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Specific interactions and binding energies between thermolysin and potent inhibitors: Molecular simulations based on *ab initio* molecular orbital method

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ABSTRACT

Biochemical functions of the metalloprotease thermolysin (TLN) are controlled by various inhibitors. In a recent study we identified 12 compounds as TLN inhibitors by virtual screening and *in vitro* competitive binding assays. However, the specific interactions between TLN and these inhibitors have not been clarified. We here investigate stable structures of the solvated TLN–inhibitor complexes by classical molecular mechanics simulations and elucidate the specific interactions between TLN and these inhibitors at an electronic level by using *ab initio* fragment molecular orbital (FMO) calculations. The calculated binding energies between TLN and the inhibitors are qualitatively consistent with the experimental results, and the FMO results elucidate important amino acid residues of TLN for inhibitor binding. Based on the calculated results, we propose a novel potent inhibitor having a large binding affinity to TLN.

1. Introduction

Thermolysin (TLN) is a Zn metalloprotease secreted by the gram-positive thermophilic bacterium *Bacillus thermoproteolyticus*. TLN has high thermal stability [1] assisted by four structural Ca ions, and catalyses the hydrolysis of peptide bonds containing hydrophobic amino acid residues [2]. One catalytic Zn ion is required for the hydrolysis function of TLN [3]. In addition to these ions, the binding of a wide variety of inhibitors controls efficiently the chemical activity and biochemical functions of TLN. Previous experiments [2,4,5] elucidated that amino acids of the four sub-pockets in the substrate binding region of TLN (S1, S1', S2 and S2') provide a significant contribution to the specificity of ligand binding.

TLN has been widely used by the chemical industry, including for the synthesis of a precursor of the artificial sweetener aspartame ZDFM [6–10]. Reducing synthetic costs by enhancing the enzymatic activity of TLN is therefore of considerable industrial value. For this purpose, its catalytic mechanisms and recombinants have been widely studied both by experimental [11–16] and theoretical [17–22] studies. However, the mechanism has not yet been clarified at atomic and electronic levels.

TLN, which is the prototype of the M4 family of proteinases, and other bacterial enzymes of the M4 family are believed to be important for suppressing or avoiding the innate immune system of the infected host during pathogenesis [23]. The inhibition of the activity of TLN and these enzymes is expected to be a novel strategy in developing the new generation of antibacterial drugs [24,25]. Zn metalloproteinases with close structural and functional similarities to TLN are named thermolysin-like proteinases (TLPs). They contain the consensus amino acid sequence HExxH forming a part of the Zn-containing catalytic domain [10]. Several TLPs play important roles for controlling different physiological functions. For example, the TLP angiotensin-converting enzyme (ACE) is involved in the control of hypertension, and ACE inhibitors have a therapeutically potential in the treatment of hypertension, heart failure and diabetic nephropathy [26]. For developing potent inhibitors of TLPs with therapeutically potentials, it is indispensable to elucidate the specific interactions between the enzymes and various ligand molecules. The determination of 3-dimensional (3D) structures of TLPs and their complexes with ligand is still not straight forward, and molecular modeling may be an alternative for gaining structural insight. Due to structural similarities among TLPs, crystallographic data of TLN and various TLN–inhibitor complexes have also been used to construct model structures of other TLPs. In addition, the functional similarity between TLN and TLPs

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indicates that TLN inhibitors also may inhibit the activity of other TLPs. Therefore, various TLN inhibitors are developed and experimentally tested [27,28].

By structure-based virtual ligand screening (VLS) techniques, we have previously identified 22 molecules as putative TLN inhibitors, and 12 of them were confirmed as TLN binders by *in vitro* competitive binding assays [29]. Furthermore, by use of the molecular modeling program ICM (Internal Coordinate Mechanics) [30–32], the binding modes and affinities of these inhibitors to TLN were predicted, giving a good correlation ($R^2 = 0.78$) between the ICM scoring value and the experimental detected IC_{50} values [29]. These predictions and our more recent studies [33,34] indicated that the binding modes and affinities are largely dependent on the type of inhibitor, although their chemical structures are quite similar to each other. The origin for this large dependency of binding affinity has not been clarified at the electronic level. In the present study, we further investigate the binding of five of the inhibitors from the VLS study to TLN by studying stable structures of solvated TLN–inhibitor complexes by classical molecular mechanics (MM) simulations. The specific interactions between TLN and the inhibitors are elucidated by *ab initio* fragment molecular orbital (FMO) calculations with solvating water molecules considered explicitly. The calculated binding energies are qualitatively consistent with the observed trend of the experimental inhibition [29]. We furthermore elucidate which amino acid residues in TLN are important for the specific binding between TLN and inhibitors at an electronic level.

