Structure-Based Design of High Affinity Streptavidin Binding Cyclic Peptide Ligands Containing Thioether Cross-Links

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Abstract: High affinity cyclic thioether-cross-linked streptavidin binding ligands were designed from the crystal structure of a complex between streptavidin and a high affinity cyclic disulfide-cross-linked peptide ligand, *cyclo*-Ac-[CHPQGPPC]-NH_2, originally discovered by phage display. Determination of the affinities of two chemically synthesized thioether ligands by surface plasmon resonance indicated affinities similar to the disulfide-cross-linked ligand from which they were designed. The crystal structures of the streptavidin−cyclic thioether complexes show that the nonlinker segments are bound in the same conformation as in *cyclo*-Ac-[CHPQGPPC]-NH_2 and make the same binding interactions with streptavidin as the disulfide-linked cyclic peptide. The structures, conformations, and dihedral energetic constraints of the thioether- and disulfide-cross-linked ligands are described and compared. Advantages of thioether cross-links over disulfide cross-links are discussed.

Introduction

The conversion of peptide leads into high affinity, bioactive, organic molecules by structure-based design represents a challenge that spans the disciplines of organic chemistry, medicinal chemistry, and structural biology. Peptides or proteins are the normal ligands or substrates for many pharmaceutically relevant macromolecular targets. As part of a research program to address the principles involved in the conversion of peptide into non-peptide ligands, the crystal structures of streptavidin−peptide complexes are used to design unnatural high affinity streptavidin binding peptides containing various functionalities and cyclizing linkages.

Streptavidin is a good model system for structure-based ligand design, since the affinities and high resolution crystal structures of several streptavidin−ligand complexes have been determined.[1−4] Streptavidin binding peptide sequences have been discovered by screening peptide libraries displayed on phage[5,6] or by screening synthetic peptide libraries[7] and the structural basis for the affinities of the peptide ligands has been probed crystallographically.[1,2,8] Hydrogen bonding and van der Waals interactions mediating the binding of linear and cyclic peptides containing the HPQ sequence were identified in crystal structures of the complexes. The streptavidin-bound crystal structures of four peptide ligands containing the HPQ sequences show a common conformation and common binding interactions involving the HPQ segment.[1,2]

Disulfide-constrained cyclic peptide ligands discovered by phage display bind streptavidin with several hundred-fold greater affinity than their linear counterparts.[6] The role of the disulfides is believed to result from a decrease in conformational entropy in the unbound cyclic ligand compared to that in linear ones. In this paper we investigate the effect on the ligand affinity and protein-bound ligand structure of replacing the ligand disulfide cross-link with thioether cross-links.

One impetus for replacement of disulfides with thioethers was the observed instability of disulfide-bridged cyclic peptide streptavidin binding ligands with respect to oligomerization via disulfide interchange under some conditions. Both of the streptavidin binding disulfide-bridged cyclic peptides studied crystallographically[1] exhibited instability toward oligomerization.[2] This type of instability may be a general problem for small disulfide-bridged peptide ligands discovered or developed for other protein targets. Thioether-cross-linked peptides, readily prepared via literature procedures,[9,10] do not undergo such oligomerizations.

Bridging thioether groups were designed from the crystal structure of a streptavidin-bound disulfide-bridged peptide (*cyclo*-Ac-[CHPQGPPC]-NH_2).[1] In this paper we report the affinities for streptavidin of two designed thioether-bridged cyclic peptides (Figure 1) and describe their streptavidin-bound crystal structures. Their affinities and structures are compared to those for the corresponding disulfide-bridged peptide (Figure 1) from which they were designed.

Experimental Section

Modeling of Streptavidin−Thioether Complexes. The thioether linkages were chosen based on two criteria. First, they were intended to span a distance similar to that by the disulfide cross-link in *cyclo*-Ac-[CHPQGPPC]-NH_2[1] (Figure 1). Secondly, the compounds containing the thioether linkages were intended to be synthetically feasible. Three-dimensional models of streptavidin-bound thioether-containing ligands were built with Insight[11] using the crystal structure of the streptavidin-bound disulfide bonded cyclic peptide, *cyclo*-Ac-[CHPQG-

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(1) Katz, B. A. Submitted to *Biochemistry*.

(2) Katz, B. A.; Stroud, R. M., Collin, N.; Liu, B.; Arze, R. Submitted to *Chem. Biol.*

(3) Weber, P. C.; Pantoliano, M. W.; Thompson, L. D. *Biochemistry* **1992**, *31*, 9350-9354.

(4) Weber, P. C.; Ohlendorf, D. H.; Wendoloski, J. J.; Salemme, F. R. *Science* **1989**, *234*, 85−88.

(5) Devlin, J. J.; Panganiban, L. C.; Devlin, P. E. *Science* **1990**, *249*, 404.

(6) Giebel, L. B.; Cass, R.; Milligan, D. L.; Young, D.; Arze, R.; Johnson, C. R. Submitted to *Biochemistry*.

(7) Lam, K. S.; Salmon, S. E.; Hersh, E. M.; Hruby, V. J.; Kazmierski, W. M.; Knapp, R. J. *Nature* **1991**, *352*, 82.

(8) Weber, P. C.; Pantoliano, M. W.; Simons, D. M.; Salemme, F. R. *J. Am. Chem. Soc.* **1994**, *116*, 2717−2724.

(9) Barker, P. L.; Bullens, S.; Bunting, S.; Burdick, D. J.; Chan, K. S.; Deisher, T.; Eigenbrot, C.; Gadek, T. R.; Gantzos, R.; Lipari, M. T.; Muir, C. D.; Napier, M. A.; Pitti, R. M.; Padua, A.; Quan, C.; Stanley, M.; Struble, M.; Tom, J. Y. K.; Burnier, J. P. *J. Med. Chem.* **1992**, *35*, 2040−2048.

(10) Roby, F. A.; Fields, R. L. *Anal. Biochem.* **1989**, *77*, 373−377.

0002-7863/95/1517-8541$09.00/0

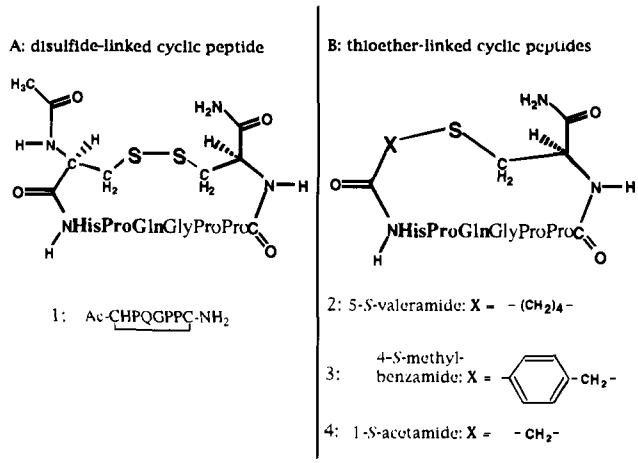

Figure 1. (a) Chemical structure of disulfide-linked *cyclo*-Ac-[CHPQGPPC]-NH_2 compared with (b) chemical structures of designed thioether-linked streptavidin binding ligands.

