Ligand Exchange between Proteins

EXCHANGE OF BIOTIN AND BIOTIN DERIVATIVES BETWEEN AVIDIN AND STREPTAVIDIN*

Received for publication, March 25, 2002, and in revised form, May 29, 2002
Published, JBC Papers in Press, June 7, 2002, DOI 10.1074/jbc.M202874200

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We have studied the structural elements that affect ligand exchange between the two high affinity biotin-binding proteins, egg white avidin and its bacterial analogue, streptavidin. For this purpose, we have developed a simple assay based on the antipodal behavior of the two proteins toward hydrolysis of biotinyl p-nitrophenyl ester (BNP). The assay provided the experimental basis for these studies. It was found that biotin migrates unidirectionally from streptavidin to avidin. Conversely, the biotin derivative, BNP, is transferred in the opposite direction, from avidin to streptavidin. A previous crystallographic study (Huberman, T., Eisenberg-Domovich, Y., Gitlin, G., Kulik, T., Bayer, E. A., Wilchek, M., and Livnah, O. (2001) J. Biol. Chem. 276, 32031–32039) provided insight into a plausible explanation for these results. This data revealed that the non-hydrolyzable BNP analogue, biotinyl p-nitroanilide, was almost completely sheltered in streptavidin as opposed to avidin in which the disordered conformation of a critical loop resulted in the loss of several hydrogen bonds and concomitant exposure of the analogue to the solvent. In order to determine the minimal modification of the biotin molecule required to cause the disordered loop conformation, the structures of avidin and streptavidin were determined with norbiotin, homobiotin, and a common long-chain biotin derivative, biotinyl e-aminocaproic acid. Six new crystal structures of the avidin and streptavidin complexes with the latter biotin analogues and derivatives were thus elucidated. It was found that extending the biotin side chain by a single CH₂ group (i.e. homobiotin) is sufficient to result in this remarkable conformational change in the loop of avidin. These results bear significant biotechnological importance, suggesting that complexes containing biotinylated probes with streptavidin would be more stable than those with avidin. These findings should be heeded when developing new drugs based on lead compounds because it is difficult to predict the structural and conformational consequences on the resultant protein-ligand interactions.

The interaction of egg white avidin and bacterial streptavidin with biotin has evolved into an indispensable tool for general use in the biological sciences and as a model for the study of the interaction of a ligand with a protein. Both avidin and streptavidin bind biotin with an essentially immeasurably high affinity constant (2).

During the course of our studies on the avidin/streptavidin-biotin complex, we sought to examine whether biotin or its derivatives can be exchanged between the two proteins. Such a transfer can be of both practical and theoretical value. If practical application is desired, one can envision a situation where one of the proteins is first introduced into an experimental system followed by addition of the second biotin-binding protein. The question then arises whether one protein replaces the other or whether the effect will be cumulative. Such a situation may indeed occur upon in vivo application of the avidin-biotin system for localization and treatment of cancers (3, 4).

From the theoretical point of view, it would be intriguing to determine whether a high affinity ligand added to one protein can be transferred to another (in this case, can biotin be transferred from avidin to streptavidin and vice versa?). Moreover, if indeed such an exchange takes place, it is of interest whether the exchange is unidirectional or bidirectional and whether free biotin and its derivatives behave similarly.

To address this problem, we exploited the previously reported difference in the behavior of avidin and streptavidin toward the hydrolysis of biotinyl p-nitrophenyl ester (BNP).¹ We have recently shown that egg white avidin hydrolyzes BNP at intermediate pH values to give biotin and p-nitrophenol, whereas streptavidin protects BNP against basic hydrolysis even under exceptionally high alkaline conditions (5). The opposing properties of the two proteins allowed us to assess the exchange of BNP and biotin molecules between the two proteins, following defined periods of incubation. Consequently, in the current study we incubated avidin or streptavidin with biotin or BNP and examined whether the rival protein is capable of removing the ligand from the respective binding site. It was found that avidin can remove biotin from streptavidin but not BNP; conversely, streptavidin can remove BNP from avidin but not biotin.

