Interaction of 7-Hydroxy-8-(phenylazo)1,3-naphthalenedisulfonate with Bovine Plasma Albumin

SPECTROSCOPIC STUDIES*

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Interaction of Orange G (OG) with bovine plasma albumin (BPA) has been investigated using NMR, UV-visible absorption, CD, and fluorescence techniques. The bound conformation of OG is a compact structure with N9-N10 bond in a non-planar syn conformation. The binding causes a decrease in the 478-nm absorption band of OG. The analysis of the binding isotherm generated from UV-visible absorption measurements gives a dissociation constant of 10 μM and stoichiometry 1:1 for BPA/OG complex. Dissociation constant is invariant in the pH range 5.0–8.0 and is ~20 times higher at pH 4.0 than its value at pH 7.0. Near and far UV-CD studies indicate alterations in the helical content and in the tertiary structure of the protein on complexation. The binding induces (−) and (+) CD at 335 nm and 465 nm, respectively. The binding also results into an increase in the steady state fluorescence anisotropy of OG without affecting emission maximum and quantum yield. Fluorescence data indicate that quenching of Trp fluorescence by OG is static in nature and OG selectively binds to Trp residues on the target proteins. Albumins are the principal proteins in blood (10). Possibly, they serve as vehicles for regulating levels of drugs and metabolites in blood (10).

The mode of interaction of various sulfonated azo compounds including 7-hydroxy-8-(phenylazo)1,3-naphthalenedisulfonate, commonly known as Orange G (OG)† (Fig. 1B) with amino acids such as Arg, Lys, and His has been reported (11). To the best of our knowledge, there is no report on the interaction of OG with any protein. Detailed knowledge of the OG-albumin interaction is relevant to the understanding of interaction of sulfonated azo dyes with enzymes and with specific binding proteins. We have therefore undertaken detailed investigations on the mode of interaction of OG and plasma protein called bovine plasma albumin (BPA).

In this report, we have characterized BPA-OG complex using a variety of spectroscopic techniques such as nuclear magnetic resonance (NMR), circular dichroism (CD), fluorescence, etc. In addition, the conformation of BPA and of OG in BPA-OG complex is discussed.

EXPERIMENTAL PROCEDURES

Materials—Bovine plasma albumin was purchased from Armour Pharmaceutical Co. and Orange G from Sigma. Concentration of BPA was determined using molecular mass of 66 kDa and A278 = 0.667 mg⁻¹ ml cm⁻¹ (12) and that of OG using ε200 = 19900 M⁻¹ cm⁻¹. Other chemicals used were of AnalAR grade. 20 mM phosphate buffer, pH 7.2, was used. A mixture of acetate and phosphate (20 mM) was used to obtain pH in the range 4–8.

NMR—NMR measurements were carried out on a Varian Unity Plus 600-MHz spectrometer. Chemical shifts were measured with respect to sodium trimethylsilylethanol. One-dimensional spectra were recorded with 16,000 data points, 6499-Hz spectral width, 64 transients, and a 2-s relaxation delay at 25 °C. A 200-ms mixing time was used in the nuclear Overhauser effect spectroscopy (NOESY) (13) experiment. For 13C-T1 measurements, inversion recovery pulse sequence was incorporated in gradient enhanced heteronuclear single quantum coherence spectroscopy sequence (14). A relaxation time of 3 s was used. The 1H spin lattice relaxation times (T1) were measured using inversion recovery pulse sequence (15) using 12-s relaxation delay. The solvent resonance was suppressed by irradiation during relaxation delay and recovery time. T1 was calculated using built-in software.

UV-visible and CD Measurements—UV-visible absorption measurements were done on a Shimadzu UV-2100 spectrometer. CD spectra were recorded on a Jasco J-600 spectropolarimeter at 25 °C. Stoichiometry and dissociation constant of the complex were determined according to the standard method (16). The method assumes n equivalent binding sites of macromolecule and that binding of first ligand does not influence the binding of additional ligands.

$$\frac{1}{n} = \frac{K_d}{n[L_i]} \frac{1 - \Delta \phi_{\text{rms}}}{\Delta \phi_{\text{max}}}$$

(Eq. 1)

$\Delta \phi_{\text{rms}}$ is the change in the observed physical property, $\Delta \phi_{\text{max}}$ is the maximum change in the observed physical property, $K_d$ is the dissociation constant of the complex, and n is the number of ligand binding sites in the macromolecule. The molar ratio, v, of bound ligands to the macromolecule is defined as $(\Delta \phi_{\text{rms}}/\Delta \phi_{\text{max}}) \times ([L_i]/[L_i^M])$, where $[L_i]$ is the albumin; $T_1$, longitudinal relaxation time; MEM, maximum entropy method; DAS, decay-associated spectrum; NOESY, nuclear Overhauser effect spectroscopy.
total ligand concentration and \([M]_0\) is the total concentration of macromolecules added.

