J|A|C|S
COMMUNICATIONS

Published on Web 04/19/2006

Site-Selective Binding and Dual Mode Recognition of Serum Albumin by a Squaraine Dye

Vadakkancheril S. Jisha, Kalliat T. Arun, Mahesh Hariharan, and Danaboyina Ramaiah*
Photosciences and Photonics, Regional Research Laboratory (CSIR), Trivandrum 695019, India
Received February 23, 2006; E-mail: rama@csrrltrd.ren.nic.in; d_ramaiah@rediffmail.com

Serum albumin is the major protein constituent of blood plasma which facilitates the disposition and transport of various exogenous and endogenous ligands to the specific targets.[1] The specific delivery of ligands by serum albumin originates from the presence of two major and structurally selective binding sites, namely, site I and site II, which are located in three homologous domains that form a heart-shaped protein.[2] The binding affinity offered by site I is mainly through hydrophobic interactions, whereas site II involves a combination of hydrophobic, hydrogen bonding, and electrostatic interactions.[3] Recently, it has been reported that molecules possessing higher affinity for serum albumin and showing preferential binding at site II are found to exhibit efficient photodynamic therapeutical applications (PDT).[4] Therefore, detection and understanding of the nature and selective binding interactions of ligands with serum albumin is important for the design of efficient drugs and sensitizers for use in PDT.

Squaraines form a class of novel dyes possessing sharp and intense absorption in the red to near-infrared region and hence are the object of intense investigations as molecular components of technological applications.[5] These include electrophotography, optical data storage,[5] solar cells,[5] ion and molecular sensors,[6,7] and nonlinear optics.[8] Recently, we have proposed squaraines as a possible new class of photosensitizers for PDT because of their highly favorable photophysical and in vitro photobiological properties.[9] Herein we report the spectroscopic and microscopic evidence for the interactions of a squaraine dye (SQ), having polyhydroxyl groups with bovine serum albumin (BSA), and demonstrate that this dye binds with protein site selectively and with high association constant involving a combination of hydrophobic, hydrogen bonding, and electrostatic interactions. To the best of our knowledge, this is the first report wherein site-selective binding can be visualized through dual mode recognition of visible color change and "turn on" fluorescence,[10] and hence SQ can be used for noncovalent labeling of serum albumin.

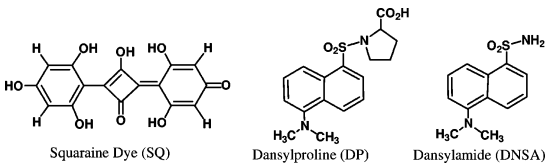

Figure 1A shows the absorbance changes of SQ with addition of BSA. The initial addition of BSA (up to 1.5 μM) led to the decrease in absorbance at 584 nm, corresponding to the squaraine chromophore. However, the subsequent additions resulted in a gradual increase in absorbance, but with the formation of a new band at 610 nm. The formation of bathochromic-shifted band interestingly resulted in visualization of color change from pinkish-red (SQ alone) to bluish in the presence of BSA (Figure 1B). Half-reciprocal analysis of the absorption data (inset of Figure 1A) gave a 1:1 stoichiometry for the complex between SQ and BSA, with an association constant of $(1.4 \pm 0.1) \times 10^6$ M^{-1} and change in

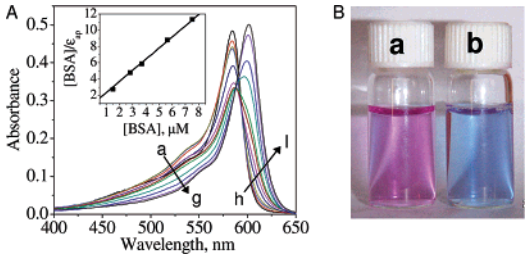

Figure 1. (A) Change in absorbance of SQ (3.0 μM) with increase in addition of BSA in phosphate buffer. [BSA] (a) 0, (g) 1.5, (h) 1.8, (l) 7 μM. Inset shows the half-reciprocal plot. (B) Visible detection of BSA, (a) pinkish-red, SQ alone; (b) bluish in color, SQ in the presence of BSA (7 μM).

