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Molecular design of orthogonal stacking system at the complex interface of HtrA PDZ domain with its peptide ligands

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Abstract: The high temperature requirement A (HtrA) protease plays a crucial role in protein quality control and cell fate. The enzyme contains a catalytic protease domain and a regulatory PDZ domain; the latter determines the substrate specificity of the former by specifically binding to the C-terminal hydrophobic stretch of its partner proteins. Previously, a pentapeptide ligand H3C1 was identified as the potential binder of HtrA PDZ domain using phage display technique. Here, an orthogonal π–cation–π stacking system at the crystal domain–peptide complex interface was analysed by integrating theoretical calculations and experimental assays. It was demonstrated that there is a strong (positive) synergistic effect between the two wings of the stacking system; breaking of cation–π interaction in one wing can largely impair the interaction strength of another wing. The π-electron contributes primarily to the synergistic effect, although geometric property is also (marginally) responsible for it. Next, the systematic combinations between the four aromatic amino acids (Phe, Tyr, Trp and His) plus one non-aromatic amino acid (Ala) at the two wings of π–cation–π stacking were investigated. It was found that two aromatic substitutions (Phe-4Tyr and Phe-4Trp) at a wing can considerably and moderately improve peptide affinity by 3.2- and 1.5-fold, respectively, whereas the non-aromatic mutations at each wing and at both of them can significantly reduce the affinity with K_d increase from 1.8 (wild type) to 34 μM and 160 μM (single-point mutations), as well as 210 μM (double-point mutation), suggesting that just breaking of one wing can substantially undermine the synergism of orthogonal π–cation–π stacking.

Keywords: HtrA protease; HtrA PDZ domain; peptide ligand; orthogonal π–cation–π stacking; synergistic effect.

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INTRODUCTION

The high temperature requirement A (HtrA) protease is a key enzyme involved in protein quality control, stress response, and cell fate,[1] which is also functionally related to a variety of biological events such as cell proliferation, migration and apoptosis.[2] The enzyme was initially identified in *E. coli* by two phenotypes of corresponding null mutants and named accordingly. Mutants either did not grow at elevated temperatures (HtrA for high temperature requirement) or failed to digest misfolded protein in the periplasm (DegP).[3] Subsequently, prokaryotic HtrA has been linked to the tolerance against various folding stresses as well as to pathogenicity. The protein is characterized by the presence of a catalytic serine protease domain (PD) followed by a regulatory post-synaptic density 95, Drosophila discs large, zona occludens-1 (PDZ) domain, which has been shown to act as substrate specificity determinant by binding to the C-terminal hydrophobic stretch of its protein partners, leading to structural change in PD domain and enzyme activation.[4]

HtrA PDZ–partner interaction has been recognized as a new and attractive druggable target; molecular disruption of the interaction can inactivate the serine protease, which can be a potential therapeutic strategy for a variety of relevant diseases such as cancer, arthritis, neurodegenerative disorder and macular degeneration.[5,6] Previously, Runyon *et al.* have identified a pentapeptide ligand H3C1 (Phe_{-4}-Gly_{-3}-Arg_{-2}-Trp_{-1}-Val_{0}-COOH) of HtrA PDZ domain using phage display technique.[7] The H3C1 peptide is bound in the active pocket of HtrA PDZ domain to competitively block the domain interaction with its cognate partners. However, the peptide can only bind weakly to the domain with a moderate affinity. Later, Liu *et al.* described a rational halogenation strategy to improve biological activity for the peptide ligand, which introduced a geometrically satisfactory halogen bond at the domain–peptide complex interface by systematically optimizing the combination of halogen types and their substitution positions at the indole moiety of peptide Trp-1 residue.[8,9] However, chemical synthesis of non-natural halogenated peptides is technically sophisticated. In addition, the designed halogen bond is vulnerable to its protein environment as this type of noncovalent force is structurally exquisite and highly specific.[10]

