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Multiple binding modes of a small molecule to human Keap1 revealed by X-ray crystallography and molecular dynamics simulation

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ABSTRACT

Keap1 protein acts as a cellular sensor for oxidative stresses and regulates the transcription level of antioxidant genes through the ubiquitination of a corresponding transcription factor, Nrf2. A small molecule capable of binding to the Nrf2 interaction site of Keap1 could be a useful medicine. Here, we report two crystal structures, referred to as the soaking and the cocrystallization forms, of the Kelch domain of Keap1 with a small molecule, Ligand1. In these two forms, the Ligand1 molecule occupied the binding site of Keap1 so as to mimic the ETGE motif of Nrf2, although the mode of binding differed in the two forms. Because the Ligand1 molecule mediated the crystal packing in both the forms, the influence of crystal packing on the ligand binding was examined using a molecular dynamics (MD) simulation in aqueous conditions. In the MD structures from the soaking form, the ligand remained bound to Keap1 for over 20 ns, whereas the ligand tended to dissociate in the cocrystallization form. The MD structures could be classified into a few clusters that were related to but distinct from the crystal structures, indicating that the binding modes observed in crystals might be atypical of those in solution. However, the dominant ligand recognition residues in the crystal structures were commonly used in the MD structures to anchor the ligand. Therefore, the present structural information together with the MD simulation will be a useful basis for pharmaceutical drug development.

1. Introduction

Living organisms have specific defense systems against various environmental stresses. Of all these stresses, the oxidative stress have been a focus of constant attention in medicine because it is related to many pathologies including cancer [1,2], cardiovascular disease [3,4], diabetes [5,6], neurodegenerative disease [7,8], chronic arthritis [9,10] and aging [11,12]. Understanding of the antioxidant response system is important to develop medical treatments for these pathologies [13]. The antioxidant response is accomplished by the sensing of oxidants and the subsequent activation of antioxidant genes [14]. The sensing of oxidants such as reactive oxygen species and electrophilic xenobiotics is carried out by the Kelch-like ECH-associated protein 1 (Keap1) [15]. On the other hand, the nuclear factor erythroid 2-related factor 2 (Nrf2) protein [16] is responsible for the transcriptional regulation of about 200 antioxidant proteins including many enzymes/transporters for drug metabolism and Nrf2 itself [17]. Keap1 is susceptible to a posttranslational modification by oxidants at certain reactive cysteine residues, allowing Keap1 to sense them [18,19]. Keap1 and other proteins assemble to make the Cullin 3-based E3 ubiquitin ligase complex that binds and ubiquitinates Nrf2. After the ubiquitination, Nrf2 is rapidly degraded by proteasome to keep the lower level of intracellular Nrf2, and therefore the transcription of antioxidant genes is suppressed in the quiescent cells [19,20]. The oxidative-stress-induced modification of Keap1 at specific cysteine residues inhibits the Nrf2 ubiquitination, thereby elevating the intracellular Nrf2 level [19]. As a result, the intact Nrf2 activates the transcription of antioxidant genes through making a complex with the small Maf protein and subsequent binding

Abbreviations: DTT, dithiothreitol; Keap1, Kelch-like ECH-associated protein 1; MD, molecular dynamics; Nrf2, Nuclear factor erythroid 2-related factor 2; PDB, Protein Data Bank

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to a cis-acting DNA sequence termed the antioxidant response element located in the promoter regions of target genes [16].

The Keap1 molecule mainly consists of the N-terminal domain, the C-terminal Kelch domain and the intervening region located in-between the two domains. A single particle analysis of electron microscopy confirmed that Keap1 in solution state forms a homodimer assembled at two cognate N-terminal domains [21]. The N-terminal domain also mediates interactions with other components of the Cullin 3-based E3 ubiquitin ligase complex [22], and contains a cysteine residue Cys151 that senses the oxidative stress [19]. The other sensing cysteine residues Cys273 and Cys288 present in the intervening region [18]. The Kelch domain is responsible for the interaction with Nrf2. As the binding site to Keap1, the ETGE motif located in the N-terminal region of Nrf2 was found first [23]. The DLG motif in the N-terminal region was identified later, as the second binding site required for the ubiquitination/degradation of Nrf2 [24]. Biophysical analyses using nuclear magnetic resonance and isothermal titration calorimetry revealed that the DLG and ETGE motifs interact independently with the Kelch domain by different dissociation constants of 0.5 μM and 8 nM, respectively [25]. Based on analyses in molecular biology, McMahon et al. proposed a two-site interaction model so-called "tethering" mechanism in which two Kelch domains of the Keap1 dimer recognize a single molecule of Nrf2 to facilitate the Cullin-mediated ubiquitination of Nrf2 [26].

