Preparation of a Protein-Dimerizing Ligand by Topochemistry and Structure-Based Design

Bradley A. Katz*

Arris Pharmaceutical Corporation
385 Oyster Point Boulevard, Suite 3
South San Francisco, California 94080

Received November 2, 1995

Recently we showed that streptavidin crystals of space group *I*222 mediate a disulfide interchange dimerization reaction involving streptavidin-bound cyclic disulfide-bonded peptide ligands presented next to one another in the crystal lattice.[1] The lattice-mediated dimerization of the protein-bound *cyclo*-Ac-[CHPQGPPC]-NH_2 ligand (discovered by phage display[2]), involving breaking and forming of covalent disulfide bonds, is the first example of a chemical reaction mediated topochemically by a protein crystal lattice. The symmetry and packing in the *I*222 streptavidin crystal structure was thus utilized to produce a "head-to-head" disulfide-bonded peptide dimer ligand from the neighboring bound peptide monomers. Here we report the use of a different crystal form of streptavidin (space group $I4_122$) to direct, topochemically, formation of a "head-to-tail" peptide ligand dimer from the same monomer (*cyclo*-Ac-[CHPQGPPC]-NH_2) ligand bound in the $I4_122$ crystal.

Analysis of the $I4_122$ crystal structure of a complex with streptavidin of a related ligand, *cyclo*-Ac-[CHPQFC]-NH_2,[3,4] showed that disulfides of neighboring ligands bound in the crystal lattice are separated by 8.6 Å. This observation suggested that in $I4_122$ crystals of streptavidin complexes with the larger ligand, *cyclo*-Ac-[CHPQGPPC]-NH_2, disulfides of neighboring ligands would contact one another and might undergo a topochemically controlled disulfide interchange reaction, as they do in *I*222 streptavidin crystals, to produce a streptavidin-bound ligand dimer, and to simultaneously dimerize streptavidin. The resulting ligand was expected to be a "head-to-tail" disulfide-bonded dimer based on the symmetry and packing in $I4_122$ streptavidin crystals.

To determine whether or not *cyclo*-Ac-[CHPQGPPC]-NH_2 ligands bound in $I4_122$ streptavidin undergo disulfide interchange, the ligand monomer[3] was incubated with $I4_122$ streptavidin crystals grown as described.[3] Resulting samples were analyzed by high-performance liquid chromatography (HPLC) on a Hewlett-Packard 1090 analytical HPLC instrument using a Vydec C18 reverse phase column (15 × 1.0 cm). Separation was achieved with a linear gradient over 15 min from 0 to 35% acetonitrile in aqueous solutions containing 0.05% TFA. Selected samples were further characterized with HPLC (Michrom BioResources ultrafast microprotein analyzer) coupled to electrospray ionization mass spectrometry (Finnigan MAT SSQ710). For comparison HPLCs were also run for solutions in which the peptide was incubated with *I*222 streptavidin crystals or incubated in buffer alone. Before HPLCs were run on crystals, the crystals were harvested by centrifugation, washed with 1.4 mL of peptide-free synthetic mother liquor, centrifuged, and washed again.

The HPLC chromatogram for freshly dissolved *cyclo*-Ac-[CHPQGPPC]-NH_2 shows one monomer peak with a retention time of 9.78 ± 0.15 min.[5] Incubation of the monomer in buffer alone (pH 7.5) for 5 days results in formation of a relatively small amount of dimer (Figure 1A). By contrast, incubation for 5 days with $I4_122$ streptavidin crystals causes formation of a relatively large amount of dimer (retention time = 11.97 ± 0.14 min), concomitant with a large decrease in the monomer within the crystals (Figure 1B). Mass spectrometry yields a molecular weight of 877.4 ± 0.3[5] for the monomer (M^+_{calcd} = 877.35) and of 1753.9 ± 0.6 for the dimer (M^+_{calcd} = 1753.7). Incubation of the peptide monomer with *I*222 crystals directs formation of the "head-to-head" dimer in the crystals,[1] with a somewhat different retention time (11.47 ± 0.12 min) and broader peak from that of the dimer formed in the $I4_122$ crystals (Figure 1C). The chemically synthesized "head-to-head" dimer also shows a broad peak with the same retention time as that formed from the monomer in *I*222 streptavidin crystals.[1] Coinjection of $I4_122$ crystals, incubated with monomer, with chemically synthesized head-to-head dimer[1] yields two dimer peaks (data not shown).

