Topochemistry for preparing ligands that dimerize receptors

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Background: The cyclic, disulfide-containing peptide, *cyclo*-Ac-[Cys-His-Pro-Gln-Gly-Pro-Pro-Cys]-NH_2, binds to streptavidin with high affinity. In streptavidin–peptide cocrystals of space group I222, cyclic peptide monomers are bound on adjacent streptavidin tetramers related by a crystallographic two-fold symmetry axis. We set out to determine whether the disulfide bonds of the peptide, presented close to one another in the crystal, could undergo disulfide interchange to form a dimer.

Results: When juxtaposed, the disulfides of neighboring peptides undergo disulfide interchange, breaking and forming covalent disulfide bonds, to produce a peptide dimer adopting the symmetry of the crystal. This is the first example of a chemical transformation mediated by a protein crystal lattice. The structure of the streptavidin-bound monomer, and that of the dimer that was eventually produced from it in the crystal, were both determined from the same single crystal studied at different times. The two-fold symmetric peptide dimer was independently synthesized and shown to form crystals of dimerized streptavidin.

Conclusions: We have shown that formation of a covalently linked peptide dimer can be mediated by a protein crystal lattice. The dimer thus produced dimerizes its target, streptavidin, suggesting that solid-state (or topochemical) reactions of this kind may be broadly useful for the preparation of ligands that can dimerize other protein targets.

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Introduction

Dimerization is a trigger for many important biological processes [1–4] including activation of intracellular kinases by membrane-bound cell surface receptors [1]. Dimerization is also important in transduction of signals from T-cell receptors [1], in the activity of transcription factors [5] and immunophilins [6,7], and in protein degradation [8]. Signaling via the human growth hormone receptor requires homodimerization of cytokine-binding domains to produce a cytokine–cytokine receptor complex with approximate C2 symmetry [9]. Monoclonal antibodies that dimerize this receptor are potent agonists [10], indicating that proximity induced by dimerization suffices to elicit the intracellular response. Receptor homo- or heterodimerization is also induced by erythropoietin [11], where the symmetry of the dimerized receptor may also be quasi-C2-symmetric (due to homology within the cytokine receptor class, reviewed in [1,2]).

Since ligand-induced protein dimerization is such a ubiquitous signaling mechanism in biology, development of small molecules that dimerize target proteins may lead to compounds of pharmaceutical value. Streptavidin is a protein with a well characterized, high-resolution crystal structure. To gain insight into the structural and stereochemical criteria and constraints involved in designing ligands that promote protein dimerization, we have used streptavidin as a model system with which to study ligand-mediated dimerization of streptavidin tetramers.

Screening of libraries of disulfide-linked cyclic peptides displayed on phage led to discovery of a high-affinity (K_d = 670 nM) cyclic, disulfide-crosslinked streptavidin ligand, *cyclo*-Ac-[Cys-His-Pro-Gln-Gly-Pro-Pro-Cys]-NH_2 [12]. The structural basis for the affinity of this ligand was studied crystallographically (B.A.K., unpublished data). Under some conditions this peptide undergoes disulfide interchange in solution to produce a dimer with two intermonomer disulfide bonds. In crystals of the streptavidin–peptide complex of space group I222, *cyclo*-Ac-[Cys-His-Pro-Gln-Gly-Pro-Pro-Cys]-NH_2 monomers are bound to adjacent streptavidin tetramers related by a crystallographic two-fold symmetry axis. The disulfides of the peptide monomers are presented close enough to one another in the crystal to expect that a disulfide interchange reaction could occur within the crystal.

When the pH of the streptavidin–peptide monomer I222 crystals was maintained at 5.0 or higher for several weeks, we observed interchange between the disulfides on adjacent, two-fold symmetry related streptavidin–*cyclo*-Ac-[Cys-His-Pro-Gln-Gly-Pro-Pro-Cys]-NH_2 monomers to produce a C2-symmetric peptide dimer with two intermolecular disulfide bonds passing through one of the crystal's two-fold symmetry axes (Fig. 1). We have thus established a strategy for producing a streptavidin-dimerizing ligand, by presentation of ligand monomers to each other within a protein

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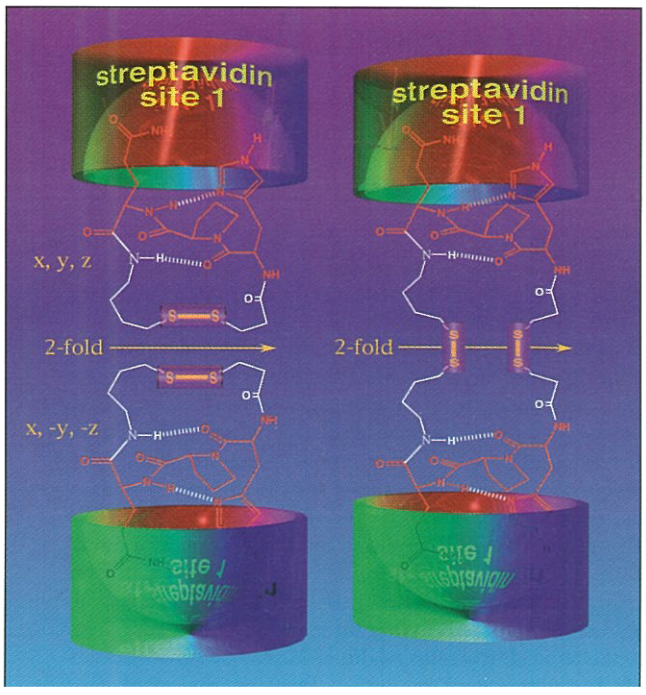

Fig. 1. Schematic diagram showing the disulfide interchange between peptide ligands and the resulting streptavidin–peptide dimerization occurring in I222 crystals of the streptavidin–peptide monomer complex. The peptide segments before and after the amino-acid sequence His-Pro-Gln-Gly are schematic (some atoms are not shown).

crystal lattice in an orientation that encourages a specific crosslinking reaction. A ligand dimer that can in turn dimerize the protein is thus produced.

