UV Resonance Raman Study of Streptavidin Binding of Biotin and 2-Iminobiotin: Comparison with Avidin

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Received 30 January 2001; revised 19 June 2001; accepted 19 June 2001

ABSTRACT: UV resonance Raman (UVRR) spectroscopy is used to study the binding of biotin and 2-iminobiotin by streptavidin, and the results are compared to those previously obtained from the avidin–biotin complex and new data from the avidin–2-iminobiotin complex. UVRR difference spectroscopy using 244-nm excitation reveals changes to the tyrosine (Tyr) and tryptophan (Trp) residues of both proteins upon complex formation. Avidin has four Trp and only one Tyr residue, while streptavidin has eight Trp and six Tyr residues. The spectral changes observed in streptavidin upon the addition of biotin are similar to those observed for avidin. However, the intensity enhancements observed for the streptavidin Trp Raman bands are less than those observed with avidin. The changes observed in the streptavidin Tyr bands are similar to those observed for avidin and are assigned exclusively to the binding site Tyr 43 residue. The Trp and Tyr band changes are due to the exclusion of water and addition of biotin, resulting in a more hydrophobic environment for the binding site residues. The addition of 2-iminobiotin results in spectral changes to both the streptavidin and avidin Trp bands that are very similar to those observed upon the addition of biotin in each protein. The changes to the Tyr bands are very different than those observed with the addition of biotin, and similar spectral changes are observed in both streptavidin and avidin. This is attributable to hydrogen bond changes to the binding site Tyr residue in each protein, and the similar Tyr difference features in both proteins supports the exclusive assignment of the streptavidin Tyr difference features to the binding site Tyr 43. © 2001 John Wiley & Sons, Inc. Biopolymers (Biospectroscopy) 62: 307–314, 2001

Keywords: UV resonance Raman; streptavidin; biotin; 2-iminobiotin; avidin

INTRODUCTION

Streptavidin is a bacterial, tetrameric protein that binds biotin extremely tightly and has a structural motif similar to that of avidin. This exceptionally strong binding affinity was the object of fundamental studies of protein–substrate interactions and the basis of numerous biotechnological applications.[1,2] The binding of biotin to avidin and streptavidin has three major components: hydrophobic interactions, especially with tryptophan (Trp); hydrophilic interactions via an extensive complimentary hydrogen bonding network; and the closure of a flexible loop around the substrate.[3] Despite the similarities between streptavidin and avidin, there are important differences between these proteins. Avidin is a glycoprotein that contains one disulfide bridge and two methionine residues whereas streptavidin is

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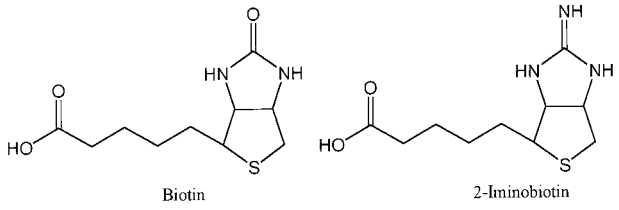
Biopolymers (Biospectroscopy), Vol. 62, 307–314 (2001)

nonglycosylated and has no sulfur-containing residues. These differences make streptavidin more easily manipulated using recombinant DNA technology, and the X-ray structure of the wild-type protein[4,5] allows rational design of mutants to investigate the various components of biotin binding.[6,7] For example, Trp to phenylalanine (Phe) site directed mutants revealed the importance of hydrophobic interactions with biotin,[8–10] and the role of hydrogen bonds to biotin in the binding pocket was addressed by various mutant protein studies.[11,12] The flexible loop was also deleted in one study to produce a mutant protein with greatly reduced biotin binding ability,[13] and a minimum-sized core streptavidin was also engineered that had higher accessibility to biotinylated macromolecules and possible use in biotechnological applications.[14]