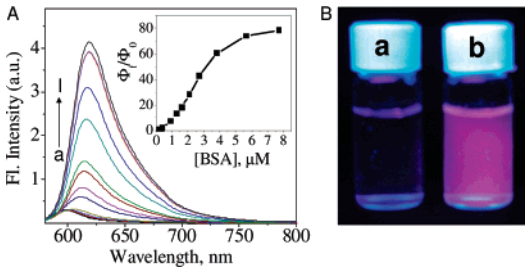

Figure 2. (A) Change in fluorescence intensity of SQ (3.0 μM) with the addition of BSA in phosphate buffer. [BSA] (a) 0, (g) 1.5, (h) 1.8, (l) 7 μM. Excitation wavelength, 560 nm. Inset shows the corresponding fluorescence quantum yields. (B) Detection of BSA through turn on fluorescence, (a) SQ alone and (b) SQ in the presence of BSA (7 μM).

free energy of -35 kJ/mol. Figure 2A shows the corresponding changes in fluorescence spectra. Addition of BSA gave initially a gradual enhancement in fluorescence intensity, with a bathochromic shift in emission from 600 to 620 nm. However, the subsequent additions yielded significant changes, and finally, ca. 80-fold enhancement in fluorescence quantum yields was observed at 7 μM of BSA (inset of Figure 2A). This significant turn on intensity with a bathochromic shift of around 20 nm led to the visual observation of fluorescence, as shown in Figure 2B. Picosecond time-resolved fluorescence analysis indicated that SQ alone exhibits a monoexponential decay with a lifetime of 121 ps, whereas biexponential decay with significantly increased lifetimes of 0.5 ns (60%) and 1.5 ns (40%) was observed in the presence of BSA. On the basis of the fluorescence quantum yields and lifetimes, we calculated nonradiative decay rate constants (k_{nr}) that are found to be 8.3×10^9 and 7.6×10^8 s^{-1}, respectively, in the absence and presence of BSA (Figure S1, Table S1, Supporting Information).

The complex formation between SQ and BSA was further confirmed by circular dichroism (CD), cyclic voltammetry (CV), and ^{1}H NMR techniques.[11] Increase in addition of SQ resulted in decrease in intensity of CD signals at 208 and 217 nm, corresponding to BSA, with formation of a new band at 230 nm and an induced CD signal at 610 nm, corresponding to the SQ moiety. In the ^{1}H NMR spectrum, an upfield shift of about $\Delta\delta$ 0.1 ppm and

10.1021/ja061301x CCC: $33.50

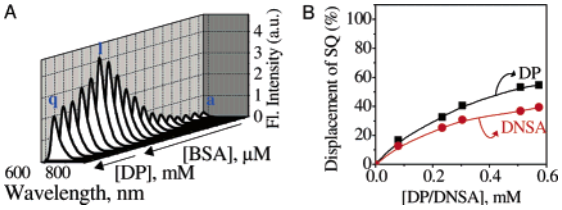

Figure 3. (A) Fluorescence spectra of SQ with the addition of BSA (a) 0 and (l) 7 μM, followed by the addition of DP (l) 0 and (q) 0.6 mM. Excitation wavelength, 560 nm. (B) Displacement of SQ (%) from the BSA−SQ complex by the addition of DP (black, ■) and DNSA (red, ●).

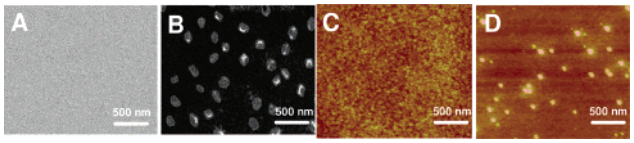

Figure 4. SEM (A,B) and AFM (C,D) images. A and C: BSA (18 μM) alone. B and D: BSA (18 μM) in the presence of SQ (9.4 μM).

broadening of peaks, corresponding to SQ aromatic protons, were observed in the presence of BSA. Similarly, the cyclic voltammogram of SQ showed a significant decrease in current intensity of 7.6 μA (37%) with the addition of BSA (Figures S2−S4, Supporting Information). The observation of induced CD signal, upfield shift of protons, and the decrease in current intensity of SQ in the presence of BSA confirms the formation of a stable and noncovalent complex between SQ and BSA.

