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Communications

Enantioselective Quenching of Room-Temperature Phosphorescence Lifetimes of Proteins: Bovine and Human Serum Albumins

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Enantioselective quenching of the room-temperature phosphorescence (RTP) lifetime of proteins was demonstrated due to the effects of various external chiral quenching agents. In the absence of quenchers, the RTP lifetimes for bovine serum albumin (BSA) and human serum albumin (HSA) were found to be 5.0 ± 0.2 and 4.0 ± 0.1 ms, respectively. The addition of various chiral quenchers (three pairs of binaphthols and two pairs of β-blockers) into the deoxygenated sample solutions containing BSA and HSA reduced their RTP lifetimes significantly, i.e., from ca. 4−5 ms (in the absence) to an average lifetime of ca. 1−2 ms (in the presence) of the chiral quenchers. For the R and S enantiomers examined, marked differences in RTP lifetimes were observed, i.e., ranging from ca. 20−29% for the binaphthols to ca.14−16% for the β-blockers. Such findings could lead to a better understanding of the relationship between chirality, dynamics/conformational changes, and biological functions of proteins.

Introduction

Since the first observation of protein phosphorescence at room temperature by Saviotti and Galley,[1] there has been increasing interest in the use of room-temperature phosphorescence (RTP) as an alternative/complementary spectroscopic technique for probing the structure, dynamics, and function of biological systems, mainly based on the measurement of the phosphorescence from tryptophan residues buried within the cores of protein molecules.[2,3] In contrast to tryptophan fluorescence decays, RTP lifetimes of proteins are primarily governed by the local microenvironments (e.g., flexibility) of the tryptophan residues. And importantly, the extension of the excited-state decay time scale from the nanosecond to the sub-millisecond−second range allows for valuable information concerning protein structure and dynamics to be obtained with extreme sensitivity.[4]

Chirality is an intrinsic property of many biological systems, and the unique chiral microenvironments provided by various host molecules, ranging from proteins[5] to cyclodextrins[6] to lanthanide complexes,[7] have allowed for chiral discrimination to occur in the triplet excited states, based on RTP detection of the phosphorescent chiral analytes. To the best of our knowledge, however, the exploitation of the tryptophan RTP of proteins for the chiral discrimination of enantiomeric molecules as well as for the study of protein structure and dynamics, based on stereoselective quenching of RTP lifetimes of proteins by chiral molecules, has never been reported. Note that the use of achiral quenchers with different physicochemical properties (e.g., sizes, charges, spin multiplicities, etc.) to alter tryptophan RTP lifetimes has been demonstrated to be an effective approach for studying protein microenvironments, structure, and dynamics.[2−4]

Experimental Section

Enantiomer standards were obtained from Sigma-Aldrich (St. Louis, MO). Sample solutions were prepared by adding appropriate volumes

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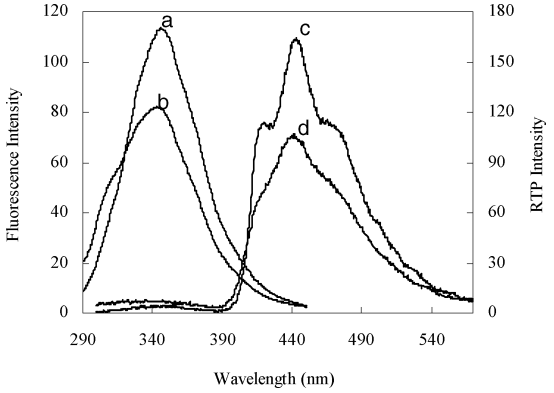

Figure 1. Fluorescence emission spectra of (a) BSA (1 μmol/L) and (b) HSA (2 μmol/L). RTP emission spectra of (c) BSA (5 μmol/L) and (d) HSA (10 μmol/L). RTP: λ_{ex} = 288 nm, delay time = 0.04 ms, and gate time = 2.00 ms.

