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Site-directed mutagenesis studies of the high-affinity streptavidin–biotin complex: Contributions of tryptophan residues 79, 108, and 120

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ABSTRACT **We report the functional characterization of site-directed biotin binding-site mutants of recombinant core streptavidin. The mutagenesis studies were aimed at characterizing the contributions of Trp residues known to contact biotin that have been postulated to control the exceptional binding affinity observed in this system. The functional properties of single site-directed mutants replacing Trp residues with Phe or Ala at positions 79, 108, and 120 were investigated by quantitating the EC_{50} binding parameters of these mutants to biotin and 2-iminobiotin in an ELISA format. The biotin EC_{50} for all mutants was the same as wild-type streptavidin, demonstrating that their ΔK_a values relative to wild type were $<10^6$. The conservative W79F and W108F mutants displayed only a 2- to 3-fold increase in EC_{50} for 2-iminobiotin, corresponding to an estimated ΔK_a <10, while the W120F mutant displayed a much greater alteration in 2-iminobiotin EC_{50}, corresponding to an estimated ΔK_a of 10^2. These ΔK_a values are likely to reflect similar changes for biotin. The 2-iminobiotin EC_{50} values for the Ala mutants fell outside the accessible concentration range of the ELISA assay, demonstrating that these mutations lowered the K_a by a factor of 10^4 to 10^6. Direct estimation of biotin K_a values for W79A, W120A, and W120F in an ultrafiltration binding assay yielded K_a values of $4.3 \times 10^7\ M^{-1}$, $8.6 \times 10^6\ M^{-1}$, and $>5 \times 10^9\ M^{-1}$, respectively, in excellent agreement with the ELISA estimates of ΔK_a with 2-iminobiotin as a reporter ligand. The results of these preliminary functional studies suggest that these aromatic side chains contribute significantly to the streptavidin–biotin binding free energy.**

Streptavidin is a tetrameric protein that binds biotin with an affinity that is among the highest displayed for noncovalent interactions between a ligand and protein ($K_a \approx 10^{13}\ M^{-1}$) (1). Both streptavidin and the homologous protein avidin have been studied as paradigms of strong ligand–protein interactions (2–10). In particular, the x-ray crystallographic studies of streptavidin by Weber *et al.* (9, 10) and Hendrickson *et al.* (11, 12) have provided considerable insight into the structural origins of the high affinity of the biotin–streptavidin system. The structure–function origins of this unusually high-affinity interaction, however, have yet to be elucidated. Streptavidin displays a number of commonly observed molecular recognition motifs in the interaction with biotin: these include hydrophobic and van der Waals dispersive interactions that are largely mediated by the aromatic side chains of Trp residues (13–18), hydrogen bonding networks mediated by donor/acceptor side chains (19–22), and disorder-to-order transitions mediated by the ordering of surface polypeptide loops upon ligand binding (23–30). Miyamoto and Kollman (31, 32) have reported a computational study that emphasizes the importance of van der Waals and hydrophobic attractions between ligand and protein, as first postulated by Green (5). Their results suggest that these interactions contribute ≈18 kcal/mol (1 cal = 4.184 J) to the absolute free energy of binding, while the electrostatic energy term (which includes hydrogen bonding interactions) contributes only ≈3 kcal/mol. In this communication, we report the design and construction of a synthetic gene for core streptavidin and initial site-directed mutagenesis studies aimed at characterizing the role of three of the Trp residues in the biotin-binding site. We have initially determined the concentration-dependent biotin and 2-iminobiotin binding isotherms of W79A/F, 108A/F, and 120A/F mutants in an ELISA assay and, independently, estimated the streptavidin–biotin K_a for the W79A, W120A, and W120F mutants. The dissection of the factors governing this interaction should provide insight into the molecular requirements for high-affinity drug design. In addition, the generation of a library of streptavidin mutants spanning a range of affinities for biotin and its derivatives will improve upon existing biotechnological applications for this widely used system (33).

METHODS

Design and Construction of Synthetic Gene. The program GCG (34) was used to generate the designed synthetic gene for core streptavidin (35), which is similar to one reported by Thompson and Weber (36). The gene incorporates favorable *Escherichia coli* codon usage (37), a consensus ribosome binding site (38), an initiating methionine codon, translational stop codons, and a number of unique restriction endonuclease recognition sites. The core streptavidin gene was constructed in three segments flanked by the following restriction endonuclease recognition sites: *Eco*RI/*Xba* I, *Xba* I/*Hin*cII, and *Hin*cII/*Hin*dIII. Individual oligodeoxyribonucleotides were 5′-phosphorylated, annealed, and ligated into a pUC18 plasmid that had been linearized with the appropriate pair of restriction endonucleases. The three DNA segments making up the core streptavidin gene were isolated and ligated into a single pUC18 plasmid. The entire gene was subsequently sequenced to confirm the nucleotide sequence.