To understand the site-selective binding of SQ with BSA, we employed ligand displacement cum fluorimetry using known site-selective binding ligands, such as dansylamide (DNSA, for site I) and dansylproline (DP, for site II).[4b] Addition of DP to a solution of the BSA−SQ complex resulted in gradual decrease of fluorescence intensity (Figure 3A) and then reached saturation at 0.6 mM of DP, with an effective displacement of about 60%. Similar results were obtained with DNSA; however, the effective displacement was found to be around 40% (Figure 3B). The titrations of DP and DNSA with SQ alone showed negligible changes in absorption and emission properties, indicating thereby that the changes observed with the BSA−SQ complex were mainly due to the displacement of SQ from the complex by the binding ligands, such as DP and DNSA (Figure S5−S9, Supporting Information).

Figure 4 shows scanning electron (SEM) and tapping mode atomic force (AFM) microscopic images of BSA in the absence and presence of SQ. SEM images of BSA alone showed a regular structure, which drastically changed upon complexation with SQ to evenly distributed features with a width of 100 ± 10 nm. Similar observations were made with AFM, wherein the regular structure[12] with a mean roughness of 2 ± 0.1 nm changed to evenly distributed features with a width of 75 ± 10 nm and a mean roughness of 8.5 ± 1.5 nm, on complexation with SQ.

Squaraine dye, SQ, is an interesting system, which is quite soluble in phosphate buffer and interacts efficiently with BSA. At low concentrations of BSA (up to 1.5 μM), we observed decrease in absorbance of SQ, which we attribute to the formation of a tight complex with BSA at site I, involving π-stacking and hydrophobic interactions. This interpretation is based on the following facts: (i) the site I has a relatively small cavity size of 2.53 Å; (ii) tryptophan, the aromatic amino acid (Trp 214),[2] is located only at site I; (iii) we observed a minor component (40%) with relatively longer lifetime of 1.5 ns; and (iv) titration with the site I specific binding ligand, such as DNSA, led to 40% of SQ displacement from the complex. The small cavity size of site I and presence of tryptophan permits the formation of a tight BSA−SQ complex, involving π-stacking and hydrophobic interactions, which results in the longer lifetime of SQ bound at this site.

The observation of significant increase in absorbance and fluorescence yields of SQ at and above 1.5 μM concentrations of BSA could be attributed to the binding of SQ at site II with a relatively larger cavity size of 2.6 Å and involving a combination of hydrophobic, hydrogen bonding, and electrostatic interactions. The absence of tryptophan residue in the hydrophobic cavity of site II and the formation of a relatively loose complex at this site led to the observation of significant bathochromic shifts in absorption and fluorescence emission. This interestingly enabled us to visualize the binding of protein at site II through dual mode recognition. Evidence for the loose binding of SQ predominantly at site II comes from the fact that we observed a major component (60%) with a relatively shorter lifetime of 0.5 ns. Moreover, the titration with the site II specific binding agent, such as DP, led to 60% displacement of SQ from the complex.

In conclusion, we demonstrated that SQ acts as a dual mode recognition system for serum albumin in buffer. The significant enhancement in emission quantum yields and lifetimes suggests that SQ can act as a novel noncovalent label in immunochemical assay and biophysical studies. The uniqueness of this molecular system is that it is quite soluble in buffer and interacts with protein selectivity at site II and signals the event by visual color change and turn on fluorescence. Further studies are in progress to understand the site-selective interactions of various squaraine dyes and their potential PDT and diagnostic applications.

Acknowledgment. This work was funded by CSIR and DST, Govt. of India. This is contribution no. PPD-209 from RRLT.

