Single Amino Acid Substitution in the Vicinity of a Receptor-Binding Domain Changes Protein–Peptide Binding Affinity

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INTRODUCTION

Understanding specific protein–peptide binding reactions and the corresponding structure–activity relationship (SAR) is a key to understanding and controlling protein–protein interactions and the associated signal pathways. Typically, through combinations of X-ray-based structural characterization and suitable affinity assays such as phage display,1,2 one-bead-one-compound (OBOC) screening,3 or synthetic peptide combinatorial library (SPCL),4 one can identify a short sequence of amino acids on a peptide that serves as the receptor-binding domain (RBD) to a protein receptor, such that substituting any amino acids on a peptide that serves as the receptor-binding domain on a peptide affects its binding to a protein. Replicates of 802 linear peptides consisting of the variants of WTHPQFAT and LQWHPPAGK, GKFPIPLGKQSG, and NGQFQVWIPGAQK, different by one amino acid, were synthesized on a glass slide with a maskless photolithography. Using a microarray-compatible label-free optical sensor, we measured the binding curves of streptavidin with the synthesized peptides and extracted the streptavidin–peptide affinity constants. We found that (a) the substitution of one residue in the HPQ motif reduces the affinity constant \( K_a \) from \( 10^8 \) M\(^{-1} \) by at least 3–4 orders of magnitude, with an exception of HPM; (b) substitution of the immediate flanking residue on the Gln side also causes the affinity to decrease by up to 3–4 orders of magnitude, depending on the substituting residue and the second-neighboring flanking residue; (c) substitution of the flanking residues on the His side has no significant effect on the affinity, possibly due to the strong binding of streptavidin to HPQF and HPQAG motifs. We also found that some of amino acid residues located close to the C-terminus (and the solid surface) improve the yield of peptide synthesis on a glass surface and can be exploited in the fabrication of peptide microarrays.

Substituting single amino acid residues in/around the binding domain on a peptide changes affinity of a protein to the peptide

ABSTRACT: Using a microarray-based assay, we studied how the substitution of amino acids in the immediate vicinity of the receptor-binding domain on a peptide affects its binding to a protein. Replicates of 802 linear peptides consisting of the variants of WTHPQFAT and LQWHPPAGK, GKFPIPLGKQSG, and NGQFQVWIPGAQK, different by one amino acid, were synthesized on a glass slide with a maskless photolithography. Using a microarray-compatible label-free optical sensor, we measured the binding curves of streptavidin with the synthesized peptides and extracted the streptavidin–peptide affinity constants. We found that (a) the substitution of one residue in the HPQ motif reduces the affinity constant \( K_a \) from \( 10^8 \) M\(^{-1} \) by at least 3–4 orders of magnitude, with an exception of HPM; (b) substitution of the immediate flanking residue on the Gln side also causes the affinity to decrease by up to 3–4 orders of magnitude, depending on the substituting residue and the second-neighboring flanking residue; (c) substitution of the flanking residues on the His side has no significant effect on the affinity, possibly due to the strong binding of streptavidin to HPQF and HPQAG motifs. We also found that some of amino acid residues located close to the C-terminus (and the solid surface) improve the yield of peptide synthesis on a glass surface and can be exploited in the fabrication of peptide microarrays.

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show that such an assay platform is useful for exploring the amino acid residues outside the RBD on a peptide for desirable as well as undesirable binding behaviors with a specific protein receptor. Our main finding is that the flanking residues up to the second neighbor can have a profound influence on the stability of a protein–peptide complex depending on the chemical identity and the structural attribute of the residues.

Peptide ligands to streptavidin are typically discovered through the phage library screening or one-bead-one-compound (OBOC) screening, followed by microcalorimetry and X-ray crystallographic studies of those with high affinity to protein. High-affinity ligands usually contain common motifs such as HPQ or HPM that bind to the same pocket to which biotin binds. The affinity of the HPQ-containing peptides has been shown, albeit in a limited way, to depend on the peptide conformation and the flanking residues. For example, cyclic peptides are found to have affinity that is orders of magnitude higher than that of their linear counterparts due to a smaller entropic cost of the complex formation; the stability of streptavidin–HPQF motif benefits from the hydrophobic interaction of the Phe residue with a likewise pocket on streptavidin; the binding to a HPQN motif is assisted by Asn that forms extra H-bonds; whereas the streptavidin–HPQGG complex is strengthened by the flexibility of the Pro–Pro sequence that facilitates an optimal peptide conformation.

