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Autor: Srivastava, Alaka / Darszon, Alberto / Strasser, Reto J.

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THE INFLUENCE OF WATER ON THE STABILITY AND ACTIVITY OF PHOTOSYNTHETIC COMPLEXES, MEMBRANES AND CELLS IN APOLAR SYSTEMS

BY

Alaka SRIVASTAVA[1], Alberto DARSZON[2] & Reto J. STRASSER[1]

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ABSTRACT

The Influence of Water on the Stability and Activity of Photosynthetic Complexes, Membranes and Cells in Apolar Systems. - Water plays a fundamental role in determining the reaction rates and the thermodynamics of biological systems. Reverse micelles offer the possibility of modulating the amount of water to which enzymes and multienzymatic complexes are exposed. We have searched for a biological source of photosynthetic complexes amenable for transfer to reverse micelles in a functional state. Fast fluorescence induction kinetics of chlorophyll *a* (Chl *a*) have been used as an assay for the functional transfer into reverse micelles of photoactive complexes from higher plant (spinach thylakoid membrane), green algae (*Chlamydomonas reinhardtii*) and purple bacteria (*Rhodospirillum rubrum*) cells. Spinach thylakoid membranes and *C. reinhardtii* cells could be solubilized in the HTS (Hexadecane-Tween-Span) reverse micellar system, and showed water dependent increases in variable fluorescence, however they were unstable after few minutes of incubation in this system. The photosynthetic activity of thylakoid membranes and *C. reinhardtii* cells was also unstable in reverse micelles formed with phospholipids (PLCs). In contrast, bacterial cells could be transferred in a stable functional form either to HTS or to PLC reverse micelles. The stable bacterial photosynthetic complexes in HTS or in PLC reverse micelles could be used to further understand the influence of water on the organization and function of photosynthetic complexes.

Key-words: Chlorophyll *a* fluorescence, Reverse micelles, Photosynthetic activity in Low Water System.

Abbreviations: ABS, absorption; Chl *a*, chlorophyll *a*; F_0 and F_M, initial and maximum Chl *a* fluorescence; φ_{P_0}, maximum quantum yield of primary photochemistry; HTS, hexadecane-tween-span; J, I, intermediate steps of Chl *a* fluorescence rise between F_0 and peak (P); LED, light emitting diode; PLC, protein lipid complex; PSII, photosystem II; Q_A and Q_B, primary and secondary bound plastoquinone; RC, reaction center; TR, energy flux for trapping; V_J, relative variable fluorescence at 2 ms.

[1] Bioenergetics Laboratory, University of Geneva, CH-1254, Jussy, Geneva, Switzerland.

[2] Institute of Biotechnology, Dept. Molecular Genetics and Physiology Universidad Nacional Autónoma de México, Cuernavaca, Morelos, Mexico.

Corresponding Address: Alaka Srivastava, Bioenergetics Laboratory, University of Geneva CH-1254, Jussy-Geneva Switzerland. Telephone No. ++41 22 759 19 44, Fax No.: ++41 22 759 19 45, e-mail: strasser@uni2a.unige.ch darszon@ibt.unam.mx

INTRODUCTION

Water is a key molecule in cellular organization, protein stability and function (KAUZMAN, 1959; RUPLEY & CARERI, 1991; MAKHATADZE & PRIVALOV, 1993). The activity of water soluble enzymes, and to a lesser extent membrane proteins, in reverse micelle solutions have been studied extensively (see reviews by LUISI & MAGID, 1986; MARTINEK *et al.*, 1986; LUISI *et al.*, 1988; DARSZON & SHOSHANI, 1992). It has been shown that reverse micellar systems are able to house nucleic acids, plasmids, organelles and even various types of cells (HAERING *et al.*, 1985; DARSZON *et al.*, 1988; HOCHKOEPPLER *et al.*, 1989; PFAMMATTER *et al.*, 1989; reviewed in PFAMMATTER *et al.*, 1992, ESCAMILLA *et al.*, 1992; FAMIGLIETTI *et al.*, 1992). Because the suspension becomes transparent when the cells or organelles are transferred to reverse micelles, the process has been operationally referred to as "solubilization", although this is not strictly occurring. Reverse micelles are formed in an organic apolar solvent from various types of amphipathic molecules such as detergents and phospholipids, and determined amounts of water (DARSZON & SHOSHANI, 1992). They allow the possibility of controlling how much water is available to proteins, organelles and cells housed in their interior (LUISI *et al.*, 1988).

We have become interested in how water influences the function and organization of photosynthetic complexes. In the present paper we have searched for a biological source of photosynthetic complexes amenable for transfer to reverse micelles in a functional state.

Fluorescence induction kinetics has been used to evaluate the photosynthetic capacity of cells and organelles. This light induced transients measure the kinetics of the filling of the plastoquinone pool and the heterogeneity associated with it (see reviews by PAPAGEORGIOU, 1975; LAVOREL & ETIENNE, 1977; GOVINDJEE & SATOH, 1986; GOVINDJEE, 1995). In a green tissue, the kinetics of the Chl *a* fluorescence rise indicates the filling up of electron acceptor pools (Q_A, Q_B and PQ, etc.) with electrons originating from water on the donor side of PSII. Three different rise components have been distinguished when Chl *a* fluorescence induction curves are measured *in vivo* (SCHREIBER & NEUBAUER, 1987; NEUBAUER & SCHREIBER, 1987; STRASSER *et al.*, 1995, 1999; SRIVASTAVA *et al.*, 1995a). At 600 Wm^{-2} of 650 nm light, as used in this paper, the successive steps in the Chl *a* fluorescence rise curves are: O to J at about 2 ms, J to I at about 30 ms and I to P at about 500 ms. From the P level, the transient decays via S to M toward the terminal steady state T (PAPAGEORGIOU, 1975).

We have used different signals of Chl *a* fluorescence induction transients as an assay for the functional transfer into reverse micelles of photoactive complexes from spinach chloroplasts, and cells of *Chlamydomonas reinhardtii* and *Rhodospirillum rubrum*. Although spinach thylakoid membranes, and green algae *C. reinhardtii* can be "solubilized" in various reverse micellar systems, only the bacterial cells could be transferred either to HTS-reverse micelles or to PLC-reverse micelles in a stable functional form. We also report that the rate of primary photochemistry, measured as

Chl *a* fluorescence induction kinetics, is water dependent, and thus this non-conventional system may allow a better understanding of how water modulates this process. Photosynthetically active bacterial cells in HTS or bacterial photosynthetic complex in PLC could have interesting applications in Biotechnology, such as analysis by dry chemistry methods. A summary of this work was published in an abstract (SRIVASTAVA *et al.*, 1997) and in a proceeding (OBREGON *et al.*, 1998).

MATERIALS AND METHODS

Materials

Thylakoid membranes were prepared according to SRIVASTAVA *et al.* (1995b). Wild type *Chlamydomonas reinhardtii* cells were grown mixotrophically in tris-acetate phosphate medium at pH 7.0 in a growth chamber at 20°C and illuminated for 12 hours with fluorescent white light. All the experiments were done with cells obtained during the log phase of their optimal growth (about 3 to 4 days after incubation). About 1 ml of the cell suspension was dark adapted for 5 min in a microcentrifuge tube and then centrifuged. The supernatant was discarded and the internal wall of the tube carefully wiped with soft tissue paper to remove excess water. The pellet containing the cells was resuspended in HTS - reverse micelles containing either 0 %, 2 %, 4 % or 6 % water. The final chlorophyll concentration in the measuring system was ~15-20 µg/ml (measured according to PORRA *et al.*, 1989).

Wild type cells of *Rhodospirillum rubrum* were grown anaerobically in Sistrom medium (SISTROM, 1960) photo-heterotrophically with succinate as carbon source, as described earlier (GHOSH *et al.*, 1994). During their optimum growth period ~ 25 ml of cell suspension was centrifuged and the cells were collected as a pellet. The pellet was resuspended in 200 µl of M-medium. An aliquot of 5 µl of cell suspension was used for each measurements.

HTS Reverse Micelles

A yellow transparent solution was obtained by dissolving Tween 85 (77.4 mM) and Span 80 (8.6 mM) in hexadecane. Water concentration in the HTS system was pre-adjusted by adding resuspension medium (200 mM sorbitol, 20 mM Tricine, 5 mM NaCl; pH 7.5) for thylakoids and M-medium and tris-acetate phosphate medium for *R. rubrum* and *C. reinhardtii* respectively.

CHO Reverse Micelles

Micelles were prepared by suspending 200 mM of cetyl trimethyl ammonium bromide (CTAB) in n-octane and hexanol in a 8:6.1 (V/V) ratio. The system became transparent after adding 1.5 % of H_2O.

Phospholipid Reverse Micelles

Soybean phospholipids were dissolved (10 mg) in isooctane (1 ml). Thylakoids, or *C. reinhardtii* or *R. rubrum* cells, equivalent to about 1 mg protein in ~70 ml, was added

to 1 ml phospholipid reverse micelles. The mixture was sonicated for 3-5 min in a water bath sonicator (Bandelin Sonorex Super RK-102H) under dim light at ~3-4°C. After sonication, 100 ml of ice cold 1 M $MgCl_2$ was added to the emulsion, which was then vortexed for 1-2 min. The preparation was further centrifuged for 3-5 min at 4000 rpm (Universal/K2S, Whitish, Tuttlingen, Germany), and the organic fraction containing the protein-lipid -complexes (PLCs) was collected from the top of the tube.

Viability Test of C. reinhardtii in Low Water System

To test the viability of *C. reinhardtii* cells in low water system, about 10 ml of the cells suspension, growing in their log phase (about 2-3 days old), were centrifuged in a test tube. After removing most of the water content from the test-tube the pellets were resuspended and incubated in 5 ml of HTS containing 0, 4 or 6 % water and kept for 5 min. Later on, these treated cells were layered on top of a centrifuge tube containing mixotroph medium, and centrifuged at 2000 RPM for 5 min. After centrifugation, the upper organic phase and the water phase were discarded and the pellet was washed once again with tris-acetate phosphate medium. From this pellet an aliquot of cells containing 20 µg of chlorophyll was added to 50 ml of mixotroph medium and kept in a growth chamber with proper shaking. Thereafter, every day 1 ml of the cell suspension was taken out and Chl *a* fluorescence and chlorophyll concentration (PORRA *et al.*, 1989) measured.

