Noncooperativity of Biotin Binding to Tetrameric Streptavidin

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ABSTRACT: Streptavidin tetramers have been separated according to their biotin content by anion exchange chromatography. Biotin-free and biotin-saturated streptavidin were coincubated. Streptavidin at intermediate ligation levels, *i.e.*, with one, two, or three molecules of bound biotin, accumulates over time. A steady state distribution of ligation levels is reached after 2 days. When biotin was allowed to redistribute starting from homogeneous populations containing two molecules of biotin per tetramer, a similar steady state distribution of ligation levels was observed, thereby demonstrating an equilibrium distribution. Quantification of this equilibrium indicates that biotin binds to streptavidin with no cooperativity.

Macromolecules interact with a heterogeneous array of surrounding species. Affinities range from weak interactions with assorted solutes to extremely tight binding of specific ligands. Ligand binding is critical to all biochemical processes, and binding mechanisms are of fundamental importance. Considerations include energetics, kinetics, binding site topology, and the structural changes imposed by binding. In cases where mechanistic, structural, and functional features are well defined, the binding process can be an attractive target for research and biomedical applications.

We are studying the binding of biotin to avidin and streptavidin as a model for the mechanism, evolution, and engineering of tight protein−ligand interactions. Biotin is a small carboxylic acid vitamin that binds to these proteins extremely tightly (Green, 1975, 1990; Wilchek & Bayer, 1989; Bayer & Wilchek, 1990). Estimated dissociation constants are 10^{-14} M to streptavidin (Chaiet & Wolf, 1964; Green, 1990) and 10^{-15} M to avidin (Green, 1975, 1990). Both proteins are antimicrobial agents that act by reducing the concentration of free biotin. Avidin is secreted into egg white, and streptavidin is secreted by the bacterium *Streptomyces avidinii* into the surrounding soil or medium. The lack of other functions suggests that the development of tight binding was the dominant evolutionary force, with minimal complication from other requirements.

The crystal structures of biotin-free and biotin-saturated streptavidin (Hendrickson et al., 1989; Weber et al., 1989) and avidin (Livnah et al., 1993; Pugliese et al., 1993, 1994) are available. The two proteins are generally similar. Four molecules of biotin bind to a tetramer that is constructed from identical subunits. Each subunit is a single domain consisting of an eight-stranded, antiparallel, up−down β-barrel. In the absence of biotin, each binding site is an open cavity within a β-barrel. Structural changes caused by binding include the movement of a tryptophan side chain [each Trp 120 of streptavidin (Weber et al., 1989) or Trp 110 of avidin (Livnah et al., 1993)] across a quaternary interface into a biotin site that is otherwise located in another subunit. Overall, biotin binding closes off sites, which is consistent with the slow dissociation of the bound ligand (Green, 1975, 1990; Piran & Riordan, 1990).

We address here whether biotin binding to streptavidin is cooperative. The quaternary structural changes are consistent with the possibility of cooperativity. Indeed, there is a previous report of positive cooperativity (Sano & Cantor, 1990a). We take advantage of the tight binding to implement a column chromatography separation of streptavidin at intermediate ligation levels. Biotin slowly equilibrates between tetramers. The equilibrium biotin distribution indicates that binding is not cooperative.

EXPERIMENTAL PROCEDURES

Materials. Biotin was from Calbiochem. [^{14}C]Biotin was from Amersham. Aminoethylbiotinamide was from Molecular Probes. Sulfo-NHS-biotin (sulfosuccinimidobiotin) and avidin were from Pierce Chemical Co. Streptavidin was from U.S. Biochemicals. Prepacked columns (Q-Sepharose and Mono Q) and other chromatography media were from Pharmacia.

Biotin and Biotin Analogs. Biotin was weighed and dissolved in 10 mM Tris base to make a 5 mM stock solution and then stored at −15 °C. [^{14}C]Biotin (57 mCi/mmol) was dissolved with 10 mM Tris Cl, (pH 8) to a nominal concentration of 1 mM and then stored at −70 °C. Aminoethylbiotinamide was dissolved in 2 mM monobasic sodium phosphate to 0.75 mM, as determined by titration into streptavidin (see the following).

Biotinamide was synthesized by reacting an activated biotin ester, sulfo-NHS-biotin, overnight with 50 mM ammonium chloride (pH 8.5). Products were applied to a DEAE-Sephacel anion exchange column equilibrated in 20 mM Tris/ 30 mM NaCl (pH 8.0). Biotin-like species were detected and quantified by their ability to decrease the intrinsic fluorescence of avidin [$E^{1\%}_{282}$ = 15.4 (Green, 1975)], with excitation at 295 nm and emissions at 390 nm. The major product (yield > 50%) eluted near the included volume, which is consistent with the expected absence of charge in biotinamide. This product was absent after a control reaction in which the ammonium chloride was replaced with 10 mM sodium bicarbonate/40 mM sodium

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chloride. In the control reaction the major product was eluted with salt, which is consistent with the expected hydrolysis of the activated ester to biotin.

