Binding Constant Measurement by Hyper-Rayleigh Scattering: Bilirubin−Human Serum Albumin Binding as a Case Study

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In this paper, a new application of the hyper-Rayleigh scattering technique in determining multiple binding constants of a small molecule like bilirubin to a macromolecule like the protein human serum albumin has been demonstrated. Human serum albumin has two binding sites for bilirubin, and the binding constants have been measured by carrying out a second harmonic titration of the protein against bilirubin and vice versa. The measured binding constants $K_1 = 1.5 \pm 0.43 \times 10^7\ M^{-1}$ and $K_2 = 1.01 \pm 0.16 \times 10^6\ M^{-1}$ agree well with the reported values obtained by other methods.

Introduction

Binding of small organic molecules and ligands to large macromolecules such as proteins and nucleic acids through noncovalent interactions is central to many biological processes.[1] High selectivity mediated by specific intermolecular interaction is a characteristic of biological binding phenomena. The ability of small molecules to discriminate between host proteins and large binding constants are important for sequestering and sensor activities. Modification of catalytic sites of an enzyme, distribution ratios between aqueous and nonaqueous phases, and interaction of a drug with a specific target are some of the areas of current interest. To develop a quantitative understanding of interactions of protein with small molecules, binding constants and binding energies of a large number of protein host−organic guest complexes have been evaluated and compared.[2]

Various spectroscopic methods such as absorption,[3] fluorescence, circular dichroism (CD),[4] NMR,[5] and electron spin resonance (ESR)[6] have been adopted in the past for measuring the binding constants of small molecules with large macromolecules. In all these methods, the free components (for example, the protein, P, and the ligand, L) and the bound complex (PL) are spectroscopically distinct and the difference in their response to a particular probe is exploited to derive the binding constant or the association constant, K_a, for the process. In absorption spectroscopy, binding generally produces a shift in the absorption spectrum and the difference in absorbance is monitored as a function of ligand concentration. It is assumed that the fractional change in absorbance is linearly proportional to the fractional saturation of binding sites, an assumption that breaks down in many cases. Of the many available techniques, fluorescence spectroscopy is widely used for binding constant determination.[7,8] In this technique, fluorescence intensity of the aromatic amino acids of the protein, extrinsic probes, or the ligand, which may alter upon binding, is monitored. This method is often complicated by the inner filter effect, energy transfer between various intrinsic chromophores of the protein, and the presence of an external fluorophore used in cases where both the protein and the ligand are nonfluorescent. For the magnetic resonance based spectroscopies, the required concentrations for binding measurements are generally high, of the order of 10^{-3}−10^{-4} M.

The binding of the small molecule bilirubin, a yellow pigment produced by the catabolism of hemoglobin from the red blood cells,[9] and the protein human serum albumin (HSA) in the blood plasma has been extensively studied in the past.[10] The literature reports on the number of binding sites vary from one to three, and up to three association constants from 10^4 to $10^7\ M^{-1}$ have been measured for this pair. For example, a CD investigation[11] reported three binding sites with binding constants $K_1 = 6.7 \times 10^6\ M^{-1}$, $K_2 = 3 \times 10^5\ M^{-1}$, and $K_3 = (2-3) \times 10^4\ M^{-1}$ in the absence of salt. The fluorescence quenching method[12] applied to the bilirubin−HSA binding yielded two binding constants with values of $6.4 \times 10^7\ M^{-1}$ and $2.6 \times 10^6\ M^{-1}$, whereas a fluorescence enhancement technique[13] showed only one binding constant of $1.31 \times 10^7\ M^{-1}$. A chemical method[14] based on peroxidase oxidation of the free bilirubin at equilibrium with HSA, estimated two binding constants which were in close agreement to the fluorescence measurements.