To systematically explore the roles of flanking residues outside the RBD in a peptide, we use a real-time-detected synthetic peptide microarray platform. Although the throughput of 10^5–10^8 per microarray is lower than that of the phage display or the OBOC screening assays, a lithographically fabricated peptide microarray has systematically modified and spatially registered peptide targets on a solid surface and enables the real-time detection of the protein–peptide association and dissociation during the reactions. As a result, one can measure the peptide–protein affinity over a large dynamic range.

**Materials and Procedures**

**Microarray Synthesis and Layout.** The peptide microarray was synthesized with the maskless photolithography on glass slides by NimbleGen Inc. The array consists of 4524 square features in a 52 × 87 arrangement. Each feature has a size of 108 × 108 μm². The gap between the features is also 108 μm. The entire microarray occupies a rectangular area of 11.1 × 18.7 mm². The feature size is chosen to ensure that the optical scanner (as described later) with a beam width of 6 μm adequately resolves the features. In each feature, a variant of the following four peptides is synthesized: WTHPQPAT (linear octapeptides), LQWHPQAAGK (linear nonapeptides), GKFIPPLGKQS, and NGQFQVWPAGQK. The last two peptides do not contain the HPQ motifs but are included as negative controls. In all of the cases, the C-terminus is at the right end of the sequence. Each variant is different from the original peptide by only one amino acid. Twenty natural amino acids are used to produce variants: Ala (A), Arg (R), Asn (N), Asp (D), Cys (C), Gln (Q), Glu (E), Gly (G), His (H), Ile (I), Leu (L), Lys (K), Met (M), Phe (F), Pro (P), Ser (S), Thr (T), Trp (W), Tyr (Y), and Val (V). For the original sequences, there are 65 replicates of WTHPQPAT, 36 replicates of LQWHPQAAGK, 48 replicates of GKFIPPLGKQS, and 52 replicates of NGQFQVWPAGQK in the microarray. For variants, four replicates of each are synthesized in “random” locations in the microarray. In total, we have 802 distinct peptide variants, including the original sequences, and a total of 3537 peptide replicates of interest. The remaining features are included for alignment and fabrication controls.

**Streptavidin–Peptide Binding Reaction Procedures.** The glass slide bearing a peptide microarray is assembled with a custom fluidic chamber. The assembly is initially filled with 1× phosphate-buffered saline (PBS). To block the portion of the glass surface that has no fabricated features, we incubate the microarray in 2 μM of bovine serum albumin (BSA) solution in 1× PBS for 30 min. From the real-time binding curve measurements, we observe no evidence of BSA binding to the glass surface, indicating that the feature-free part of the glass surface is protected from nonspecific protein binding. For the reaction with streptavidin at room temperature, we incubate the microarray in fresh 1× PBS for 10 min to obtain a baseline for binding curves. We then replace the buffer with a 3 μM solution of streptavidin in 1× PBS and incubate the microarray under the flow condition for 60 min (the association phase); afterward, we replace the streptavidin solution with 1× PBS again and incubate under a flow condition for another 120 min (the dissociation phase).

**Microarray-Compatible Label-free Optical Detection.** To measure 4524 association–dissociation curves during the reactions of streptavidin with the synthetic peptide microarray having as many features, we use an ellipsometry-based label-free sensor (oblique-incidence reflectivity difference (OI-RD)) that has been described previously. With this sensor, we measure the phase change in a reflected optical beam due to the presence of a biomolecular layer on a solid support during the reaction. Similar to a surface-plasmon-resonance (SPR) sensor, the phase change in OI-RD detection is proportional to the surface mass density Γ of the biomolecular layer. The advantage of an OI-RD-based sensor is that it is compatible with microarray-based assays and capable of simultaneously detecting in real time tens of thousands reactions on a solid support. The label-free feature of the detection also enables one to examine the quality of the microarray before the reaction for analysis and optimization.