The reasons for this distinctive behavior of avidin and streptavidin were clarified by three-dimensional structure analysis of the two proteins. Because our previous structural data (5) indicated that the interaction of BNP with the two proteins is related to differential conformational change in the L3,4 loop, the structural consequences of the tight dimensions of the binding site and its relationship to the critical loop were probed by determining their structures with norbiotin and homobiotin. The latter biotin analogues represent a single

¹ The abbreviations used are: BNP, biotinyl p-nitrophenyl ester; BNA, biotinyl p-nitrophenyl anilide; BCAP, biotinyl e-aminocaproic acid; HABA, 2-(4’-hydroxyazobenzene) benzoic acid.

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The atomic coordinates and structure factors (codes 1LDO, 1LDQ, 1LLE, 1LCV, 1LCW and 1LCZ) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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methylene group subtraction and addition, respectively, in the valeric acid side chain of biotin. The structures of the resultant complexes were compared with those of biotin. This approach enabled us to determine the minimal structural requirement for loop distortion and concomitant promotion of ligand exchange. In addition, the structures of the two proteins were determined with a long-chain biotin derivative, biotinyl e-aminocaproic acid, which is commonly used as a reagent for (strept)avidin-biotin technology. In this context, it was interesting to determine the status of the L3,4 loop in the respective protein upon binding the long-chain ligand.

In previous work, more than 80 crystal structures for streptavidin and its complexes with various ligands have been determined (6). In contrast, only six structures have been reported for avidin (7–9). Despite the numerous structures available, very little is known about the structural implications of the interactions of the two proteins with biotinyl analogues and derivatives modified at the carboxyl terminus of the ligand. In this work, we have determined six new structures of avidin and streptavidin complexes with two biotin analogues and a commonly used biotin derivative. The structures provide us with new insight into the interaction of these two proteins with the carboxylic acid side chain of the biotin molecule and the importance of the critical L3,4 loop of the protein.

MATERIALS AND METHODS

Exchange of BNP—Subsaturating quantities of BNP (100 µl, 800 µM) were added to either avidin or streptavidin (1.78 mg/ml) in 50 mM sodium acetate buffer, pH 4. After 30 min, an equivalent amount of the rival protein in the same buffer was added. The competition interaction was allowed to proceed for desired time intervals, after which an equivalent volume of 1 M sodium carbonate buffer, pH 10, was added. After 30 min, hydrolysis of BNP was determined by following the release of p-nitrophenol (ε<sub>403</sub> = 17,500 cm<sup>−1</sup> M<sup>−1</sup>). This approach effectively measured the relative amount of BNP available to avidin and protected by streptavidin.

Exchange of Biotin—A modification of this approach was used to measure transfer of biotin. Biotin-blocked forms of avidin or streptavidin (1.78 mg/ml) in 50 mM sodium acetate buffer, pH 4, were prepared by incubating the protein in the presence of an excess of biotin, followed by overnight dialysis against the same buffer with several buffer changes. An equivalent amount of the rival protein in the same buffer was then added. Competition for the bound biotin was allowed to proceed for desired time intervals, after which BNP (125 µl, 800 µM) was added. After 30 min of incubation, an equivalent volume of 1 M sodium carbonate buffer, pH 10, was added. After 20 min, BNP hydrolysis was assessed spectrophotometrically as described above. In this case, BNP was used as a postinteraction probe to measure the relative amount of the biotin derivative protected by streptavidin.

Crystallization and Data Collection of Avidin Complexes—Crystals of deglycosylated avidin (10) were obtained via the vapor-diffusion hanging drop method at 20 °C in which a 3-µl drop contained 1.5 µl of deglycosylated avidin (4.2 mg/ml) and 1.5 µl of the reservoir solution containing 12% polyethylene glycol 1000 and 0.1 M imidazole malate buffer, pH 5.8. Crystals appeared after 4 days. The crystals belonged to the orthorhombic space group P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub> with two avidin monomers in the asymmetric unit. Crystals of avidin complexed with norbiotin, homobiotin, and biotinyl p-aminocaproic acid (BCap) were obtained by soaking the crystals in a solution of the respective ligand for 10–20 days. Crystallographic data were collected at room temperature from a single crystal for each complex on a R-Axis IV++ image plate mounted on a Rigaku RU-H3R rotating anode generator with confocal MAXFLUX<sup>TM</sup> optics. The cell parameters for the avidin-norbiotin, -homobiotin and -BCap complexes were in Å: a = 71.1, b = 79.8, c = 43.1; a = 71.8, b = 79.8, c = 42.9; and a = 71.0, b = 80.7, c = 43.1, respectively. Data were integrated, reduced, and scaled using the HKL suite (11).