Preparation of Streptavidin and Ligand-Saturated Forms of Streptavidin. Streptavidin subunits were quantified using $E_{282} = 56\ mM^{-1}\ cm^{-1}$ (Green, 1975). Stoichiometries are expressed in terms of tetramers, *i.e.*, per four binding sites.

Streptavidin was further purified on a Q-Sepharose column as described under Results and in Figure 1. S-4[1] (streptavidin saturated with biotin) was prepared by incubating streptavidin with excess biotin (or [^{14}C]biotin) for >15 min, followed by size exclusion chromatography on Sephadex G-25. Streptavidin and free biotin were baseline resolved. The column buffer, 0.1 M Tris Cl (pH 8.5), allowed for quantitative binding of streptavidin to the subsequent Mono Q strong ion exchange column and was chosen to keep the protein farther from its isoelectric point and at higher ionic strength than in the Mono Q column buffer. Streptavidin was saturated with each biotin analog by the same method.

Analysis of Biotin Binding. Fluorescence titrations were performed on an SLM AmincoBowman AB2 spectrophotofluorometer, with excitation at 290 nm, 2 nm excitation, and 4 nm emission band passes, using 2 mL stirred samples that were thermostated at 22 °C. Streptavidin (1 μM in subunits) was made 0.1 M in Tris Cl and 0.05 M in total NaCl (pH 8.48). Emissions were measured at 360 nm, where saturation of S-0 with biotin decreases the fluorescence by about 50% (Kurzban et al., 1990). Biotin solutions for titrating peaks 0, 1, 2A, 2B, and 3 (as labeled in Figure 4) were prepared such that saturation required approximately 120 μL. For peak 4, the same stock solutions and volumes were employed as for peak 0. The fluorescence was corrected for dilution and then plotted against the amount of added biotin. Break points, corresponding to saturation with biotin, were assessed subjectively.

Wavelength maxima of emissions (corrected for buffer blanks and instrument response factors and then smoothed) were taken as the wavelength where the first derivative was 0.

Preparation of Streptavidin Tetramers Containing Two Biotins, S-2A and S-2B. A 4-day incubation of an equimolar mixture of S-0 and S-4 was applied to the Mono Q column (as per Figure 2). Subsequent manipulations were performed at 4 °C because biotin redistribution was negligibly slow at this temperature. Pools corresponding to the peaks labeled 2A and 2B in Figure 4 were made 0.1 M in NaCl and 0.1 M in Tris Cl (pH 8.5), concentrated by ultrafiltration, and then dialyzed against 0.1 M Tris Cl (pH 8.48) and 3 mM NaCl. This buffer exactly matches the conditions used in the time course described in Figure 3. Each S-2 sample was recovered and confirmed as being homogeneous on the Mono Q column. The redistribution of biotin was then initiated by raising the temperature to 22 °C.

RESULTS

Preparation of Homogeneous Streptavidin. Streptavidin is secreted into the growth medium of *S. avidinii* as a 159 residue polypeptide. Treatment with any of several proteases removes only nonbarrel residues and produces highly protease-resistant "core streptavidin" that starts at residue 13 or 14 and ends at residue 138 or 139 (Bayer et al., 1989; Pähler et al., 1987).

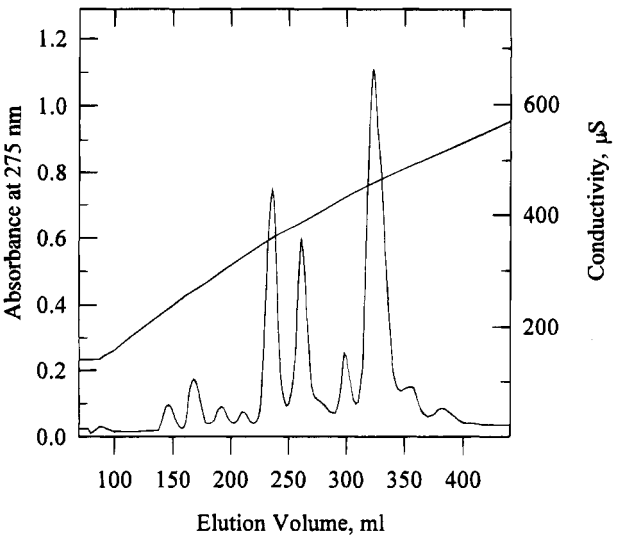

FIGURE 1: Anion exchange chromatography of core streptavidin. Streptavidin (25 mg) was made 0.1 M in Tris Cl (pH 8.5) and applied to a 20 mL column of Q-Sepharose. Chromatography was in 10 mM Tris Cl, (pH 7.3), at 60 mL/h, with a 1.2 mM/bed gradient of NaCl. The last streptavidin peak, eluting in 12 mM NaCl, was used for all subsequent studies.

