Shielding of Tryptophan Residues of Avidin by the Binding of Biotin†

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ABSTRACT: The binding of biotin to tetrameric avidin changes the environment of tryptophan residues. Binding reduces the total tryptophan fluorescence by 34%, shifts the emission peak from 337 to 324 nm, and reduces the fluorescence bandwidth from 61 to 46 nm. These changes are consistent with the movement of tryptophans to a nonpolar, internal environment. In the absence of biotin, iodide readily quenches the fluorescence of 20–29% of the initial fluorescence, which likely corresponds to one tryptophan located in a positively charged environment. Iodide may have weak access to additional fluorescence, corresponding to perhaps one additional tryptophan. Acrylamide, in the absence of biotin, has good access to three-fourths or more of the fluorescence, but the remainder, due to one or two tryptophans, is well shielded. The binding of biotin completely prevents iodide quenching and decreases acrylamide access dramatically. The data indicate that biotin binding shifts two or three tryptophans to an internal, hydrophobic, shielded environment.

Avidin is a protein found primarily in the egg white and oviduct of birds, amphibians, and reptiles (Elo & Korpela, 1984) and is presumed to function as an antibacterial agent through its ability to reduce the free biotin concentration (Green, 1975). Avidin is heavily employed in biotechnology (Wilchek & Bayer, 1988). Its applied value derives from three properties: the avid binding of biotin [approximately 10^{-15} M (Green, 1963a, 1975)] allows for the formation of essentially irreversible complexes; four biotin binding sites allow for multiple attachments and amplification; and, facile modification of biotin's carboxylate functional group allows for a wide range of conjugates to be constructed.

Avidin is a tetramer of four essentially identical subunits (Green, 1964a; DeLange & Huang, 1971). The amino acid sequence of the 128-residue subunit has been established (DeLange & Huang, 1971). Heterogeneous carbohydrates, seven to nine residues, are attached at asparagine-17 (DeLange, 1970; Bruch & White, 1982). The only cysteines, residues 4 and 83, form an intrachain disulfide (Green, 1963c; DeLange, 1970). Avidin is basic, containing 12 acidic and 18 basic residues. The secondary structure is predominantly comprised of β-structure, with little or no α-helix (Green & Melamed, 1966; Honzatko & Williams, 1982; Argarana et al., 1986; Hunt & Barker, 1989).

Avidin has four tryptophans per subunit (DeLange & Huang, 1971). Early chemical modification studies indicated that at least a subset of tryptophans was vital to biotin binding [reviewed in Green (1975)]. Most recently, modification of tryptophan-70 or -110 by 2-hydroxy-5-nitrobenzyl bromide was observed to cause a complete loss of biotin binding, implying that these residues are within the binding site (Gitlin et al., 1988). Biotin binding alters both the fluorescence emission spectrum (Green, 1964b) and the ultraviolet absorbance spectrum (Green, 1963b) of avidin. The spectral changes have been interpreted in terms of the movement of tryptophans into a more hydrophobic environment (Green, 1975).

We are interested in the interplay between biotin binding and conformational events. Here, we report studies of avidin's fluorescent tryptophans. The four tryptophan residues can be divided into several categories. The binding of biotin shifts two or three tryptophans to an internal, hydrophobic, shielded environment.

Experimental Procedures

Avidin. Affinity-purified hen egg white avidin was purchased from Sigma (A-9275). Avidin concentration was assessed by the absorbance at 282 nm [$E_{subunit}$ = 24 mM^{-1} cm^{-1}, $E_{1\%}$ = 15.4 cm^{-1} (Green, 1975)]. Titrations of avidin with biotin, using the displacement of 2-anilinonaphthalene-6-sulfonic acid (Mock et al., 1985, 1988) or of 2-(4-hydroxyazobenzene)benzoic acid (Green, 1970), or changes in the intrinsic fluorescence intensity of avidin or dansylated avidin each indicated the presence of four vacant biotin sites per tetramer, and thus the absence of bound biotin.

Fluorescence Spectroscopy. Avidin, in 50 mM sodium phosphate, pH 6.8, was excited at 295 nm, and fluorescence spectra or emissions at a single wavelength (near emission maxima) were recorded.