Figure 1 shows the arrangement of an OI-RD-based detection of a peptide microarray in a fluidic chamber. The differential phase change δ is proportional to the surface mass density Γ′ with a proportionality constant C = −1.1 × 10^4 rad cm²/g, assuming that the volume mass density and refractive indices of peptides and protein probes are independent of the amino acid composition.

We acquire an OI-RD image at 10 μm steps in both x and y direction. Figure 2a shows the image of a fresh peptide microarray in 1× PBS before the reaction. Four replicates of a peptide variant are highlighted. For the end points of the streptavidin reactions with the synthesized peptides, we capture the OI-RD images of the microarray before the association phase and after the dissociation phase of the reaction. We take the difference as the measure of captured streptavidin or, more precisely, nondissociated streptavidin–peptide complexes after 2 h. This is shown in Figure 2b. It reveals the amount of captured streptavidin by those peptides with sufficiently high affinity.

For the association–dissociation curve measurements, we record the signals only from the center of each feature and a point midway between the two neighboring features (a reference pixel). We take the difference between the signal from a peptide variant and the average of the signals from the two neighboring reference pixels at one time point. Each time
step takes 18 s. By scanning the microarray repeatedly every 18 s, we acquire the association–dissociation curves for all of the 4254 peptide variants and controls; 1393 of them are variants of WTHPQFAT and LQWHPQAGK. Figure 3 shows the association–dissociation curves for the three variants of LQWHPQAGK. For each of the 802 peptide variants, regardless of whether it forms a stable complex with streptavidin that survives the dissociation phase, we have a set of association–dissociation curves acquired from its replicates. We globally fit the curve set to one-site Langmuir reaction model. From the fit, we obtain the affinity constant given by $K_a = k_{on}/k_{off}$. We use $K_a$ instead of the end points of the reaction in our analysis because $K_a$'s obtained from the binding curve sets have a much larger dynamic range and include those reactions with large dissociation rate constants and are, thus, easily missed in the differential image (Figure 2b).

### RESULTS

Figure 4 displays the affinity constants of 153 variants of WTHPQFAT with streptavidin. There are eight panels, and each panel displays $K_a$ of the 20 variants that are different by one amino acid at a fixed location on WTHPQFAT. For example, the third panel shows the affinity constants of those peptides that have the form of WT(X)PQFAT, with X being one of the 20 natural amino acids. Figure 5 displays the affinity...
constants of 172 variants of LQWHPQAGK, and there are nine panels for as many residue locations on the peptide. The error bar on each data point is the variance obtained from four or more duplicate targets of the same peptide sequence. When the captured streptavidin is below the detection limit in the present study, only the upper bound is reported. For a few peptide ligands, the dissociation rates $k_{off}$ are below the detection limit; in these cases, we report only the lower bound of $K_a$.

When a peptide sequence is synthesized on a solid support, the motif of interest is purposely located at a distance from the surface through a spacer (in this case, a few nonfunctional amino acids) to minimize the effect of the surface. Figures 4 and 5 show that as few as two to three 3 amino acid residues are sufficient to buffer the motif from the presence of the solid surface, indicating that the flanking residues beyond the second neighbor do not have a significant effect on the peptide–protein binding. We now examine the details revealed by these two figures.

**HPQ** is the Receptor-Binding Domain (RBD) of a Linear Peptide for Complex Formation with Streptavidin.
Article

Stable Complex Formation of Streptavidin with Peptides Containing HPQF and HPQAG. Panels (1) and (2) in Figure 4 show that the substitution of the first and second flanking residues on the His side of the HPQ motif does not significantly change the affinity of (XX)HPQFAT. Similarly, panels (1) and (2) in Figure 5 show that the substitution of the first and second flanking residues on the His side of the HPQ motif has little effect on the affinity of L(XX)HPQAGT. One of the two possibilities is at work here: the substitution of the flanking residues on the His side generally has no substantial effects on the affinity of a HPQ-containing linear peptide to streptavidin, or HPQF and HPQAG motifs provide the needed stability of a linear peptide with the protein so that other residues, including the flanking residues on the His side, are rendered insignificant in affecting the complex formation. From the studies reported by Giebel et al.15 and Lam et al.,3 both possibilities seem to be at work.