We chose to use core streptavidin in this study. There are no reported differences in biotin binding or subunit interactions between the full-length and core species. Core streptavidin generally is preferred for physical studies because the full-length form tends to aggregate (Bayer et al., 1986, 1989; Pähler et al., 1987; Sano & Cantor, 1990b) and is sensitive to adventitious proteolysis. We also chose to use core streptavidin because Sano and Cantor (1990a) used it in their work regarding cooperativity. Lastly, pilot experiments indicated that the full-length species has poor recovery from the Mono Q column.

Development of an ion exchange separation of S-0 and S-4 (see the following) was initially hampered by the heterogeneity of the streptavidin. At pH 8.6, core streptavidin elutes from a Mono Q anion exchange column in a single peak at about 120 mM NaCl (data not shown). Heterogeneity becomes apparent at more acidic conditions, pH 7.3−7.6 (Figure 1). Heterogeneity has been observed previously by isoelectric focusing (Dittmer et al., 1989; Kurzban et al., 1991) and by PAGE (Sano & Cantor, 1990a; Kurzban et al., 1991) and would interfere with the chromatographic resolution of S-0 and S-4. A plausible source of heterogeneity is in the exact sites of the amino and carboxyl terminals, compounded by the presence of four subunits. Other sources of heterogeneity cannot be excluded.

Homogeneous core streptavidin, defined by chromatography on the Mono Q column, was prepared by anion exchange chromatography (Figure 1). Nine peaks were recovered and compared. All were similar with respect to fluorescence emissions ($\lambda_{max} = 335.5 \pm 0.5$ nm). In the presence of excess biotin, fluorescent emissions were similar for all peaks ($\lambda_{max} = 329.4 \pm 0.25$ nm). Titrations of the intrinsic fluorescence with biotin indicated that all had approximately four available sites per tetramer. These findings are consistent with the absence of bound biotin and the presence of four competent subunits. Thus, the hetero-

[1] Abbreviations: S-0, S-1, S-2, S-3, and S-4 are tetramers of streptavidin containing zero, one, two, three, and four molecules of bound biotin, respectively.

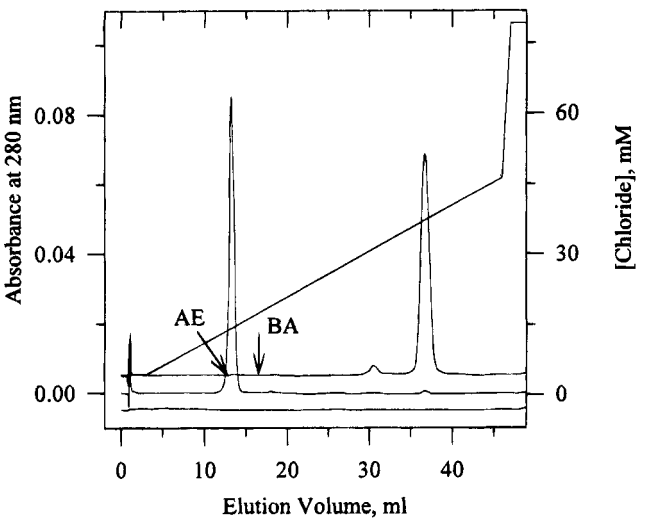

FIGURE 2: Anion exchange chromatography separating biotin-free from biotin-saturated streptavidin. The chromatograms are, from bottom to top, 0.1 M Tris Cl buffer (pH 8.5), biotin-free streptavidin (S-0) and streptavidin saturated with biotin (S-4). The arrows marked AE and BA show the elution positions of tetramers that were saturated with aminoethylbiotinamide and biotinamide, respectively. The 1 mL Mono Q column was equilibrated in 5 mM Tris Cl, (pH 7.3) and eluted with a linear gradient of NaCl at 1 mM/mL and 1 mL/min at room temperature. The detector employed a 1 cm light path.

geneity is not due to the presence of various amounts of bound biotin.

The major peak at 330 mL in Figure 1 was used for the remainder of this study. This peak was chosen because it is a major, well-resolved species. Streptavidin from U.S. Biochemicals was used because it was composed entirely of core streptavidin and because this last peak was a large fraction of the total sample. Alternate sources of streptavidin had variable amounts of core streptavidin (data not shown). Chromatograms of streptavidin obtained from other sources were qualitatively similar to that shown in Figure 1. Some peaks were present in streptavidin from more than one commercial source, albeit in varying ratios (data not shown).

Development of Anion Exchange Resolution of Streptavidin by Ligation Level. Biotin-free streptavidin (S-0) and biotin-saturated streptavidin (S-4; prepared as described under Experimental Procedures) were used to develop conditions for their separation on a Mono Q strong anion exchange column. Resolution was minimal at pH 8.6 (data not shown), but was excellent at pH 7.3 (Figure 2). The fastest flow rate and steepest salt gradient that did not seriously compromise the resolution at pH 7.3 were then chosen for further studies. Figure 2 also shows the homogeneity of S-0 and S-4.

