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Biomolecular Engineering
16 (1999) 57–65

Biomolecular Engineering

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Streptavidin-binding and -dimerizing ligands discovered by phage display, topochemistry, and structure-based design

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Abstract

Structural and mechanistic determinants of affinity of streptavidin-binding peptide ligands discovered by phage display are reviewed along with the use of streptavidin as a paradigm for structure-based design. A novel way of producing protein-dimerizing ligands in the streptavidin model system is discussed, in which crystal packing topochemically mediates or even catalyzes dimerization of adjacent bound ligands whose reactive ligating groups are presented toward one another in productive orientations in the crystal lattice. Finally, through crystallography on a set of streptavidin complexes with small molecule and peptide ligands at multiple pHs in two space groups, the mechanism by which ligands enhance intersubunit stabilization of the streptavidin tetramer is probed. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Streptavidin-binding and dimerizing ligands; Phage display; Structure-based drug design; Crystallographically determined pK_a shifts

1. Introduction

The remarkably high affinity ($K_d \sim 10^{-14}$ M, [1]) of the vitamin biotin for streptavidin has fascinated theoreticians and experimentalists alike for decades in both academic and industrial labs throughout the world. Extensive use in bioanalytical, biotechnological, diagnostic and therapeutic applications ([2] and references therein) has rendered streptavidin the target of protein engineering endeavors that have yielded dimeric streptavidins with potential advantages over the tetramer in some applications [3], tetramers with enhanced stability toward subunit dissociation [4,5], and tetramers with distinct ligand binding sites within single molecules that have reversible and irreversible biotin binding sites [2].

Streptavidin is also a well studied model system for discovering unnatural peptide ligands through phage display [6–10], for probing the structural basis for high affinity protein–ligand interactions [11–13], and for developing and testing structure-based design strategies [14]. High resolution crystal structures of apostreptavidin and complexes of streptavidin with biotin [15] and with other small molecules [16] have revealed the various structural features that mediate high affinity binding of biotin. In addition, thermodynamic [16–18] and free energy perturbation [19,20] comparisons of the binding of biotin to streptavidin and streptavidin mutants with the binding of other ligands have allowed further dissection of the structural and physicochemical factors responsible for the impressive affinity of biotin for native streptavidin.

A common challenge in drug design is to convert proteins or peptides that are either the natural ligands or substrates of macromolecular targets, or that are discovered or engineered by phage display, into tight binding, bioactive, organic molecules. Streptavidin is well characterized, commercially available, readily crystallizable, and both small molecule and peptide ligands for streptavidin are known, affording the opportunity to probe the structural relationships between bound peptide and non-peptide ligands. Thus we chose this protein as a model system for charting discovery routes to peptide and non-peptide drug leads that bind and dimerize cytokine receptors. This review focuses on development of streptavidin-binding and -dimerizing ligands through iterations of design, synthesis, assay, and structure determination in a paradigm commonly used for structure-based drug design initiated with leads discovered by phage display. The review also summarizes structural and mechanistic aspects of the binding to streptavidin of HPQ-containing peptides and of the

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PII: S1050-3862(99)00036-4

stabilization of the streptavidin tetramer toward subunit dissociation by binding of biotin.

2. Small, high affinity, cyclic peptide ligands discovered by phage display, utilize a β-turn structure to recognize streptavidin

By screening cyclic peptide libraries displayed on phage, Giebel et al. [21] discovered several cyclic, disulfide-bonded, peptide ligands for streptavidin. In complexes with *cyclo*-Ac-[CHPQGGPC]-NH_2, *cyclo*-Ac-[CHPQFC]-NH_2, and linear FSHPQNT, the HPQ recognition module of the bound peptides adopts a common conformation [22] that is the same as that in the bound linear *Strep*-tag affinity peptide (AWRHPQFGG, $K_d = 37$ μM, [13]), with a type I β-turn stabilized by a hydrogen bond between the His main chain carbonyl oxygen and the main chain amide NH of the $i+3$ residue in the bound cyclic peptides and in bound AWRHPQFGG. In bound *cyclo*-Ac-[CHPQFC]-NH_2 there is an additional hydrogen bond, indicative of α-helix, between the main chain carbonyl and the main chain of the $i+4$ (C-terminal) Cys [amide NH group]. In bound AWRHPQFGG the Phe side chain superimposes well on that in bound *cyclo*-Ac-[CHPQFC]-NH_2. In both complexes the peptide Phe side chain is sandwiched between the peptide Gln side chain and Trp120 of a neighboring subunit, and also makes hydrophobic interactions with the Leu25, Trp108, and Leu110 side chains. However, the Phe carbonyl and the main chain amide of the C-terminal Cys in bound *cyclo*-Ac-[CHPQFC]-NH_2 do not superimpose on the corresponding atoms in bound AWRHPQFGG, the peptide backbone of which exhibits 3_{10}-helical conformation [13]. The linear and disulfide-bridged cyclic peptides make common hydrogen bonds and hydrophobic interactions with streptavidin through the His, Pro, and Gln residues (Fig. 1a), although significant differences in structures and interactions occur for flanking residues among the complexes. Binding of these peptides is mediated by tetrahedrally coordinated water molecules. The directionalities of all hydrogen bonds (a total of 11 for streptavidin-FSHPQNT) between the peptides and streptavidin could be unambiguously inferred [23] (Fig. 2).