Runyon *et al.* fused H3C1 peptide into the C-terminus of HtrA PDZ domain and then solved the crystal structure of the fusion protein system (PDB: 2P3W),[7] which characterizes a homodimer of two H3C1 peptide-fused HtrA PDZ domains in biological assembly form (Fig. 1A), where the C-terminal H3C1 peptide of one domain is tightly bound to the active pocket of another (Fig. 1B). It was revealed that the H3C1 peptide contains two aromatic residues Trp-1 and Phe-4; they can form an orthogonal π–cation–π stacking with the Arg360 residue of HtrA PDZ domain (Fig. 1C). Previously, we have successfully designed several protein–peptide interactions using computational modeling and affinity assay,

including amphiphysin SH3–PPII peptide[11] and EGFR–mig-6 peptide.[12] Here, an attempt was made to investigate the synergistic effect between the two wings of the stacking by systematically mutating the two aromatic residues to other three natural aromatic amino acids (Phe, Tyr, Trp and/or His) and one non-aromatic amino acid (Ala), as well as their impacts on the domain–peptide binding. Instead of halogenation strategy,[13] the mutation does not introduce non-natural amino acids and thus the designed peptide mutants can be readily obtained *via* standard phase peptide synthesis. In addition, the stacking interaction is insensitive to its protein environment and has only low geometric requirement. These features largely extend the applicability of the mutation strategy in rational peptide design. It was demonstrated that combinatorial optimization of the synergistic effect can effectively enhance the interaction potency of HtrA PDZ domain with its peptide ligands, and fluorescence spectroscopy assays to substantiate the computational findings were performed.

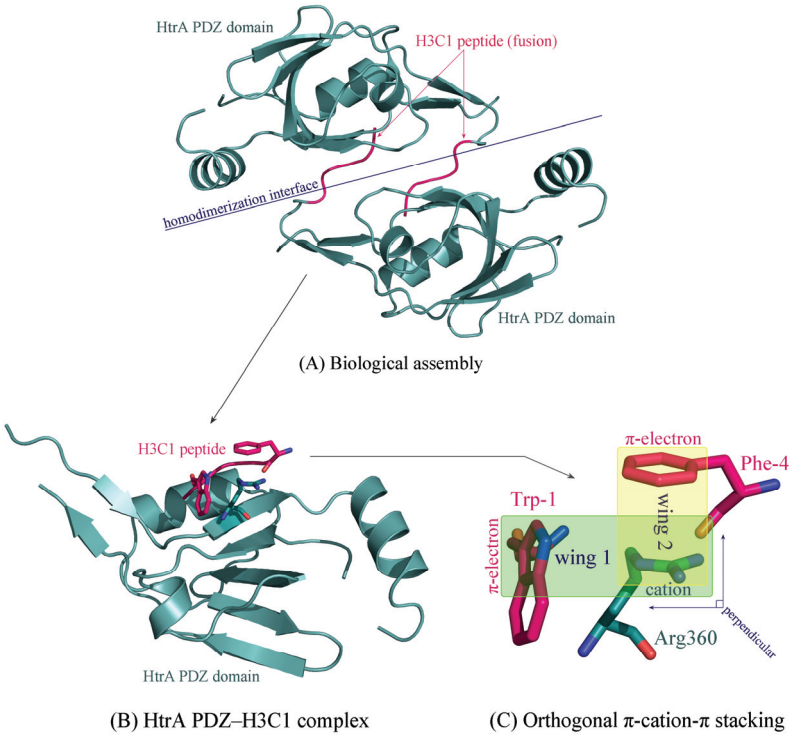

Fig. 1. A) Homodimer of two H3C1 peptide-fused HtrA PDZ domains in the crystal structure of biological assembly (PDB: 2P3W). B) Complex structure of HtrA PDZ domain with H3C1 peptide extracted from the biological assembly. C) An orthogonal π–cation–π stacking is formed across the complex interface by domain positively charged residue Arg360 and peptide aromatic residues Trp-1 and Phe-4. The stacking consists of two approximately perpendicular wings 1 and 2; each represents a cation–π interaction.

