Biochimica et Biophysica Acta, 682 (1982) 75–85
Elsevier Biomedical Press

BBA 41165

LOW-INTENSITY SUBNANOSECOND FLUORESCENCE STUDY OF THE LIGHT-HARVESTING CHLOROPHYLL *a*/*b* PROTEIN

WILLIAM T. LOTSHAW [a], RANDALL S. ALBERTE [a,*] and GRAHAM R. FLEMING [b]

Departments of Biology [a] and Chemistry [b], The University of Chicago, Chicago, IL 60637 (U.S.A.)

(Received January 21st, 1982)
(Revised manuscript received June 15th, 1982)

Key words: Photosynthesis; Energy transfer; Chlorophyll-protein complex; Photon counting; Fluorescence; Light-harvesting complex

The fluorescence decay characteristics of the isolated light-harvesting chlorophyll *a*/*b* protein have been studied using low-intensity subnanosecond-resolution time-correlated single-photon counting. In the monomeric state in detergent micelles, the chlorophyll *a*/*b* protein exhibits biexponential decay ($\tau_1 = 1.2$ ns, $\tau_2 = 3.3$ ns) with the two components having very similar weights. The decay parameters do not depend on emission wavelength. These results are discussed in relation to the Van Metter-Knox-Shepanski model (Van Metter, R.M. (1977) Biochim. Biophys. Acta 462, 642–657; Shepanski, J.S. and Knox, R.S. (1982) Isr. J. Chem., in the press) of the chlorophyll *a*/*b* protein, and a kinetic analysis of the energy-transfer processes. The influence of detergent composition and concentration on the fluorescence decay of the chlorophyll protein is also described.

Introduction

The Chl *a*/*b* protein is the dominant chlorophyll-binding protein in the chloroplasts of green plants and algae, accounting for up to 60% of the total chlorophyll of these organelles [1]. This protein functions as the principal component of the 'antenna' chlorophyll pigment arrays which act to increase the absorption cross-sections of specialized chlorophyll molecule(s) called reaction centers or traps. These are the sites of charge transfer from water (Photosystem II) to physiological reductant (Photosystem I) which are driven by absorbed photons. Current evidence suggests that all of the chlorophyll in photosynthetic membranes is protein bound, and that the photosynthetic apparatus is composed of some number of unique pigment-binding proteins [2]. It is proposed that there are two Chl *a*-containing pigment-protein complexes each possessing a trap species (Photosystem I or II) and antenna Chl *a*-binding proteins. One Chl *a* complex is associated with the oxidation $H_2O \rightarrow \frac{1}{2}O_2 + 2H^+ + 2e^-$ (Photosystem II) and the other with the reduction of a physiological reductant (Photosystem I). The latter complex has been isolated from algae and higher plants by detergent solubilization of photosynthetic membranes, and is called the P-700-Chl *a* protein. The remaining chlorophyll is found in a macromolecular aggregate of the Chl *a*/*b* protein, which interacts with the trap-containing complexes via electronic excitation transfer.

Electronic excitation energy originating in the antenna chlorophyll is commonly thought to migrate through the antenna array to the traps via the transition dipole-dipole interaction proposed by Förster [3]. This interaction results in the transfer of a virtual photon from an excited (I) to an

* To whom correspondence should be addressed.
Abbreviation: Chl, Chlorophyll.

unexcited (J) molecule with a rate constant $F_{I,J}$.

$$F_{I,1;J,1} = (K)\frac{\left[(\hat{\mu}_I \cdot \hat{\mu}_J) - 3(\hat{\mu}_I \cdot r_{I,J})^2(\hat{\mu}_J \cdot \hat{r}_{I,J})\right]^2}{\| R_{I,J} \|^6} \times \int \frac{f(\nu)\epsilon(\nu)}{\nu^4} d\nu \quad (1)$$

Here, $f(\nu)$ and $\epsilon(\nu)$ are the frequency-dependent emission and absorption cross-sections, K a numerical constant dependent upon the choice of units, $\mu_{I,J}$ unit vectors in the direction of the transition dipole moments to the first excited states of molecules I,J and $r_{I,J} = R_{I,J}/\| R_{I,J} \|$ is a unit vector in the direction of the vector $\bar{R}_{I,J}$ which connects the origins of μ_I and μ_J.

The dynamics of electronic excitation migration in the photosynthetic chlorophyll array can be represented by the coupled first-order differential equations.

$$\dot{p}_{I,i} = \sum_{J \neq I} F_{I,i;J,j}\delta_{I,i}\delta_{I,j}p_{J,j} - \left[\sum_{I \neq J} F_{J,j;I,i}\delta_{I,i}\delta_{I,j} + k_{D,I}\delta_{I,i}\right] p_{I,i} \quad (2)$$

where the $F_{I,i;J,j}(i,j = 1)$ are time-independent transfer rate constants between the first electronic excited states of the Ith and Jth molecules. The $k_{D,I}$ is the molecular excited state decay rate for the first excited state of molecule I, and the $p_{I,i}(\dot{p}_{I,i})$ the normalized population densities (and their derivatives) of the ith state of the Ith molecule when each molecule is treated as a two-level system [4,5]. This is the starting point for most treatments of excitation transfer in solution or on lattices in the incoherent transfer limit. Since the relative orientation and separation between any two molecules in an array will affect the magnitude of the $F_{I,i;J,j}$, the parameters must be weighted according to the probability of particular relative orientations and separations between the Ith and Jth molecules. Calculations using Eqn. 2 to model transfer dynamics in solution employ random distributions of molecules and orientations [5–7], while orientation averaged isotropic $F_{I,i;J,j}$ are commonly employed for calculations modeling dynamics on lattices [4,8]. The results of recent experiments on the fluorescence depolarization and donor fluorescence decay in homogeneous and donor-acceptor dye solutions are in agreement with the theoretical predictions that the energy transfer is diffusive after very short times at moderate or high concentrations [6,9]. The effect of a nonrandom distribution of intermolecular distances and orientations in a small (approx. 10 molecules) array on the dynamics of excitation migration within the isolated array, or an array which is some multiple of that unit, has received little attention.