The binding of biotin to avidin and streptavidin was investigated by a number of spectroscopic techniques, including UV absorption, circular dichroism (CD), and fluorescence spectroscopy.[1,15–19] The UV absorption and fluorescence data indicate that the binding site Trp residues undergo a change to a more hydrophobic environment upon the addition of biotin, and the CD data indicate a small change in the secondary structure when biotin is added. These systems were also studied by IR and visible/near-IR Raman spectroscopy. IR studies of avidin revealed secondary structural changes with the addition of biotin and evidence for the lengthening of the biotin ureido carbonyl group upon complex formation.[20,21] IR studies of streptavidin revealed increased protein thermostability and conformational changes with biotin binding,[22] and a hydrogen–deuterium exchange experiment followed by IR spectroscopy revealed slower exchange when biotin is bound.[23] The first Raman study of avidin using visible wavelengths[24] revealed changes in the protein secondary structure, and more recent visible and near-IR Raman studies on avidin and streptavidin further refined this analysis and also examined small spectral changes due to protein binding site Trp and tyrosine (Tyr) residues upon complex formation with biotin and other biotin analogues.[25–29] These recent Raman studies, however, examined the lyophilized protein complexes that are likely to be different in overall secondary structure than the proteins in solution. (Large reversible secondary structural changes in a range of proteins were shown to occur upon lyophilization with a substantial increase in the β-sheet content and a lowering of the α-helix and disordered content.[30])

UV resonance Raman (UVRR) spectroscopy has the advantage in many biological applications of providing specific enhancement from the Trp and Tyr residues; therefore, avidin and streptavidin, which have multiple aromatic residues in their binding sites, represent excellent systems for study in the development of this relatively new technique. We recently published a study examining the avidin–biotin complex in solution using UVRR spectroscopy with 244-nm excitation.[31] A wealth of spectral changes were observed in the difference spectrum between the complex and the apo-protein, which could be interpreted to provide information about the binding site Trp and the single Tyr 33 residues.

In this contribution we extend our previous avidin work to present the first UVRR study of the interaction of biotin and 2-iminobiotin with streptavidin and the results are compared to those previously obtained from the avidin–biotin complex and new data from the avidin–2-iminobiotin complex. The biotin binding site of streptavidin is very similar to that of avidin in terms of the positions of the binding site aromatic residues except that the Phe 79 in avidin is replaced by Trp 92 in streptavidin, giving it four binding site Trp residues. Streptavidin also has four other nonbinding site Trp residues and a total of six Tyr residues, of which only one (Tyr 43) is present in the binding site. However, the present study shows that the UVRR technique can easily detect the changes to the binding site Trp and Tyr 43 residues, even in this more complex protein.



MATERIALS AND METHODS

Biotin and 2-iminobiotin were purchased from Sigma, and streptavidin and avidin were purchased from Vector Labs. Protein solutions were prepared in phosphate buffered saline at pH 7.5 to which 5 m*M* KNO_3 was added to serve as an internal intensity standard for Raman difference

spectroscopy. Solid biotin or 2-iminobiotin was added in an approximate 10-fold excess to the streptavidin solution, gently shaken for 5 min, and kept on ice for 1 h. It was not possible to add a small amount of a concentrated solution of biotin or 2-iminobiotin to the protein solutions due to the poor solubility of the substrate at neutral pH. The addition of solid biotin also kept dilution of the protein solution to a minimum, which allows more accurate difference spectra to be obtained.

Raman spectra were obtained using 2 mW of 244-nm radiation at the sample from an intracavity frequency-doubled argon ion laser (Coherent Innova 300 FreD) and acquired using a Renishaw micro-Raman system 1000 spectrometer modified for use at 244 nm.[32] To minimize photodegradation the protein samples were circulated through a fused silica capillary tube (0.2-mm i.d.) by a miniature peristaltic pump (0.5-mm tubing i.d., P625, Camlab Limited) from a reservoir. The total sample volume was typically 200 μL, although volumes as small as 100 μL can be examined using the miniature peristaltic pump system. Spectra were accumulated over 30 min (60 $\times$ 30 s integration) and analyzed using GRAMS 32 (Galactic Industries Corporation). Accurate difference spectra were produced using the 1048 cm^{-1} band of potassium nitrate as an internal reference standard. The integrity of nitrate as an internal intensity standard was tested by accumulating two sequential protein spectra without the addition of substrate, after which a featureless, zero baseline difference spectrum was obtained.