EXPERIMENTAL

Geometric description of orthogonal π–cation–π stacking system

The geometric configuration of orthogonal π–cation–π stacking across the complex interface of HtrA PDZ domain with H3C1 peptide is schematically illustrated in Fig. 2. The wings 1 and 2 represent two perpendicular cation–π interactions of domain Arg360 residue with peptide Trp-1 and Phe-4 residues, respectively. The angle between wings 1 and 2 is δ. The guanidyl group of domain Arg360 residue, the indolyl group of peptide Trp-1 residue and the phenyl group of peptide Phe-4 residue are assigned within planes 1, 2 and 3, respectively. The dihedral angle between planes 1 and 2 is θ_1, and between planes 1 and 3 is θ_2. The distance between Arg360 C_ζ atom and Trp-1 aromatic ring center is d_1, and between Arg360 C_ζ atom and Phe-4 aromatic ring center is d_2.

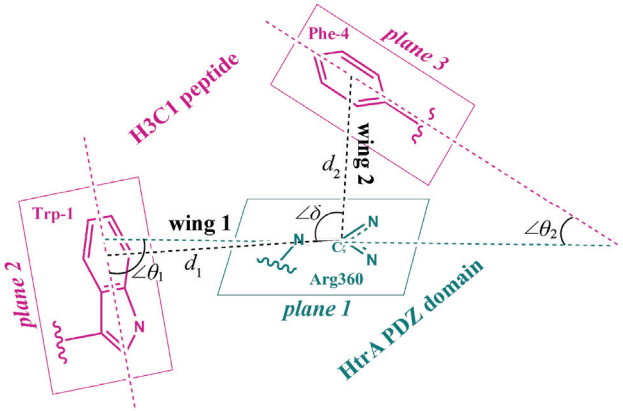

Fig. 2. Schematic representation of the geometric parameters of orthogonal π–cation–π stacking across the complex interface of HtrA PDZ domain with H3C1 peptide.

Quantum mechanics/molecular mechanics analysis

The residues Trp-1 and Phe-4 of H3C1 peptide in crystal complex structure with HtrA PDZ domain (PDB: 2P3W) can be computationally mutated to other aromatic amino acids by using the rotamer-based Scwrl4 program.[14] The mutated complex structures were then subjected to structural optimization with a two-layered hybrid quantum mechanics/molecular mechanics (QM/MM).[15] This method enables different levels of theory to be applied to different parts of a large biomolecular system. The QM/MM calculations were carried out using ONIOM algorithm.[16] A scheme described in our previous work[17] was employed to partition the PDZ–peptide system, that is, the domain cationic residue Arg360 as well as the peptide aromatic residues Trp-1 and Phe-4 involved in the π–cation–π stacking were included in high-level QM layer, while rest of the system was in low-level MM layer. Hydrogen atoms were used as link atoms to saturate the dangling bonds. The QM layer was modeled by the semi-empirical AM1 theory,[18] while the MM layer was described with biomolecular AMBER force field.[19] Other methods such as PM6[20] and B3LYP[21] were not considered here since we have previously demonstrated that the AM1/AMBER hybrid combination is comparable with or even better than that of first principle-based scheme, but has a low computational cost.[22] The QM/MM calculations were carried out with Gaussian 09 suite of programs;[23] the inputs and outputs were prepared/examined using GaussView GUI.[24]

The intermolecular interaction energy (ΔU) of PDZ–peptide complex was estimated with a strategy described by Zhou *et al.*[25-27] This was accomplished by performing single-point energy calculation twice; one on the complex system ($U_{complex}$) and another on the same system but its members (protein and peptide) were separated from each other by a sufficiently large distance (U_{domain} and $U_{peptide}$). In this way, the interaction energy can be expressed as $\Delta U = U_{complex} - (U_{domain} + U_{peptide})$.[28-30] The Poisson-Boltzmann/surface area (PB/SA) method was used to account for solvent effect associated with the PDZ–peptide binding.[31] In PB/SA procedure, the change of total desolvation Gibbs energy (ΔG_{slv}) was estimated from the polar (ΔG_{plr}) and nonpolar (ΔG_{nplr}) desolvation energies. The polar aspect was calculated by finite difference solutions to the nonlinear Poisson–Boltzmann equation in Delphi program,[32] while nonpolar contribution was determined by summing up the weighted surface area of solute molecule, *i.e.*, $\Delta G_{nplr} = \gamma \Delta A$, where $\gamma = 0.0072$ kcal*/(mol·Å^2) and ΔA is the change in solute's surface area upon the PDZ–peptide binding.[33–35]