Here we describe the exploitation of packing interactions and symmetry in streptavidin crystals to mediate, within the crystal, a chemical reaction controlled 'topochemically' by the appropriate presentation of reactive ligand groups. Topochemical reactions have been observed in small-molecule crystals. Stereospecific chemical reactions in chiral crystals have been used to provide a method of asymmetric synthesis [13]; such reactions include lattice-controlled photorearrangements [14–16], heterogeneous reactions [17] and thermal and photochemical reactions [18]. Other crystal-mediated, lattice-controlled reactions in small-molecule crystals include X-ray induced cycloaddition [19] or racemization [20], photochemical cycloaddition, dimerization and polymerization [14,21–24], and a thermal dimerization [25].

When crystal packing forces direct the reactions in small-molecule crystals, single crystal-to-single crystal chemical transformations can occur [19,23,26]. Such transformations involve conversion of reactants to products within a crystal. We are aware of no other examples of such lattice-controlled transformations involving covalent chemistry within protein crystals, however. The protein crystal mediated transformation illustrated in Figure 1 is the first example of such a reaction.

Results and discussion

Two distinct structures determined in one crystal

The structure, symmetry, and $(2|F_o|-|F_c|)$, α_c density for the two neighboring monomers in the I222 crystal structure of streptavidin–*cyclo*-Ac-[Cys-His-Pro-Gln-Gly-Pro-Pro-Cys]-NH_2 monomer is shown in Figure 2a.

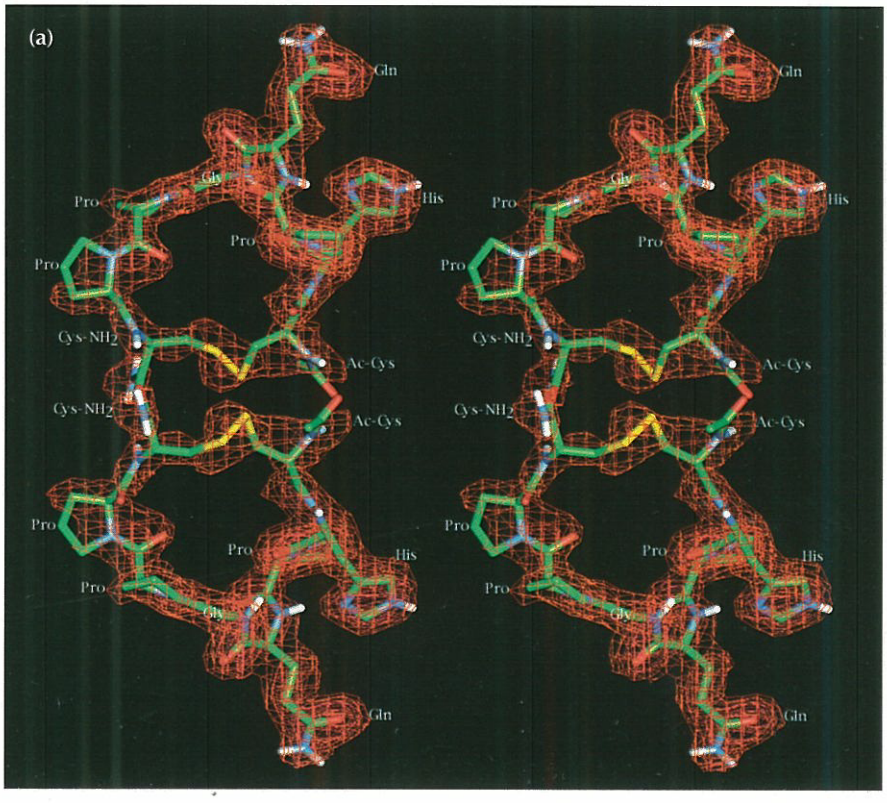

Fig. 2. Stereoimages showing the X-ray crystal structures of peptide ligands in complex with streptavidin. **(a)** $(2|F_o|-|F_c|)$, α_c map for the neighboring streptavidin-bound monomers of *cyclo*-Ac-[Cys-His-Pro-Gln-Gly-Pro-Pro-Cys]-NH_2 ligands. (Fig. 2 continued on following page) ➤

After six days of data collection on this crystal followed by six weeks of incubation, the same crystal was re-analyzed crystallographically. A large contraction in the cell volume (by 32 900 ± 5800 Å^3, Table 1) occurred, and the crystal diffracted to better resolution than initially. Another complete data set was collected. Subsequent refinement established that peptide monomers were crosslinked across a crystallographic two-fold axis. The