These studies imply that a small molecule capable of binding to the Nrf2 interaction site on the Keap1 Kelch domain could be a useful medicine to activate the cellular defense to the oxidative stress through inhibiting the ubiquitination of Nrf2. Although several candidates for such compounds were reported, no one was applied in practical use [27–29]. Precise structural information, for instance, from the X-ray crystallography at high resolution, is indispensable for the structure-based drug design. To date, several crystal structures were reported: the Kelch domain [30], the Kelch domain in complex with the Nrf2 peptide containing the ETGE motif [31,32] or the DLG motif [33,34]. However, structural information of Keap1 complexes with small molecule ligands is still limited; only two complex structures have been reported recently [35,36]. Structural comparison between multiple crystal structures of Keap1 complexes with different small molecule ligands would be useful for the more effective design of Keap1 inhibitors [28]. Here we determined a crystal structure of the Kelch domain of human Keap1 in complex with a small ligand referred to as Ligand1 that has a novel chemical scaffold. Interestingly, two different binding modes were observed in the Keap1–Ligand1 complex crystals.

2. Results and discussion

2.1. Screening and characterization of Ligand1

To search for the candidate compounds, an *in silico* screening was performed on the crystal structure of the Kelch domain of human Keap1 in complex with the ETGE peptide of Nrf2 (PDB entry 2flu) [32]. The in-house and commercially available compounds were docked against the Nrf2 peptide binding site. Based on the docking score and the predicted affinity against Keap1, 65 compounds were selected. Then, the binding ability of these compounds was evaluated using a surface plasmon resonance-based solution assay at 50 μM of the compound concentration. We found 27 active compounds including the LigandX (Fig. 1).

Based on the structural comparison between the docking pose of LigandX and the ETGE peptide of Nrf2 in 2flu, we designed and synthesized the Ligand1 in which the phenol moiety of LigandX was modified by the oxyacetic acid group that intended

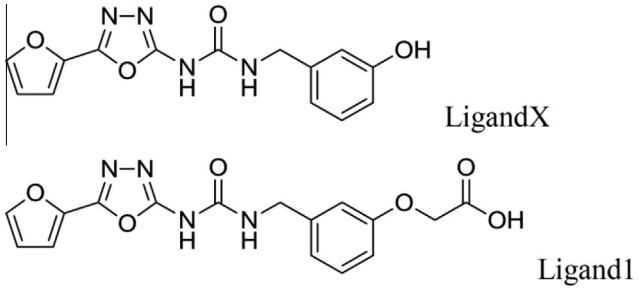

Fig. 1. Chemical structure of ligands.

to mimic the sidechain of the first (N-terminal) glutamic acid residue in the ETGE peptide (see Section 4 for the synthesis of Ligand1). The association constant of Ligand1 to the human Keap1 Kelch domain was estimated as the third to the fourth power of ten from the equilibrium affinity analysis of a surface plasmon resonance-based solution experiment (Fig. 2). Unfortunately, the limited solubility of Ligand1 (less than 1 mM) hampered further analyses on binding properties including the precise association constant, the number of binding sites, and the cooperativity. The Keap1–Ligand1 interaction was also confirmed by another assay using AlphaScreen (PerkinElmer Inc.), a bead-based, amplified luminescent proximity homogeneous assay, which revealed the competitive effect of Ligand1 on the interaction between the Nrf2 peptide and the Kelch domain of Keap1 (Supplemental Fig. S1).

2.2. Quality of crystal structures

Two crystal structures of the human Keap1 Kelch domain in complex with Ligand1, the soaking form and the cocrystallization form, were determined at 2.1 Å resolution (Table 1). In these two forms, the asymmetric unit contained a Kelch domain and a Ligand1 molecule. The final models of the Kelch domain covered the amino-acid residues 322–609 with well-defined electron densities, while the N-terminal 21 residues (20 His-tag residues and Ala321) were not included due to a structural disorder, as observed in the crystal structure of the same Kelch domain reported (PDB entry 1u6d) [30]. The temperature factor (*B*) values calculated from final models (average) were comparable to the Wilson *B* values from corresponding diffraction data. The soaking form crystal was isomorphous to 1u6d with the space group $P6_522$, whereas the cocrystallization form revealed a different crystal packing with the other space group $P2_12_12_1$. The stereochemistry analysis revealed no residue in generously allowed or disallowed regions of the Ramachandran plot, except for Arg336 and His516 in the generously allowed region. These two residues were found in well-defined electron densities without steric clashes. All atoms comprising Ligand1 were identified in electron density maps with reasonable individual *B* values comparable to those of neighboring protein atoms, indicating high occupancy of the ligand (Table 1). Thus we fixed the occupancies of all atoms comprising Ligand1 to 1.0 and did not refine them. In addition, annealed $2F_o-F_c$ omit maps for Ligand1 in both the forms provided clear electron densities comparable to those of corresponding final $2F_o-F_c$ maps (Supplemental Fig. S2), confirming the existence of ligands bound.

2.3. Recognition of Ligand1

The overall structural architecture of the human Keap1 Kelch domain in our structures is the same as that in the PDB entry 1u6d that represents the β-propeller structure composed of a six-fold repeat of all β domain called "blade" [30]. The Rmsd values