# Protein–Flavonol Interaction: Fluorescence Spectroscopic Study

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***ABSTRACT*** **Recent studies have shown that various synthetic as well as therapeutically active naturally occurring flavonols possess novel luminescence properties that can potentially serve as highly sensitive monitors of their microenvironments in biologically relevant systems. We report a study on the interactions of bovine serum albumin (BSA) with the model flavonol 3-hydroxyflavone (3HF), using the excited-state proton-transfer (ESPT) luminescence of 3HF as a probe. Upon addition of BSA to the flavonoid solutions, we observe remarkable changes in the absorption, ESPT fluorescence emission and excitation profiles as well as anisotropy ($r$) values. Complexation of 3HF with protein results in a pronounced shift (20 nm) of the ESPT emission maximum of the probe (from $\lambda_{em}^{max}$ = 513 nm to $\lambda_{em}^{max}$ = 533 nm) accompanied by a significant increase in fluorescence intensity. The spectral data also suggest that, in addition to ESPT, the protein environment induces proton abstraction from 3HF leading to formation of anionic species in the ground state. Fairly high values of anisotropy are observed in the presence of BSA for the tautomer ($r$ = 0.25) as well as anion ($r$ = 0.35) species of 3HF, implying that both the species are located in motion-restricted environments of BSA molecules. Analysis of relevant spectroscopic data leads to the conclusions that two binding sites are involved in BSA–3HF interaction, and the interaction is slightly positively cooperative in nature with a similar binding constant of $1.1 - 1.3 \times 10^5\ M^{-1}$ for both these sites. Proteins 2001; 43:75–81.** © 2001 Wiley-Liss, Inc.

**Key words: 3-hydroxyflavone; bovine serum albumin; fluorescence; excited-state-proton transfer**

## INTRODUCTION

The flavonoids are a large group of polyphenolic natural products which are widely distributed in higher plants.[1–5] Such compounds are increasingly being recognized as possessing a broad spectrum of biological activities and important therapeutic applications,[1,6–8] including novel features, e.g., as anticancer, antitumor, anti-inflammatory, and anticoagulant drugs.[1,8,9] Interestingly, many biologically active flavonoids appear to have effects on various proteins, including enzymes.[10] For example, quercetin, a naturally occurring flavonoid of widespread occurrence, can bind to human plasma proteins. Another naturally occurring flavonoid, fisetin, has also gained attention for its inhibitory action on protein kinase C (PKC), a signal-transducing enzyme[11] and HIV-1 proteinase, a virally encoded protein that is indispensable for maturation and processing of acquired immunodeficiency syndrome (AIDS) virus and a viable target for anti-human immunodeficiency virus (HIV) therapy.[12] The microenvironments of the binding sites of such flavonoid molecules inside the living cell are expected to be complex in nature and essentially an unexplored area until now.

In previous articles, we have shown that the intramolecular excited-state proton transfer (ESPT) and dual fluorescence properties of natural[13,14] as well as synthetic[15–17] flavonoids can be used as highly sensitive probes for exploring their microenvironments in various biomembrane mimetic organized assemblies, namely micelles, reverse micelles, and liposomes. However, very few reports have appeared so far on the prospective uses of the emission characteristics of such flavonoids for probing the microenvironments of binding sites of proteins.[18,20] The present work was primarily motivated by long-term objectives of using the intrinsic fluorescence emission properties of flavonoids in unraveling the nature of their interaction with different enzymes. For a critical assessment of the potentialities of such work, 3-hydroxyflavone (3HF), a simple synthetic prototype flavonol (structure shown in scheme 1) and a model protein, bovine serum albumin (BSA), which is frequently used as a testing ground for initial characterization of novel optical probes for proteins[21,22] were chosen for the present investigation. We chose 3HF both as a representative ESPT fluorescence probe and as a model for biologically significant flavonols.[23] We report that upon addition of BSA to 3HF solution, remarkable changes are observed in the ESPT emission and excitation profiles, as well as in anisotropy values.

The present research provides perspectives highlighting the sensitivity of the fluorescence emission properties of 3HF to its local environment, which makes it an excellent probe for investigating the nature of its binding sites in the

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Dedicated to Professor Michael Kasha on the occasion of his 80th birthday.