The mechanism of the resolution was probed. S-0 elutes from the Mono Q column before S-4. A plausible mechanism is that biotin adds negative charge to the streptavidin and that the column is reading the resultant difference in charge between the species. This is consistent with the structure of biotin, the faster migration of S-4 in PAGE (Sano & Cantor, 1990a; Kurzban et al., 1991), and the lower isoelectric point of S-4 (Kurzban et al., 1991). Alternatively, the column might be reading conformational differences between S-0 and S-4. To address this distinction, streptavidin was saturated with an uncharged biotin analog, biotinamide, or with a positively charged analog, aminoethylbiotinamide. S-0 and the three biotin-saturated species each eluted as a single peak. The sequence of elution was the aminoethylbiotinamide–streptavidin (arrow marked AE in Figure 2), S-0, the biotinamide–streptavidin (marked BA in Figure 2), and then biotin–streptavidin (S-4).

The fluorescence emission spectra of the streptavidin that contained either of the analogs (before and after anion exchange chromatography) were similar to that of S-4, and the addition of biotin did not alter their fluorescence (data not shown). These data indicate that the dominant species in each case was at the S-4 ligation level. Thus, biotin and each analog were bound tightly during size exclusion and anion exchange chromatography.

The elution positions suggest that the charge of the ligand is the dominant factor in chromatography. Specifically, biotin binding resulted in far tighter binding to the column, but the neutral biotinamide caused only a small change. However, the positively charged aminoethylbiotinamide had only a small effect on elution position. This can be explained by two contrasting mechanisms. One possibility is that when only the positively charged analog binds, the total charge change for the protein is small due to changes in the pK_a's of protein groups and/or the ligand. Alternatively, conformational changes imposed by each ligand are different and alter details of the interactions of the protein with the column. The slight shift in elution position caused by the binding of the neutral biotinamide is consistent with some contribution of conformational information to the chromatography.

The mobility of the four samples was also compared by PAGE at pH 8.5 in a Tris–glycine buffer (Kurzban et al., 1991) (data not shown). The relative electrophoretic mobilities were similar to the relative elution positions: S-0 and the two forms saturated with the biotin analogs essentially coelectrophesed, but S-4 migrated substantially faster. The PAGE results are consistent with the conclusion that the Mono Q column is mostly reading the added negative charge of the biotin carboxylate. The lack of a significant change in electrophoretic mobility with aminoethylbiotinamide supports the idea that this ligand either loses its charge upon binding or causes a compensating change in the charge of the protein.

Redistribution of Bound Biotin. S-0 and S-4 were combined to an approximately 1:1 stoichiometry and then subjected to anion exchange chromatography. The chromatogram was identical to the sum of the S-0 and S-4 chromatograms shown in Figure 2 (data not shown). After 3 h at room temperature, two new peaks were observed that eluted between S-0 and S-4 (Figure 3A). Progressively, the S-0 and S-4 peaks declined, the new peaks increased, and an additional doublet accumulated in the middle of the profile (a 1 day incubation is shown in Figure 4). The distribution of streptavidin among the six peaks was the same after 2 (data not shown), 3 (Figure 3B), and 6 days (data not shown).

Control 6 day incubations of S-0 and S-4 each had unaltered chromatography (data not shown). The recovery of protein was generally quantitative, and there was no evidence for the selective loss of any species.

The six peaks were characterized. S-4 was prepared using [^{14}C]biotin and then combined with an equimolar amount of S-0. After 1 day, the sample was applied to the Mono Q column (Figure 4). Each peak (as identified in Figure 4) was characterized by the amounts of streptavidin and bound biotin (Table 1). The peaks from left to right are tetramers