PPC]-NH_2, and energy minimized using Discover[11] with the Amber force field,[12] or using Xplor.[13] Initially the Cα atoms of the HPQGPP residues were restrained to their positions in the refined structure of streptavidin-*cyclo*-Ac-[CHPQGPPC]-NH_2. In the final minimizations, all atoms in the complex were unrestrained, and the energy was minimized until convergence (gradient < 0.001 kcal/Å).

Chemical Synthesis of Streptavidin-Binding Ligands. Synthesis of *cyclo*-Ac-[CHPQGPPC]-NH_2 (**1**) has been described.[1] Syntheses of *cyclo*-[5-*S*-valeramide-HPQGPPC]-NH_2 (**2**) and *cyclo*-[4-*S*-methylbenzamide-HPQGPPC]-NH_2, (**3**) outlined below, followed literature procedures.[9,10] Preparative high performance liquid chromatography (HPLC) separations were carried out on a Gilson 715 instrument. The HPLC column used was 25 × 250 mm Vydac (C_{18}, 10 μm particle size). A linear gradient of 0.1% TFA[14] in water versus MeCN (95.0 → 2.0%) was used for elution over a time period of 45 min at a flow rate of 10.0 mL/min with UV detection at 214 nm. Electrospray mass spectra (EMS) were recorded on a Finnigan SSQ 710 instrument. For analytical HPLC separations, a Michrom BioResources Ultrafast Microprotein analyzer (C18 reverse phase column) was employed. Analytical capillary zone elecrophoresis (CZE) analysis was done with a Hewlett Packard 3D-CE system.

HPQGPPC-NH-PAL-PEG-PS Resin. PAL-PEG-PS resin was purchased from Millipore. Fmoc-amino acids purchased from Novabiochem had the His, Gln, and Cys side chains protected with trityl groups. Fmoc-PAL-PEG-PS resin (1.0 g, 0.21 mmol) was loaded into an automated peptide synthesizer (Millipore 9600), and the peptide was synthesized using standard Fmoc/Boc protocols. The Nα-Fmoc was removed by incubation in 30% piperidine in DMF for 20 min. The Fmoc-amino acids (5.0 equiv) were preactivated with PyBOP (5.0 equiv), HOBT (5.0 equiv), and NMM (5 equiv) in 0.20 M DMF. Coupling times were 2−4 h. The resin was washed with DMF, CH_2Cl_2, and MeOH and dried under vacuum.

***Cyclo*-[5-*S*-valeramide-HPQGPPC]-NH_2 (2).** A solution of 5-bromovaleric acid (0.181 g, 1.00 mmol) and DIPC (172 μL, 1.10 mmol) in 4.0 mL of 1 vol:1 vol DMF:CH_2Cl_2 was stirred at room temperature for 5 min. After addition of NMM (121 μL, 1.10 mmol), the solution was filtered and added to 0.40 g of HPQGPPC-NH-PAL-PEG-PS resin. After 1.5 h the Kaiser ninhydrin test[15] was negative, and the resin was washed with DMF, CH_2Cl_2, and MeOH and dried under vacuum. The 5-bromovaleramide-peptide was cleaved from the resin by treatment with 5 mL of 3% triethylsilane in TFA for 1 h and isolated by precipitation in 1 vol:1 vol ether:*tert*-butyl ether. The solid was collected by filtration and dissolved in 1 vol:1 vol acetonitrile:water (40 mL). After adjustment of the pH to 9.5−10.0 with concentrated aqueous ammonia, the peptide was cyclized by stirring under nitrogen overnight. Following a negative Ellman's test,[16] the solution was frozen and lyophilized. HPLC purification (C18 reverse-phase gradient, 0.1% TFA in water to 0.1% TFA in acetonitrile) gave a single peak yielding 9 mg. CZE indicated a purity of greater than 95%. EMS yielded a peak at *m/z* 816 (calc. MW = 817).

***Cyclo*-[4-*S*-methylbenzamide-HPQGPPC]-NH_2 (3).** This cyclic ether was synthesized as above using α-bromo-*p*-toluic acid (0.215 g, 1.00 mmol). HPLC purification gave a single peak yielding 16 mg. CZE indicated a purity of 70%. EMS yielded a peak at *m/z* 850 (calc. MW = 851).

***Cyclo*-[1-*S*-acetamide-HPQGPPC]-NH_2 (4).** Attempts to synthesize this compound according to the procedures described above for **2** and **3** failed.

Determination of Binding Affinities of Thioether Ligands Using Surface Plasmon Resonance. The affinities of chemically synthesized streptavidin binding peptides were determined by surface plasmon resonance[17,18] using real-time biospecific interaction analysis (BIAcore).[19] The BIAcore system, sensor chip, and coupling reagents[14] NHS, EDC, and ethanolamine-hydrochloride were from Pharmacia Biosensor AB, Uppsala, Sweden. Affinities for each peptide ligand were determined under identical conditions in a competition assay involving a cyclic, disulfide-containing streptavidin binding peptide, *cyclo*-Ac-AE[CHPQGPPC]IEGRK-NH_2 (whose binding affinity has been previously determined[6] by BIAcore) immobilized on the sensor chip surface via the ε-amino group of the C-terminal lysine residue using standard amine immobilization chemistry.[20,21]

Immobilization on the sensor chip surface was done at a flow rate of 5 μL/min, 25 °C. Between injections of reagents, the sensor chip was continuously washed with 10 mM HEPES, pH 7.5, 150 mM NaCl, 3.4 mM EDTA, and 0.005% surfactant P20. The surface was activated by a 2 min pulse of a mixture containing 0.050 M NHS and 0.20 M EDC. The *cyclo*-Ac-AE[CHPQGPPC]IEGRK-NH_2 peptide solution (0.1 mM in 0.1 M sodium borate, pH 8.5) was then injected for 2 min followed by a 7.0 min injection of 1.0 M ethanolamine-HCl to inactivate any remaining NHS ester groups. In order to wash out any noncovalently bound peptide, the immobilization concluded with a 2 min pulse of 6.0 M guanidine-HCl (pH 2.1).

The competition assay involved preincubation of streptavidin (167 nM) with increasing concentrations (0−2500 μM) of each of the different peptide ligands. Each streptavidin-peptide ligand solution was injected over the immobilized peptide surface as well as over a (blank) surface identical except lacking the immobilized peptide, at a flow rate of 5.0 μL/min for 7.0 min. After subtracting the nonspecific response for the blank surface from the specific signal of the immobilized peptide surface, the resulting streptavidin binding response was plotted as a function of the competing peptide concentration to produce the inhibition curves shown in Figure 2. In order to regenerate the peptide surface before assay of each peptide concentration, 6.0 M guanidine-HCl (pH 2.1) was injected to dissociate remaining bound streptavidin.