Incoherent second harmonic light scattering or hyper-Rayleigh scattering (HRS), which occurs in solution due to instantaneous orientational fluctuations of molecular dipoles,[15,16] is forbidden in centrosymmetric structures under the electric dipole approximation but nonzero otherwise. Any molecule which has an anisotropic electronic charge distribution over its length is likely to show second harmonic scattering in solution. The magnitude of the intensity of light depends on how anisotropic is the charge distribution and how low is its symmetry. The HRS technique has been employed, in the past, to measure a variety of physical properties such as the dissociation constant of a weak organic acid,[17] partition coefficient of a compound distributed in two immiscible solvents,[18] stability constant of cation binding to functionalized crown ethers,[19] and critical micelle concentration of surfactants.[20] The second harmonic (SH) scattered light intensity of a molecule in solution depends on its structure and electronic charge distribution over the entire backbone. If two noninteracting molecules scatter SH light from solution ($I_{2\omega}$), the response should be additive and one could

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TABLE 1: Measured Hyperpolarizabilities of Various Compounds

molecule	β [10^{-30} esu]	λ_{max} [nm]
HSA	430 ± 10	280
bilirubin	215 ± 5	432
pNA (MeOH)	22.4 ± 0.4	380
pNA (Water)	20 ± 0.2	372

write the following in terms of the molecular hyperpolarizability of the various species involved:[21]

$$\frac{I_{2\omega}}{I_{\omega}^{2}} = g[N_1\beta_1^{2} + N_2\beta_2^{2}] \quad (1)$$

where N's are the number densities of the respective species and β's are their first hyperpolarizabilities, g is the instrument factor, and I_ω is the incident light intensity. However, when a small molecule binds to a large molecule through noncovalent interaction, the second harmonic intensity generated by the bound complex is expected to be different (higher or lower) from that of the sum total of the individual components.

In this paper, we report a new application of the HRS technique where the binding constants of bilirubin with HSA have been measured. Both bilirubin and HSA show large second harmonic response on their own. We have shown how the second harmonic response from the complex can be analyzed to yield the two binding constants.

Experimental Section

Materials. HSA and crystalline bilirubin (bovine gall stone) were obtained from Sigma, USA. All other reagents used for the study were of analytical grade and were obtained locally. The concentration of HSA was determined spectrophotometrically using the absorbance at 280 nm and molecular weight 66 kDa.[22] Bilirubin solution was prepared by dissolving the crystals in 0.1 M sodium phosphate buffer (pH = 7.4), and its concentration was determined using the absorbance at 440 nm and the reported extinction coefficient $\epsilon = 47\,500\ M^{-1}\ cm^{-1}$.[23] There was no band observed in the UV spectrum around 490 nm, indicating that there was no colloid or aggregate formation in solution. Respective dilutions were made from the stock solutions. All solutions were kept in the dark and used within 3 h.

Experimental Setup. The HRS method has been described in detail previously.[24] In brief, the fundamental of a Q-switched Nd:YAG laser (LAB 170, Spectra Physics) with a 10 Hz rep. rate and 8 ns pulses was used as the light source. The laser power was kept below 18 mJ/pulse. The sample was taken in a cylindrical sample cell (volume = 20 mL), and the laser beam was focused 2−3 cm outside the cell using a plano-convex lens. The scattered photons at 532 nm were collected at 90° geometry using the monochromator (Jobin Yvon TRIAX 550) and a visible PMT (Photonis XP2262B). The signal from the PMT was averaged over 128 laser shots in a storage oscilloscope (Tektronix TDS3000B). Solutions of varying number densities of the order of 10^{15} molecules/cm^3 were prepared for measurements. The quadratic dependence of the observed second harmonic signal was checked (data not shown). The standard external reference method was employed for the measurement of first hyperpolarizability of the protein and bilirubin using *para*-nitroaniline (pNA) in water as an external reference[25] (Table 1). Water used for the measurements was double distilled. Two-photon fluorescence (TPF) contribution to the SH signal was checked by scanning the monochromator through the second harmonic (532 nm) wavelength. No TPF contribution to the second harmonic signal was observed.