In the cyclic peptide complexes the disulfides, located on the surface of the protein, do not make any interactions with it. Thus the increase in affinity, by up to 7000-fold for *cyclo*-Ac-AE[CHPQFC]IEGRK-NH_2 ($K_d = 0.023$ μM) compared with that of linear Ac-AEFSHPQNTIEGRK-NH_2 ($K_d = 160$ μM) at pH 7.3 [23]), reflects a decrease in entropy of the unbound cyclic peptides compared with that of the linear ones.

3. The pK_a of the bound HPQ-containing peptide ligands is < 3.0

Through crystallography and plasmon resonance binding measurements on a set of HPQ-containing cyclic peptides at multiple pH values, the mechanism of binding of such peptides was probed [23]. The decrease in affinity of the peptides with decrease in pH implicated a group with a pK_a of ~ 6.3 involved in binding. The pK_a of the His side chain, when solvent exposed, is 6.3 [24]. In crystal structures of complexes at pHs as low as 2.5, the electron density for the peptide His side chain is strong and well defined (Fig. 1a). An intrapeptide hydrogen bond accepted by the Nδ1 atom of the peptide His from the peptide Gln amide N atom (Fig. 1a) indicates that in the crystals the imidazole of the bound peptide is uncharged at this low pH. The presence of the $N\delta1_{His}$–N_{Gln} hydrogen bond along with the pH dependence of the affinities of the peptides demonstrate that deprotonation of the peptide His is required for high affinity binding of HPQ-containing peptides to streptavidin both in the crystals and in solution.

The peptide His side chain is not involved in salt bridge interactions, but it makes two hydrogen bonds ($N\delta1_{His}$–N_{Gln} and $N\varepsilon2_{His}$–$O\gamma2_{Thr90}$) at pH values ≥ 2.5 (Fig. 1a). Although the Cδ2 atom of the imidazole of the peptide His is accessible to solvent from one direction, the other imidazole atoms are shielded from solvent by the rest of the bound peptide and by Ala86, Trp79, Ser88, Leu110, and Trp120 of a neighboring subunit (Fig. 1a). Shifts in pK_a values of ionizable groups can be effected by a variety of factors [24–27], including hydrogen bonding interactions [28], hydrophobic interactions [29], and desolvation [30–32], all of which may contribute to the decrease in the pK_a of the

Fig. 1. Structure of I222 streptavidin-*cyclo*-[5-S-valeramide-HPQGPPC]K-NH_2, pH 2.5, 1.45 Å resolution [23] superimposed on the $2|F_o| - |F_c|$ electron density map, contoured at 0.9 σ (yellow) and 1.8 σ (red) at site 1 (a, top), or at site 2 (b, bottom). The cyclic peptide is shown in thick sticks. Peptide residues are labeled in yellow and protein residues in white. Hydrogen bonds mediating peptide binding are in white; intrapeptide hydrogen bonds are in yellow. Note that L110 is discretely disordered. (b) The peptide His side chain is rotated 180° about the Cβ–Cγ bond with respect to the conformation in (a) to allow Nδ1 to make a hydrogen bond (shown in cyan) with a water molecule. This change in the His conformation increases the $N\varepsilon2_{His}$–$O\gamma_{Ser88}$ distance from 3.05 to 3.21 Å, and decreases the $N\varepsilon2_{His}$–$H\varepsilon2_{His}$–$O\gamma_{Ser88}$ angle from 163 to 108°, indicating a weakening or loss of the hydrogen bond involving $N\varepsilon2_{His}$ and $O\gamma_{Ser88}$. However, an alternate hydrogen bond, $C\varepsilon1_{His}$–$O\gamma_{Ser88} = 2.98$ Å, $C\varepsilon1_{His}$–$H\varepsilon1_{His}$–$O\gamma_{Ser88} = 126°$ (shown in cyan), similar to those observed in other protein crystal structures [52], is now possible.

Fig. 2. Hydrogen bonding network in streptavidin-FSHPQNT. Protein residues are green, peptide residues yellow, bound waters red, and direct and water-mediated protein–peptide hydrogen bonds white.