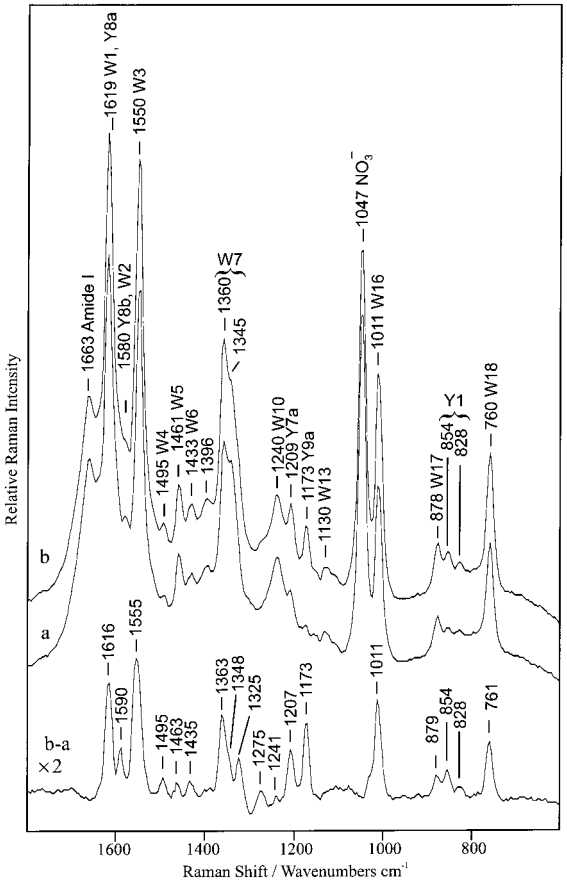


Figure 1. UVRR spectra at pH 7.5 of avidin (spectrum a), avidin–2-iminobiotin complex (spectrum b), and the difference spectrum (spectrum b-a). The intensities of spectra a and b are normalized to the internal standard NO_3^- peak at 1048 cm^{-1}, and the difference spectrum is scaled by a factor of 2 for clarity.

RESULTS AND DISCUSSION

Avidin–2-Iminobiotin Complex

Figure 1 shows the UVRR spectra of avidin and the avidin–2-iminobiotin complex in solution at pH 7.5 and the difference spectrum. The protein spectra are dominated by contributions from the four avidin Trp residues and single Tyr residue (Tyr 33) with moderate intensity from the amide I band at 1650 cm^{-1}.[33] Upon binding of 2-iminobiotin a number of relative intensity changes occur to the avidin UVRR spectrum that are more clearly highlighted in the difference spectrum and can be attributed to both the Trp and Tyr 33 bands. Similar to biotin, 2-iminobiotin does not exhibit Raman bands of significant intensity and thus makes no contributions to the Raman spectra.

The Trp contributions to the difference spectrum in Figure 1 are essentially the same as previously observed in the avidin–biotin study. The intensity enhancement for the Trp bands is likely to be due to a change to a more hydrophobic environment for the binding site Trp residues. An UVRR study of Tyr and Trp residues using 229-nm radiation excitation revealed an increase in the relative Raman intensity, especially the Trp Raman bands, upon decreasing the water exposure.[34–36] The present study used 244-nm excitation, which is far removed from the Trp B_b electronic transition at ~220 nm, that is probed by the use of 229-nm excitation. The Trp B_b absorption band is known to redshift with a near