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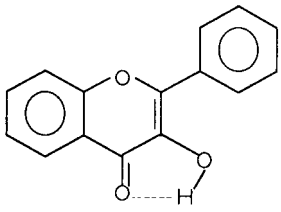

3-Hydroxyflavone

protein. It is noteworthy that the presence of more than one tryptophan residue in BSA (the exact number of tryptophan was reported as two in earlier studies[24] and three in more recent studies [http://www.ncbi.nlm.nih.gov, accession no. CAA76847. PID g3336842]) makes the system more complex than HSA, which contains only one tryptophan residue in it, and was used in previous studies of protein–3HF interactions. Furthermore, unlike human serum albumin (HSA), the three-dimensional structure of BSA is not clearly known, due to the unavailability of suitable crystals for X-ray diffraction (XRD) studies.[21] In contrast to previous work (on HSA) that relied on fluorescence emission profiles and energy transfer data,[23] we have used fluorescence emission as well as excitation spectra and anisotropy measurements. These studies provide critical insights regarding the nature of binding of 3HF with the protein (BSA), in both qualitative and quantitative detail.

## MATERIALS AND METHODS

3HF was obtained from Aldrich. The purity of the sample was checked by comparing the electronic absorption and emission spectra with published data.[25,26] Water was quartz-distilled. BSA and urea were obtained from Sisco Research Laboratories (India). HEPES buffer was a Sigma product; 50 mM HEPES buffer solution was prepared in water, and pH was adjusted to 7.0 and used in all studies of 3HF in proteins. Concentrated stock solutions of 3HF were first prepared in spectrograde ethanol (Merck) and then diluted with HEPES buffer to obtain a final 3HF concentration 5 μM (final ethanol concentration in buffer was <1%). This concentration of 3HF was maintained for all the spectroscopic measurements. Stock BSA solution was prepared in 50 mM HEPES buffer, pH 7.0; 8 M urea solution prepared in HEPES buffer was used to denature BSA.

Steady-state absorption and fluorescence spectra were recorded with a Hitachi model U-3300 spectrophotometer and with a model F-4010 spectrofluorometer, respectively. The fluorescence spectra were corrected for the wavelength dependence of the sensitivity of the apparatus.[27] The fluorescence anisotropy ($r$) values were obtained from the expression $r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH})$, where $I_{VV}$ and $I_{VH}$ are the vertically and horizontally polarized components of 3HF emission with excitation by the vertically polarized light at 340 nm and 420 nm for neutral and anionic species of 3HF, respectively, and $G = I_{HV}/I_{HH}$.[27]

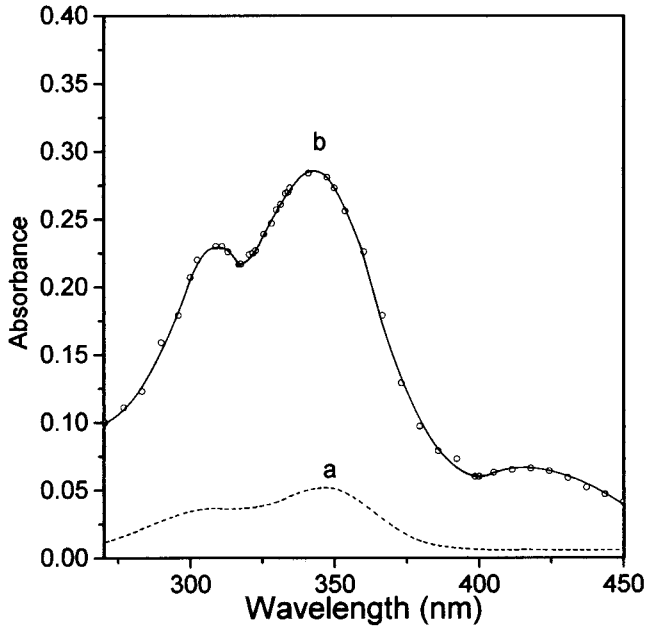


Fig. 1. Absorption spectrum of 3-hydroxyflavone (3HF) in 50 mM HEPES buffer pH 7.0, (**a**) [bovine serum albumin (BSA)] = 0 and (**b**) [BSA] = 60 μM.