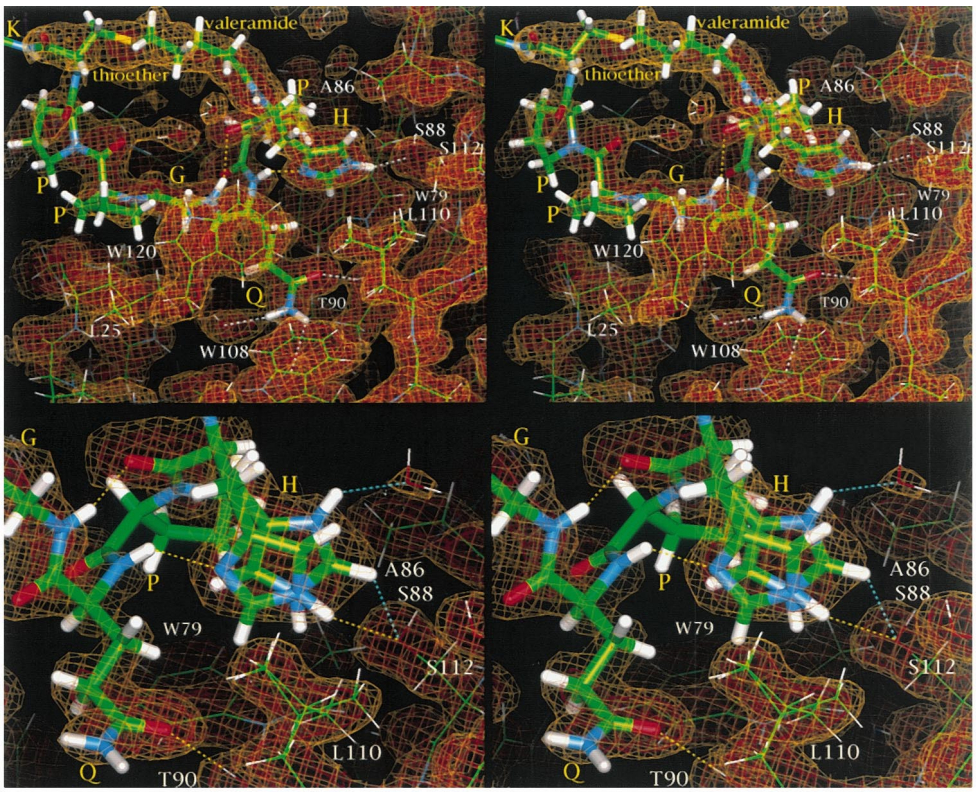

Fig. 1

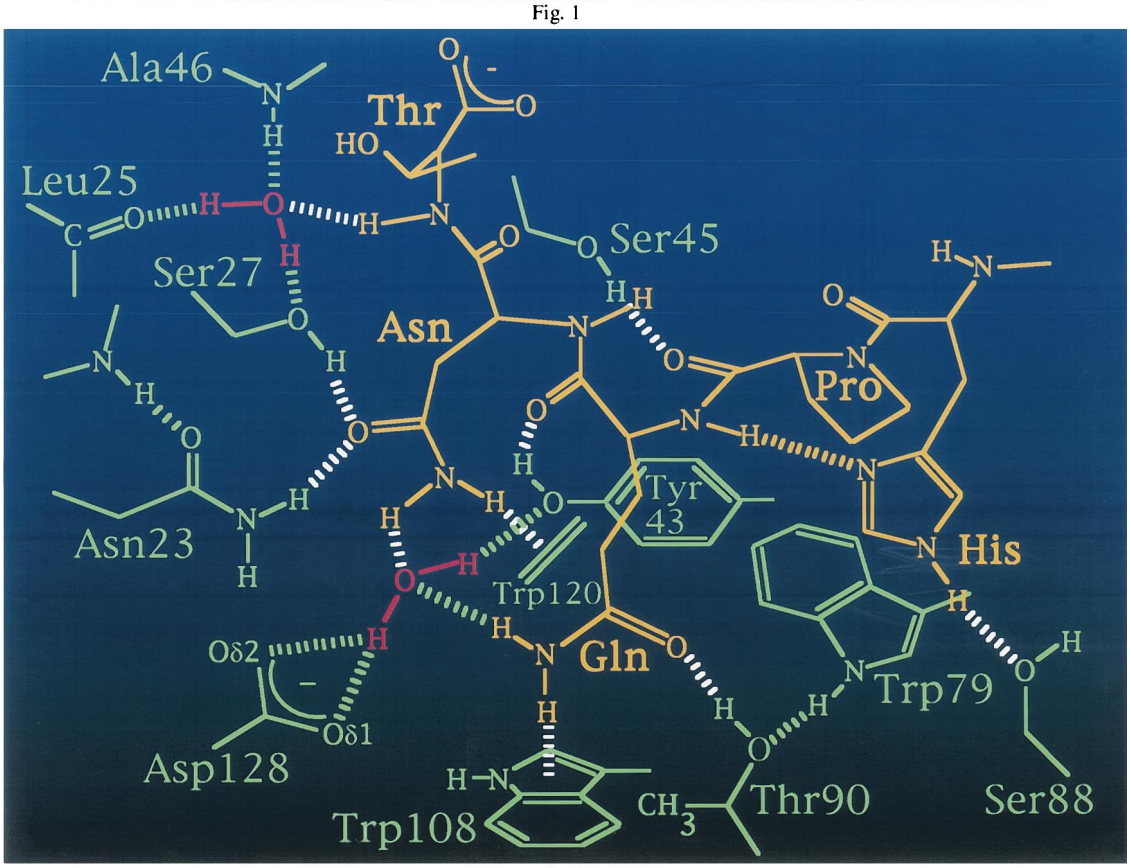

Fig. 2

Figs. 1 and 2.

His of the streptavidin-bound HPQ-containing peptide ligands.