The change of total binding Gibbs energy of a peptide ligand to its domain receptor can be expressed as follows:[36]

$$\Delta G = \Delta U_{int} + \Delta G_{plr} + \Delta G_{nplr} \quad (1)$$

Ab initio *electron correlation calculation*

The π–cation–π stacking region (*i.e.*, PDZ residue Arg360 and peptide residues Trp-1 and Phe-4) was split from the whole QM/MM-optimized domain–peptide complex system, which was then used to derive the change of interaction energy (ΔE) of each wing of the stacking at a high electron correlation theory level of Møller–Plesset second order perturbation (MP2) in conjunction with a Dunning's correlation consistent basis set aug-cc-pVDZ. This electron-correlation theory has been shown to accurately capture the dispersive energy involved in noncovalent complex system.[37] In addition, the basis set superposition error (*BSSE*) was eliminated by the standard counterpoise method of Boys and Bernardi.[37] The electron correlation calculations were carried out in Gaussian 09 suite of programs.[23]

Fluorescence polarization assay

The H3C1 peptide and its five mutants (see Table I) were synthesized by GBiochem using Fmoc solid-phase chemistry. The binding affinity between the PDZ and peptide was determined using a fluorescence polarization (FP) protocol modified from our previous work.[12] Synthetic peptides were labeled with conjugated fluorescein (FITC). Titrations were performed by monitoring FP as a function of increasing protein amounts of HtrA PDZ domain (residues 354–453) added to 10 μM FITC-peptides in a buffer containing 50 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA and 1 mM dithiothreitol (DTT). FP analysis was measured using a Perkin–Elmer spectrofluorimeter. The dissociation constants (K_d) were determined by fitting titration curves to the equation:

$$F = \frac{F_0 + F_\infty (c_{PDZ} / K_d)}{1 + (c_{PDZ} / K_d)} \quad (2)$$

where the c_{PDZ} is the PDZ protein concentration at each measurement point, F is the observed FP value at a given protein concentration, F_0 is the FP value of free peptide, and F_∞ is the maximal FP value saturated with protein. Each assay was performed in duplicate.

* 1 kcal = 4186 J

RESULTS AND DISCUSSION

Synergistic effect of geometric and energetic properties between two wings

The orthogonal π–cation–π stacking at the complex interface of HtrA PDZ domain with its H3C1 peptide ligand contains two individual cation-π interactions that are approximately perpendicular to each other (δ = 76°). The positively charged guanidyl group of domain residue Arg360 serves as the central cation that can separately form a T-shaped cation–π interaction with the aromatic indolyl group of peptide Trp-1 residue (wing 1) and a parallel cation–π interaction with the aromatic phenyl group of peptide Phe-4 residue (wing 2, Fig. 1). Considering that the two interactions are tightly bound together and share a common cation, they are expected to exert direct influence on each other. Here, the peptide π-electron residues Trp-1 and Phe-4 are separately mutated to non-aromatic amino acid Ala (*i.e.*, Trp-1Ala or Phe-4Ala mutation) to examine the synergistic effect of geometric and energetic properties between the two orthogonal wings. Firstly, the three complex structures of HtrA PDZ domain with wild-type H3C1 peptide (crystal structure) as well as its Trp-1Ala and Phe-4Ala mutants (Scwrl4--modeled structures) were separately subjected to QM/MM optimization to reach at conformation-refined state. Next, the π–cation–π stacking region was stripped from the optimized complex structures, and the interaction energies ΔE of domain residue Arg360 with peptide residues -1 (Trp-1 or Ala-1) and -4 (Phe-4 or Ala-4) were separately calculated using *ab initio* electron correlation MP2/aug--cc-pVDZ theory with BSSE correction.

