Photophysical Consequences of Coupling Bacteriochlorophyll *a* with Serine and its Resulting Solubility in Water¶

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ABSTRACT

We investigated the dependence on solvents of optical absorption and emission of the bacteriochlorophyll *a*-serine (BChl-ser), a water soluble bacteriochlorophyll (BChl) derivative. Comparison between the experimental data and those collected for BChl in nonaqueous solvents shows that only a minor interaction takes place between serine and the macrocycle's π-electron system. Nevertheless, the coupling with serine results in a small enhancement of the nonradiative relaxation rate from the first excited singlet state S_1. In buffered aqueous solution (pH = 7.4), the Stokes shift of the BChl-ser fluorescence and its nonradiative relaxation rate are enhanced compared with those in nonaqueous solutions (Scherz, A., S. Katz, Y. Vakrat, V. Brumfeld, E. Gabelmann, D. Leupold, J. R. Norris, H. Scheer and Y. Salomon (1998) *Photosynthesis: Mechanisms and Effects*, Vol. V (Edited by G. Garab), pp. 4207–4212. Kluwer Academic, Dordrecht.), probably as a result of a hydrogen bonding between the BChl macrocycle and the water molecules. In aprotic solvents, without hydrogen bonds, the permanent dipole moment of the first excited singlet state in both BChl and BChl-ser is increased compared with the ground state by at least 2.5 Debye.

INTRODUCTION

The photophysics of bacteriochlorophyll *a* (BChl)† has received considerable attention during the last three and a half decades to understand the functions of the molecule as a component in antennae and in reaction centers of photosynthetic bacteria. BChl is responsible for the intense absorption and the very fast energy transfer through the antenna system and eventually to the reaction center, as well as for the following ultrafast charge separation across the photosynthetic membrane.

In addition to the ongoing research of BChl in the context of photosynthesis, there is a growing interest in the medical applications of BChl and its derivatives, in particular, in considering the spectroscopic and photochemical properties of BChl, which make it a promising sensitizer for photodynamic therapy (PDT) (1,2).

However, the poor solubility of the native compound in water limits its application to micellar or liposomic formulation. Furthermore, the hydrophobicity of the sensitizer may also slow down its clearance from normal tissue (3,4).

Recently it became possible to render BChl water-soluble by esterification with a polar amino acid such as serine methyl ester (5). Initial tests (3,4) have indeed shown that a 17^3 ester between bacteriochlorophyllide *a* and serine methyl ester (BChl-ser) is an improved PDT sensitizer compared with BChl, which calls for basic photophysical investigations of this new molecule.

BChl is known to show a different solvatochromism in polar and nonpolar solvents (6). Here we focused on a series of polar solvents, including water, and investigated the dependence of the Q_x and Q_y transition energies, the fluorescence lifetime and the fluorescence quantum yield of BChl-ser on the solvent tested. In interpretating the data we aimed to separate the influences of: (1) the serine interactions with the BChl moiety; and (2) the effect of an aqueous environment on the photophysical behavior of BChl. We show that the spectroscopic properties of the BChl moiety are practically not affected by the serine residue. The evaluation of this data allows, for the first time, to investigate the photophysical properties of monomeric BChl in an aqueous environment.

MATERIALS AND METHODS

Materials. BChl-ser was synthesized as described in Fiedor *et al.* (5). All solvents used were of spectroscopic grade and were acquired from the following sources. Diethylether, dichloromethane, acetone, dimethylsulfoxide (DMSO), ethanol, chloroform, methanol, tetrahydrofuran (THF) and dioxane from Merck Uvasol; isobutanol, acetophenone and pyridine from Aldrich; quinoline (distilled before measuring) from Chemapol; phosphate-buffered solution (PBS, pH = 7.4) from Sigma.
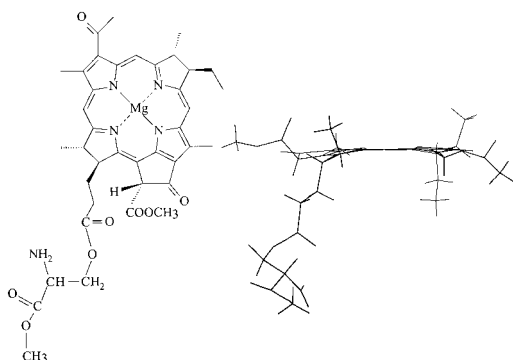
Figure 1. Structural formula of BChl-ser (left) and conformation (side view, right) calculated with a molecular mechanics force field (30).