First- and Second-Neighboring Flanking Residues Adjacent to His of the HPQ Motif Have Little Effect on Stable Complex Formation of Streptavidin with Peptides Containing HPQF and HPQAG. Panels (1) and (2) in Figure 4 show that the substitution of the first and second flanking residues on the His side of the HPQ motif does not significantly change the affinity of (XX)HPQFAT. Similarly, panels (1) and (2) in Figure 5 show that the substitution of the first and second flanking residues on the His side of the HPQ motif has little effect on the affinity of L(XX)HPQAGT. One of the two possibilities is at work here: the substitution of the flanking residues on the His side generally has no substantial effects on the affinity of a HPQ-containing linear peptide to streptavidin, or HPQF and HPQAG motifs provide the needed stability of a linear peptide with the protein so that other residues, including the flanking residues on the His side, are rendered insignificant in affecting the complex formation. From the studies reported by Giebel et al.15 and Lam et al.,3 both possibilities seem to be at work.

First- and Second-Neighboring Flanking Residues Adjacent to Gln of HPQ Have Profound Effects on Formation of Peptide–Streptavidin Complexes. From panels (6) and (7) of Figure 4 and panels (1) and (2) of Figure 5, we can conclude that the substitution of any one residue in the HPQ motif (except for HPM) causes the affinity constant to decrease from $10^8$ M$^{-1}$ to below $10^5$ M$^{-1}$ (the detection limit under the current assay condition). This reaffirms what is known in the literature that HP(Q/M) are necessary for a peptide to bind strongly to streptavidin at the pocket where a biotin binds.

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Figure 5. Affinity constants of 172 variants of LQWHPQAGK to streptavidin. The unit for the horizontal axes is log$_{10}$(K) and K is in M$^{-1}$. Panel (1) lists those for (X)QWHPQAGK; panel (2) for L(X)WHPQAGK; panel (3) for LQ(X)HPQAGK; panel (4) for LQW(X)PQAGK; panel (5) for LQW(H)XQAGK; panel (6) for LQWH(X)QAGK; panel (7) for LQWHP(X)AGK; panel (8) for LQWHPA(X)K; and panel (9) for LQWHPQA(X)K. X is one of the 20 natural amino acids.
DISCUSSION

The roles of flanking residues outside the HPQ motif in the complex formation of peptides with streptavidin have been reported and discussed in the literature.\textsuperscript{5,6,15} By screening the cyclic hexa/hepta/octapeptide phage libraries, Giebel and co-workers found that (a) isolated peptides (in solution phase as opposed to those attached to a substrate such as a solid support) that strongly bind to streptavidin all have an HPQ motif; (b) the HPQ motif is almost always immediately adjacent to the N-terminal cysteine; and (c) these cyclic peptide ligands have either HPQ(G, F, V, N) motifs or HPQ(SG) motif. For hexapeptides, only CHPQFC is found after three rounds of biopanning. The fact that cyclic hepta- and octapeptide ligands almost always have the HPQ sequence close to the N-terminal was attributed by these authors to the constraint on the short cyclic peptides and the need for an optimal display of HPQ side chains. In the light of our present study, it seems equally likely (if not more so) that cyclic hepta- and octapeptide ligands need to have suitable flanking residues adjacent to the Gln side to have peptide streptavidin complexes. This can be accommodated only if the HPQ sequence is close to the N-terminal cysteine, making room for flanking residues such as F, G, V, N, and SG on the Gln side. This explanation is consistent with our observation that linear peptides with HPQ(G, F, V, N) motif or HPQ(SG) motif and yet no constraint brought about by the cyclic structure also bind to streptavidin with high affinity. It is also consistent with our observation that flanking residues on the His side seem less important than the residues on the Gln side in stabilizing the complexes.