The impacts of peptide mutation Phe-4Ala (in wing 2) or Trp-1Ala (in wing 1) on the change of interaction energy, ΔE, distance d and dihedral angle θ of wings 1 and 2 are investigated, respectively. As can be seen, mutation of aromatic residue to Ala in one wing would considerably impair the interaction strength of another wing, although the mutation is not involved in the calculated wing itself. For example, the peptide residue Trp-1 only participates in the cation–π interaction of wing 1, but the interaction energy of wing 2 is reduced largely upon the Trp-1Ala mutation, with ΔE_2 change from –6.7 to –4.2 kcal/mol for wing 2. A similar phenomenon can be observed in wing 2 Phe-4Ala mutation on wing 1 interaction energy (with ΔE_1 change from –4.8 to –2.9 kcal/mol). In addition, the two mutations Phe-4Ala (in wing 2) and Trp-1Ala (in wing 1) have also a moderate effect on the geometric configuration of non-self-wing; which causes the wing distance to increase from 5.64 to 6.37 Å (d_1) and from 4.12 to 4.97 Å (d_2), respectively, and induces the wing dihedral angle shifting from 72 to 67° (θ_1) and from 12 to 16° (θ_2), respectively. Both the energetic and geometric analyses suggest that there is a strong synergistic effect between the two wings of orthogonal π–cation–π stacking, that is to say, presence of cation-π interaction in one wing can substantially enhance the interaction strength in another wing. This

is expected because the π-electron conjugated effect and delocalization energy can be maximized only by forming the complete stacking system.

The synergistic effect can also be found in the binding of peptide ligand to HtrA PDZ domain. The change of total binding Gibbs energies, Δ*G*, of wild-type C3H1 peptide and its two single-point mutations Trp-1Ala and Phe-4Ala as well as one double-point mutation Trp-1Ala/Phe-4Ala were calculated with QM/MM and PB/SA. As shown in Fig. 3, the wild-type peptide binds to domain with Δ*G* of –12.5 kcal/mol, and the single-point mutation can considerably impair the binding potency to –8.4 (Trp-1Ala) or –9.1 (Phe-4Ala) kcal/mol. The Trp-1 residue should not only participate in cation–π interaction, but also tightly pack against its surroundings to form other nonbonded chemical forces such as van der Waals and hydrophobic contacts, although the wing 1 seems to be tighter than wing 2 as the former is formed by a short, parallel cation–π stacking, while the latter is defined by a long, T-shaped stacking. Simultaneous double-point mutation of the two residues (Trp-1Ala/Phe-4Ala) can further reduce the binding potency to –8.0 kcal/mol, which is quite modest relative to single-point mutations, suggesting that just a single mutation on one wing can substantially undermine the synergism of π–cation–π stacking.

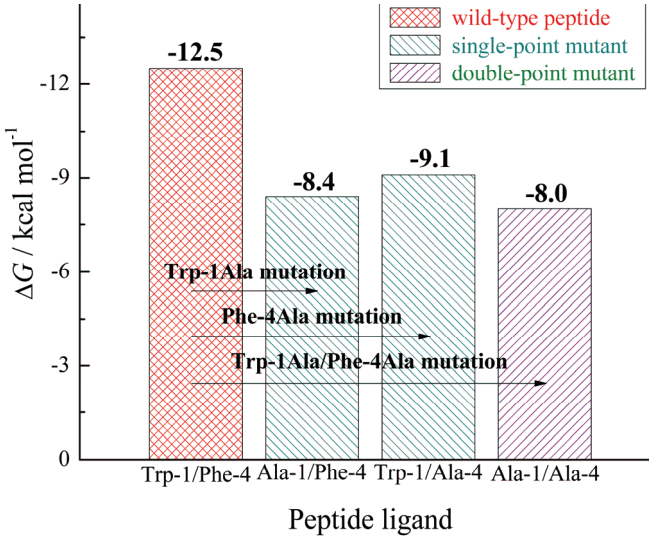

Fig. 3. The impacts of peptide single-point mutation Trp-1Ala or Phe-4Ala and double-point mutation Trp-1Ala/Phe-4Ala on the change of total binding Gibbs energy, Δ*G*, of peptide ligands to HtrA PDZ domain.