Figure 2. Absorption spectra of BChl and BChl-ser dissolved in acetone.

Experimental. Absorption spectra were recorded using a Lambda-19 spectrometer from Perkin–Elmer.

A Fluorolog model FL-112 (Spex Industries) fluorimeter was used to measure the steady-state fluorescence. To avoid reabsorption effects, all fluorescence data were taken from solutions with low optical densities (OD < 0.1 at the absorption maximum). BChl in diethyl ether (Φ_{fl} = 0.19) was used as a standard to calculate the absolute values of the fluorescence quantum yields Φ_{fl} in other solvents and of other pigments (7). The fluorescence emission was excited at 740 nm.

Fluorescence lifetimes were measured using a nitrogen laser (MSG 850/LTB Berlin)–pumped dye laser (pulse duration 400 ps, excitation wavelength 770 nm) as an excitation source and a boxcar-integrator detection system (8).

RESULTS AND DISCUSSION

Solvent effects on the absorption

The photophysical properties of BChl-ser (see Fig. 1) are mainly determined by the macrocycle's π-electron system and the coordinated central Mg atom. This part of the molecule has a basically planar structure.

At first, the absorption behavior of BChl-ser will be compared with that of BChl. Although BChl is insoluble in water it can be dissolved in several organic solvents (*e.g.* [6]). The UV–visible–near-IR absorption spectrum of BChl in acetone is shown in Fig. 2. It consists of four main bands termed Q_y, Q_x, B_x, B_y in the order of increasing energy. The last two comprise the Soret band. The first two result from transitions to the first and second excited singlet states, respectively (9,10). The absorption spectra of BChl-ser and BChl in the same polar solvent are similar (*cp* Fig. 2).

Limantara *et al.* (6) investigated the optical absorption of BChl in numerous solvents. In accordance with the theory of Nagae *et al.* ([11], *cp* also [12]), they observed a linear dependence of the Q_y and Q_x absorption energies on the parameter $R(n) = (n^2 - 1)/(n^2 + 2)$, determined by the refractive index *n*. Different slopes were obtained in polar and nonpolar solvents. The Q_y transition energies of BChl (taken from Limantara *et al.* [6]) and of BChl-ser (this work) are compared for polar solvents in Fig. 3. Apparently, the $S_0 \rightarrow S_1$ transition energy of BChl-ser and BChl follows the same dependence on $R(n)$. Note that $R(n)$ of BChl-ser in aqueous solution (PBS, *cp* below) fits well into the general $R(n)$ dependence. The linear regression in Fig. 3 is faithfully represented by
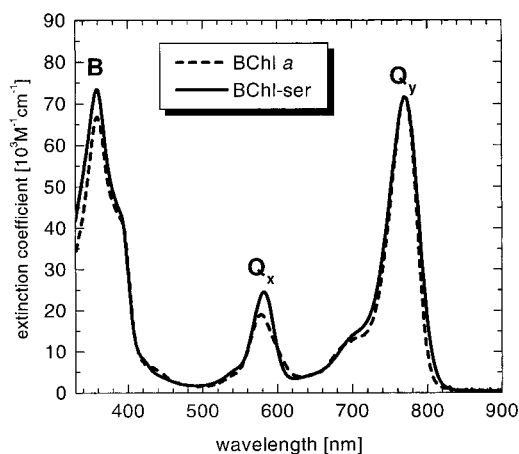
$$\nu Q_y[cm^{-1}] = 1895 \cdot R(n) + 13380, \sigma = 47.5,$$

where ν is the wavenumber of the Q_y absorption maximum. The coefficients correspond to the data given by Limantara *et al.* (6). The extrapolation to aqueous solvents indicates that the position of the Q_y-band can also be used to derive a local, isotropic *n* for protein environments, as long as there are no pigment–pigment interactions or specific interactions with particular amino-acid residues.