of KI and Na_2SO_3 into the bovine serum albumin (BSA) or human serum albumin (HSA) stock solution (1×10^{-4} mol/L), followed by the addition of appropriate amounts of chiral quenchers and dilution to the mark of a volumetric flask (10 mL) using Tris-HCl buffer (0.1 mol/L). The sample solutions were let stand for approximately 10 min and transferred into a 1 cm quartz cell. The fluorescence and RTP spectra were obtained using a LS-50B spectrofluorimeter (Perkin-Elmer), and all measurements were carried out at 20 °C. The excitation and emission slits were typically set at 5 and 10 nm, respectively. For RTP measurements, the delay time and the gate time were set at 0.04 and 2.00 ms, respectively. RTP lifetimes were measured in the time domain using a TimeMaster lifetime spectrometer (Photo Technology International, PTI). The light source consisted of a nitrogen laser (pulse width 600 ps, luminescent frequency 15 Hz, transient power 15−20 kW, main output wavelength 337.1 nm), which was used to pump a dye laser (rhodamine 580), and its emission was passed through a frequency doubler to generate pulsed energy with a wavelength of 290 nm for sample excitation. The phosphorescence decay curves and lifetimes were analyzed and determined using a multiexponential fitting software (TimeMaster) provided by PTI.

Results and Discussion

Figures 1a and 1b show the fluorescence emission spectra of BSA and HSA, respectively, with the spectral profiles similar to those previously reported for the tryptophan fluorescence of various proteins (e.g., the emission maximum centered at ca. 345 nm).[8] Due to experimental difficulties encountered in the detection of RTP signals, the phosphorescent quantum yield of BSA was enhanced via the addition of iodide to introduce the so-called "heavy atom" effect and the use of sodium sulfite for chemical deoxygenation.[9] As shown in Figure 1c, relatively strong phosphorescent signal intensity can be obtained for the RTP emission spectrum of BSA, with spectral characteristics very similar to those previously reported for tryptophan RTP of various proteins (in the absence of iodide),[1,8] i.e., the appearance of RTP emission between ca. 400 and 570 nm, and the major and minor peak maxima located at ca. 443 nm and 424/469 nm, respectively. Similarly, Figure 1d shows that the RTP emission spectrum of HSA and the heavy atom induced RTP spectral profile of HSA were found to be similar to those of BSA as shown in Figure 1c. Importantly, the good match in spectral characteristics between tryptophan RTP of proteins as reported in the literature[1,8] and those shown in Figures 1c and 1d supported that the addition of iodide was effective in enhancing RTP emission intensity of tryptophan residues present in BSA and HSA.

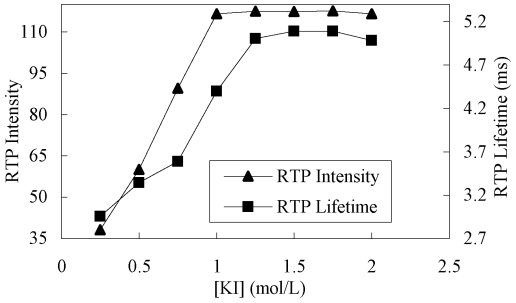

Figure 2. Plots of the RTP intensity and the lifetime of BSA as a function of KI concentration

Figure 2 shows the changes in RTP intensity and lifetime of BSA as a function of increasing KI concentration, and the results indicated that both of these parameters increased markedly once the KI concentration exceeded ca. 0.5 mol/L but remained relatively constant at concentrations between 1 and 2 mol/L. (Similar results were also obtained for HSA.) The effect of sodium sulfite concentration was also investigated, and the RTP intensity and lifetime of BSA/HSA were found to remain constant at concentrations between 1 and 3 mmol/L. The optimal concentrations of KI and Na_2SO_3 chosen for the RTP study of both BSA and HSA were 1.5 mol/L and 2 mmol/L, respectively.