As the smallest integral unit of chlorophyll organization in higher plants [1] (six to seven chlorophyll molecules; three of Chl *b*, three to four of Chl *a*) the Chl *a/b* protein is sufficiently simple to be amenable to detailed theoretical treatment and experimental investigation. Evidence from other laboratories suggests that the Chl *a/b* protein may be involved in the physiological control of the distribution of absorbed photons between the two photosystems [10,11]. For these reasons the Chl *a/b* protein is a reasonable starting point for the experimental investigation of photosynthetic energy migration and trapping. Van Metter [12] and Knox and Shepanski [13] have carried out a number of steady-state spectroscopic studies of this protein and have proposed a model for its structure.

Materials and Methods

Sample preparation

Chloroplast membranes were prepared as described in Ref. 14 from the leaves of 10–20-day-old *Zea mays* (DeKalb var. XL-43), except as noted below. The well washed membranes were homogenized by hand in cold (4°C) buffer containing 1% SDS (w./w.) (SDS/chlorophyll, ratio 10:1) and stirred for 1 h. The homogenate was then centrifuged to remove particulate matter and loaded onto a hydroxyapatite column and developed as described [14] with several modifications. (1) The buffers used in the sample preparation were kept free of $MgCl_2$ except for the leaf tissue homogenization buffer. (2) The leaf tissue homogenization buffer was 0.4 M in sorbitol, rather than 0.5 M in sucrose. (3) Buffers used in elution of the sample were 0.05% SDS (w./w.) and the Chl *a/b* protein was eluted as a single band at a

phosphate concentration of 0.24 M. (4) The isolated sample was passed through a Sepharose CL-4B (Pharmacia Fine Chemicals, Inc.) column equilibrated with 0.05% SDS to remove phosphate. The sample collected from this column was made no greater than 0.02% (w/w) in Triton X-100. Changes in the concentration of detergent could be made through the last modification. The resulting sample was observed to be stable for 2–3 days after isolation when stored at 4°C between experiments, as described below. Samples older than 3 days were discarded and replaced with freshly prepared material. The sample identity was confirmed by (i) visual inspection of the room-temperature optical absorption and fluorescence spectra obtained on an Aminco DW2 spectrophotometer or SPF-500 corrected spectrum fluorometer and, (ii) gel electrophoresis of the sample on native [15] and denaturing [16] gel systems. The chromophore composition of the sample was determined by sucrose column chromatography of the 80% acetone extracts [17].

Spectroscopic analysis

The time-correlated single-photon counting apparatus consisted of a synchronously pumped picosecond dye laser and data acquisition and processing system similar to that described in detail by Robbins et al. [18]. The detector used was a water-cooled red-sensitive photomultiplier tube (Amperex PM 31000) which resulted in an instrumental response to the laser pulse input of approx. 300 ps for the full-width at half-maximum. The repetition rate of the laser was modified by a variable frequency dividing circuit which triggered a Pockels cell at frequencies which were integral divisions of the ion laser mode locker driving frequency. This resulted in an exciting pulse repetition rate of 11–91 kHz with an extinction ratio of passed to blocked pulses which was greater than 500:1. A polaroid was set at the magic angle to the excitation beam polarization between the sample and detector to exclude the fluorescence depolarization contribution from the observed decay [19]. Colored-glass filters and interference filters were inserted between the polaroid and detector to sample portions of the fluorescence spectrum and to eliminate scattered excitation light. The stop pulse counting rate was kept below 2% of the start pulse counting rate by attenuating the excitation beam through calibrated neutral density filters placed in front of the final excitation beam polarizer. Typical pulse energies incident on the sample were no greater than 0.6 pJ, corresponding to photon densities of $9.5 \cdot 10^{10}/\text{cm}^2$ at 650 nm. A typical sample had a clorophyll concentration of $1.7 \cdot 10^{16}$ molecules/ml, so that excitation was within the Beer's law domain. All analyses were conducted at room temperature (298 K).

The sample was held in a stationary quartz cuvette or flowed in a flow cell by a peristaltic pump. Approx. 10-nm sections of the fluorescence decay spectrum were observed in each experiment on the Chl *a*/*b* protein. The system performance was evaluated by comparing the measured fluorescence decay of the subnanosecond standard rose bengal dissolved in methanol to literature results ($\tau = 530$ ps) [20]. The fluorescence decay of a 10^{-5} M solution of oxazine-725 perchlorate (Exciton) in methanol was measured on the instrument after results within experimental error for rose bengal were obtained.

Data analysis

The observed decay in a photon-counting experiment is a convolution of the instrumental response and the sample impulse response function [16]:

$$i(t) = \int_0^t g(t')h(t-t')\mathrm{d}t'$$

where $g(t')$ is the instrumental response to a δ-function (here an approx. 6 ps pulse) input and $h(t-t')$ the sample impulse response function. The observed decays are fitted to a convolution of the measured instrumental response and the predicted form of the sample impulse response (decay law) which we have chosen to be a sum series of one to three exponential terms. The fitting is accomplished by an iterative least-squares method [16] using a VAX-11/780 computer interfaced to a multichannel analyzer. The goodness of fit was evaluated by the reduced χ-squared test, which for values near 1.00 indicates that the residuals of the data curve about the fitted curve are normally distributed [21].

