In Crystals of Complexes of Streptavidin with Peptide Ligands Containing the HPQ Sequence the pK$_{a}$ of the Peptide Histidine Is Less than 3.0*

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The pH dependences of the affinities for streptavidin of linear and cyclic peptide ligands containing the HPQ sequence discovered by phage display were determined by plasmon resonance measurements. At pH values ranging from 3.0 to 9.0, the $K_d$ values for Ac-A-EFSHPQNTIEGRK-NH$_2$, cyclo-Ac-AE[CHPQGPPC]IEGRK-NH$_2$, and cyclo-Ac-AE[CHPQFC]IEGRK-NH$_2$, were determined by competition, and those for cyclo-[5-S-valeramide-HPQGPPC]K-NH$_2$ were determined directly with equilibrium affinity measurements. The $K_d$ values of the ligands increase by an average factor of 3.0 ± 0.8 per decrease in pH unit between pH 4.5 and pH 6.3. Below pH 4.5 there is a smaller increase in $K_d$ values, and above pH 6.3 the $K_d$ values become relatively pH-independent. We determined the crystal structures of complexes of streptavidin with cyclo-[5-S-valeramide-HPQGPPC]K-NH$_2$ at pH 1.5, 2.5, 3.0, and 3.5, and with cyclo-Ac-[CHPQFC]-NH$_2$ at pH 2.0, 3.0, 3.6, 4.2, 4.8, and 11.8, with cyclo-Ac-[CHPQGPPC]-NH$_2$ at pH 2.5, 2.9, and 3.7, and with FSHPQQT at pH 4.0 and compared the structures with one another and with those previously determined at other pH values. At pH values from 3.0 to 11.8, the electron density for the peptide His side chain is strong, flat, and well defined. A hydrogen bond between the Nδ1 atom of the His and the peptide Gln amide group indicates the His of the bound peptide in the crystals is unchanged at pH 3.0. By determining selected structures in two different space groups, I222 with two crystallographically inequivalent ligand sites and I4122 with one site, we show that below pH 3.0, the pK$_{a}$ of the bound peptide His in the crystals is influenced by crystal packing interactions. The presence of the Nδ1...Nε$_2$ hydrogen bond along with pH dependences of the peptide affinities suggest 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.

Screening of peptide libraries either displayed on phage by molecular biology or produced by combinatorial chemical synthesis is an effective method for discovery of peptide ligands for diverse protein targets. Owing to the remarkably high stability and high affinity of streptavidin for its natural ligand biotin ($K_d$ ~ 10$^{-15}$ M) (1), together with widespread bioanalytical, diagnostical, and therapeutic applications (2–5), this protein has been extensively used to develop and validate such screening methodologies. High affinity unnatural ligands have been discovered by screening linear (6–11) and cyclic (6) peptide libraries. Streptavidin also provides an ideal paradigm for probing the structural basis of high affinity protein-ligand interactions (12–16) and for introducing or improving properties by protein engineering (2, 17, 18). Finally, the high resolution crystal structures of apo-streptavidin and of streptavidin-ligand complexes provide a powerful basis for developing structure-based ligand design strategies (12, 19–23).

Recently we described the structures of streptavidin-bound linear and cyclic peptide ligands containing the HPQ sequence (13) discovered by phage display and probed the structural basis for the higher affinities of the cyclic ligands compared with the corresponding linear ones. These structures enabled the successful design of cyclic peptide ligands conformationally constrained with designed thioether cross-links (22), of streptavidin dimerizing peptide ligands (19–21), and of a streptavidin-binding small molecule ligand (12).

The binding to streptavidin and avidin of certain small molecule and peptide ligands is pH-dependent. The affinities for avidin of biotin derivatives, 2-iminobiotin and diaminobiotin, decrease dramatically as the pH is lowered (24), as does the affinity for streptavidin of a linear peptide discovered by phage display, FSHPQQT (25). The extent of topochemical catalysis of disulfide formation and the resulting dimerization of designed streptavidin-bound HPQ-containing ligands whose thiols are presented next to one another in the crystal lattice also depend on pH (20).

Determination of the pH dependence of ligand binding or of changes in properties incurred by ligand binding often yields insight into the mode of action (26–28) or mechanism of binding (29–36) in biological and chemical processes. Appraisal of the ionization states of groups in a protein-bound ligand or at the ligand binding site of the protein target may reveal some of the determinants of high affinity binding crucial for structure-based ligand design. To this end we determine the pH dependences of binding to streptavidin of linear and cyclic peptide ligands containing the HPQ sequence and probe the structural basis for the dependences through crystallographic determination of complexes at multiple pH values. The affinities for streptavidin at pH 3.0, 4.0, 5.0, 6.0, 6.2, 6.4, 7.0, 7.3, 8.0, and 9.3 of linear Ac-AEFSHPQNTIEGRK-NH$_2$, cyclo-Ac-AE[CHPQGPPC]IEGRK-NH$_2$, cyclo-Ac-AE[CHPQFC]IEGRK-NH$_2$, and cyclo-[5-S-valeramide-HPQGPPC]K-NH$_2$ are determined by plasmon resonance measurements. We also determine the streptavidin-bound crystal structures of cyclo-[5-S-valeramide-HPQGPPC]K-NH$_2$ at pH 1.5, 2.5, 3.0, and 3.5 and of smaller versions of the other ligands, cyclo-Ac-[CHPQGPPC]-NH$_2$ at pH 2.0, 3.0, 3.6, 4.2, 4.8, and 11.8, cyclo-Ac-[CHPQFC]-NH$_2$ at pH 2.5, 2.9 and 3.7, and FSHPQQT at pH 4.0. Several structures are determined in two space groups, I222 and I4122. The
structures of the complexes are compared with those previously determined at other pH values. The crystal structures of the streptavidin-bound linear and cyclic peptide ligands determined over a range of pH values show that the pKₐ of the peptide His is greatly reduced in the crystals. Together with the pH dependences of the peptide affinities, the structures of the complexes over a range of pH values suggest that deprotonation of the peptide His is required for high affinity binding both in the crystals and in solution.