Fluorescence Measurements

Chl *a* fluorescence induction kinetics were measured with a fluorometer (Plant Efficiency Analyzer (PEA), Hansatech Ltd., King's Lynn, Norfolk, England) with 650 nm of 600 Wm^{-2} light intensity. Light was provided by an array of 6 LED (peak, 650 nm), focused on the sample surface to provide a homogeneous illumination over the exposed area (4 mm diameter). Chl *a* fluorescence signals were detected using a PIN photocell after passing through a long pass filter (>730 nm, 50 % transmission). For measuring the bacterial fluorescence the regular long pass filter was changed by an 890 nm interference filter.

Absorption Changes Measurements

Light induced absorbance changes around 820 nm were measured by combining the Hansatech $P700^+$ measuring system with the PEA-fluorometer. This was done by replacing one of the red LEDs (out of 6) from the PEA head with a broad far red LED, which was covered by an interference filter (peak 820 nm). The measuring beam modulated at 4 KHz was provided through this far red LED. The transmitted light was monitored at the opposite side of the sample by a photodiode screened as well by a 820 nm interference filter. Actinic light was provided by red LEDs through PEA. The signal from the detector was simultaneously plotted in a chart recorder and digitized and stored with a 12-bit resolution in a computer.

RESULTS AND DISCUSSION

Limited functional studies of active membrane-bound enzymes have been performed in reverse micelles. The catalytic cycle of cytochrome c oxidase (ESCAMILLA, *et al.*, 1989; KORNBLATT & KORNBLATT, 1992) and of the sarcoplasmic reticulum ATPase (BARRABIN, 1993) involve intermediates with distinct water requirements. Water movements at the catalytic site of the chloroplast CF1 have been reported to occur in chloroplast membranes during photo-phosphorylation (ZOLOTAREVA *et al.*, 1990). Also, the activity of the chloroplast ATPase in reverse micelles depends on the water content (KERNEN *et al.*, 1997). These results prompted us to search for a biological system from which functional photosynthetic complexes could be transferred into reverse micelles where the influence of water on phototransduction can be explored.

Fast fluorescence induction kinetics of Chl *a* were used as a tool to evaluate the photosynthetic activity of cells and organelles transferred to various reverse micellar systems. When dark-adapted green samples are exposed to saturating light the Chl *a* fluorescence transient starts from F_0 intensity and increases to a maximum peak (P or M). Chl *a* fluorescence signals plotted on a log time scale reveal two intermediate steps between F_0 and F_P labeled as J (F_J) and I (F_I). This variable fluorescence signal is defined as: $F_V = F_M - F_0$, where F_0 and F_M are the minimum and the maximum fluorescence.

(A) HTS- Reverse Micellar System

It is surprising that complex structures as large as cells can be housed in reverse micellar systems. It is thought that medium sized proteins (50-100 Å) are hosted in the water pool of reverse micelles, which have similar dimensions. However, it is difficult to envisage how cells and organelles that have diameters in the range of mm, are accommodated in reverse micelles whose size has never been found to be this large. In this regard, considering our ignorance about the mechanisms involved in housing large structures in reverse micellar systems, we simply refer to their transfer to the system. We chose to use the HTS micellar system because it has been perhaps the most successful in housing functional cells, probably due to its hydrophobic-hydrophilic balance (PFAMMATTER *et al.*, 1992).

(a) C. reinhardtii Cells and Spinach Thylakoid Membranes in HTS Reverse Micellar System: Dark Adapted cells of *C. reinhardtii* in aqueous media showed regular O-J-I-P Chl *a* fluorescence kinetics, when exposed to actinic light (Fig. 1A). In contrast when the cells were transferred to HTS without water (0 %), the Chl *a* fluorescence transient almost leveled off at the J level and lost a large fraction of the variable fluorescence Fv. The transient became of the O-J-P type. Interestingly, the Chl *a* fluorescence transient of cells transferred to HTS became closer to that in all aqueous media as the amount of H_2O in the micelles was increased. The changes in variable fluorescence were found to depend on the incubation time (see Fig. 3) and water content in the system. After incubating cells in HTS containing 6 % and 4 % water for 2 min, about 86 % and 73 %

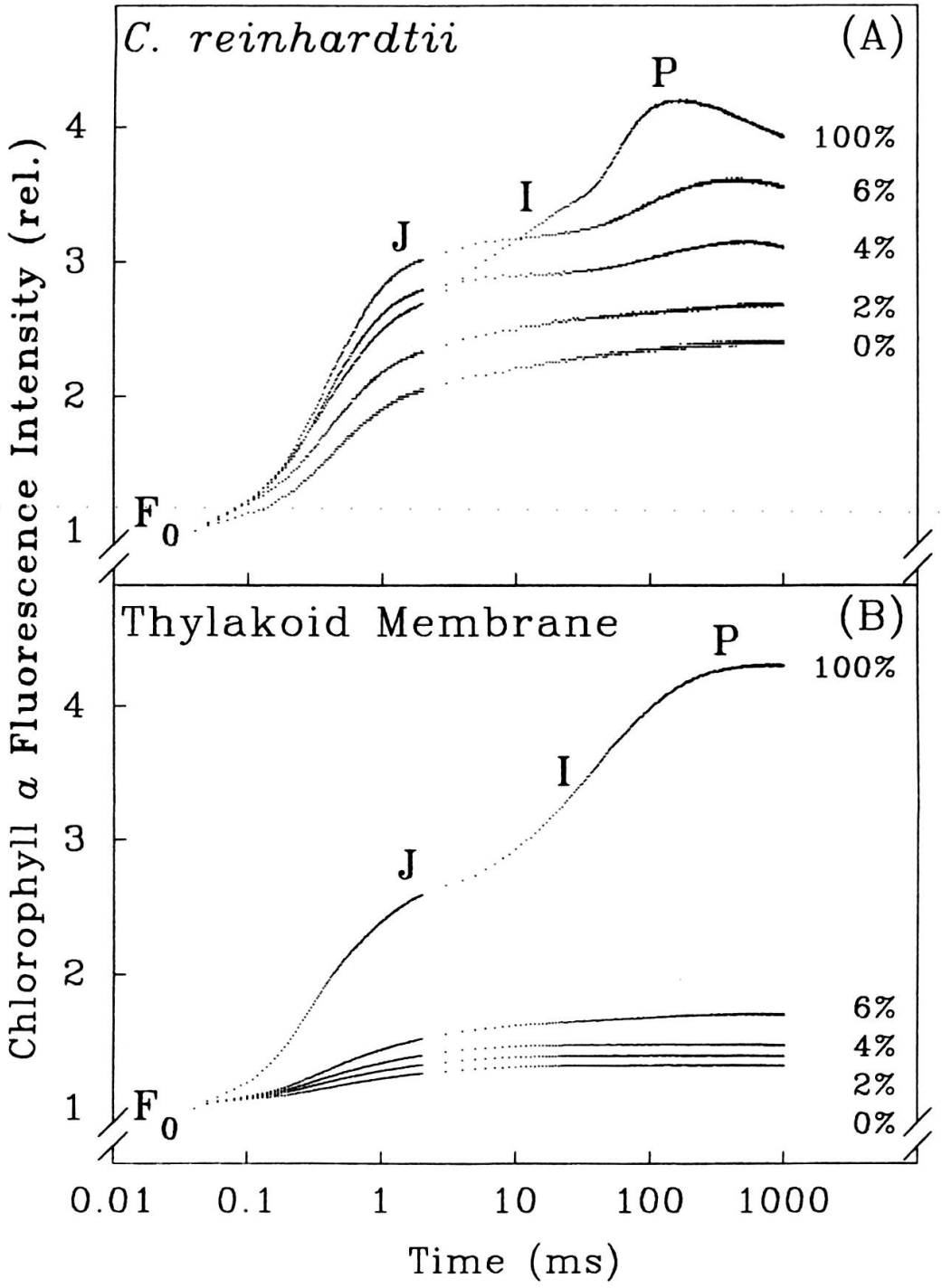

FIG. 1.

Effect of water content on the fast Chl *a* fluorescence induction kinetics of *C. reinhardtii* cells (A) and spinach thylakoid membranes (B) in the HTS system. Cells and thylakoid membranes were prepared, transferred to HTS reverse micelles as described in Methods and exposed to 600 Wm^{-2} red actinic light after 2 min of incubation in aqueous media (100 %), or in HTS micelles containing 6, 4, 2 and 0 % water.

of variable fluorescence was observed respectively, in comparison to control cells in buffer (Fig. 1A).

Like *C. reinhardtii* cells, isolated spinach thylakoid membranes exposed to actinic light displayed well defined Chl *a* fluorescence induction kinetics (Fig. 1B). However when the membranes were suspended in HTS micelles with 0 % water, a sharp decrease in variable Chl *a* fluorescence was observed. An increase in F_0 and a decrease in F_M, which became more pronounced after longer incubation times in HTS (not shown), resulted in a very small variable Chl *a* fluorescence (Fig. 1B). Addition of 6 % water to HTS allowed the recovery of a small but reproducible fraction of variable Chl *a* fluorescence, however it still decreased with time and vanished in 5 min. Apparently the HTS micellar system can partially dissolve antenna chlorophyll, leading to increases in F_0.

To better understand why the variable Chl *a* fluorescence signal improved with the water content of the HTS micelles, the extracted pigments were quantified both for the transferred thylakoid membranes and the *C. reinhardtii* cells (Fig. 2). After a 5 min incubation in HTS micelles without water, more than 86 % of the pigment was extracted from thylakoid membranes. As the water content in HTS micelles was increased, the extracted pigments decreased (see Fig. 3). Pigment extraction into HTS micelles also increase with time (data not shown). In comparison to thylakoid membranes, the amount of pigment extracted when the *C. reinhardtii* cells were incubated in HTS-reverse micelles was much smaller, and in presence of 6 % H_2O insignificant amount of pigment got extracted (Fig. 2).

(b) Quantitative Criteria of the Influence of Water on Chl a Fluorescence Induction Kinetics of Isolated Thylakoid Membranes and C. reinhardtii Cells in HTS Reverse Micellar System: The *C. reinhardtii* cells and thylakoid membranes incubated in HTS with different concentrations of water showed major changes in the levels of Chl *a* fluorescence kinetics (FJ, FI and FM) with respect to the HTS water content and time of incubation as shown in Fig. 3. The results indicate that: (a) thylakoid membranes are more sensitive to water than the *C. reinhardtii* cells, (b) total variable fluorescence decreases by lowering the HTS water content and by increasing the incubation time, (C) a sharp decrease in I to F_M level occurs as less water is present and as the incubation period is extended, (D) although the J level decreased with the HTS water content and time incubation in the low water system, it is less sensitive than the I and the F_M levels, (E) in the presence of 6 % water, and after 5 min of incubation, insignificant differences in between J, I and F_M levels were observed. In HTS system, the transients obtained from both thylakoid membranes and from *C. reinhardtii* cells exhibit a shape reminiscent of the transients recorded in the presence of DCMU, a herbicide that blocks the steps after Q_A^- (STRASSER *et al.*, 1995, SRIVASTAVA *et al.*, 1995a,b). Possibly by removing pigments, HTS micelles without water prevent the electrons to go further than Q_A^-.