Results and Discussion

Characteristics of the Chl a/b protein

Typical room temperature (298 K) absorption and fluorescence spectra of the isolated Chl *a/b* protein used in fluorescence decay measurements are shown in Fig. 1. The average Soret peak absorptions for Chl *a* and Chl *b* are at 438.3 ± 0.9 and 470.2 ± 1.5 nm, respectively. The average Q_y absorption peaks of Chl *a* and Chl *b* are at 671.8 ± 1.0 and 652.7 ± 1.0 nm, respectively. The pigment analysis of the sample shows that there are approximately three molecules of Chl *b* for every three to four molecules of Chl *a* in agreement with published values [14]. The Chl *a/b* protein isolated by hydroxyapatite chromatography migrates as a single band on a 'native' electrophoretic gel system as described previously [14]. Denaturing gel electrophoresis resolved the sample into two polypeptides of nearly equivalent molecular weight; the faster migrating band was much less intensely stained than the more slowly migrating band.

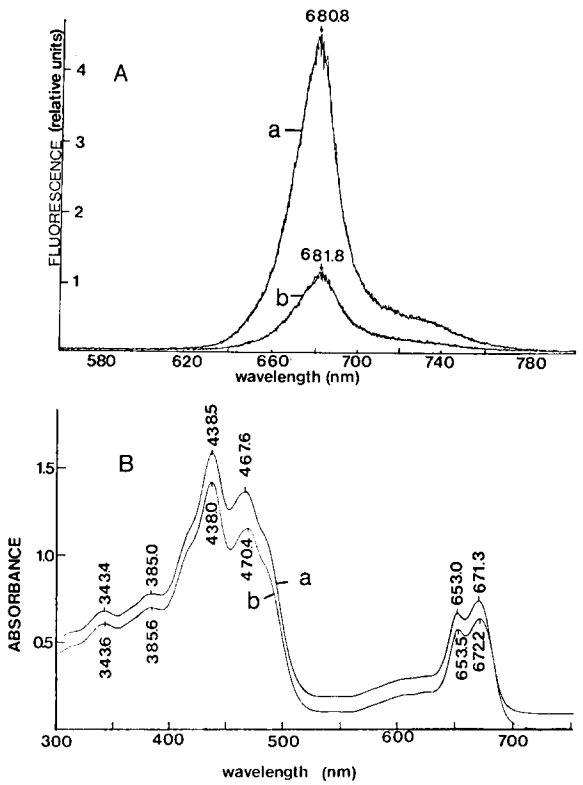

Fig. 1. (A) Steady-state emission spectra (298 K) of the hydroxyapatite chromatography-isolated Chl *a/b* protein excited at 435.0 nm (excitation slit, 2.0 nm; emission slit, 0.5 nm). (a) Sample in 50 mM Tris-HCl, pH 8.0, containing 0.05% SDS (w/w) and 0.02% Triton X-100 (w/w). (b) Same as above (a) except that no Triton X-100 was present. (B) Absorption spectra (298 K) of the samples in a and b as described above.

Instrument performance

The results of the fluorescence decay measurements on Rose Bengal and oxazine are summarized in Table I. The results of eleven separate experiments on oxazine show the decay to be first order with a lifetime (1/e time) of 835 ps, and to be independent of excitation wavelength. When the oxazine decays were fitted to a sum of two exponential terms, the values of the 1/e times of each term converged to the single exponential result. Oxazine was chosen as a secondary subnanosecond lifetime standard, since its fluorescence spectrum overlaps those of Chl *a* and *b*.

Fluorescence decay of the Chl a/b protein

The fluorescence decay data of the Chl *a/b* protein could not be fitted with a normal distribution of residuals to a single exponential decay law. Addition of a second exponential term to the decay law resulted in normally distributed residuals. The 1/e times and weights of the exponential terms were not observed to vary with the wavelength of observation at a single excitation wavelength. Therefore, the results of experiments at a single excitation wavelength were averaged to constitute a single result at that wavelength. The results of the fluorescence decay experiments are presented in Table I. As a function of excitation wavelength, the lifetimes of one exponential term varied from 1200 to 1500 ps and from 3300 to 4000 ps for the other term. These 1/e values are for samples in a low-salt buffer system which favors a monomeric aggregation state of the chlorophyll protein [10]. The lifetimes of both terms appear to increase slightly with increasing excitation wavelength, while the weight of each term remains constant. We note that researchers in another laboratory [22] have recently published results on the fluorescence decay of the Chl *a/b* protein usi. g streak camera detection of fluorescence excited by the second harmonic of a modelocked Nd/YAG laser. We feel that the lower

TABLE I

RESULTS OF FITTING THE HYDROXYAPATITE-ISOLATED Chl *a*/*b* PROTEIN FLUORESCENCE DECAY TRACES, WHEN EXCITING AT VARIOUS WAVELENGTHS, TO THE DECAY LAW $A_1e^{-t/\tau_1} + A_2e^{-t/\tau_2}$ AS DESCRIBED IN THE TEXT

A_1 and A_2 are the relative weights of each term, and τ_1 and τ_2 are the 1/e times of each term in ps. The oxazine and rose bengal results are included to establish the instrument performance.