Site-Directed Mutagenesis of Core Streptavidin. W79A, W79F, W108A, and W108F site-directed mutants of wild-type (WT) streptavidin were created by PCR mutagenesis (39). W120A and W120F were created by cassette mutagenesis. Oligodeoxyribonucleotides spanning the *Mlu* I–*Hin*dIII endonuclease restriction sites with degenerate codons at residue 120 incorporating the Trp → Ala/Phe mutations were annealed and ligated into *Mlu* I/*Hin*dIII-linearized pUC18 containing the core streptavidin gene. DNA sequencing was used to identify clones containing either the Phe or Ala mutation.

Expression of Streptavidin and Mutants in *E. coli*. Expression protocols were slightly modified from those reported by Sano and Cantor (40) for the cloned streptavidin gene. BL21(DE3) (Novagen) cells harboring the streptavidin gene in

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Abbreviations: WT, wild type; BSA, bovine serum albumin.
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pET-21a were cultured overnight at 37°C. The cell pellet was subsequently resuspended in 10 ml of fresh Luria–Bertani medium and used to inoculate 6.5 liters of 2× YT medium supplemented with ampicillin (100 μg/ml) in shaker flasks. The culture was incubated with shaking at 37°C until the OD_{600} reached 1.0, at which point isopropyl β-D-thiogalactoside was added (1 mM) to induce protein expression. Cells were cultured for a further 3 h, after which they were harvested by centrifuging at 4500 × *g* for 10 min.

Isolation and Purification of Expressed Streptavidin and Mutants. The isolation and refolding protocols were modified from Sano and Cantor (40). The cell pellet was resuspended in 200 ml of 50 mM Tris·HCl, pH 8.0/0.75 M sucrose/1 mM phenylmethylsulfonyl fluoride and ruptured by sonication. The lysed cells were incubated for 15 min with DNase I (10 μg/ml)/RNase A (10 μg/ml)/$MgCl_2$ (10 mM) and centrifuged at 22,000 × *g* for 30 min. The insoluble fraction was washed with 50 mM Tris·HCl, pH 8.0/10 mM EDTA/1.5 M NaCl/1 mM phenylmethylsulfonyl fluoride/0.5% Triton X-100 and then washed four times without the Triton X-100 in the buffer. The inclusion bodies were dissolved in 6 M guanidine hydrochloride (500 ml, pH 1.5) to a concentration ≤50 μM (streptavidin monomer) and dialyzed against 20 liters of 50 mM Tris·HCl, pH 8.0/150 mM NaCl/10 mM EDTA/0.1 mM phenylmethylsulfonyl fluoride/0.5 mM benzamidine hydrochloride for 24 h with one 20-liter change of dialysis buffer. The dialysate was centrifuged, vacuum-filtered through filters (0.45 μm, pore size), and concentrated in a stirred ultrafiltration cell (Amicon).

WT streptavidin and the Trp → Phe mutants were purified by affinity chromatography with iminobiotin-agarose (Pierce) (41, 42). The lowered affinity of the Trp → Ala mutants for iminobiotin precluded its use. Instead, the samples were applied to a DEAE-Sepharose FF column (1.5 × 5 cm) (Pharmacia) equilibrated with 20 mM Tris·HCl (pH 7.0). Under these conditions, streptavidin does not bind to the column and is eluted in the void volume. The streptavidin-containing fractions were pooled, concentrated, equilibrated in 20 mM Tris (pH 8.5), and passed over a DEAE-Sepharose FF column (1.5 × 5 cm) equilibrated in the same buffer. Streptavidin was then eluted by applying a linear 0–0.3 M NaCl gradient.

