cys270 and Cys297 form a disulfide bond in the same way. We thus changed Cys into its deprotonated state and added the connection between the deprotonated Cys residues. These disulfide bonds are considered to be important for keeping the active conformation of PLN.

2.2. Structure optimizations for PLN+inhibitor complex

The structure of the inhibitor shown in Figure 1a was optimized in vacuum by the B3LYP/6-31G(d,p) method of the *ab initio* molecular orbital (MO) calculation program Gaussian16 (G16) [9]. The charge distribution of the optimized structure was evaluated by the restrained electrostatic potential (RESP) analysis [10], using the HF/6-31G(d) method of G16. Based on this charge distribution, atomic charge parameters of the force fields employed in the MM optimizations were constructed for the inhibitor.

The initial structure of the complex was fully optimized in water using the classical MM and molecular dynamics (MD) simulation program AMBER18 [11]. In order to properly consider the solvation effect on the complex, we added water molecules with a layer of 8 Å around the complex. The total number of water molecules considered was 2205. In the MM optimizations, the FF14SB force field [12], TIP3P model [13] and the generalized AMBER force field (GAFF) [14] were used for PLN, water molecules and inhibitor, respectively. The threshold value of energy gradient for convergence in the MM optimization was set as 0.0001 kcal/mol/Å.

2.3. FMO calculations for PLN+inhibitor complex

To elucidate the specific interactions between PLN and inhibitor, the electronic properties for the solvated structure of the complex were investigated by the *ab initio* FMO method [15]. We employed 62 water molecules existing within 8 Å from the inhibitor explicitly, to consider the effect of water molecules on the interactions between PLN and inhibitor. In FMO calculations, a target molecule is divided into units, each of which is called "fragment", and the electronic properties of the target molecule are estimated from the electronic properties of the monomers and the dimers of the fragments. The specific interactions between the fragments can be investigated from the interaction energies obtained by the FMO calculation. In the present FMO calculations, the inhibitor, each PLN residue and each water molecule were assigned as separate fragments, because this fragmentation enables us to evaluate the interaction energies between the inhibitor and each PLN residue. It is noted that Zn ion and some residues coordinated with Zn were considered in the same fragment, because they are strongly interacting with each other. In fact, Zn ion was included in the same fragment as Hid140, Hid144 and Glu164. Furthermore, Cys30 and Cys58 were included in the same fragment, while Cys270 and

Cys297 are also included together in the same fragment, because these Cys residues form disulfide bonds.

The FMO calculation program ABINIT-MP Ver.6.0 [16] was used in the present study. The *ab initio* MP2[17, 18]/6-31G method of FMO was employed to accurately investigate the π–π stacking, NH–π and CH–π interactions as well as the hydrogen-bonding and electrostatic interactions between the inhibitor and the PLN residues. In addition, to elucidate which residues contribute to the binding of the inhibitor, we investigated the inter fragment interaction energies (IFIE) [19] obtained by the FMO calculations.

3. Results and discussion

3.1 Optimized structure of PLN+inhibitor complex

To determine the most preferable protonation states of His223 and Glu141 in PLN, we first optimized the structure of the PLN+inhibitor complex in water and compared the optimized structure with the PDB structure (PDB ID: 6FZX [3]). It is expected that the structure having the smaller deviation from the PDB structure is the more reliable for the solvated PLN+inhibitor complex.

At first, we compared the structure around the inhibitor in the PDB and the optimized structures. The atoms contributing to the interactions between PLN and the inhibitor were selected and the distances between them were analyzed. The assignments of these atoms are shown in Figure 1b, indicating that the distances between Zn and O1/O2 of inhibitor and between OE1/OE2 of Glu141 and O1/N2 of inhibitor are main factors for determining the interactions between inhibitor, PLN and Zn. We therefore analyzed the distances in the optimized structures for the six types of protonation states of His223 and Glu141. As listed in Table 2, when His223 had Hid and Glu141 had Glh-1 or Glh-2 protonation, the distance between Zn and O1 of inhibitor deviated significantly (1.9 Å) from that (2.19 Å) in the PDB structure. In contrast, when His223 had Hid and Glu141 had Glu protonation, all distances listed in Table 2 were similar to those in the PDB structure. It is thus considered that the Glh-1 and Glh-2 protonation states of Glu141 are not realistic and that the Glu141 prefers to have Glu protonation state in the PLN+inhibitor complex. A similar trend occurred when His223 had Hie protonation, as indicated in Table 2, although the deviation was smaller compared with that of the Hid protonation.