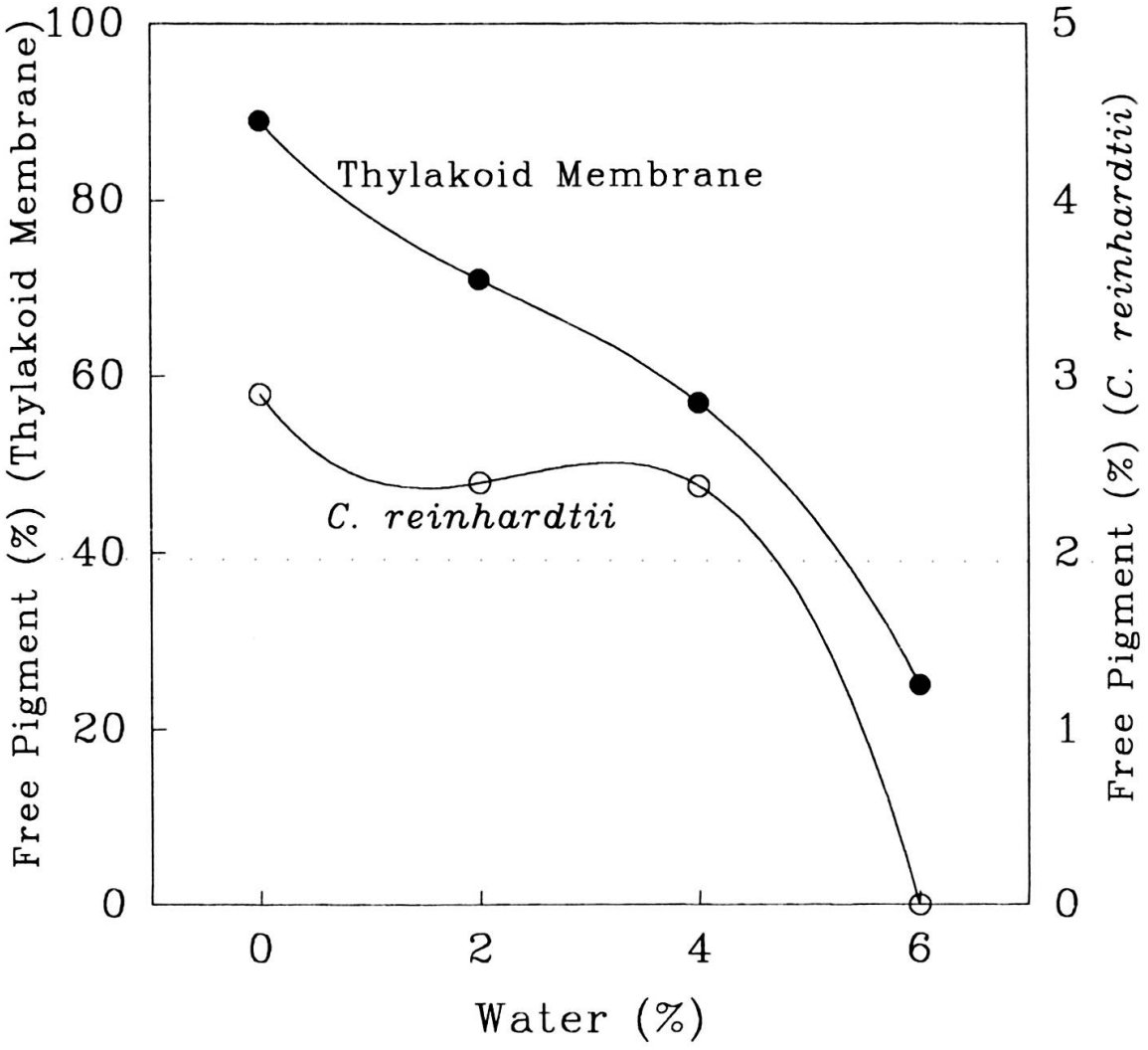

FIG 2.

Water dependence of the extracted pigment from thylakoid membrane and the *C. reinhardtii* cells after incubating them for 5 min in HTS reverse micelles. *C. reinhardtii* cells and thylakoid membranes equivalent to about 25 μg of chlorophyll were incubated in HTS reverse micelles containing different concentration of water. After 5 min, the preparations were centrifuged and the spectra measured (600 nm to 750 nm) from the green supernatant. Changes in the absorption peak at 665 nm (after correcting them at 750 nm) were used to quantify the pigment. One hundred percent free pigment was obtained by dissolving 25 μg of total chlorophyll in methanol. Chlorophyll concentration was also measured in the pellet after dissolving it in methanol. Notice the larger Y-axis values for *C. reinhardtii* than for thylakoid membranes.

(c) Does water affect photosynthetic activity and/or the transfer and stability of membrane-protein complexes in micelles? Since the Chl *a* fluorescence rise represents the filling of the electron acceptor pool of PSII (SCHREIBER & NEUBAUBER, 1987; STRASSER *et al.*, 1995), the water dependence of F_V could indicate that this step is water sensitive. Alternatively, increasing the water pool in the micellar system could be simply protecting the cells from deleterious detergent effects.

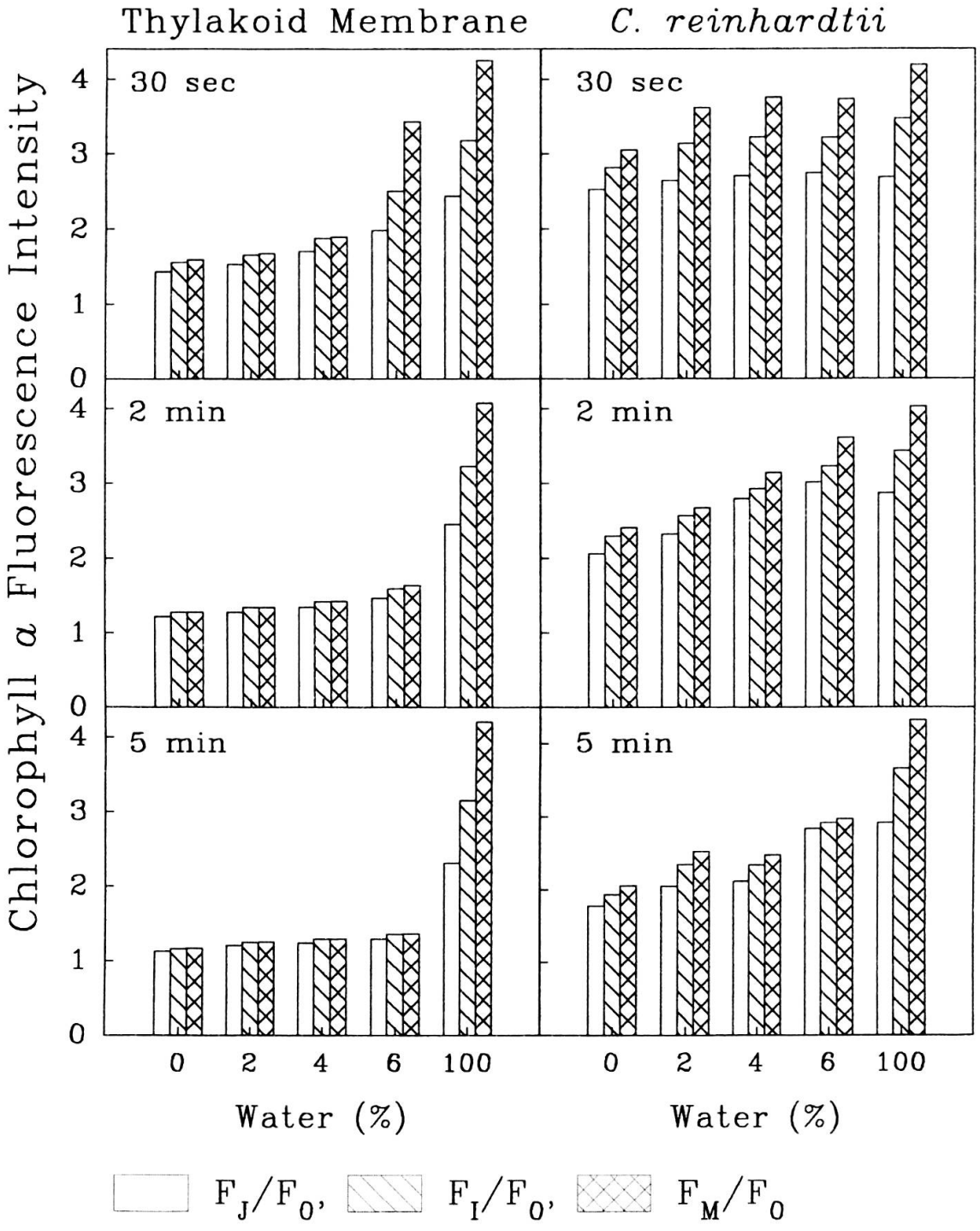

FIG. 3

Effect of time of incubation of spinach thylakoid membrane (left panel) or *C. reinhardtii* cells (right panel) in HTS reverse micelles containing different water concentrations on F_J, F_I and F_M levels. Experimental conditions are as in Fig. 1. Data are normalized by their F_0 values.

To evaluate the contribution of each of the two explanations to the effect of HTS-water-content on F_V, *C. reinhardtii* cells were incubated in presence of 2 % water, allowed to stabilize for 5-6 min, and thereafter, supplemented with 2 or 4 % water. An increase in F_V was observed, which was again found to be water dependent (Fig. 4). However, even after rising the HTS micellar water content to 6 %, the F_V recovery did not match that found in cells directly transferred to HTS with 6 % water (Fig. 4). Though these results confirm that water influences the photosynthetic activity of the cells in HTS reverse micelles, this solvent also affects the transfer process itself.