The spectral position of the Q_x absorption band of BChl depends strongly on the coordination state of the central metal atom. This is mainly because of electron density migration between the ligand and the metal that modifies the metal covalent radius and the electrostatic interaction of the metal with the electron density at the π-atoms in the frontier molecular orbitals (13). When penta-coordinated, the Q_x-band is around 570 nm and the Mg is localized outside the plane of the macrocycle. If the Mg is hexa-coordinated, it is positioned in the plane of the macrocycle (14) and the Q_x-band shifts to around 600 nm. This assignment, which was originally based on the solvent dependence of the absorption
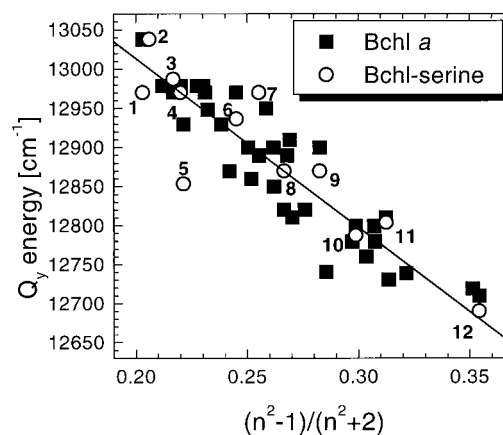

Figure 3. Absorption maxima of the Q_y band of BChl (from Limantara *et al.* [6]) and BChl-ser. Only those solvents that were used in this study for BChl-ser are marked by numbers: 1, methanol; 2, PBS; 3, diethyl ether; 4, acetone; 5, ethanol; 6, THF; 7, dichloromethane; 8, chloroform; 9, DMSO; 10, pyridine; 11, acetophenone; 12, quinoline.

Table 1. Coordination number N of the central Mg and spectral position of the Q_x absorption band (in nm, in parentheses) of BChl (from Limantara *et al.* [6]) and of BChl-ser

Solvent	BChl *a*	BChl-ser
Methanol	6 (607)	6 (605)
Diethyl ether	5 (573)	5 (580)
Acetone	5 (577)	5 (580)
Ethanol	6 (607)	6 (606)
DMSO	6 (608)	6 (607)
	5 (580)	5 (583)
Isobutanol	6 (604)	6 (599)
Chloroform	5 (580)	5 (581)
PBS (pH = 7.4)	—	6 (607)

(15), has been supported by Raman spectroscopy (6,14,16) and extended X-ray absorption fine structure (13). Although there are further variations depending on the ligand strength (between 575 and 585 nm if the Mg is penta-coordinated and 593–610 nm if it is hexa-coordinated), it is possible to assign the coordination state by absorption spectroscopy.

Following this approach, the coordination numbers of the Mg atom in BChl and BChl-ser are identical in each of a variety of solvents (Table 1), indicating that the serine residue has no influence on the coordination of the central Mg.

Taking this result together with the already described fact that the Q_y transition energy of BChl-ser shows the same dependence on polar solvents as that of BChl, we have concluded that there is a negligible influence of the serine bound to the C-17-propionic acid side chain, on the π-electron system of the macrocycle. This allows us, for the first time, to obtain experimental information on the optical absorption and related molecular parameters of monomeric BChl in an aqueous environment by measuring the absorption properties of BChl-ser in aqueous solution. In this environment, the absorption band of the Q_x transition is located at 607 nm, suggesting that the Mg is hexa-coordinated in water and located in the plane of the macrocycle. The Mg interacts with two water molecules that are ligated along the z-axis. Brereton and Sanders (17) showed by NMR spectroscopy that the hexa-coordinated BChl is more stable against oxidation than the penta-coordinated molecule (17). Nevertheless, it is noteworthy that BChl-ser in PBS is not (photo)chemically stable if the solution is equilibrated with air. Even exposure to the measuring light of the spectrophotometer was found degrading and Ar bubbling was required to inhibit (photo)chemistry.