In the *I*222 crystal, dimerization occurs at only half of the ligand sites (the sites that are close to a crystallographic 2-fold axis), and thus after incubation the ratio of dimer to monomer in the crystals is 1:1 (by weight) and does not increase further[1] (Figure 1C). In the $I4_122$ incubations, however, more than 90% of the monomer is converted to dimer after 2 weeks (data not shown). Treatment with 10 mM DTT of $I4_122$ and of *I*222 crystals of the streptavidin−peptide dimer complex yielded identical HPLC chromatograms (data not shown).

In the supernatant of incubations of peptide solution with $I4_122$ crystals (and also with *I*222 crystals),[1] the amount of dimer produced is small, equivalent to that produced in the absence of crystals. The absence of significant amounts of dimer in the supernatant could reflect a lack of dimer release from the crystal due to the expected high affinity of such a dimer.[1] It could also reflect a thermodynamic tendency of the dimer to revert back to the monomer by disulfide interchange when the ligand dissociates from the crystal, since there is no significant dimer formation in the absence of crystals (Figure 1A).

To determine the structure of the peptide dimer that forms within $I4_122$ streptavidin crystals, the crystals were soaked in monomer solution (10 mg/mL) at pH 7.5 for several days, mounted, and left for 4 weeks to ensure quantitative dimer formation before data collection. Crystallographic data collection, data reduction, and solution and refinement[6] of the structure were carried out by described procedures.[3] The *R*-factor for the crystallographically refined structure is 17.1% (1.92 Å resolution).

Figure 2 shows the $(2|F_o| - |F_c|)$, α_c electron density maps[7] for the $I4_122$ streptavidin-bound *cyclo*-Ac-[CHPQGPPC]-NH_2 "head-to-tail" dimer produced by topochemistry in the crystal. Strong density is observed for the ligand CHPQGP segment. Weaker density and higher temperature factors for the remaining ligand residues, which do not make any interactions with the protein, reflect higher mobility. The C-terminal sulfurs of the "head-to-tail" disulfides are not well determined by density. The structure and symmetry are incompatible with "head-to-head" dimerization; the well defined Cα atoms of symmetry-related N-terminal peptide cysteines (Figure 2) are much too far apart (9.8 Å) to allow a disulfide between them.[8]

Since peptide dimerization occurs at all four ligand binding sites of the streptavidin tetramer in the $I4_122$ crystals, strepta-

* Phone: (415) 737-1643. FAX: (415) 737-8590. E-mail: bak@arris.com.

(1) Katz, B. A.; Stroud, R. M.; Collins, N.; Liu, B.; Arze, R. *Chem. Biol*. **1995**, *2*, 591−600.

(2) Giebel, L. B.; Cass, R. T.; Milligan, D.; Young, D.; Arze, R.; Johnson, C. *Biochemistry* **1995**, *34*, 15430−15435.

(3) Katz, B. A. *Biochemistry* **1995**, *34*, 15421−15429.

(4) Katz, B. A.; Cass, R. T.; Liu, B.; Arze, R.; Collins, N. *J. Biol. Chem.* **1995**, *270*, 31210−31218.

(5) Standard deviations are derived from 7−15 independent determinations.