Quencher stocks were 50 mM in sodium phosphate, at a final pH of 6.8. After the sample's fluorescence was measured, aliquots of quencher were added, and the fluorescence was again measured. With iodide, the stock, avidin, and *N*-acetyltryptophanamide (NATA)[1] were made 1 mM in sodium thiosulfate.

Fixed-wavelength studies were performed on an L-format spectrofluorometer (SLM Instruments, Inc., Urbana, IL) equipped with Jarrell Ash (Jarrell Ash, Waltham, MA) quarter-meter monochromators and a Spex digital photometer (Spex Industries, Inc., Metuchen, NJ). Corrections were made for buffer fluorescence, and then for the inner filter effect and dilutions. The inner filter effect was calculated from the

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[1] Abbreviation: NATA, *N*-acetyltryptophanamide.

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combined absorbance at 295 nm of the quencher and protein (or NATA) according to the equation of McClure and Edelman (1967).

Emission spectra were recorded on an SLM Instruments SPF-500C spectrofluorometer, with an excitation band-pass of 5 nm and an emission band-pass of 7.5 nm. Technical spectra were taken at each concentration of quencher, in both the presence and absence of avidin, and corrected for instrument response factors. Buffer spectra were then subtracted from spectra of avidin. Net spectra were then corrected for dilutions and the inner filter effect. With iodide, a 10 mm × 10 mm cuvette was employed. With acrylamide, the inner filter effect can be substantial (E_{295} approximately 0.25 M^{-1}) and was reduced by employing a 2-mm excitation path (2 mm × 10 mm cuvette).

The Raman peak at 330 nm could potentially interfere with the emission spectra of avidin. Its magnitude was minimal; in the worst case, the ratio at 330 nm of avidin fluorescence to Raman emissions was 38:1.

Quantum yields of avidin were calculated from the total fluorescence emissions (305–450 nm) and were normalized to the fluorescence of NATA [quantum yield = 0.15 (Kirby & Steiner, 1970)]. The quantum yields reported by Burstein et al. (1973) have been adjusted to a quantum yield of 0.14 for aqueous tryptophan (Eisinger, 1969).

Analysis of Quenching. Fluorescence quenching can be considered to be of two types, collisional and static (Eftink & Ghiron, 1976, 1981). If only one fluorophore is present (and is itself homogeneous), or if multiple fluorophores are homogeneous, then quenching can generally be described by a Stern–Volmer type equation:

$$F_0/F = (1 + K_{sv}[Q]) \exp(V[Q]) \quad (1)$$

where F and F_0 are fluorescence intensities in the presence and absence of a quencher, respectively, [Q] is the concentration of quencher, V is the static quenching constant, and K_{sv} is the collisional Stern–Volmer constant. K_{sv} is equal to $k_q\tau_0$, where k_q is the bimolecular rate constant for the quenching process and τ_0 is the fluorescence lifetime in the absence of the quencher.

For multiple, heterogeneous fluorophores, eq 1 can be summed to

$$F/F_0 = \sum_{i=1}^{n} f_i/\{(1 + K_{sv_i}[Q]) \exp(V_i[Q])\} \quad (2)$$

where f_i is the fraction of the initial fluorescence of the ith fluorophore. Heterogeneity will bend Stern–Volmer plots (F_0/F vs [Q]) downward while the static components will bend plots upward.

Avidin contains four tryptophans per monomer. Assuming each to be in a single environment, eq 2 can be rewritten as

$$F/F_0 = \sum_{i=1}^{i=4} f_i/\{(1 + K_{sv_i}[Q]) \exp(V_i[Q])\} \quad (3)$$

where f_i, K_{sv_i}, and V_i are each attributable to a single tryptophan residue.

Data were fit to eq 3 by using a microcomputer and spreadsheet software (Lotus 1-2-3) and were evaluated by examining Stern–Volmer plots of the observed and calculated data. Unless stated otherwise, collisional quenching constants were set at approximately 10 times the static constant of the same component (Eftink & Ghiron, 1981).