The structure and binding interactions of the imidazole of the His of the bound HPQ-containing peptides were determined at multiple pH values at three crystallographically inequivalent binding sites: the two crystallographically independent sites in space group I222 or the unique one in space group $I4_122$. At pH values $\leq$ 2.5 differences in the protonation state of the peptide His are observed at crystallographically different sites. Fig. 1a and Fig. 1b compare the structure of one of the bound cyclic peptides at the more solvent shielded site (site 1) with that at the more solvent exposed site 2, respectively, at pH 2.5 in space group I222. Unlike at site 1, the imidazole density at site 2 is elongated in a direction corresponding to the presence of a second protonated histidine conformer that is rotated 17° about the Cα–Cβ bond with respect to the unprotonated conformer. The occupancies of the protonated and unprotonated conformers are 20 and 80%, respectively, yielding a calculated pK_a at site 2 of 1.9. By contrast, at site 1, the occupancies of any unprotonated components at pH $\geq$ 2.5 are not high enough to observe or to resolve. Thus the pK_a of the bound peptide His is detectably lower at site 1 than at site 2. (It is also lower at the unique site in space group $I4_122$). Inequivalence of the two peptide binding sites in I222 complexes is also manifested by lower temperature factors at site 1 than at site 2, except at very low pH where they become large at both sites. The protonation state of the peptide His at the more solvent exposed site 2 more accurately reflects its ionization state in solution. However, the observation that long range crystal packing interactions can perturb the pK_a of the His of streptavidin-bound peptides should be considered a caveat in inferring the pK_a values in solution from the corresponding ones determined in crystals.

4. Structure-based design of high affinity cyclic peptide ligands containing thioether crosslinks

High affinity cyclic peptide thioether-crosslinked peptides, *cyclo*-[5-S-valeramide-HPQGPPC]-NH_2 (K_d = 0.68 μM), and *cyclo*-[4-S-toluamide-HPQGPPC]-NH_2 (K_d = 1.75 μM), were designed from the streptavidin-bound structure of the disulfide-bonded ligand, *cyclo*-Ac-[CHPQGPPC]-NH_2, discovered by phage display [33]. In the structures of the streptavidin-bound thioether crosslink-containing ligands (Fig. 1a) the HPQ segments adopt the same conformation as that of the bound disulfide-containing parent peptide and make the same binding interactions with streptavidin.

Thioether crosslinked ligands are advantageous over disulfide-linked counterparts because they do not dimerize or oligomerize, and are thus a simpler chemotype for further design. They are also parent molecules for facile preparation of two additional sulfoxide diastereomer analogs, one of which invariably showed enhanced potency in cyclic RGD-containing ligands for the glycoprotein IIB/IIa adhesion molecule [34]. The absence in thioether-crosslinked ligands of the high, 8 kcal/mol, χ^3 dihedral energy barrier present in disulfides increases the conformational diversity. Finally, greater chemical and conformational diversity in libraries of thioether-crosslinked molecules is possible because the crosslinks are not restricted to naturally occurring amino acids.

5. Structure-based design of azobenzene ligands

From the structure of streptavidin-bound 2-(4′-hydroxyphenylazo)benzoic acid (HABA, K_d = 140 μM, [16]), subsequent azobenzene ligands were designed with affinities enhanced by up to 165-fold (K_d = 0.83 μM) from that of the parent [14]. Measured thermodynamic parameters indicated that increase in ligand affinities were due primarily to favorable entropic contributions. Crystal structures of streptavidin-ligand complexes suggested that contributions to overall favorable entropy changes upon ligand binding included displacement of water from the ligand binding site and retention of ligand flexibility upon binding [14].

6. Structure-based design of a small molecule peptide-biotin hybrid ligand

Comparison of the structures of streptavidin-HPQ-containing ligands with that of streptavidin–biotin shows that four contiguous Gln side chain atoms (Cβ, Cγ, Cδ, and Nε2) in the bound cyclic peptides superimpose well on four contiguous atoms (C1″, C2, S1, and C5) of bound biotin (Fig. 3), suggesting a potential ligand that is a hybrid of biotin and the Gln side chain [35]. In the fine chemical database we identified a molecule, glycoluril, that incorporates this aspect of the design (Fig. 3). The structure of streptavidin-glycoluril [35,36] shows that glycoluril (K_d = 2.5 μM) makes interactions common to both streptavidin–biotin and streptavidin-HPQ-containing peptides from which it was designed. The contribution to binding of the valerate group present in biotin and absent in glycoluril was estimated to be 11.8 kcal/mol.

7. Preparation of streptavidin-dimerizing ligands via topochemistry mediated by crystal packing

Dimerization triggers many biologically relevant processes [37–41]. Drug development for diseases such as

