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TIGHT-BINDING STREPTAVIDIN LIGANDS FROM A CYCLIC PEPTIDE LIBRARY

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Abstract: During the screening of a soluble library of cyclo(AXXXXXAE)K-$CONH_2$, a cyclic peptide cyclo(AHPQFPAE)K-$CONH_2$ was identified as a tight-binding ligand (IC_{50} = 128 nM) and found to bind 1000-fold more tightly than its linear peptide to streptavidin. The results of this study suggest that library screening of conformationally constrained cyclic peptides can be an effective means for the discovery of high affinity ligands.

Combinatorial library methods have been widely accepted as useful tools in studies of antigenic determinants, receptor-binding ligands, enzyme substrates and inhibitors.[1,2] Currently, the focus of combinatorial chemistry for drug discovery is on peptidomimetics and small organic compounds[3–8] since ligands selected from linear peptide libraries are inferior drug candidates, in part due to their poor oral availability and rapid enzymatic degradation. Cyclic peptides are constrained peptidomimetics with reduced flexibility in conformation, have been reported to better resist unwanted proteolysis, and enhance binding affinity to various receptor molecules.[9,10] Cyclic peptide libraries can be a convenient tool in rapid identification of peptidomimetic lead compounds for further pharmacological and pharmaceutical research. For example, recent studies on cyclic peptide libraries have led to the discovery of various inhibitors for chymotrypsin,[11] serine protease,[12] α-glucosidase,[13] cyclin-dependent kinase 2 (Cdk2),[14] pp60c-src,[15] and HIV-1 protease.[16] Endothelin antagonists,[17] epitopes of somatostatin,[18] and ionophores[19,20] were also identified from cyclic peptide libraries.

Intramolecular cyclization of peptides can be readily achieved by the formation of disulfides, lactams, thioethers, or other covalent linkages.[21–25] Disulfide-bridged cyclic peptides are known to undergo oligomerization via the disulfide interchange under certain conditions[26] and are not always metabolically stable in vivo. In this paper we constructed a soluble library of cyclic peptide lactams (via *N*-to-side chain cyclization) to screen for ligands that bind tightly to streptavidin (from *Streptomyces avidinii*). Streptavidin was used as a model receptor in this study because of its useful biochemical applications[27] and established crystal structures of various streptavidin-ligand complexes.[28–31] Also, streptavidin has recently been reported as an ideal model for the development of structure-based ligand design.[32]

Several screening studies of peptide libraries expressed on the phage surface or solid support have been carried out to define peptides that specifically bind to streptavidin.[33–39] Most screened linear peptides that bind to streptavidin, with reported mM to μM affinities, share the consensus tripeptide sequence HPQ with little amino acid sequence conservation observed on either side of this tripeptide.[34,35] Giebel et al. screened disulfide-bridged cyclic peptide libraries of various ring sizes on the surface of filamentous phage M13 against streptavidin and confirmed that all identified tight-binding cyclic peptides also contained HPQ motif and bound the receptor protein with an increased affinity with respect to its linear counterparts.[33] Based on the crystal

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structure of a disulfide-bridged cyclic peptide-streptavidin complex, Katz et al. rationally designed two thioether-bridged cyclic peptides and showed that these cyclic peptides retained high affinity binding with streptavidin.[26] Here we wish to report our initial effort in the search for tight binding ligands to biological receptors from conformationally constrained libraries. A cyclic peptide lactam library of cyclo(AXXXXXAE)K-$CONH_2$ was screened against streptavidin and one ligand, cyclo(AHPQFPAE)K-$CONH_2$ (**1**), was found to bind streptavidin with an affinity (IC_{50} = 128 nM) higher than that of previously reported linear and cyclic peptide ligands.