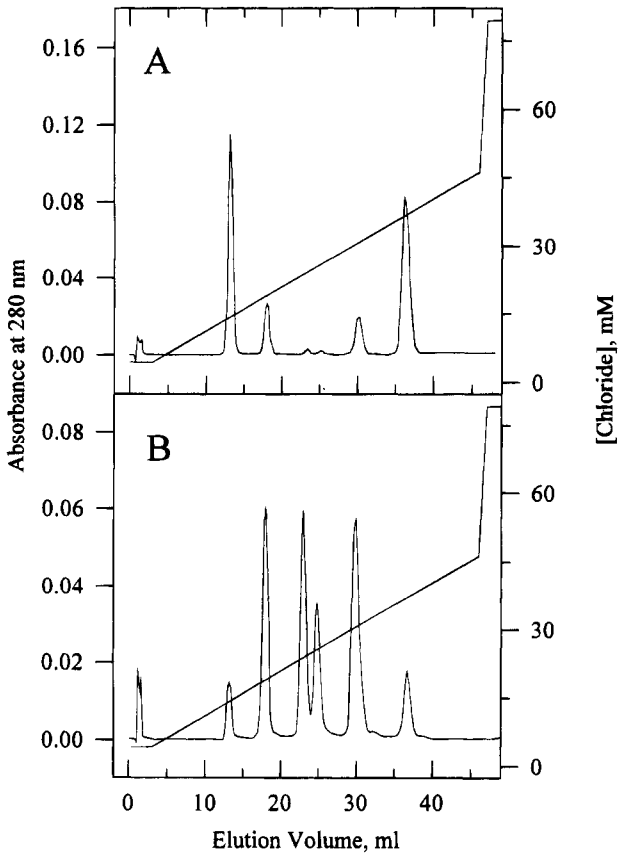

FIGURE 3: Redistribution of biotin produces intermediate ligation levels. S-0 and S-4 were combined in an approximately 1:1 stoichiometry. The final buffer composition was 0.1 M Tris Cl, (pH 8.48) and 3 mM NaCl. The sample was incubated at room temperature in the dark. (A) 3 h after mixing; (B) 3 days after mixing.

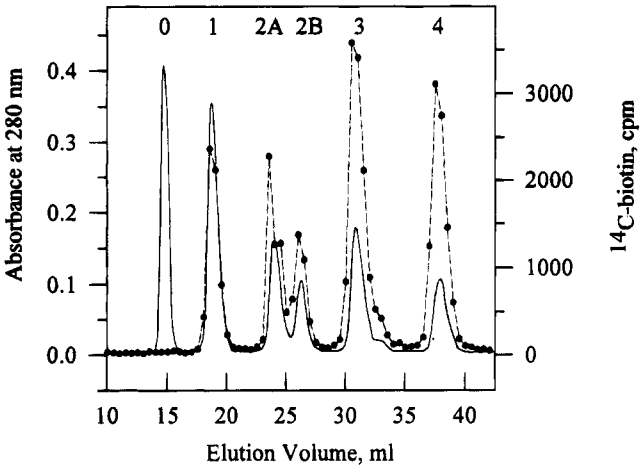

FIGURE 4: Characterization of the biotin content of streptavidin peaks. S-4 was prepared by using [^{14}C]biotin and incubated for 20 h at a 1:1 stoichiometry with S-0 prior to chromatography. The biotin content of each peak was determined by liquid scintillation counting (---•---). Note that the distribution of streptavidin is intermediate between that seen 3 h after mixing (Figure 3A) and that seen 2−6 days later (Figure 3B).

containing zero, one, two, two, three, and four molecules of bound biotin (Table 1, Figure 3) and are referred to as S-0, S-1, S-2A, S-2B, S-3, and S-4, respectively.

Additional properties are consistent with the peak assignments. Saturation with biotin decreases and blue shifts streptavidin's intrinsic tryptophan fluorescence (Kurzban et al., 1990). As the content of [^{14}C]biotin increased, fluorescence emissions progressively decreased (Table 1) and shifted to shorter wavelengths (data not shown), as expected for progressively higher biotin contents. Saturation with biotin made the emission spectra of all six species similar in both intensity and wavelength maxima. The fluorescence of S-4 was unaffected by biotin, as expected (data not shown). The number of available biotin sites was determined by titrating the intrinsic fluorescence with biotin (Table 1). The results are consistent with the peak identifications and indicate that all recovered species retain four functional biotin sites per tetramer.

Table 1: Properties of Resolved Forms of Streptavidin[a]

peak	bound biotin per tetramer	available biotin sites per tetramer	biotin-dependent decrease in fluorescence	amount (%)
0	0.003	4.00	2.05	5.3 ± 0.54
1	1.05	2.99	1.79	22.0 ± 0.42
2A	1.94	2.12	1.48	22.9 ± 0.21
2B	1.97	2.02	1.41	13.9 ± 1.37
3	2.91	1.00	1.22	27.2 ± 0.52
4	4.00	0	1.01	8.6 ± 0.83

[a] Peaks were recovered from the chromatogram shown in Figure 4 to determine biotin binding and fluorescence properties and are identified along the top of the figure. Bound biotin per tetramer was determined by quantifying the [^{14}C]biotin content by liquid scintillation counting and the streptavidin content by absorbance at 282 nm. Results have been normalized to a value of 4 for peak 4. Calculated stoichiometries were 5.2 for peak 4 and 5.0 for S-4 prepared by incubating S-0 in excess [^{14}C]biotin followed by size exclusion chromatography as described under Experimental Procedures. Available binding sites per tetramer were determined by titrating the intrinsic fluorescence with biotin, as described under Experimental Procedures. Stoichiometries have been normalized to a value of 4 for peak 0. Differences between the calculated stoichiometries (4.94 for peak 0 and 5 for concentrated, highly purified streptavidin recovered from the initial Q-Sepharose column) presumably reflect systematic errors in assigning concentrations of biotin stocks and/or in the extinction coefficient of streptavidin. The biotin-dependent decrease in fluorescence is the ratio of the intrinsic fluorescence at 360 nm before and after the biotin titration. The last column records the amount of each peak in the experiment described in Figure 3. Each chromatogram was enlarged on a copying machine, peaks were cut out, and the amount of each peak was assessed as being proportional to the mass of the paper. The percentage of streptavidin in each peak was calculated separately for each of the 2, 3 and 6 day time points and are reported as the mean and standard deviation of the three determinations.