Characterization of Streptavidin and Mutants. SDS/PAGE analysis used precast Mini-Protean 10–20% gradient gels (Bio-Rad) with a discontinuous buffer system (43). Native PAGE was performed by omitting SDS in the sample application buffer and the gel running buffer and omitting the heat denaturation of proteins before electrophoresis. The concentration of WT streptavidin was determined by using an extinction coefficient (ε_{280}) of 34,000 $M^{-1}\cdot cm^{-1}$ for the subunit (40). Concentrations of the mutants were determined by the method of Gill and von Hippel (44) with the ε_{280} of WT streptavidin as reference. Protein electrospray mass spectrometry was carried out on an API-III electrospray mass spectrometer (PE Sciex, Thornhill, Ontario, Canada). The biotin-binding stoichiometry of WT streptavidin and mutants were determined in solution by the quantitative quenching in the fluorescence of fluorescein-biotin (Molecular Probes) upon titration with protein (A.C. and P.S.S., unpublished results).

ELISA Assays. A modified version of the assay reported by Bayer *et al.* (45) was used to quantitate the binding of biotin and 2-iminobiotin. Biotin/bovine serum albumin (BSA) (Pierce) or iminobiotin/BSA at 10 μg/ml was adsorbed to microtiter plates overnight at 4°C in 15 mM Na_2CO_3 (pH 9.6). Microtiter plates were incubated with blocking buffer at pH 8.0 or pH 10.0 (50 mM sodium phosphate, pH 8.0 or 50 mM Na_2CO_3, pH 10.0/100 mM NaCl/0.5% BSA/0.05% Tween 20) for at least 2 h at room temperature and then incubated in serial 1:10 dilutions of WT streptavidin or mutants starting at 100 μg/ml for 2 h at room temperature, rinsed with blocking buffer at pH 8.0 or pH 10.0, and incubated for 1 h at room temperature in a 1:2000 dilution of primary anti-streptavidin antibodies (Sigma) in blocking buffer at pH 8.0 or pH 10.0. The plates were rinsed thrice with blocking buffer, incubated for 1 h at room temperature in a 1:20,000 dilution of secondary anti-IgG alkaline phosphatase conjugate (Sigma) in blocking buffer, rinsed thrice with blocking buffer, and assayed for alkaline phosphatase activity at pH 10.0. Every plate assayed had triplicates for each protein concentration. The data were processed on MATHCAD (Mathworks) to determine the EC_{50} values by using a published four-parameter nonlinear fitting algorithm (46).

Equilibrium Binding of Biotin. [^{3}H]Biotin at 2 nM (WT, W79A, or W120A) or 0.2 nM (WT or W120F) was incubated in aliquots of serially diluted protein for 2 h. The free ligand was then separated from the protein-bound ligand by either Microcon-30 or Centriprep-30 centrifugal ultrafiltration devices (Amicon). Typically, 0.1–1 ml of the free ligand solution or the protein–ligand mixture was added to 18 ml of liquid scintillation solution (Ecolume, ICN) and assayed on a liquid scintillation counter (Beckman). The K_a was determined from a nonlinear curve fit of the fraction of protein-bound ligand vs. the free protein concentration.

RESULTS

Amino acid compositional analysis and N-terminal sequencing of refolded affinity-purified WT streptavidin agreed with the calculated composition and sequence of core streptavidin. SDS/PAGE of heat-denatured protein showed that the monomers for each Trp mutant are the same size as WT streptavidin monomers and that the purification methods employed for WT streptavidin and the Trp mutants yield homogeneous protein. Electrospray mass spectrometry of WT streptavidin agreed with the calculated mass to within 2 Da. The mass difference between WT streptavidin and the Trp mutants demonstrated that the mutation is the desired one in all cases. The similar rates of migration of WT streptavidin and the Trp mutants in native PAGE demonstrate that the refolded mutants form native tetramers in solution (results not shown). The binding stoichiometry of fluorescein-biotin to WT streptavidin and all of the Trp mutants ranged from 0.85 to 1.1 (on a subunit basis), within experimental error of the ratio of 1.0 predicted for binding of one biotin per streptavidin subunit.

With biotin as the ligand, the concentration-dependent binding isotherms of WT streptavidin and all Trp mutants are identical in the ELISA assay at both pH 8.0 and 10 (Fig. 1*A* shows the binding isotherm of WT streptavidin and the W79A/F mutants). However, with iminobiotin/BSA as the ligand (Fig. 1*B* for WT streptavidin and the W79A/F mutants), the binding isotherms indicate marked differences in the affinities of WT streptavidin and the mutants, with the EC_{50} values in the order WT < Phe < Ala. The absolute and relative EC_{50} for the binding of the WT streptavidin and the Trp mutants to iminobiotin, at pH 8.0 and 10.0, are summarized in Table 1 and Fig. 2.