## RESULTS AND DISCUSSION

Figure 1 presents a typical absorption spectrum of 3HF in the presence of BSA along with the reference spectrum in HEPES buffer at pH 7.0. Upon complexing with protein, a band with $\lambda_{abs}^{max} \approx 417$ nm appears. From the spectral position, this band can be assigned to the anionic species of 3HF.[28,29] Thus, in the presence of BSA, both the neutral ($\lambda_{abs}^{max} = 345$ nm) and anion ($\lambda_{abs}^{max} = 417$ nm) forms contribute to the probe (3HF) absorption. The presence of a large spectral shift between the absorption maxima of these two species of 3HF permits selectively exciting each species and separately studying their characteristic emission behaviors as well as binding properties with BSA.

Figure 2a shows emission spectra of the neutral species of 3HF (in buffer) containing varying concentrations of added BSA. In the absence of BSA, well-resolved dual emission bands are seen, with a blue-violet normal fluorescence of $\lambda_{em}^{max} = 410$ nm and a green ESIPT tautomer fluorescence of $\lambda_{em}^{max} = 513$ nm. Interestingly, the dual emission profile shows dramatic changes with variation of BSA concentration. The effect is especially pronounced in case of the tautomer band. Figure 2a (inset) and 2b shows the variation of $\lambda_{em}^{max}$ and relative intensity, respectively, as a function of BSA concentration. Upon gradual addition of BSA, $\lambda_{em}^{max}$ of 3HF changes from 513 nm ([BSA] = 0) to 533 nm ([BSA] = 60 μM) accompanied by increased fluorescence intensity. Interestingly, the shift in $\lambda_{em}^{max}$ with change in BSA concentration is extremely rapid up to [BSA] = 10 μM, beyond which the emission spectrum of 3HF consists of predominantly the green band with $\lambda_{em}^{max} =$ 533 nm. It should be noted that the position of this emission maximum remains essentially unchanged with

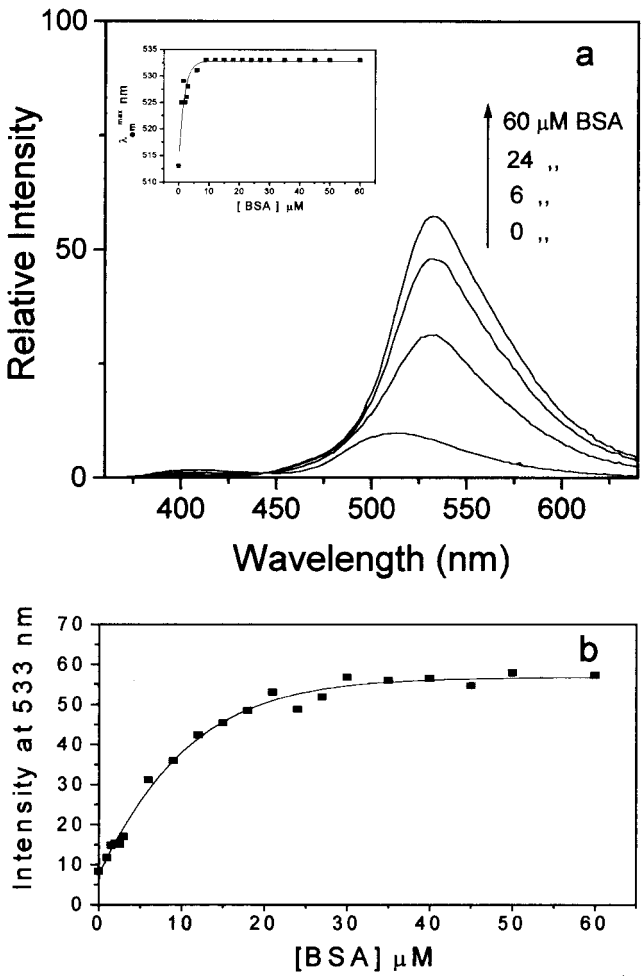


Fig. 2. **a:** Emission spectra of 3-hydroxyflavone (3HF) (neutral species) in buffer containing varying concentration of bovine serum albumin (BSA). Inset, variation of the $\lambda_{em}^{max}$ of 3HF at the same BSA concentrations. $\lambda_{ex}$ = 340 nm. **b:** Variation of relative fluorescence intensity of 3HF (neutral) in buffer containing varying concentration of BSA. $\lambda_{ex}$ = 340 nm and $\lambda_{em}$ = 533 nm.