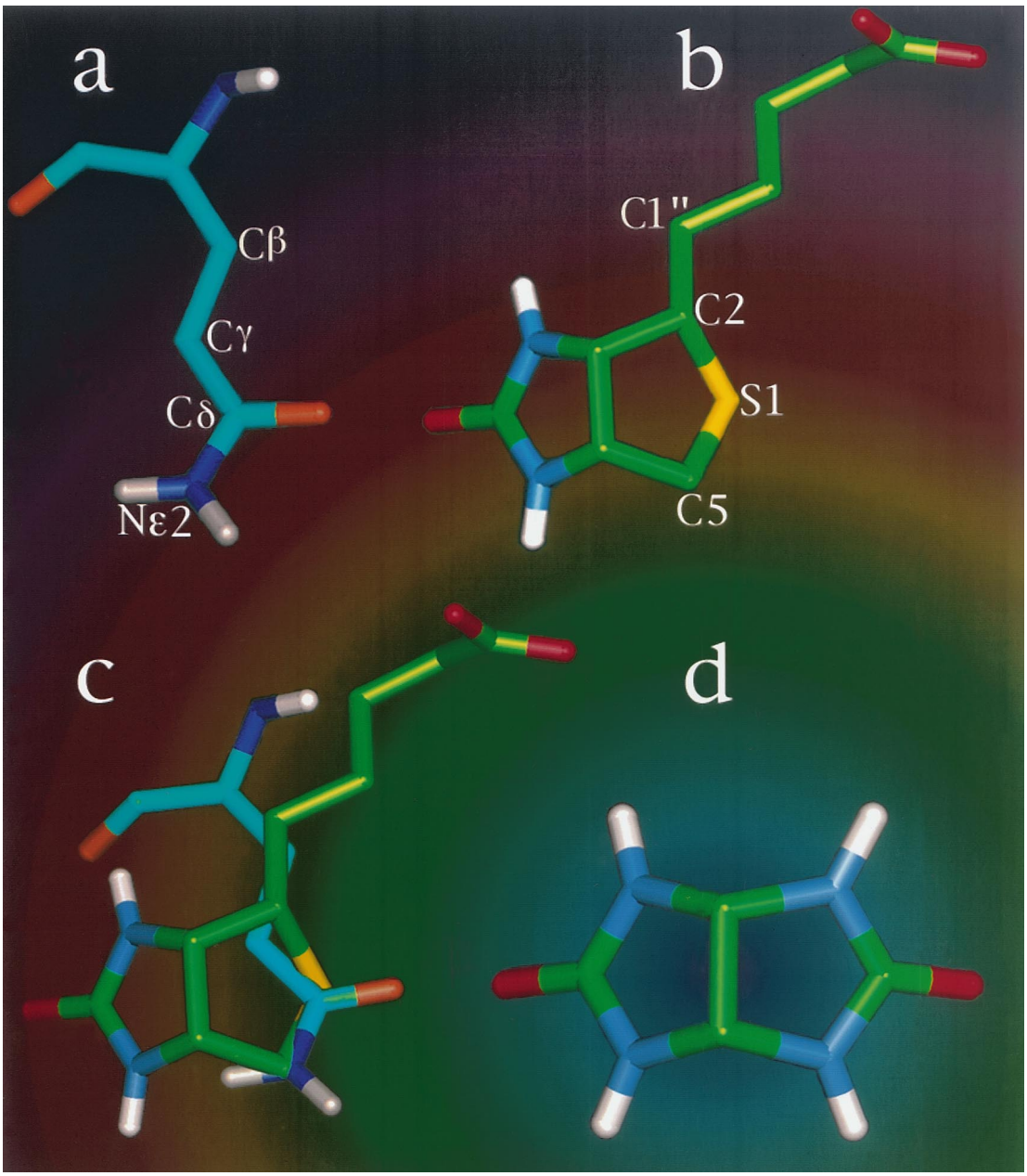

Fig. 3. Design of glycoluril ligand. (a) Structure of Gln residue in bound HPQ-containing ligands. (b) Structure of bound biotin. (c) Relationship of bound biotin and Gln of bound HPQ-containing ligands after superposition of complexes. (d) Glycoluril.

dwarfism and anemia involves design or discovery of ligands that dimerize human growth hormone receptor [42–44] or erythropoietin receptor [45–47] whose mechanism of action involves receptor dimerization. To gain insight into the design of ligands that dimerize receptors, we used streptavidin as a model system for designing streptavidin-dimerizing ligands and crystallographically studying the dimerization [48,49].

In streptavidin crystals of space group I222 at pH $\geq$ 5.0 after several days, a novel crystal lattice-mediated interchange occurs between the peptide disulfides presented toward one another on adjacent, two-fold related, streptavidin-bound *cyclo*-Ac-[CHPQGPPC]-NH_2 monomers to produce a C2-symmetric, head-to-head, bound peptide dimer, and to simultaneously dimerize streptavidin [48] (Fig. 4a). Dimerization is associated with a decrease in crystal volume and a closer proximity, by 1.0 Å, of the dimerized streptavidin tetramers. The decrease in crystal volume that leads to more crystal packing interactions may drive the reaction.

Amazingly, crystal packing of the same protein, streptavidin, in a different space group, $I4_122$, is also appropriate for topochemically mediating dimerization of the same peptide, *cyclo*-Ac-[CHPQGPPC]-NH_2, to

produce a different, C2-symmetric, head-to-tail, streptavidin-peptide dimer [49] (Fig. 4b). The two-fold symmetry of the streptavidin dimers produced in space group I222 and $I4_122$ is the same as that for the complex involving two erythropoietin receptors and a peptide dimer discovered by phage display [47]. Because peptide dimerization in space group $I4_122$ occurs at all four ligand binding sites of the streptavidin tetramer, streptavidin tetramers are polymerized in three dimensions by the ligand dimer (Fig. 5a). The insolubility of these crystals in water, methanol, and dimethyl sulfoxide reflects both the polymeric nature of the crystals