EXPERIMENTAL PROCEDURES

**Peptide Synthesis**—Peptides for crystallography, FSHPQNT, cyclo-Ac-[CHPQGPPC]-NH₂, cyclo-Ac-[CHPQFC]-NH₂, and cyclo-[5-S-valeramide-HPQGPPC]-NH₂, were synthesized as described (6, 13, 22). For the BIAcore analysis, corresponding peptides Ac-AEFSHPQNTIEGRK-NH₂, cyclo-Ac-AE[CHPQGPPC]IEGRK-NH₂, and cyclo-[5-S-valeramide-HPQGPPC]-NH₂ were synthesized as described (6, 22). The first three of these are longer than the corresponding core versions; the corresponding one for crystallography contains N-terminal AE synthesized as described (6, 22). The BIaCore analysis, corresponding peptides Ac-AEFSHPQNTIEGRK-NH₂, cyclo-Ac-AE[CHPQGPPC]IEGRK-NH₂, and cyclo-[5-S-valeramide-HPQGPPC]-NH₂ were synthesized as described (6, 22). The first three of these are longer than the corresponding ones for crystallography; they contains N-terminal AE sequence and C-terminal IEGRK for reasons independent of the present study (6).

**Crystallization of Streptavidin-Peptide Complexes**—Apostreptavidin, purchased from Calbiochem, was crystallized by vapor diffusion in 40 % sitting drops under conditions described for crystals of space group I222 (13, 37). Streptavidin-FSHPQNT was coocrystallized (13) in the I222 and I4122 (13, 37). Streptavidin-FSHPQNT was co-crystallized (13) in the I222 and I4122 (13, 37) from 1.00 to 1.50 °/frame, 20–45 min/frame. For highly diffracting crystals, data were collected with an R-AXIS IV image plate and associated data reduction software (Biotex) were from image plate system equipped with mirrors mounted on the Rigaku generator upgraded to operate at 50 kV, 100 mA. The nonmonochromated x-rays were filtered with 0.004 cm of Be. Diffraction data were systematically identified with the program Peak-pick, which was written in house, and analyzed. Water structure was determined with Peak-pick, X-sight, or X-solvate from Molecular Simulations, Inc. (San Diego, CA) and refined according to published procedures (40). Waters with temperature factors greater than 50 Å² were examined in the context of the corresponding difference Fourier maps and kept only if there was significant density for them. The coordinates corresponding to the conformations of discretely disordered residues (such as the peptide histidine in some structures) were simultaneously refined along with the rest of the structure, followed by simultaneous refinement of temperature factors and then of occupancies. Refinement of coordinates, temperature factors, and occupancies was iterated until the parameters and occupancies converged. Peak-pick, X-sight, and X-solvate statistics are given in Table I. The determination of affinities as a function of pH by BIAcore Measurements—the affinities of linear and cyclic streptavidin binding peptides were determined as a function of pH by surface plasmon resonance. The BIAcore 2000 system, sensor chip, amine coupling kit containing N-hydroxysuccinimide, N-ethyl-N'-(3-diethylaminopropyl)-carbodiimide, and ethanolamine-HCl were from Pharmacia Biotech Inc. Buffers containing 150 mM NaCl, 3.4 mM EDTA, and 0.005% surfactant P20 ranging from pH 3.0 to 8.0 were 10 mM potassium acetate, pH 3.0; 10 mM sodium acetate, pH 4.0, 5.0, and 6.0; 10 mM bis-Tris, pH 6.2 and 6.4; 10 mM HEPES, pH 7.0 and 7.3; and 10 mM Tris, pH 8.0 and 9.0.

The peptide, cyclo-Ac-AE[CHPQGPPC]IEGRK-NH₂, was coupled directly to the sensor chip surface (41) to the e-amino group of the lysine residue under a continuous HEPES buffer flow of 10 μl/min. The sensor chip surface was activated with a 2-min pulse of a solution of 50 mM N-hydroxy succinimide, 200 mM N-ethyl-N'-(3-diethylaminopropyl)-carbodiimide. The peptide surface was made by injection of 100 μM peptide in 100 mM sodium borate, pH 8.5. To inactivate any remaining N-hydroxysuccinimide ester groups, the immobilization procedure was completed by an 8-min injection of 1.0 M ethanolamine-HCl followed by a 2-min pulse of 0.6 M guanidine-HCl, pH 2.1, to wash out any noncovalently bound peptide. The Kᵣ values of cyclo-Ac-AE[CHPQGPPC]IEGRK-NH₂, cyclo-AE[CHPQFC]IEGRK-NH₂, and Ac-AEFSHPQNTIEGRK-NH₂ were determined by described procedures (13, 20, 22) as a function of pH through competition for streptavidin with cyclo-Ac-AE[CHPQGPPC]IEGRK-NH₂ immobilized on the surface. Direct Kᵣ values were determined for cyclo-[5-S-valeramide-HPQPPC]-NH₂ immobilized on the BIaCore chip. At each pH, two flow cell surfaces with different relative amounts of immobilized peptide were made by varying the peptide injection time. The cell with the lower density surface was used as a blank. At each pH, triplicate data sets were analyzed by the method of multispot sensing (41), which relies on equilibration of the affinity measured on two surfaces containing different ligand densities. To minimize perturbations to the apparent affinity constants caused by avidity or ligand binding to the surface more than once, it was necessary to use surfaces whose peptide densities corresponded to below 15 resonance units. Affinities with experimental error of those previously determined by other methods (6) were thus obtained.