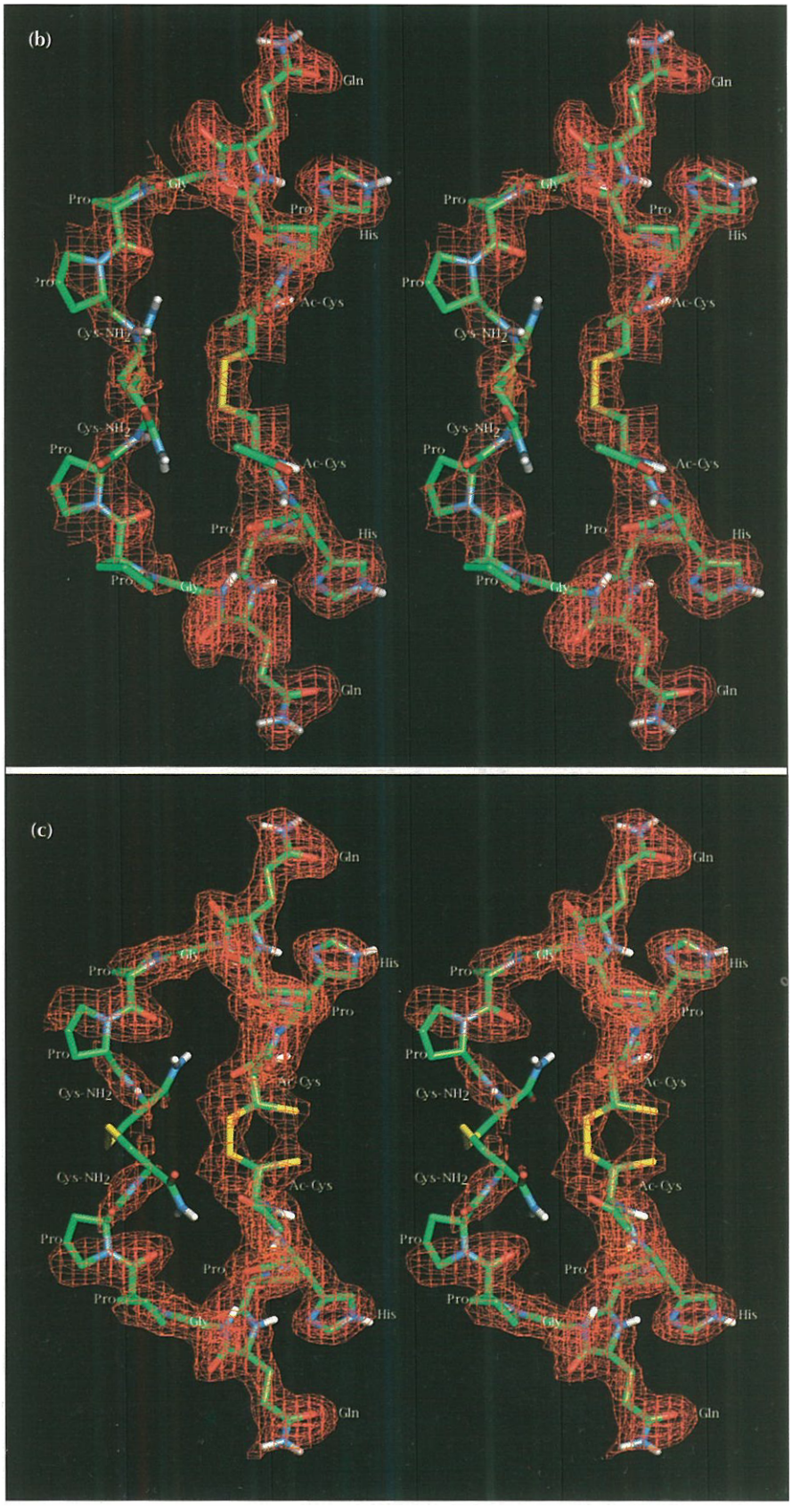

Fig. 2 (cont.). **(b)** $(2|F_o|-|F_c|)$, α_c map for streptavidin-*cyclo*-Ac-[Cys-His-Pro-Gln-Gly-Pro-Pro-Cys]-NH_2 dimer produced by disulfide interchange in the crystal used for the structural determination of the streptavidin–peptide monomer complex in (a) above, superimposed on the refined structure. Both of the structures in (a) and (b) were determined using the same single crystal. **(c)** $(2|F_o|-|F_c|)$, α_c map for the streptavidin-bound *cyclo*-Ac-[Cys-His-Pro-Gln-Gly-Pro-Pro-Cys]-NH_2 dimer ligand, chemically synthesized and then soaked into the crystals. An essentially identical structure was obtained when the dimer peptide was cocrystallized with streptavidin instead.

Table 1. Unit cell characteristics.

	n[a]	a (Å)	b (Å)	c (Å)	Volume (Å^3)
Sulfate, pH 5.6	5	95.43 (29)	106.12 (11)	47.50 (22)	481 000 (2700)
Monomer, pH 5.0	5	97.65 (63)	107.61 (44)	49.46 (26)	519 700 (4900)
Dimer, same crystal	5	95.30 (30)	105.49 (28)	48.42 (24)	486 800 (3100)
Dimer, cocrystal, pH 6.0	12	95.42 (41)	105.39 (23)	47.78 (22)	480 500 (3200)
Dimer, soaked, pH 6.0	18	95.60 (71)	106.84 (60)	48.45 (20)	494 800 (5000)

Lattice parameters and cell volumes are for apostreptavidin (streptavidin sulfate), and streptavidin–*cyclo*-Ac-[Cys-His-Pro-Gln-Gly-Pro-Pro-Cys]-NH_2 monomer and dimer.
[a]n is the number of orientation matrices (determined from different batches of data) that were used to calculate averages and corresponding standard deviations (shown in parentheses).

structure, symmetry and $(2|F_o|-|F_c|)$, α_c density of the streptavidin-bound peptide dimer produced after disulfide interchange in a crystal initially composed of the streptavidin–peptide monomer complex are shown in Figure 2b. One of the new crosslinks, the Cys1–Cys1' disulfide (on the right of Fig. 2b) is unambiguously defined by density. The Cys8–Cys8' disulfide is not as well defined by density, suggesting that it may be disordered. Mass spectrometry (see below) shows that the sulfurs are not modified; the molecular weight of the dimer is within 0.3 mass units expected for the M+1 species of a dimer with two disulfides.

Synthetic peptide dimer dimerizes streptavidin

To test the ability of a synthetic dimer to dimerize receptors, a 'head-to-head' *cyclo*-Ac-[Cys-His-Pro-Gln-Gly-Pro-Pro-Cys]-NH_2 peptide dimer was synthesized and cocrystallized with streptavidin. We determined and refined the structure of the streptavidin–peptide dimer cocrystal and also that of the same complex produced by soaking the pre-made head-to-head dimer into crystals. The structure of the protein–peptide dimer complex in these determinations is nearly identical to the structure resulting from dimerization of the monomer within the crystal.