Construction of the Cyclic Peptide Libraries. Although the combinatorial library approach is molecular diversity, a focused library can enhance the relevance of the library to the receptor-ligand binding and best represent potential ligand candidates. Also, it simplifies the chemical synthesis of libraries and the effort in screenings. In this research, using streptavidin as a model receptor protein, a focused and conformationally constrained library of cyclic peptide lactams was studied to search for tight-binding ligands. This soluble library of cyclo(AXXXXXAE)K-$CONH_2$ (X = H, P, Q, Y, G, F, D and I) (8^5 = 32,768) was chosen for six reasons. First, linear peptides can assume many different conformations in solution and only few of which may be recognized by streptavidin. In general, weak- to moderate-affinity ligands may be discovered. Constraining the conformational freedom by cyclization and thus decreasing the entropy of unbound peptides in a library should, in principle, result in isolation of higher-affinity ligands. This entropy-mediated gain in binding affinity is well documented in literature and has been successfully applied to several biochemical systems,[11–20,40] including disulfide- and thioether-bridged peptides-streptavidin bindings.[26,33] Second, the lactam linkage in cyclic peptide lactam libraries prepared by solid-phase synthesis is more stable than the disulfide bond in disulfide-bridged phage or chemical libraries.[26] Third, the library screening was carried out in solution, so that nonspecific binding interactions potentially observed in library screening on beads are eliminated. Fourth, among eight chosen amino acids in this focused library, H, P, Q, F, and G were included because they were part of the binding motif previously discovered from both linear and cyclic disulfide-bridged peptide libraries.[33–39] The proline and glycine residues were also known to facilitate peptide cyclization on solid support.[41] Y, D, and I are aromatic, charged, and hydrophobic amino acids, respectively. Fifth, the library was flanked with two alanines fixed as a template in order to minimize the peptide sequence dependence in cyclization between the *N*-terminus of alanine and the side chain of glutamic acid.[41] The lysine residue was included in every peptide to ensure greater, if not complete, precipitation of libraries in cold ethyl ether upon release from PAL resin by trifluoroacetic acid. Finally, the procedure of library deconvolution in solution is straightforward.

Peptide libraries were prepared on the PAL resin using standard Fmoc chemistry and the method of split synthesis.[2] Unlike the library screening on beads, no linkers between peptide and the solid support were needed.[42] The *N*-to-side chain peptide cyclization was carried out as previously described.[41] The libraries of cyclic peptide lactams were released from the solid support using trifluoroacetic acid, precipitated by cold ethyl ether, and lyophilized two times prior to screening. As a control, the corresponding linear peptide library, Ac-AXXXXXAEK-$CONH_2$, was prepared in order to confirm that the increase in binding affinity to streptavidin was indeed due to conformational constraint.

Iterative Deconvolution of Libraries. It is known that nonspecific binding interactions may occur upon screening libraries on beads. Except the high-throughput screening of soluble libraries prepared by array

synthesis, most of the soluble library screenings were performed using the approach of iterative deconvolution.

Since streptavidin is a biotin-binding protein having no enzymatic activity, a streptavidin-alkaline phosphatase (SAP) conjugate was used and a capture ELISA assay was developed for the library screening.[43] A streptavidin-binding peptide Ac-HPQFG[44] immobilized on TentaGel resin was employed as the capture ligand to assess the inhibition ability of each sublibrary of both cyclic and linear peptides.[45] If binding ligands were present in the soluble libraries, less unbound SAP conjugate could be captured onto beads. The degree of inhibition was monitored, at 405 nm, by the alkaline phosphatase-catalyzed hydrolysis of a soluble substrate *p*-nitrophenyl phosphate (*p*NPP) after complete washing of the peptide libraries and SAP. The most active amino acid in each randomized position of peptides was determined by the corresponding sublibraries that gave the highest inhibition (i.e., the lowest reading at 405 nm). Based on results from the screening of libraries of cyclo($AX_1X_2X_3X_4X_5AE$)K-$CONH_2$, where H sublibrary offered the highest inhibition, new sublibraries were synthesized by fixing X_1 with the most active amino acid residue (i.e., H) in this position. This deconvolution process was repeated four times until the final sequences were discovered. Results of this deconvolution are shown in Figure 1. In brief, maximum inhibition was observed from the H sublibrary at X_1 position, the P sublibrary at X_2 position, the Q sublibrary at X_3 position, the F sublibrary at X_4 position, and the P- and G-peptides at X_5 position. This deconvolution method used for the library screening in solution led to the discovery of two new cyclic peptides, **1** and cyclo(AHPQFGAE)K-$CONH_2$ (**2**), as potential high-affinity ligands to streptavidin.