Results

Fluorescence Spectra of Avidin. Fluorescence emission spectra of avidin are shown in Figure 1 and are similar to those

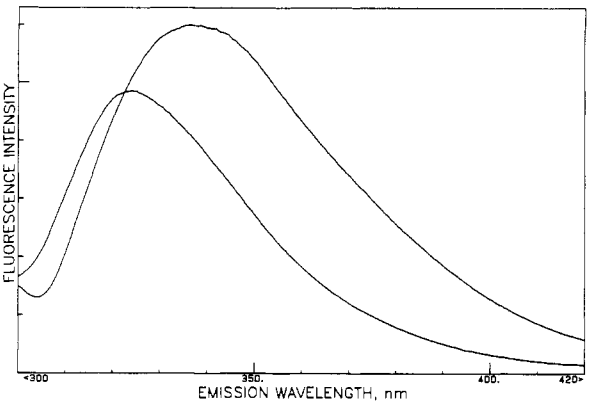

FIGURE 1: Fluorescence spectra of avidin. Top spectrum, avidin, 30 μM in subunits (0.5 mg/mL). Bottom spectrum, avidin after the addition of 150 μM biotin, corrected for the small dilution.

reported by Green (1964b). In the absence of biotin (top spectrum), the maximum fluorescence is at 337 nm, and the peak has a bandwidth at half-height of 61 nm. Biotin binding dramatically alters the emission spectrum (Figure 1, bottom spectrum). Total fluorescence (305–450 nm) is reduced by 34%, and peak fluorescence is reduced by 19%. The peak is blue-shifted from 337 to 324 nm, and the spectral bandwidth is reduced from 61 to 46 nm. the average quantum yield of the four tryptophans (305–450 nm) was estimated as 0.135 and 0.089 in the absence and presence of biotin, respectively, by comparison to the tryptophan model compound NATA [quantum yield = 0.15 (Kirby & Steiner, 1970); maximum fluorescence at 354 nm; bandwidth = 75 nm (not shown)].

A key distinction is whether biotin binding moves tryptophans into a buried environment or selectively places polar tryptophans into proximity to a quencher. Biotin, 0–0.6 M, does not quench the fluorescence of the tryptophan model compound, NATA (data not shown). Similarly, the quantum yield of indole is not altered by covalent attachment (three- or four-carbon spacer) to biotin (Liu & Leonard, 1979). The lack of quenching of NATA does not rule out the possibility that biotin quenches avidin's tryptophans, since tryptophans within the biotin site could be exposed to extremely high local biotin concentrations. It is also conceivable that polar tryptophans are shifted by biotin binding into proximity to a quencher other than biotin.

The emission spectra (Figure 1) provide unmistakable evidence that the dominant effect of biotin is that of altering the average tryptophan environment. Biotin binding decreased fluorescence above 323 nm, but increased fluorescence at lower wavelengths (Figure 1). If biotin binding resulted in quenching, then there should have been a decrease in fluorescence at all wavelengths. The higher fluorescence of the avidin–biotin complex below 323 nm directly implies a movement of tryptophans from a polar to a nonpolar environment.

Accessibility of Tryptophan Residues to Iodide. Iodide is a highly efficient quencher of tryptophan fluorescence. It is a large, polar anion and is considered to have access only to surface tryptophans (Lehrer, 1971; Eftink & Ghiron, 1981; Lakowicz, 1983).

The quenching of the fluorescence of avidin and of NATA by iodide is shown in Figure 2 as a Stern–Volmer plot. In the absence of biotin, iodide partially quenches the fluorescence of avidin. Biotin binding confers complete protection against quenching. Presuming that biotin binding did not itself quench tryptophans (see above), then accessible tryptophan's must have been shifted to a protected environment.

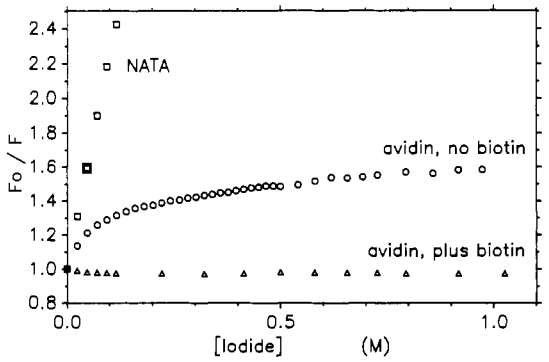

FIGURE 2: Quenching of the fluorescence of avidin by iodide. (□) NATA (*N*-acetyltryptophanamide); (○) avidin, no biotin; (△) avidin with biotin bound. Avidin was 3 μM in subunits, and [NATA] was 12 μM.