Proof of Equilibrium. A steady state distribution of the five ligation levels was reached after 2 days when starting from a mixture of S-0 and S-4 (Figure 3, Table 1). To test whether this was an equilibrium biotin distribution, we allowed the system to approach this putative equilibrium from an opposed starting situation, homogeneous S-2A and homogeneous S-2B.

S-2A and S-2B had similar behavior. The S-1 and S-3 peaks were observed at early time points, and the S-0 and S-4 peaks accumulated later (data not shown). Chromatograms taken after 3 (data not shown) and 6 days (Figure 5) were indistinguishable, indicating that the two samples had achieved steady state biotin distributions. The distributions were highly similar to those seen when starting with a mixture of S-0 and S-4 (Figures 3 and 5). These results provide compelling evidence that the system had reached equilibrium.

Analysis of Cooperativity. If the equilibrium distribution of a ligand between multiple binding sites is known, then

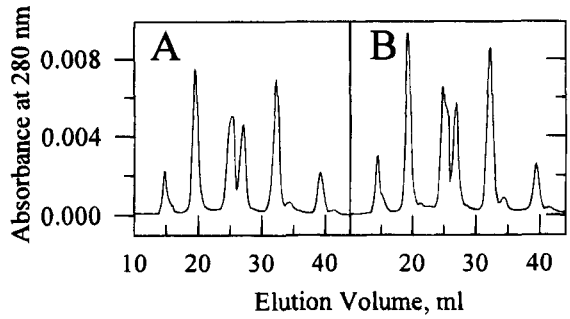

FIGURE 5: Reequilibration of biotin from streptavidin tetramers containing two molecules of biotin. S-2A (A) and S-2B (B) (labeled 2A and 2B, respectively, in Figure 4) were prepared as described under Experimental Procedures and then incubated at room temperature. Chromatography of 6 day incubates was as described for Figure 2.

Table 2: Relative Binding Affinity of Biotin to Streptavidin at Different Ligation Levels[a]

	dissociation constant	cooperativity	
Adair constant	formula	formula	$\Delta\Delta G$ (kcal mol^{-1})
k_1	$=4K_1 = 4\frac{[S\text{-}0][biotin]}{[S\text{-}1]}$	(reference state)	
k_2	$={}^3/_2K_2 = {}^3/_2\frac{[S\text{-}1][biotin]}{[S\text{-}2]}$	$-RT\ln\frac{8[S\text{-}0][S-2]}{3[S\text{-}1][S\text{-}1]}$	-0.04 ± 0.04
k_3	$={}^2/_3K_3 = {}^2/_3\frac{[S\text{-}2][biotin]}{[S\text{-}3]}$	$-RT\ln\frac{6[S\text{-}0][S-3]}{[S\text{-}1][S\text{-}2]}$	-0.04 ± 0.07
k_4	$={}^1/_4K_4 = {}^1/_4\frac{[S\text{-}3][biotin]}{[S\text{-}4]}$	$-RT\ln\frac{16[S\text{-}0][S-4]}{[S\text{-}1][S\text{-}3]}$	-0.12 ± 0.12

[a] Values of $\Delta\Delta G$ are the mean and standard deviation of the 2, 3, and 6 day determinations and are expressed relative to k_1. Positive cooperativity would be reflected in a negative value of $\Delta\Delta G$.

the intrinsic (microscopic) dissociation constants can be calculated. The multiple equilibria between biotin and streptavidin are as follows:

$$S{-}0 + B \overset{K_1}{\rightleftharpoons} S{-}1 + B \overset{K_2}{\rightleftharpoons} S{-}2 + B \overset{K_3}{\rightleftharpoons} S{-}3 + B \overset{K_4}{\rightleftharpoons} S{-}4$$

where K_1, K_2, K_3, and K_4 are the macroscopic dissociation constants and B is biotin. The intrinsic (Adair) dissociation constants are related to the macroscopic dissociation constants, as shown in Table 2 (Voet & Voet, 1990). Calculation of the intrinsic binding constants is not possible here because the concentration of free biotin is unknown. However, the intrinsic dissociation constants can still be compared by using the equilibrium distribution of ligation levels (Table 2).