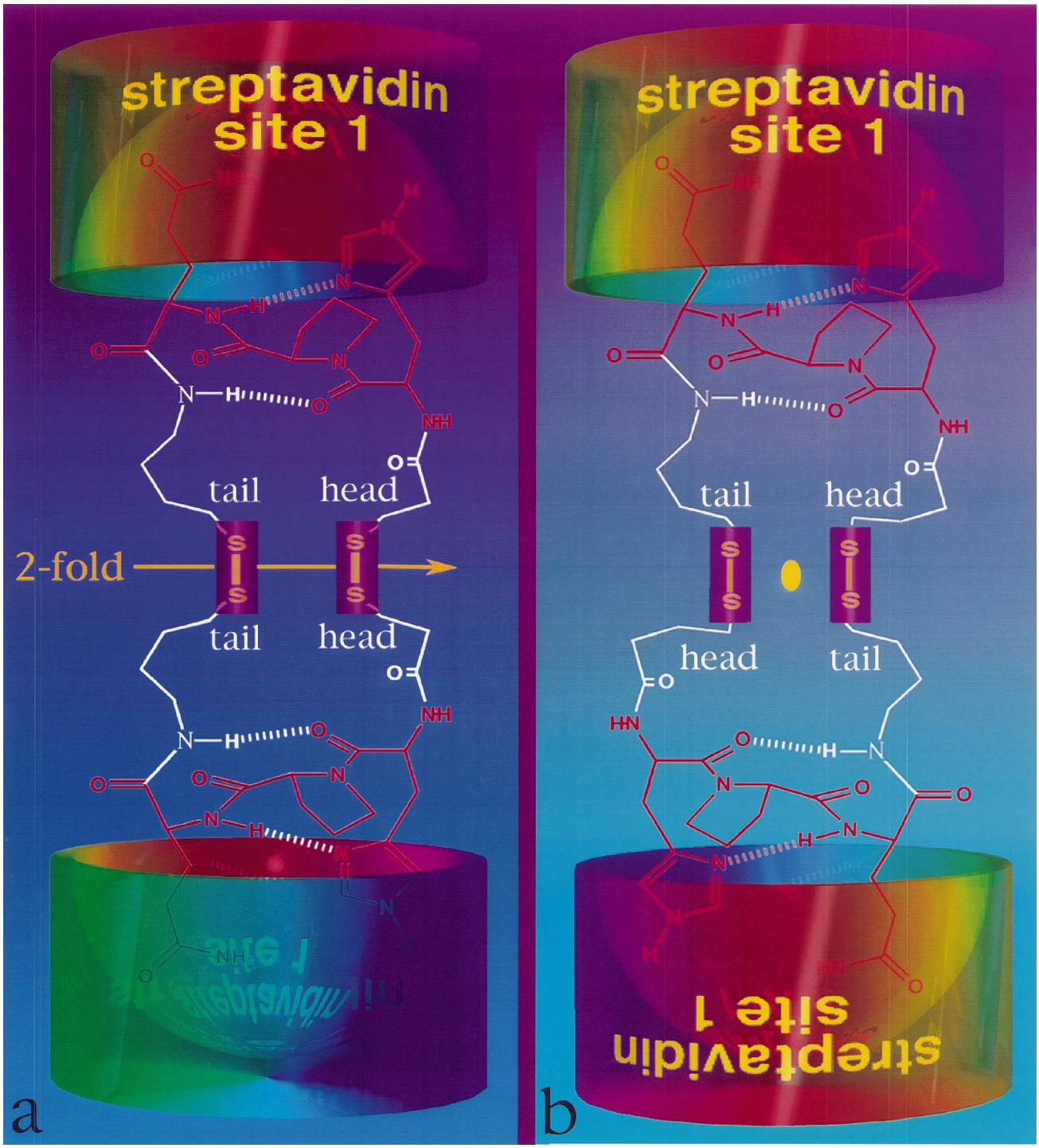

Fig. 4. (a, top) Symmetry of the streptavidin-head-to-head peptide dimer complex produced by topochemical reaction in I222 crystals. The HPQ segments of the peptides are shown in red. The peptide segments before and after the HPQ segments are schematic (some atoms are not shown). (b, bottom): Symmetry of the streptavidin-head-to-tail peptide dimer complex produced in space group $I4_122$.