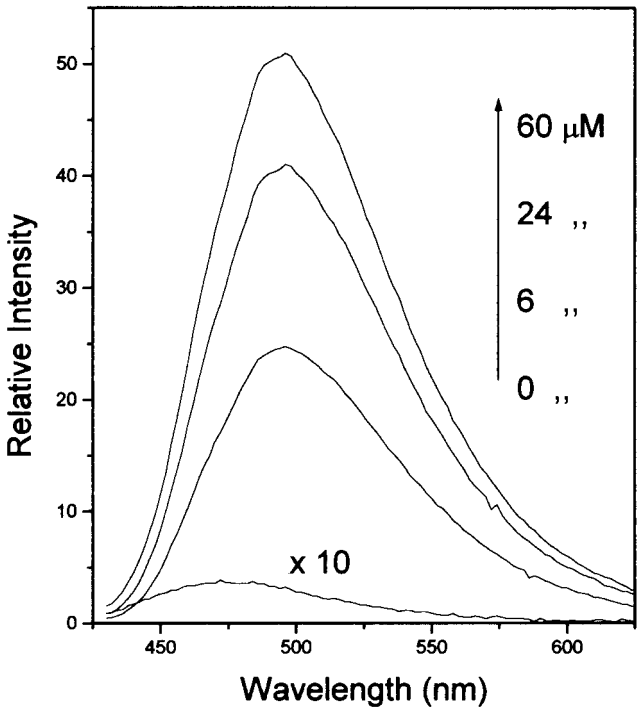


Fig. 3. Emission spectra of 3-hydroxyflavone (3HF) (anionic species) in buffer containing varying concentration of BSA. $\lambda_{ex}$ = 420 nm.

further increase in BSA concentration. It is of interest to note that the $\lambda_{em}^{max}$ corresponds to that for the polar aprotic solvent diethyl ether ($\lambda_{em}^{max}$ = 532 nm), as reported by Wolfbeis et al.[28] Previous workers[21,26,27] have shown that the appearance of the blue-violet emission from the normal non-proton-transferred form of 3HF molecules requires external H-bond interaction, which interferes with the ESPT process. In hydrocarbon as well as polar aprotic solvents, such as *n*-heptane and diethyl ether,[28] respectively, external H-bonding perturbation is absent, resulting in an efficient ESPT process. Therefore, in such solvents, the spectrum of 3HF consists of a single emission band from the ESPT tautomer with high quantum yield.[28]

Figure 3 shows that upon exciting the anionic species of 3HF ($\lambda_{ex}$ = 420 nm), relative emission intensity (which is practically negligible in buffer) increases with increasing BSA concentration as in the case of the neutral species. However, it should be noted that the position of this emission maximum ($\lambda_{em}^{max}$ = 496 nm) (which closely matches the previously published result for the emission maximum of 3HF anionic species[28]) remains essentially unchanged with increased BSA concentration. It would therefore be reasonable to assign the observed pronounced sensitivity in emission spectrum to the binding effect of BSA on the photophysics of 3HF. The change in emission behavior observed for both tautomer and anionic species with increase in BSA concentration implies that more and more 3HF molecules bind with appropriate sites of BSA.

More interesting changes are seen from a comparison of the emission spectra of 3HF–BSA complex ([BSA] = 6 μM) in the presence and absence of 8 M urea, while exciting the neutral and anionic species of 3HF separately (Fig. 4). In the presence of urea, the intensity of both the tautomer and anionic bands sharply decreases, with the tautomer band undergoing a shift from 533 nm to 513 nm (note that $\lambda_{em}^{max}$ = 513 nm corresponds to the value for aqueous environment[28,29]). It is apparent that, in the presence of urea, denaturation of the protein causes release of 3HF molecules to the aqueous (buffer) medium, which would account for the lowering of emission intensity as well as the shift in the emission maximum. These observations further support the binding phenomenon of 3HF with BSA in native form.