Crystallization and Data Collection of Streptavidin Complexes—Crystals of core streptavidin were obtained by the vapor-diffusion hanging drop method, using crystallization conditions previously reported (5, 8) at a temperature of 20 °C in which a 3-µl drop contained 1.5 µl of the protein (30 mg/ml) and 1.5 µl of the reservoir solution. The 1-ml reservoir solution contained 1.2 µM ammonium sulfate and 0.1 M sodium acetate, pH 4.2. Crystals appeared within 2 days and reached the final size after 4 more days. Complexes of core streptavidin with norbiotin, homobiotin, and BCap were obtained by first soaking the crystals with the dye HABA (12) until the crystals became red (8). The red crystals were then transferred to solutions containing the desired ligand. A slow decolorization was observed while the respective ligand displaced HABA from the binding site until the crystals became colorless. As reported previously (5,13), core streptavidin crystals belonged to the orthorhombic space group I222, with two streptavidin monomers in the

FIG. 1. Exchange of BNP between avidin and streptavidin. Avidin (circles) or streptavidin (squares) was loaded with BNP at pH 4 and incubated either in the absence or presence of the competing protein. The pH was then raised to 10. The observed signal is proportional to the amount of avidin-borne BNP, because BNP is protected by complexation with streptavidin. The extent of exchange in each case was calculated by the amount of BNP hydrolyzed by avidin and was standardized by determining the maximum amount of BNP hydrolyzed by avidin in the absence of streptavidin. The results indicate that upon addition of streptavidin to solutions of the avidin-BNP complex, extensive ligand exchange occurs. In contrast, addition of avidin to the streptavidin-BNP complex fails to induce ligand exchange.

FIG. 2. Exchange of biotin between avidin and streptavidin. Avidin (circles) or streptavidin (squares) was loaded with biotin and incubated either in the absence or presence of the competing protein. The amount of free binding sites in avidin was determined by adding an equivalent amount of BNP and raising the pH to 10. When bound to avidin, the BNP probe is subject to hydrolysis, whereas when bound to streptavidin the probe is protected. The extent of exchange at each time point was calculated by the amount of added BNP hydrolyzed and was standardized by determining the maximum amount of BNP hydrolyzed by avidin in the absence of streptavidin. The results indicate that addition of avidin to the streptavidin-biotin complex results in a reduction of signal, due to the transfer of biotin to avidin and consequent protection of the BNP probe when bound to the vacated binding sites in streptavidin. In contrast, addition of streptavidin to solutions of the avidin-biotin complex fails to generate an increase in signal that would have been expected had biotin been transferred from avidin to streptavidin.
asymmetric unit. The cell parameters for the streptavidin-norbiotin, homobiotin, and -BCAP complexes were in Å: \( a = 47.3, b = 95.3, c = 105.3; a = 47.3, b = 95.8, c = 105.6; \) and \( a = 47.5, b = 94.9, c = 105.5, \) respectively. Diffraction data for each complex were collected at room temperature. The streptavidin-norbiotin and -homobiotin complexes were collected on a RAXIS-IIc image plate mounted on a Rigaku RU-300 rotating anode generator with Charles Supper mirrors. Data for the streptavidin-BCAP complex were collected on a Rigaku RAXIS IV++ image plate mounted on a RU-H3R rotating anode generator with confocal MAXFLUX™ optics. Data were integrated, reduced, and scaled using the HKL suite (11). In all experiments, the x-ray generators were operated at 50 kV and 100 mA.

RESULTS

Ligand Exchange between Avidin and Streptavidin—The distinctive properties of avidin and streptavidin with respect to hydrolysis of BNP were used to study the exchange of biotin or BNP between avidin and streptavidin. We assumed that upon prior incubation of BNP with avidin (under conditions where BNP alone is not hydrolyzed), followed by introduction of streptavidin, the amount of intrinsic hydrolyzable BNP would be reduced if ligand exchange took place, because the BNP molecules that were transferred from avidin to streptavidin would now be resistant to alkaline hydrolysis. On the other hand, if BNP were first incubated with streptavidin before the introduction of avidin, ligand exchange would result in an increase of BNP hydrolysis.

Fig. 1 shows the influence of streptavidin on BNP-borne avidin and vice versa. The figure clearly demonstrates the transfer of BNP from avidin to streptavidin, as shown from the reduction in released p-nitrophenol. The effect was completed following 1 day of interaction. In contrast, no movement of BNP from streptavidin to avidin could be observed, even up to 9 days of incubation. Increase in the amount of avidin failed to change this behavior.