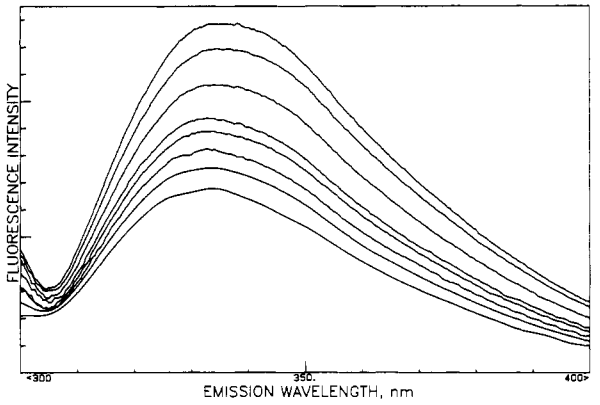

FIGURE 3: Emission spectra of biotin-free avidin at various concentrations of iodide. Additions of potassium iodide were made to 0.10 mg/mL avidin. Corrections were made for instrument response factors, buffer fluorescence, dilutions, and the inner filter effect as described under Experimental Procedures. From the top, iodide concentrations were 0, 0.015, 0.05, 0.15, 0.3, 0.8, 1.5, and 2.5 M.

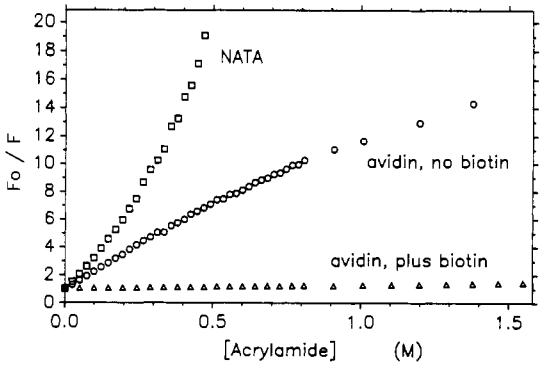

FIGURE 4: Quenching of the fluorescence of avidin by acrylamide. Avidin was 3 μM in subunits, and [NATA] was 12 μM. (□) NATA (*N*-acetyltryptophanamide); (○) avidin, no biotin; (△) avidin with biotin bound.

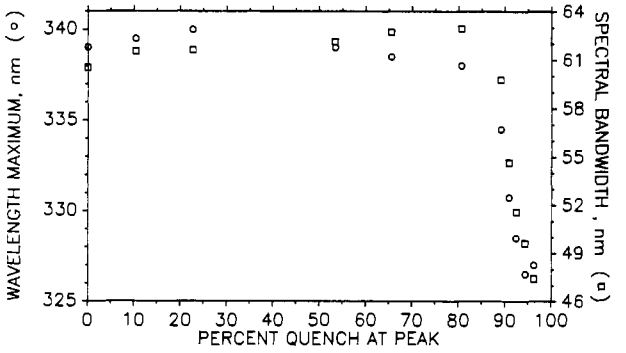

FIGURE 5: Effects of acrylamide upon the emission spectrum of biotin-free avidin. Spectra were corrected for instrument response factors, buffer fluorescence, dilutions, and the inner filter effect upon successive additions of acrylamide to 0.5 mg/mL avidin. The inner filter effect was reduced by using a 2-mm excitation path length. Emission maxima and spectral bandwidths are shown as a function of the amount of quenching. From the left, acrylamide concentrations were 0, 0.01, 0.025, 0.08, 0.13, 0.25, 0.6, 1.2, 2, 3, and 4 M.

The fluorescence of NATA, a model compound for tryptophan, was readily quenched, as expected for a completely exposed species (Figure 2). The collisional quenching constant was approximately 13 M^{-1}, and static quenching was negligible.

The quenching of avidin by iodide, in the absence of biotin, is complex. The downward curvature of the Stern–Volmer plot (Figure 2) indicates heterogeneity in accessibility. The maximum observed quenching, at 1.37 M iodide, was only 42%. The maximum accessible fraction could not be extracted from a Lehrer-style plot (Lehrer, 1971) due to curvature near the *y* axis (not shown).