We furthermore investigated the deviation of the His223 structure from the PDB structure for the six types of the protonation states of His223 and Glu141. As indicated in Figure 2, the optimized structure of the aromatic ring of His223 side-chain significantly deviates from the PDB structure. In particular, for the Hie-Glh-1 and the Hie-Glh-2

protonation states, the deviation is rather large compared with the other protonation states. It is noted that the deviation was larger than 1.0 Å when Glu141 had the Glh-1 or Glh-2 protonation state, indicating that the Glh-1 and Glh-2 states for Glu141 are not realistic for the examined PLN+inhibitor complex.

In contrast, as shown in Figure S3 of the Supporting information, the optimized structure of Glu141 side-chain was at most 1.1 Å deviated from the PDB structure, and the size of the deviation was similar for the six types of protonation states. Therefore, it is elucidated that the structure of Glu141 side-chain is not affected so significantly by the change in its protonation state.

As for the structure of the inhibitor, Figure 3 clarifies that the atoms of the hydroxamic acid group and Cl atoms at the other side have rather large deviation. In particular, when Glu141 had Glh-1 or Glh-2 protonation state, O1, O3, Cl-1, Cl-2 as well as C atoms of the aromatic ring had large deviation from the PDB structure. On the other hand, when Glu141 had Glu protonation state, the deviation was rather small.

From the above mentioned analysis on the deviation of PLN and inhibitor structures from the PDB structure, it is elucidated that Glu141 prefers to have non-protonated Glu state rather than the protonated Glh-1 and Glh-2 states in the PLN+inhibitor complex. Hereafter, we employed the Glu state for Glu141. On the other hand, His223 can have both Hie and Hid protonation states, because the deviation of the structure from PDB is similar for the both protonation states. Accordingly, we will determine the more preferable His223 protonation state precisely at an electronic level, based on the *ab initio* FMO calculations in the same manner as our previous study [4].

3.2. Electronic states of PLN+inhibitor complex evaluated by FMO

Total energies, total inter-fragment interaction energies (IFIEs) between inhibitor and all PLN residues as well as Zn, and interaction energies between inhibitor and the Zn group of PLN were evaluated for the optimized structures of PLN+inhibitor, using *ab initio* FMO calculations. As listed in Table S1 of Supporting information, the total energy of the complex is almost the same for both the structures with Hid223 or Hie223 protonation, indicating that His223 can have both the protonation states. To investigate the binding affinity between PLN and inhibitor, we moreover evaluated the total IFIEs to find that the size of total IFIE is 64.5 kcal/mol larger when His223 has Hie protonation. The interaction energy between the Zn group and inhibitor has similar trend as the total IFIE; the size of the interaction energy is 36.1 kcal/mol larger when His223 has Hie protonation. Consequently, it is elucidated that the binding affinity between PLN and inhibitor is significantly affected by the change in protonation state of His223.

To elucidate the reason why the His223 protonation state affects so significantly

on the binding affinity between PLN and inhibitor, we investigated the IFIEs between inhibitor and the PLN residues for the two types of His223 protonation. As revealed in Figures 4a and 4b, inhibitor binds most strongly to the Zn group of PLN. This group is composed of Zn, Hid140, Hid144 and Glu164 and has main contribution (about 80% of total IFIE) to the total binding of PLN to inhibitor, as indicated in Table S1. Accordingly, it is expected that inhibitors with strong binding to these residues as well as Zn can be potent inhibitors against PLN.