(6) Structures were refined with Xplor (Brünger, A. T. *X-PLOR Manual*, version 3.0; Yale University: New Haven, CT, 1992) and with difference Fourier methods (Chambers, J. L.; Stroud, R. M. *Acta Crystallogr*. **1979**, *B33*, 1861). Water structure was determined and refined according to published procedures (Finer-Moore, J. S.; Kossiakoff, A. A.; Hurley, J. H.; Earnest, T.; Stroud, R. M. *Proteins* **1992**, *12*, 203−222).

(7) The following abbreviations and definitions are used: F_o and F_c, observed and calculated structure factors: α_c, calculated phases.

0002-7863/96/1518-2535$12.00/0

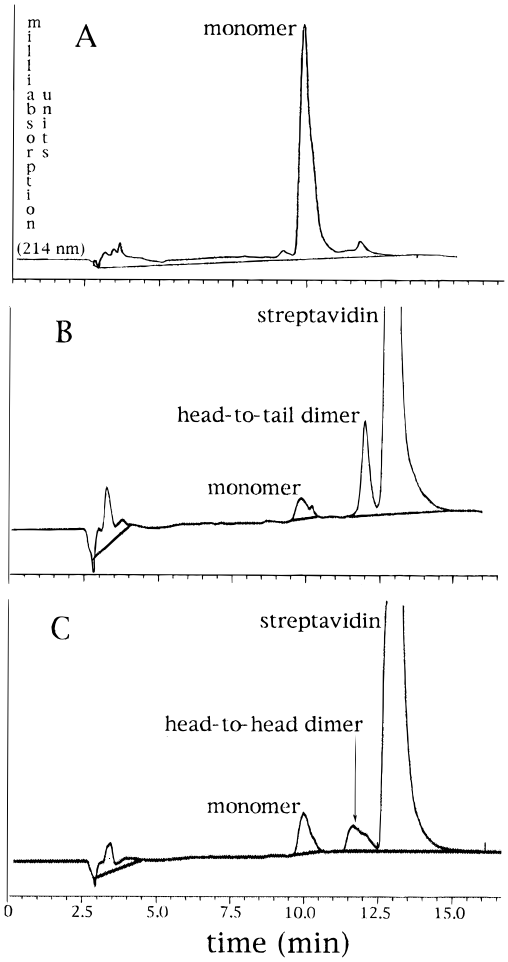

Figure 1. HPLC chromatograms for (A) *cyclo*-Ac-[CHPQGPPC]-NH_2 monomer (1.0 mM) incubated in buffer (50% saturated ammonium sulfate, 50% 1.0 M Tris, pH 7.5) for 5 days; (B) crystals of $I4_122$ streptavidin after 5 days of incubation with the monomer in buffer; (C) crystals of $I222$ streptavidin after 5 days of incubation with the monomer.