**RESULTS**

**Affinities of Core Peptides Used for Crystallography Are Similar to Affinities of Corresponding Longer Peptides Used for BIaCore**—Because of the large amounts of peptides required for both crystallography and BIaCore at low pH where the binding is weak, two sets of peptides were used for most of this study: core peptides for crystallography and longer peptides synthesized for a previous study (6) for BIaCore. The core peptides were used for crystallography because the C- and N-terminal extensions in the streptavidin-bound longer versions are expected to be disordered or to make crystal cracking from peptide soaking more likely. The affinities of the longer peptides were determined to be within experimental error of the corresponding core versions; the Kᵣ of cyclo-Ac-[CHPQGPPC]-NH₂, pH 7.3, is 310 mM compared with 230 mM for cyclo-Ac-AE[CHPQGPPC]IEGRK-NH₂, pH 7.3 (22), and the Kᵣ of FSHPQNT is 78 mM (6) or 125 mM (25) at pH 7.3 compared with 150 mM for FSHPQNTIEGRK-NH₂, pH 7.3 (12) or 160 mM determined here for Ac-AEFSHPQNTIEGRK-NH₂, pH 7.3. Similarly the Kᵣ values of the other core peptides in this study were determined to lie within experimental error of the corresponding longer versions.
### TABLE I
Crystalllography of streptavidin-peptide complexes at various pH values

|                  | X-1000 | X-1000 | R-AXIS IV | R-AXIS | X-1000 |
|------------------|--------|--------|----------|--------|--------|
| **Parameters, cyclo-Ac-[CHPQFC]-NH$_2$** |        |        |          |        |        |
| **Area detector system** | R-AXIS | R-AXIS | X-1000 | R-AXIS | R-AXIS |
| **pH** | 2.00 | 3.00 | 3.60 | 4.20 | 4.77 |
| **Space group** | I222 | I222 | I4 122 | I222 | I4 122 |
| **No. atoms (including disorder)** | 10497 | 10533 | 2153 | 2199 | 22552 |
| **No. waters (including disorder)** | 65 | 62 | 45 | 34 | 77 |
| **No. residues with refined occs** | 4 | 6 | 10 + 4 | 10 + 4 | 10 + 4 |
| **Diffraction statistics** |        |        |          |        |        |
| **Resolution (Å)** | 1.79 | 1.81 | 1.33 | 1.33 | 1.32 |
| **No. observations** | 49532 | 49331 | 126387 | 85713 | 61076 |
| **No. merged reflections** | 12424 | 12200 | 26406 | 28233 | 20636 |
| **Average redundancy** | 3.9 | 4.0 | 4.8 | 3.0 | 3.0 |
| **Rp(merge) (%)** | 5.3 | 6.0 | 8.1 | 9.4 | 9.3 |
| **Refinement statistics** |        |        |          |        |        |
| **Refinement resolution** | 7.5–1.86 | 7.5–1.87 | 7.5–1.50 | 7.5–1.85 | 7.5–1.85 |
| **No. merged reflections** | 10046 | 10462 | 18080 | 13205 | 10040 |
| **Fo/Fe cut-off (%)** | 2.0 | 2.0 | 2.7 | 1.7 | 2.6 |
| **Rmerge (%)** | 19.1 | 17.9 | 20.3 | 19.8 | 20.5 |
| **Free Rmerge (%)** | 20.9 | 20.9 | 24.6 | 24.3 | 25.0 |
| **Overall completeness (%)** | 75.0 | 79.3 | 74.2 | 65.3 | 80.5 |
| **At highest resolution (%)** | 28.9 | 36.4 | 32.6 | 38.5 | 60.5 |
| **Highest resolution shell** | 1.94–1.86 | 1.95–1.87 | 1.57–1.50 | 1.67–1.60 | 1.93–1.85 |
| **Root mean square deviations** |        |        |          |        |        |
| **Bond lengths (Å)** | 0.019 | 0.018 | 0.018 | 0.017 | 0.019 |
| **Bond angles (°)** | 3.0 | 3.0 | 4.2 | 4.3 | 4.4 |
| **Torsion angles (°)** | 28.5 | 28.4 | 25.8 | 25.3 | 25.1 |

|                  |        |        |          |        |        |
| **Parameters, cyclo-Ac-[CHPQGPC]-NH$_2$** |        |        |          |        |        |
| **Area detector system** | R-AXIS | R-AXIS | X-1000 | R-AXIS | R-AXIS |
| **pH** | 2.50 | 2.85 | 3.67 | 2.50 | 3.50 |
| **Space group** | I222 | I222 | I4 122 | I4 122 | I4 122 |
| **No. atoms (including disorder)** | 10497 | 10422 | 1143 | 2107 | 2248 |
| **No. waters** | 60 | 54 | 76 | 56 | 58 |
| **No. residues with refined occs** | 6 | 7 | 10 + 7 | 10 + 7 | 10 + 7 |
| **Diffraction statistics** |        |        |          |        |        |
| **Resolution (Å)** | 1.35 | 1.38 | 1.84 | 1.33 | 1.33 |
| **No. observations** | 34357 | 103204 | 38381 | 65678 | 45610 |
| **No. merged reflections** | 17544 | 21313 | 12118 | 25029 | 26827 |
| **Average redundancy** | 2.0 | 3.8 | 3.2 | 2.6 | 1.7 |
| **Rp(merge) (%)** | 8.0 | 6.0 | 8.0 | 6.0 | 5.9 |