Since the concentration of the *cyclo*-Ac-AE[CHPQGPPC]IEGRK-NH_2 peptide immobilized on the sensor chip is high (~110 μM), the

(11) Insight and Discover are molecular modeling programs licensed from Biosym Technologies, Inc., 10065 Barnes Canyon Road, San Diego, CA 92121.

(12) Weiner, S. J.; Kollman, P. A.; Case, D.; Singh, U. C.; Ghio, C.; Alagona, G.; Profeta, S., Jr.; Weiner, P. *J. Am. Chem. Soc.* **1984**, *106*, 765−784.

(13) Brünger, A. T. Xplor Manual, version 2.1; Yale University, New Haven, CT 06511, 1990.

(14) The following abbreviations and definitions are used: FMOC, 9-fluorenylmethoxycarbonyl; BOC *tert*-butoxycarbonyl; Acm, acetamidomethyl; PyBOP, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; HOBt, *N*-hydroxybenzotriazole·H_2O; NMM, *N*-methylmorpholine; DIPC, *N,N'*-diisopropylcarbodiimide; DMF, dimethylformamide; MeCN, methyl cyanide; MeOH, methanol; TFA, trifluoroacetic acid; HPLC, high performance liquid chromatography; EMS, electropray mass spectrometry; CZE, capillary zone electrophoresis; NHS, *N*-hydroxysuccinimide; EDC *N*-ethyl-*N'*-(-3-diethylaminopropyl)carbodiimide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid kV, kilovolt; mA, milliamp; F_o and F_c, observed and calculated structure factors; α_c, calculated phases; rms, root-mean-square.

(15) Kaiser, E.; Colescot, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 595−598.

(16) Ellman, G. L. *Arch. Biochem., Biophysics* **1959**, *82*, 70−77.

(17) Fagerstam, L. *Tech. Protein Chem.* **1991**, *2*, 65−71.

(18) Malmqvist, M. *Nature* **1993**, *361*, 186−187.

(19) Jonsson, U.; Malmqvist, M. *Advances Biosensors* **1992**, 2, 291−336.

(20) Fagerstam, L.; Frostell, A.; Karlsson, R.; Kullman, M.; Larsson, A.; Malmqvist, M.; Butt, H. *J. Mol. Recog.* **1990**, *3*, 208−214.

(21) Johnsson, B.; Lofas, S.; Lindquist, G. *Anal. Biochemistry* **1991**, *198*, 268−277.

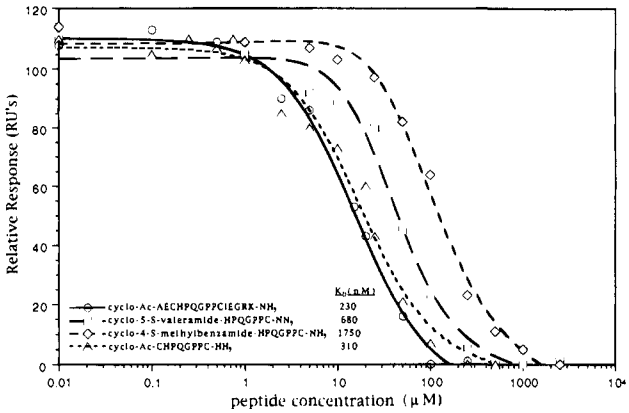

Figure 2. Inhibition curves for the competition for streptavidin between *cyclo*-Ac-AE[CHPQGPPC]IEGRK-NH_2 immobilized on the sensor chip and *cyclo*-Ac-AE[CHPQGPPC]IEGRK-NH_2, *cyclo*-Ac-[CHPQGPPC]-NH_2, *cyclo*-[4-*S*-methylbenzamide-HPQGPPC]-NH_2, or *cyclo*-[5-*S*-valeramide-HPQGPPC]-NH_2 in the solution flowing over the sensor chip. The IC_{50}s for all ligands were converted to K_ds by applying the factor relating the IC_{50} determined for *cyclo*-Ac-AE[CHPQGPPC]-IEGRK-NH_2 determined here with the actual K_d determined in a separate BIAcore experiment (Giebel, L. B.; Cass, R.; Milligan, D. L.; Young, D.; Arze, R.; Johnson, C. R. Submitted to Biochemistry). The IC_{50}s were determined by curve fitting the data to the function: RU = $(A - D)/(1 + (IC_{50}/\text{conc})^B) + D$, where RU is the BIAcore response, conc is the concetration of the free peptide ligand, and A, B, IC_{50}, and D are the parameters derived from the curve fit.

IC_{50} value for each peptide does not reflect the true equilibrium dissociation constant (K_d). Calculation of the true K_d's of the peptide ligands relied on determining the relationship between the apparent K_d (IC_{50}) in this study and the true K_d, determined previously,[6] for *cyclo*-Ac-AE[CHPQGPPC]IEGRK-NH_2, and applying the appropriate correction factor to the IC_{50}'s for all the peptide ligands in this study. (Thus, for this purpose, the IC_{50} for competition of the nonimmobilized *cyclo*-Ac-AE[CHPQGPPC]IEGRK-NH_2 solution ligand with sensor-bound *cyclo*-Ac-AE[CHPQGPPC]IEGRK-NH_2 was determined in this study.) Similar protocols for determining true K_d's from the results of competition assays involving high concentrations of ligands immobilized on the sensor chip have been described.[22,23]

Cocrystallization of Streptavidin−Thioether Ligand Complexes. Apostreptavidin was purchased from Calbiochem and cocrystallized with thioether ligands (10.0 mM) by vapor diffusion in 40 λ sitting drops at pH 4.0 under conditions described.[1,24] Some set-ups were seeded with crystals of apostreptavidin. Cocrystals of both streptavidin−thioether complexes were very thin. Crystals of the complexes were transferred to synthetic mother liquor at pH 6.0 containing 10.0 mM ligand before mounting.

Crystallographic Data Collection of Streptavidin−Thioether Complexes. X-ray diffraction data from single crystals of streptavidin-bound thioethers were collected on a Siemens IPC area detector coupled to a Siemens three-circle goniometer mounted on a Rigaku rotating anode target tube operating at 50 kV, 60 mA. Data were indexed and reduced to produce integrated intensities and structure factors with the programs Sadie and Saint supplied by Siemens. Lattice parameters and cell volumes are listed in Table 1. The following settings were used: crystal to detector distance = 8.4 cm; $2\theta = -22.5°$; oscillation widths in $\omega = 0.085°$; exposure time per frame = 200 or 400 s. Sweeps of 90° in ω at φ values of 0.0°, 45.0°, and 90.0° were collected.