These experimental results suggested that the Ala mutants should be experimentally accessible in equilibrium biotin binding assays. A spin-column assay with a 30-kDa cutoff filtration membrane was used to quantitate the partitioning of [^{3}H]biotin between the free and bound states. While this assay is not strictly an equilibrium technique because it requires centrifugation to separate the free ligand, it does provide a useful estimate of the K_a when the affinities lie between 10^6 and 10^9 M^{-1}. The concentration-dependent binding isotherm for W120F was at the tight-binding limit ($K_a > 10^9$ M^{-1}) at the lowest experimentally accessible total biotin concentration (0.2 nM) and identical to WT streptavidin (results not shown). While the W108A mutant protein proved to be unstable in this assay at the necessary concentrations, the W79A and W120A mutants yielded complete concentration-dependent binding

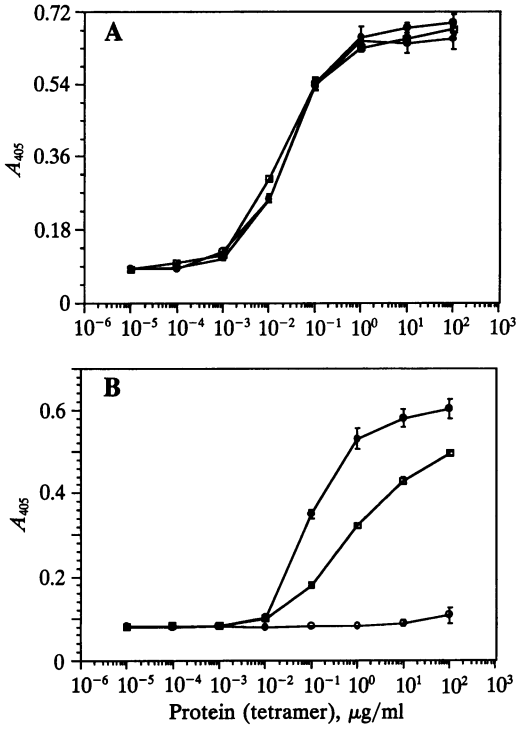

FIG. 1. Concentration-dependent binding of WT streptavidin, W79A, and W79F to biotin/BSA at pH 10.0 (*A*) and 2-iminobiotin/BSA at pH 10.0 (*B*) measured in an ELISA. ●, WT streptavidin; □, W79F; ○, W79A.

isotherms from which the K_a was determined to be 4.3×10^7 M^{-1} and 8.6×10^6 M^{-1}, respectively. These experimentally determined K_a estimates provide independent confirmation of the ΔK_a values estimated from the ELISA assays as discussed below.

The important observations from the ELISA assays are summarized as follows: (*i*) With biotin as the ligand, no differences are observed in the ELISA binding isotherms and, consequently, between the EC_{50} values of WT streptavidin and the Trp mutants. (*ii*) With iminobiotin as the ligand, differences are observed in the binding behavior of the Trp mutants with respect to WT streptavidin. The relative EC_{50} values of the mutants follow the trend: Ala > Phe > Trp (WT). The

Table 1. EC_{50} results for binding of WT streptavidin and Trp mutants by 2-iminobiotin at pH 8.0 and pH 10.0

Protein	pH	Absolute EC_{50}, μg/ml	Relative EC_{50} (mutant/WT)
WT*	8/10	0.03 ± 0.02	1.0
WT	8	0.046 ± 0.03	1.0
WT	10	0.052 ± 0.013	1.0
WxA†	8/10	≥100	≥2000
W79F	8	8.27 ± 3.01	180
W79F	10	0.14 ± 0.01	2.7
W108F	8	9.6 ± 0.74	209
W108F	10	0.12 ± 0.07	2.3
W120F	8	10.27 ± 0.5	223
W120F	10	4.03 ± 1.91	78

Absolute EC_{50} values were derived from the nonlinear least squares fit of at least three measurements of the binding isotherm. The relative EC_{50} values are normalized to the average (absolute) EC_{50} of WT streptavidin at pH 8 or pH 10.

*Biotin is the ligand. Six isotherms of WT binding to biotin/BSA were fitted to determine the EC_{50} value.

†The complete binding isotherm of the Trp → Ala mutants could not be determined, leading to a lower bound of their EC_{50} values.