About one-fourth of the total fluorescence was readily quenched by iodide (Figure 2; 25% quenching at $F_0/F = 1.33$). The initial slope of the Stern–Volmer plot was 5.7 M^{-1}. Omitting static quenching for the moment, this slope is equal to the average K_{sv}, weighted by the fluorescence yield of each of avidin's four tryptophans, as shown in eq 3 (see Experimental Procedures). Since about three-fourths of the fluorescence is poorly quenched by iodide, the K_{sv} of the most accessible fraction is substantially greater than the observed average. A more detailed analysis is presented under Discussion.

To further characterize the accessibility of tryptophans to iodide in the absence of biotin, emission spectra were recorded at several iodide concentrations (Figure 3). The first addition of iodide, to 0.015 M, reduced the fluorescence by 7%. Assuming the absence of conformational changes, the difference between spectra at 0 and 0.015 M iodide should be nearly equivalent to the spectrum of the most accessible component. The 0–0.015 M difference spectrum had an emission maximum at 337 nm and a bandwidth of 55 nm (not shown). These are properties of an only moderately polar tryptophan residue (Burstein et al., 1973).

The fluorescence wavelength maximum decreased only slightly, from 337 nm in the absence of iodide to 333–334 nm above 0.05 M iodide. The spectral bandwidth changed more, decreasing steadily from 60 nm at 0.015 M iodide to 53 nm at 2.5 M iodide (Figure 3). Difference spectra for each increment of iodide above 0.015 M had bandwidths of 58–66 nm. The selective effect upon the spectral bandwidth may reflect a decrease in the heterogeneity of tryptophan environments (due to quenching) but may also be due to effects of iodide upon avidin and/or water structure.

Accessibility of Tryptophan Residues to Acrylamide in the Absence of Biotin. Acrylamide is an efficient quencher of tryptophan fluorescence. Acrylamide is polar, but uncharged, and has good access to all but the most highly buried tryptophan residues (Calhoun et al., 1986).

The quenching of avidin and NATA by acrylamide is shown in Figure 4. The Stern–Volmer plot of the tryptophan model compound bends upward, indicating the presence of static quenching. The data are consistent with $K_{sv} = 18.4$ M^{-1} and $V = 1.37$ M^{-1}.

In the absence of biotin, acrylamide has access to all of avidin's fluorescence (Figures 4 and 5). The Stern–Volmer plot (Figure 4) curves downward, indicating heterogeneity among tryptophans. The slope of the Stern–Volmer plot was

12 M^{-1} initially but declined to 7 M^{-1} at 1–1.4 M acrylamide (7–9% remaining fluorescence). A more detailed analysis is presented under Discussion.

To further characterize the accessibility of tryptophans, emission spectra of avidin (no biotin) were recorded at several concentrations of acrylamide. Low concentrations slightly increased the wavelength maximum and spectral bandwidth (Figure 5). High concentrations of acrylamide were required to exceed 80% quenching. As the remaining, highly shielded component was quenched, the wavelength maximum and spectral bandwidth decreased markedly. At 4 M acrylamide, the 3% remaining fluorescence had maximum emission at 327 nm, with a bandwidth of 47 nm. These are the spectral properties of buried tryptophans (Burstein et al., 1973) and are similar to those of the avidin–biotin complex (Figure 1; maximum at 324 nm, bandwidth = 46 nm). It is not clear whether the initially shielded component has the spectral properties observed at 4 M acrylamide, since the acrylamide may have induced conformational changes by either a specific or a nonspecific interaction with avidin. Acrylamide, in general, does not appear to be a structural perturbant (Eftink & Ghiron, 1976, 1977).

Accessibility of Tryptophan Residues to Acrylamide in the Presence of Biotin. Quenching of the avidin–biotin complex by acrylamide is shown in Figure 4. Acrylamide still has access to the tryptophans, but the slope of the Stern–Volmer plot is less than 1 M^{-1}, far lower than in the absence of biotin.

Emission spectra, in the presence of biotin, were taken at several concentrations of acrylamide (not shown). Total fluorescence intensity (305–450 nm) was reduced by 59% at the highest concentration of acrylamide (4 M). Peak positions were unchanged. The spectral bandwidth at half-height was slightly reduced with each addition of acrylamide, ranging from 46 nm in the absence of acrylamide to 44 nm at 4 M acrylamide. Difference spectra, representing the fluorescence of the quenched species, had wider bandwidths than the unquenched species [48 nm for 0–0.25 M; 47 nm for 0–4 M (not shown)]. The small changes in spectral bandwidth may reflect some heterogeneity between tryptophans but could also be ascribed to secondary effects of the high acrylamide concentrations.