linear dependence when changing the solvent composition from 100% water/0% propanol to 0% water/100% propanol.[34] The effect on the Trp 244-nm excited Raman band cross sections under similar conditions are not precisely known; however, clearly the addition of biotin to avidin perturbs the electronic structures of the binding site Trp residues, resulting in increased UVRR scattering. A change to a more hydrophobic environment for the binding site Trp residues is more clearly supported by the extra intensity to the 1363 cm^{-1} W7 Fermi doublet component over the 1348 cm^{-1} component. The ratio of the W7 Fermi doublet is known to be a good indicator of the hydrophobic natured Trp.[33,37]

The broad feature at 1555 cm^{-1} in the difference spectrum in Figure 1 is assigned to W3, which is known to exhibit a strong correlation to the torsional angle of Trp[38] and may indicate a reorientation of binding site Trp residues upon the addition of 2-iminobiotin. The similar pattern of intensity enhancement observed for the Trp Raman bands with the addition of biotin and 2-iminobiotin shows that both these compounds make similar interactions with the avidin binding site Trp residues.

The changes to the Tyr 33 Raman bands are however notably different than those previously observed with adding biotin to avidin. The features at 1616, 1590, 1207, 1173, 854, and 828 cm^{-1} in the difference spectrum are assigned to the intensity enhancement from Y8a, Y8b, Y7a, Y7b, and the Y1 Fermi doublet, respectively. There is also a likely contribution from W1 to the difference feature at 1616 cm^{-1}. The intensity enhancement of the Tyr features are most likely due to a shift to a more hydrophobic environment for Tyr 33 when 2-iminobiotin is added. This is supported by a recent study examining the ratio of the Y9a/Y7a Raman bands of Tyr in various solvents using 244-nm excitation, which shows this ratio increasing as the solvent becomes more hydrophobic.[39] The increase in the intensity of the Y9a band is almost as large as that of some of the Trp Raman bands in Figure 1 and far greater than that observed for Y7a. The nature of the intensity enhancement for the 2-iminobiotin complex is notably different than that previously observed for the avidin–biotin complex, reflecting the difference in local hydrogen bonding and noncovalent interactions between the Tyr 33 of avidin and 2-iminobiotin.

The Y1 Fermi doublet at ~850 and ~830 cm^{-1} is known to report on the degree of hydrogen bonding of the Tyr phenolic oxygen.[40–43] The relative increase in intensity of the 850 cm^{-1} component of the Y1 Fermi doublet upon the addition of 2-iminobiotin indicates a weakening of the hydrogen bonding to the Tyr 33 residue of avidin, which is consistent with a previous near-IR Raman study.[27]

The guanidinium group of 2-iminobiotin forms a hydrogen bond with the phenolic oxygen of the avidin Tyr 33 residue side chain. An X-ray crystal structure study of the binding of 2-iminobiotin to streptavidin found that only the nonprotonated, neutral form of 2-iminobiotin is bound, even at low pH values well below the guanidinium group pK_a. Streptavidin binds biotin in a similar manner to avidin, and it is reasonable to assume that avidin also binds 2-iminobiotin in a manner similar to streptavidin.

A previous near-IR Raman study of the avidin–2-iminobiotin complex reports small changes to the Trp contributions and a similar change to the Y1 Fermi doublet at 854 and 828 cm^{-1}.[27] However, these studies examined the lyophilized protein complex, which is likely to have a different overall secondary structure than that found in solution. A FTIR study revealed large reversible secondary structural changes in a range of proteins, and lyophilization substantially increased the β-sheet content and lowered the α-helix and disordered content.[30] The UVRR method has the advantage of allowing direct analysis of protein Tyr and Trp residues in the native protein in solution.