The extinction coefficient, ϵ, at the Q_y maximum (in PBS) was determined to be ϵ = 42 000 M^{-1} cm^{-1} by the following procedure: BChl-ser was dissolved in a defined volume of acetone and the absorption was recorded. After evaporation of the acetone under a stream of Ar and redissolving the BChl-ser in an identical volume of PBS, the optical density was recorded again, and ϵ was calculated from the absorbance ratio and the known ϵ in acetone. The extinction coefficient of BChl-ser dissolved in acetone, determined by atomic absorption spectroscopy (A. Scherz, unpublished), provided the same value (71 500 M^{-1} cm^{-1} at the Q_y maximum) as BChl dissolved in acetone (18). The absorption of BChl-ser followed Lambert–Beer's law up to the maximum concentration used in the present study, $c = 5 \times 10^{-5}$ M.
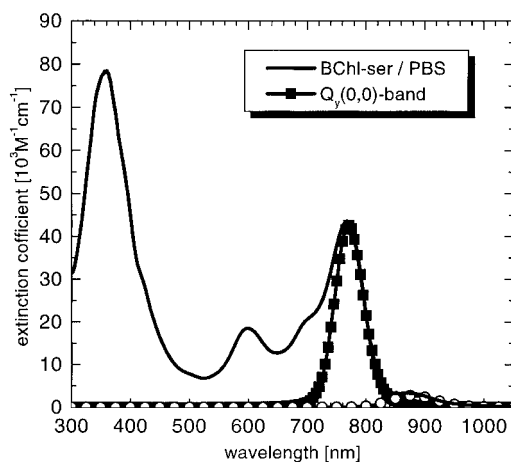
Figure 4. Absorption spectrum of BChl-ser in PBS, pH = 7.4 (solid line), and band analysis of the 0–0 component of the Q_y-band by a Voigt-curve (squares, see text for details).

No concentration dependence could be observed; that means there is no indication of aggregated BChl-ser. These results are corroborated by recent studies of Katz (19).

As already shown in Fig. 2, BChl and BChl-ser differ somewhat in their absorption line-shapes. Therefore, we analyzed in more detail, the absorption line-shape of the Q_y region of BChl-ser in PBS. The 0–0 absorption band of the $S_0 \rightarrow S_1$ transition was filtered out using a Voigt-profile I_{Voigt}. We started the analysis at 760 nm in order to minimize the influence of higher vibronic transitions on the Q_y(0,0)-band. The resulting band is shown in Fig. 4. Besides this band and the mentioned higher energy vibronic transitions, there is also a redshifted minor absorption band at 875 nm of yet unidentified origin.

The transition dipole moment μ of the Q_y band (in Debye) has been determined by the following equation (16)

$$|\mu_i|^2 = \frac{9.18 \cdot 10^{-3} \cdot 9n}{(n^2 + 2)^2} \int_{\nu_1}^{\nu_2} \frac{\epsilon_i}{\nu} \, d\nu, \qquad (1)$$

where n is the refractive index, ν the wavenumber and ϵ_i the molar extinction coefficient for transition i. In this equation, the Lorentz correction factor is used (20). Using this formula, we found that integration over the Voigt-type absorption band previously described, provides a transition dipole moment of (5.2 ± 0.5) D for BChl-ser in PBS, pH = 7.4. This increases to (6.6 ± 0.5) D if we consider the vibrational shoulder as well. The transition dipole moment of the entire Q_y band of BChl-ser in acetone, determined in the same way, has a value of μ = (6.8 ± 0.5) D. This value is comparable, within the experimental error, to that of BChl in acetone (μ = 6.5 D), which confirms the value determined in Scherz and Parson (16). Note that the dipole moment of the $S_0 \rightarrow S_1$ absorption transition of BChl-ser in PBS is comparable to that of the $S_0 \rightarrow S_1$ absorption transition of BChl-ser in acetone, although the maximum extinction coefficient in PBS is significantly smaller, because of the absorption band broadening in water.