The differences in affinity of biotin binding to S-0, S-1, S-2, and S-3 fall within a 0.12 kcal/mol range (see the last column of Table 2). For comparison, the positive cooperativity for oxygen binding to hemoglobin has $\Delta\Delta G = -8$ kcal mol^{-1} between k_1 and k_4 (Englander et al., 1992). Thus, binding affinity to streptavidin is essentially identical for each ligation species, and biotin binding is not cooperative.

DISCUSSION

Experimental Design and Validity. The anion exchange chromatography reported here derives from the separation of S-0 from S-4 by PAGE, as developed by Sano and Cantor (1990a). Isoelectric focusing can also separate S-0 and S-4 (Kurzban et al., 1991). These reports are consistent with the conclusion that the biotin carboxylate adds net negative charge to the streptavidin. We reasoned that ion exchange chromatography should also be able to separate streptavidin tetramers according to their biotin content. In principle, a charge-based technique should also be able to resolve streptavidin tetramers that contain intermediate amounts of bound biotin. Further, chromatography offers the ability to recover substantial amounts of each resolved species.

Experiments employing biotin analogs indicate that the anion exchange separation is indeed predominantly due to the added charge of the bound ligand. Streptavidin elutes earliest when it contains the positively charged aminoethylbiotinamide, later when it contains the uncharged biotinamide, and yet later when it contains the negatively charged biotin. Furthermore, PAGE and anion exchange chromatography gave similar results.

The positively charged aminoethylbiotinamide caused streptavidin to elute only slightly earlier than in the absence of any ligand. This is interpretable in terms of compensatory charge changes in either the analog or the protein upon binding this biotin analog. Fortunately, such compensatory changes did not compromise the studies performed here with biotin binding.

The quality of the anion exchange chromatography improved as the pH was decreased from 8.6 to 7.3, presumably because the total negative charge of the protein was reduced. With four Arg, four Lys, two His, five Glu, four Asp, and unblocked amino and carboxyl terminals, the net charge on core streptavidin in this pH range is likely to be between 0 and -3 per subunit. The addition of the biotin carboxylate thus could confer significant and highly pH-dependent changes in global charge.

The anion exchange chromatography allowed species at each ligation level to be recovered and analyzed. Recoveries were excellent. There is some disassociation of biotin during chromatography. For example, a small peak of S-3 is seen in chromatograms of S-4 (Figure 2). Another concern is that biotin might accumulate on the column and then reassociate with streptavidin during that chromatography or during a subsequent application. This does not appear to be a problem. Only small amounts of S-1 are observed in chromatograms of S-0 (*e.g.*, Figure 2). Further, since streptavidin was either dialyzed or subjected to size exclusion chromatography prior to the ion exchange chromatography, the only significant source of free biotin was the dissociation of biotin during chromatography.

Biotin Binds to Streptavidin without Cooperativity. Coincubation of S-0 and S-4 produced four new peaks that elute between those of S-0 and S-4 (Figure 3). These peaks contain one, two, or three molecules of biotin per tetramer (Figure 4 and Table 1). Elution positions and the time course are consistent with the peak identifications. While the identity of the new peaks is clear, the mechanism by which biotin redistributes is not known. Biotin may dissociate from intact tetramers and reassociate to other tetramers. Alternatively, redistribution may involve subunit dissociation and reassociation. Our lack of information concerning how biotin redistributes, however, is of no consequence to the question of cooperativity so long as conclusions are derived from considerations of equilibrium thermodynamics.

The redistribution of biotin between S-0 and S-4 reached a steady state after 2 days. Similar distributions were observed when the incubations started with either of the two

peaks at the S-2 ligation level. That is, the final biotin distribution was independent both of time and history. We therefore conclude that the system achieved an equilibrium biotin distribution.

Quantification of this equilibrium indicates that the binding energy is very similar for S-0, S-1, the summed behavior of the S-2 peaks, and S-3 (Table 2). Thus, there is no indication of cooperativity in biotin binding.

Two Peaks at the S-2 Ligation Level. Why are there two peaks of S-2? There are three topologically unique biotin distributions for S-2, but only one for each of the other ligation levels. A simple explanation is that all three forms of S-2 are present and that the Mono Q column resolves them into two peaks. This is consistent with the absence of cooperativity, such that the three forms should be equally populated, resulting in an overall peak ratio of 2:1, which is similar to that seen experimentally.

Previous Reports Concerning Cooperativity. Green (1964, 1975) concluded from studies of the quenching of the fluorescence of avidin by dinitrophenylbiotin that binding was random. Green also prepared avidin with an average of 1, 2, or 3 equiv of bound biotin/tetramer and showed that the enthalpy change per subsequently added biotin was the same for all samples, which is consistent with the absence of cooperativity (Green & Melamed, 1966). Chignell et al. (1975) concluded from studies of the interactions of nitroxide free radical biotin analogs with avidin that the initial binding was random, but that rearrangements placed probes preferentially adjacent to vacant sites.