|                  |        |        |          |        |        |
| **Parameters, cyclo-[5-S-valeramide-HPQGPC]-K-NH$_2$** |        |        |          |        |        |
| **Area detector system** | R-AXIS | R-AXIS | X-1000 | R-AXIS | R-AXIS |
| **pH** | 1.50 | 2.50 | 2.50 | 3.00 | 3.50 |
| **Space group** | 1222 | 1222 | 14.22 | 1222 | 14.22 |
| **No. atoms (including disorder)** | 4352 | 4554 | 2223 | 4680 | 2266 |
| **No. waters** | 146 | 139 | 64 | 34 | 77 |
| **No. residues with refined occs** | 11 | 20 + 9 | 10 + 2 | 20 + 9 | 10 + 4 |
| **Diffraction statistics** |        |        |          |        |        |
| **Resolution (Å)** | 1.32 | 1.35 | 1.33 | 1.33 | 1.32 |
| **No. observations** | 89532 | 97859 | 127962 | 82155 | 108525 |
| **No. merged reflections** | 41504 | 36788 | 28160 | 41763 | 27343 |
| **Average redundancy** | 2.2 | 2.7 | 4.5 | 2.0 | 4.0 |
| **Rp(merge) (%)** | 5.6 | 5.4 | 10.7 | 7.9 | 6.8 |

|                  |        |        |          |        |        |
| **Refinement statistics** |        |        |          |        |        |
| **Refinement resolution** | 7.5–1.50 | 7.5–1.45 | 7.5–1.70 | 7.5–1.45 | 7.5–1.50 |
| **No. merged reflections** | 25381 | 29499 | 11983 | 28570 | 17621 |
| **Fo/Fe cut-off (%)** | 1.8 | 1.8 | 2.5 | 1.6 | 2.0 |
| **Rmerge (%)** | 20.2 | 20.7 | 19.5 | 20.5 | 20.5 |
| **Free Rmerge (%)** | 23.5 | 23.7 | 22.3 | 24.0 | 24.6 |
| **Overall completeness (%)** | 65.7 | 68.8 | 71.6 | 66.8 | 72.4 |
| **At highest resolution (%)** | 34.3 | 43.3 | 48.4 | 33.6 | 39.7 |
| **Highest resolution shell** | 1.57–1.50 | 1.52–1.45 | 1.78–1.70 | 1.52–1.45 | 1.57–1.50 | 1.52–1.45 |
An engineered thioether cross-linked ligand (22), cyclo-[5-S-valeramide-HPQGPPC]K-NH₂, was used both for crystallography and BLAcore.

pH-dependent Affinities Implicate Ionization of a Group with a pKₐ of ~6.3 upon Binding—The pH dependences of affinities of Ac-AEFSHPQNTIEGRK-NH₂, cyclo-Ac-AE[CHPQFC]IEGRK-NH₂, and cyclo-[5-S-valeramide-HPQGPPC]K-NH₂ are shown in Table II and plotted in Fig. 1. The affinity of the linear peptide is several hundred fold lower than the affinities of the cyclic peptides at all pH values of this study. The Kᵰₐ values of the ligands increase at roughly the same rate as the pH is lowered from ~6.5 to ~4.5, by an average factor of 3.0 ± 0.8 per decrease in pH unit. Below pH ~4.5 the increase in Kᵰₐ values becomes smaller, and above pH ~6.3 the Kᵰₐ values become relatively pH-independent. The apparent pKᵰₐ of ~6.3 is most clearly seen in the data for cyclo-[5-S-valeramide-HPQGPPC]K-NH₂, for which direct Kᵰₐ values (and associated standard deviations from triplicate data sets) at pH 5.0, 6.0, 6.2, 6.4, 7.0, 7.3, 8.0, and 9.0 were determined. Thus ionization of a group (or groups) with an apparent pKᵰₐ of ~6.3 in the unbound state is implicated in the binding process.

An Intrapeptide Hydrogen Bond between N₁His and N₁Gln Is Preserved at Low pH—The (2Fᵢ – |Fᵢ|) map superimposed on the refined structures of I222 streptavidin-cyclo-[5-S-valeramide-CHPQFC]K-NH₂, pH 2.5, at one of the two crystallographically independent ligand binding sites is shown in Fig. 2A. The refined N₁His–N₁Gln distance and associated angles (Table III) indicate a hydrogen bond between N₁Gln and the unprotonated N₆¹ atom of the uncharged His. The imidazole density is strong, well defined, and flat, typical for a well ordered histidine. Similar density and an N₁His–N₁Gln distance of 2.6 Å (for reflections from 7.5 Å to the highest resolution) is the observation of the intensity of reflection h 61.

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. Occupancies (occs) for poorly defined groups of atoms were refined. Discretely disordered groups are not included in this category.

TABLE I—continued

| pH   | Cyclo-Ac-AE[CHPQFC]IEGRK-NH₂ | Cyclo-[5-S-valeramide-HPQGPPC]K-NH₂ | Ac-AEFSHPQNTIEGRK-NH₂ |
|------|-----------------------------|-----------------------------------|-----------------------|
| 3.0  | 2300                        | × 10⁻⁹                            | × 10⁻⁹                |
| 4.0  | 2200                        | ND                                | ND                    |
| 5.0  | 1400                        | 1500                              | 3100 (350)            |
| 6.0  | 380                         | 500                               | 1730 (400)            |
| 6.2  | ND*                         | ND                                | 1400 (460)            |
| 6.4  | ND*                         | ND                                | 687 (239)             |
| 7.0  | ND*                         | 45                                | 284 (60)              |
| 7.5  | 130                         | 23                                | 256 (25)              |
| 9.0  | ND*                         | 47                                | 322 (50)              |
|      | 373 (87)                    | ND                                |                      |

a ND, not determined.

Standard deviations determined from three measurements are in parentheses.
this low pH, but the density of the imidazole is well defined enough to resolve two imidazole components. The Nδ1 atom of one component is not within hydrogen bonding distance of NγGln and is surely protonated. Although the refined Nδ1His-NγGln distance (2.92 ± 0.13 Å) of the other component suggests a hydrogen bond, the NγGln-HδGln-Nδ1His angle is poor (123 ± 5°), lower by 4.1° than the average for the other complexes above pH 2.0 (Table III). Thus this other component may be largely protonated as well, despite the small Nδ1His-NγGln distance.