Solvent effects on fluorescence

In water, BChl-ser has an emission maximum at λ_{em} = 801 nm, corresponding to a Stokes shift of ν_{St} = 540 cm^{-1}.
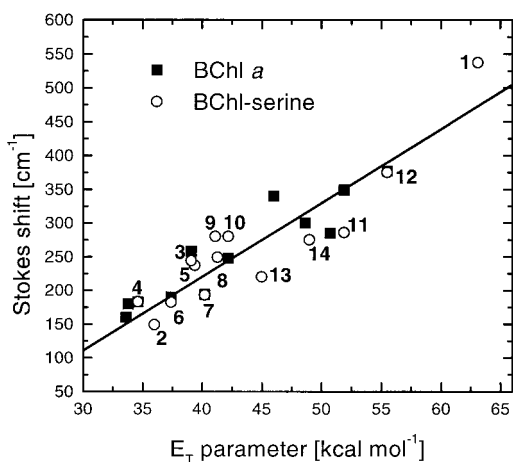
Figure 5. Dependence of the Stokes shift of BChl (from Connolly *et al.* [18]) and BChl-ser in different solvents on the Dimroth–Reichardt parameter E_T (21) of these solvents. Linear parameters of the fit for BChl-ser are $\Delta\nu_{St} = 10.7 \cdot E_T - 209$ (with a correlation coefficient of 0.91). The numbers give only those solvents in which BChl-ser was measured: 1, PBS; 2, diethyl ether; 3, THF; 4, quinoline; 5, chloroform; 6, pyridine; 7, dichloromethane; 8, acetophenone; 9, acetone; 10, isobutanol; 11, ethanol; 12, methanol; 13, dioxane; 14, DMSO.

Figure 6. Stokes shift of BChl-ser and BChl as function of the refractive index n and the dielectric constant ϵ (Lippert plot). The numbers give the solvents in which BChl-ser was investigated: 1, PBS; 2, diethyl ether; 3, THF; 4, quinoline; 5, chloroform; 6, pyridine; 7, dichloromethane; 8, acetophenone; 9, acetone; 10, isobutanol; 11, ethanol; 12, methanol; 13, dioxane; 14, DMSO.

The fluorescence properties of the molecule are determined by the permanent dipole moments of the ground and excited states, which in turn depend on the interaction between the fluorophore and the solvent molecules. These interactions result from the additive effect of electrostatic and electrodynamic forces, including dispersion, dipole–dipole interactions and hydrogen bonds. The Dimroth–Reichardt parameter E_T is a widely used global solvent polarity parameter (21) that accounts for this cumulative effect. The scatter plot in Fig. 5 shows the dependence of the Stokes shift ν_{St} on the E_T values of the corresponding solvents.

For both BChl and BChl-ser, the Stokes shift has the same linear dependence on E_T. BChl-ser dissolved in PBS has a Stokes shift that fits this linear dependence within the limits of uncertainty of the E_T parameter. Linear fits were also found for ν_{St} as a function of α (hydrogen bonding donor ability), π^* (ability to stabilize a charge distribution) and β (hydrogen bonding acceptor ability) (22–24), with comparable quality. It is therefore likely that the fluorescence properties of BChl-ser in PBS are determined by the interaction between BChl and the solvent molecules and not by special effects, for example, the formation of aggregates, or a folding-back of the serine over the macrocycle, which even seems to be absent in vacuum following force-field calculations.

A frequently used expression to describe the effect of the solvent on the Stokes shift of fluorophores is the Lippert equation (12)

$$\nu_{St} = \nu_a - \nu_{fl}$$

$$= \frac{2}{hc}\left(\frac{\epsilon - 1}{2\epsilon + 1} - \frac{n^2 - 1}{2n^2 + 1}\right)\frac{(\mu^* - \mu)^2}{a^3} + \text{const}, \quad (2)$$

where h is Plancks constant, c the speed of light, a the radius of the cavity in which the fluorophore resides, μ^* the permanent dipole moment of the excited electronic state and μ
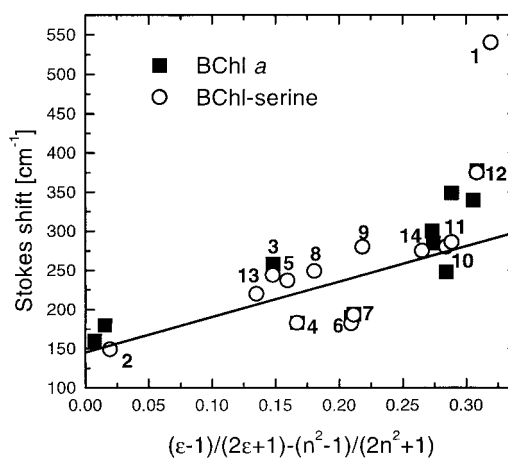
that of the ground state. The values ν_a and ν_{fl} represent the absorption and fluorescence maxima (in cm^{-1}), respectively. The term contains the static dielectric constant ϵ, $(\epsilon - 1)/(2\epsilon + 1)$, which reflects both the reorientation of the solvent dipoles and the redistribution of the electrons in the solvent molecules, whereas the term $(n^2 - 1)/(2n^2 + 1)$, that contains the refractive index n, accounts only for the redistribution of electrons.