Figure 5a and 5b displays typical excitation spectra (for BSA = 6 μM) monitored at the two emission bands (λ = 535 nm and 480 nm), respectively. In each case, the reference spectrum in buffer is included for comparison. Excitation spectra of 3HF in buffer, monitored at both λ = 535 nm and 480 nm show smooth (i.e., without vibrational structure) profiles with $\lambda_{ex}^{max}$ = 340 nm and insignificant

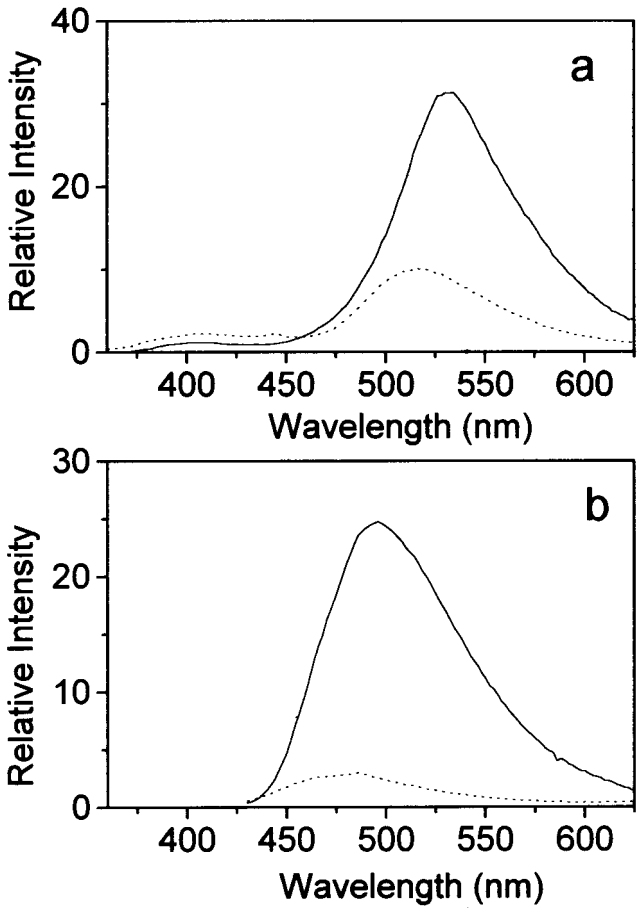


Fig. 4. Comparison of the emission behaviors of (a) neutral and (b) anionic species of 3-hydroxyflavone (3HF) in native (unbroken curve) and denatured (dotted curve) forms (obtained by addition of urea) of bovine serum albumin (BSA).