The structure of the chemically synthesized *cyclo*-Ac-[Cys-His-Pro-Gln-Gly-Pro-Pro-Cys]-NH_2 dimer soaked into a streptavidin crystal is shown in Figure 2c. The disulfide bond between Cys1 and Cys1' is again clearly defined. Density around Cys8 and Cys8' remains weak or absent, again suggesting some disorder in this region of the peptide.

In $(|F_o|-|F_c|)$, α_c maps of the streptavidin-bound peptide dimer where the sulfur atoms were omitted from the refinement and map calculations, and in $(2|F_o|-|F_c|)$, α_c maps, the density for the disulfide involving the amino-terminal cysteines residues is well defined (Fig. 2c). The refined, unrestrained distance between the sulfur atoms of the amino-terminal cysteine residues is 1.90 Å, in close agreement with the accepted value of 2.02 Å for a disulfide bond [27]. The refined, unrestrained dihedral angle about the S–S bond ($\chi 3$) for this disulfide conformation is 86°, which is close to the optimal value for a right-handed disulfide [28]. The additional density connecting the amino-termini in Figure 2c shows that the amino-terminal disulfide also adopts a second, alternative conformation. Refined occupancies for both conformations give a ratio of 3:2. This is also the major conformation adopted by the single disulfide bond formed when a peptide that contains only one cysteine residue (either Phe-Cys-His-Pro-Gln-Asn-Thr-NH_2 or Ac-Cys-His-Pro-Gln-Asn-Thr-NH_2), forms a homodimer in a cocrystal complex with streptavidin (B.A.K., R.T. Cass, B.L., N.C. & R.A., unpublished data).

In both the streptavidin-bound monomer and dimer crystal structures, Pro7 and the two cysteines of the ligand are on the surface of the protein and do not interact with the protein. The density for these residues is not as well defined as that for the other ligand residues, which interact strongly with, and are as well defined as, the protein. Weak density and high temperature factors for Pro7 and the two cysteines reflects the mobility and/or disorder of these residues. Table 2 compares the temperature factors of some of the least mobile ligand atoms with those of the disulfide sulfurs in the streptavidin-bound peptide monomer and dimer crystal structures.

Disulfide interchange induces crystal packing changes

The lattice parameters and volumes for the streptavidin–peptide monomer complex are significantly larger than those for the streptavidin–peptide dimer complex or for apostreptavidin (Table 1). The crystal structure of the streptavidin–peptide dimer complex produced by dimerization of the peptide monomer within the crystal, or by cocrystallization or soaking in the presence of chemically synthesized peptide dimer solution, show that the peptide monomers and the associated bound streptavidin tetramers are about 1.0 Å closer together in the protein–peptide dimer complex than in the protein–peptide monomer complex.

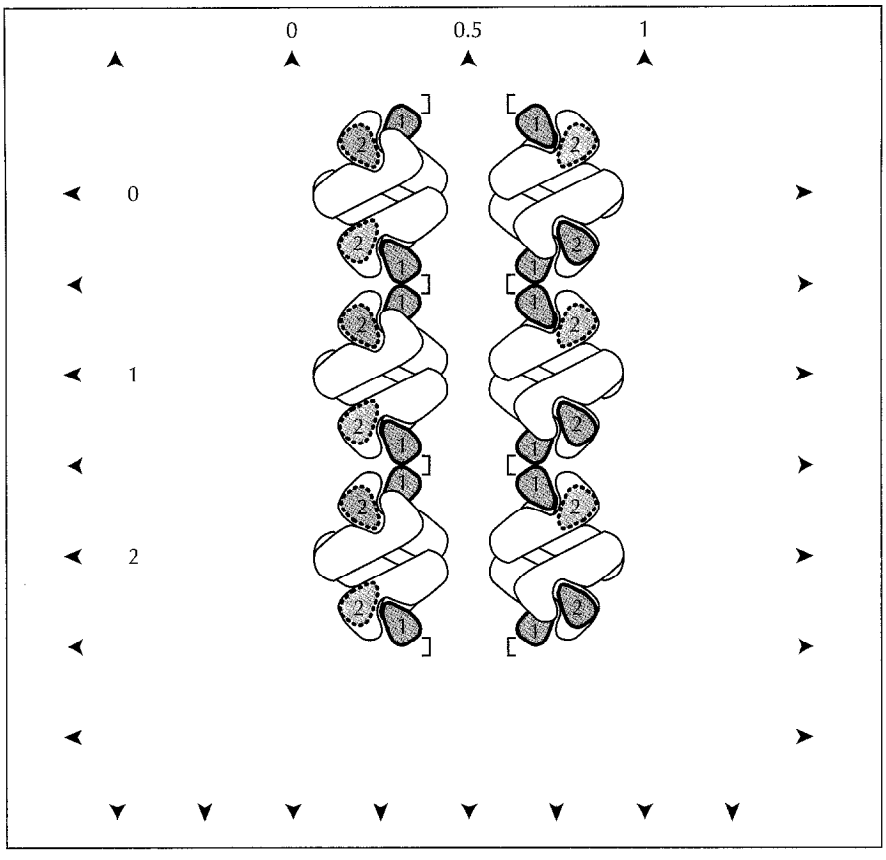

Fig. 3. Schematic packing diagram for the streptavidin–peptide dimer crystal structure produced from disulfide interchange within the crystal. The a-axis of the lattice is horizontal and the c-axis vertical. Dimerization occurs at the site 1–site 1 binding sites. The site 2 binding sites are separated by a larger distance and are not crosslinked. The two-fold axes in the a–c plane are represented by arrows. The fractions of unit cell dimensions along the a and c directions are given. The peptides bound at site 2 that are obscured by the protein are represented by dotted lines.