Structure Determinations of Streptavidin−Thioether Complexes. The initial model for both thioether complexes was the crystal structure of streptavidin-*cyclo*-Ac-[CHPQGPPC]-NH_2[1] in which the N-terminal cysteine and disulfide cross-link were omitted. After each thioether dataset was refined to convergence with Xplor, electron density maps calculated with coefficients ($|F_o|$-$|F_c|$) or ($2|F_o| - |F_c|$) and phases (α_c) enabled determination of the structure of the thioether cross-link.

Table 1. Crystallography of Streptavidin-Bound Cyclic Thioether-Containing Ligands

	5-*S*-valeramide pH 6.0	4-*S*-methyl-benzamide pH 6.0
Parameters[a]		
no. atoms (including disorder)	2143	2139
no. waters (including disorder)	191	178
no. discretely disordered groups[b]	3	4
no. discretely disordered waters	4	3
no. side chains with refined occs[c]	24	21
Diffraction Statistics		
resolution (Å)	50−1.83	50−1.83
no. observations	39329	58107
no. unique obs	15322	20583
average redundancy	2.6	2.8
R_{merge} (%)[d]	29.9	23.1
no. observations (filtered)[e]	23407	35882
no. unique obs (filtered)[e]	12880	16499
redundancy (filtered)[e]	1.8	2.2
R_{merge} (%)(filtered)[e]	10.5	10.3
Refinement Statistics		
refinement resolution	8.5−2.00	7.5−1.92
no. merged reflections	9366	12408
$\|F_o\|/\sigma$ cutoff	1.0	1.5
R_{cryst} (%)[f]	18.0	18.5
overall completeness (%)	55.9	66.6
and at highest resolution (%)	35.4	34.3
RMS Deviations[g]		
bond lengths (Å)	0.019	0.018
bond angles (deg)	3.6	3.4
torsion angles (deg)	27.7	27.5

[a] Restrained, isotropic temperature factors were refined for all structures. Bulk solvent contributions were included for all structures. [b] Not including waters. [c] Also includes ligand groups. Density for all side chain atoms or for terminal atoms in these groups was weak or absent and temperature factors were high. Discretely disordered groups are not included in this category. Occupancies for poorly defined groups of atoms were refined. [d] $R_{merge} = \sum_h\sum_i|I(h)_i - \langle I(h)\rangle|/_h\sum_i I(h)_i$, where $I(h)_i$ is the ith observation of the intensity of reflection h. [e] Data with $R_{sym} > 50\%$ were rejected along with data with values $>3.5\ \sigma$ from the mean for a bunch of symmetry equivalents. [f] $R_{cryst} = \sum(||F_o| - |F_c||)/\sum|F_o|$ (for reflections from 7.5 Å to the highest resolution). [g] Root mean square deviations from ideal bond lengths and bond angles.

Crystallographic Refinement and Analysis of Streptavidin−Thioether Complexes. Models of the streptavidin-thioether complexes were built with Quanta[25] or Insight[12] and refined with Xplor[13] and with difference Fourier methods.[26] In ($|F_o| - |F_c|$), α_c maps, positive and negative peaks whose magnitudes were greater than 2.8−3.0 σ were systematically identified (using the program peak-pick written in house) and analyzed. Water structure was determined and refined by published procedures.[27] Included waters were based on significant density in both ($|F_o| - |F_c|$), α_c and ($2|F_o| - |F_c|$), α_c maps in stereochemically reasonable locations and were generally rejected if their refined temperature factors exceeded 60 Å^2. Deviations of bond lengths from ideality in the final structures were typically 0.018 Å (Table 1). Because of the small size of the streptavidin-*cyclo*-[5-*S*-valeramide-HPQGPPC]-NH_2 crystal, the high resolution data were weak (R_{sym}'s were large) and were not used in the refinement (Table 1).

Comparison of Structures of Streptavidin−Ligand Complexes. For comparison of streptavidin structures, all structures were superim-

(22) Felder, S.; Zhou, M.; Hu, P.; Urena, J.; Ullrich, A.; Chaudhuri, M.; White, M.; Shoelson, S.; Schlessinger, J. *Mol. Cell. Biol.* **1993**, *13*, 1449−1455.
(23) Payne, G.; Shoelson, S.; Gish, G.; Pawson, T.; Walsh, C. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 4902−4906.
(24) Pähler, A.; Hendrickson, W. A.; Kolks, M. A. G.; Argaraña, C. E.; Cantor, C. R. *J. Biol. Chem.* **1987**, *262*, 13933−13937.
(25) Quanta is a molecular modeling program licensed from Molecular Simulations, 16 New England Executive Park, Burlington, MA 01803.
(26) Chambers, J. L.; Stroud, R. M. *Acta Crystallogr.* **1979**, *B33*, 1861.
(27) Finer-Moore, J. S.; Kossiakoff, A. A.; Hurley, J. H.; Earnest, T.; Stroud, R. M. *Proteins* **1992**, *12*, 203−222.