Ionization State of the Peptide Histidine Depends on Crystal Packing—In I222 streptavidin-peptide complexes one of the two crystallographically independent binding sites (site 1) is near a 2-fold related crystallographically equivalent site and is thus more shielded from solvent than site 2. The unique site in I4,22 streptavidin is also solvent-shielded from a nearby 2-fold related equivalent site. At many pH values the density of the bound peptide in the complexes is better defined at the unique site in I4,22 complexes and at site 1 in I222 complexes than at site 2. The conclusions regarding the protonation states of the His of the bound peptides are essentially the same for site 1 and site 2 of I222 complexes and for the unique site of I4,22 complexes at pH values > 2.5 where the density is well defined, the temperature factors relatively low, and the Nδ1His-NγGln distances and associated angles (Table III) clearly indicate an Nδ1His-NγGln hydrogen bond at all three sites. However, at pH values ≤ pH 2.5, differences in the protonation state of the peptide His are observed at crystallographically different sites.

Fig. 2 (A and B) compares the structures of the bound peptide at site 1 and site 2, respectively, for I222 streptavidin-cyclo-[5-S-valeramide-HPQGPPC]K-NH₂, pH 2.5. The imidazole density at site 2 is distinctly different from that at site 1; it 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 distance and angle parameters of the unprotonated conformer indicate an Nδ1His-NγGln hydrogen bond (Table III) shown in yellow in Fig. 2B. The occupancies of the protonated and unprotonated conformers are 20 and 80%, respectively, yielding a calculated pKₐ at site 2 of 1.9. For the same I222 complex at pH 3.0, similar inequivalence of the two sites is observed, with resolvable protonated and unprotonated conformers at site 2. The occupancies of the protonated and unprotonated conformers at site 2 are 15 and 85%, respectively, at pH 3.0, yielding a calculated pKₐ of 2.2. By contrast, at site 1, the occupancies of any unprotonated components at pH ≥ 2.5 are not high enough to observe or to resolve. Thus the pKₐ,
of the bound peptide His is detectably lower at site 1 than at site 2.

In the conformation of the protonated component at site 2 in I222 streptavidin-cyclo-[5-S-valeramide-HPQGPPC]-K-NH₂, pH 2.5, shown in Fig. 2B, the side chain is rotated 180° about the Cβ-Cγ bond with respect to the conformation of the unprotonated component to allow N61 to make a hydrogen bond (shown in cyan) with a water molecule. This change in the His conformation increases the N61-δGln hydrogen bond length over a range of pH values in the bound linear and cyclic peptides. The peptide His is thus reduced to a value of less than 2.5. Therefore the decrease in binding affinity in solution as the pH decreases is attributed to the cost of deprotonating the peptide His at low pH. The deprotonation and formation of the N61His-δGln hydrogen bond are required for high affinity binding both in the crystals and in solution.

Table III provides the N61His-δGln hydrogen bond lengths and associated angles for various HPQ-containing peptide ligands at various pH values for this and other investigations (13, 19–22). This hydrogen bond is also observed in complexes of streptavidin with HHPQGPH (linear) and Ac-CHPQGPPC-NH₂. These data suggest that for other HPQ-containing linear peptide ligands such as HDHPQNL and SHHPQGPS and disulfide-bonded cyclic peptide ligands such as cyclo-[CHPQFSNC], cyclo-[CHPQPC], and cyclo-[CHPQFNC] (6), the same hydrogen bond forms in the complexes with streptavidin at pH values above 2.5.

Long Range Crystal Packing Interactions Perturb the pKₐ of the Histidine of the Bound Peptide Ligands—Inequivalence of the two crystallographically independent sites in I222 streptavidin results in significant differences in some of their properties. The temperature factors of bound peptide ligands are often lower and the density better defined at site 1 than at site 2 (21). Two of the hydrogen bonds to the ureido oxygen of biotin are systematically shorter over a range of pH values at site 1 than at site 2.² In

² B. A. Katz, unpublished observations.

| Ligand                        | pH | Space group | Resolution | Length  | Angle 1°  | Angle 2°  | Reference |
|------------------------------|----|-------------|------------|---------|-----------|-----------|-----------|
| Cyclo-[CHPQFC]-NH₂           | 2.00 | I4, 22     | 1.50       | 2.65    | 157       | 144       | this work |
| Cyclo-[CHPQGPPC]-NH₂         | 2.50 | I222       | 1.50       | 2.80    | 141       | 158       | this work |
| Cyclo-[5-S-valeramide-HPQGPPC]-K-NH₂ | 1.50 | I222       | 1.50       | 2.86 (10) | 152 (5)   | 160 (4)   | this work |
| [4-S-toluidide-HPQGPPC]-NH₂  | 6.00 | I222       | 1.50       | 2.86 (10) | 152 (5)   | 160 (4)   | this work |
| FCPFQNT                      | 4.00 | I222       | 1.50       | 2.86 (10) | 152 (5)   | 160 (4)   | this work |
| Ac-CPFQNT-β-NH₂ dimer        | 7.00 | I222       | 1.50       | 2.86 (10) | 152 (5)   | 160 (4)   | this work |

² Imidazole centroid, δ1His-δN1His-δGln.

³ N61His-δGln-δN61His.

Table III provides the N61His-δGln hydrogen bond lengths and associated angles for various HPQ-containing peptide ligands at various pH values for this and other investigations (13, 19–22). This hydrogen bond is also observed in complexes of streptavidin with HHPQGPH (linear) and Ac-CHPQGPPC-NH₂, and linear Ac-CHPQFC-NH₂. These data suggest that for other HPQ-containing linear peptide ligands such as HDHPQNL and SHHPQGPS and disulfide-bonded cyclic peptide ligands such as cyclo-[CHPQFSNC], cyclo-[CHPQPC], and cyclo-[CHPQFNC] (6), the same hydrogen bond forms in the complexes with streptavidin at pH values above 2.5.