Discussion

Overview. Avidin has several intriguing properties which remain unexplained. The avidin–biotin interaction is perhaps the tightest known noncovalent binding of a small ligand, at 10^{-15} M (Green, 1963a, 1975), but is not well understood. Avidin, even in the absence of biotin, is unusually resistant to denaturation; studies have been thwarted by marked hysteresis between unfolding and refolding (Green, 1963c, 1975). Third, the available evidence is that there is no cooperativity in biotin binding (Green, 1975). This is hard to reconcile with the tight binding of biotin and the multiple interactions between subunits necessary to stabilize quaternary structure. Understanding these aspects of avidin is important and may improve avidin's utility in biotechnology.

Fluorescence Spectra of Avidin. In the absence of biotin, the maximum wavelength (337 nm) (see Figure 1) indicates that the average tryptophan environment is moderately polar (Burstein et al., 1973). The bandwidth at half-height (61 nm) is wider than that expected if all tryptophans were in a moderately polar environment (53–55 nm; Burstein et al., 1973), suggesting heterogeneity in polarity among tryptophans.

Biotin binding dramatically altered the fluorescence emission spectrum of avidin, and the wavelength maximum and quantum yield after binding biotin match those of tryptophans buried in a nonpolar environment (Burstein et al., 1973). The spectral bandwidth is quite narrow, suggesting that all remaining fluorescent tryptophans are in similar environments.

Biotin could act either by itself quenching nearby tryptophans or by causing a conformational change which moves tryptophans into proximity to some other quencher. Two observations compel the rejection of the possibility that biotin binding results in quenching. First, biotin does not reduce the quantum yield of indoles (this report; Liu & Leonard, 1979). Second, spectra of avidin with and without biotin bound had an isoemissive point at 323 nm (Figure 1). The increase in fluorescence below 323 nm is incompatible with quenching but is consistent with the movement of tryptophans from a polar to a nonpolar environment, which is consistent with results from ultraviolet spectroscopy (Green, 1963b, 1975).

Interactions with Iodide and Acrylamide in the Absence of Biotin. Iodide had limited accessibility to tryptophans, and the quenchable fluorescence had the emission spectrum of a moderately polar tryptophan residue. Acrylamide had access to all tryptophan fluorescence, but heterogeneity was evident.

The Stern–Volmer plots for both iodide and acrylamide can be fit to eq 3, as developed under Experimental Procedures. With 4 tryptophans, there are 12 fitting parameters, and deducing a unique set of parameters is generally not possible (Eftink & Ghiron, 1981). However, the presence of a small fraction of the fluorescence with either much higher or much lower quenching constants (for iodide and acrylamide, respectively) limited the range of reasonable fits and allowed us to reach conclusions as to the accessibility of tryptophans to iodide and to acrylamide (see below).

Two limitations to the curve fitting approach should be noted. Equation 3 assumes that each tryptophan is in a singular environment, such that a single K_{sv} and a single V are sufficient to describe its response to a quencher. In fact, individual tryptophans can be heterogeneous in their interactions with quenchers, presumably due to the presence of multiple protein conformers [see Eftink and Waslewski (1989)]. Thus, a tryptophan may have multiple sets of quenching constants; the nature of the distribution of values cannot presently be stated.

Second, it is possible that the quencher perturbs the structure of the macromolecule, thus altering the quenching parameters of individual tryptophans. The available data on acrylamide argue against it being a structural perturbant (Eftink & Ghiron, 1976, 1977). Potassium iodide, however, is a moderately strong perturbant of protein structure (von Hippel & Wong, 1964). Avidin, though, is remarkably resistant to denaturation (Green, 1975). The continued shielding of fluorescence from the highest concentrations of iodide is consistent with the retention of nativelike structure.

Quenching of avidin by iodide can be fit to an accessible component of 20–29% of the fluorescence. The K_{sv} of this component ranged from 24 M^{-1} at 29% accessible to 46 M^{-1} at 20% accessible. A second component, with a K_{sv} of 0.2–3.9 M^{-1}, may comprise 13–25% of the initial fluorescence. The remaining fluorescence has yet smaller quenching constants, which mostly affect the behavior at the higher iodide concentrations.