Specific to our present microarray-based study, we found that linear peptides containing HPQ(F, G, N, V) motifs form stable complexes with streptavidin as their cyclic counterparts. In addition, we found that the linear peptides containing HPQ(A) motif also form stable complexes with streptavidin—a motif not reported in previous studies. Furthermore, similar to the observation by Giebel et al. on short cyclic peptides,\textsuperscript{15} we found that linear peptides containing HPQ(SG) motif form stable complexes with streptavidin as well. More importantly, we discovered the reason for the high affinity of HPQ(SG) motif: as the second flanking residue on the Gln side, the Gly residue acts to stabilize the complex formation of streptavidin with HPQ(SG) and nine other linear peptides with HPQ(D, E, I, L, M, N, Q, R, T, W, Y)G motifs. It seems that HPQF and HPQAG motifs sufficiently stabilize the peptide—streptavidin complexes, rendering the flanking residues on the His side irrelevant. In the absence of suitable flanking residues on the Gln side, Lam et al. found 12 HPQ-containing linear pentapeptide ligands to streptavidin with flanking residues (MY, RE, IQ, GN, TV, IG, WM, GA, PL, AI, AA, and TP) only on the His side.\textsuperscript{3} Their observation indicates that the stabilizing

Figure 6. Surface mass densities (in unit of $10^{-7}$ g/cm$^2$) of 153 variants of WTHPQFAT synthesized on a glass slide. Panel (1): (X)THPQFAT; panel (2): W(X)HPQFAT; panel (3): WT(X)PQFAT; panel (4): WTH(X)QFAT; panel (5): WTHP(X)FAT; panel (6): WTHPQ(X)AT; panel (7): WTHPQF(X)T; and panel (8): WTHPQFA(X). X is one of the 20 natural amino acids.
Table 1. Summary of the mean equilibrium dissociation constants $K_d$ (in M) of 172 variants of LQWHPQAGK synthesized on a glass slide. Panel (1): (X)QWHXHPQAGK; panel (2): L(X)WHXHPQAGK; panel (3): LQ(X)HPQAGK; panel (4): LQW(X)HPQAGK; panel (5): LQWH(X)AGK; panel (6): LQXHP(X)AGK; panel (7): LQWHXHPQ(X)GK; panel (8): LQWHXHPQ(X)K; and panel (9): LQWHXHPQAGK. X is one of the 20 natural amino acids.

| Flanking Residue | $K_d$ (M) |
|------------------|-----------|
| (X)QWHXHPQAGK    | 10$^{-7}$ |
| L(X)WHXHPQAGK    | 10$^{-7}$ |
| LQ(X)HPQAGK      | 10$^{-7}$ |
| LQW(X)HPQAGK     | 10$^{-7}$ |
| LQWH(X)AGK       | 10$^{-7}$ |
| LQXHP(X)AGK      | 10$^{-7}$ |
| LQWHXHPQ(X)GK    | 10$^{-7}$ |
| LQWHXHPQ(X)K     | 10$^{-7}$ |
| LQWHXHPQAGK      | 10$^{-7}$ |

Figure 7. Surface mass densities (in unit of 10$^{-7}$ g/cm$^2$) of 172 variants of LQWHPQAGK synthesized on a glass slide. Panel (1): (X)QWHXHPQAGK; panel (2): L(X)WHXHPQAGK; panel (3): LQ(X)HPQAGK; panel (4): LQW(X)HPQAGK; panel (5): LQWH(X)AGK; panel (6): LQXHP(X)AGK; panel (7): LQWHXHPQ(X)GK; panel (8): LQWHXHPQ(X)K; and panel (9): LQWHXHPQAGK. X is one of the 20 natural amino acids.

Effect of these flanking residues on the His side (not encountered in our present study) is also significant.

**Constraints on Linear Peptides Synthesized on a Solid Support Increases the Affinity to Streptavidin Due to Reduced Entropy Cost.** Affinity constants $K_d$ for the majority of synthesized linear peptides that bind to streptavidin are between 10$^6$ and 10$^9$ M$^{-1}$. This means that the equilibrium dissociation constants $K_d$ are between a few nM and 100 nM, much higher than those for isolated HPQ-containing linear peptides with same flanking residues. This is expected, as constraints on the conformation of linear peptides synthesized on a solid support reduce the entropy cost when forming complexes with streptavidin and thus increase affinity constants from those for solution-phase linear peptides.