Sample	λ_{ex} (nm)	A_1	τ_1 (ps)	A_2	τ_2 (ps)
Chl *a*/*b* protein [a]	600	0.49 (±0.03)	1208.6 (±85.1)	0.50 (±0.03)	3278.0 (±76.4)
	620	0.41 (±0.01)	1289.5 (±9.9)	0.59 (±0.01)	3839.1 (±75.6)
	640	0.46 (±0.03)	1387.5 (±105.5)	0.54 (±0.03)	3763.3 (±129.6)
	650	0.45 (±0.05)	1400.5 (±159.5)	0.55 (±0.05)	3642.6 (±162.8)
	660	0.42 (±0.03)	1273.1 (±175.7)	0.58 (±0.03)	3806.8 (±326.1)
	670	0.48 (±0.02)	1465.0 (±49.6)	0.52 (±0.02)	4060.3 (±223.7)
Oxazine-725 perchlorate	650	–	839.5 (±19.3)	–	–
(10^{-5} M) in methanol	600–650	–	831.3 (±30.1)	–	–
Rose bengal (10^{-5} M) in methanol	570–590	–	529	–	–

[a] Measured in 50 mM Tris-HCl (pH 8.0) containing 0.02% Triton X-100 and 0.05% SDS.

precision of the streak camera data prevented these workers from observing the biexponential behavior of the sample fluorescence decay. Earlier streak camera work by Searle and Tredwell [23] on the digitonin membrane fragment FIII found a single exponential decay of 4 ns for the fluorescence. Some of the data from samples in the 'monomer' state described above could not be fitted with normally distributed residuals throughout the decay when a biexponential impulse response (decay law) was used. The deviation in the residual distribution occurred exclusively at early times of the decay (see Fig. 2) and could be eliminated by the addition of a third exponential term to the decay law. The lifetime of the new term invariably was less then 80 ps, and contributed less than 4% to the area of the integrated decay curve. The values of the lifetimes of the other two components increased slightly and remained approximately equal in weight. This 'short' component varied widely from one decay to the next at a single excitation wavelength, but was generally shorter (10–20 ps) for excitation in the 640–670 nm range and longer (70 ps) in the 600–620 nm range. However, biexponential fits to the fluorescence decays with normally distributed residuals throughout the decay were observed in most experiments, and deviations from the above results are thought to arise from an observation wavelength dependence in the instrumental response to a δ-function input. As described below, contributions from fluorescence depolarization to the observed decay resulting from an improper setting of the magic-angle polaroid did not alter the values of the weights or 1/e times of the terms in the decay law. The filter combinations used to isolate sections of the sample fluorescence spectrum reduced the photomultiplier counting rate to the dark current level when a highly scattering suspension of nondairy coffee whitener was placed at the sample position. For these reasons, these alternative explanations of the occasional observation of triple exponential decay laws under the described experimental conditions have been ruled out.

Detergent effects

A recent investigation [24] of the Chl *a*/*b* protein by phase fluorometry showed that the fluorescence decay time (assumed single exponential) varied with the concentration of detergent in the sample buffer. Specifically, it was reported that at low detergent concentrations (no greater than 0.01%, w/w) the lifetime was short (approx. 1 ns), while at higher concentrations (0.05% or greater, w/w) the lifetime became long (5 ns). Treatment of our preparation in a similar manner resulted in

TABLE II

RESULTS OF FITTING THE Chl *a*/*b* PROTEIN FLUORESCENCE DECAY TRACES, WHEN EXCITED AT VARIOUS WAVELENGTHS, UNDER VARYING ISOLATION AND SAMPLE BUFFER CONDITIONS

The decay law used is: $A_1e^{-t/\tau_1} + A_2e^{-t/\tau_2} + A_3e^{-t/\tau_3}$, where A_1, A_2 and A_3 are relative weights of each term and τ_1, τ_2, and τ_3 are 1/e times of each term in ps.

Chl *a*/*b* protein [a]	Age [b]	A_1	τ_1 (ps)	A_2	τ_2 (ps)	A_3	τ_3 (ps)	λ_{ex} (nm)
Chromatographically isolated								
(a) no additions	30 min	0.49	164.1	0.34	837.3	0.17	2749.0	670
(b) no additions	7 h	0.56	165.3	0.34	717.1	0.10	2618.8	670
(c) no additions	24 h	0.59 (±0.05)	146.7 (±52.1)	0.35 (±0.05)	669.8 (±39.2)	0.06 (±0.001)	2813.0 (±75.6)	640
(d) +0.1% SDS	7 h	–	–	0.47 (±0.05)	959.7 (±27)	0.53 (±0.05)	3413.9 (±188.2)	660
(e) +0.1% Triton	7 h	–	–	0.15	1154.6	0.85	5277.6	660
Electrophoretically isolated								
(a) +0.01% SDS	24 h	0.63 (±0.01)	171.5 (±17.9)	0.26 (±0.02)	825.6 (±14.8)	0.11 (±0.01)	2854.1 (±80.5)	660
(b) +0.01% SDS and 0.01% Triton	24 h	0.33	294.7	0.43	1191.8	0.25	3257.8	660

[a] Sample in 50 mM Tris-HCl (pH 8.0) containing 0.025% SDS upon isolation.