Combinatorial optimization of the synergistic effect between two wings

In order to explore the synergistic effect on domain–peptide binding, the systematic combinations between the four aromatic amino acids (Trp, Phe, Tyr and

His) plus one non-aromatic amino acid (Ala) at the residues -1 and -4 of C3H1 peptide were investigated, totally resulting in 25 (5×5) peptide ligands, in which one is wild-type C3H1 peptide, fifteen are peptide mutants with aromatic amino acid substitutions at one or both of the residues –1 and –4 (and thus can form the complete π–cation–π stacking), eight are peptide single-point mutations with an aromatic substitution at one residue and a non-aromatic Ala substitution at another (and thus can only form one wing cation–π interaction), and one is a peptide double-point mutation with two Ala substitutions at both the residues -1 and -4 (and thus cannot form any wing cation-π interaction). These mutant domain––peptide complex structures were computationally modeled using Scwrl4 method[14] based on the crystal structure of HtrA PDZ domain in complex with wild-type C3H1 peptide (PDB: 2P3W), refined with QM/MM optimization, and their binding energies (ΔG) were then derived by QM/MM and PB/SA analyses.

The systematic peptide binding energy profile is visualized as a heatmap in Fig. 4A. At first glance, a distinct difference between the 16 aromatic-substituted peptides (surrounded by a frame in the heatmap) and others 9 Ala-substituted peptides; the former generally has higher binding potency as compared to the latter, imparting that formation of the complete π–cation–π stacking is critical for domain–peptide binding, and breaking of just one wing cation–π interaction would largely impair the peptide binding potency due to the synergistic effect between two wings. At residue-1, the Trp is most favorable for the binding. A previous competitive assay also identified that the wild-type Trp-1 residue plays a crucial role in the domain–peptide recognition,[7] indicating that the residue should

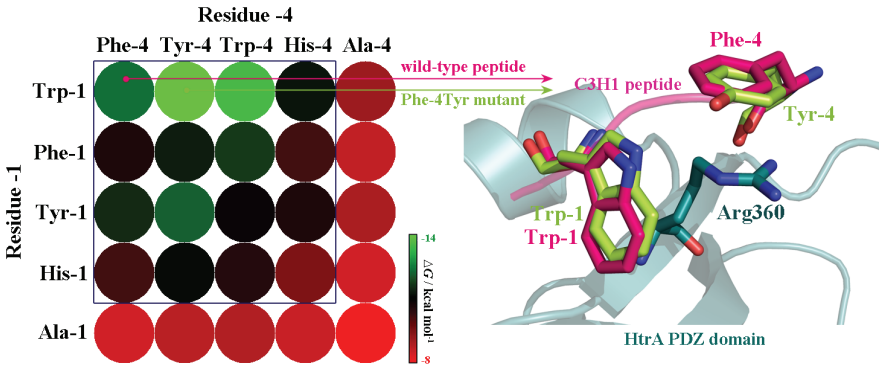

Fig. 4. A) Heatmap of peptide binding energy profile regarding the systematic combinations between the four aromatic amino acids (Trp, Phe, Tyr and His) plus one non-aromatic amino acid (Ala) at the residues -1 and -4 of C3H1 peptide. The block surrounded by a frame represents those aromatic amino acid combinations that can form the orthogonal π–cation–π stacking. B) Superposition of the residues Trp-1 and Tyr-4 of affinity-improved peptide Phe-4Tyr mutant onto the residues Trp-1 and Phe-4 of wild-type C3H1 peptide. The structures of both the wild-type and mutant domain–peptide complexes are refined by QM/MM optimization.

not only participate in π–cation–π stacking, but also interact with its protein environment to provide additional contribution to the binding. For residue –4, the Tyr and Trp substitutions can improve peptide binding capability from wild-type Phe. Tyrosine has a polar phenolic hydroxyl group, while tryptophan is π-electron rich system; both of them can effectively interact with the guanidinium cation of domain Arg360 residue. The His substitution at either residue -1 or -4 would reduce peptide affinity, albeit the decreased affinity is moderate, suggesting that histidine is not a good choice for the π–cation–π stacking system since its imidazolyl moiety may be partially protonized (and thus cause electrostatic repulsion with Arg 360 cation).