A modification of the above approach is also appropriate for

![Avidin-norbiotin](image1)

![Avidin-homobiotin](image2)

![Avidin-BCAP](image3)

**Fig. 3.** Electron density maps of avidin and streptavidin complexes. Stereoview of the \( F_o-F_c \) electron density maps calculated at the respective resolutions indicated in Table I, after the initial rigid body and simulated annealing refinement steps with no ligand in the model. The maps were constructed at 2.2 \( \sigma \) with superimposed coordinates from the final models.
evaluating the exchange of biotin using extrinsically added BNP as a probe. If biotin is first incubated with avidin followed by the introduction of streptavidin, ligand exchange can be detected by the subsequent addition of known levels of BNP. Thus if biotin is transferred from avidin to streptavidin, some hydrolysis of BNP would be anticipated, according to the amount of biotin that vacated the binding site of avidin. On the other hand, if biotin is first incubated with streptavidin followed by introduction of avidin, ligand exchange would result in a reduction of hydrolysis of added BNP compared with that of control samples that lack the biotin-primed streptavidin.

In order to examine the exchange of biotin between the two proteins, the desired protein was primed with the free vitamin followed by successive additions of the rival protein and BNP. As can be seen from Fig. 2, the biotin molecule formed a very stable complex with avidin and failed to undergo transfer to streptavidin. Consequently, no increase in released p-nitrophenol could be detected even after 10 days of incubation. On the other hand, a slow but consistent exchange of biotin from streptavidin to avidin was observed, which became more pronounced upon longer periods of incubation.

A possible explanation for these unexpected findings may be evident from the distinctive three-dimensional structures of avidin and streptavidin with reference to the previously demonstrated differential binding of the BNP analogue, biotinyl p-nitroanilide (BNA) (5). We therefore analyzed in further detail the interaction of binding site residues and the critical L3,4 loop of the two proteins with a homologous series of biotin analogues and a commonly used biotin derivative.

**Structure Determination and Refinement of Avidin and Core Streptavidin Complexes**—Because both avidin and streptavidin crystals belong to space groups already reported for the apo forms, molecular replacement methods were not required, and the solution was initiated at the stage of rigid body refinement.
The initial goals were to place the avidin and streptavidin monomers in the correct position in the unit cell and to determine via electron density maps the presence of the ligands in the respective binding sites. The structures were initially refined using the rigid body protocol in the crystallography NMR graphics program O (16). The initial goals were to place the avidin and streptavidin complexes are shown in Table I. The model of the avidin-norbiotin complex consists of residues 3–123 for both monomers. The model of the avidin-homobiotin complex consists of residues 3–123 for monomer 1 and 3–134 for both monomers. The model of the avidin-homobiotin complex consists of residues 3–36 and 45–123 for monomer 1 and 3–36 and 46–123 for monomer 2. The model of the avidin-BcAP complex consists of residues 3–36 and 45–123 for monomer 1 and 3–36 and 43–123 for monomer 2. The model of the streptavidin-norbiotin complex consists of residues 15–135 for monomer 1 and 16–133 for monomer 2. The model of the streptavidin-homobiotin complex consists of residues 16–134 for both monomers. The model of the streptavidin BcAP complex consists of residues 16–134 and 16–133 for monomers 1 and 2, respectively. The coordinates of the norbiotin, homobiotin, and BcAP complexes with avidin (1LDO, 1LDQ, and 1LEL, respectively) and streptavidin (1LCV, 1LCW, and 1LCZ, respectively) are available at the Research Collaboratory for Structural Bioinformatics protein data bank (17).

In all the avidin and streptavidin complexes, the overall folds of the monomers and their tetrameric arrangements are similar to those of the structures of the proteins in the apo- and ligand-complexed states described earlier (8, 9, 18, 19). Each monomer of avidin and streptavidin is constructed of eight anti-parallel β-strands, which form a classic β-barrel with the binding site for biotin, its analogue, or derivative at the wide end of the barrel (Fig. 4). The main structural difference between the avidin and streptavidin monomers lies in the size and conformation of the hairpin loops that connect the adjacent β-strands. The critical loop for the present study connects strands β3 to β4, denoted L3,4. Loop L3,4 in avidin is three residues larger than that of streptavidin and shows higher structural flexibility upon comparison of the available structures (6 avidin structures, 85 streptavidin structures) (8, 20).