Streptavidin–Biotin Complex

Figure 2 shows the UVRR spectra of streptavidin and the streptavidin–biotin complex in solution at pH 7.5 and the difference spectrum. The spectra are dominated by the contributions from the eight Trp residues in streptavidin with moderate contributions from the six Tyr residues and the amide I band at 1662 cm^{-1}.[33] When biotin is bound a number of spectral changes occur to the streptavidin UVRR spectrum that are clearly visible in the difference spectrum and can be attributed to both Tyr and Trp vibrational bands. The Trp and Tyr contributions to the difference spectrum are of near equal intensity, which is in contrast to the previously reported difference spectrum of the avidin–biotin interaction in which the Trp contributions dominated.[31] In streptavidin

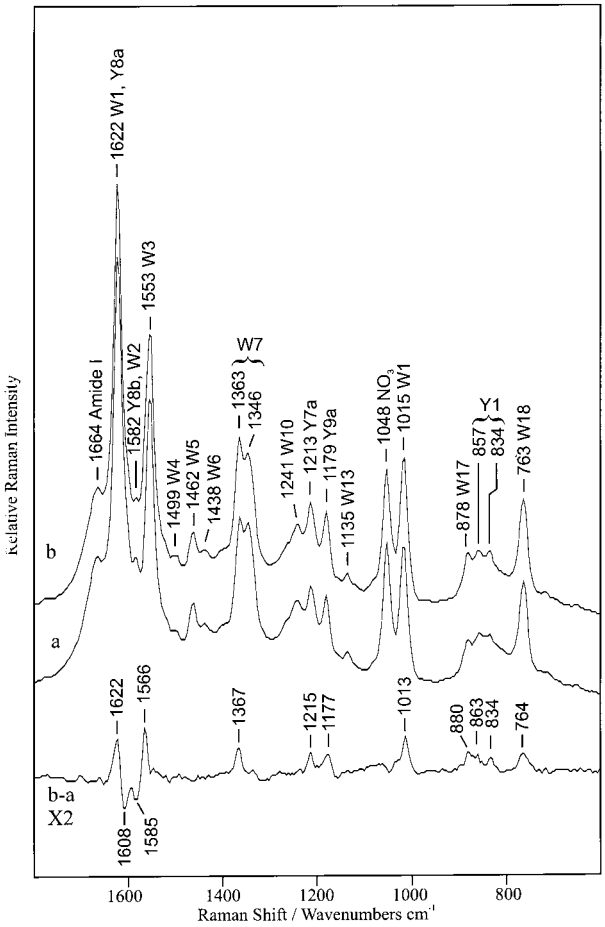


Figure 2. UVRR spectra at pH 7.5 of streptavidin (spectrum a), streptavidin–biotin complex (spectrum b), and the difference spectrum (spectrum b-a). The intensities of spectra a and b are normalized to the internal standard NO_3^- peak at 1048 cm^{-1} and the difference spectrum is scaled by a factor of 2 for clarity.

four of the eight Trp residues are found in the binding site (Trp 79, 92, 108, and 120) and in avidin three out of the four are in the binding site.

The peak at 1367 cm^{-1} in the difference spectrum is due to an intensity enhancement of the high wavenumber component of the W7 Fermi doublet, indicating a change to a more hydrophobic environment.[33,37] This is consistent with a fluorescence study of the streptavidin–biotin complex that showed that biotin induced a conformational change that moves the Trp's into a more hydrophobic environment, causing a reduction in the quantum yield and fluorescence lifetime.[19]

The W3 peak frequency is known to exhibit a strong correlation to the torsional angle of Trp,[38] and in this protein the band at 1553 cm^{-1} for both holo- and apo-streptavidin represents an average $|\chi^{2,1}|$ value of 100°. In the difference spectrum the feature at 1566 cm^{-1} is assigned to W3 and may be due to a small reorientation of one or more Trp residues upon the addition of biotin. The average degree of hydrogen bonding of the Trp residues does not appear to change with the biotin binding. A change in the degree of hydrogen bonding would be reflected in a change in the frequency in the W17 band.[38,44]