In addition, we analyzed the difference in IFIEs between the Hid and the Hie protonation states. As shown in Figure 4c, the Zn group binds 36 kcal/mol more strongly to inhibitor, when His223 has Hie protonation state, while Asn112 and His223 interact 13.9 and 10.6 kcal/mol more strongly with inhibitor, respectively. It is therefore elucidated that the interactions between inhibitor and the Zn group, Asn112 and His223 of PLN are enhanced significantly by the change in His223 protonation state from Hid to Hie.

To clarify the reason for this significant effect of His223 protonation on the IFIE between inhibitor and the PLN residues as well as Zn, we compared the interacting structures between inhibitor, Zn and these residues in the PLN+inhibitor complexes with the Hid223 or Hie223 protonation state. As shown in Figure 5a, when His223 has Hid protonation, the two oxygen atoms of the hydroxamic acid group of inhibitor are strongly coordinated with Zn at 1.9 and 2.1 Å distance, respectively, while the inhibitor has no remarkable interaction with the PLN residues. In contrast, when His223 has Hie protonation state, the three oxygen atoms of inhibitor interact strongly with the side-chain of Hie223 as well as Zn, as clearly indicated in Figure 5b. In fact, the proton added to the ε-site of the imidazole ring of Hie223 has strong electrostatic interactions with the two oxygen atoms of ligand. Furthermore, Asn112 gets closer to inhibitor to interact electrostatically with the central part of inhibitor. These additional interactions between inhibitor and Hie223 and Asn112 are the main reasons for the strong interactions of these residues with the inhibitor.

As for the interaction between inhibitor and the Zn group composed of Zn, Hid140, Hid144 and Glu164, the interaction energy for the Hie223 protonation state is 36.1 kcal/mol larger than that for the Hid223 protonation state, as shown in Table S1. It is thus expected that the interacting structures between inhibitor and Zn as well as these PLN residues are significantly different for the PLN+inhibitor complex with the two different His223 protonation states. In contrast, Figures 5a and 5b reveal the similar interacting structures around Zn for the both protonation states. It is likely that the other PLN residues around the Zn group may affect the interactions between inhibitor and the

Zn group of PLN.

From the above mentioned results, on specific interactions between the inhibitor and the PLN residues as well as Zn, we revealed that His223 can both have Hid and Hie protonation states, and as indicated in Figure 5b the Hie223 protonation state has a significant effect to enhance the interactions between inhibitor and PLN residues in the PLN+inhibitor complex.

4. Conclusions

In the present molecular simulations, we considered six types of protonation states for His223 and Glu141 in PLN and determined which state is the most realistic, using the MM optimizations and the *ab initio* FMO calculations. The MM optimizations in explicit waters reveal that Glu141 prefers to have non-protonated state, while the *ab initio* FMO calculations elucidate that inhibitor binds more strongly to PLN with the Hie223 protonation state. The present results clearly demonstrate that the protonation states of His and Glu residues existing around the inhibitor-binding pocket of PLN should be considered precisely for investigating the specific interactions between PLN and its inhibitor.

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References

[1] S. Sjøli, E. Nuti, C. Camodeca, I. Bilto, A. Rossello, J.-O. Winberg, I. Sylte, O. A. Adekoya, Synthesis, experimental evaluation and molecular modelling of hydroxamate derivatives as zinc metalloproteinase inhibitors, Eur. J. Med. Chem. 108 (2016) 141–153.

[2] O. A. Adekoya, S. Sjøli, Y. Wuxiuer, I. Bilto, S. M. Marques, M. A. Santos, E. Nuti, G. Cercignani, A. Rossello, J.-O. Winberg, I. Sylte, Inhibition of pseudolysin and thermolysin by hydroxamate-based MMP inhibitors, Eur. J. Med. Chem. 89 (2015) 340–348.

[3] A. M. Kany, A.Sikandar, S. Yahiaoui, J. Haupenthal, I. Walter, M. Empting, J. Kohnke, R. W. Hartmann, Tackling *pseudomonas aeruginosa* virulence by a hydroxamic acid-based LasB inhibitor, ACS Chem. Biol. 13 (2018) 2449–2455.