Next, the binding affinity of wild-type C3H1 peptide and its two predicted affinity-improved peptide mutants Phe-4Tyr and Phe-4Trp as well as its three Ala-substituted mutants Trp-1Ala, Phe-4Ala and Trp-1Ala/Phe-4Ala (served as controls) to the recombinant protein of HtrA PDZ domain was determined using fluorescence polarization assays (Fig. 5). The obtained affinity K_d values as well as calculated geometric and energetic parameters for the π-cation-π stacking of these domain–peptide complexes are listed in Table I.

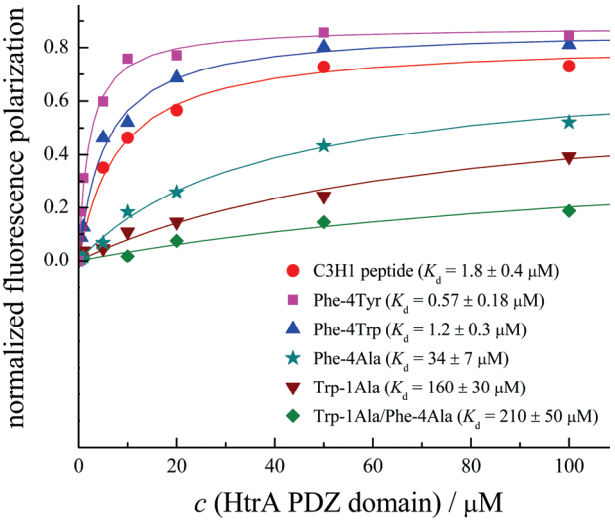

Fig. 5. The normalized binding curves of wild-type C3H1 peptide and its five designed mutants to HtrA PDZ domain.

As might be expected, the wild-type peptide can bind to domain with a moderate affinity (K_d = 1.8 μM), which is basically in line with a previously reported value (K_d = 1.1 μM) for the peptide measured using isothermal titration calorimetry (ITC).[7] The Phe-4Tyr and Phe-4Trp mutations can considerably and moderately improve the affinity by 3.2-fold and 1.5-fold (K_d change from 1.8 to 0.57 and 1.2 μM), respectively; this is basically consistent with theoretical prediction

(ΔG change from –12.5 to –14.2 and –13.7 kcal/mol, respectively). However, separate breaking of wings 1 and 2 can largely reduce peptide affinity with K_d change from 1.8 (wild type) to 160 μM and 34 μM (single-point mutations Trp--1Ala and Phe-4Ala), respectively, and simultaneous breaking of the two wings can further (moderately) reduce the affinity to 210 μM (double-point mutation Trp-1Ala/Phe-4Ala), confirming that the completeness of π–cation–π stacking system is fundamentally important to guarantee the high affinity of peptide ligands.

TABLE I. Geometric and energetic parameters of orthogonal π–cation–π stacking system formed across the complex interface of HtrA PDZ domain with wild-type C3H1 peptide and its five designed mutants

Wing 1				Wing 2				δ	ΔG[b]	K_d[c]
Residue -1	d_1 Å	θ_1 °	ΔE_1[a] kcal mol^{-1}	Residue -4	d_2 Å	θ_2 °	ΔE_2[a] kcal mol^{-1}	°	kcal mol^{-1}	μM
Trp-1[d]	5.64	72	–4.7	Phe-4[d]	4.12	12	–6.7	76	–12.5	1.8±0.4
Trp-1	5.37	75	–5.2	Tyr-4	3.98	14	–7.6	83	–14.2	0.57±0.18
Trp-1	5.49	70	–5.0	Trp-4	4.03	19	–7.2	79	–13.7	1.2±0.3
Trp-1[e]	6.37	67	–2.9	Ala-4[e]	–	–	–2.1	–	–9.1	34±7
Ala-1[e]	–	–	–1.3	Phe-4[e]	4.97	16	–4.2	–	–8.4	160±30
Ala-1[f]	–	–	–1.1	Ala-1[f]	–	–	–1.7	–	–8.0	210±50