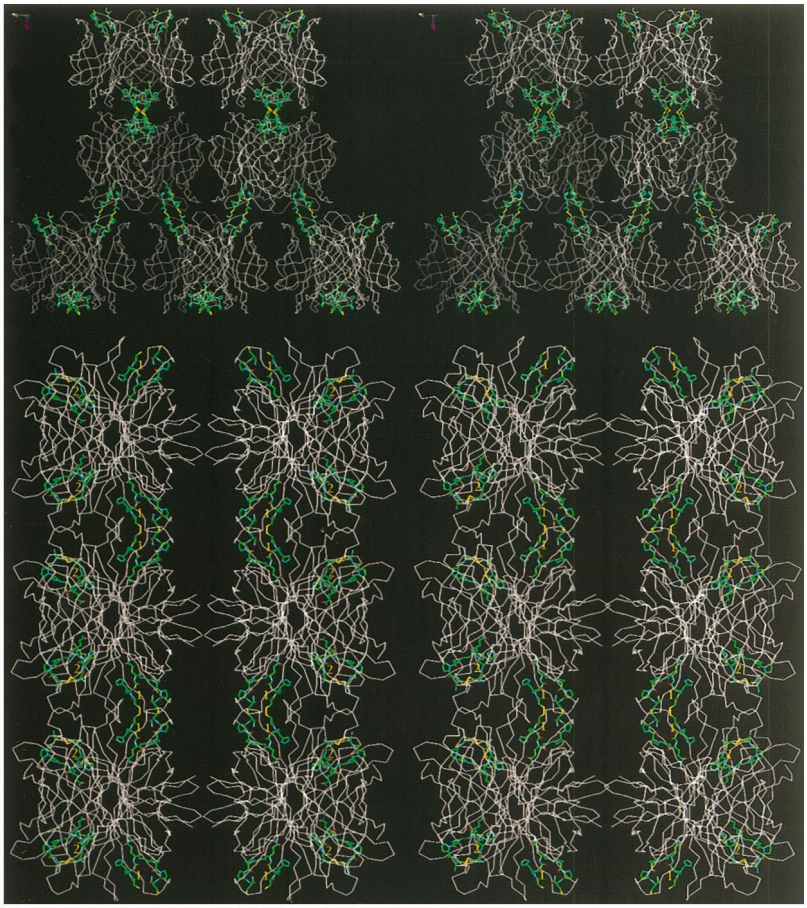

Fig. 5

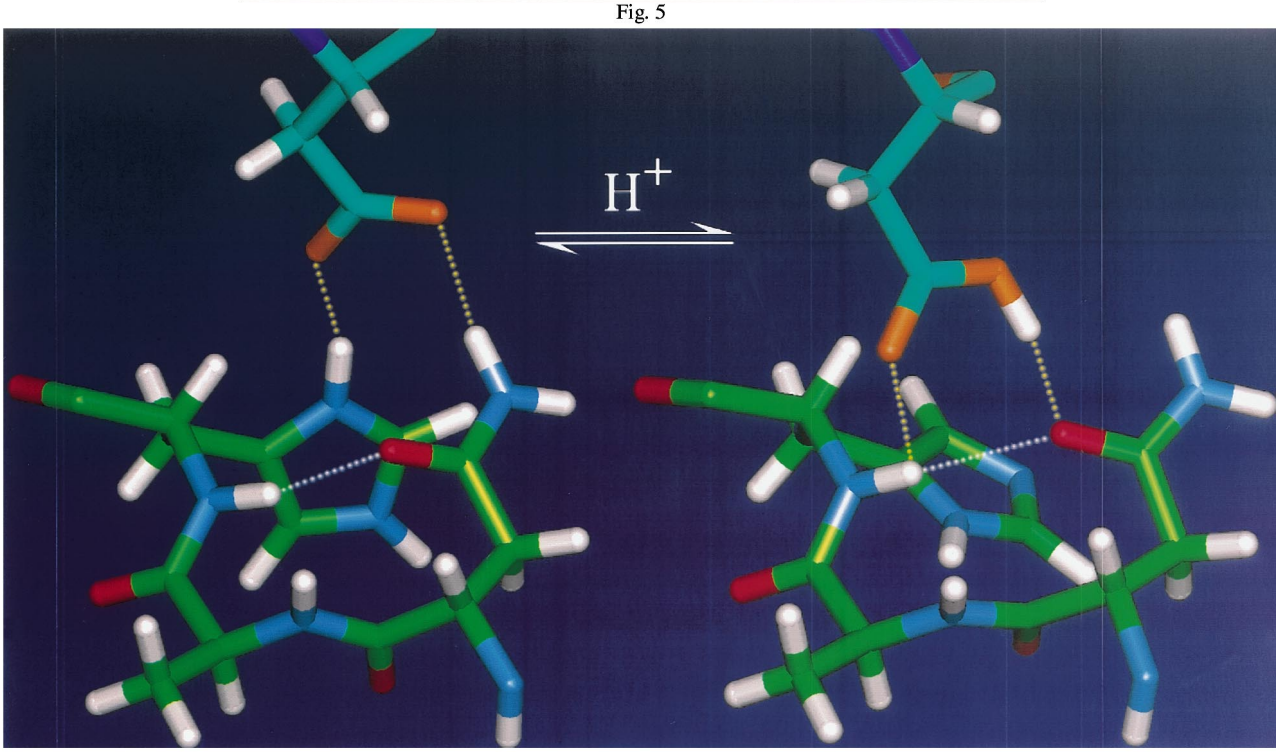

Fig. 6

Fig. 5. Crystal packing in space group $I4_122$ (a, top) and in space group I222 (b, bottom).
Fig. 6. Change in intersubunit hydrogen bonds upon protonation of Asp61. At appropriate pH values there are significant fractions of protonated and unprotonated Asp61 components that are well separated and well resolved. Refined occupancies allow calculation of pK_a of Asp61 for various complexes.

and the high affinity of the peptide dimer for them. By contrast, dimerization at only one of the two independent sites in space group I222 yields crystals that are polymerized in two dimensions (Fig. 5b) which are stable at lower salt concentrations and/or higher methanol concentrations than uncrosslinked crystals.

The crystal lattice-mediated dimerization of the peptide ligands in streptavidin crystals suggests a general strategy for preparing ligands that dimerize protein targets in a precise orientation. First, a ligand for the monomeric protein is designed or discovered. Given the structure of the protein–ligand complex and the relationship between the protein monomers in the desired protein dimer complex, crosslinkable groups can be introduced at sites in the ligand that do not interfere with ligand binding and that when crosslinked span the distance required to produce a biologically active dimer. Dimerization of the ligand monomer at the introduced crosslinking groups affords a double-headed ligand dimer capable, in principle, of dimerizing the protein target.