The respective quaternary structure of avidin and streptavidin has been described as a dimer of dimers (21). The quaternary contact areas of avidin and streptavidin are very similar and consist of three intermonomeric interface regions. The interface previously defined as 1–2 (8) is crucial for the high affinity binding of biotin and other ligands. In this 1–2 interface, a tryptophan residue (Trp-110 in avidin and Trp-120 in streptavidin) is donated from one monomer to the biotin-binding site of its neighbor, thus contributing one of the aromatic residues which together form a tight hydrophobic cage (8). This conserved tryptophan contributes an essential component to the biotin-binding site of both proteins, and the conformation of this critical residue in both proteins is retained upon binding biotin. Site-directed mutagenesis of Trp-110 in avidin (22) and Trp-120 in streptavidin (23) has indeed substantiated the significance of this particular residue to the high affinity exhibited by each protein toward biotin and the contribution of this binding site residue to the stability of the quaternary structure (24, 25).

### Table I

<table>
<thead>
<tr>
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<th>Streptavidin</th>
<th>Avidin</th>
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<tbody>
<tr>
<td>Complex</td>
<td>Norbiotin</td>
<td>I222</td>
</tr>
<tr>
<td>Resolution</td>
<td>50–2.3</td>
<td>50–2.2</td>
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<tr>
<td>Unique reflections</td>
<td>10,095</td>
<td>11,861</td>
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<tr>
<td>Redundancy</td>
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<tr>
<td>R*&lt;sub&gt;sym&lt;/sub&gt;(I&lt;sup&gt;a&lt;/sup&gt;)</td>
<td>9.4 (35.3)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.5 (32.8)</td>
</tr>
<tr>
<td>Completeness</td>
<td>92.2 (93.4)</td>
<td>94.3 (98.6)</td>
</tr>
<tr>
<td>I/o&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.7 (2.3)</td>
<td>12.2 (4.0)</td>
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<tr>
<td>Number of protein atoms</td>
<td>1787</td>
<td>1784</td>
</tr>
<tr>
<td>Number of solvent atoms</td>
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<td>106</td>
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<tr>
<td>Number of ligand atoms</td>
<td>30</td>
<td>34</td>
</tr>
<tr>
<td>Number of carbohydrate atoms</td>
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<td>None</td>
</tr>
<tr>
<td>R-factor (R&lt;sub&gt;sym&lt;/sub&gt;)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>18.5% (23.4%)</td>
<td>18.3% (23.3%)</td>
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<tr>
<td>rmsd&lt;sup&gt;e&lt;/sup&gt; from ideality</td>
<td>0.006</td>
<td>0.005</td>
</tr>
</tbody>
</table>

<sup>a</sup> R<sub>sym</sub>(I) = Σ[I(–I)]/ΣI.

<sup>b</sup> Outer shell resolution range for streptavidin-norbiotin (2.38–2.3 Å), homobiotin (2.28–2.2 Å), BcAP (2.02–2.95 Å), and avidin-norbiotin (2.24–2.2 Å), homobiotin (2.75–2.7 Å), BcAP (3.0–2.9 Å).

<sup>c</sup> Test set is 5% of data for all complexes.

<sup>d</sup> rmsd, root mean square deviation.

### Data collection and refinement statistics of the avidin and streptavidin complexes

Calculations of the Ramachandran plot were performed using PROCHECK (31).

### Calculations of the Ramachandran plot

<table>
<thead>
<tr>
<th></th>
<th>Streptavidin</th>
<th>Avidin</th>
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</thead>
<tbody>
<tr>
<td>Number of protein atoms</td>
<td>1787</td>
<td>1784</td>
</tr>
<tr>
<td>Number of solvent atoms</td>
<td>95</td>
<td>106</td>
</tr>
<tr>
<td>Number of ligand atoms</td>
<td>30</td>
<td>34</td>
</tr>
<tr>
<td>Number of carbohydrate atoms</td>
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</tr>
<tr>
<td>Favored</td>
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</tr>
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</tr>
<tr>
<td>Disallowed</td>
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<td>0.0</td>
</tr>
</tbody>
</table>

- Factor (R<sub>sym</sub>)
- rmsd<sup>e</sup> from ideality
- Bond length (Å)
- Bond angle (<°>)
- Redundancy
- Unique reflections
- Resolution
- Completeness
- I/o
- Number of protein atoms
- Number of solvent atoms
- Number of ligand atoms
- Number of carbohydrate atoms
- Test set is 5% of data for all complexes.
The complexes of streptavidin with norbiotin, homobiotin, and biotin exhibit entirely different conformational changes.