Peptide dimers crosslink streptavidin tetramers

The packing diagram for the streptavidin–peptide dimer complex (Fig. 3) shows the two crystallographically independent streptavidin subunits, each with a bound peptide. Binding site 1 on one subunit is close to a crystallographic two-fold axis, whereas site 2, on the crystallographically non-equivalent subunit, is not. Thus the streptavidin–cyclic peptide dimer complex has two symmetry-related sites at which the peptide dimer crosslinks the proteins, so that the crystals are composed of networks of crosslinked streptavidin tetramers. The other binding site (site 2) is not involved in protein oligomerization in the crystal, and is more solvent-exposed. These relationships are shown schematically in Figure 3.

HPLC and LC–MS studies of disulfide interchange

The crystal-induced dimerization of *cyclo*-Ac-[Cys-His-Pro-Gln-Gly-Pro-Pro-Cys]-NH_2 was confirmed by high performance liquid chromatography (HPLC) or by HPLC coupled to mass spectrometry (LC–MS). Incubation of the monomer for nine days in the presence of crushed I222 streptavidin crystals results in two prominent peaks in HPLC chromatograms (Fig. 4a). Electrospray and ion desorption mass spectrometry show that the first major peak is monomer ($MW_{experimental}$ = 877.3 ± 0.3 (15 measurements); $MW_{calculated}$ (M+1) = 877.35), and that the second major peak is characteristic of a dimer with two disulfides (MW_{exptl} = 1754.0; $MW_{calc.}$ (M+1) = 1753.7). The fraction of dimer is ~25 %.

Table 2. Temperature factors for selected atoms.

	Gln4 Oε1	Gln4 Nε2	His2 Nε2	His2 Nδ1	Cys1 Sγ	Cys8 Sγ
Monomer pH 5.0	7.5	15.1	12.1	12.1	68.3	69.8
Dimer, same crystal, pH 5.0	12.3	14.1	17.2	15.8	67.8	80.2
Dimer, cocrystal, pH 6.0	2.0[a]	4.9	28.7	27.7	55.0	90.6
Dimer, soaked, pH 6.0	15.2	17.3	16.2	16.4	41.4[b] 43.8	88.0

Temperature factors are given in Å^2 for selected atoms in the *cyclo*-Ac-[Cys-His-Pro-Gln-Gly-Pro-Pro-Cys]-NH_2 monomer and dimer ligands.
[a]Minimum temperature factor allowed by Xplor [27].
[b]Refined occupancy in major conformation of 0.60 corresponds to B = 41.4 Å^2; refined occupancy in minor conformation of 0.40 corresponds to B = 43.8 Å^2.

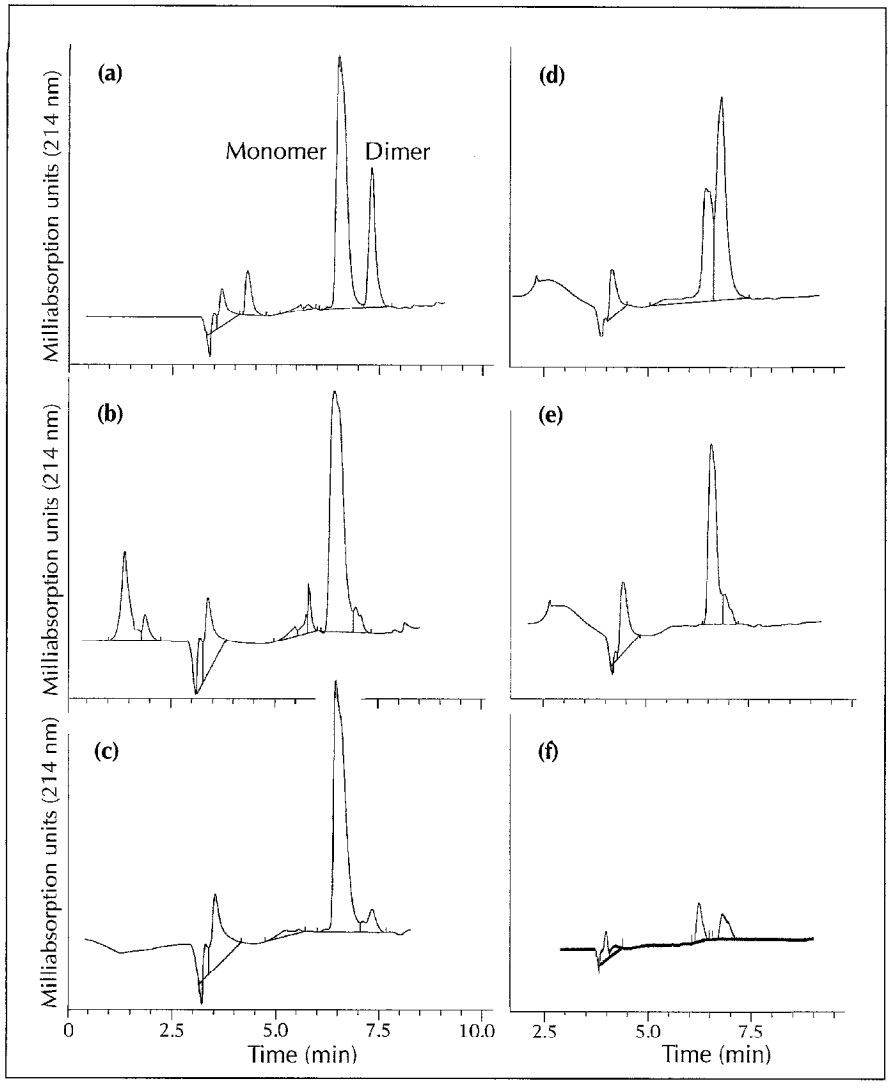

Fig. 4. HPLC chromatograms after incubation of *cyclo*-Ac-[Cys-His-Pro-Gln-Gly-Pro-Pro-Cys]-NH_2 monomer (1.5×10^{-8} moles peptide at a concentration of 0.30 mM) for nine days in: **(a)** buffer containing finely crushed I222 apostreptavidin crystals from two sitting-drop crystallization setups. (The maximum possible amount of the crystals, based on the sitting-drop crystallization conditions, is 1.0×10^{-8} moles of subunits); **(b)** 50 % saturated ammonium sulfate, 50 % buffer, pH 7.0; **(c)** buffer containing a similar amount of non-crystalline streptavidin. **(d)** HPLC chromatogram resulting when the sample used for (a) was coinjected with chemically synthesized dimer, showing that the synthetic dimer comigrates with the dimer produced from the monomer in the crystals. **(e)** HPLC chromatogram of the supernatant from the sample used for (a). **(f)** HPLC chromatogram of crystals alone of sample (a) after thorough washing of the crystals.