In some cases topochemistry could be a powerful strategy to achieve the dimerization. In streptavidin the topochemisty utilized the close proximity of neighboring bound peptides in the crystal lattice. In many cases, however, the biologically relevant protein dimer can be formed in solution in the absence of ligand by protein–protein association at high protein concentration where the protein dimer could bind and present the ligands toward one another in orientations favorable for simultaneous dimerization of ligand and protein in a biologically relevant way in solution.

The streptavidin crystal lattice-mediated peptide dimerizations are not catalytic over the period of time studied, 40 days, probably because the affinity of the dimer is too high to allow dissociation. We showed that topochemical catalysis is feasible, however, by designing weaker binding, linear peptides with cysteine thiols that are directed toward one another upon peptide binding. The ensuing dimerization is *catalyzed* by the crystal lattice [50].

8. Modulation of intersubunit salt bridge stability by ligand binding

Binding of biotin increases the stability of the streptavidin tetramer toward subunit dissociation [51]. To gain insight into the mechanisms by which small molecule or peptide ligands can stabilize protein–protein interactions, whose modulation can control both physiological and pathological processes [37–41], the role of ligand binding in intersubunit stabilization was probed [36]; the crystal structures of apostreptavidin and complexes with biotin, glycoluril, 2-iminobiotin, -*cyclo*-Ac-[CHPQFC]-NH_2, and -*cyclo*-[5-S-valeramide-HPQGPPC]K-NH_2 were determined and compared over a large range of pH values, from 1.4 to 10.4.

Low pH induces a large change in the conformation and intersubunit hydrogen bonding interactions involving the loop comprising Asp61 to Ser69. The hydrogen bonded salt bridge between $O\delta2_{Asp61}$ and $N\delta1_{His87}$, at neutral and high pH, is replaced with a strong hydrogen bond between $O\delta1_{Asp61}$ and $O\delta1_{Asn85}$ at low pH (Fig. 6). Through crystallography at multiple pH values, the pH where this conformational change occurs, and thus the pK_a of Asp61, was determined in crystals of space group I222 and/or $I4_122$ of apostreptavidin and complexes.

The decrease in pK_a of Asp61 (by 10.9 σ in 2.9 M $(NH_4)_2SO_4$) from 3.66 ± 0.01 in $I4_122$ apostreptavidin to 2.46 ± 0.11 in $I4_122$ streptavidin–biotin is associated with an ordering of the flexible flap comprising residues Ala46 to Glu51, that in turn orders the Arg84 side chain of a neighboring loop through resulting hydrogen bonds ($O\delta1_{Asn49}$–$N\eta1_{Arg84}$ and $O\varepsilon1_{Glu51}$–$N\varepsilon_{Arg84}$). Ordering of Arg84 in close proximity to the strong intersubunit interface appears to stabilize the conformation associated with the $O\delta2_{Asp61}$–$N\delta1_{His87}$ hydrogen-bonded salt bridge. Thus the route of propagation of long range structural perturbations from biotin binding on one side of the strong intersubunit interface to the other side, where the ionization–conformational equilibrium of Asp61 is modulated, could be deduced.

Sano et al. [3] demonstrated that biotin stabilizes this strong interface; a dimeric streptavidin in which the weaker interface was removed by protein engineering is stable when biotin is bound but in the absence of biotin gradually dissociates into non-functional monomers that are then incapable of binding biotin. Thus biotin appears to play a dual role in imparting affinity and enhancing stability toward subunit dissociation; in addition to stabilizing the tetramer by direct mediation of intersubunit interactions at the weak interface through contact with Trp120 [17,18], biotin also stabilizes the tetramer at the strong interface more indirectly, apparently by ordering loop residues that lead to preservation of the $O\delta2_{Asp61}$–$N\delta1_{His87}$ hydrogen-bonded salt bridge at low pH [36]. Glycoluril and 2-iminobiotin also exert such salt bridge stabilization. In the peptide complexes the flaps, which are disordered at both low and high pH, can not adopt the closed conformation observed in streptavidin–biotin and other complexes, as this conformation is occluded by the bound peptides. The peptides therefore do not stabilize the intersubunit salt bridge. This study demonstrated that when an ionization and conformational equilibrium are coupled, structural perturbations that differentially stabilize one of the conformations involved in the ionization equilibrium can correspondingly shift the ionization equilibrium.

9. Conclusions

Streptavidin has afforded a powerful paradigm for combining the complementary disciplines in drug discovery and development of phage display and structure based-design. Streptavidin crystals have also served as ideal systems for developing strategies to dimerize proteins by topochemistry. Dimerizations of the streptavidin–cyclic peptide complex in two space groups, involving breaking and forming of covalent disulfide bonds, are the first examples of a chemical reaction directed by a protein crystal lattice. Further structure-based design allowed demonstration of catalytic dimerization of linear thiol containing ligands, representing the first example of crystal lattice-mediated catalysis. Through crystallography at multiple pH values on small molecule and peptide complexes of streptavidin, insights into the mechanism of binding to streptavidin of HPQ-containing peptides and into the stabilization by biotin of the streptavidin tetramer towards dissociation have emerged.

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