Steady-state Fluorescence—Steady-state fluorescence intensity and anisotropy measurements were carried out using Spex Fluorolog 1681 T format spectrophotofluorometer. Tryptophan residues were selectively excited with 295 nm radiation. Correction was done for inner filter effect using Equation 2 (17),

\[
F_{\text{cor}} = F_{\text{obs}}10^{\frac{t}{m}}
\]

(Eq. 2)

where \(F_{\text{obs}}\) and \(F_{\text{cor}}\) are the observed and corrected fluorescence intensity, \(A_x = (A_{x1} + A_{x2})/2\), and \(A_{x1}\) and \(A_{x2}\) refer to the absorbances at excitation and emission wavelengths, respectively. For determination of stoichiometry, emission was monitored at 344 nm.

Time-resolved Fluorescence—Time-resolved fluorescence studies were performed using tunable picosecond dye-laser pulse, from synchronously pumped cavity-dumped dye (Rhodamine 6G) laser driven by frequency-doubled output at 532 nm of the CW mode-locked Nd-YAG laser system. Fluorescence decay profiles were collected using a time-correlated single photon counting set-up coupled to a microchannel plate photomultiplier. The tunable output of the dye laser was frequency-doubled to generate second harmonic beam at 295 nm and was used to excite the samples. The instrument response function was obtained by collecting scattered light at 295 nm from the milk powder suspension in water. Before starting data collection, the instrument response was checked by measuring the lifetime of N-acetyltryptophanamide. Emission profiles were collected at a magic angle of 54.7° to eliminate the contributions from the anisotropy decay. To get the fluorescence decay curves, 10^4 counts at the peak were collected in 512 channels with channel width of 76 ps.

The intensity decay data were fitted to a sum of exponential terms using least squares reconvolution procedure (18),

\[
I(t) = \sum_{i=1}^{3} a_i \exp(-t/\tau_i), \sum_{i=1}^{3} a_i = 1
\]

(Eq. 3)

where \(a_i\) is the amplitude and \(\tau_i\) is the fluorescence lifetime of the \(i\)th component. The goodness of the fit was determined from the randomness of the weighted residual distribution and \(\chi^2\).

Analysis of the fluorescence decay profiles was also carried out using maximum entropy method (MEM) (19) by choosing a distribution of amplitude \((a(t))\) that yields maximum entropy (S), which is defined as shown by Equation 4 (20, 21),

\[
S = -\int a(t)\log\frac{a(t)}{m(t)} dt
\]

(Eq. 4)

where the starting model, \(m(t)\), is a flat distribution of the amplitudes in log \(t\) scale. Maximization of the function \(S\) was carried out under the \(\chi^2\) constraint defined as shown in Equation 5,

\[
\chi^2 = \frac{1}{M}\sum_{m=1}^{M} \frac{(F_{\text{cal}} - F_{\text{exp}})^2}{\sigma_m^2} = 1.0
\]

(Eq. 5)

where \(F_{\text{cal}}\) and \(F_{\text{exp}}\) are the calculated and observed intensities, respectively, at time \(t\), \(\sigma_m^2\) is the variance of the \(m\)th channel, and \(M\) is total number of data points. The optimized amplitude distribution represents maximum probable distribution of amplitudes among the different lifetime components.

Fluorescence Quenching Mechanism—Fluorescence quenching can be represented by a Stern-Volmer plot (17, 22), shown in Equation 6,

\[
\frac{F_0}{F} = 1 + K_{q}[Q]
\]

(Eq. 6)

where \(F_0\) and \(F\) are the fluorescence intensities in the absence and in the presence of quencher, respectively, \(K_q\) is the quenching constant, and \([Q]\) is the molar concentration of the quencher.