Through the use of optimal RTP conditions in terms of the heavy atom (iodide) and oxygen scavenger (sulfite) concentrations as well as sample pH (9.55 for BSA and 7.40 for HSA), the RTP lifetimes for BSA and HSA were found to be 5.0 ± 0.2 and 4.0 ± 0.1 ms, respectively. The addition of chiral quenchers into the deoxygenated BSA sample solutions reduced the RTP lifetimes significantly, i.e., from ca. 5 ms (in the absence) to an average lifetime of ca. 2 ms (in the presence) of an external chiral quencher. As shown in Table 1, differences in the RTP lifetimes were obtained for all of the pairs of R and S enantiomers examined. For the three pairs of chiral binaphthols (commonly employed as chiral reagents in asymmetric synthesis), 1,1′-bi-2-naphthol (BNOH), 1,1′-binaphthyl-2,2′-diylhydrogenphosphate ($BNPO_4$), and 1,1′-binaphthyl-2,2′-diamine ($BNNH_2$), the percentage lifetime differences ranged from 20.4% to 29.4%, whereas for the two pairs of chiral cardiovascular drugs (β-blockers), atenolol and propranolol, the percentage lifetime difference was smaller, in the range between 15.6% and 16.6%.

The decrease in RTP lifetime for BSA due to the addition of the various chiral agents was examined by fitting the data to the Stern−Volmer equation, $\tau_0/\tau = 1 + K_{SV}[Q]$, where τ_0 and τ are the RTP lifetimes of BSA in the absence and the presence of a quencher (Q), respectively. A plot of τ_0/τ versus [Q] yielded the corresponding Stern−Volmer quenching constants (K_{SV}) as shown in Table 1 for BSA in the presence of different chiral quenchers. For the three pairs of binaphthols, the lifetimes for all of the R isomers were shorter than those of the S isomers, which are consistent with the comparatively higher K_{SV} values obtained for all R isomers. Similar results were also obtained for HSA; e.g., the RTP lifetime decreased markedly from ca. 4 to 1 ms (average) upon the addition of chiral quenchers; the average percentage lifetime differences for the three binaphthols (data not shown) and two β-blocker drugs were ca. 22% and 17%, respectively, and the K_{SV} ratios for the chiral agents fell in the range between 1.2 and 1.6. Note that good correlation coefficients (falling in the range between 0.990 and 0.999) were obtained for both BSA and HSA systems, indicating that the quenching data (decrease in RTP lifetimes) fitted well with the Stern−Volmer treatment within the concentration ranges studied (typically between ca. 1 and 40 μmol/L, except for atenolol,

Table 1. RTP Lifetime Differences and Stern−Volmer Quenching Constants of BSA/HSA in the Presence of Different Chiral Quenchers

	chiral quenchers (μmol/L)[a]	RTP lifetime (ms)[b] R isomer	RTP lifetime (ms)[b] S isomer	lifetime difference (%)	K_{SV} (L mol^{-1}) R isomer	K_{SV} (L mol^{-1}) S isomer	K_{SV}(R)/K_{SV}(S) ratio
BSA	BNOH (8)	1.2 ± 0.1	1.5 ± 0.1	20.4	3.4×10^5	2.6×10^5	1.3
	$BNPO_4$ (5)	2.1 ± 0.1	2.8 ± 0.1	26.7	2.9×10^5	2.1×10^5	1.4
	$BNNH_2$ (8)	1.2 ± 0.1	1.6 ± 0.1	29.4	2.3×10^5	1.2×10^5	1.9
	atenolol (300)	3.5 ± 0.2	3.0 ± 0.1	15.6	3.6×10^3	4.1×10^3	0.87
	propranolol (20)	1.2 ± 0.1	1.1 ± 0.1	16.6	3.0×10^5	5.9×10^5	0.51
HSA	atenolol (400)	1.1 ± 0.1	1.3 ± 0.1	13.6	4.5×10^3	3.9×10^3	1.2
	propranolol (22)	0.8 ± 0.0	1.0 ± 0.1	20.8	1.4×10^5	1.1×10^5	1.3

[a]Concentrations of chiral quenchers at which maximum lifetime differences were observed. [b] An average of five determinations. [BSA] = 5×10^{-6} mol/L. [HSA] = 1×10^{-5} mol/L. RTP lifetimes were measured using a TimeMaster lifetime spectrometer (Photon Technology International), consisting of a nitrogen-pumped dye laser system and a frequency doubler (λ_{ex} = 290 nm, λ_{em} = 443 nm).