[b] Time between isolation and measurement.

a qualitatively similar response (Table II). At low SDS concentrations the decay cannot be fitted by a sum of two exponential terms, requiring a third to yield normally distributed residuals. The lifetime of this term was approx. 150 ps. A large decrease in the lifetime of the 1.30 ns component of the 'monomeric' sample to approx. 850 ps was characteristic of this treatment. High (0.1% SDS or greater, w/w) concentrations in the sample buffer result in a decay which is fitted well by a biexponential decay law with weights and lifetimes close to those of the monomeric sample (Table I). The high detergent concentration in the sample results in a loss of Chl *a* to pheophytin *a* within a few hours at room temperature and 4°C. An SDS concentration of 0.05% on a weight basis in the sample buffer corresponds to a $1.81 \cdot 10^{-3}$ M solution. If the detergent exists predominantly in micelles of approx. 100 molecules [25] the micellar concentration is $1.81 \cdot 10^{-5}$ M. This is nearly equimolar to the concentration of Chl *a/b* protein calculated from the Chl *a* and *b* concentrations measured in 80% acetone extracts of the sample and assuming that there are six chlorophyll molecules per protein unit. At very low SDS concentrations the Chl *a/b* protein precipitates out of solution as an amorphous solid. We have found that the addition of the detergent Triton X-100 to a final concentration of 0.02% or less (w/w) to a sample already 0.05% in SDS preserves the absorption, fluorescence, fluorescence decay, and chemical properties of the Chl *a/b* protein for 2–3 days. This effect is shown graphically in Fig. 1. The difference in the fluorescence intensity for samples of equal absorbance is diagnostic. While the physical mechanism of this preservation is unknown, we conjecture that the detergent Triton X-100 contributes to a mixed detergent/protein micelle in which SDS is prevented from decomposing the protein while maintaining a 1 : 1 protein/micelle state of organization. This preservative effect has also been observed in the SDS-prepared P-700-Chl *a* protein by another worker in our laboratory [26]. It is to be noted that an aggregation effect similar to that described in Ref. 10 under high-salt conditions can be induced by low detergent concentrations, and results in reduced excited state lifetimes (Table II) and fluorescence intensity (Fig. 1).

If Triton X-100 is added to a final concentration nearly equal to or greater than that of SDS, or if it replaces SDS in the sample buffer at any concentration, a loss of structure in the absorption spectrum is observed in both the Soret and Q_y transitions. The 470 nm Soret peak of Chl *b* is blue shifted to 465 nm, and the prominent shoulder at 480 nm is lost. The fluorescence spectrum of such a sample is blue shifted approx. 7 nm and the fluorescence decay becomes nearly first order with a 1/e time of 5.2 ns (Table II). Upon rechromatography on hydroxyapatite, or gel electrophoresis of these samples, the chlorophyll is not found to be uniquely associated with protein, as it appears in the fast migrating 'free' pigment zone in an electrophoretogram. Samples prepared by preparative gel electrophoresis [14,27] exhibit the same behavior toward detergent as those prepared by hydroxyapatite chromatography (Table II).

Nonexponential fluorescence decay

A typical fluorescence decay of the Chl *a/b* protein is shown in Fig. 2. The decay is seen to be

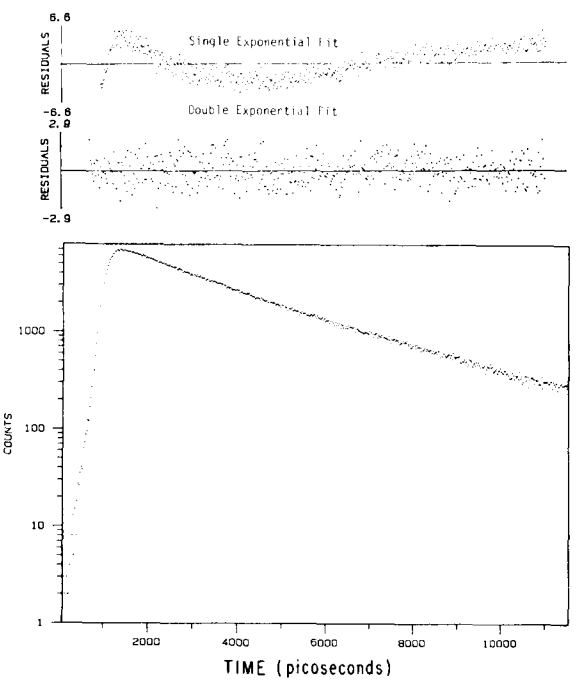

Fig. 2. The fluorescence decay of the isolated Chl *a/b* protein excited at 650 nm. The residuals of both the double-exponential and a single-exponential fit are shown. The observation wavelength is 680±5 nm.

nonexponential, and is fitted well by a sample impulse response (decay law) which is a sum of two exponential terms, both with positive preexponential factors (Table I). In order to determine if this behavior can be attributed to long-lived excited states of chlorophyll (e.g., triplets) or of some other species quenching singlet excitations in the sample, we performed the fluorescence decay measurements on flowing and stationary samples. For stationary samples, the laser repetition rate was varied between 11 and 91 kHz, corresponding to repetition periods of approx. 80 and 10 μs, respectively. At a laser repetition rate of 45 kHz the sample was flowed with sufficient linear velocity to limit exposure of the sample to 193 cycles of the laser in a single traversal of the excitation beam. The sample volume was sufficient to make the sample circulation period 104 s. In neither experiment were the lifetimes or weights observed to vary beyond sampling error. Assuming a sample absorbance of 1.00 at the excitation wavelength a single selected pulse from the Pockels cell excited approx. 0.003% of the Chl *a/b* protein complexes within the beam. Of these, approx. 0.7 may intersystem cross to triplet states based on solution triplet yields [28]. If we assume (i) that these triplet states accumulate and do not decay during the sample traversal of the excitation beam, and (ii) that there is negligible depletion of the ground singlet state, the probability of singlet excitation of a protein carrying a triplet chlorophyll state resulting from earlier pulses in the pulse train is less than 0.002 for a single passage through the excitation beam. We therefore consider this an unlikely mechanism for the observed biexponential decay.