The Tyr residues make a significant contribution to the UVRR spectra of streptavidin at 1662, 1213, 1179, 850, and 830, which are assigned to Y8a, Y7a, Y9a, and the Y1 Fermi doublet, respectively; the difference spectrum also reveals that most of these bands are affected by the addition of biotin. The derivative feature at 1608 and 1622 cm^{-1} is assigned to Y8a, which is shifted up in frequency, resulting in the pair of positive and negative bands in the difference spectrum. The 1608 cm^{-1} band may also have Trp W1 contributions. The difference features at 1215 and 1177 cm^{-1} are due to intensity enhancement from the Y7a and Y9a bands, and the lack of change in the Y9a/Y7a ratio indicates a lack of change in the hydrophobic environment for the Tyr 43 binding site residue.[39] It appears likely that all the UVRR Tyr spectral changes in streptavidin that are highlighted in the difference spectrum can be assigned to Tyr 43 because the difference features are almost identical to those found for the avidin–biotin interaction and avidin has only one Tyr residue. The Tyr 33 of avidin is found in a region with a sequence identical to the Tyr 43 of streptavidin, and both Tyr residues make hydrogen bonds to bound biotin. Thus, the technique of UVRR difference spectroscopy in this particular example disentangles the changes to a single Tyr residue in the binding site from the other Tyr residues present in the protein.

The Y1 Fermi doublet at 857 and 834 cm^{-1} is known to report on the degree of hydrogen bonding of the Tyr phenolic oxygen.[40–43] The UVRR data in Figure 2 do not show a significant change in this ratio, suggesting that Tyr 43 does not change its degree of hydrogen bonding when biotin binds, which is in agreement with previous near-IR Raman studies.[26,28] The X-ray structures of streptavidin reveal that the binding site of the apo form contains several water molecules that are displaced by biotin, which makes van der Waals contact with the binding site Trp residues

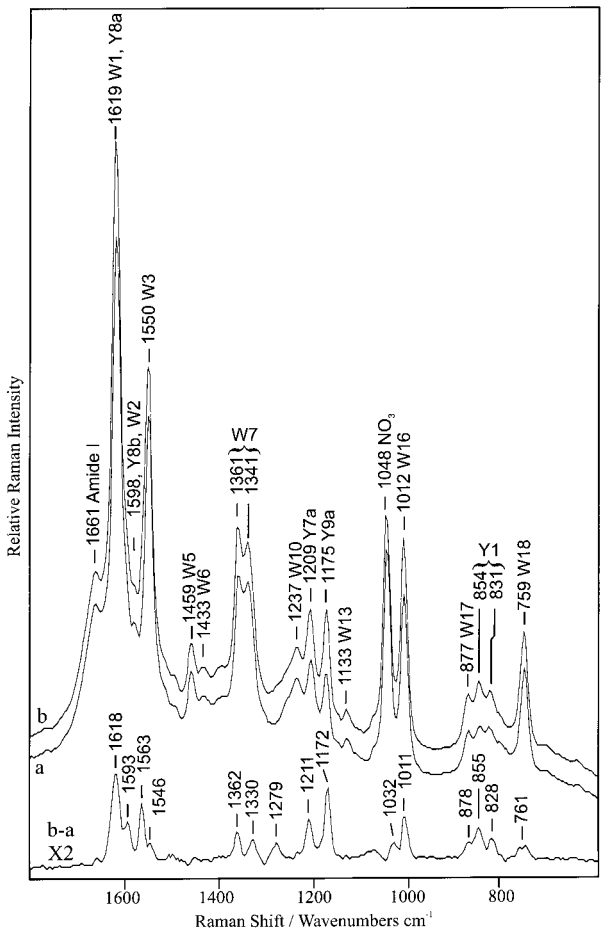


Figure 3. UVRR spectra at pH 7.5 of streptavidin (spectrum a), streptavidin–2-iminobiotin complex (spectrum b), and the difference spectrum (spectrum b-a). The intensities of spectra a and b are normalized to the internal standard NO_3^- peak at 1048 cm^{-1}, and the difference spectrum is scaled by a factor of 2 for clarity.