Decay-associated Spectra—The decay-associated emission spectra were computed from steady-state emission spectrum and fluorescence decay parameters (23, 24), as shown by Equation 7,

\[
F_k(\lambda) = \frac{a_k(\lambda)\tau_k}{\sum_k a_k(\lambda)\tau_k}
\]

(Eq. 7)

where \(F_k\) and \(F\) are the fluorescence intensities in the absence and in the presence of quencher, respectively, \(K_q\) is the quenching constant, and \([Q]\) is the molar concentration of the quencher.

\[
F_k(\lambda) = F_{\text{obs}}(\lambda) \frac{a_k(\lambda)\tau_k}{\sum_k a_k(\lambda)\tau_k}
\]

(Eq. 7)

where \(r_{t}\) is the rotation correlation time and \(t_{r}\) is the limiting anisotropy at zero time. The data were fitted to a single exponential, and the goodness of the fit was examined by random distribution of weighted residuals and \(\chi^2\) value.

RESULTS AND DISCUSSION

NMR Studies—NMR chemical shifts, line widths (\(T_2\)), and the resonance intensities of bound molecules are different from those of free ones and are influenced by the dynamics of exchange between free and bound species as well as their respec-
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![Fig. 2. NOESY spectrum of BPA-OG complex (BPA = 0.20 mM; [OG] = 4.0 mM). Acquisition parameters were as follows: 2000 data points in F2 and 300 experiments in F1 dimension with 32 transients in phase-sensitive mode, mixing time 200 ms, and a relaxation delay of 2 s.

Table 1

Longitudinal relaxation time ($T_1$) of Orange G (10 mM) in phosphate buffer and in presence of BPA (0.5 mM)

|       | $^{13}$C $T_1$ (ms) | $^1$H $T_1$ (ms) |
|-------|---------------------|------------------|
|       | In buffer | With BPA | In buffer | With BPA |
| C2H   | 1085      | 556      | 6504      | 1951      |
| C4H   | 928       | 496      | 2453      | 1056      |
| C5H   | 670       | 476      | 1362      | 818       |
| C6H   | 1155      | 568      | 2627      | 896       |
| C12, 16H | 939   | 626      | 2136      | 894       |
| C13, 15H | 1160  | 824      | 1820      | 764       |
| C14H  | 831       | 670      | 2003      | 815       |

Attempts have been made to obtain information about the conformation of OG in the bound and the free form using NOESY experiments. The NOESY spectrum of OG alone shows only weak and negative cross-peaks. This is true even for proton pairs such as H-4–H-5 and H-5–H-6, which are fairly close in space and should give rise to strong nuclear Overhauser effects. This is expected since OG is a small molecule and weak nuclear Overhauser effects. This is expected since OG is a small molecule and weak nuclear Overhauser effects. However, on binding with BPA, rotational correlation times for the inter-proton vectors will be much larger since OG-BPA complex will tumble like the BPA itself as shown by $T_1$ measurements. This is indeed what is observed in the NOESY spectrum. Fig. 2 depicts the NOESY spectrum of OG in presence of BPA. The spectrum shows several cross-peaks between the proton pairs of OG including those between protons of ring A and ring B. It is clear that the conformation of bound OG is significantly different from that found in x-ray crystal structure (11). In fact, if the crystal structure was maintained in the bound OG then none of the nuclear Overhauser effects between protons in ring A and ring B should have been observed. Obviously, the conformation of the bound OG is such that these two rings come close to each other and possibly stack as manifested in the chemical shift changes. A rotation around the N-N bond by 180° (anti → syn conformation) results in serious overlap between H-16 and O-7. Thus, the bound conformation arises from changes in torsional angles around the bonds H-8–N-9 and the N-10–C-11 leading to a fairly compact structure for the bound OG with N-9–N-10 bond in a non-planar syn conformation.

UV-visible Absorption Studies—Difference absorption spectroscopy has been used to quantify the stoichiometry and affinity of ligand binding. The absorption spectrum (not shown) of OG consists of three bands centered at 240, 330, and 478 nm. The band at 478 nm, which is free from contamination due to protein absorption, has been monitored to follow binding. Binding isotherm generated from the absorption measurements ($\Delta$A plotted against [BPA]) at 298 K attain saturation at $-45 \mu$M of BPA (Fig. 3A). A double inverse plot (Fig. 3A, inset) of change in absorption ($1/\Delta A$ versus protein concentration (1/[BPA])) has been used to get maximum change in absorption ($\Delta A_{\text{max}}$). The value of $\Delta A_{\text{max}}$ (0.22) thus obtained has been used in Equation $\nu = (\Delta A_{\text{max}}/[\text{protein}])/(\Delta A_{\text{max}}/[\text{BPA}])$ and free concentration ($1/\Delta A_{\text{max}}$) is shown in Fig. 3B. The $K_d$ and $n$ obtained from the slope and intercept of the straight line are $10 \pm 3 \mu$M and $1.3 \pm 0.3$, respectively. The low value of $K_d$ indicates high affinity of BPA to OG.