Although the disulfide involving the carboxy-terminal cysteines showed weak or absent density in the dimer structures (Fig. 2b,c), the mass obtained for the peptide dimer is within experimental error of that expected for a dimer with two disulfide crosslinks. Moreover, removal of a disulfide would require reduction of the peptide; since no reductants were present, and the solutions were accessible to oxygen from the air, it is highly unlikely that the second disulfide is not present. It is much more likely simply to be disordered in the crystal.

The HPLC chromatogram of the monomer incubated at pH 7.0 in 50 % saturated ammonium sulfate for nine days in the absence of streptavidin (Fig. 4b) shows a predominant monomer peak and very little dimer. In the negative control involving incubation of the monomer for nine days in the presence of non-crystalline streptavidin (Fig. 4c) the minor dimer peak comprises only ~5 % of the sample. These HPLC experiments demonstrate that disulfide interchange and peptide dimerization occur in the absence of X-ray irradiation.

It is important to note that the peptide dimer that eventually forms in the controls could be chemically different from that produced in the crystals, even though they comigrate on HPLC. (It is conceivable that the 'head-to-tail' dimer, or an inseparable mixture of the two possible dimers, might form in the controls described above.) This would only emphasize the important role of the crystal in directing the reaction, however. We did not investigate the identity of the disulfide bonds formed in solution in the absence of I222 streptavidin crystals.

Figure 5 shows the rate of crosslinked peptide dimer formation during incubation of the peptide monomer in the presence of streptavidin (in a cocrystallizing solution) (trace A), of I222 streptavidin crystals (trace B), and of buffer alone (trace C). In the presence of I222 streptavidin crystals, transition from monomer to dimer occurs within three days (Fig. 5, trace B). Under crystallization conditions in the presence of streptavidin (Fig. 5, trace A), the appearance of the dimer peak after 12 days coincided with crystallization. The larger fraction of dimer in trace A (~50 %) compared with trace B (~25 %) is attributed to a greater amount of streptavidin in the former sample.

Protein crystal lattices can catalyze chemical reactions

The chemical transformation induced in the crystal lattice of the streptavidin–*cyclo*-Ac-[Cys-His-Pro-Gln-Gly-Pro-Pro-Cys]-NH_2 monomer complex has many features of

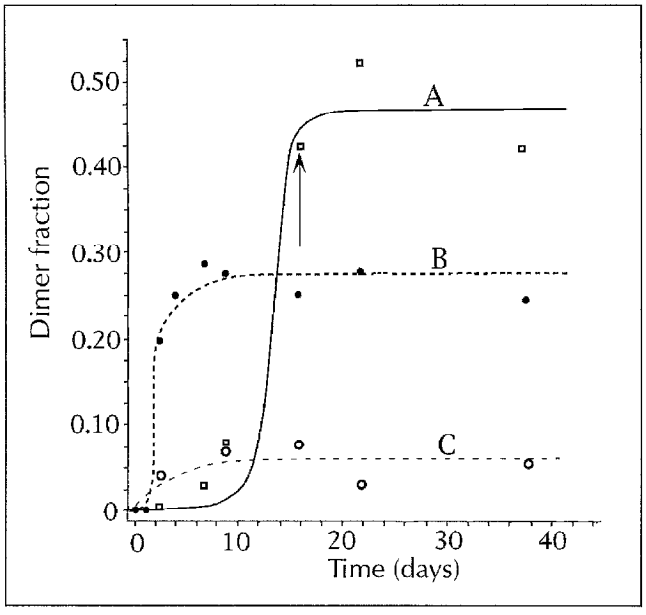

Fig. 5. Fraction of peptide dimer as a function of time determined by integration of HPLC peaks for incubations of the monomer in the presence of streptavidin under conditions for producing I222 crystals (trace A), I222 streptavidin crystals (trace B), or buffer alone (trace C). At the time point indicated by the arrow, streptavidin microcrystals were observed.

reactions catalyzed by enzymes. The adjacent, bound cyclic peptide monomers are in productive orientations for the ensuing reaction, the effective concentration of the reactant is greatly raised, and binding involves H-bonds, ordered water molecules, and hydrophobic interactions at several sub-sites away from the receptor site.

Unlike enzymatic catalysis, however, the crystal packing interactions in I222 streptavidin crystals are unlikely to significantly stabilize the transition state for the disulfide interchange reaction. In addition, the I222 streptavidin crystals cannot catalyze more than one round of dimerization of the cyclic peptide monomer 'substrate' under these conditions (Fig. 5). The fraction of dimer after nine days (curve B in Fig. 5) presumably reflects conversion of all monomer molecules initially bound at site 1, which are the limiting reagent of the reaction. No further increase in dimer concentration is observed after 39 days, and more than half of the monomer remains throughout the time course.

The successful preparation of the streptavidin–peptide dimer by soaking streptavidin crystals in dimer solution demonstrates that diffusion of the dimer into (and out of) the crystal lattice is possible, and thus product release, allowing catalytic turnover, is theoretically feasible. The expected affinity of the peptide dimer for the two adjacent two-fold related binding sites in the crystals is high, however (~4.5×10^{-13} M, on the order of the square of that for the monomer for a single site, 6.7×10^{-7} M [12]). Thus the protein crystals are poorly optimized for product release and catalytic turnover.