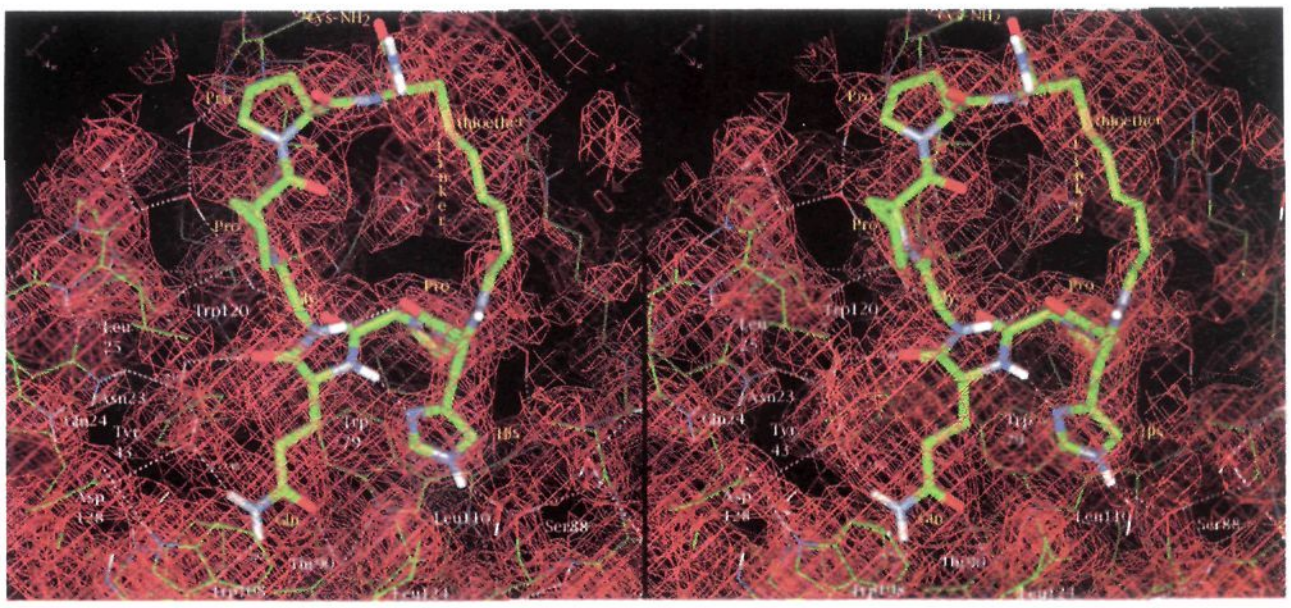

Figure 3. Refined structure of the complex between streptavidin and the 5-*S*-valeramide-containing cyclic thioether ligand superimposed on the $(2|F_o| - |F_c|)$, α_c map. The ligand is presented by "licorice" sticks. Ligand residues or groups are labeled with yellow fonts, and streptavidin residues are labeled with white fonts. The density in the foreground in front of the peptide Gln residue corresponds to part of one surface of the Trp120 side chain of a neighboring subunit of the tetramer. Within the bound peptide there is an H-bond between Nδ1 of His and the main chain Gln amide NH, and an H-bond between the Gly main chain amide NH and the His main chain carbonyl, defining a type 1 β-turn. There are three waters that mediate binding to streptavidin of the three cyclic peptides in this study. There is an H-bonding interaction between the peptide Gln side chain amide NH group and the Trp108 π ring system similar to those described by Burley, S. K.; Petsko, G. A. *FEBS Lett.* **1986**, *203*, 139−143. There are also van der Waals interactions between the peptide Gln residue and Trp79 and between the Cα of the peptide Gly and Trp120 of a neigboring subunit.

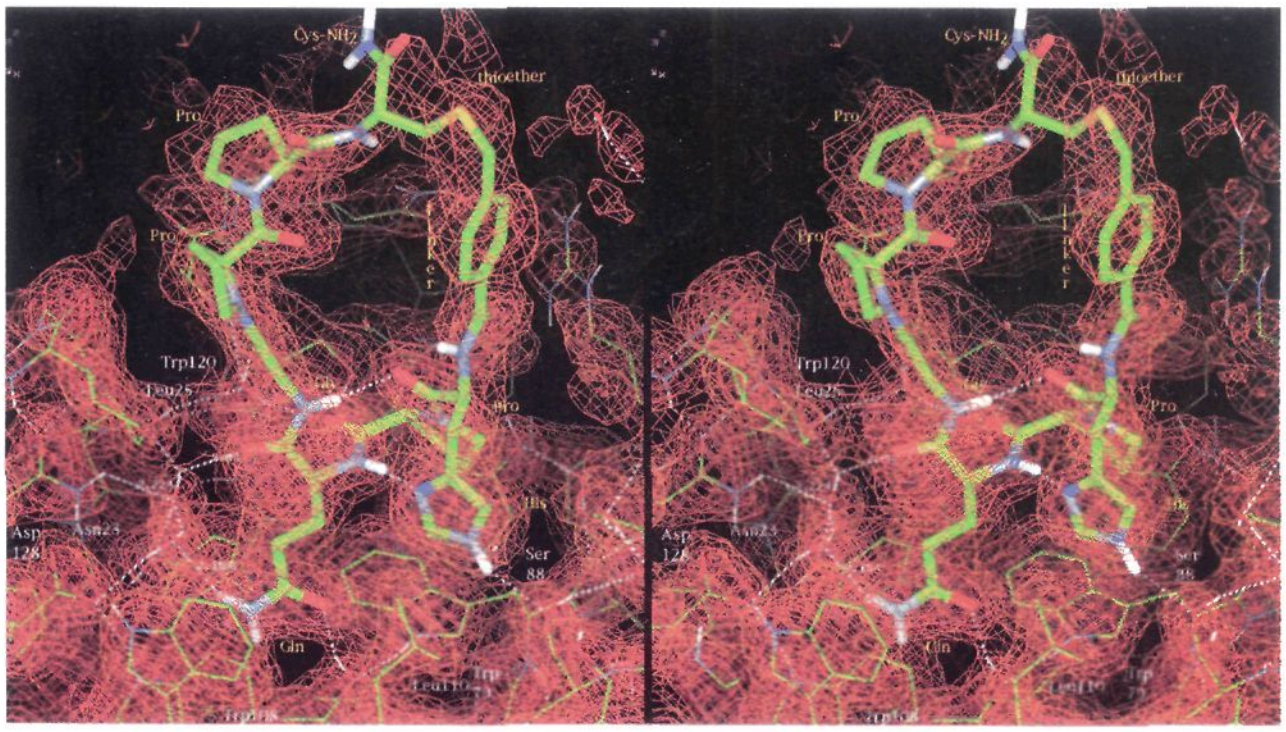

Figure 4. Refined structure of the complex between streptavidin and the 4-*S*-methylbenzamide-containing cyclic thioether ligand superimposed on the $(2|F_o| - |F_c|)$, α_c map. Representation and labeling scheme are similar to that in Figure 3.

posed with matrices determined from corresponding pairs of non-hydrogen atoms involving 46 well ordered ($B < 20$ Å^2) residues spread throughout the molecule. The following pairs of atoms were used: 29−31, 38−39, 54−57, 73−79, 86−97, 104−114, and 124−130.

Results

Plasmon Resonance Measurements Indicate High Affinity Binding of Both Designed Cyclic Thioether Ligands. Both designed thioethers bind to streptavidin with high affinities, similar to the affinity of the *cyclo*-Ac-[CHPQGPPC]-NH_2 (K_d = 310 nM) cyclic peptide ligand from which they were designed. The K_d for the ligand containing the 5-*S*-valeramide cross-link is 680 nM; for the ligand with the 4-S-methylbenzamide thioether the K_d is 1750 nM. The affinities of the cyclic thioethers are several hundred-fold greater than for linear peptides containing the HPQ sequence.[6] Thus the conformational constraints provided by the thioether cross-links are effective in maintaining high affinity of the streptavidin binding ligands.

In both Streptavidin-Bound Cyclic Thioethers the Structure of the Nonlinker Part of the Ligand Is the Same as That in the Streptavidin-Bound Cyclic Disulfide-Bonded Peptide. Figure 3 shows the $(2|F_o| - |F_c|)$, α_c map superimposed on the refined structure of the streptavidin-*cyclo*-[5-*S*-valeramide-HPQGPPC]-NH_2 complex, pH 6.0. Strong density defines virtually the entire streptavidin-bound cyclic ligand. The streptavidin-bound *cyclo*-[4-*S*-methylbenzamide-HPQGPPC]-NH_2 complex, pH 6.0 (Figure 4), is also equally well defined by the electron density.