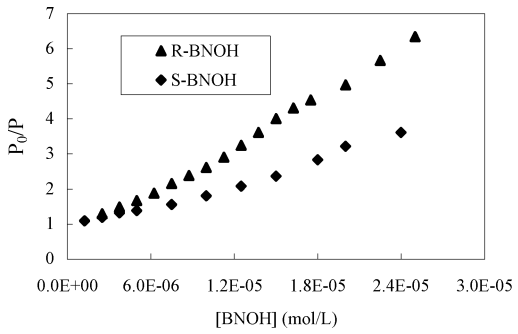

Figure 3. Stern−Volmer plot. Ratio of RTP intensity of BSA as a function of the chiral quenchers (BNOH enantiomers)

which was between ca. 25 and 700 μmol/L). For comparison, the addition of a pair of geometric isomers, coproporphyrin I and III (of similar size as the various optical isomers shown in Table 1), into the deoxygenated sample solutions under optimized RTP conditions were also found to induce to a lifetime difference, but the magnitudes (ca. 8.6% and 5.5% for BSA and HSA, respectively) were markedly smaller than those of the enantiomers and were much closer to the experimental uncertainty of ca. 5%.

The relatively large lifetime differences observed for both groups of chiral quenchers (binaphthols and β-blockers) in comparison to the geometric isomers (coproporphyrin I and III) suggested that the larger lifetime differences obtained for the optical isomers were based on some types of enantioselective perturbations that occurred between the residues within BSA and HSA upon interactions with the R and S isomers of the chiral quenchers. Figure 3 shows a decrease in the RTP intensity of BSA (P_0 in the absence, P in the presence of the chiral quencher) to the concentration of the chiral quencher (R−BNOH or S−BNOH) by fitting the data to Stern−Volmer equation, $P_0/P = 1 + K_{SV}$[BNOH]. It can be seen that an upward curvature appeared in the Stern−Volmer plots for both chiral quenchers. In the case of fluorescence quenching studies with proteins, upward curving Stern−Volmer plots tend to suggest that in addition to a collisional-based quenching process other quenching mechanism, e.g., based on static quenching, have to be taken into consideration.[10,11] In the present RTP quenching studies with BSA and HSA, the interpretation of quenching data such as that shown in Figure 3 is not straightforward since the observed RTP lifetime reduction could arise from a number of factors in addition to nonradiative energy transfer, including an alteration in the accessibility to the heavy atom (iodide), an influence on the local flexibility of the tryptophan microenvironment, as well as the number of tryptophan residues within the proteins.

For the two chiral drugs (atenolol and propanolol) studied, it is interesting to note from Table 1 that in the presence of BSA the lifetimes of the R isomers were longer than those of the S isomers, corresponding to the comparatively smaller K_{SV} values obtained for the R isomers (K_{SV} ratios <1). In contrast, when the RTP emitting protein was HSA, the lifetimes of the R isomers were shorter than those of the S isomers, consistent with the comparatively higher K_{SV} values obtained for all R isomers (K_{SV} ratios >1). These findings appear to indicate that different quenching/binding mechanisms may be involved in the stereoselective interactions of these two chiral drugs with BSA and HSA. Note that although the chiral interactions of small molecules (such as binaphthols and β-blockers) with serum proteins have been thoroughly studied and it is known that there is only one tryptophan residue in HSA, whereas two tryptophan residues can be found in BSA,[12] the exact chiral resolution/discrimination mechanisms for various chiral analytes are still not well understood, especially for BSA.[13]

Concluding Remarks

Stereoselective quenching of RTP lifetimes of proteins such as BSA and HSA, due to the effects of external chiral quenchers, is reported for the first time. Such findings should stimulate the further development and optimization (especially in terms of the magnitude of lifetime differences) of tryptophan RTP lifetime spectroscopy for the rapid chiral discrimination of a wide range of enantiomers (including nonfluorescent/phosphorescent chiral analytes) as well as the fundamental investigation of mechanisms involved in the chiral quenching of the RTP lifetimes of various proteins. However, the sensitivity of tryptophan RTP lifetimes toward the chirality of external quenchers should allow for novel insights to be gained (within the millisecond or longer time scale) associated with slow dynamic processes, such as conformational changes induced by protein interactions with chiral drugs and substrates. Hopefully, such information could lead to a better understanding of the relationship between chirality, dynamics/conformational changes, and biological functions of proteins.

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