Long Range Crystal Packing Interactions Perturb the pKₐ of the Histidine of the Bound Peptide Ligands—Inequivalence of the two crystallographically independent sites in I222 streptavidin results in significant differences in some of their properties. The temperature factors of bound peptide ligands are often lower and the density better defined at site 1 than at the more solvent exposed site 2. Topochemical lattice-mediated disulfide interchange occurs between neighboring bound cyclo-Ac-[CHPQGPPC]-NH₂ ligands at site 1, which is close to a 2-fold related equivalent site, but not at site 2 (21). Two of the hydrogen bonds to the ureido oxygen of biotin are systematically shorter over a range of pH values at site 1 than at site 2.² In
this investigation site 1 and site 2 were also shown to differ because of the observed effect of crystal packing on the pK values of the peptide His at site 1 in I222 streptavidin complexes and at the more solvent-exposed site 2 in I222 space group. The protonation state of the peptide His at the more solvent-exposed site 2 in I222 streptavidin-peptide crystals more accurately reflects its state in solution. However, because of the observed effect of crystal packing on the pK values of the peptide His at site 1 in I222 streptavidin complexes and at the unique site in I4122 streptavidin complexes, solvent shielding to a lesser extent by crystal packing at site 2 in I222 streptavidin complexes must also be considered as a potential factor that could perturb the pK values of the peptide His at this site from the pK in solution. Thus the crystallographically determined pK values of the peptide His at site 2 in I222 streptavidin should be taken as a lower limit to the corresponding value in solution.

**Directionalities of Hydrogen Bonds Involving Linear and Cyclic Peptide Ligands Are Unambiguous**—Because the proton atoms of protein-ligand complexes are normally not visible by x-ray crystallography, directionalities of hydrogen bonding interactions can not always unambiguously be determined by this technique. In some cases, however, from the environments of residues or groups participating in hydrogen bond networks, directionalities of some hydrogen bonds can be inferred (44). For the complexes of streptavidin with the linear and cyclic peptide ligands discussed here, the directionality of every hydrogen bond involving every atom of each ligand is uniquely determined at each pH value > 2.5.

In complexes of streptavidin with HPQ-containing peptide ligands, the orientation of the peptide Gln side chain amide group is unambiguous based on the better geometry of hydrogen bonds in one orientation (Fig. 3A) versus the alternate one in which this amide is rotated by 180° (Fig. 3B). In the less favorable orientation (Fig. 3B), the vector between Ne2Gln and Oγ2Thr90 is directed right between the Gln Nε2 hydrogens, and there are two proton donors to Oγ2Thr90 (Ne2Gln and Ne1Trp79). In the more favorable orientation, the Oγ2 proton of Thr90 is directed at Oc1Gln, whereas one of the Ne2Gln hydrogens is directed at the oxygen of Wat600. In this arrangement Oγ2Thr90 receives a proton from Ne1Trp79 and donates a proton to Oc1Gln. The angles associated with these hydrogen bonds are more favorable in this orientation than in the alternate one. The angle between the hydrogen bonds received and provided by Oγ2Thr90 is ~90°. The peptide Gln side chain is also uniquely oriented because of its interaction (d = 3.51 ± 0.15 Å, determined from 12 structures) with the Trp108 ring, which reflects an NH → π aromatic ring system hydrogen bond similar to those described (45).

**FSHPQNT**—Fig. 4 shows the hydrogen bonding network that connects the linear peptide to streptavidin at pH 4.0 and 5.6. The orientation of the peptide Asn side chain is unambiguous because of the hydrogen bond between its O61 atom and Nδ2Asn23. Asn23 is in turn uniquely oriented due to the hydrogen bond between its O61 atom and Nε2Thr87. The peptide Asn side chain is also uniquely oriented because of an Nδ2Asn23 → πTrp120 interaction (d = 3.64 ± 0.04 Å, determined from four structures). Because the peptide His Nε1 atom accepts a proton from the peptide Gln main chain NH group, the peptide His Nε2 atom must donate a proton to Oγ2Ser89. The two other peptide groups, Oγ1Trp and Nε3Trp, involved in hydrogen bonds must be an acceptor and donor, respectively. Thus the directionalities of all 11 peptide-protein hydrogen bonds in streptavidin-FSHPQNT are unambiguous, as well as the directionality of the intrapeptide hydrogen bond.

**Cyclic Peptides**—Fig. 5 shows the hydrogen bonding interactions for streptavidin-cyclo-Ac-[CHPQFC]-NH2, pH 3.0. The directionalities of the hydrogen bond interactions involving this cyclic peptide are also unambiguous based on considerations similar to those described above for the streptavidin-bound linear peptide. The same hydrogen bonding network is observed at pH 11.8, 7.5, 4.8, 4.2, 3.6, and 2.0 with a protonated His component at the latter pH. Likewise, the hydrogen bond directionalities involving the binding of cyclo-Ac-[CHPQG-PPC]-NH2 to streptavidin at pH 2.5, 2.9, 3.7, 5.0, and 7.5 are unambiguous (Fig. 6). The same network is observed in complex with cyclo-[5-S-valeramide-HPQGPPC]-KH2 at pH 1.5, 2.5, 3.0, and 3.5; cyclo-[5-S-valeramide-HPQGPPC]-NH2, pH 6.0 (22); cyclo-[4-S-toluamide-HPQGPPC]-NH2, pH 6.0 (22); head-to-head cyclo-[CHPQGPPC]-NH2 dimer, pH 2.5, 3.5, 5.0 (21), and 6.0 (21); and head-to-tail cyclo-[CHPQGPPC]-NH2 dimer, pH 2.5, 3.5, and 7.3 (19), with protonated peptide His components at pH values ≤ 2.5 in these complexes.

