CHARACTERIZATION OF THE FLUORESCENCE DECAYS OF THE CHLOROPHYLL A/B PROTEIN

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INTRODUCTION

Characterization of chlorophyll excited state lifetimes provides a sensitive means for investigating the excitation migration and trapping kinetics in the photosynthetic apparatus. The fluorescence decay of intact photosynthetic membranes is complex and the interpretation of these decays is nontrivial. We have been using biochemical purification of photosynthetic subcomponents and genetic manipulation of whole cells to simplify the analysis and to resolve features that may be hidden in the in vivo decays.

The Chl a/b protein was examined by using both of these approaches in order to characterize more precisely this ubiquitous protein. In the first stage of experiments the protein was isolated from barley and characterized under a variety of ionic and denaturing conditions in vitro. Secondly, we studied the protein in vivo using the Chlamydomonas mutant, C2, which does not have either reaction center I or II. We have found that 1) the in vitro protein has an ionic strength dependent absorption spectrum that does not seem to affect the fluorescence decay; 2) the time resolved fluorescence of the in vitro protein is a biexponential decay of the form \( f(t) = 15\exp(-t/0.7 \pm 0.15 \text{ ns}) + 85\exp(-t/3.2 \pm 0.2 \text{ ns}) \) demonstrating that \( >90\% \) of the steady state fluorescence is due to the 3.2 ns component; and 3) the in vivo decay of the C2 mutant is a single exponential with a lifetime of 2.4 \( \pm 0.1 \text{ ns} \).

MATERIALS AND METHODS

The Chl a/b protein was isolated from barley by the method of Lotshaw et al. (2) with the following modifications: 1) the washed membranes were homogenized in a solution of 1% sodium dodecyl sulfate in 20 mM tricine (pH
7.2) (1 ml/mg chlorophyll) for 2.5 minutes and immediately centrifuged to remove insoluble material; 2) the green eluate of the hydroxylapatite column was collected in fractions, each fraction was brought to 0.31 mM Triton X-100, those fractions which had a minimal chlorophyll b emission were combined, and the combined fractions were desalted by passage through a Sephadex G-25 column equilibrated with 1.8 mM SDS, 0.31 mM Triton X-100, 20 mM Tricine (pH 7.4). The C. reinhardii strain C2 was grown in partial light to a typical cell density of 1x 10^5 cells/ml in an acetate containing medium. The C2 strain has acquired a secondary mutatuion, heritable starch granule, that is characterized by one extremely large starch granule incasing the pyrenoid (E. Beasley and L. Mets personal communication). C2 cultures in late log phase the starch accumulation becomes so extreme that normal division is blocked and the cells clump.

Steady state absorption measurements were performed on an Aminco DW 2 and the steady state fluorescence measurements were performed with a corrected Aminco SPF 500. Fluorescence decays were determined by time correlated single photon counting (1).

Results and Discussion

The Chlamydomonas mutant C2 is a PSI PSII strain that has no detectable P700 activity, does not show fluorescence induction, and lacks the reaction center proteins which are normally detectable upon gel electrophoresis (L. Mets unpublished). The C2 strain can thus be used to establish the behavior of the Chl a/b protein in the thylakoid membrane. The absorption spectrum of C2 (Fig. 2) is identical to that of the in vitro Chl a/b protein. The fluorescence decay of the C2 mutant fits well to a single exponential decay with a lifetime of 2.4 + .07 ns. The lifetime of 2.4 ns is independent of excitation and emission wavelengths at the experimental excitation wavelengths
of 652 and 665 nm and the emission range of 680 to 720 nm. We attribute the very slight nonexponentially observed in our previous study (1) to cultures which contained nonphysiological cells (see Materials and Methods).

Figure 1 shows the absorption spectra of the isolated Chl a/b protein from barley at 0.24 M and 0.12 M M Na HPO and in salt-free buffer (pH 7.2). As the salt concentration is raised the Chl a and Chl b absorption peaks broaden but do not shift in wavelength. The two simplest explanations for this salt effect are that either the number of peptides per micelle increases with increasing ionic strength or that specific ionic interactions exist between the protein and the ions. Since we can reproduce the same effects with MgCl₂, NaCl, or Na HPO and since the same broadening occurs at nearly equal ionic strengths of the solutions, the existence of either a specific cationic or anionic interaction with the protein seems unlikely. To understand the characteristics of a salt-dependent aggregation of the protein in the micelle, the details of a salt-dependent aggregation of the protein must be considered.