2. Methods of calculations

2.1. Classical MM optimizations for solvated TLN–ligand complexes

TLN–ligand complexes from our previous study [29] were used as initial structures for the calculations. These complexes were obtained by the ICM-Docking program [30–32]. The structures of the five ligands (Nx, x = 1–5) employed here are shown in Fig. 1. Hydrogen atoms were added to the complexes, and the positions of the hydrogen atoms were optimized by the classical MM program package AMBER9 [35]. In order to obtain a solvated structure of TLN–ligand complex, solvating water molecules (about 4500) were added within 8 Å from the surface of the complex. The positions of only the solvating water molecules were optimized to reduce steric clashes between the water molecules and the complex. Since the ligand N3 is so small giving a wide space around N3 in the ligand-binding pocket of the solvated TLN–N3 complex, the structure of the solvated TLN–N3 complex was fully optimized by AMBER9 to obtain more stable conformation of N3. The PARM99 [36] and TIP3P [37] force fields were used for the complex and the water molecules, respectively. The charges for each atom of the ligands were determined from the restrained electrostatic potential (RESP) analysis [38] based on the MP2/6-31G(d,p) method [39] of the *ab initio* MO program Gaussian03 [40]. The force field parameters for Zn and Ca ions in TLN were constructed by using the antechamber program of AMBER9 [35]. The charges of these ions were set to 2.0.

2.2. FMO calculations for solvated TLN–ligand complexes

In order to elucidate the specific interactions between TLN and ligands at an electronic level, electronic properties of the solvated TLN–ligand complexes were investigated by the multilayer FMO (MFMO) method [41–46] implemented in the ABINIT-MP ver.4.1 program [47]. MFMO calculations of fully solvated TLN–ligand complexes are not practical to perform, since these complexes contain around 4500 solvating water molecules. Water molecules existing far away from the ligand and the Zn ion do not have a large influence on the TLN–ligand interactions, and therefore only water molecules existing within 5 Å from the ligand or Zn ion were considered in the MFMO calculations. In FMO calculations, the target molecule is divided into units called fragments, and the electronic properties of the target molecule are estimated from the electronic properties of the monomers and dimers of the fragments. Since the electronic properties of the dimers are calculated in FMO, we can obtain the interaction energies between specific fragments with the effect from the other fragments considered. This pair interaction energy, also called inter-fragment interaction energy (IFIE) [48–50], is somewhat similar to the simple pair interaction energy computed by classical force field methods. However, in the FMO evaluation of the pair interaction energy, the influence from other fragments is taken into account as a direct coulomb interaction. In addition, the charge redistribution around the binding site induced by ligand binding is considered in FMO calculations, whereas classical MM method uses a fixed charge parameter for ligand-free and ligand-bound proteins. In the present study, the fragment size was set as one amino acid residue or one water molecule. We thus investigated the interaction energies between the ligand and each amino acid residue of TLN or each solvating water molecule, in order to elucidate which residues in TLN are important for ligand binding. The effect of solvating water molecules on specific ligand interactions was also elucidated. To describe electronic states around the Zn ion more precisely, the Zn ion was included in the same fragment as His142, His146 and Glu166 existing near the Zn ion, as explained in our previous studies [51,52].

In the present MFMO calculations, the electronic properties around the ligand were evaluated accurately by MP2/6-31G method which considers electron correlation effects, while the effect of the amino acid residues existing far from the ligand was treated by HF/6-31G method, because the electron correlation effects of these residues are negligible. In fact, the ligand, the amino acid residues of TLN and water molecules existing within 5 Å from the ligand were treated by MP2/6-31G, while the other residues and water molecules were treated by HF/6-31G.

In order to investigate the binding energy between TLN and the ligand, the solvated TLN–ligand structure was divided into the following three structural domains: TLN–ligand complex containing solvating water molecules (TLN + ligand + water), TLN containing solvating water molecules (TLN + water) and ligand. The structures of TLN + water and ligand were extracted from the optimized structure of TLN + ligand + water. From their total energies (T.E.) obtained by the MFMO calculations, the binding energy (B.E.) between TLN and the ligand mediated by solvating water molecules was estimated as B.E. = T.E. (TLN + ligand + water) − T.E. (TLN + water) − T.E. (ligand). It is noted that the basis set superposition error was neglected in the analysis of binding energy between TLN and the ligands.

In order to elucidate binding affinity between TLN and the ligands quantitatively, binding free energy should be evaluated. However, it is not practical to calculate free energies for the solvated TLN–ligand complexes by *ab initio* FMO method. In the present study, we thus investigated the binding energies between TLN and the ligands to estimate the binding affinity between them qualitatively, on the assumption that the entropic effects for the solvated TLN–ligand complexes are almost similar.

3. Results and discussion

3.1. Optimized structures of solvated TLN–ligand complexes

Of the 12 compounds confirmed as TLN inhibitors in our previous study [29], five were selected and investigated for their specific TLN interactions in the present study. The structures of the five