In a previous study of the binding of streptavidin to FSH-PQNT, it was suggested that enhanced binding at neutral compared with acidic pH reflects an equilibrium between alternate unprotonated states of the peptide His in alternate equi-energetic hydrogen bond networks involving the protein at neutral pH (25). Thus an increase in binding at neutral pH was proposed to reflect an increase in entropy (25). In the present study, however, we were able to establish one unique hydrogen bonding network wherein the directionalities of all hydrogen bonds between FSHPQNT and streptavidin, even those mediated by water molecules, are essentially unambiguous at pH 4.0 and 5.6. Similarly, unique, lowest energy hydrogen bond networks were delineated for all the streptavidin-cyclic peptide complexes at pH values > 2.5. In this investigation, no disorder in the His occurs at low pH (< 2.5), not neutral or basic pH. Therefore the pH dependence of binding of any of these peptides to streptavidin is probably not due to such an entropy effect. The structures and affinities of the linear and cyclic HPQ-containing peptide ligands determined over a large range of pH values in two space groups are most consistent with deprotonation of the peptide His upon binding as a major determinant of the pH dependence of ligand binding.
interactions, it makes two hydrogen bonds (N→H bonds). The peptide segments before and after the HPQG segment are schematic (atoms are missing).

**Structural Basis for the Large Perturbation of the pKₐ of the Peptide Histidine**—The experimentally determined pKₐ values of certain noncatalytic residues in naturally occurring proteins are shifted by as much as 2.5 units (46). Larger shifts in the pKₐ values often occur in functionally important residues, such as those at active sites where buried charged residues often participate in catalysis (47–51). For example, the pKₐ of active site Asp²⁰⁶ in reduced thioredoxin is elevated by more than 5 units (52). The pKₐ values of charged residues engineered into hydrophobic cores of protein mutants are also perturbed by as much as 3.9 units (53–55). Much of the theoretical work that involves prediction of pH-dependent properties of proteins is based on the assumption that ionization equilibria in proteins are influenced primarily by electrostatic interactions (43, 56–59) and hydrophobic interactions (60) may also play roles in determining pKₐ values. Large shifts in pKₐ values can also be effected simply by desolvation, the dominant factor shifting the pKₐ of the buried lysine introduced into staphylococcal nuclease (53).

Although the peptide His side chain of streptavidin-bound HPQ-containing peptide ligands is not involved in salt bridge interactions, it makes two hydrogen bonds (N→H bonds) at pH values ≥ 2.5. Thus, hydrogen bonding interactions together with desolvation within a protein cavity of low dielectric constant are among the factors perturbing the pKₐ in the streptavidin-HPQ-containing peptide complexes. Although the C62 atom of the imidazole of the peptide His is solvent-accessible from one direction, the other imidazole atoms are shielded from solvent by the rest of the bound peptide and by Trp⁷⁹, Leu¹⁰⁰, Ser⁸⁸, Ala⁴⁸, and Trp²⁰⁰ of a neighboring subunit. Upon protonation at low pH a small rotation about χ₁ of the peptide His and a 180° rotation about χ₂ allows N⁶¹ to hydrogen bond with a solvent molecule (Fig. 2B).

**Conclusions**—Through plasmon resonance measurements combined with crystallography at multiple pH values on a set of HPQ-containing ligands, features of the mechanism of high affinity binding to streptavidin have been delineated. High resolution crystal structures at pH values as low as 1.5 yield insight into the nature of the structural rearrangements that occur in the bound peptide upon protonation of the His at three crystallographically different binding sites in two space groups. Observation of perturbations to the pKₐ values of the peptide His from long range crystal packing interactions should be taken as a caveat in extrapolation of pKₐ values determined in crystals to the corresponding ones in solution. The determination of the greatly reduced pKₐ of the His in streptavidin-bound HPQ-containing peptides and of the difference in pKₐ at sites with different extents of solvent shielding should provide valuable structural data for testing and improving theoretical models directed at predicting pH-dependent properties of proteins and of protein-ligand complexes.

**REFERENCES**

1. Green, N. M. (1975) Adv. Protein Chem. 29, 85–143
2. Chilkoti, A., Schwartz, B. L., Smith, R. D., Long, C. J., and Stayton, P. S. (1995) Biotechnology 13, 1198–1204
3. Green, N. M. (1999) Methods Enzymol. 184, 51–67
4. Wilchek, M., and Bayer, E. A. (1990) Methods Enzymol. 184, 5–13
5. Wilchek, M., and Bayer, E. A. (1990) Methods Enzymol. 184, 14–45
6. Giebel, L. B., Cass, R. T., Milligan, D., Young, D., Arze, R., and Johnson, C. (1995) Biochemistry 34, 15430–15435
7. Saggio, I., and Laufer, R. (1993) Biochem. J. 293, 613–616
8. Kay, B. K., Adey, N. B., He, Y.-S., Manfredi, J. P., Mataragnon, A. H., and Fowlkes, D. M. (1993) Gene 129, 59–65
9. Roberts, D., Guelguel, K., and Winter, J. (1993) Gene (Amst.) 128, 67–69
10. Lam, K. S., Salmon, S. E., Hersh, E. M., Hruby, V. J., Kazmiwerski, W. M., and Knapp, R. J. (1993) Nature 354, 82–84
11. Devlin, J. J., Panganiban, L. C., and Devlin, P. E. (1990) Science 249, 404–406
12. Katz, B. A., Liu, B., and Cass, R. (1996) J. Am. Chem. Soc. 118, 7914–7920
13. Katz, B. A. (1995) Biochemistry 34, 15421–15429
14. Weber, P. C., Wendoloski, J. J., Pantoliano, M. W., and Salemme, F. R. (1992) J. Am. Chem. Soc. 114, 3197–3200
15. Hendrickson, W. A., Pahler, A., Smith, J. L., Satow, Y., Merritt, E. A., and Phizackerley, R. P. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 2190–2194
Streptavidin-Peptide Binding and Structure at Multiple pHs