and forms a hydrogen bond to the phenolic oxygen of Tyr 43.[45]

Streptavidin–2-Iminobiotin Complex

In the 2-iminobiotin molecule the carbonyl group of biotin is replaced by a guanidino group, and the effect of this structural difference on the UVRR difference spectrum of the 2-iminobiotin–streptavidin system is shown in Figure 3. The Trp contributions to the difference spectrum in Figure 3 are essentially identical to those of the biotin complex in Figure 2 and the relative intensities of the difference features at 1563, 1362, 1011, 878, and 761 cm^{-1} are very similar. This is similar to those found for the avidin, and the UVRR data also indicates that the interactions of the streptavidin binding site Trp residues with biotin and 2-iminobiotin are nearly identical.

The most striking contrast between the two difference spectra in Figures 2 and 3 are the contributions that can be assigned to Tyr 43, which dominate the difference spectrum in Figure 3. There is overall intensity enhancement of the Tyr UVRR peaks that are most notable at 1618, 1211, and 1172 cm^{-1}, which can be assigned to Y8a, Y7a, and Y9a respectively. The 1616 cm^{-1} feature has a strong contribution from the Y8a mode, as well as a contribution from W1. Unlike the difference spectrum in Figure 2, there is no shift in frequency for Y8a, revealing the feature at 1590 cm^{-1} that is assigned to a combination of Y8b and W2. The relative increase in intensity of the Y9a band over the Y7a band indicates a shift to a more hydrophobic environment for the Try 43 residue.[39] The Y1 Fermi doublet ratio was also clearly changed by the addition of 2-iminobiotin. The increase in the 855 cm^{-1} peak over that of the 828 cm^{-1} peak indicates that the phenolic oxygen of Tyr 43 is a weaker hydrogen bond donor when 2-iminobiotin is bound compared with water in the apo form or biotin in the holo form. 2-Iminobiotin is bound to avidin in its nonprotonated form even at low pH values because there is insufficient space in the binding pocket where the guanidino group resides for the extra proton to be accommodated.[46] Streptavidin can donate only two hydrogen bonds to the guanidino group of 2-iminobiotin compared with three to the ureido group of biotin. 2-Iminobiotin can donate one hydrogen bond to avidin via its guanidino group. The change in the local hydrogen bonding network together with the difference in local dipole–dipole interactions of biotin and 2-iminobiotin with streptavidin account for the difference in the Tyr 43 contributions to the UVRR difference spectra.

CONCLUSIONS

The UVRR technique is particularly well suited to study the avidin– and streptavidin–biotin and –2-iminobiotin complexes because the binding site Trp and Tyr residues are resonantly enhanced at 244 nm. This allowed for direct optical probing of the binding pocket using difference spectroscopy, which reveals a wealth of informa-

tion concerning the reorientation, change in environment, and hydrogen bonding status of the binding site Tyr and Trp residues upon substrate binding. The hydrophobic interactions of biotin and 2-iminobiotin with the Trp residues were found to be similar; however, significant differences in the environment of Tyr 43 were observed. UVRR spectroscopy clearly resolves both the Trp and Tyr contributions to the binding of biotin and 2-iminobiotin to streptavidin and highlights the differences in the avidin Tyr 33 and streptavidin Tyr 43 environments between these complexes. Despite the fact that streptavidin contains many Trp and Tyr residues, as well as those in the binding pocket, the UVRR difference technique allowed the binding site to be probed exclusively. Although one cannot rule out effects on Trp and Tyr residues distant from the binding or interaction sites in every case, UVRR difference spectroscopy appears to be a very promising technique for probing specific residues and regions in some proteins without the need for the preparation of a range of mutants.

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