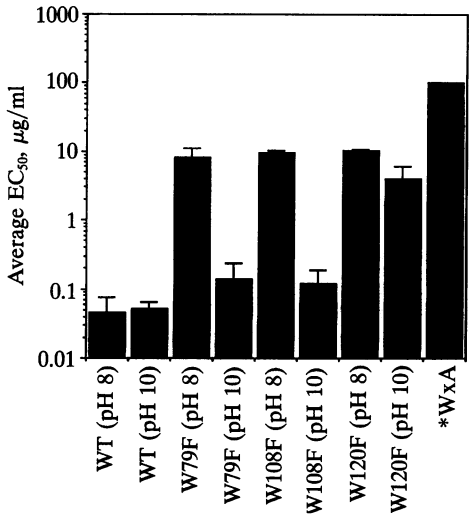

FIG. 2. Average EC_{50} values for the binding of WT streptavidin and Trp mutants to iminobiotin, at pH 8.0 and 10.0. Each mutant with its assay pH is identified. The asterisk on WxA indicates that the reported EC_{50} value is a lower bound.

binding of the Ala mutants to iminobiotin is weakened to the extent that a complete ELISA binding isotherm cannot be obtained. (*iii*) The Phe mutants display pH-sensitive binding behavior when iminobiotin is the ligand; i.e., the EC_{50} at pH 8.0 > EC_{50} at pH 10.0, consistent with the inverse pH dependence in the affinity of iminobiotin for streptavidin (41, 42). W120F displays the largest EC_{50} of the Phe mutants at pH 10 (relative $EC_{50} \approx 10^2$), while the relative EC_{50} of W79F and W108F is only 2–3 at pH 10.0. W120F also displays weaker pH dependence in binding iminobiotin than W79F or W108F; thus, the EC_{50} values of all Trp → Phe mutants are approximately similar at pH 8 (relative $EC_{50} \approx 10^2$), despite the two orders of magnitude greater EC_{50} of W120F at pH 10 compared to W79F and W108F. (*iv*) The absolute EC_{50} values for the binding of WT streptavidin to both biotin and iminobiotin (at both pH 8.0 and 10.0) are similar, indicating that a $K_a < 10^7$ M^{-1} is necessary to observe differences with this assay.

DISCUSSION

Order of magnitude determinations of ΔK_a for the mutants can be estimated from the EC_{50}, given the known K_a values of some of these ligand–protein partners. The subsequent analysis is based on the experimentally determined K_a of 2.5×10^{13} M^{-1} for biotin–WT streptavidin (1) and the K_a of $\approx 10^8$ M^{-1} for iminobiotin–WT streptavidin at pH 10.0, estimated from the experimentally determined value for iminobiotin–avidin (2). These numbers are further supported by the independently determined biotin-binding affinities of the W79A and W120A mutants, which agree well with the ELISA estimates.

Our ELISA assay is insensitive to the ΔK_a in ligand–protein binding when the K_a values are higher than $\approx 10^7$ M^{-1}; i.e., the ELISA binding isotherms for ligand–protein partners are indistinguishable when the K_a values are in the range of 10^7 to 10^{13} M^{-1}. This assumption is supported by the following observations: the experimentally measured EC_{50} for WT streptavidin–biotin is identical to the EC_{50} of WT streptavidin–iminobiotin at pH 10.0, despite the marked differences in the K_a of WT streptavidin–biotin (2.5×10^{13} M^{-1}) and of iminobiotin–WT streptavidin at pH 10.0 ($\approx 10^8$ M^{-1}). The similar EC_{50} values for these two ligands suggest that ELISA is insensitive to ΔK_a in the range of 10^8 to 10^{13} M^{-1}. Furthermore, the binding of WT streptavidin by iminobiotin is pH-sensitive, and the K_a of WT streptavidin–iminobiotin is likely to be an order of magnitude lower at pH 8.0 than at pH 10.0, as is the

experimentally determined ΔK_a for the closely related iminobiotin–avidin system. Thus, an upper limit of 10^7 M^{-1} for the sensitivity of ELISA toward ligand K_a can be established. The similar biotin EC_{50} values measured for WT streptavidin and all of the Trp mutants similarly suggest that the K_a values of the Trp mutants for biotin are in the range of 10^7 to 10^{13} M^{-1}.