16. Weber, P. C., Ohlendorf, D. H., Wendolski, J. J., and Salemme, F. R. (1989) Science 241, 85–88
17. Sano, T., Pandori, M. W., Chen, X., Smith, C. L., and Cantor, C. R. (1995) J. Biol. Chem. 270, 28204–28209
18. Reznik, G., Vajda, S., Smith, C. L., Cantor, C. R., and Sano, T. (1996) Nat. Biotechnol. 14, 1007–1011
19. Katz, B. A. (1996) J. Am. Chem. Soc. 118, 2535–2536
20. Katz, B. A., Cass, R. T., Liu, B., Arze, R., and Collins, N. (1995) J. Biol. Chem. 270, 31210–31218
21. Katz, B. A., Stroud, R. M., Collins, N., Liu, B., and Arze, R. (1995) Chem. Biol. 2, 591–600
22. Katz, B. A., Johnson, C., and Cass, R. T. (1995) J. Am. Chem. Soc. 117, 8541–8547
23. Weber, P. C., Pantoliano, M. W., Simons, D. M., and Salemme, F. R. (1994) J. Am. Chem. Soc. 116, 2717–2724
24. Green, N. M. (1966) Biochem. J. 101, 774–780
25. Weber, P. C., Pantoliano, M. W., and Thompson, L. D. (1992) Biochemistry 31, 9350–9354
26. Zheng, R.-L., and Kemp, R. G. (1994) J. Biol. Chem. 269, 18475–18479
27. Qamar, R., and Cook, P. F. (1993) Biochemistry 32, 6802–6806
28. Knowles, J. R. (1976) CRC Crit. Rev. Biochem. 4, 165–173
29. Swint-Kruse, L., and Robertson, A. D. (1996) Biochemistry 35, 171–180
30. Raghavan, M., Bonagura, V. R., Morrison, S. L., and Bjorkman, P. J. (1995) Biochemistry 34, 14649–14657
31. D'Souza, U. M., and Strange, P. G. (1995) Biochemistry 34, 13635–13641
32. Cheng, Y., Mason, A. B., and Woodworth, R. C. (1995) Biochemistry 34, 14879–14884
33. Persson, E., Ebzan, M., and Shymko, R. M. (1995) Biochemistry 34, 12775–12781
34. Huang, S. L., and Klingenberg, M. (1995) Biochemistry 34, 349–360
35. Fay, S. P., Habersett, R., Domalewski, M. D., Posner, R. G., Houghton, T. G., Piersson, E., Muthukumaraswamy, N., Whitaker, J., Haughland, R. P., Freer, R. J., and Morris, J. A. (1994) Cytometry 15, 148–153
36. Boniface, J. J., Albritton, N. L., Reay, P. A., Cantor, C. R., Stryer, L., and Davis, M. M. (1993) Biochemistry 32, 11761–11768
37. Pahler, A., Hendrickson, W. A., Kolks, M. A. G., Argarâne, C. E., and Cantor, C. R. (1987) J. Biol. Chem. 262, 13933–13937
38. Brünger, A. T. (1992) in Xplor Version 3.1: A System for X-ray Crystallography and NMR, pp. 187–206, Yale University Press, New Haven, CT
39. Chambers, J. L., and Stroud, R. M. (1979) Acta Crystallogr. Sec. B 33, 1681–1687
40. Finer-Moore, J. S., Kossiakoff, A. A., Hurley, J. H., Earnest, T., and Stroud, R. M. (1992) Proteins 12, 203–222
41. Karlsson, R., and Stahlberg, R. (1995) Anal. Biochem. 228, 77–80
42. Derewenda, Z. S., Lee, L., and Derewenda, U. (1995) J. Mol. Biol. 252, 248–262
43. Antosiewicz, J., McCammon, J. A., and Gibson, M. K. (1994) J. Mol. Biol. 238, 415–436
44. McDonald, I. K., and Thornton, J. M. (1994) Protein Eng. 8, 217–224
45. Bünig, R., and Petsko, G. A. (1983) FEBS Lett. 203, 139–143
46. Bashford, D., and Karplus, M. (1990) Biochemistry 9, 327–335
47. Kossiakoff, A. A. (1983) Annu. Rev. Biophys. Bioeng. 12, 159–182
48. Baldwin, J., and Chothia, C. (1979) J. Mol. Biol. 129, 175–220
49. Gelin, B. R., and Karplus, M. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 801–805
50. Perutz, M. F. (1978) Science 201, 1187–1191
51. Kraut, J. (1978) Annu. Rev. Biochem. 46, 313–358
52. Wilson, N. A., Barbar, E., Fuchs, J. A., and Woodward, C. (1995) Biochemistry 34, 8931–8939
53. Stites, W. E., Gitsis, A. G., Lattman, E. E., and Shortle, D. (1991) J. Mol. Biol. 221, 7–14
54. Dao-pin, S., Anderson, D. E., Baase, W. A., Dahlquist, F. W., and Matthews, B. W. (1991) Biochemistry 30, 11521–11529
55. Varadarajan, R., Lambright, D. G., and Boxer, S. G. (1989) Biochemistry 28, 3771–3781
56. Antosiewicz, J., and McCammon, J. A. (1996) Biochemistry 35, 7819–7833
57. Gelin, B. R., and Karplus, M. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 801–805
58. Perutz, M. F. (1978) Science 201, 1187–1191
59. Warshel, A., and Russel, S. (1984) Q. Rev. Biophys. 17, 283–422
60. Urry, D. W., Gowda, D. C., Peng, S., Parker, T. M., Jing N., and Harris, R. D. (1994) Biopolymers 34, 889–986
61. Rosemann, M. G., Leslie, A. G. W., Abdel-Meguid, S. S., and Tsukihara, T. (1979) J. Appl. Crystallogr. 12, 570–581
62. Brünger, A. T. (1992) Nature 355, 472–474