In the streptavidin-BCAP complex, the L3,4 loop maintains a closed (ordered) conformation, in contrast to that of the analogous avidin complex (Fig. 6). Consequently, the H-bonding network of the streptavidin-BCAP complex is generally preserved, compared with that of its complex with biotin (Fig. 5G). However, the two BCAP molecules in the asymmetric unit have different conformations, such that the amide linkage between the biotin side chain and caproic acid moieties is rotated in the two monomers by almost 180°. The corresponding caproyl side chains exhibit entirely different conformations, resulting in a displacement of the respective carbohydrate group (not shown).

In contrast to the avidin-homobiotin and -BCAP complexes, in which the disordered L3,4 loop conformation results in the loss of a critical H-bond interaction with a ureido ring nitrogen, the bond is retained in the complexes analogous with streptavidin because of the closed conformation of the loop.

**DISCUSSION**

Egg white avidin and its bacterial analogue streptavidin are well known for their tenacious binding to the vitamin biotin. The affinity constant for avidin has been estimated at $10^{15} \text{ M}^{-1}$ and that for streptavidin at 1–2 orders of magnitude lower (2, 26). In this context, the rate constant for biotin binding was measured only once for the avidin-biotin complex. Subsequent estimates of dissociation constants for streptavidin-biotin and for the interaction of either protein with other biotin analogues and derivatives have all been based on the original rate constant for the avidin-biotin complex. Consequently, the estimated dissociation constants are only approximate. Interestingly, the rate of association for the avidin-biotin complex is similar to that of many other protein-ligand interactions. Thus, the definitive factor for the affinity constant is the rate of dissociation. In fact, experimentally, it is much easier to measure the half-life of the avidin/streptavidin-ligand complex. For biotin, the complex with avidin was determined to be 200 days at pH 7, whereas that for streptavidin was only 2.9 days (2).

The results of the ligand exchange experiments described in this work indicate that biotin is transferred unilaterally from streptavidin to avidin and not vice versa. Conversely, the directionality of transfer of the biotin derivative, BNP, is re-
Fig. 5. Schematic representation of the hydrogen-bonding network in the avidin and streptavidin complexes with various biotin-related ligands. The biotin complexes with avidin (A) and streptavidin (B) show the H-bond network of the respective proteins with the native ligand. The biotin ring system shows a similar network of interactions in both proteins, whereas the respective biotin carboxylates show distinct differences. In avidin (A) one of the carboxylate oxygens forms three H-bond interactions with residues of the L3,4 loop and the other forms two H-bonds with Ser-73 and Ser-75. However, in streptavidin (B) the L3,4 loop is three residues shorter, and only one H-bond interaction is thus formed with the biotin carboxylate. The norbiotin complex with streptavidin (D) forms an identical set of H-bond interactions compared with those of the biotin complex (B). In the avidin-norbiotin complex (C), the L3,4 loop is also in the closed conformation. Norbiotin, which lacks a single methylene group, forms two H-bond interactions with residues of the L3,4 loop, thus stabilizing its closed conformation. In the avidin-homobiotin complex (E), the L3,4 loop is also in the closed conformation. Homobiotin, which lacks a single methylene group, forms two H-bond interactions with residues of the L3,4 loop, thus stabilizing its closed conformation. In the avidin-homobiotin complex (F), the L3,4 loop is also in the closed conformation. Homobiotin, which lacks a single methylene group, forms two H-bond interactions with residues of the L3,4 loop, thus stabilizing its closed conformation. In the avidin-homobiotin complex (G), the L3,4 loop is also in the closed conformation. Homobiotin, which lacks a single methylene group, forms two H-bond interactions with residues of the L3,4 loop, thus stabilizing its closed conformation. In the avidin-homobiotin complex (H), the L3,4 loop is also in the closed conformation. Homobiotin, which lacks a single methylene group, forms two H-bond interactions with residues of the L3,4 loop, thus stabilizing its closed conformation.
Ligand Exchange between Avidin and Streptavidin