BPA is known to experience a conformational transition around pH ~ 4.5 from native state to “fast state,” a conformer that moves faster in the gel electrophoresis (26). We have...
estimated dissociation constant in the pH range 4–8 to find out the binding characteristics of the other conformer and the possibility of the involvement of ionizable groups of the side chain of amino acids present in BPA. It is observed that dissociation constant is more or less independent of pH in the range of 5–8 (Table II). However, a large increase in the dissociation constant (470 $\mu$M) occurs at pH 4.0. This is probably due to alterations in the conformation of the binding site in the fast state of BPA, which causes loose binding of OG.

Thermodynamic parameters have been calculated using temperature dependence studies of dissociation constant. The negative value of enthalpy of dissociation ($-35.3$ kJ mol$^{-1}$) indicates that the dissociation of BPA-OG complex is an exothermic process. The entropy of dissociation ($-200$ J mol$^{-1}$ K$^{-1}$) with a negative sign indicates unfavorable dissociation of the complex. The free energy of the dissociation (25 kJ mol$^{-1}$), again indicates the high stability of the complex.

**CD Studies**—Far-UV CD has been used to quantify the gross secondary structure of the protein (27–30). Far-UV CD spectrum of BPA and its complex with OG is given in Fig. 4A. The percentage of $\alpha$-helix has been calculated using ellipticity at 222 nm (31). The calculated value of the helicity (55%) is in good agreement with that reported in the literature. The CD of BPA-OG complex (1:1) (Fig. 4A (ii)) shows 10% increase in the helical content of the protein. The observed increase in the helical content may be due to an induced optical activity of far UV bands of bound OG or because of possible changes occurring in the CD of aromatic residues as a result of OG binding or both. The characteristics of the spectrum remain unchanged for higher concentration of OG.

The near-UV CD spectra of OG (i), BPA (ii), and OG-BPA (iii) complex are shown in Fig. 4B. OG itself has no CD in the 250–300 nm region; hence, it does not interfere with the interpretation of protein spectrum. One observes definite changes in the spectrum of protein on complexation with OG. It is possible that the apparent changes are due to the induced activity of 240-nm band of the bound OG. Alternatively these changes can be correlated to conformational alterations in BPA.

CD can also provide information about the environment of the ligand bound to protein (32). In many cases the free ligand is optically inactive, but on binding to the protein it may get immobilized in the asymmetric environment and thus give rise to induced CD signals in the characteristic absorption regions. OG is CD-inactive in the UV-visible region (Fig. 5). However, when BPA was added to a fixed concentration of OG (25 $\mu$M) there is a progressive induction of (−) and (+) CD at wavelengths 335 and 478 nm, respectively. This may be due to the induced asymmetry in the conformation of OG on binding to BPA (30, 33). It may be noted that NMR results show that the ligand bound to protein (32). In many cases the free ligand is optically inactive, but on binding to the protein it may get immobilized in the asymmetric environment and thus give rise to induced CD signals in the characteristic absorption regions. OG is CD-inactive in the UV-visible region (Fig. 5). However, when BPA was added to a fixed concentration of OG (25 $\mu$M) there is a progressive induction of (−) and (+) CD at wavelengths 335 and 478 nm, respectively. This may be due to the induced asymmetry in the conformation of OG on binding to BPA (30, 33). It may be noted that NMR results show that the conformation of OG changes significantly on binding with BPA. The analysis of binding isotherm (Fig. 5, inset) constructed from the CD at 335 nm using Equation 1 gives dissociation constant of 4 $\mu$M and stoichiometry of 1:0.95. These values are in reasonable agreement with those calculated from the absorption data.

**Steady-state Fluorescence Studies**—Trp and Tyr present in the proteins can act as intrinsic fluorescent probes (34). BPA has two Trps located in loop 3 and 4, respectively, at positions 135 and 214. These Trps are primarily responsible for BPA fluorescence. The steady-state fluorescence spectrum of BPA

![Figure 3](image1.png)

**Fig. 3.** A, change in the optical density (ΔA) of OG (25 $\mu$M) for varying concentrations of BPA in phosphate buffer of pH 7.2. Inset is the ΔA versus 1/[BPA] plot. B, inverse plot between molar ratio of bound OG to BPA (1/ν) and free [OG] (1/(1 − x)[OG]).