In both streptavidin-bound cyclic thioethers, the HPQGPP residues adopt a conformation similar to that in streptavidin-*cyclo*-Ac-[CHPQGPPC]-NH_2 and make the same interactions with the protein as in the cyclic disulfide-bonded peptide. Figure 5 shows the superposition of the two streptavidin−thioether complexes onto the structure of the disulfide-bonded cyclic peptide from which they were designed. The intricate hydrogen bonding network and van der Waals interactions involving *cyclo*-Ac-[CHPQGPPC]-NH_2 and streptavidin have been previously described[1] as well as the water structure that mediates binding. A brief summary of the interactions is provided in the caption to Figure 3.

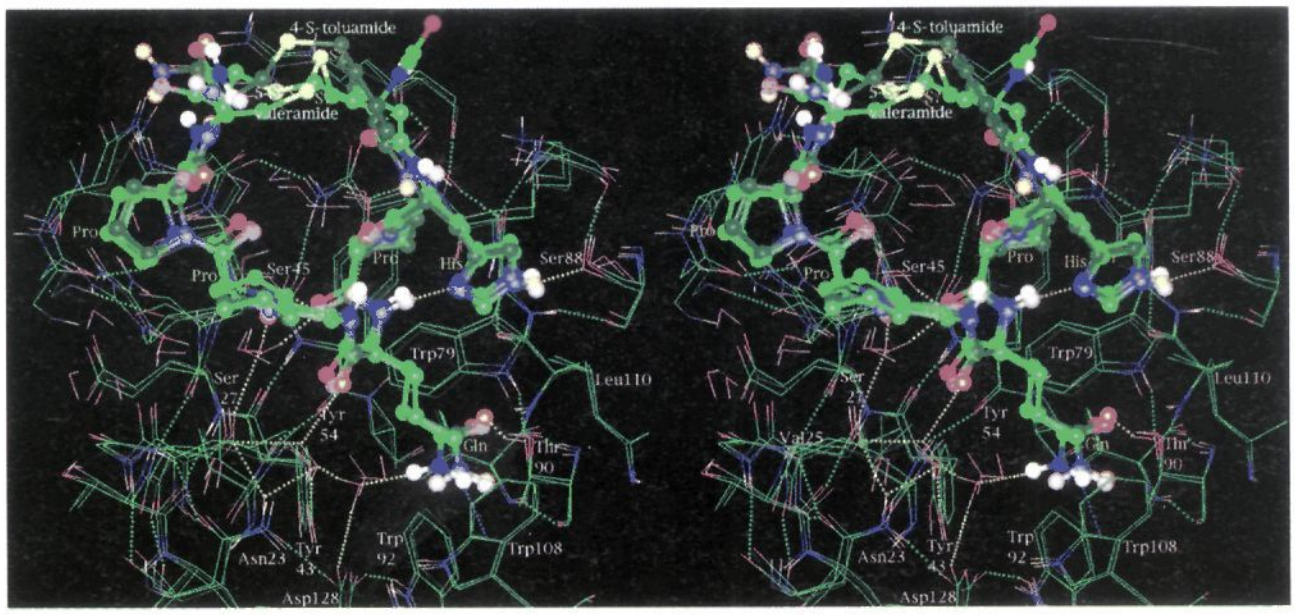

Figure 5. Superposition of the crystal structures of streptavidin-Ac-[CHPQGPPC]-NH_2, streptavidin-*cyclo*-[5-*S*-valeramide-HPQGPPC]-NH_2, and streptavidin-*cyclo*-[4-S-methylbenzamide-HPQGPPC]-NH_2. Ligands are represented by balls and sticks. In the streptavidin-*cyclo*-Ac-[CHPQGPPC]-NH_2 structure the coloring scheme is conventional: carbons are green, oxygens red, nitrogens blue, hydrogens white, and the disulfide Sγ atoms are labeled in yellow. In the streptavidin-*cyclo*-[5-*S*-valeramide-HPQGPPC]-NH_2 structure carbons are cyan, oxygens pink, nitrogens light blue, and hydrogens light pink. In the *cyclo*-[4-*S*-methylbenzamide-HPQGPPC]-NH_2 structure carbons are dark green, oxygens orange, nitrogens purple, and hydrogens light orange. Ligand residues are labeled in yellow font, and streptavidin residues are labeled in white font. Direct or water mediated hydrogen bonds involving the ligands are shown in yellow.

Thioether Cross-Links Are More Flexible Than Their Disulfide Analogs. A comparison of the structures of the thioether and disulfide cross-links is shown in Figure 6, along with the dihedrals that define their structures and conformations. The conformation of the disulfide cross-link in *cyclo*-Ac-[CHPQGPPC]-NH_2 is defined by the $\chi1$, $\chi2$, $\chi3$, $\chi2'$, and $\chi1'$ dihedrals labeled in Figure 6c. The corresponding dihedral energy barriers are 4.0, 2.0, 8.0, 2.0, and 4.0 kcal/mol, respectively.[12] The dihedral with the largest barrier, $\chi3$, involves rotation about the Sγ−Sγ bond (represented by the red rotation arrow). The dihedrals with relatively low (2.0 kcal/mol) energy barriers involving rotation about the RCH_2−SR bonds in the thioether cross-links are represented by green rotation arrows in Figure 6a,b. Within the thioether cross-links there are no large barriers to rotation corresponding to that involving $\chi3$ in a disulfide, and thus thioether cross-links are more flexible than their disulfide analogs.

The disulfide dihedrals in streptavidin-bound *cyclo*-Ac-[CHPQGPPC]-NH_2 are all near their optimal values[12,28] with the exception of $\chi2$ (−137°), which is close to its maximal dihedral energy (occurring at 0°, ±120°). Since the $\chi2$ and $\chi2'$ dihedral energy barriers are lower than the other dihedral energy barriers in a disulfide group, $\chi2$ and $\chi2'$ are expected to be the most variable. This variability in dihedrals about RCH_2−SR bonds is observed for both disulfides and methionines in high resolution protein crystal structures, where values of maximal energy are sometimes observed.[28]

The dihedrals defining the streptavidin-bound 4-*S*-methylbenzamide thioether cross-link are shown in Figure 6b. Like the $\chi2$ value for one of the RCH_2−SR dihedrals in the streptavidin-bound *cyclo*-Ac-[CHPQGPPC]-NH_2 cyclic peptide, both RCH_2−SR dihedrals (128° and 19°) in this thioether are near a maximum in the dihedral energy function. The other dihedrals are of low energy.