Supporting Information Available: Experimental and calculation details, Table S1 and Figures S1−S9 showing photophysical, CD, and CV properties of SQ in the presence and absence of BSA, DNSA, and DP. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Peters, T. *All about Albumin*: *Biochemistry, Genetics and Medicinal Applications*; Academic Press: San Diego, CA, 1996. (b) Kumar, C. V.; Buranaprapuk, A.; Opiteck, G. J.; Moyer, M. B.; Jockusch, S.; Turro, N. J. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 10361. (c) Kumar, C. V.; Buranaprapuk, A. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 2085.
(2) (a) He, X. M.; Carter, D. C. *Nature* **1992**, *358*, 209. (b) Dockal, M.; Carter, D. C.; Ruker, F. *J. Biol. Chem.* **1999**, *274*, 29303.
(3) (a) Lhiaubet-Vallet, V.; Sarabia, Z.; Bosca, F.; Miranda, M. A. *J. Am. Chem. Soc.* **2004**, *126*, 9538. (b) Jimenez, M. C.; Miranda, M. A.; Vaya, I. *J. Am. Chem. Soc.* **2005**, *127*, 10134.
(4) (a) Szacilowski, K.; Macyk, W.; Drzewiecka-Matuszek, A.; Brindell, M.; Stochel, G. *Chem. Rev.* **2005**, *105*, 2647. (b) Pandey, R. K.; Constantine, S.; Tsuchida, T.; Zheng, G.; Medforth, C. J.; Aoudia, M.; Kozyrev, A. N.; Rodgers, M. A. J.; Kato, H.; Smith, K. M.; Dougherty, T. J. *J. Med. Chem.* **1997**, *40*, 2770. (c) Bonnett, R. *Chemical Aspects of Photodynamic Therapy*; Gordon and Breach Science Publishers: The Netherlands, 2000.
(5) (a) Fabian, J.; Nakazumi, H.; Matsuoka, M. *Chem. Rev.* **1992**, *92*, 1197. (b) Law, K. Y. *Chem. Rev.* **1993**, *93*, 499.
(6) (a) Zhong, Z.; Anslyn, E. V. *J. Am. Chem. Soc.* **2002**, *124*, 9014. (b) Wallace, K. J.; Gray, M.; Zhong, Z.; Lynch, V. M.; Anslyn, E. V. *Dalton Trans.* **2005**, 2436.
(7) (a) Ros-Lis, J. V.; Garcia, B.; Jimenez, D.; Martinez-Manez, R.; Sancenon, F.; Soto, J.; Gonzalvo, F.; Vallendecabres, M. C. *J. Am. Chem. Soc.* **2004**, *126*, 4064. (b) Martinez-Manez, R.; Sancenon, F. *Chem. Rev.* **2003**, *103*, 4419.
(8) Kolev, T. M.; Yancheva, D. Y.; Stoyanov, S. I. *Adv. Funct. Mater.* **2004**, *14*, 799.
(9) (a) Arun, K. T.; Ramaiah, D. *J. Phys. Chem. A* **2005**, *109*, 5571. (b) Jyothish, K.; Arun, K. T.; Ramaiah, D. *Org. Lett.* **2004**, *6*, 3965. (c) Ramaiah, D.; Eckert, I.; Arun, K. T.; Weidenfeller, L.; Epe, B. *Photochem. Photobiol.* **2004**, *79*, 99. (d) Ramaiah, D.; Eckert, I.; Arun, K. T.; Weidenfeller, L.; Epe, B. *Photochem. Photobiol.* **2002**, *76*, 672.
(10) de Silva, A. P.; Gunaratne, H. Q. N.; Gunnlaugsson, T.; Huxley, A. J. M.; McCoy, C. P.; Rademacher, J. T.; Rice, T. E. *Chem. Rev.* **1997**, *97*, 1515.
(11) Neelakandan, P. P.; Hariharan, M.; Ramaiah, D. *Org. Lett.* **2005**, *7*, 5765.
(12) Xu, L. C.; Rodriguez, V. V.; Logan, B. E. *Langmuir* **2005**, *21*, 7491.

JA061301X