[a]ΔE, the interaction energy of HtrA PDZ domain residue Arg360 with peptide residue -1 or -4, calculated using *ab initio* electron correlation MP2/aug-cc-pVDZ with BSSE correction; [b]ΔU, the total binding energy of peptide ligand to HtrA PDZ domain, calculated using QM/MM and PB/SA; [c]K_d, the binding affinity (dissociation constant), determined by fluorescence spectroscopy assay; [d]wild-type C3H1 peptide; [e]control (only one wing cation–π interaction formed); [f]control (no wing cation–π interaction formed)

The geometric profile is changed moderately as compared to energetic and affinity properties upon the mutation, where the distances d_1 and d_2 seem to vary more significantly than dihedral angles θ_1 and θ_2. It is worth noting that the angles δ (76, 83 and 79°) of three π–cation–π stackings in wild-type peptide as well as its Phe-4Tyr and Phe-4Trp mutants are all roughly close to 90°, indicating that the orthogonality of these stacking systems can be maintained during the aromatic substitution. Superposition of the residues Trp-1 and Tyr-4 of high-affinity peptide Phe-4Tyr mutant onto the residues Trp-1 and Phe-4 of wild-type peptide is shown in Fig. 4B. As can be seen, the location and configuration of the two residues are well overlapped between the two peptides, although the mutation considerably improves peptide affinity K_d from 1.8 to 0.57 μM, indicating that the stacking geometry and orthogonality are not vulnerable to the affinity-improved mutation.

CONCLUSION

An orthogonal π–cation–π stacking system was identified at the co-crystallized complex interface of HtrA PDZ domain with its pentapeptide ligand C3H1. The stacking system was characterized rigorously *via* crystal structure dissection,

QM/MM calculation and energetics analysis, revealing a strong synergistic effect involved in the system; breaking of one cation–π interaction would largely impair the interaction strength of another. The π-electron contributes primarily to the synergistic effect, although geometric property is also responsible for it. A number of C3H1 mutants were designed based on the computational investigations, from which two mutants were identified to have increased affinity relative to the wild-type peptide, which may be used as lead molecular entities to develop HtrA PDZ-targeted agents for disease therapy.

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ИЗВОД

МОЛЕКУЛСКИ ДИЗАЈН ОРТОГОНАЛНОГ СИСТЕМА ПАКОВАЊА НА ДОДИРНОЈ ПОВРШИНИ ИЗМЕЂУ HtrA PDZ ДОМЕНА И ЊЕГОВИХ ПЕПТИДНИХ ЛИГАНАДА

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Протеаза А зависна од високе температуре (HtrA) има кључну улогу у контроли квалитета протеина и судбине ћелије. Ензим се састоји од каталитичког протеазног домена и регулаторног PDZ домена, који одређује супстратну специфичност протеазе након везивања С-терминалног хидрофобног дела протеина-партнера. Пентапептидни лиганд H3C1 је раније идентификован као потенцијални везујући партнер за HtrA PDZ домен користећи технику експримирања у фагу. У овом раду је анализиран ортогонални π-катјон–π систем паковања на међуповршини кристалног комплекса домен–пептид користећи теоријска израчунавања и експерименталне податке. Показано је да постоји јак (позитиван) синергистички ефекат између два крака система паковања; прекид у катјон–π интеракцији у једном краку у великој мери спречава интеракцију у другом. π-Електрон је најодговорнији за синергистички ефекат, мада и геометрија има делимичан допринос. Даље је систематично испитана комбинација четири ароматичне аминокиселине (Phe, Tyr, Trp и His) и једне неароматичне (Ala) на два крака π–катјон–π паковања. Нађено је да присуство две ароматичне аминокиселине (Phe-4Tyr и Phe-4Trp) на краку може повећати афинитет према пептиду 3,2, односно 1,5 пута, док мутације са неароматичним аминокиселинама на једном или оба краја доводе до значајног смањења афинитета, повећавајући K_d од 1,8 (немутирана форма) до 34 μM и 160 μM (једна тачкаста мутација), односно 210 μM (две мутације), указујући да промена у једном краку може значајно умањити синергизам ортогоналног π–катјон–π паковања.

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