The cooperativity of streptavidin was studied by Sano and Cantor (1990a). They established that S-0 and S-4 could be separated by PAGE. The addition of one, two, or three molecules of biotin per tetramer produced only species that coelectrophesed with the biotin-free and biotin-saturated forms. They concluded that the absence of intermediate ligation levels was due to positive cooperativity.

The tight binding of biotin complicates analysis of all previous reports. Rapid association and subsequent slow dissociation can result in stable, nonequilibrium distributions of the ligand. Consider events upon adding biotin to an excess of (strept)avidin with relatively slow mixing. Tetramers near the point of addition will see an excess of biotin and should become saturated rapidly. As mixing proceeds, additional (strept)avidin will come into contact with a local excess of biotin and should also become saturated. When the biotin has mixed with a stoichiometric amount of the (strept)avidin, the population will contain a mixture of biotin-free and biotin-saturated tetramers, and the concentration of free biotin will be very low. Because dissociation is slow, the system should now be trapped. If the biotin distribution is examined, then positive cooperativity may be deduced from the high levels of biotin-free and biotin-saturated species, even if cooperativity is absent. Alternatively, if different initial stoichiometries of biotin are compared, then subsequent additions of biotin will produce identical physical changes in the system, even if the system is markedly cooperative.

This trapping artifact may account for Sano and Cantor's observation (Sano & Cantor, 1990a) that biotin initially distributes into only two electrophoretically distinguishable species, one of which contains biotin. However, additional caveats may be relevant. First, there is no evidence of whether the electrophoretic analysis can detect intermediate ligation levels. Thus, while much of the streptavidin accumulated as S-0, the presence and amount of S-1, S-2, and S-3 in their study cannot be stated. Second, the likelihood of the trapping artifact depends upon three parameters that are unknown under the given set of experimental conditions: the rate of mixing of biotin with streptavidin and the rates of biotin association and dissociation. Third, as demonstrated herein, streptavidin is heterogeneous. The two studies may then have employed different forms of streptavidin, with an unknown effect upon the system's behavior.

It is also plausible that streptavidin has positive cooperativity at the time of the addition of biotin to S-0, despite our unambiguous finding of noncooperativity at later times. Biotin-free streptavidin may be in a conformational state that is characterized by positive cooperativity. Biotin binding may then drive conformational changes (Weber et al., 1989) that ultimately allow the system to access an alternate, noncooperative state. Relevant to this possibility is that subunit exchange may be significant during the long experimental time frame.

Rationale and Implications of the Absence of Cooperativity. Biotin binds to streptavidin without apparent cooperativity, despite the large binding energy and the known conformational changes imposed by binding. The recently reported crystal structures of avidin (Livnah et al., 1993; Pugliese et al., 1993, 1994) show that the functional similarities between avidin and streptavidin are accompanied by a high level of structural homology. The absence of cooperativity in avidin is thus likely. The protocols reported here for streptavidin in principle can be adapted to avidin, although the tighter binding of biotin to avidin and avidin's high isoelectric point [p$I \cong 10$ (Woolley & Longsworth, 1942)] may present technical difficulties.

The absence of cooperativity can be rationalized according to streptavidin's biological function. In order to deprive competing organisms of free biotin, streptavidin needs to be present in excess of ligand. As a result, the lower ligation levels are likely to be important. In this situation, positive cooperativity would arguably be detrimental. Similar reasoning can be applied to hen egg white avidin.

In the absence of cooperativity, why are these proteins tetramers? The primary drive in their evolution presumably was to develop tight biotin binding. Even a monomeric β-barrel progenitor with weak binding could have supported the antimicrobial function. The quaternary structure then could have developed so as to allow Trp 120 to participate in biotin binding by a quaternary contact within the binding sites (Hendrickson et al., 1989; Livnah et al., 1993; Pugliese et al., 1993; Weber et al., 1989). Modification of Trp 120 of streptavidin (Gitlin et al., 1988b) or the homologous Trp 110 of avidin (Gitlin et al., 1988a) with Koshland's reagent eliminates biotin binding. However, the contribution of unmodified Trp 120 to the overall affinity is not known.

A second proposed role for the quaternary structure is to stabilize the global structure. Stabilization could improve binding by rigidifying binding sites and contribute to the remarkable resistance of avidin (Green, 1975) and streptavidin (Kurzban et al., 1991) to denaturation, thereby improving function and longevity under a variety of extracellular conditions. Evolution even to a dimer might have been critical, since one pair of quaternary interfaces is more extensive than the other two pairs (Hendrickson et al., 1989;

Livnah et al., 1993; Pugliese et al., 1993; Weber et al., 1989). The interface that allows for the quaternary Trp contact is also substantial and could also contribute to global stabilization. An alternative evolutionary scheme has similar principles, but starts from a multimeric β-barrel progenitor, which then evolved biotin binding capability.

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