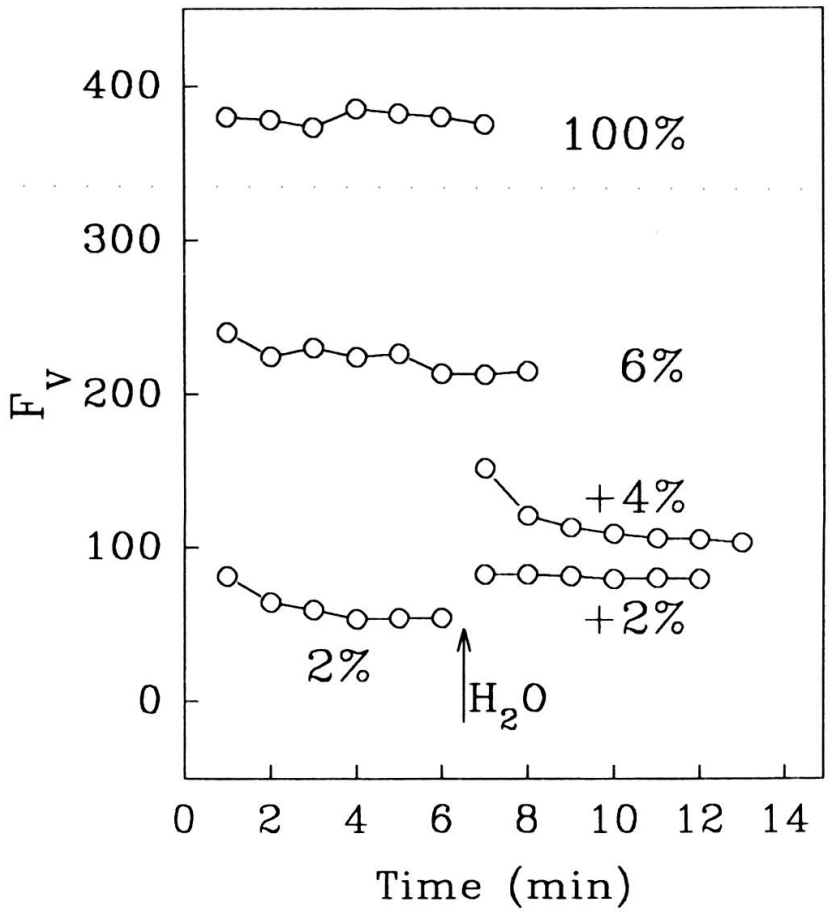

FIG. 4.

Variable fluorescence changes of *C. reinhardtii* cells in 2 % water containing HTS micelles after a further addition of 2 and 4 % water. Fluorescence induction kinetics of cells in 2 % water HTS micelles were measured, as indicated in Fig. 1 and in Methods, after 1 min dark intervals for 6 min. Thereafter, cells were supplemented with water, and recordings continued for another 6 to 7 min. Changes in the F_V (F_M-F_0) are shown in the figure.

(d) Analysis of the Alterations in PSII of Spinach Thylakoids and C. reinhardtii Cells in HTS Reverse Micellar System Using the JIP-test: The fast phase of the fluorescence induction kinetics have been suggested to be controlled by photochemical charge separation (the photochemical phase) leading to the reduction of the quencher Q_A to

Q_A^-, however, this process is also affected by the donor side (SCHREIBER & NEUBAUER, 1987; NEUBAUER & SCHREIBER, 1987; STRASSER *et al.*, 1995). The J to I and I to P levels reflect certain heterogeneity, which is due to the existence of fast and slow reducing plastoquinone centers, as well as due to different redox states of the RC which reduce the plastoquinone. The maximum rate of photochemical reaction, when all RCs are open, can be determined precisely by measuring the initial slope of the variable Chl *a* fluorescence ($dV/dt_0 = (F_{300\mu s} - F_{50\mu s}) / (F_M - F_0)$). For the dark adapted samples, assuming that all RCs are open, the ratio of the maximum exciton trapping flux (TR_0) and the absorption flux (ABS) can be calculated from F_0 and F_M measurements for any sample theoretically as:

(1) The link between variable fluorescence ($F_V = F_M - F_0$) and maximum yield of excitation energy trapping $\varphi_{Po} = TR_0 / ABS$, and quenching phenomenon by quinones, has been derived (KITAJIMA & BUTLER, 1975):

$$\varphi_{Po} = TR_0 / ABS = 1 - F_0 / F_M = F_V / F_M \qquad (1)$$

(2) The link between variable fluorescence (F_V) and energy cycling between the RC and the core antenna (for detail see BUTLER & STRASSER, 1977) leads to the same expression (BUTLER & KITAJIMA, 1975):

$$\varphi_{Po} = TR_0 / ABS = 1 - F_0 / F_M = F_V / F_M = T \qquad (2)$$

where T is the probability that an exciton in the core antenna reaches a closed RC and excites again a core antenna chlorophyll.

(3) Considering energy cycling between the RC and the core antenna (trapping T) as well as between the core antenna and the light harvesting complex (cycling C), leads to the same expression (BUTLER & STRASSER, 1977):

$$\varphi_{Po} = TR_0 / ABS = 1 - F_0 / F_M = F_V / F_M = T / (1 - C) \qquad (3)$$

(4) If in addition to trapping T and coupling C, cooperativety between photosynthetic units (grouping G) is considered, the maximum quantum yield of excitation energy trapped has been expressed as (STRASSER, 1978, 1986):

$$\varphi_{Po} = TR_0 / ABS = 1 - F_0 / F_M = F_V / F_M = T / (1 - C) * (1 - G) \qquad (4)$$

(5) The actual quantum yield for any time and state between zero and maximum was derived by PAILLOTIN (1976) as:

$$\varphi_{Pt} = TR_t / ABS = 1 - F_t / F_M = \varphi_{Po} * ((F_M - F_t) / (F_M - F_0)) \qquad (5)$$

The same equation has been derived and experimentally tested later (GENTY *et al.*, 1989). HAVAUX *et al.* (1991) provided the theoretical link between biophysical expressions like φ_{P_0} and the fluorescence signals.

The maximum yield of excitation energy trapping ($\varphi_{P_0} = TR_0 / ABS$) was found to be lower in the absence of water (Fig. 5A). It is also clear from Fig. 5A that the φ_{P_0}

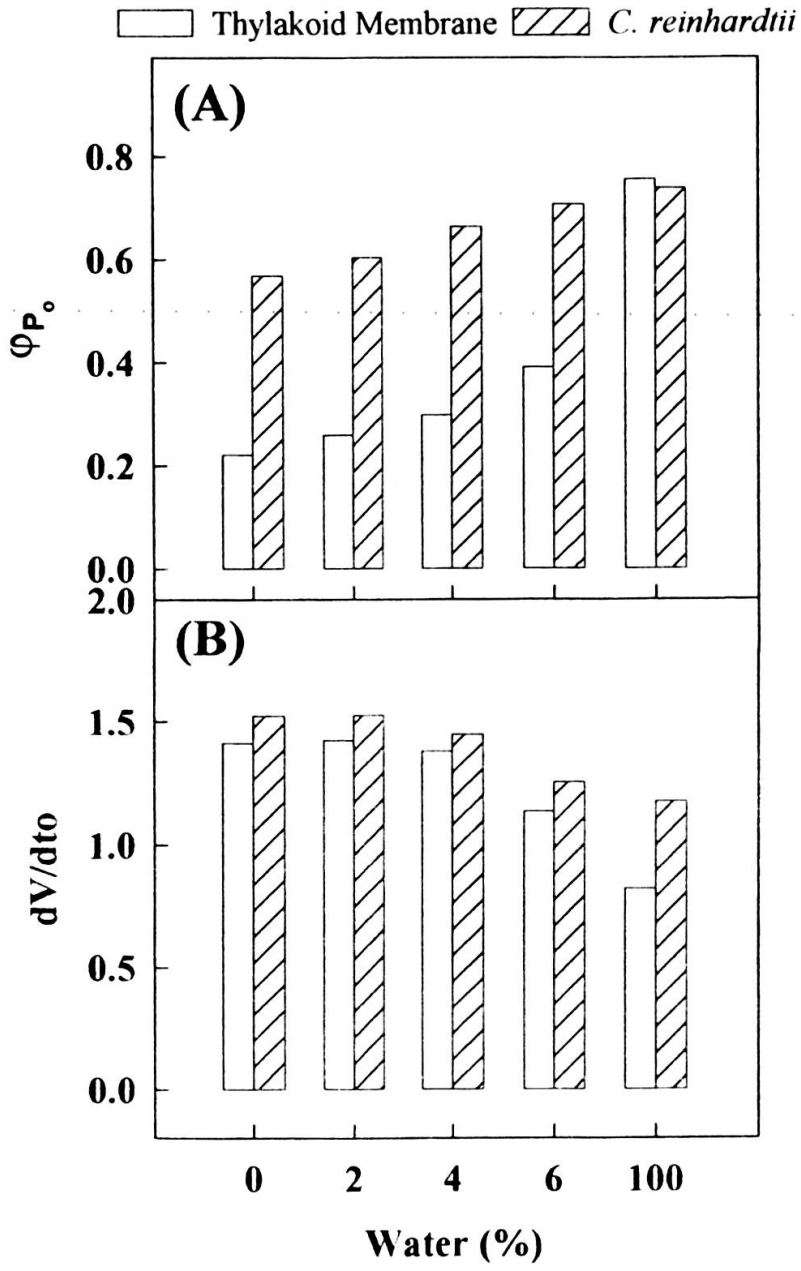

FIG. 5.

The water content of HTS micelles to which *C. reinhardtii* cells or thylakoid membranes are transferred influences their yield of primary photochemistry (φ_{P_0}) (A) and rate of initial fluorescence change (dV/dt_0) (B). *C. reinhardtii* cells and thylakoid membranes were transferred to HTS micelles containing different amounts of water and incubated for 2 min. Experimental conditions as described in Fig. 1 and Methods.

of *C. reinhardtii* cells was less affected by the lack of water than it was in thylakoid membranes. It further confirms that thylakoid membranes are more sensitive to water in low water systems than the *C. reinhardtii* cells. The initial slope of the relative variable fluorescence transient ($dV/dt_0 = (F_{300\mu s}-F_{50\mu s})/F_V$) was higher in cells or membranes incubated in low water system (Fig. 5B) than in the aqueous solution.