![Figure 4](image2.png)

**Fig. 4.** A, far-UV CD spectrum of (i) BPA (1 $\mu$M), (ii) BPA complexed with OG (1:1). B, near-UV CD spectrum of (i) OG (25 $\mu$M), (ii) BPA (25 $\mu$M), and (iii) BPA complexed with OG (1:1). The experimental parameters are: bandwidth 1 nm, sensitivity 10 millidegrees, step resolution 0.2 nm/datum, scan speed 50 nm/min, and number of scans 4.
shows strong emission centered at 344 nm (Fig. 6A (a)). Addition of equimolar OG leads to 30% quenching of Trp fluorescence (Fig. 6A (b)) without apparent shift in emission maximum. On further addition of OG, the intensity of the fluorescence continues to decrease until BPA:OG ratio reaches 1:3 and levels off thereafter (Fig. 6A (c)). Out of two Trps in BPA, Trp-135 is more exposed to hydrophilic environment, whereas Trp-214 is buried in the hydrophobic core. It is concluded, therefore, that the residual fluorescence intensity is most likely due to inaccessibility of OG to the buried Trp. For the estimation of the stoichiometry, BPA has been added to a fixed concentration of OG and Trp fluorescence has been monitored. The experiments have been carried out for two concentrations of OG, e.g. 5 μM and 15 μM. The fluorescence intensity is plotted against [BPA]/[OG] (Fig. 6B). The stoichiometry obtained from the extrapolation of two linear portion of the curve is close to 1:1 for both concentrations. OG in aqueous solution when excited at 335 nm gives a weak fluorescence at 465 nm. On addition of equimolar or more BPA, no change in the fluorescence characteristics (e.g. emission maximum or intensity) can be observed. This indicates that quantum yield of OG does not change on binding to BPA. The binding isotherm generated by anisotropy measurement at different concentration of BPA (data not shown) is similar to the binding isotherm generated by absorption and CD studies.

Time-resolved Fluorescence Studies—To get more insight
into the mechanism of binding of OG to BPA, time-resolved fluorescence studies have been done. The data has been analyzed using an unbiased MEM to get distribution of lifetimes as described under “Experimental Procedures.” The results reveal two sets of lifetime distributions for native BPA (Fig. 7A), one centered at 6.2 ns contributing 70% of the total fluorescence, and other centered at 2.1 ns contributing the rest of the fluorescence. These two lifetimes have been assigned to the two Trps present in BPA on the basis of the following argument. Human serum albumin, which has a Trp at the conserved position 214, gives only 0.33 of total BPA fluorescence (35). Thus, Trp-135 contributes more to the fluorescence of BPA than Trp-214. Hence, the intense peak in the lifetime distribution pattern centered at 6.2 ns corresponds to Trp-135 and the other peak (centered at 2.2 ns) is due to Trp-214. Addition of OG to BPA (1:1) causes a decrease in the amplitude of longer lifetime component with the appearance of a shorter lifetime component at 0.4 ns (Fig. 7B), which suggests the binding of OG near Trp-135. For BPA:OG (1:2), the contribution of the longer lifetime component decreases further and the full width at half maximum of Trp-214 reduces considerably (Fig. 7C). This decrease in the FWHM indicates a less heterogeneous environment of Trp-214 in BPA:OG complex than in BPA itself.

A detailed analysis of the above data has been carried out using Equation 3. Fig. 8 is the decay profile of Trps present in BPA. The data has been fitted to a bi-exponential function giving rise to lifetimes of 6.16 and 2.13 ns with the amplitudes of 0.69 and 0.31, respectively, for two Trps. The $\chi^2$ value for this fit was 1.02. To understand the localization and influence of OG on lifetime, experiments have been carried out by varying the concentration of OG and analyzing the fluorescence decay at each concentration. The results are summarized in Fig. 9. It is observed that the lifetimes of the two species having lifetimes of 6.16 and 2.13 ns do not vary much with [OG]. However, the average lifetime decreases from 4.66 ns to 1.64 ns (Fig. 9A). This may be due to the decrease in the contribution of species with longer lifetime to total fluorescence intensity. On increasing the concentration of OG, a third lifetime component could also be seen. The lifetime of this component first increases slowly and later levels off after a value of ~0.7 ns.