complex (E), however, the L3,4 loop is completely disordered. Thus, one of the uredio ring nitrogens exhibits no H-bond interaction because the distance to Thr-35 (Oy) is 5.7 Å. Moreover, the homobiotin carboxylate forms no interactions with the protein. In contrast, in the streptavidin-homobiotin complex (F), the L3,4 loop is in the closed conformation, and interaction with one of the carboxylate oxygens is maintained. However, the interaction with Ser-88 is lost, and the homobiotin carboxylate oxygens interact with two solvent molecules. In the avidin-BCAP complex (G), the L3,4 loop is disordered, resulting in an H-bonding network similar to that of the homobiotin complex. Particularly, the H-bond interaction of Thr-35 with the uredio ring nitrogen is lost. In the streptavidin-BCAP complex (H), the L3,4 loop is in the closed conformation in both monomers, whereas a minor difference is observed in the BCAP carboxylate. In monomer 1, the caproic acid carboxylate and Ser-112 form an H-bond interaction, whereas in monomer 2 (monomer 1 is portrayed in the figure). The H-bonding interactions with the amide group are conserved compared with respect to native avidin, yet the valeric acid side chain was exposed to solvent and lacked the hydrophobic and polar interactions. One would therefore expect a decrease in the affinities of avidin toward biotinyl derivatives and analogues that induce disorder of the L3,4 loop. Indeed, the reported affinity constants of avidin for BNP and other biotin derivatives are several orders of magnitude lower than that for biotin (27, 28).

BNP, however, can be exchanged from avidin to streptavidin but not in the reverse direction. In a previous work (5), we described the comparative three-dimensional structures of a stable BNP analogue, BNA, complexed to avidin and streptavidin. The structures revealed that in avidin the BNA molecule was partially exposed to solvent, because of disordering of the L3,4 loop of the protein. Moreover, the disordered loop in the avidin-BNA complex resulted in the loss of four H-bonds that were formed with biotin. In contrast, the analogous loop in streptavidin was in the fully ordered (consensus) conformation, maintaining the H-bond network as in the biotin complex (Fig. 5) such that the BNA molecule essentially remained buried in the binding site. Thus, the BNP molecule can undergo displacement from avidin and transfer to streptavidin but not vice versa.

The structures of the avidin and streptavidin complexes with norbiotin and homobiotin revealed the consequences of minor conformational adaptations of the respective protein toward hydrophobic interactions by aromatic residues (7, 8) involved in biotin binding of both proteins, is consistent with the observed differences in affinities. In avidin, the biotin carboxylate forms five H-bonds, whereas in streptavidin only two are observed. The L3,4 loop in the closed conformation forms a lid that embraces biotin in the binding site. The L3,4 loop of avidin is three residues larger than that in streptavidin and forms three H-bonds with one of the biotin carboxylate oxygens, whereas in streptavidin only one H-bond is formed.

Loop L3,4 in streptavidin was previously shown to have a crucial role in the high affinity toward biotin. When the loop was omitted (residues 47–51) from the structure, a substantial decrease in the affinity constant toward biotin was observed ($K_a = 10^7$ M$^{-1}$) (26). Structural studies indicated that the H-bonding network of the biotin bicyclic ring system was conserved with respect to native streptavidin, yet the valeric acid side chain was exposed to solvent and lacked the hydrophobic and polar interactions. One would therefore expect a decrease in the affinities of avidin toward biotinyl derivatives and analogues that induce disorder of the L3,4 loop. Indeed, the reported affinity constants of avidin for BNP and other biotin derivatives are several orders of magnitude lower than that for biotin (27, 28).

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**Fig. 6.** Comparison of the conformations of the L3,4 loop in the apo forms of avidin and streptavidin and in their respective ligand complexes. The left and right panels represent the avidin and streptavidin structures, respectively. The arrows represent the edges of the disordered L3,4 loop in the relevant structures. In the apo form of streptavidin, the L3,4 loop is disordered, whereas upon binding biotinyl analogues or derivatives the loop adopts a closed consensus conformation. The BCAP complex shown in the figure represents the ligand conformation as observed in monomer 1. In contrast to the streptavidin complexes, in the avidin complexes the structural stability of the L3,4 loop shows a higher susceptibility to the size and disposition of the biotinyl side chain. In the apo form, the loop is disordered, but upon binding to biotin or norbiotin the loop adopts a closed conformation. The addition of a single methylene group to the biotin side chain (i.e., homobiotin) results in the complete disorder of the loop. In the avidin-BCAP complex, where biotin is conjugated to the commonly used caproic acid spacer, the loop is also disordered. Structural representations were generated using MIDAS (33).