The structure and dihedrals for the streptavidin-bound 5-*S*-valeramide cyclic thioether are shown in Figure 6a. All dihedrals are of low energy except for the one labeled in red (128°) which is close to the 4.0 kcal/mol maximum energy for this type of dihedral (involving rotation about an RCH_2−CH_2R bond[12]). Since the density defining the ligand is weakest in this region, this value may be of limited accuracy.

(28) Katz, B. A.; Kossiakoff, A. A. *J. Biol. Chem.* **1986**, *261*, 15480−15485.

Discussion

The novel thioether-containing cyclic peptide ligands, designed from the streptavidin-bound crystal structure of a cyclic disulfide-bonded ligand (*cyclo*-Ac-[CHPQGPPC]-NH_2), retain high affinity binding. The K_d for the 5-*S*-valeramide- and the 4-*S*-methylbenzamide-cross-linked thioether ligands are 680 and 1750 nM, respectively, compared with 310 nM for the disulfide-cross-linked ligand from which they were designed. The thioether ligands make the same H-bonds and van der Waals interactions that the corresponding disulfide-cross-linked peptide ligand makes with the protein.

The 5-*S*-valeramide thioether linkage is much less constrained than the 4-*S*-methylbenzamide linkage. In the latter linkage the 4-methylene carbon and benzamide atoms are all constrained to lie in a plane. Thus the entropy-mediated enhancement of binding should be greater for the 4-*S*-methylbenzamide cross-linked peptide than for the 5-*S*-valeramide cross-linked peptide. However the observed relative binding affinities for the two thioether ligands are opposite to that predicted based on expected differences in conformational entropy; the less constrained thioether ligand binds streptavidin somewhat more tightly than the more constrained one. Other factors, such as differences in desolvation energy or internal conformational energy, must be involved. The 4-*S*-methylbenzamide cross-link contains a total of three more carbon atoms, and all cross-link atoms, including an aromatic ring, are in the solvent region. The fewer hydrophobic atoms in the solvent region for the 5-*S*-valeramide ligand may be one of the determinants of its better binding affinity. Also, since the 5-*S*-valeramide group does not have the planar constraints of the 4-*S*-methylbenzamide group, its conformation may be of lower internal energy.

Tighter binding of cyclic peptide streptavidin ligands compared with corresponding linear ones is believed to reflect a reduction of the conformational entropy of the cyclic ligand in the unbound state.[6] This entropy-mediated enhancement of ligand binding through conformational constraints has been applied to several systems,[29−36] one also involving thioether cross-links.[9] The realization of high affinity binding in the

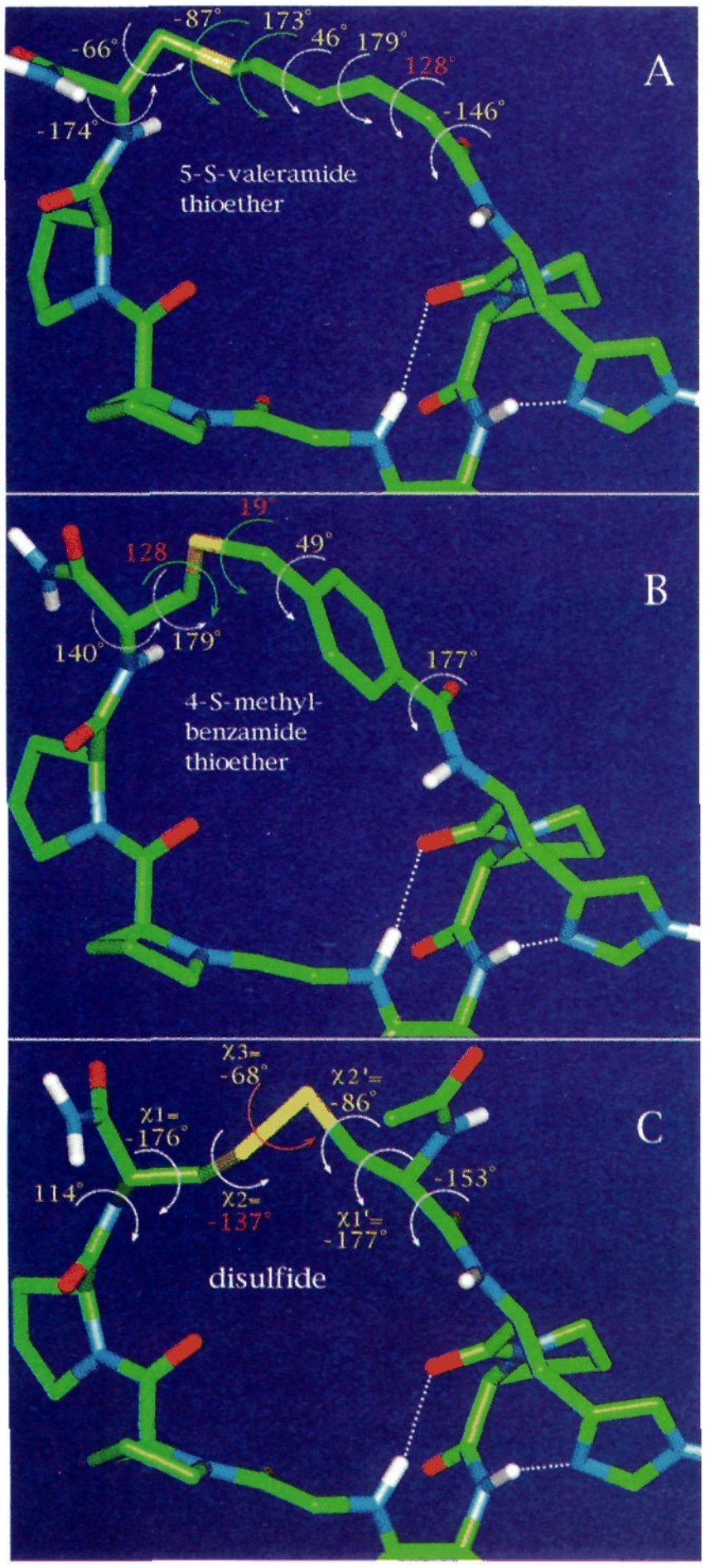

Figure 6. (a) Streptavidin-bound structure of *cyclo*-[5-*S*-valeramide-HPQGPPC]-NH_2 showing the dihedral angles for the cross-link. The dihedrals with low (2.0 kcal/mol) energy barriers to rotation about the RCH_2-SR bonds are represented by the green rotation arrows. The high energy dihedral (128°) is labeled red. (b) Streptavidin-bound structure of *cyclo*-[4-*S*-methylbenzamide-HPQGPPC]-NH_2 showing the dihedral angles for the cross-link with same color scheme as in (a). (c) Streptavidin-bound structure of *cyclo*-Ac-[CHPQGPPC]-NH_2 showing the dihedral angles for the cross-link with same color scheme as in (a).

streptavidin-binding HPQ ligand system and in the glycoprotein IIb/IIIa adhesion molecule binding RGD system[9] indicates that thioether cross-links are an effective alternative to disulfides for providing conformational constraints in ligands.