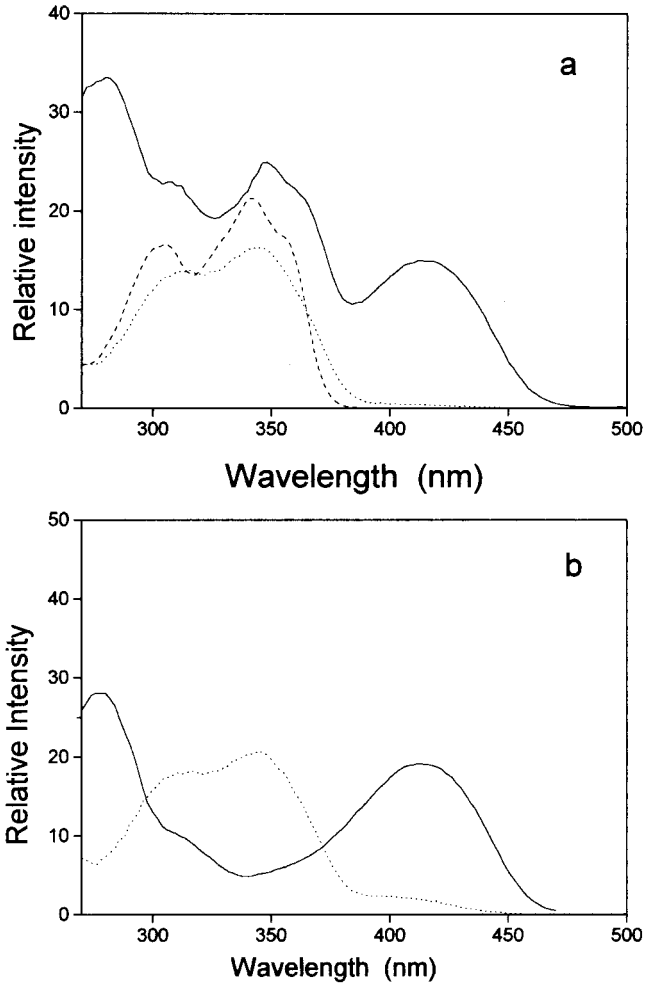


Fig. 5. **a:** Excitation spectra of 3-hydroxyflavone (3HF) in bovine serum albumin (BSA) (unbroken curve), ethyl acetate (dashed curve), and buffer (dotted curve) for the neutral species. $\lambda_{em}$ = 535 nm. **b:** Excitation spectra of 3HF in BSA (unbroken curve), and buffer (dotted curve) for the anionic species. $\lambda_{em}$ = 480 nm.

contribution at ~410 nm. These spectra are found to be very similar, suggesting the presence of a single type of ground-state species of 3HF in buffer solution. But in the presence of BSA, several interesting observations are noted. The excitation spectrum obtained upon monitoring the emission at 535 nm contains a vibrationally resolved band with $\lambda_{ex}^{max} \approx 345$ nm, and a conspicuous shoulder at 363 nm. The appearance of such a vibrational shoulder is consistent with a predominantly aprotic environment,[25,30] which sharply contrasts with the smooth, structureless excitation band typically observed in polar protic environments.[31] In contrast, the excitation profile obtained upon monitoring the emission at $\lambda$ = 480 nm shows only a strong band with $\lambda_{ex}^{max}$ = 410 nm. According to Wolfbeis et al.,[28,29] the band at 410 nm corresponds to the anionic species of 3HF. (Note that the 410-nm excitation peak is also present in the excitation profile monitored at the tautomer emission peak at 535 nm. This is due to significant overlap between the tautomer and anion emission region). The foregoing results indicate that in the presence of BSA, the two emission bands originate from the two different ground state populations of 3HF, one from the neutral and the other from the anionic species. Furthermore, it is noteworthy that in the presence of BSA, a band with $\lambda_{ex}^{max} \approx 280$ nm (corresponding to tryptophan) appears in the excitation profiles obtained upon monitoring the emission at 535 nm and 480 nm. This provides strong evidence for the occurrence of Forster-type energy transfer between the tryptophan (donor) present in BSA to the 3HF molecule (acceptor).

Figure 6 presents the variation of anisotropy ($r$) with increase in BSA for tautomer fluorescence of 3HF. The plot shows the pronounced increase in anisotropy of 3HF tautomers with increasing BSA. The change is sharp up to [BSA] = 10 $\mu$M (where $r$ = 0.25, compared with the initial value $r$ = 0.05 in the absence of BSA). Beyond this, the plot tends to level off with no significant change in r with further addition of BSA. In contrast to this behavior obtained for the tautomer, the anisotropy ($r$) for the anionic species shows no significant dependence with variation of protein concentration and remains at a constant value of $r$ = 0.35. The continuous increase in