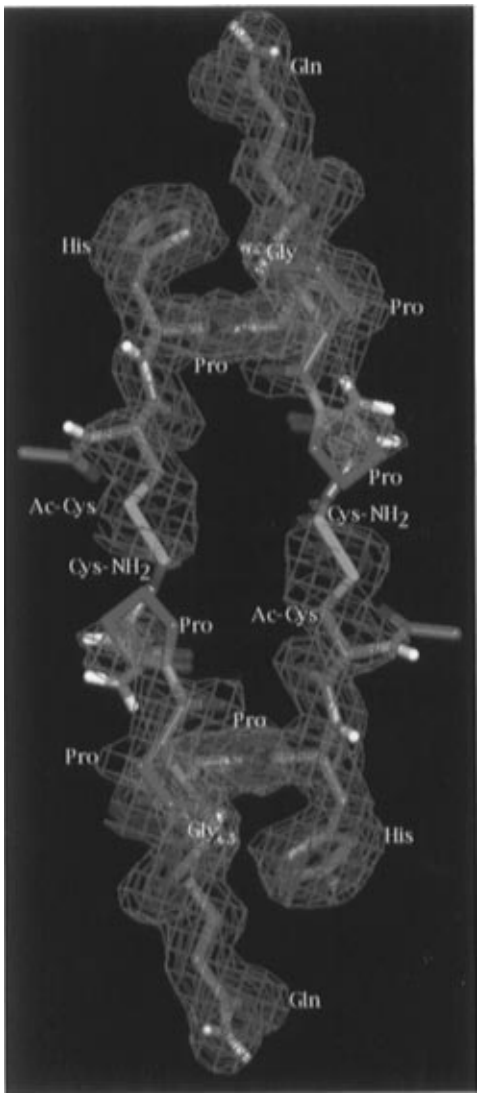

Figure 2. $(2|F_o| - |F_c|)$, α_c map for the streptavidin-bound head-to-tail peptide dimer superimposed on the refined structure.

vidin tetramers are polymerized by the ligand dimer. These crystals are insoluble in water, methanol, and dimethyl sulfoxide, reflecting both the polymeric nature of the crystals and the high affinity of the peptide dimer for them.

Topochemical reactions have been studied for many years in small-molecule crystals: Such lattice-controlled reactions include asymmetric synthesis achieved by lattice-controlled photorearrangements,[9] by heterogeneous reactions,[10] or by thermal and photochemical reactions.[11] Also included are X-ray-induced cycloaddition[12] or racemization,[13] photochemical cycloaddition, dimerization, and polymerization,[9,14,15] and a thermal dimerization.[16] Observation of lattice-directed ligand dimerization in not only the $I4_122$ but also the $I222$ streptavidin crystal lattice suggests that the scope of the diverse topochemical reactions seen in small-molecule crystals can be extended to protein crystals.

Topochemical reactions could also occur in the absence of a crystal lattice,[1] mediated instead by associated protein monomers within a dimer complex which binds ligand monomers with ligating groups presented next to one another. Preparation of protein-dimerizing agents by topochemistry in the streptavidin model systems provides insight into strategies to prepare ligands that dimerize other proteins whose biological activity depends on dimerization. Such ligand agonists may have pharmaceutical uses.

JA9536936

(8) The average Cα−Cα distance for disulfide-bonded residues in the Brookhaven Data Base is 5.45 (0.63) Å (Katz, B. A.; Kossiakoff, A. A. *J. Biol. Chem.* **1986**, *261*, 15480−15485).

(9) Suzuki, T.; Fukushima, T.; Yamashita, Y.; Miyashi, T. *J. Am. Chem. Soc.* **1994**, *116*, 2793−2803.

(10) Chenchaiah, P. C.; Holland, H. L.; Richardson, M. F. *J. Chem. Soc., Chem. Commun.* **1982**, 436−437.

(11) Addadi, L.; Ariel, S.; Lahav, M.; Leiserowitz, L.; Popovitz-Biro, R.; Tang, C. P. In *Chemical Physics of Solids and Their Surfaces*; Roberts, M. W., Thomas, J. M., Eds.; Specialist Periodical Reports; The Royal Socity of Chemistry: London, 1980; Vol. 8, Chapter 7, pp 202−244.

(12) Mori, A.; Kato, N.; Takeshita, H.; Kurahashi, Y.; Masahito, I. *J. Chem. Soc., Chem. Commun.* **1994**, 869−870.

(13) Ohashi, Y. *Acc. Chem. Res.* **1988**, *21*, 268−274.

(14) Enkelmann, V.; Wegner, G.; Novak, K.; Wagner, K. B. *J. Am. Chem. Soc.* **1993**, *115*, 10390−10391.

(15) Nakanishi, H.; Hasegawa, M.; Sasada, Y. *J. Polym. Sci., Polym. Phys. Ed.* **1977**, *15*, 173−191.

(16) Miller, E. J.; Brill, T. B.; Rheingold, A. L.; Fultz, W. C. *J. Am. Chem. Soc.* **1983**, *105*, 7580−7584.