**Mechanisms for Complex Formation of Streptavidin with Linear Peptides Having Various HPQ-Containing Motifs.**

With limited X-ray-based structural data available, it is difficult and premature to draw a general rule on how flanking residues help to stabilize peptide−streptavidin complexes. Yet, based on the work of Katz,$^7$ we make an attempt here to propose a few likely scenarios as guides to understand the protein−peptide complex formation in general. On the role of the first flanking residue, it seems that (a) the HPQG motif stabilizes the linear peptide−protein complex by the Gly main chain amide forming an intrapeptide hydrogen bond with the His main chain carbonyl, thus defining a favorable type I $\beta$-turn in the peptide, as found by Katz in the CHPQGPPC−streptavidin complex; (b) the HPQN motif stabilizes the complexes by Asn residue forming additional direct and water-mediated hydrogen bonds with the protein, as found in $\text{F}_5\text{H}_2\text{Q}_{\text{NT}}$−streptavidin complex by Katz; (c) HPQ(F,V,A) motifs achieve the same feat by (F,V,A) enacting hydrophobic interaction with a likewise pocket on the protein, as in CHPQFC−streptavidin complexes. Because the hydrophobic side chain on Ala is much smaller, its stabilizing effect is expected to be somewhat reduced. This would explain why linear peptides with HPQ(C, I, K, L) motifs have a much lower affinity to streptavidin than those with HPQF(C, I, K, L) motifs.

The roles of the second-neighboring flanking residues on the formation of peptide−streptavidin complexes have not been studied before. As such, there is no direct or indirect X-ray structural data available that would shed light on what they bring to the complex. Yet, the influence of these flanking residues is clearly not negligible. The positive effect is illustrated in panel (6) of Figure 4 and panel (7) of Figure 5: the Gly side chain on Ala is much smaller, its stabilizing effect is clearly not negligible. The positive effect is illustrated in panel (6) of Figure 4 and panel (7) of Figure 5: the Gly residue stabilizes most peptides with HPQ(A) motifs with streptavidin, whereas the Ala residue does a much poorer job for peptides with HPQ(X)A motifs. On the other hand, the fact that linear peptides with HPQA(C, I, K, L) motifs have affinities at least 3 orders of magnitude less than those with HPQA(X, X ≠ C, I, K, L) motifs demonstrates the destabilizing effect of C, I, and K L as the second-neighboring flanking residues.

Finally, we should note that the O1-RD image of a peptide microarray before the reaction enables us to examine the quality of the array. Figure 2a shows that the surface mass density of the synthesized peptides varies within a square feature and from one feature to another. This image ensures that the negative “hit” is not due to the absence of target materials. In further analyzing the image, we notice that the sequence of a peptide, particularly the amino acid residues close to the C-terminus, affects the efficiency of the synthesis and, in turn, the peptide target number density. Figure 6 shows the
surface mass densities of the variants of WTHPQFAT and Figure 7 shows the variants of LQWHPQSGK. We find that some residues close to the C-terminus, and thus the solid surface, lead to significantly higher peptide densities, whereas some others do just the opposite. For example, the linear peptides with Asp (D) and Glu (E) having negatively charged side chains and being close to the C-terminus have significantly higher surface number densities than the linear peptides of otherwise same sequences. In contrast, Trp (W) and Tyr (Y), having bulky aromatic side chains, have significantly smaller surface number densities when they are close to the C-terminus. These tendencies hold true even when these residues are farther away from the C-terminus. One can then use the residues such as D, E, W, and Y to optimize the number density of synthesized peptides for subsequent binding assays.

# CONCLUSIONS

The quest for understanding protein–protein interactions and devising peptide-based drugs to modulate these interactions calls for the identification and functional characterization of key receptor-binding domains (RBD) on a peptide.9,16,17 Our present study, in combination with earlier studies by others, shows that the flanking residues outside an RBD, at least up to the second neighbors, are crucial in stabilizing or destabilizing the peptide–protein complex formation and their roles should be systematically investigated and explored. A photolithographically synthesized peptide microarray on a glass slide detected with a compatible label-free sensor is an efficient assay platform for such an investigation and exploration. If it generally holds that only up to the second neighboring flanking residues are important in peptide–protein binding, one can then explore residues at positions beyond the second neighbors that add other desirable attributes of drug candidates, such as resistance against proteinase, circulation time, plasma membrane penetration, and low toxicity,9,16,17 or simply enable efficient and controllable synthesis on a solid support.11

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