After excitation, a redistribution of electrons occurs in the excited electronic state, to which the surrounding solvent molecules respond by redistribution of their electrons. This process, termed solvent relaxation, influences the Stokes shift (12).

The Lippert plot for both BChl and BChl-ser can be fitted with the same linear parameters for most of the solvents (Fig. 6), indicating that serine introduces only a minor pertubation of the solvent shell around the BChl. The only exception is the value for BChl-ser dissolved in PBS, which lies significantly outside the fitted curve. However, the fit of BChl in methanol is already somewhat out of the linear curve. A likely reason for this discrepancy is the hydrogen bonding ability of water and, although less pronounced, methanol. For (Eq. 2) it is assumed that solvent molecules are treated as a continuum. Hence, specific solvent effects like hydrogen bonding are not considered, and with water the limits of the applicability of the Lippert equation are reached. The plot in Fig. 6 clearly shows the importance of the hydrogen bonds for the interaction between BChl-ser and water.

The similarity between the Stokes shifts of BChl and BChl-ser in the same solvents, combined with the absorption data, shows that the energy of the excited states of both molecules and the electronic density distribution in the frontier orbitals are the same. This means that the serine does not affect the spectral position of the $S_0 \rightarrow S_1$ and $S_1 \rightarrow S_0$ transitions.

An initial estimate of the change of the dipole moment from the ground state to the first excited state in solvents without hydrogen bonds can be computed using the Lippert

Table 2.   Fluorescence data of BChl-ser (this work) and BChl (from Connolly *et al.* [18], in parentheses)

Solvent	λ_{em} (nm)	Φ_{fl}	τ_{fl} (ns)	τ_0 [ns]*	ν_{St} (cm^{-1})	$k_{IC} + k_{ISC}$ ($\times 10^8$ s^{-1})
PBS	801	0.04	1.1	27.5	540	8.7
Methanol	795 (793)	0.11 (0.12)	2.1 (2.32)	19.1 (19.3)	375 (377)	4.2 (3.8)
Ethanol	795 (795)	0.14 (0.14)	2.0 (2.5)	14.3 (17.8)	286 (349)	4.3 (3.44)
Isobutanol	797	0.14	2.08	14.9	275	4.1
DMSO	791	0.17	2.86	16.8	220	2.9
Acetone	788 (785.5)	0.14 (0.18)	2.5 (3.14)	17.8 (17.5)	280 (248)	3.4 (2.7)
Acetophenone	797	0.17	2.3	13.5	249	3.6
Dichloromethane	788	0.08	2.34	29.75	280	3.9
Pyridine	794 (793)	0.20 (0.26)	2.9 (3.53)	14.5 (13.8)	193 (194)	2.75 (2.1)
Quinoline	803	0.15	2.4	16	237	3.54
Chloroform	792 (796)	0.09 (0.17)	1.7 (2.6)	18.9 (15.3)	244 (258)	5.3 (3.2)
Tetrahydrofuran	782 (782.5)	0.20 (0.24)	3.0 (3.56)	15.0 (14.8)	166 (190)	2.66 (2.1)
Dioxan	782	0.20	3.1	15.5	149	2.6
Diethyl ether	781 (782)	0.14 (0.19)	2.66 (3.0)	19.0 (16.0)	183 (183)	3.2 (2.5)

equation. The linear dependence of ν_{St} = (500 ± 180) cm^{-1}·$f(n, \epsilon)$ + const. was determined from the plot in Fig. 6 for BChl-ser (without considering the values in PBS and methanol because of their strong hydrogen bonding character) and BChl. It results in a dipole change $\Delta\mu$ = (2.5 ± 0.3) D for a minimal cavity radius of a = 5 Å. If a value of a = 8 Å is used as an upper estimate, a dipole change of $\Delta\mu$ = 5.1 D is obtained (25,26).