Little is known about the macrochemistry of mixed detergent solutions and even less on mixed detergent-protein solutions but it is known that the micelles formed are a mixture of both SDS and Triton X-100 (3). The Triton X-100 concentration of the protein solution is 0.31 mM which is in excess of the critical micelle concentration (cmc) of Triton X-100 (0.24 mM, 3). Furthermore, the ionic strength of a solution has an inverse effect on the cmc (the number of detergent monomers per micelle). Therefore, all of the detergent-salt buffers have detergent in excess of the cmc. One possible effect of an increased ionic strength is to favor the formation of larger micelles. Thus these larger micelles could contain more more peptides. Another possibility is that salt-induced aggregation of the Chl a/b protein could be occurring. Aggregation of the protein is known to occur at high salt
concentrations and is exploited in the precipitation of the protein as crystals (4). Whether the number of peptides present in a micelle increases under higher ionic strengths is presently being investigated.

The fluorescence spectra of the isolated Chl a/b protein and the C2 mutant are shown in Figure 2. The fluorescence spectrum is a sensitive measure of the status of the in vitro protein. When the protein denatures the efficient energy transfer from Chl b to Chl a no longer occurs and Chl b fluorescence is observed. The C2 mutant (Fig. 2) shows that the Chl a/b protein in its native environment has no Chl b emission, whereas our best isolates of the Chl a/b protein have a small shoulder at 652 nm that is attributed to Chl b emission. The emission spectra of the high salt and salt free protein solutions are indistinguishable.

The fluorescence decay of the isolated Chl a/b protein fits well to a biexponential function of the form \( f(t) = 15\exp(-t/0.7 \pm 0.15 \text{ ns}) + 85\exp(-t/3.2 \pm 0.2 \text{ ns}) \). Over 97% of the steady state fluorescence is accounted for by the 3.2 ns component in our best preparations. Further, the integrity of the samples can be checked by time resolved fluorescence measurements. Depending on the degree of denaturation of the protein via time or heat, the fits to the fluorescence decays change. The long component increases in lifetime while the short component decreases and the inclusion of a third component becomes necessary to achieve a good reproduction of the data. As the protein denatures two possible effects occur which disrupt the Forster transfer of energy from one chromophore to another: 1) the chlorophylls may dissociate from the protein and become free in the core region of the micelles; and/or 2) the chlorophylls may remain associated with the denatured protein but assume new positions relative to each other that would lead to either a higher probability of nonradiative quenching (decreased separation) or a lower
probability of both energy transfer and nonradiative quenching (increased separation). The changes on denaturation are very similar to those observed by Ide et al. (1986), where the authors isolated the Chl a/b protein from pea seedlings and maintained the protein in a n-octyglycoside detergent solution. We propose that the biexponential fluorescence behavior of the \textit{in vitro} Chl a/b protein is most likely due to a small amount of denaturing of protein inherent in the isolation procedure.

The disparity between the C2 mutant lifetime of 2.4 ns and the lifetime of \textit{in vitro} protein from barley of 3.2 ns is not fully understood. Two possibilities exist which are presently being investigated. The first possibility is that the Chl a/b protein from barley and \textit{Chlamydomonas} are inherently different. An experiment is underway to answer this question by isolating the protein from C2 and measuring its fluorescence decay. A second possibility is that the macroscopic environment of the protein in the micelle causes the protein to have a slightly different structure, aggregation states, or boundary interactions and these effects lead to the increased lifetime. To test this possibility a technique was developed that of reconstitutes the protein into mixed lipid vesicles containing the lipids sulfoquinovosyldiacylglycerol, monogalactosyldiacylglycerol and digalactosyldiacylglycerol. These vesicles, when formed with the proper ratio of the lipids retain the steady state fluorescence properties of the native sample (Figure 3). By determining the fluorescence decays of these Chl a/b protein vesicles we will be able to establish if the protein must be in a thylakoid lipid environment to emulate \textit{in vivo} conditions.