Other correlations between fluorescence signals and energy fluxes for photochemistry can be derived using the JIP-test (STRASSER & STRASSER 1995; STRASSER *et al.*, 1996). TR_0 / RC expresses the rate at which an exciton is trapped by an open RC and at which it reduces Q_A to Q_A^-. If we consider that at time zero all the RCs are open, the following expressions can be derived for activity per RCs:

(a) Trapping per reaction center

$$TR_0 / RC = (dV / dt_0) / (V_J) \quad (6)$$

where $dV / dt_0 = 4 * ((F_{300\mu s} - F_{50\mu s}) / (F_M - F_0))$. This is the initial slope of the relative variable fluorescence and has been expressed as the initial increment per ms
and $V_J = (F_{2ms} - F_{50\mu S}) / (F_M - F_0)$

TR_0 / RC is also affected by the electron donation from the oxygen evolving complex at PSII.

(b) Average absorption per RC, the ABS/RC can be derived as

$$TR_0 / RC = (TR_0 / ABS) * (ABS / RC)$$
$$= \varphi_{Po} * (ABS / RC) \quad (7)$$

$$\text{or } ABS / RC = (TR_0 / RC) * (1 / \varphi_{Po}) \quad (8)$$

ABS stands for the photons absorbed by the sample per unit of time, which is proportional to the concentration of absorbing pigments. Therefore, ABS/RC indicates an averaged antenna size expressed as a relative number of absorbing chlorophylls per one active RC of PSII present in the sample.

According to figure 6 and Table 1, the stability, activity, and amount of photosynthetic complexes is highly dependent on the HTS-micellar water content at the time when the spinach thylakoid membranes are transferred. Table 1 shows the distribution of free chlorophyll in the supernatant following centrifugation in HTS and bound chlorophyll (pellet dissolved in methanol), after transferring thylakoid membranes to HTS reverse micelles and incubating for 5 min. Free and bound chlorophyll absorption (ABS_{free} and ABS_{bound}) was measured separately using a spectroscopic method (see Table 1 for details). The fact that the sum of ABS_{free} + ABS_{bound} coincide well with the measured ABS_{total} concentration shows that practically no chlorophyll is degraded.

TABLE 1

Effect of water on the partitioning of bound/free pigments and active RC of spinach thylakoid membrane in HTS-low water system. Membranes, equivalent to 25 µg of chlorophyll were incubated in HTS-reverse micelles containing different concentration of water. After 5 min, they were centrifuged and from the green supernatants the spectra were measured ranging from 600nm to 750nm. The spectra were also measured from pellets after dissolving them in methanol. Changes in the peak at 665nm (after correcting them at 750nm) were used to quantify the relative pigment concentration in bound and free from in pellets and supernatant respectively. 100% free pigment was obtained by dissolving 25 µg of chlorophyll in methanol. Since total variable is coming from bound chlorophyll (data not shown), all the active RCs are in pellet. The rel.ABS_{bound}/RC has been calculated as rel.ABS_{bound}/RC = (rel.ABS_{total} /RC)* (% of bound chlorophyll in the pellet).

% H_2O	rel.ABS_{total}/RC	µg Chl/ml	ABS_{bound} of pellet in Methanol OD/ml	ABS_{free} of supernat. in HTS OD/ml	ABS_{total} Pellet+ Supernat Methanol + HTS OD/ml	Total Chlorophyll %	rel.ABS_{bound}/RC	% RC_{bound}/pellet = RC/ABS_{total}
0	8.62	25	0.225 (12%)	1.620 (89%)	1.845 (101%)	101	103	11.7
2	5.22	25	0.39					

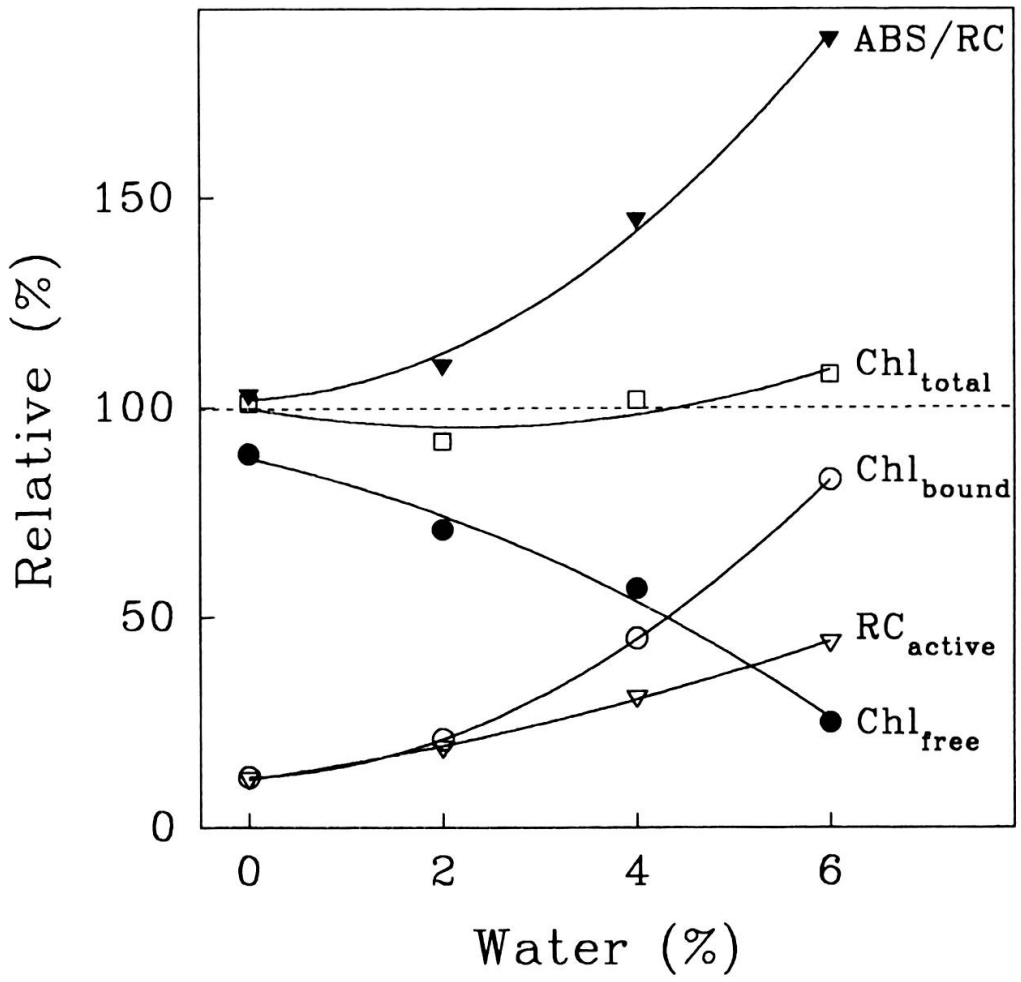

FIG. 6.

Effect of water on bound/free pigments of thylakoid membranes after 5 min of incubation in HTS reverse micelles. The role of water on the % of active RC and ABS/RC is also shown (see Table 1 for details).

Fluorescence measurements have shown that all variable fluorescence in the HTS belongs to the fraction, which can be pelleted by centrifugation. No variable fluorescence was found in the supernatant (data not shown). Therefore, we conclude that the ABS_{total} per ml in HTS preparation is constant and the number of active RCs per ml HTS is equal to the number of active RCs found in the pellet. In this way the fraction of active RCs as a function of water content can be calculated as:

$$\% \text{ of active RCs bound in the pellet} = RC / ABS_{total} \quad (9)$$

This value is accessible through fluorescence measurement as derived in equation 8 (for detail see STRASSER *et al.*, 1999).

Taking into account that all active RCs are found in the pelleted complexes, an average antenna size of the active complexes can be calculated as follow:

$$ABS_{bound} / RC = \text{fraction of chlorophyll in pellet} * ABS_{total} / RC \quad (10)$$

The results show that: (1) by increasing the water in HTS reverse micelles (0 to 6 %) more active RCs of PSII (10 to 45 %), and more chlorophyll (10 to 85 %) is preserved in the pelletable macrocomplex; (2) the photochemically active complexes exhibit bigger antennas (100 to 200 %) when the water content increased from 0 to 6 % (Figure 6 and Table 1).

Therefore, the water content determines the quantity and quality (integrity) of the photochemical active units present in the micelles.

(e) Viability of C. reinhardtii Cells in HTS Reverse Micellar System: The complete process of transfer, and incubation of *C. reinhardtii* cells in HTS micelles, and back transfer to aqueous media, to test viability took about 15 min (5 min incubation, 5 min centrifugation and about 5 min for other processes). It can be seen from the upper panel of Fig. 7 that the cells incubated in HTS micelles containing 2 and 4 % water totally lost their variable fluorescence in 15 min. When these cells were transferred into liquid mixotroph medium, they could not grow further and died within 24 hours (data not shown). In contrast, cells incubated in HTS with 6 % water maintained their variable fluorescence at least up to the J level. The J to I and I to P phase totally disappeared during the incubation process. In spite of this, when these cells were returned to aqueous medium, they partially recovered the J to I and the P phases, as can be seen in the lower panel of Fig. 7 (1 day trace). After 2 days of incubation more than 50 % of variable fluorescence was restored and the traces showed the normal OJIP transient. On the 3rd day, the cells behaved like the normal cells.

The growth rate of the cells was also monitored by measuring their chlorophyll concentration. The inset in Fig. 7B illustrates that control cells started to grow just after incubation, and maximum cell growth was observed after the first 3 days of inoculation. Nevertheless, cells transferred to HTS micelles containing 6 % water showed slower growth rates within the first 2 days, but from the 2nd day onward a sharp increase in chlorophyll concentration was recorded. These results suggest that *C. reinhardtii* cells have the capacity to adapt to the low water system when it contains at least 6 % water. Possibly the cells are simply dormant i.e. they are able to display metabolic processes, but mitosis is delayed. If true, this would indicate that the microemulsion system stabilizes the cells in a state which lasts but that does not allow cell division. Although it has been reported earlier that yeast cells and the cyanobacterial cells can remain viable in water-in-oil microemulsion (PFAMMATTER *et al.*, 1989), this is the first report, to our knowledge, where the algal cells can maintain their viability (at least for 15 min) in reverse micelles, and when returned to aqueous media restore their inhibited photosynthetic activities.