The K_{sv} of the accessible component is greater than that of iodide toward NATA (24–46 vs 13 M^{-1}, respectively). Since K_{sv} is the product of the fluorescence lifetime and the bimolecular collisional constant, the high K_{sv} can be some combination of a long lifetime and/or a high bimolecular collisional constant. The lifetimes of protein tryptophans are within a factor of 2.5 of the lifetime of NATA (Lakowicz, 1983).

Therefore, the bimolecular collisional constant between iodide and the readily quenched component is probably at least as great as between iodide and NATA.

It was also possible to fit the iodide quenching data with the most accessible component having $V > K_{sv}$ (V approximately 20 M^{-1}). This tryptophan has either a large collisional quenching constant or a substantial static quenching constant. In either case, it is implicit that the accessible component is in a positively charged environment, which increases the local concentration of iodide.

Curve fitting can also further define the heterogeneity in accessibility of tryptophans to acrylamide. Successful fits required dividing tryptophans into an accessible set and a poorly accessible component. Three-fourths or more of the fluorescence is readily quenched, with K_{sv} of 8–13 M^{-1}. The remaining fluorescence is difficult to quench, with K_{sv} of 0–1.4 M^{-1}. When the accessible tryptophans were 85–94% of the initial fluorescence, fits were made with $K_{sv-i} \gg V_i$. As the accessible fraction decreased below 85%, fits increasingly required higher static quenching constants and lower collisional constants (e.g., at 84% accessible, V = 2.2–3.5 M^{-1} and K = 10–13 M^{-1}; at 78% accessible, V = 3.9–6.5 M^{-1} and K = 8–10.5 M^{-1}).

Emission spectra taken at several concentrations of acrylamide (Figure 5) were consistent with the presence of shielded tryptophans, and the acrylamide-resistant fluorescence has the emission spectrum of a hydrophobic, buried tryptophan.

How Many Tryptophans Are Accessible to Iodide or Shielded from Acrylamide? If all four tryptophans were equally fluorescent, then the iodide-accessible 20–29% would correspond to one tryptophan. Heterogeneity in environment will result in varied quantum yields for tryptophan residues (Burstein et al., 1973). Buried tryptophans will generally have lower quantum yields (0.077 vs 0.14–0.21 for more polar residues). The acrylamide quenching data implied a buried component of a fourth or less of the initial fluorescence. If this were to correspond to two tryptophans, then an iodide-accessible tryptophan could contribute 28–42% of the fluorescence. If there was a single buried tryptophan, then an iodide-accessible tryptophan could contribute 22–37% of the fluorescence. Thus, iodide could have high accessibility to only one tryptophan whether one or two tryptophans are shielded from acrylamide. It is also possible for a tryptophan residue to be nonfluorescent. If avidin possessed a nonfluorescent tryptophan, then the quenching data would also be interpretable as there being one iodide-accessible tryptophan and only one buried (and fluorescent) tryptophan.

Iodide may have modest accessibility toward an additional 13–25% of avidin's fluorescence. By the same arguments as above, this could correspond to a single tryptophan. The remaining fluorescence is well shielded from iodide, indicating only that these residues are not highly solvent-exposed.

How many tryptophans are shielded from acrylamide? The curve fitting indicated a wide range for the fraction of the initial fluorescence attributable to shielded residues. With the use of the quantum yields of tryptophans suggested by Burstein et al. (1973) (0.077, 0.21, and 0.14 for buried, moderately polar, and polar residues, respectively), a single buried tryptophan would contribute 11–15% of the initial fluorescence (assuming no silent tryptophans). If we stipulate that collisional quenching is predominant for the accessible fraction, then the shielded fraction is not likely to be greater than 15%, and would be attributable to a single tryptophan.

The Stern–Volmer plot could be fit with greater than 15% of the fluorescence shielded from acrylamide if accessible tryptophans were assigned high static quenching constants (e.g., V = 2.2–3.3 M^{-1} at 16% shielded and V = 3.9–6.5 M^{-1} at 22% shielded). In this case, it is more likely that two tryptophans are buried. A high static quenching constant is entirely plausible. The amide portion of acrylamide has the potential to form two sets of hydrogen bonds with the biotin site due to its similarity to the ureido portion of biotin, which is critical to binding (Green, 1975). Acrylamide binding may be somewhat looser than the binding of urea [K = 28 M^{-1} (Green, 1963b, 1975)], which can potentially form three sets of hydrogen bonds at the ureido binding site. Since there are two or more tryptophans within the biotin binding site (Green, 1975; Gitlin et al., 1988), acrylamide, bound as a weak biotin analogue, might quench two tryptophans by a static mechanism.