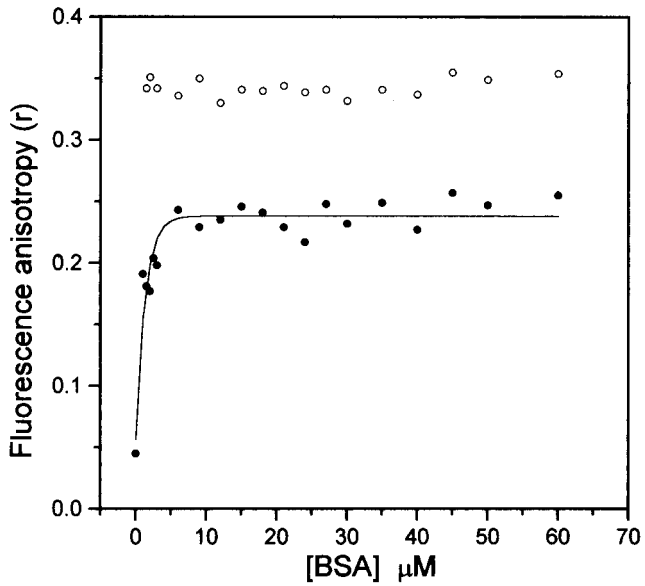


Fig. 6. Variation of fluorescence anisotropy of 3-hydroxyflavone (3HF) with bovine serum albumin (BSA) concentration. For neutral species (●), $\lambda_{ex}$ = 340 nm, $\lambda_{em}$ = 535 nm and for anionic species (○), $\lambda_{ex}$ = 420 nm, $\lambda_{em}$ = 495 nm.

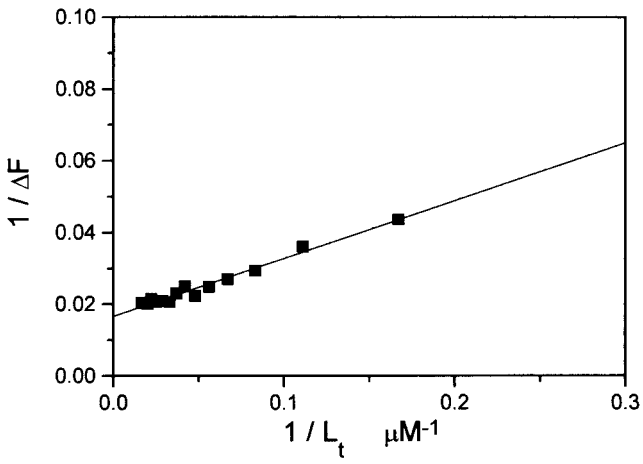


Fig. 7. Plot of $1/\Delta F$ versus $1/L_t$, where $L_t$ is the concentration of bovine serum albumin (BSA) added to 3-hydroxyflavone (3HF) (neutral species).

anisotropy of tautomer emission is consistent with the picture that more and more 3HF molecules are getting bound with BSA, while in the case of anionic emission, the anisotropy data suggest the significant occurrence of the anionic species only in the presence of BSA. The relatively high anisotropy values imply that both the species of 3HF are bound to BSA in motion-constrained environment of BSA molecules. (The differences in magnitude observed between the anisotropy values for the two species could be due to possible differences in the corresponding limiting anisotropies.)

The anisotropy results along with the emission and excitation characteristics suggest that the neutral and anionic forms of 3HF molecules are bound with the BSA, predominantly in two different sites. Binding of 3HF to BSA, would lead to the removal of water molecules from the immediate neighborhood of 3HF at the binding sites of the protein. As a result, external H-bonding perturbation would decrease, which in turn would increase the probability of successful intramolecular ESPT process. Furthermore, the peak position of the tautomer emission ($\lambda_{em}^{max}$ = 533 nm) is indicative of an aprotic polar environment for the corresponding binding site. This appears to be another (besides exclusion of water molecules) contributing factor for the environment of the ESPT process in 3HF. The significant enhancement of the emission intensity for the anionic species, as well as the high anisotropy value, observed upon binding of 3HF molecules to BSA, indicate the presence of a second binding site that is predominantly rich in amino acids that can act as proton acceptors at the specific pH used (pH 7.0). This may be the plausible explanation for obtaining a significant amount of ground-state anionic species of 3HF in the presence of BSA.

The presence of a 280-nm band (attributable to tryptophan) in the excitation profile (Fig. 5a,b) for both types of 3HF–BSA complex provides evidence for the occurrence of a Forster-type energy transfer between intrinsic tryptophan (donor) residue(s) of BSA, with the 3HF (acceptor) molecules in the BSA–3HF complex.