(f) R. Rubrum Cells in HTS Reverse Micellar System: Figure 8 and its inset show the bacteriochlorophyll fluorescence induction transient of *R. rubrum* cells during the log

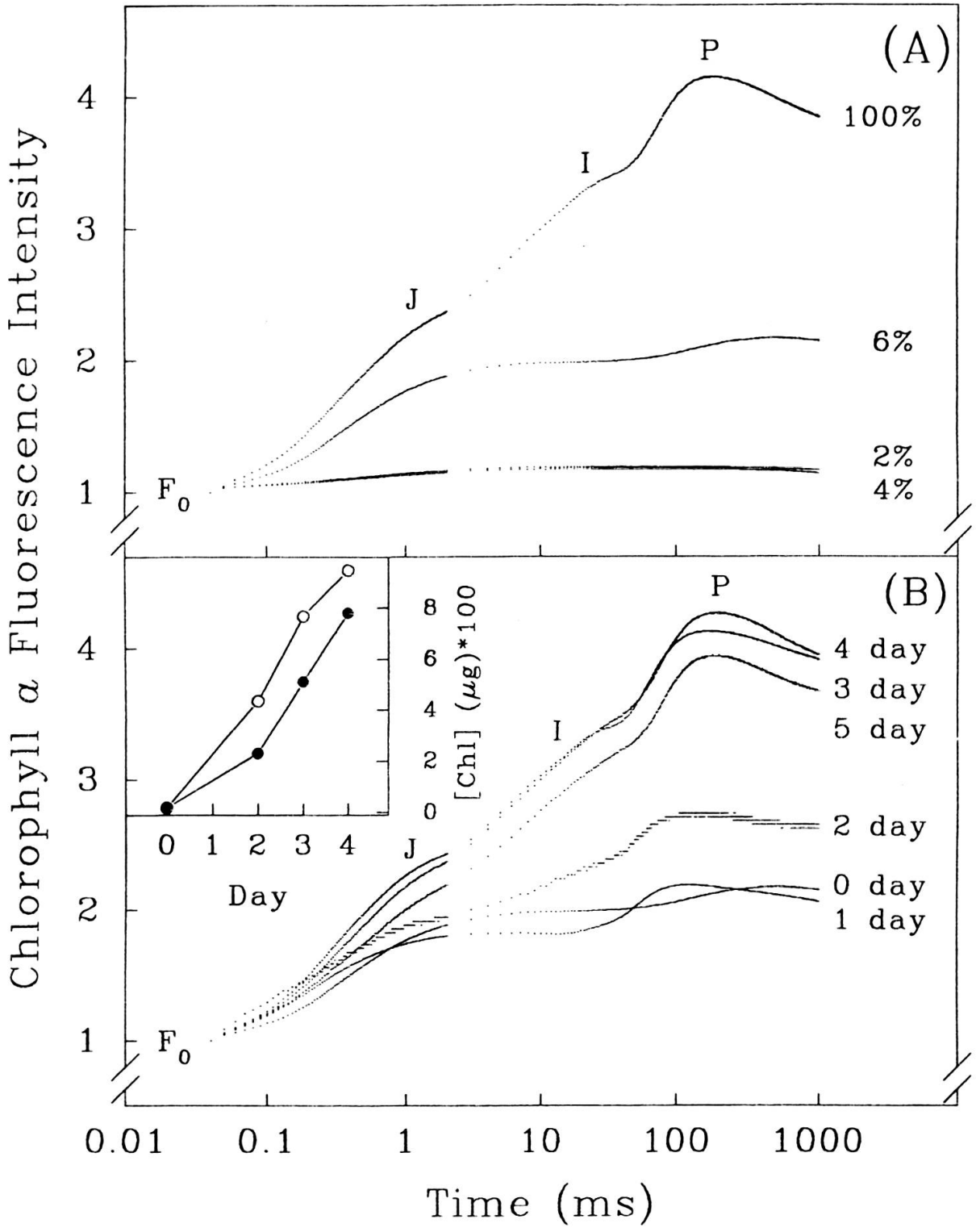

FIG 7.

Viability of *C. reinhardtii* cells in HTS-reverse miceller system. First the cells were incubated in HTS-reverse micellar system containing either 2 %, 4 % or 6 % water for 5 min. Thereafter, an aliquot of the HTS-system was layered on top of mixotroph medium and centrifuged for 5 min. The pellets were washed with mixotroph medium and further incubated in the same growth medium. Chl *a* fluorescence transients shown in panel A were obtained from the cells just after transferring them to mixotroph

phase of their growth incubated either in water (100 %) or in HTS reverse micelles containing 0 and 6 % water. Cells were exposed to light after 1 min of incubation in the respective solutions. The shape of the transient of bacteriochlorophyll fluorescence differ widely, depending on the physiological state and the growth conditions of the cells (STRASSER & GHOSH, 1995). When *R. rubrum* cells were exposed to actinic light, a fast fluorescence induction curve was recorded (Fig. 8). The cells incubated in HTS-reverse micelles with 6 % water behaved almost like the normal cells, they lost only a very small fraction of their variable fluorescence. But the cells incubated in HTS micelles without water lost some of the variable fluorescence, and after longer exposures showed a linear fluorescence decrease (Fig. 8). It is possible that light induces the formation of a kind of quencher, which decreases fluorescence during longer exposure of cells in HTS micelles in absence of water.

To test if this putative quencher induced by light recovered during dark adaptation, cells were incubated either in buffer or in HTS reverse micelles with 0, 2, 4 or 6 % of water and exposed several times for 10 sec, at 100 sec dark intervals, immediately after mixing (Fig. 9). In the absence of water, exposure of the cells to light shifted the whole fluorescence transient and decreased the F_0 level (Fig. 9A). The variations in the F_0 levels after different exposures are shown in Fig. 10. The amplitude of this shift decreased by increasing the water content of the HTS micelles (Fig. 9, 10), and can be explained due to the spectroscopic adjustment of the sample during the first few seconds. After further exposing the cells in HTS reverse micelles without water to light, a decrease in F_M and F_0 was also observed (Fig.9A). A small quenching was still observed after adding 2 % water (Fig. 9B), but in the presence of 4 % (data not shown) and 6 % (Fig. 9C) water, the full transient was reproducibly recorded even after several exposures. Although a small decrease in F_M was observed in buffer (Fig. 9D), in HTS containing 6 % water, the photochemical activity was totally reversible. These results indicate that the photochemical complexes of bacterial cells transferred to this micellar system are even more stable than in aqueous media.

(B) CHO-Reverse Micellar System

The influence of water on the catalytic activity of several enzymes has been explored using the CHO-reverse micellar system. Furthermore, KERNEN *et al.* (1997) showed that thylakoid ATP-ase activity is water dependent in this micellar system. We tried to transfer thylakoid membranes and cells of *R. rubrum* and *C. reinhardtii* to the CHO system and failed to recover any photosynthetic activity (data not shown).

medium. Fluorescence induction kinetics shown in panel (B) were obtained from the cells pre-treated in HTS low water system with 6 % water as in (A) after 1, 2, 3, 4, 5 days of inoculation. Inset in (B) shows the growth rate, measured as chlorophyll concentration (mg) of *C. reinhardtii* cells after treating them in low water HTS system with 6 % water as in (A). Open circles and closed circles represent the growth rate, measured in terms of total chlorophyll concentration, from the control cells incubated them in aqueous medium and from the cells after incubating them in HTS with 6% water respectively. Cells equivalent to about 20 µg of chlorophyll concentration were used at the time of inoculation; (µg)*100 is equivalent to 100 µg chlorophyll.

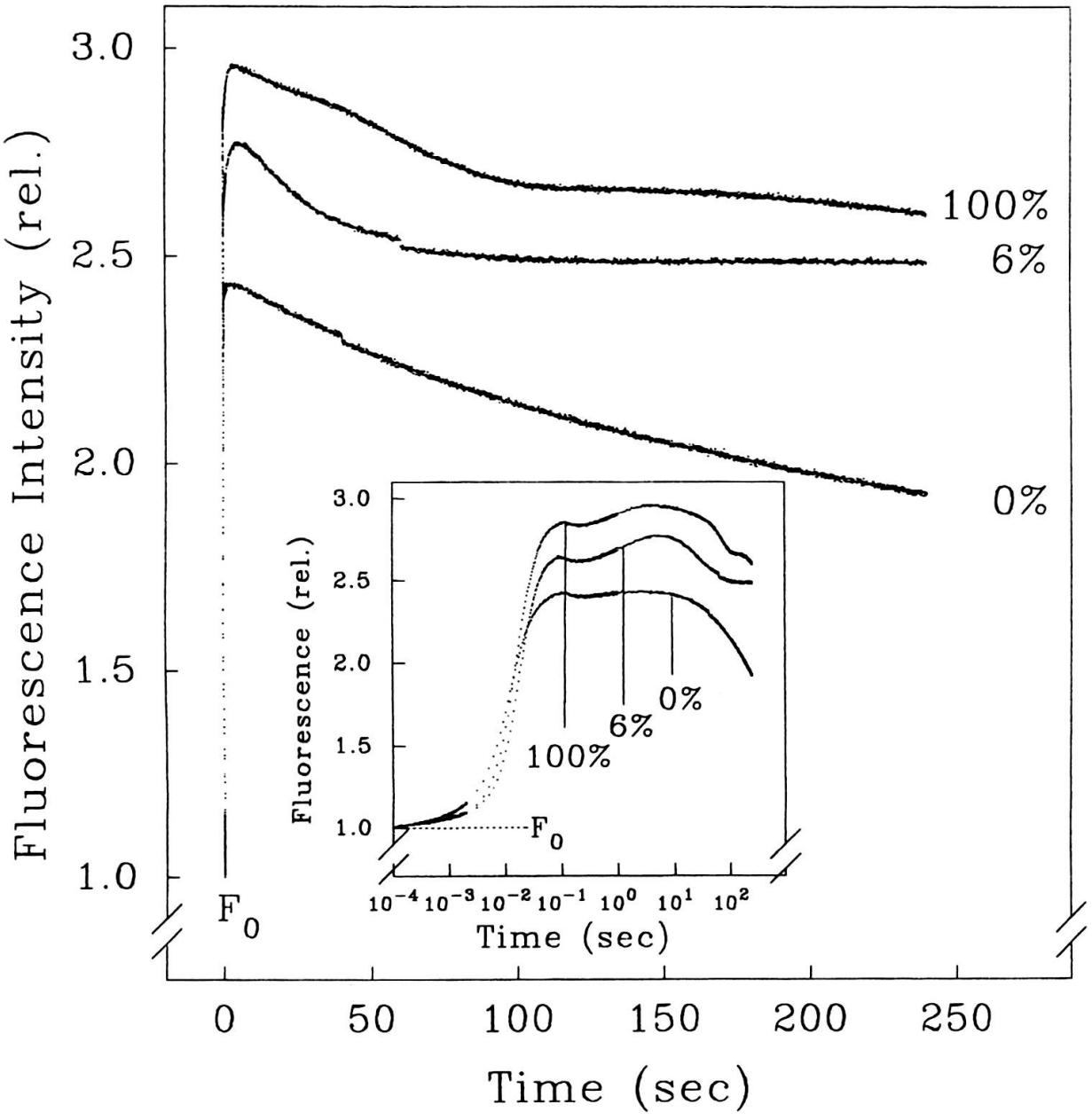

FIG. 8

Bacteriochlorophyll fluorescence induction transients of *R. rubrum* in aqueous buffer (100 % water) or in HTS reverse micelles with 0 and 6 % of water. Cells were incubated in the respective medium for 1 min before exposing them to red actinic light of 600 Wm^{-2}. Inset shows the same data but plotted on a log time scale.