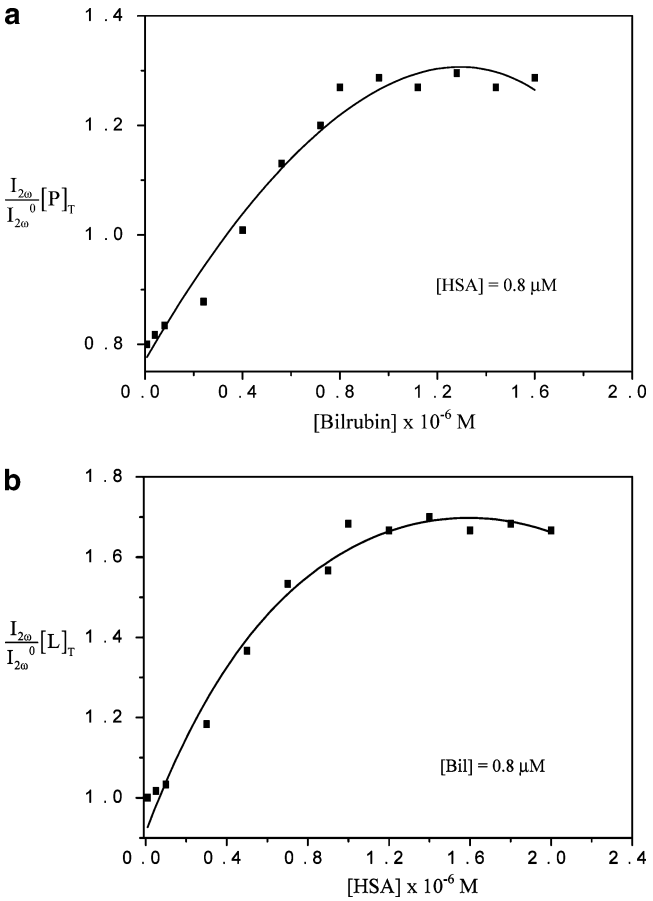

Figure 1. Second harmonic titration curves of bilirubin vs HSA and vice versa. The solid line is a nonlinear regression fit through the experimental points with $R^2 = 0.98$. (a) Initial HSA concentration at 0.8 μM. (b) Initial bilirubin concentration at 0.8 μM.

Results and Discussion

Binding of bilirubin to HSA was followed by carrying out titrations by adding aliquots of bilirubin solution to a fixed concentration of the protein. The solutions were prepared in protein/bilirubin mole ratios of 1.0:0.01−2.0 and equilibrated for an hour prior to each experiment. The SHG intensity from each solution was measured. The titration experiments were done at three different initial protein concentrations of 0.4×10^{-6}, 0.6×10^{-6}, and 0.8×10^{-6} M. A typical titration curve is shown in Figure 1a. The titration curve has a half stretched-S shape. The second harmonic scattered intensity reaches a high value at a protein/bilirubin mole ratio of 1:1 or thereafter and only increases monotonically as excess bilirubin is added. To verify the nature of the observed shape of the titration curve, reverse titrations were performed with fixed bilirubin concentrations at 0.4×10^{-6}, 0.6×10^{-6}, and 0.8×10^{-6} M and varying protein concentrations (a typical titration curve is shown in Figure 1b).

The titration curves of Figure 1a,b may be analyzed in many possible ways. For the two independent binding site model as applicable in the case of bilirubin−HSA binding, the simplest is to fit the curve with four parameters where the first hyperpolarizabilities, β's, of HSA and bilirubin measured in separate experiments have been used (Table 1). The data analysis method will be described in brief here.

Assuming that HSA has two independent binding sites for bilirubin, the two steps in the binding process are

$$P + L = PL \tag{2}$$

$$PL + L = PL_2 \tag{3}$$

The equilibrium constant for eq 2, K_1 is

$$K_1 = \frac{[PL]}{[P]_f[L]_f} \tag{4}$$

and for (3), K_2 is

$$K_2 = \frac{[PL_2]}{[PL][L]_f} \tag{5}$$

where [ ] represents concentration and the subscript "f" refers to the concentration of the "free" species in solution.