A second possibility is that the sample is heterogeneous. The sample prepared by hydroxyapatite chromatography migrates as a single band on native electrophoresis gels [15], and no free pigment band is observed. Further, there is no detectable photochemical trap activity which could quench singlet excitation and lead to a time dependence in the rate constant of the decay as described in Ref. 29. The formation of trap-like species involving chlorophyll molecules in two separate proteins by the aggregation of monomeric chlorophyll proteins could also result in deviations from simple first-order kinetics, as well as increase the quenching cross-section of a fast-migrating or long-lived excited state. We consider the last possibility remote for the reasons described above and because the exciting photon densities used for these experiments were at least four orders of magnitude below those required for singlet-singlet annihilation [30]. The observed invariance of the lifetimes and weights of the components of the fluorescence decay with observation wavelength and the invariance of the fluorescence spectrum with excitation wavelength at steady-state illumination require that any Chl *b* contribution to the sample fluorescence be either small or anomalously red shifted. Workers in another laboratory [12] have observed that the fluorescence yield is also excitation wavelength independent and that the polarization of fluorescence (at 730 nm) is low when excited at wavelengths shorter than 670 nm, and nearly zero with 650 nm excitation. This indicates that the Chl *b* transitions at this wavelength are coupled by excitation transfer to other chlorophylls in the complex. They have proposed a model of the chlorophyll arrangement on the Chl *a/b* protein which yields qualitative agreement with their measurements of the fluorescence, absorption and circular dichroism on a sample similar to ours [14]. The model is noteworthy in that it predicts a long-wavelength absorbing (665 nm) transition of Chl *b* the fluorescence of which would presumably be near 677 nm. Further, the Chl *b* molecules interact strongly, producing trimer excitonic states two of which are degenerate at 652 nm. Such long-wavelength absorbing Chl *b* transitions have been observed in systems containing Chl *b* aggregates [31]. We have used the Van Metter-Knox-Stepanski model as a starting point for our analysis of the fluorescence decay kinetics of the Chl *a/b* protein.

Decay kinetics

Systems exhibiting donor-acceptor excitation transfer [6,32] exhibit irreversibility in the transfer from donor to acceptor when the acceptor excited state is sufficiently short-lived [33,34] that acceptor to donor transfer cannot occur with significant frequency. The acceptor species in the Chl *a/b* protein, Chl *a*, has a lifetime which is long with respect to the theoretical rate of 'back transfer' [5]. Under these conditions, a 'thermal' population distribution of excitation probability among the

donor and acceptor excited states will be achieved prior to radiative decay [12,35]:

$$\frac{P_D}{P_a} \propto \exp[-\Delta E/kT] \quad (3)$$

Where $P_{D,A}$ are the excited state populations of the donor and aceptor, ΔE the energy gap between the donor and acceptor excited states, k Boltzmann's constant and T the absolute temperature. This sort of emitter heterogeneity does not result in a nonexponential decay on the time scale defined by the excited state lifetimes of the donor and acceptor molecules in dilute solutions, provided the transfer rates are rapid with respect to this time scale. We can obtain a lower limit to the transfer rate from the decay of the components of the fluorescence polarized parallel and perpendicular to the excitation polarization. These are indistinguishable from the excited state decay measured at the 'magic' angle [19] (which eliminates the contribution of the fluorescence anisotropy, $r(t) = [I_{\parallel}(t) - I_{\perp}(t)][I_{\parallel}(t) + 2I\perp(t)]$, where $I_{\parallel,\perp}(t)$ are fluorescence intensities). Since the relative positions and orientations of the chlorophyll molecules in the protein are fixed, the sample memory of the excitation polarization is lost primarily by excitation transfer among the chlorophylls, and $r(t)$ will decay on a time scale following excitation comparable to $(F_{I,i;J,j})^{-1}$. Therefore, the $F_{I,i,J,j}$ are sufficiently large that $r(t)$ may not be observed by photon counting, and excitation transfer is at least 10^2-times faster than the excited state lifetime on the chlorophyll protein. By detailed balance, the acceptor, Chl a, to donor, Chl b, back transfer rate is related to the forward rate in the same way that P_D is related to P_A in Eqn. 3. Because the Chl a/b protein contains a small number of chlorophyll molecules, Eqn. 2 may be solved exactly for the kinetic scheme (following Van Metter [12] and Shepanski and Knox [13]) shown in Fig. 3 to yield the system impulse response function for any fixed set of the rate constants $F_{I,i;J,j}$ and $k_{D,I}$ [7,36]. The relative magnitudes of the $F_{I,i;J,j}$ and the $k_{D,I}$ derived by the above argument are in qualitative agreement with those resulting from a calculation of the $F_{I,i;J,j}$ from Eqn. 1, experimentally derived $f(\nu)$, $\epsilon(\nu)$, and distribution functions for the $\overline{R}_{I,J}((F_{I,i,J,j})/k_{D,I} \approx 10^3)$ [5]. With values for the rate constants $F_{I,i;J,j}$ and $k_{D,I}$ in conformity with the above discussion and Fig. 3, the excited state decay will be single exponential for times following excitation defined by the order of magnitude of $(k_{D,I})^{-1}$. Excitation transfer will be observed through the fluorescence anisotropy $r(t)$ at times following excitation defined by the order of magnitude of the $(F_{I,i;J,j})^{-1}$. In this calculation the back transfer rate constant for Chl a to b transfer is $1/10$ the forward transfer rate from Chl b to a, corresponding to an energy gap of approx. 450 cm^{-1} between the excited states of Chl b and a. This result does not compare well to the observed fluorescence decay which is well characterized as a sum of nearly equally weighted exponential terms with $1/e$ times close to the $(k_{D,I})^{-1}$ values of the isolated molecules. The exact value of the predicted $1/e$ time for the decay of the excited state is the excitation probability weighted average of the donor, Chl b, and acceptor, Chl a, excited state lifetimes $(k_{D,I})^{-1}$. By manipulating the values of the transfer rate constants in Eqn. 2, we have found that it is necessary to isolate two groups of molecules in the protein array by setting to zero some of the transfer rate constants between them to yield a biexponential decay law with $1/e$ times within the same order of magnitude as the $(k_{D,I})^{-1}$. This will result in the irreversible accumulation of excitation in these two groups, which will then decay independently.