(C) PLC-Reverse Micellar System

Several membrane bound enzymes remain highly active in phospholipid reverse micelles (DARSZON & SHOSHANI, 1992). We have attempted to use this system for the different cells and organelles used in this work.

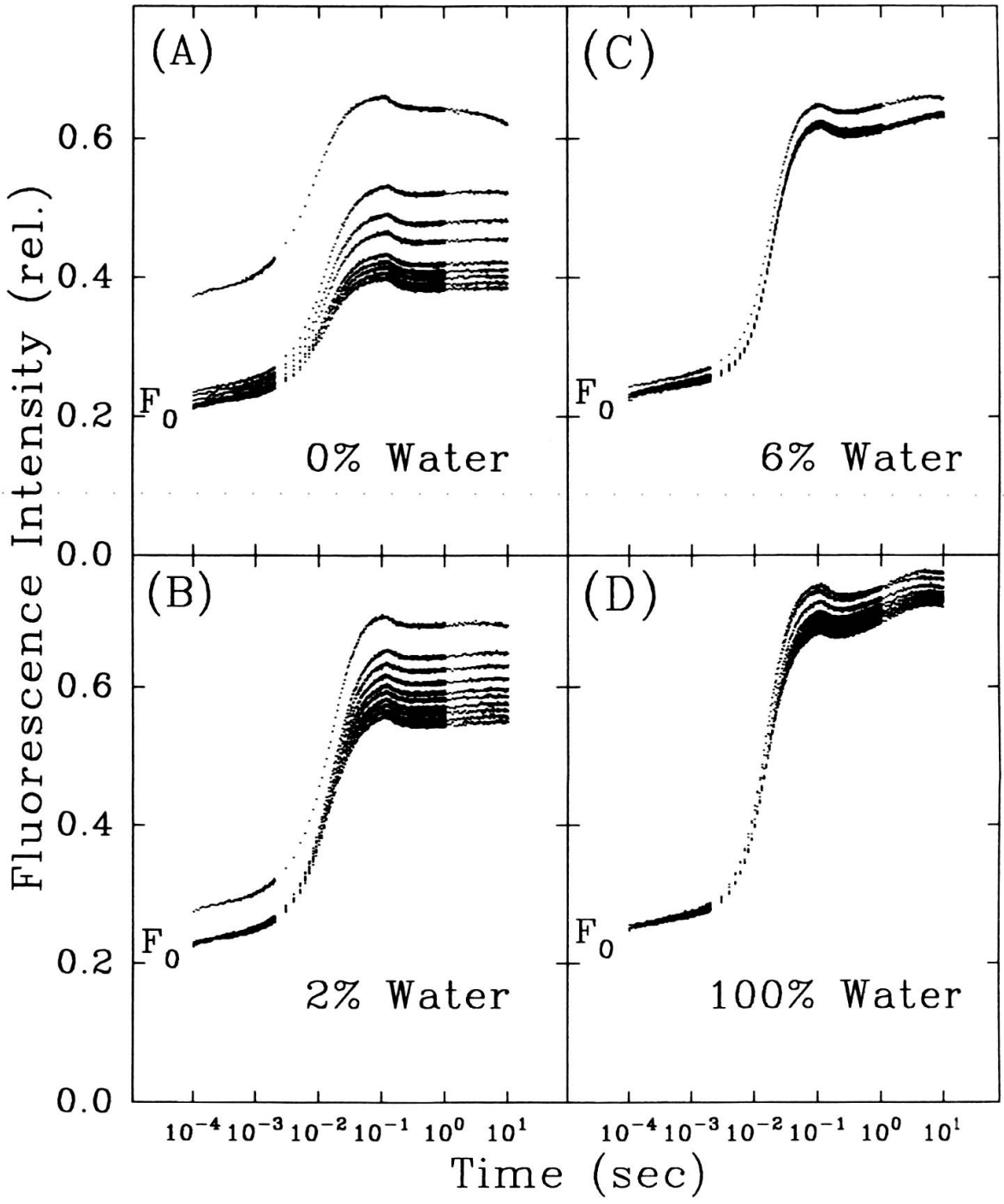

FIG. 9

Effect of water on the photochemical recovery process of *R. rubrum* cells in HTS micelles. Cells were incubated in the respective solution and exposed immediately to 600 Wm^{-2} of red actinic light for 10 sec, dark adapted for 100 sec and re-illuminated for 10 sec. The re-illumination was repeated 10 times at 100 sec intervals. Original curves are shown without any normalization or adjustment (note that there are 9 superimposable curves in top figure of right panel).

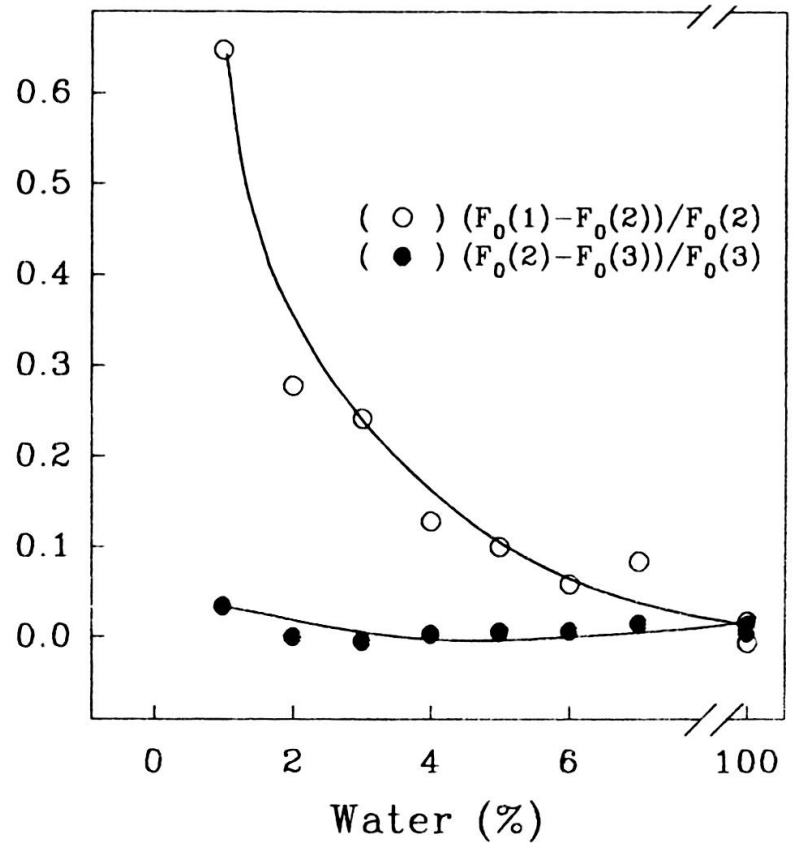

FIG. 10.

Effect of water content on the difference in the amplitude of the F_0 of *R. rubrum* cells after 1st, 2nd and 3rd exposure to light in HTS reverse micelles. Data were obtained from figure 9.

(a) Spinach Thylakoid Membranes and Cells of C. reinhardtii in PLC-Reverse Micellar System: Thylakoid membranes in PLC-reverse micelles totally lost their variable fluorescence (data not shown). The cells of *C. reinhardtii* displayed a very small, and abnormal variable fluorescence (Fig. 11A). When the *C. reinhardtii* cells in PLC were exposed to actinic light 4 times during 1 sec, waiting 100 sec in the dark in between exposures, a kind of bleaching was observed which resulted in the decrease in F_0 and F_M. Therefore, under the conditions tested, this reverse micellar system was unable to house stable functional photosynthetic complexes from green cells and thylakoid membranes.

(b) R. Rubrum Cells in PLC-Reverse Micellar System: In contrast to the green cells and thylakoid membranes, very stable and the full bacterial fluorescence induction curves were observed after the transfer of bacterial cells to the PLC reverse micelles (Fig.11B). When these PLC-reverse micelles were exposed several times to 600 Wm^{-2} for 10 sec, at 100 sec dark intervals, insignificant differences in the fluorescence induction kinetics were recorded in between each curve (Fig. 11B).