Employing the fluorescence behavior of individual species of 3HF, we estimated the affinity parameters viz. binding constant, and stoichiometry for the protein–3HF interactions. Figure 7 shows the linear plot of $1/F$ versus $1/L_t$ for the neutral species of 3HF, following the equation[32]:

$$\frac{1}{\Delta F} = \frac{1}{\Delta F_{max}} + \left(\frac{1}{K_a L_t}\right)\left(\frac{1}{\Delta F_{max}}\right)$$

where $\Delta F = F_x - F_0$, $F_x$, and $F_0$ representing the fluorescence intensity of 3HF in the presence and absence of the added total BSA concentration ($L_t$), respectively. $\Delta F_{max}$ is the maximum change in fluorescence intensity. The intercept of the above plot on the $1/F$ axis corresponding to $1/L_t = 0$ measures the $1/\Delta F_{max}$, while the slope gives the estimate for the affinity constant $K_a$.[32] Interestingly, nearly similar values are obtained for the binding affinity constants for both the neutral and anionic (not shown) species of 3HF (to BSA). This value comes out to 1.1–1.3 × $10^5$ $M^{-1}$, which markedly differs from corresponding values reported by previous workers[23] in case of interaction of 3HF with HSA. Analysis of the results of fluorimetric titration of 3HF (for both the neutral and anion species) with BSA by a Scatchard plot showed that 3HF molecules are bound to two sites of BSA corresponding to a binding stoichiometry of 2:1 (not shown). Taking into account this stoichiometry value, together with the fact that presence of energy transfer (between tryptophan (donor) and 3HF (acceptor)) is evident, as discussed earlier, it is tempting to speculate that perhaps some or all tryptophan residues are at or near the binding sites of 3HF molecules in the protein (BSA).

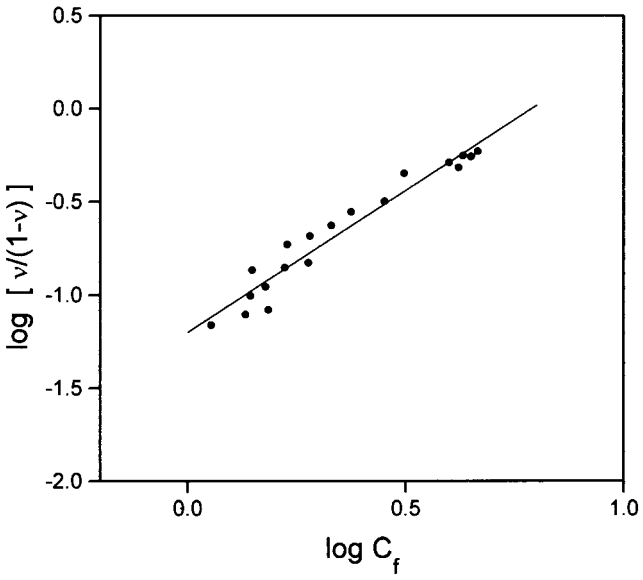


Fig. 8. Plot of log($\nu$/1 − $\nu$) versus log $C_f$ . The concentration of free 3-hydroxyflavone (3HF) ($C_f$) is expressed in microns ($\mu$M).

To ascertain whether the binding is cooperative, binding data were analyzed by the following equation[32]:

$$\log \frac{\nu}{1-\nu} = \log K_a + j \log[C_f]$$

where $\nu$, $C_f$, and j are the extent of binding, free flavone concentration and degree of cooperativity, respectively. Figure 8 plots log ($\nu$/1−$\nu$) vs log [$C_f$] for neutral species of 3HF. From the slope, the cooperativity is measured to be 1.4 (a similar value is also found when measured from anionic species; not shown), indicating that the mode of 3HF binding is slightly positive cooperative. This suggests that binding of one 3HF molecule at one binding site may somehow induce some structural changes in a second binding site of BSA, which in turn enhances the probability of binding with another 3HF molecule.

The present work shows that the emission properties of both the tautomer and anion forms of 3HF provide critical assessment of the nature of the microenvironments and the stoichiometry of binding in a model protein (BSA). However, the precise nature of the binding site remains to be elucidated clearly; we leave this issue to be resolved by more detailed studies in the future. Further extension of the present research to encompass comparative studies on different serum albumins, as well as relevant enzymes, should be of considerable interest in relation to studies on biological activities of flavonoids, including their therapeutic applications.

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