The high affinity of the peptide dimer for the I222 crystals is demonstrated by the HPLC of the non-crystalline supernatant of the peptide monomer solution incubated with crystals, which shows predominantly monomer (Fig. 4e). The amount of monomer is similar to that found when the peptide is incubated for the same amount of time in buffer alone (Fig. 4c). The dimer is localized to the crystalline phase (Fig. 4f). Of the peptide bound in the crystal, 50 % is present as dimer at site 1 and 50 % as monomer at site 2 (Fig. 4f), where dimerization does not occur.

Elucidation of the crystal structures of the streptavidin–peptide monomer and dimer complexes has enabled us to design other peptides that are dimerizable by I222 streptavidin crystals. Furthermore, these crystals are capable of catalyzing disulfide formation between ligands with a lower affinity for streptavidin than *cyclo*-Ac-[Cys-His-Pro-Gln-Gly-Pro-Pro-Cys]-NH_2. The affinity of the linear peptide, Phe-Ser-His-Pro-Gln-Asn-Thr, (K_d =125 μM) [29] is about 200-fold lower than that of *cyclo*-Ac-[Cys-His-Pro-Gln-Gly-Pro-Pro-Cys]-NH_2. Thus we reasoned that disulfide formation between linear cysteine-containing peptide analogs of Phe-Ser-His-Pro-Gln-Asn-Thr, such as Phe-Cys-His-Pro-Gln-Asn-Thr or Cys-His-Pro-Gln-Asn-Thr, might be catalyzed by the I222 streptavidin crystal lattice, since the sulfhydryls of these peptide monomers that are bound at site 1, related by a nearby two-fold axis of symmetry, would be in close proximity. The K_ds of these dimerized linear peptides for the two adjacent two-fold related binding sites in the crystals is expected to be ~0.01 μM (of the order of the square of the K_d for the monomers), or higher. This affinity is of similar magnitude to the micromolar affinity of avidin for esters of the azo dye HABA (4'-hydroxyazobenzene-2-carboxylic acid); avidin is known to catalyze hydrolysis reactions of these esters in solution [30]. We have demonstrated catalysis by I222 streptavidin crystals of disulfide formation between Phe-Cys-His-Pro-Gln-Asn-Thr monomers and determined the structure of the streptavidin–peptide dimer complex (B.A.K., R.T. Cass, B.L., N. C., & R. A., unpublished data).

Significance

We have described a novel crystal lattice mediated transformation within a single crystal of a protein. The dimerization of the streptavidin-bound *cyclo*-Ac-[Cys-His-Pro-Gln-Gly-Pro-Pro-Cys]-NH_2 monomer ligands, involving breaking and forming of covalent disulfide bonds, is to our knowledge the first example of a chemical reaction directed by a protein crystal lattice.

Agents that cause the dimerization of proteins might be useful in a number of biological systems, but biological activity often requires dimerization in a precise orientation. The crystal lattice mediated dimerization of the peptide ligand in our model system suggests a general method for preparing ligands that

dimerize protein targets. First, a ligand for a monomeric protein is developed (e.g. by structure-based design or screening). Two pieces of information are then required: the three-dimensional structure of the protein–ligand complex, and the relationship between the protein monomers in the desired protein dimer complex. With this information, one can introduce crosslinkable groups at sites in the ligand that do not interfere with ligand binding, and where a suitable crosslink could span the distance between the two protein monomers in the biologically active dimer. Subsequent crosslinking of the introduced groups, by whatever method, produces a double-headed molecule that should be able to dimerize the protein target.

In some cases, topochemistry of the kind described here may provide a powerful method of achieving the final step in this strategy. In the streptavidin model system, we relied on the fact that the ligand molecules are brought close to each other by crystal-packing interactions. In many cases, however, the biologically relevant dimer can be made to form in the absence of ligand by protein–protein interaction (for example, by increasing the protein concentration). In such cases, the dimer could bind, orient and present the bound ligands toward one another in solution.

The streptavidin model system provides an opportunity for developing strategies to dimerize this protein using peptides, peptidomimetics or small molecules produced by topochemistry and/or structure-based design. Ligands designed to dimerize other protein targets could be useful as leads for development of drugs targeted towards proteins whose biological activity involves dimerization.

Materials and methods

Peptide synthesis

The synthesis of *cyclo*-Ac-[Cys-His-Pro-Gln-Gly-Pro-Pro-Cys]-NH_2 will be described elsewhere (B.A.K., unpublished data). Synthesis of the disulfide-bonded 'head-to-head', *cyclo*-Ac-[Cys-His-Pro-Gln-Gly-Pro-Pro-Cys]-NH_2 used orthogonal cysteine-protecting groups; the amino-terminal Cys was protected with trityl, the carboxy-terminal Cys with acetamidomethyl. The disulfide between the amino-terminal cysteines was formed by incubation in aqueous 0.010 M $K_3Fe(CN)_6$ at pH 8.5 for 1 h at room temperature. The second carboxy-terminal disulfide was formed by oxidation of the acetamidomethyl-protected cysteine residues with I_2 as described [31]. Cyclization was monitored by HPLC, and the peptide was purified by HPLC. HPLC/MS analysis was performed on a Finnigan MAT Single Stage Quadrapole (SSQ710) electrospray mass spectrometer. Mass found (M+1) = 1754; calculated (M+1) = 1754. Amino-acid analysis: Cys (3.8), His (2.1), Pro (5.7), Glx (2.0), Gly (2.0).