The mass balance equations for the total reaction are

$$[P]_f = [P]_T - [PL] - [PL_2] \tag{6}$$

$$[L]_f = [L]_{add} - [PL] - 2[PL_2] \tag{7}$$

where the subscript "add" refers to the added ligand concentration which is a variable in the titration when a fixed amount of protein is titrated with small aliquots of the ligand. From eqs 4−7, eliminating [PL] and [PL_2] and neglecting terms higher than quadratic in $[L]_f$, we get

$$\left(2[P]_T + \frac{1}{K_2} - [L]_{add}\right)[L]_f^2 + \left(\frac{1}{K_2}[P]_T - \frac{1}{K_2}[L]_{add} + \frac{1}{K_1K_2}\right)[L]_f - \frac{[L]_{add}}{K_1K_2} = 0 \tag{8}$$

Equation 8 yields

$$[L]_f = \left\{-\left(\frac{1}{K_2}[P]_T - \frac{1}{K_2}[L]_{add} + \frac{1}{K_1K_2}\right) \pm \sqrt{\left(\frac{1}{K_2}[P]_T - \frac{1}{K_2}[L]_{add} + \frac{1}{K_1K_2}\right)^2 + 4\left(2[P]_T + \frac{1}{K_2} - [L]_{add}\right)\left(\frac{[L]_{add}}{K_1K_2}\right)}\right\} \Big/ 2\left(2[P]_T + \frac{1}{K_2} - [L]_{add}\right)$$

where $P_1 = 1/K_1$, $P_2 = 1/K_2$, and the ligand added is the variable in this experiment which is X; then

$$[L]_f = \{-(P_2[P]_T - P_2X + P_1P_2) \pm \sqrt{(P_2[P]_T - P_2X + P_1P_2)^2 + 4(2[P]_T + P_2 - X)(P_1P_2X)}\} \Big/ 2(2[P]_T + P_2 - X) \tag{9}$$

The total signal intensity, $I_{2\omega}$, when the protein is titrated against bilirubin is given by

$$\frac{I_{2\omega}}{I_\omega^2} = g[(N_L)_f\beta_L^2 + (N_P)_f\beta_P^2 + (N_{PL})\beta_{PL}^2 + (N_{PL2})\beta_{PL2}^2] \tag{10}$$

The above equation can be expressed in terms of the concentration of the species as

$$\frac{I_{2\omega}}{I_\omega^2} = G[\beta_L^2[L]_f + \beta_P^2[P]_f + \beta_{PL}^2[PL] + \beta_{PL2}^2[PL_2]] \tag{11}$$

where $G = g \times 6.023 \times 10^{23}$ and the concentrations are expressed in moles/liter.

In the beginning of the titration curve, the observed SH intensity ($I_{2\omega}^0$) is the contribution from the protein HSA only and is given as

$$\frac{I_{2\omega}^0}{I_\omega^2} = G\beta_P^2[P]_T \tag{12}$$

From eqs 11 and 12,

$$\frac{I_{2\omega}}{I_{2\omega}^0} = \left[\frac{\beta_L^2}{\beta_P^2}\frac{[L]_f}{[P]_T} + \frac{[P]_f}{[P]_T} + \frac{\beta_{PL}^2[PL]}{\beta_P^2[P]_T} + \frac{\beta_{PL2}^2[PL_2]}{\beta_P^2[P]_T}\right] \tag{13}$$

$$\frac{I_{2\omega}}{I_{2\omega}^0} = \left[B\frac{[L]_f}{[P]_T} + \frac{[P]_f}{[P]_T} + P_3\frac{[PL]}{[P]_T} + P_4\frac{[PL_2]}{[P]_T}\right] \tag{14}$$

Substituting [PL] and [PL_2] from eqs 6 and 7, respectively, in eq 14 and simplifying, we get

$$\frac{I_{2\omega}}{I_{2\omega}^0}[P]_T = [P]_f\left[\left(\frac{P_4}{P_1P_2}\right)[L]_f^2 + \left(\frac{B}{[P]_f} + \frac{P_3}{P_1}\right)[L]_f + 1\right] \tag{15}$$

where $B = \beta_L^2/\beta_P^2 = 0.25$, $P_1 = 1/K_1$, $P_2 = 1/K_2$, $P_3 = \beta_{PL}^2/\beta_P^2$, and $P_4 = \beta_{PL2}^2/\beta_P^2$. The value of these four parameters, P_1, P_2, P_3, and P_4, can be accurately determined from the experimental data from several measurements by plotting $(I_{2\omega}/I_{2\omega}^0)[P]_T$ vs $[Bil]_{add}$ in the forward titration (Figure 1a).