**Fig. 7.** Schematic summary of the directionality of exchange of biotin and BNP between avidin and streptavidin. Biotin undergoes exchange from the streptavidin-biotin complex to free avidin but not in the opposite direction. Conversely, BNP undergoes exchange from the avidin-biotin complex to free streptavidin.
minimal alterations in the biotin molecule. In streptavidin, the removal or addition of a single methylene group to the biotin valeric acid side chain caused insignificant alterations in the biotin-binding site. For example, in the streptavidin-homobiotin complex, the L3,4 loop adopted the consensus (closed) conformation (Fig. 6), similar to that of its complex with biotin. In the analogous avidin complex, however, the extra methylene group of homobiotin resulted in the complete disorder of the loop and subsequent loss of four hydrogen bonds and exposure of the homobiotin carboxylate to solvent. The L3,4 loop in the avidin-norbiotin complex retained the closed conformation, similar to that of the biotin complex, accompanied by the formation of two H-bonds with both norbiotin carboxylate oxygens. Like the avidin-biotin complex, it thus appears that the position of the ligand carboxylate and consequent H-bonding network are crucial to maintaining a closed conformation of the L3,4 loop.

In general, the relationship between derivatized biotins and the L3,4 loop of avidin or streptavidin appears to be different in the two proteins. In this context, the relatively short loop of streptavidin attains an ordered consensus conformation, whereas the longer loop of avidin is largely disordered. The differential status of the L3,4 loop in avidin and streptavidin with respect to biotin derivatives appears to be valid for both the bulky, aromatic derivatives (e.g. BNP and BNA) and the more commonly used aliphatic derivatives, exemplified by BCAP. Because of the disordered status of the loop in the avidin complexes, the protein-ligand interaction is deficient in its H-bonding pattern, relative to that of streptavidin. The ligand is thus more exposed to the solvent. Of crucial significance is the fact that in the avidin complexes with BNA, homobiotin, and BCAP, the disordered conformation of the L3,4 loop results in the spatial displacement of Thr-35 and the consequent loss of H-bonding interaction with a ureido nitrogen of the ligand. Hence, the derivatized biotins would be expected to display a higher propensity for exchange from avidin to streptavidin or, alternatively, avidin could be dissociated from the biotinylated target.

Such a tendency could provide the molecular basis for the observed behavior of avidin and streptavidin toward biotinylated probes using radioimmunotherapy (4, 29, 30). In this three-step procedure, biotinylated antibody against the target cancer cell is delivered first, followed by free avidin, which serves both to bind to the biotinylated target and to remove excess biotinylated antibody from the circulation. The final step involves delivery of the biotinylated radionuclide. It was found that the efficacy of this treatment could be increased tremendously if streptavidin were also administered after introduction of avidin but before the radionuclide. This phenomenon was difficult to resolve, although several explanations were suggested. One such explanation was that avidin is cleared from the system before it reaches the target cells. The results of the present study provide a plausible alternative explanation, in that avidin bound to the biotinylated antibody can be exchanged by streptavidin. It seems that avidin bound to any biotinylated molecule would be subject to exchange by streptavidin, due to the derivatization of the biotin carboxylate, which forces the L3,4 loop into an open, disordered conformation. The findings outlined in this study may have broad implications for drug design and development, which are usually based on lead compounds. Our results suggest that even minor modifications of a ligand (e.g. drug) may generate dramatic structural alterations in its interactions with the target protein. Even with the strongest known protein-ligand interaction, epitomized by the avidin/streptavidin-biotin system, such significant effects would not have been foreseen. It thus follows that similarly minor modifications of ligands in lower affinity systems, such as those that characterize drug-receptor interactions, would lead to even more dramatic and less predictable consequences to the protein-ligand interaction.

From the theoretical perspective, the present work demonstrates that strong, weak, or unusual binding properties between two molecules are not necessarily a simple function of the affinity constant. In this context, elucidation of the three-dimensional structures of a protein in the presence of appropriate ligand analogues and derivatives is required for better understanding of the behavior of interacting molecules that characterize protein-ligand and protein-protein interactions in general.

REFERENCES