However, when the ligand affinity is lowered into the accessible range of this ELISA assay by using iminobiotin as the ligand, marked changes in the EC_{50} values are observed for the Trp mutants. The relative EC_{50} results for the Trp mutants are summarized in Fig. 2 and illustrate their increased iminobiotin EC_{50} values. W79F and W108F display 2- to 3-fold greater EC_{50} values at pH 10.0 compared to WT streptavidin; when the assay pH is lowered to 8.0, their EC_{50} values dramatically increase by two orders of magnitude, consistent with the pH-dependent decrease in the affinity of iminobiotin for streptavidin. These results suggest that the affinity of W79F and W108F mutants for iminobiotin is less than one order of magnitude lower than WT streptavidin at pH 10.0 ($K_a \approx 10^7$ to 10^8 M^{-1}). If the assay pH is lowered to 8.0, the K_a decreases further to $\approx 10^6$ M^{-1}. W120F displays a significantly lower affinity for iminobiotin at pH 10.0 ($K_a \approx 10^6$ to 10^7 M^{-1}), compared to W79F and W108F, as shown by its 100-fold greater relative EC_{50} at pH 10.0, and a weaker pH dependence, shown by the smaller increase in relative EC_{50} at pH 8.0 (relative $EC_{50} \approx 200$ at pH 8).

The Trp → Ala mutation results in larger changes in 2-iminobiotin binding affinities, so that only a lower bound of their EC_{50} values can be estimated (relative $EC_{50} \geq 2000$). The EC_{50} values for the Trp → Ala mutants bound to iminobiotin are probably overestimated by the disproportionate loss of the bound protein during multiple washing steps in ELISA. Since the Ala mutants display a biotin-binding isotherm that is indistinguishable from that of WT streptavidin, which sets a maximum ΔK_a, we can estimate that the ΔK_a values of the Ala mutants fall between 10^4 and 10^6. This result is supported by the direct estimation of K_a for the W79A and W120A mutants, which places these affinities at 10^7 M^{-1}.

Significant contributions of Trp residues to binding affinity have been demonstrated in antibody–antigen interactions (e.g., ref. 47), cyclophilin–cyclosporin A interactions (16), and in human growth hormone–growth hormone receptor interactions (48). The crystal structure of streptavidin reveals that Trp-79 and Trp-108 line the biotin-binding site, with Trp-79 primarily interacting with the valeric acid side chain and Trp-108 interacting with the bicyclic ring system of biotin (Fig. 3). Trp-120 is contributed by the adjacent subunit and "caps" the tetrahydrothiophenic ring. The large decrease in biotin-binding free energy for all three Ala site-directed mutants relative to WT streptavidin implies a crucial role for these van der Waals contacts in generating the high biotin-binding affinity of streptavidin. Further characterization of multiple-site mutants will be required to determine what proportion of these apparent binding free energy reductions can be assigned directly to side-chain binding contact interactions. It is interesting that the conservative W120F mutant displays a significantly reduced binding affinity relative to W79F and W108F. This result could point to a crucial role for tryptophan at this position in regulating the exceptionally slow dissociation rate of the biotin–streptavidin complex.

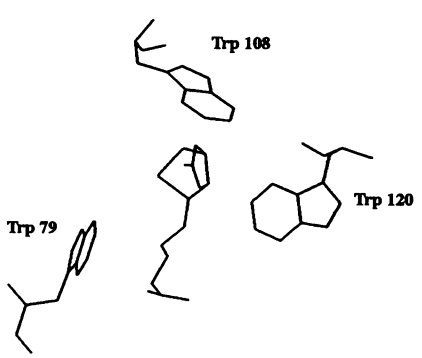

FIG. 3. Line drawing of the biotin-binding site showing the spatial relationships between biotin and Trp-79, Trp-108, and Trp-120 (which is contributed from the adjacent subunit).

The analysis presented here assumes that the EC_{50} differences for iminobiotin reflect similar differences for biotin. This assumption is supported by the calculations of Miyamoto and Kollman (31, 32), which suggest that the difference in the absolute free energies of binding for the two ligands is largely due to the differences in the solvation energies of the two ligands rather than large differences in ligand–protein interaction free energies. The ΔK_a estimates from the 2-iminobiotin ELISA binding isotherms are closely corroborated by the direct estimates of the biotin K_a for the W79A, W120A, and W120F mutants. Our results thus suggest that iminobiotin is likely to be a good reporter for intrinsic streptavidin–biotin interactions, consistent with the fact that the Trp residues do not directly interact with the structurally altered ureido moiety.

In summary, our initial mutagenesis work is consistent with the hypothesis that van der Waals and hydrophobic interactions, largely mediated by the aromatic side chains of Trp residues, are an important determinant of the binding free energy in the streptavidin–biotin system. These results suggest that the contribution of the Trp side chains to the absolute free energy of biotin binding are substantial, although the ΔK_a values measured here could well include some interaction free energy contributions. These functional interpretations are also dependent on the retention of protein structure and solvent interactions with the mutant proteins.

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