Curve fittings were carried out in Origin 6.0. This software carries out curve fitting by nonlinear regression based on the Levenberg−Marquardt algorithm. To mention in brief, initially the iteration starts with a given set of parameters. Subsequently the sum of squared deviations of the theoretical curve from the experimental points is minimized by performing a series of iterations. The final set of parameters represents the best fit for the experimental data obtained.

A similar analysis was followed for the reverse titration curves where $(I_{2\omega}/I_{2\omega}^0)[L]_T$ vs $[HSA]_{add}$ plots were made (Figure 1b).

Table 2 lists the equilibrium constants obtained from the fits of the titration results. The average values of the equilibrium constants are $K_1 = 1.5 \pm 0.43 \times 10^7$ M^{-1} and $K_2 = 1.01 \pm 0.16 \times 10^6$ M^{-1}. From the goodness of the fit ($R^2 \sim 1$), the closeness of the values of the hyperpolarizability of the PL and PL_2 complexes, and the two binding constants, K_1 and K_2, from different sets of data, we conclude that the unknowns are accurately determined. The value of R^2 close to 1 guarantees the two unknown association constants K_1 and K_2 to be obtained within a narrow range which gives a certain confidence about the reliability of our method of analysis. The association constants determined by the HRS technique are also in agreement with the reported values.

The crystal structure of HSA has revealed the presence of two hydrophobic pockets which may correspond to the binding sites for a variety of apolar ligands. Site I lies in subdomain IIA and has been suggested to overlap with the bilirubin binding locus. Site II, which can also accommodate hydrophobic small molecules, lies in subdomain IIIA.[26,27] The results of the present

TABLE 2: Binding Constants, Fitted Parameters (See Text for Details) and Hyperpolarizabilities of the Complexes

initial concn [μM]	P_1	P_2	P_3	P_4	$K_1 \times 10^7$ [M^{-1}]	$K_2 \times 10^6$ [M^{-1}]	β_{PL} [10^{-30} esu]	β_{PL2} [10^{-30} esu]	R^2
0.4 HSA	0.066 ± 0.02	1.055 ± 0.21	1.40 ± 0.47	2.10 ± 0.55	1.51	0.95	508.8	623.1	0.99
0.6 HSA	0.107 ± 0.05	1.112 ± 0.31	1.50 ± 0.13	2.30 ± 0.71	0.94	0.89	526.6	652.1	0.95
0.8 HSA	0.054 ± 0.01	1.103 ± 0.12	1.34 ± 0.2	2.18 ± 0.6	1.85	0.91	497.8	634.9	0.98
0.4 Bil	0.047 ± 0.02	0.761 ± 0.1	6.11 ± 2.3	8.54 ± 1.7	2.13	1.31	531.4	628.3	0.94
0.6 Bil	0.084 ± 0.01	0.940 ± 0.09	5.71 ± 1.2	9.07 ± 1.8	1.20	1.06	513.8	647.5	0.96
0.8 Bil	0.072 ± 0.01	1.08 ± 0.03	5.62 ± 0.9	9.14 ± 1.3	1.39	0.93	509.7	650.0	0.98

study are consistent with the existence of two sites. A more detailed understanding of the molecular interactions involved in the binding process must await the structural characterization of the complex.

Conclusion

In this report, we have extended the hyper-Rayleigh scattering method for the measurement of the binding constant of a small molecule to a large molecule with bilirubin binding to HSA as a test case. This is also an example of a ligand binding to two independent sites in a macromolecule. The values of the two association constants, K_1 and K_2, have been determined fairly accurately. This technique does not depend on the intrinsic spectroscopic properties of the molecules involved and is independent of the wavelength of measurement and thus can be applied, in principle, to study interactions between molecules which lack suitable chromophores. This is a fairly sensitive technique, and with large values of hyperpolarizability, it is possible to make measurements even at nanomolar concentrations. Unlike in absorption and fluorescence based techniques where the spectral property has to change upon binding, this technique is independent of the actual spectral properties of the species involved. This HRS binding experiment can also be extended to monitor changes in binding parameters upon variation in the ionic strength, pH, and temperature of the solution. The elucidation of noncovalent interaction in biomolecules using HRS in this work paves the way for more systems to be explored soon using this technique.

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