X-ray crystallography

Crystals of streptavidin–*cyclo*-Ac-[Cys-His-Pro-Gln-Gly-Pro-Pro-Cys]-NH_2 monomer were prepared by soaking large apostreptavidin [32] crystals in synthetic mother liquor at a peptide concentration of 10.0 mM, pH 5.0 for two days. A crystal of streptavidin–*cyclo*-Ac-[Cys-His-Pro-Gln-Gly-Pro-Pro-Cys]-NH_2 dimer was produced by allowing the mounted crystal of streptavidin-bound monomer, from which data had been collected, to undergo disulfide interchange over a period of six weeks. Other crystals of the streptavidin-bound dimer were prepared by soaking large apostreptavidin crystals in synthetic mother liquor containing 5.0 mM chemically synthesized 'head-to-head' dimer. Finally, the streptavidin–dimer complex was cocrystallized using chemically synthesized dimer. These cocrystals were small and thin.

Crystallographic data collection, data reduction, solution and refinement of structures were carried out by procedures that will be described elsewhere (B.A.K., unpublished). X-ray diffraction data from single crystals of streptavidin-bound *cyclo*-Ac-[Cys-His-Pro-Gln-Gly-Pro-Pro-Cys]-NH_2 monomer or *cyclo*-Ac-[Cys-His-Pro-Gln-Gly-Pro-Pro-Cys]-NH_2 dimer 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. The size of the small cocrystal (0.18 x 0.12 x 0.01–0.03 mm) of streptavidin–*cyclo*-Ac-[Cys-His-Pro-Gln-Gly-Pro-Pro-Cys]-NH_2 dimer allowed collection of data with reasonable statistics only to 2.65 Å.

A model of streptavidin with no inhibitor or waters near the expected binding regions of the peptide was built with Quanta (Molecular Simulations, 16 New England Executive Park, Burlington, MA 01803) from the structure of streptavidin–*cyclo*-Ac-[Cys-His-Pro-Gln-Gly-Pro-Pro-Cys]-NH_2 monomer (B.A.K., unpublished). Initial electron density maps calculated with coefficients ($|F_o|-|F_c|$) or ($2|F_o|-|F_c|$) and phases (α_c) for the model enabled determination of the structure of the streptavidin-bound cyclic peptide dimer.

Structures were refined with Xplor [27] and with difference Fourier methods [33]. Water structure was determined and refined as described [34]. Data collection and refinement statistics are given in Table 3. The structures will be deposited into the Brookhaven Data Bank.

HPLC and LC–MS measurements

A Hewlett-Packard 1090 analytical HPLC (C18 reverse-phase column) instrument was used. The linear gradient was from 0.05 % trifluoroacetic acid in water to 0.05 % in acetonitrile over 15 min. The fraction of dimer in each sample was calculated by dividing the integrated area of the dimer peak by the sum of the integrated areas of the monomer and dimer peaks. (Since the extinction coefficient of the dimer is expected to be twice that of the monomer (on a molar basis) the fraction of dimer thus calculated is on a weight basis). HPLC (Michrom BioResources Ultrafast Microprotein Analyzer) coupled to electrospray ionization mass spectrometry (Finnigan MAT SSQ710), or plasma desorption time-of-flight mass spectrometry (BioIon 20 instrument) was also done for selected samples.

Table 3. Crystallography parameters.[a]

	Monomer crystal 1 pH 5.0	Dimer crystal 1 pH 5.0	Dimer (co-crystal) pH 6.0	Dimer (soaked) pH 6.0
Parameters				
Number of atoms (including disorder)	2075	2151	2151	2111
Number of water molecules (including disorder)	150	194	194	144
Number of discretely disordered groups[b]	0	3	3	4
Number of discretely disordered waters	0	2	2	2
Number of side chains with refined occs[c]	24	24	24	26
Diffraction statistics				
Resolution (Å)	50–1.90	50–1.90	50–2.41	50–1.85
Number of observations[d]	64 474	70 104	15 363	76 657
Number of unique observations	16 042	17 597	5622	20 986
Redundancy	4.0	4.0	2.7	3.7
R_{merge} (%)[e]	7.5	6.3	11.6	9.6
Refinement statistics				
Refinement resolution	7.5–2.00	7.5–1.95	9.5–2.65	7.5–2.00
Number of merged reflections	14 948	16 025	4993	13 736
$\|F_o\|/\sigma$ cutoff	1.0	1.5	1.0	2.0
R_{cryst} (%)[f]	19.1	17.2	14.3	19.7
Free R_{cryst} (%)[g]	23.3	21.9	22.2	22.4
Overall completeness (%)	84.7	89.8	69.4	79.5
Overall completeness at highest resolution (%)	54.4	77.8	33.9	51.5
Highest resolution shell	2.09–2.00	2.04–1.95	2.77–2.65	2.09–2.00
RMS deviations[h]				
Bond lengths (Å)	0.018	0.017	0.016	0.018
Bond angles (°)	3.2	3.2	3.5	3.4
Torsion angles (°)	27.9	28.1	28.2	27.6

Crystallography statistics for streptavidin-bound *cyclo*-Ac-[Cys-His-Pro-Gln-Gly-Pro-Pro-Cys]-NH_2 monomer and dimer.
[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]Data with $R_{sym} > 50$ % were rejected along with data with values > 3.5 σ from the mean for several symmetry equivalents.
[e]$R_{merge} = \Sigma_h\Sigma_i|I(h)_i - \langle I(h)\rangle|/\Sigma_h\Sigma_i I(h)_i$, where $I(h)_i$ is the i^{th} observation of the intensity of reflection h.
[f]$R_{cryst} = \Sigma(||F_o|-|F_c||)/\Sigma|F_o|$ (for reflections from 7.5 Å to the highest resolution).
[g]Cross validation R-factor using 10 % of the data withheld from the refinement [35]. The structure was first determined and refined. Subsequently 10 % of the data was withheld and the structure re-refined to convergence.
[h]Root-mean-square deviations from ideal bond lengths and bond angles.

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