Accessibilities toward iodide and acrylamide were in sharp contrast. Iodide is a strongly hydrated anion and is considered to have access only to tryptophans located at the surface of proteins (Lehrer, 1971; Burstein et al., 1973; Lakowicz, 1983). Thus, the iodide data suggest that one or two tryptophans are at or near avidin's surface, with one in a positively charged environment. The two residues accessible to iodide are almost certainly accessible to acrylamide as well (Calhoun et al., 1986). Iodide is highly unlikely to have access to the residues protected from acrylamide. The overall picture, then, is that one tryptophan is in a moderately polar, positively charged environment; another one or two tryptophans are not deeply buried; and the remaining one or two tryptophans are in a highly shielded environment.

Can we establish which tryptophan is most likely to be the one which iodide readily quenches? Avidin is a highly basic protein (Wooley & Longsworth, 1942; DeLange & Huang, 1971). Thus, at least some of the tryptophans are expected to be in a positively charged environment. Tryptophan-110, identified as being within the biotin site (Gitlin et al., 1988), is a poor candidate. While it is immediately adjacent to lysine-111, it is also adjacent to two negatively charged residues, aspartates-108 and -109. The other three tryptophans could each be in a locally positively charged environment. Tryptophan-10 is immediately adjacent to lysine-9 but also has a nearby negative charge, aspartate-13. Tryptophan-70, identified as being within the biotin site (Gitlin et al., 1988), is immediately adjacent to lysine-71. Tryptophan-97 has two nearby positively charged residues, arginine-100 and lysine-94. A low rate of diffusion into the biotin site might conceivably result in shielding from collisional quenching. The site binds biotin rapidly (Green, 1975) and has been proposed to be an open cleft in avidin's surface (Chignell et al., 1975). On the basis of these arguments, any of tryptophans-10, -70, and -97 could reasonably both be accessible to iodide and be in a positively charged microenvironment.

Effects of Biotin Binding upon Avidin. Biotin binding shields tryptophan residues from both iodide and acrylamide. Iodide has no access to the fluorescent tryptophans of the biotin–avidin complex. Minimally, biotin binding shields the previously highly accessible tryptophans, presumably by movement of the tryptophans to a nonsurface location. Acrylamide also has poor access to the biotin-avidin complex (Figure 4). Thus, the two or three tryptophans that are accessible in the absence of biotin are moved to a shielded environment by biotin binding. The emission spectra support this picture. In the absence of biotin, the tryptophan fluorescence indicates a mix of surface and buried residues.

The avidin–biotin complex, in contrast, has the properties of solely internal tryptophans.

How does biotin shield tryptophan residues? For tryptophans located within the biotin binding site, the shielding of tryptophans may be due to biotin simply filling the binding site, rather than to a biotin-induced conformational change. If the quenchers have access to tryptophans by diffusing into the biotin site, then biotin could decrease quenching by eliminating the diffusion path. Since at least two tryptophans are within the biotin site when biotin is bound (Green, 1975, Gitlin et al., 1988), biotin could decrease quenching, as observed, by the act of binding. Thus, it is not necessary to invoke a conformational change in order to explain the quenching data. The same mechanism would account for the ability of biotin binding to protect tryptophan residues against chemical modification by *N*-bromosuccinimide (Green, 1963b; Liu & Leonard, 1979).

The spectral changes that accompany biotin binding have been interpreted as a movement of tryptophans from a relatively polar environment to a highly hydrophobic, internal environment. The binding of biotin presumably displaces water molecules from the binding site. Since biotin is relatively hydrophobic, it is plausible that the spectra of the tryptophans found in the vacant biotin site are shifted due to the difference between being in proximity to water versus biotin. We cannot be sure whether either the spectral changes or the changes in accessibility to quenchers are caused by conformational events or merely by the proximity of tryptophans to bound biotin.

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