[4] Y. Terauchi, R. Suzuki, R. Takeda, I. Kobayashi, A. Kittaka, M. Takimoto-Kamimura, N. Kurita, Ligand chirality can affect histidine protonation of vitamin-D receptor: *ab initio* molecular orbital calculations in water, J. Steroid Biochem. Mol. Biol. 186 (2019) 89–95.

[5] C. R. Søndergaard, M. H. M. Olsson, M. Rostkowski, J. H. Jensen, Improved treatment of ligands and coupling effects in empirical calculation and rationalization of pKa Values, J. Chem. Theory Comput. 7 (2011) 2284–2295.

[6] M. H. M. Olsson, C. R. Søndergaard, M. Rostkowski, J. H. Jensen, PROPKA3: Consistent treatment of internal and surface residues in empirical pKa predictions, J. Chem. Theory Comput. 7 (2011) 525–537.

[7] M. T. H. Khan, Y. Wuxiuer, I. Sylte, Binding modes and pharmacophore modelling of thermolysin inhibitors, Mini-Rev. Med. Chem., 12 (2012) 515−533.

[8] X. Hu, S. Balaz, W. H. Shelver, A practical approach to docking of zinc metalloproteinase inhibitors, J. Mol. Graph. Model., 22 (2004) 293−307.

[9] M. J. Frisch *et al.*, Gaussian 16, Revision C.01, Inc., Wallingford CT, 2016.

[10] C. I. Bayly, P. Cieplak, W. Cornell, P. A. Kollman, A well-behaved electrostatic potential based method using charge restraints for deriving atomic charges: the RESP model, J. Phys. Chem. 97 (1993) 10269–10280.

[11] P. A. K. D. A. Case, I. Y. Ben-Shalom, S. R. Brozell, D. S. Cerutti, T. E. Cheatham, III, V. W. D. Cruzeiro, T. A. Darden, R. E. Duke, D. Ghoreishi, M. K. Gilson, H. Gohlke, A. W. Goetz, D. Greene, R. Harris, N. Homeyer, S. Izadi, A. Kovalenko, T. Kurtzman, T. S. Lee, S. LeGra, AMBER 2018, Univ. California, San Francisco, 2018.

[12] J. A. Maier, C. Martinez, K. Kasavajhala, L. Wickstrom, K. E. Hauser, C.

Simmerling, ff14SB: Improving the accuracy of protein side chain and backbone parameters from ff99SB, J. Chem. Theory Comput. 11 (2015) 3696–3713.

[13] W. L. Jorgensen, J. Chandrasekhar, J. D. Madura, R. W. Impey, M. L. Klein, Comparison of simple potential functions for simulating liquid water, J. Chem. Phys. 79 (1983) 926–935.

[14] J. Wang, R. M. Wolf, J. W. Caldwell, P. A. Kollman, D. A. Case, Development and testing of a general amber force field, J. Comput. Chem., 25 (2004) 1157–1174.

[15] D. G. Fedorov, T. Nagata, K. Kitaura, Exploring chemistry with the fragment molecular orbital method, Phys. Chem. Chem. Phys. 14 (2012) 7562−7577.

[16] S. Tanaka, Y. Mochizuki, Y. Komeiji, Y. Okiyama, K. Fukuzawa, Electron-correlated fragment-molecular-orbital calculations for biomolecular and nano systems, Phys. Chem. Chem. Phys. 22 (2014) 10310−10344.

[17] Y. Mochizuki, T. Nakano, S. Koikegami, S. Tanimori, Y. Abe, U. Nagashima, K. Kitaura, A parallelized integral-direct second-order Møller–Plesset perturbation theory method with a fragment molecular orbital scheme, Theor. Chem. Acc. 112 (2004) 442−452.

[18] Y. Mochizuki, S. Koikegami, T. Nakano, S. Amari, K. Kitaura, Large scale MP2 calculations with fragment molecular orbital scheme, Chem. Phys. Lett. 396 (2004) 473−479.

[19] K. Fukuzawa, Y. Komeiji, Y. Mochizuki, A. Kato, T. Nakano, S. Tanaka, Intra- and intermolecular interactions between cyclic-AMP receptor protein and DNA: Ab initio fragment molecular orbital study, J. Comput. Chem. 27 (2006) 948–960.