The fluorescence properties of BChl-ser are summarized in Table 2, together with those of BChl (in parenthesis, if available from the literature). Although most parameters are similar, there are some significant differences. Among these, the most obvious are a lower fluorescence yield and a shorter fluorescence lifetime for BChl-ser in most solvents. BChl-ser and BChl show the same radiative lifetime $\tau_0 = \tau_{fl}\cdot\Phi_{fl}^{-1}$, taking into account an experimental error of 10%. Apparently, the nonradiative rates (intersystem crossing—k_{ISC}, or internal conversion—k_{IC}) are enhanced in BChl-ser, in comparison with BChl.

In order to distinguish between both processes, fluorescence lifetimes were also measured at 77 K for BChl and BChl-ser in diethyl ether. An enhanced ISC quantum yield for BChl-ser (Φ_{ISC} = 82%) compared with BChl (Φ_{ISC} = 77%) was determined having Φ_{ISC} = 1 − (τ_{fl}[77 K])/$\tau_{fl}\cdot\Phi_{fl}$ (27). The value for BChl is in good agreement with published data (7). The IC process is not influenced by the serine. In both molecules it is around 4%, corresponding to an IC rate of about 0.15×10^8 s^{-1}. In conclusion, the attached serine specifically enhances the ISC process. Excluding a radical pair mechanism, the enhancement probably originates from increased spin–orbit coupling, possibly by means of the bound water.

The fluorescence properties of BChl-ser dissolved in PBS can be summarized as follows: the Stokes shift is very large, with a value of 540 cm^{-1}. The fluorescence quantum yield is small with Φ_{fl} = 0.04, and the fluorescence lifetime is comparably shortened to τ_{fl} = 1.1 ns. This results in a radiative lifetime of τ_0 = (27.5 ± 3) ns, which can be determined by absorption data as well (28)

$$\tau_0^{-1} = 2.9\cdot10^{-9}n^2\langle\nu_f^{-3}\rangle^{-1}\int\frac{\epsilon(\nu)}{\nu}\,d\nu,$$

where $\langle\nu_f^{-3}\rangle^{-1} = \int I(\nu)\,d\nu/\int\nu^{-3}I(\nu)\,d\nu$ is the reciprocal of the mean value of ν^{-3} in the fluorescence spectrum. The radiative lifetime of (30 ± 3) ns thus obtained for the S_1 state agrees within the error limits.

In an additional experiment, bacteriopheophorbide-serine (BPheide-ser) was dissolved in PBS in order to evaluate the contribution of the Mg atom on the solubility of BChl-ser in water. The absorption spectrum of BPheide-ser in PBS shows two absorption maxima at around 751 and 790 nm that are characteristic of a monomer–dimer equilibrium with the aggregate absorption redshifted with respect to the monomer (16). However, no fluorescence signal could be observed for BPheide-ser, which is also indicative of dimerization (29). Hence, the Mg atom plays a key role in enhancing the pigment solubility in water.

CONCLUSIONS

We have shown that the covalently bound amino acid, serine, influences the kinetics, but not the energetics, of the excited states of bacteriochlorophyll. The energy of the $S_0 \rightarrow S_1$ transitions and the Stokes shift of both BChl and BChl-ser show the same dependence on solvent polarities, but the deactivation processes from the S_1 state are somewhat enhanced in the serine conjugate. Moreover, a somewhat stronger ISC relaxation was found in BChl-ser by means of fluorescence quantum yield and fluorescence lifetime measurements, whereas the IC is comparable in both molecules.

The water environment, accessible with BChl-ser for the first time, leads to a larger Stokes shift, a reduced extinction coefficient at the maximum of the $Q_y(0,0)$ absorption band, a broadening of the absorption bands, a reduced fluorescence quantum yield and an increased nonradiative relaxation process when compared with nonaqueous solvents.

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