The seminal work on the isolation and biochemical investigation of the Chl a/b protein by

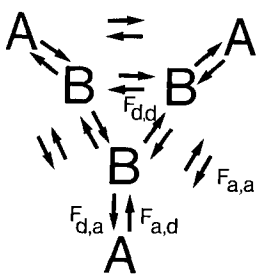

Fig. 3. Representation of the kinetic scheme of Eqn. 2 for energy transfer within the Chl a/b protein. $F_{a,a}$ and $F_{d,d}$ are the acceptor-acceptor and donor-donor transfer rate constants; $F_{d,a}$ and $F_{a,d}$ are the donor-acceptor and acceptor-donor rate constants. Not shown are the molecular excited state decay rate constants and all possible transfer rate constants connnecting all possible pairs of donors and acceptors.

Kung and Thornber [27] and Kan and Thornber [14] showed a Chl *a* to Chl *b* ratio which varied from 1.00 to 1.20. Our samples show the latter ratio, and are consistent with the model shown in Fig. 3 with three or four Chl *a* molecules. We noted earlier that the Van Metter-Knox-Shepanski model assumes an excitonic trimer in Chl *b*, implying that Chl *b* excitation is shared over the three excitonic transitions. If this model is correct, the transfer of excitation from Chl *b* to Chl *a* must be made irreversible in the forward direction to two pools of Chl *a* of equal oscillator strength to account for the observed decay when the transfer rates are fast with respect to the excited state lifetimes. A Boltzmann-weighted back transfer rate would still be 10–100-times faster than the excited state lifetime, and result in a simple first-order decay on a nanosecond time scale. Another possibility consists of two Chl *b* molecules interacting with one Chl *a*, and the remaining Chl *b* molecule (which must not transfer excitation to the other Chl *b* molecules) interacting with two Chl *a* molecules in the three-Chl *a* model. Each group of three molecules would evolve independently of the other, possessing a lifetime which would be the weighted average of the molecular lifetimes of the group. Except for details of the circular dicroism spectrum of the complex ascribed to the Chl *b* trimer, this formulation is not at variance with the Van Metter-Knox-Shepanski model. The origin of the 1.30 ns lifetime may then be ascribed to a particular physical picture. Molecular aggregates of Chl *a* in neat apolar solvents are known to irreversibly quench singlet excitations of monomeric Chl *a* in solution [37]. The interactions leading to excitonic splitting of molecular wave functions and electronic energy transfer are formally similar, if not identical, to those observed in the formation of aggregated chlorophyll species in solution, as shown by Shipman and co-workers [38,39]. The presence of a Chl *b* dimeric species in one of the chlorophyll subsets described above could easily reduce the excited state lifetime of that subset by the observed amount depending upon the magnitude of the dimer quenching rate constant. In this case, the Chl *a* and *b* molecules would exchange energy among themselves according to Eqn. 2, and irreversibility of excitation transfer would not be required. We note that the lifetime of the Chl *b* excited state in such a case would show a long-lived component that was some weighted average of the two 1/e times given in Table I. Since Chl *b* absorbs significantly more strongly than Chl *a* at 652 nm, this lifetime may be measured directly by ground state recovery [40]. The predictions for the number of exponential terms and lifetime of the Chl *b* decay in this case and the irreversible transfer case are quite different and provide a clear test of the models.

Conclusions

The isolated light-harvesting Chl *a/b* protein in what we believe is a monomeric form in a detergent micelle, exhibits surprisingly complex fluorescence decay kinetics. The decay curve is well described by the sum of two exponential decays with very similar weights and lifetimes of 1.2 and 3.3 ns. In terms of the current model for the structure of this protein and considering reasonable energy-transfer parameters, such a decay can only result from two noncommunicating pigment groups. Such structural subtlety may be required for the role of the Chl *a/b* protein in the regulation of energy transfer between the two photosystems in the intact chloroplast. Following the suggestion of Seely [41], subtle changes in the ordering of the Chl *a/b* proteins in the functional photosynthetic membrane may result in significantly distinct trapping probabilities by Photosystems I and II. This results from the rates of inter-protein energy transfer depending upon the relative orientation of two coupled proteins, and not simply the distance between them. Time-resolved absorption studies, which are in progress in our laboratory, will be of great value in unravelling the Chl *b* to *a* energy-transfer processes.