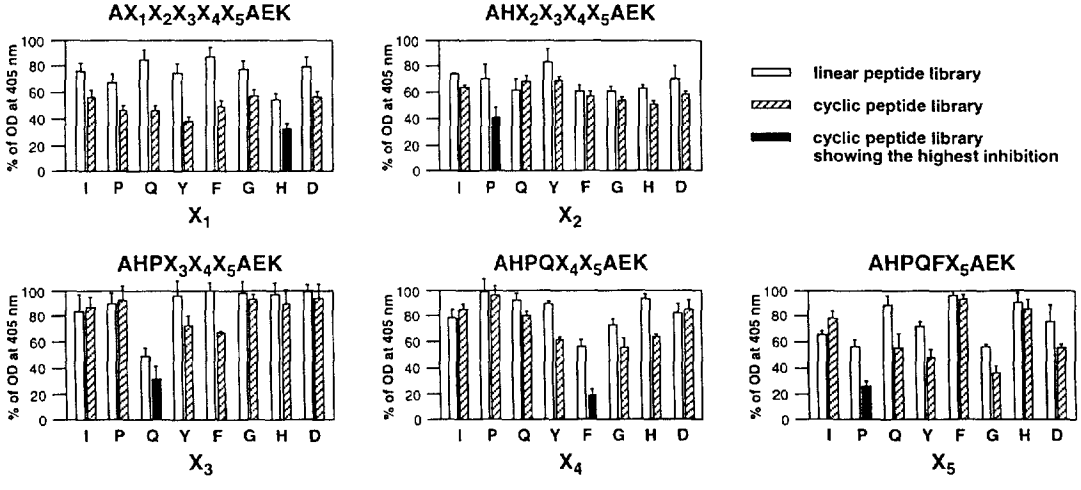

Figure 1. Deconvolution screening of a cyclic peptide lactam library, cyclo($AX_1X_2X_3X_4X_5AE$)K-$CONH_2$. X_1: first round of iterative selection for the amino acid at X_1 position; X_2: second round of iterative selection with one defined amino acid (H) at X_1; X_3: third round of iterative selection with two defined amino acids (HP) at X_1X_2; X_4: fourth round of iterative selection with three defined amino acids (HPQ) at $X_1X_2X_3$; X_5: fifth round of iterative selection with four defined amino acids (HPQF) at $X_1X_2X_3X_4$. See ref 45 for experimental conditions.

Evaluation of Streptavidin Ligands. To verify that library-selected **1** and **2** bind specifically with streptavidin, cyclic peptide lactams (**1** and **2**), their corresponding linear analogs (Ac-AHPQFPAEK-$CONH_2$ **3** and Ac-AHPQFGAEK-$CONH_2$ **4**), a disulfide-bridged ligand (Ac-AE-CHPQGPPC-IEGRK-$CONH_2$ **5**),[33] and a

control cyclic peptide lactam (cyclo(AQYGHFAE)K-CONH2 **6**)[46] were individually synthesized on PAL resin and purified by HPLC. Using a streptavidin affinity column, the two library-selected cyclic ligands, **1** and **2**, were retained on the column and could be readily eluted by biotin, demonstrating that the binding of these cyclic peptides to streptavidin was specific and the cyclic peptides compete with biotin on the same binding site of streptavidin.[47] Also, these cyclic peptide ligands exhibited no inhibition to the alkaline phosphatase-catalyzed reactions, confirming that the cyclic peptide ligands bind to streptavidin, but not alkaline phosphatase, on the SAP conjugate (data not shown).

It has been well established that streptavidin specifically recognizes linear peptides having a HPQ tripeptide sequence. In this work, our results from the library screenings of cyclic peptide lactams, cyclo(AXXXXXAE)K-$CONH_2$, showed that cyclic peptides containing a tetrapeptide sequence HPQF bind most tightly with streptavidin: a maximum inhibition was observed at the X_4 position (Figure 1). This importance of having a phenylalanine residue as part of the binding motif in the cyclic peptides has been recently realized by the study of a crystal structure of streptavidin complex with a disulfide-bridged cyclic peptide, cyclo(CHPQFC) (**7**).[29] The increase in binding affinity of **7** to streptavidin was attributed to both the change of entropy in the unbound state, as mentioned above, and the suggested hydrophobic interactions of this phenylalanine residue in **7** with L25 and W108 of one subunit and also with W120 of a neighboring subunit of streptavidin.[29] However, it is noted that among the previously reported disulfide-bridged cyclic peptide ligands (ring size = 8 amino acid residues), a binding motif HPQG has the highest binding affinity to streptavidin (e.g., **5**[33]), instead of the HPQF motif identified in our library of same ring size (**1** and **2**).

Table 1. Relative Affinities of Peptides Binding to Streptavidin

Peptide		IC_{50}[a]
cyclo(AHPQFPAE)K-$CONH_2$	**1**	128 nM
Ac-AHPQFPAEK-$CONH_2$	**3**	136 μM
cyclo(AHPQFGAE)K-$CONH_2$	**2**	19 μM
Ac-AHPQFGAEK-$CONH_2$	**4**	204 μM
AECHPQGPPCIEGRK-$CONH_2$ (S–S)	**5**	730 nM
cyclo(AQYGHFAE)K-$CONH_2$	**6**	> 5 mM

[a] IC_{50} values were determined for inhibition of streptavidin binding to the TentaGel-bound Ac-HPQFG peptide. Estimated RSD, ≤ 15%.