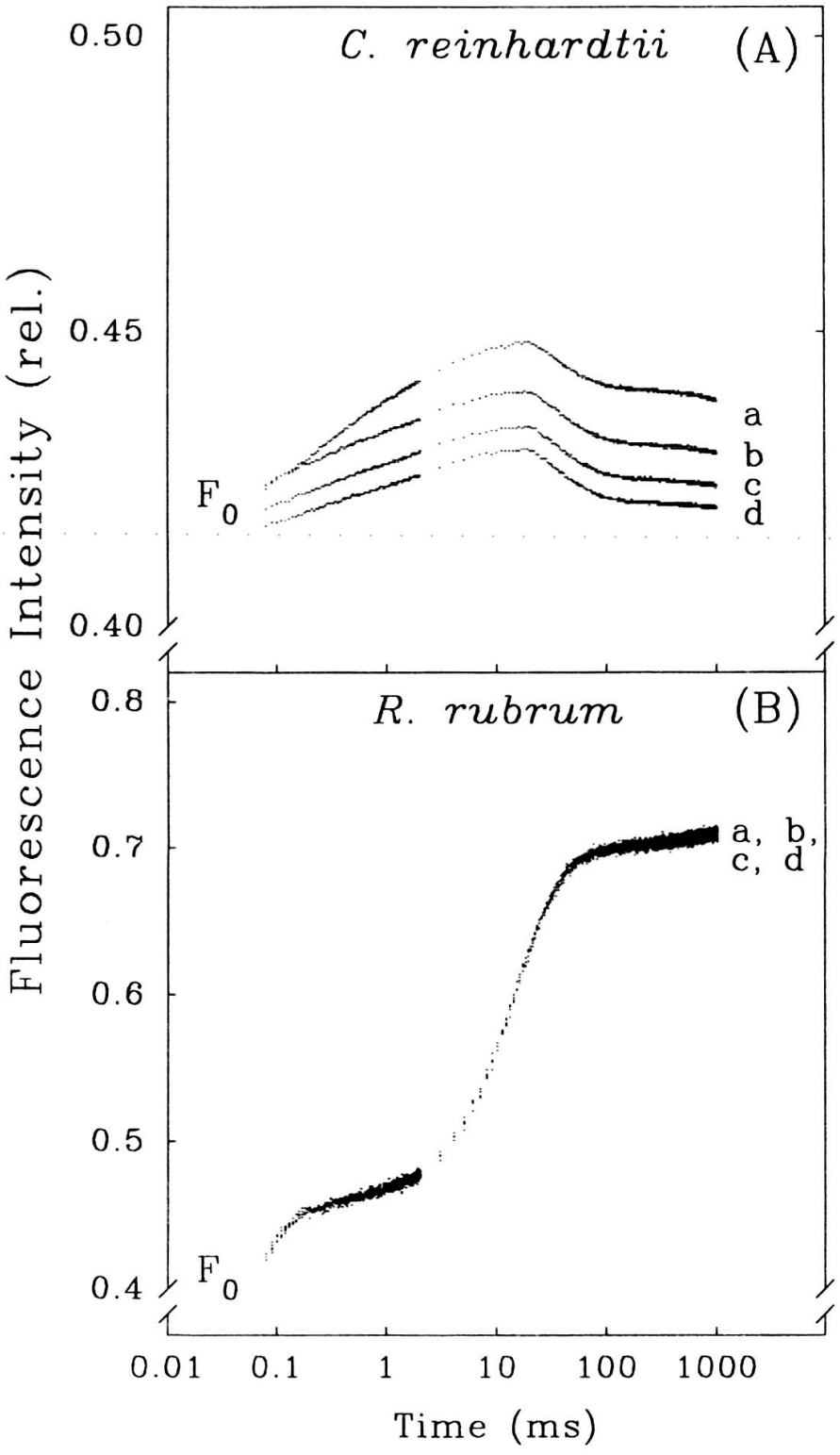

FIG. 11.

Fluorescence induction transient of *C. reinhardtii* cells (A) or *R. rubrum* cells (B) in the PLC-reverse micellar system. Cells were exposed 4 times for 1 sec to 600 Wm^{-2} red actinic light with dark intervals of 100 sec. Traces a, b, c and d corresponds to the 1st, 2nd, 3rd and 4th exposures respectively. Note that the y-axis of the *C. reinhardtii* has been magnified 4 times with respect to that of *R. rubrum*.

The fluorescence induction kinetics of the bacterial cells transferred to PLC-reverse micelles exposed to 150, 300 and 600 Wm^{-2} red actinic light demonstrated the intensity-dependent phenomenon (Fig. 12). The rate of the fluorescence increase between 1 ms to the half rise time of the maximum fluorescence ($\tau_{1/2}$)) has been calculated and plotted as ($1/\tau_{1/2}$) in the inset of Fig. 12. The expression $1/\tau_{1/2}$ is shown to be proportional to the light intensity indicating a pseudo first order photochemical reaction. It further confirms that protein complexes from *R. rubrum* cells transferred to PLC-reverse micelles display normal photochemical activity, as revealed by their full variable fluorescence, that is stable for several days.

In conclusion, the data presented in this work show that the spinach thylakoid membranes or the *C. reinhardtii* cells were found to be very unstable in HTS, CHO or PLC micellar systems. Although *C. reinhardtii* cells survived for a few min in HTS containing 6 % of water, this system cannot be used for longer time experiments.

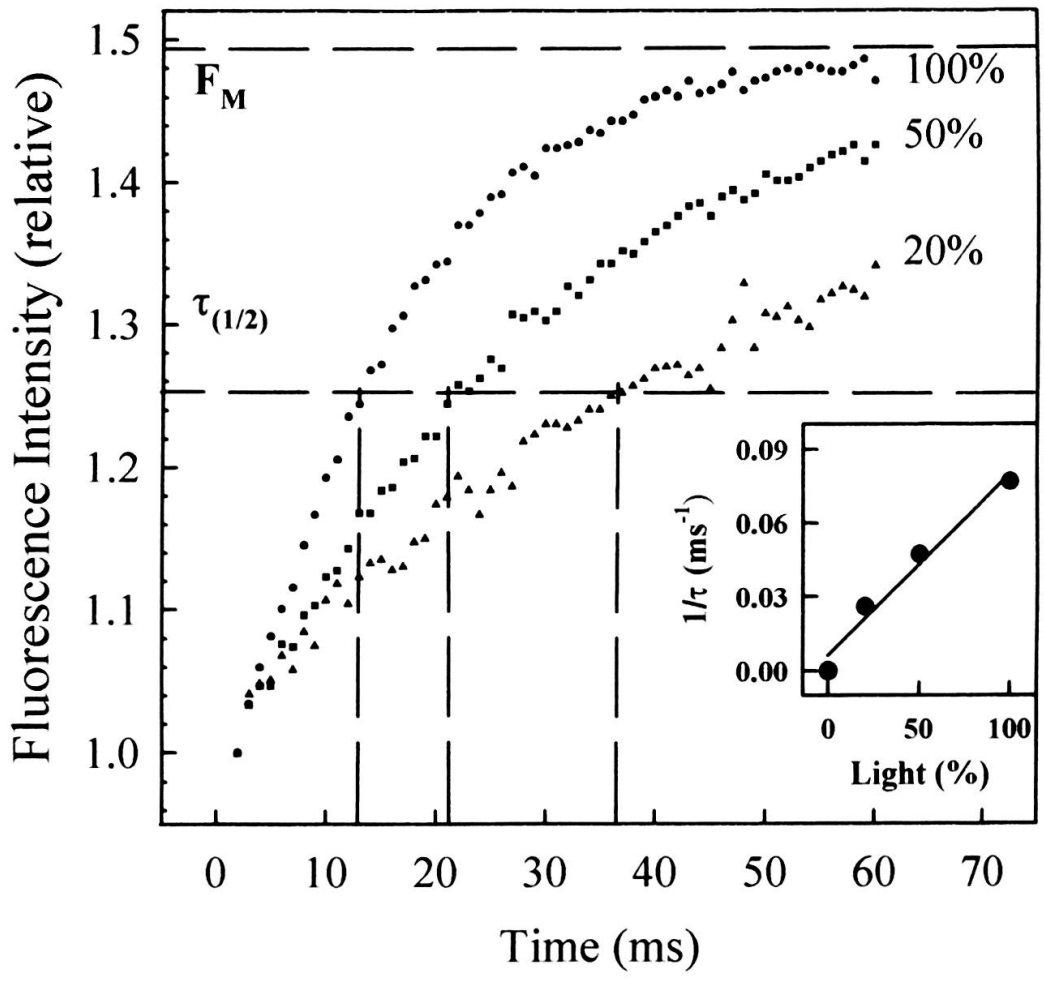

FIG. 12.

Fast fluorescence induction kinetics of *R. rubrum* cells in the PLC-reverse micellar system. Cells were exposed to 100 %, 50 % and 20 % of red actinic light (100 % = 600 Wm^{-2}). Inset shows the 1/half rise time ($1/\tau_{1/2}$) of the maximum fluorescence induction of the cells exposed to different light intensities.

Interestingly, we have found that functional photosynthetic complexes from *R. rubrum* cells can be transferred to reverse micelles formed either from HTS or from phospholipids in isooctane in functional form. Therefore, we can now use this system to ask how water influences the organization and function of these bacterial photoactive complexes. In purple bacteria, the reaction centers mediate the initial steps of a light driven proton pump, coupling transfer of electron to proton uptake. Site directed mutagenesis of this reaction centers have indicated residues close to Q_B that are important for proton transfer. Apparently the two protons involved may use different pathways. The structure of the reaction center near the Q_B site suggests that water molecules may participate, in addition to protein residues, in the proton transfer chain to Q_B as well as in stabilizing the charge on the reduced quinone (reviewed in OKAMURA & FEHER, 1995). Furthermore, a chain of 14 water molecules extending 23 Å from Q_B to the cytoplasmic surface of the reaction center were reported in the structure of *Rb. Sphaeroides* reaction centers determined by X-ray diffraction studies at 2.65 Å resolution (ERMLER *et al.*, 1994). Reverse micellar studies using the wide variety of bacterial reaction center mutants that exist could allow the identification of the specific residues involved in the steps affected by water.

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RÉSUMÉ

INFLUENCE DE L'EAU SUR LA STABILITÉ ET L'ACTIVITÉ DE COMPLEXES PHOTOSYNTHÉTIQUES, MEMBRANES ET CELLULES DANS DES SYSTÈMES APOLAIRES

L'eau joue un rôle fondamental dans la détermination des taux (vitesses) de réaction et la thermodynamique des systèmes biologiques. Les micelles inverses permettent de moduler la quantité d'eau à laquelle les enzymes et complexes multienzymes sont exposés. Nous avons recherché une source biologique de complexes photosynthétiques susceptibles d'être transférés dans des micelles inverses dans un état fonctionnel. La cinétique d'induction de la fluorescence rapide de la chlorophylle *a* (Chl *a*) a été utilisée comme test d'un transfert fonctionnel dans les micelles inverses de complexes photoactifs de cellules de plantes supérieures (membrane thylakoide d'épinard), Algues vertes (*Chlamydomonas reinhardtii*) et de bactéries pourpes (*Rhodospirillum rubrum*). Les membranes thylakoides d'épinard et les cellules de *C. reinhardtii* ont pu être solubilisées dans le système de micelles inverses HTS (Hexadecane-Tween-Span) et ont montré des augmentations, liées à l'eau, de la fluorescence variable,

cependant elles se sont révélées instables après quelques minutes d'incubation dans ce système. L'activité photosynthétique des membranes thylakoides et des cellules de *C. reinhardtii* est aussi instable dans les micelles inverses formées avec des phospholipides (PLCs). Par contre, les complexes photoactifs de cellules bactériennes peuvent être transférés en forme fonctionnelle stable, à des micelles inverses HTS ou PLC. Des complexes photosynthétiques bactériens stables en micelles inverses HTS ou PLC pourraient donc être utilisés pour mieux comprendre l'influence de l'eau sur l'organisation et la fonction des complexes photosynthétiques.

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