Acknowledgments

We thank Elizabeth Vierling for assistance with the electrophoretic procedure and Professor R.S. Knox for access to his work on the Chl *a/b* protein prior to publication. W.T.L. was supported by NIH training grant GM 07183. G.R.F. was an Alfred P. Sloan Fellow while R.S.A. was an Andrew Mellon Fellow during a portion of this research. Acknowledgment is made to the Donors

of The Petroleum Research Fund, administered by the American Chemical Society, for partial support of this research. This work was also supported by NIH GM 23944 and NSF PCM 78-10535 grants to R.S.A. and a grant from the Research Corporation to G.R.F.

References

1 Thornber, J.P., Alberte, R.S., Hunter, F.A., Shiozawa, J.A. and Kan, K.S. (1976) Brookhaven Symp. Biol. 28, 132–148
2 Markwell, J.P., Thornber, J.P. and Boggs, R.T. (1980) Proc. Natl. Acad. Sci. U.S.A. 76, 1233–1235
3 Förster, T., (1948) Ann. Phys. 2, 55–75
4 Knox, R.S. (1979) in Primary Processes in Photosynthesis (Barber, J., ed.), pp. 55–97, Elsevier-North Holland, Amsterdam
5 Shipman, L. (1980) Photochem. Photobiol. 31, 157–167
6 Millar, D.P., Robbins, R.J. and Sewail, A.H. (1981) J. Chem. Phys. 75, 3649–3660
7 Gochanour, C.R., Anderson, H.C. and Fayer, M.D. (1980) J. Chem. Phys. 70, 4254–4271
8 Hemenger, R.P., Pearlstein, R.M. and Lakatos-Lindenberg, K. (1972) J. Math. Phys. 13, 1056–1063
9 Gochanour, C.R. and Fayer, M.D. (1981) J. Phys. Chem. 85, 1989–1994
10 Burke, J.J., Ditto, C.L. and Arntzen, C.J. (1978) Arch. Biochem. Biophys. 187, 252–263
11 Lieberman, J.R., Bose, S. and Arntzen, C.J. (1978) Biochim. Biophys. Acta 502, 417–429
12 Van Metter, R.M. (1977). Biochim. Biophys. Acta 462, 642–657
13 Shepanski, J.S. and Knox, R.S. (1982) Isr. J. Chem., in the press
14 Kan, K.S. and Thornber, J.P. (1976) Plant Physiol. 57, 47–52
15 Thornber, J.P. (1970) Biochemistry 9, 2688–2697
16 Laemmli, U.K. (1970) Nature 227, 680–685
17 Strain, H.H., Cope, B.T. and Svec, W.A. (1971) Methods Enzymol. 23A, 452–476
18 Robbins, R.J., Fleming, G.R., Beddard, G.S., Robinson, G.W., Thistlethwaite, P.J. and Woolfe, G.J. (1980) J. Am. Chem. Soc. 102, 6271–6278
19 Fleming, G.R., Morris, J.M. and Robinson, G.W. (1976) Chem. Phys. 17, 91–100
20 Robbins, R.J. (1980) Thesis, University of Melbourne
21 Kendall, M.G. and Sutart, A. (1976) Advanced Theory of Statistics, Vol. 1, Ch. 16.5, 3rd edn., pp. 370–372, Hafner Publishing Co., New York
22 Nordlund, T.M. and Knox, W.H. (1981) Biophys. J. 36, 193–202
23 Searle, G.F.W. and Tredwell, C.J. (1979) in Chlorophyll Organisation and Energy Transfer in Photosynthesis, Ciba Found. Symp. 61, 257–277
24 Il'ina, M.D., Kotova, E.A. and Borisov, A.Yu (1981) Biochim. Biophys. Acta 636, 193–200
25 Helenius, A. and Simons, K. (1975) Biochim. Biophys. Acta 415, 29–79
26 Vierling, E. and Alberte, R.S. (1981) Plant Physiol. 62, 498
27 Kung, S.D. and Thornber, J.P. (1971) Biochim. Biophys. Acta 253, 285–289
28 Vander Meulen, D.L. and Govindjee (1973) J. Sci. Ind. Res. 32, 62–69
29 Altmann, J.A., Beddard, G.S. and Porter, G. (1978) Chem. Phys. Lett. 58, 54–57
30 Beddard, G.S. and Porter, G. (1977) Biochim. Biophys. Acta. 462, 63–72
31 Livingston, R., Watson, W.F. and McArdle, J. (1948) J. Am. Chem. Soc. 71, 1542–1550
32 Auerbach, R.A. and Robinson, G.W. (1980) J. Chem. Phys. 72, 3528–3538
33 Ippen, E.P., Shank, C.V. and Woerner, R.L. (1977) Chem. Phys. Lett. 46, 20–23
34 Ippen, E.P., Shank, C.V. and Bergman, A. (1976) Chem. Phys. Lett. 38, 611–614
35 Van Metter, R.M. and Knox, R.S. (1976) Chem. Phys. 12, 333–340
36 Haan, S.W. and Zwanzig, R. (1978) J. Chem. Phys. 68, 1879–1883
37 Yuen, M.J., Shipman, L.L., Katz, J.J. and Hindman, J.C. (1980) Photochem. Photobiol. 32, 281–296
38 Shipman, L.L. (1977) J. Phys. Chem. 81, 2180–2184
39 Shipman, L.L., Norris, J.R. and Katz, J.J. (1976) J. Phys. Chem. 80, 877–881
40 Beddard, G.S., Fleming, G.R. and Waldeck, D.H. (1981) Il Nuovo Cimento 63B, 151–173
41 Seeley, G.R. (1973) J. Theor. Biol. 40, 189–199