To compare binding affinities of various cyclic peptide ligands, a capture ELISA assay was employed to measure the IC_{50} values of these peptides in the inhibition of streptavidin binding to the Ac-HPQFG on beads.[48] Results in Table 1 show that library-identified **1** and **2** give IC_{50} values of 128 nM and 19 μM, respectively. As expected, their linear peptide analogs **3** and **4** bind 100- and 10-fold weaker to streptavidin (Table 1). A previously reported disulfide-bridged cyclic peptide **5** has its IC_{50} value of 730 nM. A 8-mer control cyclic peptide **6** does not bind to streptavidin up to 5 mM.

Conclusion. The new cyclic peptide lactam ligands identified from conformationally constrained peptide library bind streptavidin with high affinity. The lactam cyclization in cyclic peptides enhances the binding affinity to streptavidin compared to its linear analogs and disulfide-bridged cyclic peptides. The binding

affinity of **1** is 5 times greater than that of the disulfide-bridged cyclic peptide and 1,000 times greater than that of its linear analog. Also, the HPQF sequence was verified as a critical binding motif of cyclic peptide lactam to streptavidin. The results presented demonstrate that the conformational constraint is valuable in increasing the binding affinities and may be applicable to other biological receptor proteins.

References and Notes

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44. Ac-HPQFG-TentaGel was identified as a ligand to streptavidin from a linear peptide library prepared in our library (Yu, Z.; Chu, Y.-H. unpublished results).
45. *Screening of Libraries Using a Capture ELISA Assay.* A streptavidin-binding peptide Ac-HPQFG was synthesized on a TentaGel support and suspended in the blocking buffer (0.1% gelatin in 2× PBS containing 0.05% Tween 20, pH 7.2). Aliquots of peptide-resin suspension were evenly distributed into microtiter wells (0.25 mL; approximately 0.25 mg resin/well) and allowed to incubate overnight with the blocking buffer. Individual soluble peptide library (1 mM), used for competition, was mixed with SAP conjugate (1:1,000 dilution) at 4 °C for 12 h before screening. Subsequently, the peptide library-SAP conjugate mixture was added to the microtiter well in triplicate and allowed to incubate for 45 min. The solution in each well was removed followed by washing the resin with 1× PBST buffer (8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, and 0.1% Tween-20, pH 7.2) twice and 2× TBS buffer (2.5 mM Tris, 13.7 mM NaCl, 0.27 mM KCl, pH 8.0) once. The soluble substrate *p*-nitrophenyl phosphate (*p*NPP) was added (0.25 mL/well) and color development was allowed to develop for 30 min. The reaction of *p*NPP hydrolysis was terminated with 3 N NaOH (0.25mL) and the solution was diluted to 1.0 mL with water. Absorbance was recorded at 405 nm.
46. The amino acids in the control peptide cyclo(AQYGHFAE)K-$CONH_2$ were chosen from each deconvolution step where the specific amino acid gave low inhibition at that position.
47. Both library-identified cyclic peptide ligands **1** and **2** were used to incubate with a streptavidin-immobilized affinity column (Pierce Chemical Co.) for 30 min. After the affinity column was washed with the binding buffer (10 columns volume), a solution containing biotin (1 mM, 5 columns volume) was used to elute out the bound cyclic peptide. All eluting fractions were collected and analyzed by MALDI MS (Kratos Kompact MALDI-III). The cyclic peptide ligands were detected in the eluted fractions after the biotin-washing step, but not in the fractions after binding buffer washing step (Zang, X.; Chu, Y.-H. unpublished result).
48. *Determination of the IC_{50} Values.* Using the same ELISA method described in ref 45, peptide was added to the blocking buffer containing 1:2,500 diluted SAP conjugate. This peptide-SAP mixture was then systematically diluted using a solution containing SAP conjugate (1:2,500 dilution) to prepare solutions of peptide with concentrations ranging from 10 nM to 1mM, while keeping the SAP conjugate concentration constant. At each peptide concentration, the peptide-SAP conjugate mixture was incubated for 12 h and then mixed with the straptavidin-binding Ac-HPQFG on TentaGel resin for 30 min. After removing the peptide-SAP solution and washing the resin with 1× PBST twice and 2× TBS once, the soluble substrate *p*NPP was added (0.25 mL/well) and color development was allowed to proceed for 30 min. The hydrolysis of *p*NPP was stopped using 3 N NaOH and subsequently diluted to a final volume of 1.0 mL with water. Absorbance were recorded at 405 nm.