Both conformational and chemical considerations determine the ease of disulfide bond formation in synthetic peptides, where formation of small rings may be synthetically difficult.[37] While these considerations also hold for thioether cross-links, thioethers are not subject to the disulfide interchange reactions that have been observed in small disulfide-bonded peptides. For both disulfide-bonded cyclic peptides whose streptavidin-bound structures were determined (*cyclo*-Ac-[CHPQGPPC]-NH_2, and *cyclo*-Ac-[CHPQFC]-NH_2), disulfide interchange was observed under some conditions.[2] We expect that disulfide-bonded cyclic peptide ligands of similar size developed for other targets may also exhibit similar lability that is not possible for the corresponding thioethers. Thus the thioethers might afford a simpler chemotype for assay, structure determination, and subsequent redesign.

Another potential advantage of thioether-containing cyclic ligands is that the dihedral energy barrier (2.0 kcal/mol)[12] around the two RCH_2-SR bonds involving the thioether (represented by the green rotation arrows in Figure 6a,b) is much lower than the barrier about the $S\gamma-S\gamma$ bond in disulfides, $\chi 3 = 8.0$ kcal/mol,[12] (represented by the red rotation arrows in Figure 6c). Also, whereas there are three low energy dihedrals, ±60°, 180°, involving each RCH_2-SR bond in a thioether linkage, there are only two, ±83°, involving the $S\gamma-S\gamma$ bond in a disulfide linkage.[12,28] Thus a thioether cross-link having the same number of bonds as a disulfide cross-link should have a greater repertoire of low energy conformations than the disulfide and a better chance of adopting a low energy conformation compatible with the binding conformation of the ligand containing the cross-link.

The greater flexibility of a thioether cross-link compared with a disulfide cross-link containing the same number of rotateable bonds is expected to decrease the entropy-mediated enhancement of binding affinity. However, greater flexibility of thioethers may under some circumstances allow design of thioether cross-links with fewer rotateable bonds than corresponding disulfide cross-links. The entropy-driven affinity enhancement would be greater in the ligands with the shorter cross-links.

Greater diversity is possible in the length and chemical character of thioether cross-links than in disulfide cross-links restricted to cysteines. Greater diversity or flexibility in a cross-link may aid in the successful design of cyclic ligands whose protein-bound structures are not known. Thioether cross-links may thus be useful in the construction of cyclic peptide libraries with more chemical and conformational diversity than corre-

(29) Mosberg, H. L.; Hurst, R.; Hruby, V. J.; Gee, K.; Yamamura, H. I.; Galligan, J. J.; Burks, T. F. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 5871−5874.

(30) Morgan, B. P.; Holland, D. R.; Matthews, B. W.; Bartlett, P. A. *J. Am. Chem. Soc.* **1994**, *116*, 3251−3260.

(31) He, Y.-B.; Huang, Z.; Raynor, K.; Reisine, T.; Goodman, M. *J. Am. Chem. Soc.* **1993**, *115*, 8066−8072.

(32) Etzkorn, F. A.; Guo, T.; Lipton, M. A.; Goldberg, S. D.; Bartlett, P. A. *J. Am. Chem. Soc.* **1994**, *116*, 10412−10425.

(33) Jackson, S.; DeGrado, W.; Dwivedi, A.; Parthasarathy, A.; Higley, A.; Krywko, J.; Rockwell, A.; Markwalder, J.; Wells, G.; Wexler, R.; Mousa, S.; Harlow, R. *J. Am. Chem. Soc.* **1994**, *116*, 3220−3230.

(34) Bach II, A. C.; Eyermann, C. J.; Gross, J. D.; Bower, M. J.; Harlow, R. L.; Weber, P. C.; DeGrado, W. F. *J. Am. Chem. Soc.* **1994**, *116*, 3207−3219.

(35) Ku, T. W.; Ali, F. E.; Barton, L. S.; Bean, J. W.; Bondinell, W. E.; Burgess, J. L.; Callalan, J. F.; Calvo, R. R.; Chen, L.; Eggleston, D. S.; Gleason, J. D.; Huffman, W. F.; Hwang, S. M.; Jakas, D. R.; Karash, C. B.; Keenan, R. M.; Kopple, K. D.; Miller, W. H.; Newlander, K. A.; Nichols, A.; Parker, M. F.; Peishoff, C. E.; Samanen, J. M.; Uzinskas, I.; Venslavsky, J. W. *J. Am. Chem. Soc.* **1993**, *115*, 8861−8862.

(36) McDowell, R. S.; Blackburn, B. K.; Gadek, T. R.; McGee, L. R.; Rawson, T.; Reynolds, M. E.; Robarge, K. D.; Somers, T. C.; Thorsett, E. D.; Tischler, M.; Webb II, R. R.; Venuti, M. C. *J. Am. Chem. Soc.* **1994**, *116*, 5077−5083.

(37) Andreu, D.; Albericio, F.; Solé, N. A.; Munson, M. C.; Ferrer, M.; Barany, G. In *Methods in Molecular Biology, Vol. 35: Peptide Synthesis Protocols*; Pennington, M. W., Dunn, B. M., Eds.; Humana Press Inc.: Totowa, NJ, 1994; Chapter 7, pp 91−169.

sponding disulfide-cross-linked libraries composed of natural amino acids.

A final potential advantage of thioether cross-links compared with disulfides is that two corresponding sulfoxide diasteriomers can be readily prepared from oxidation of each thioether compound. Oxidation of the thioether provides a means of changing the solvation properties of the cross-link to modulate the binding affinity of the ligand. Indeed, for each of the ~30 synthesized thioether-containing RGD ligands that bind to the glycoprotein IIb/IIIa adhesion molecule, one sulfoxide diastereomer showed enhanced affinity compared with the corresponding thioether parent.[9]

Conclusion

The thioether-cross-linked ligands in this study represent a step in the conversion of peptide to non-peptide ligands. Thioether cross-links impart a significant enhancement in the binding of streptavidin ligands compared with linear counterparts and have advantages over disulfide-cross-linked analogs. They do not undergo disulfide interchange/dimerization reactions. Moreover, they are parent molecules for the facile preparation for two additional sulfoxide diasteriomer analogs that may exhibit increased binding affinity. The absence in thioether-cross-linked ligands of the high energy $\chi 3$ dihedral barrier present in disulfides should permit more conformational diversity than in corresponding disulfide-cross-linked ligands. Finally the potential for greater chemical and conformational diversity is inherent in this approach to cyclization that is not restricted to naturally-occurring amino acids. Thus the determination of the structural and chemical criteria and constraints involved in the design and synthesis of high affinity thioether-containing ligands in the streptavidin model system may prove useful in structure-based design of therapeutics in other systems.

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