Fig. 9B is the plot of the amplitude of different lifetime components with [OG]. The amplitude of the longer lifetime component decreases from 0.69 to 0.06, with the increase in [OG] indicating that OG selectively binds near the Trp-135 and quenches its fluorescence. The observed increase in the amplitude of Trp-214 may be due to the constraint imposed in the sum of the amplitudes, which should be unity in the process of analysis in Equation 3. The amplitude of third lifetime component increases with the increase of [OG].

The Stern-Volmer plots have been prepared at different temperatures (Fig. 10) to get the quenching mechanisms. For this purpose, OG has been added to fixed concentration of BPA (5 μM) and Trp fluorescence has been monitored. One notices that all the Stern-Volmer plots are not linear but show saturation at higher concentration of OG. Deviation of Stern-Volmer plot from the linearity is frequently observed whenever the quenching of Trp fluorescence of proteins occurs as a result of interaction of polar or charged quenchers, which do not readily penetrate the hydrophobic interior of the protein, and only the surface Trp residues get quenched (36). In BPA the two Trps are present in different environments. The quencher OG is charged, so it may not penetrate into the hydrophobic core of the protein. This indicates that Trp-214 is not accessible to OG as has been inferred from the earlier experiments. The marginal decrease in the initial slope of the Stern-Volmer plots with the increase of the temperature indicates that the quenching of BPA fluorescence by OG is static in nature. Supporting evidence is provided by $\tau_{tr}$ versus [OG] plots (Fig. 10, lower part), which do not vary with [OG] (20). Thus, it can be inferred that the formation of complex takes place in the ground state of the fluorophore.

The environment of Trp(s) has been investigated by separating the spectra of two Trps. The decay-associated spectra (DAS) were calculated from the wavelength dependence of the time-resolved fluorescence of BPA using Equation 7. The decay of the fluorescence with excitation wavelength 295 nm and emission wavelength ranging from 310 to 400 nm were analyzed using global analysis. In this analysis, the lifetime is assumed to be the same at all the wavelengths and the amplitude is the
only allowed parameter that can vary with the wavelength. Fig. 11 shows the DAS of the BPA and BPA-OG complex. In the DAS of native protein, emission maximum of Trp-135 is at 347 nm contributing 75% of the BPA fluorescence, whereas for Trp-214 it is at 330 nm (Fig. 11A). Since the emission maxima for the two Trp values are different, they must be coming from two different Trps. This indicates that the environment of Trp-135 is hydrophilic and that of Trp-214 is hydrophobic (37). In BPA-OG complex, three DAS are observed (Fig. 11B). In this case, due to the considerable quenching of Trp-135 fluorescence by OG, Trp-214 is the major contributor to total fluorescence. The large decrease in the fluorescence of Trp-135 confirms the binding of OG near to Trp-135. The emission maximum of Trp-135 (347 nm) is unaffected, whereas for Trp-214 it is red-shifted to 343 nm. The observed red-shift of 13 nm in the emission maximum indicates that Trp-214 gets more exposed to hydrophilic environment. This may be due to changes in the tertiary structure of the protein. Therefore, one may conclude that the binding of OG to BPA leads to a change in the conformation of BPA at tertiary level.

It is known that the sulfonated compounds interact with amino acids such as Arg, Lys, His, etc., via sulfonate oxygen and make direct or indirect hydrogen bonds with the side chain NH (11). A closer inspection of BPA sequence reveals the presence of such charged residues near Trp-135. This enhances the possibility of binding of OG near Trp-135. Moreover, OG, being a hydrophilic molecule with two sulfonate groups, is unlikely to penetrate deeper into the hydrophobic core of BPA and hence less likely to bind near Trp-214. For these reasons, the inference drawn that OG binds near Trp-135 seems to be more feasible.

Although BPA is a single polypeptide chain, it is considered to be a multidomain protein (38). The conformational changes in BPA structure at the quaternary level have been investigated using time-resolved fluorescence anisotropy, which is related to the rotational dynamics of the emission dipole of the fluorophore. The fluorescence anisotropy decay due to two lifetime components were analyzed (Fig. 12). The measurement was carried out with 15 μM BPA, and excitation and emission wavelength were 295 and 344 nm, respectively.
indicating that it arises from a reorientation of Trps together with the whole protein and not because of the segmental motion of the Trps residues. Further, observation of a single and more or less same value of rotational correlation time for BPA and BPA-OG complex indicates that the overall globular structure of BPA remains unaltered on binding of OG despite certain internal rearrangement indicated by CD experiments in the protein structure as discussed above.

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