

Influence of water on the primary photosynthetic activity of *Rhodospirillum rubrum* in reverse micelles

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Abstract

The effect of water on the primary photosynthetic activity of purple bacterium *Rhodospirillum rubrum* was studied in Hexadecane-Tween-Spane (HTS)- and phospholipid (PLC)-reverse micelles. Reverse micelles offer the possibility of modulating the amount of water to which enzymes and multienzymatic complexes are exposed. Fast bacteriochlorophyll (BChl) fluorescence induction kinetics and reaction centre absorption changes at 820 nm were used as an assay for the functional transfer of bacterial cells into HTS-reverse micelles and bacterial photosynthetic complexes (BPC) into PLC-reverse micelles. Both the bacterial cells and BPC showed an increase in the rate of primary photosynthetic activity by increasing the concentration of water in the reverse micelles. The bacterial cells could be kept viable for many hours in HTS-reverse micelles in presence of 6 % (v/v) water. NMR studies indicated that the photosynthetic activity was affected by the availability of water in reverse micelles. The bacterial cells in HTS or BPC in PLC reverse micelles could be used to further understand the influence of water on the organisation and function of photosynthetic complexes.

Additional key words: absorbance; bacteriochlorophyll fluorescence; hexadecane-tween-spaine reverse micelles; nuclear magnetic resonance; phospholipid reverse micelles.

Introduction

Microemulsions are optically isotropic and thermodynamically stable solutions of water, oil (hydrophobic/non-polar solvent), and surfactants. The water-in-oil microemulsions are fine dispersions of water in a non-polar solvent. Water droplets are surrounded by surfactant molecules, forming the so-called reverse micelles (Luisi *et al.* 1988). Reverse micellar systems are able to house proteins, nucleic acids, plasmids, organelles, and even various types of cells (reviewed in Haering *et al.* 1985, Martinek *et al.* 1986; Luisi *et al.* 1988, Hochköppler *et al.* 1989, Escamilla *et al.* 1992, Famiglietti *et al.* 1992, Pfammatter *et al.* 1992, Chhabra *et al.* 1997, Tuena de Gómez-Puyou and Gómez-Puyou 1998). These systems allow the possibility of controlling how much water is available to proteins, organelles, and cells housed in their interior.

Water is a key molecule in cellular organisation, protein stability, and function (Kauzman 1959, Rupley and Careri 1991, Makhatadze and Privalov 1993). The reaction centres of purple bacteria, which couple the transfer of electrons and proton uptake, have water molecules close to the Q_B site that are involved in proton transfer. These considerations lead us to explore the influence of water in the function and organisation of photosynthetic complexes. Although spinach thylakoid membranes and *Clamydomonas reinhardtii* green algae can be transferred into various reverse micellar systems, only *R. rubrum* bacterial cells display functional stability in reverse micelles formed with Hexadecane-Tween-Spane (HTS) or phospholipids (PL) (Obregón *et al.* 1998). Despite of some basic differences in the structure and function of the reaction centres of the purple bacteria

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and of the green plants, the general principles of energy transduction are the same in anoxygenic and oxygenic photosynthesis (Blankenship *et al.* 1995).

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 and 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 occur in chloroplast membranes during photophosphorylation (Zolotareva *et al.* 1990). The activity of 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 photo-transduction could be explored. Our findings

showed that from the systems tested only bacteria remained stable and functional in HTS and PLC reverse micellar systems (Srivastava *et al.* 1999).

In this paper bacteriochlorophyll (BChl) fluorescence (Strasser and Ghosh 1995) and reaction centre absorption changes at 820 nm (Woodbury and Allen 1995) have been used as tools to monitor the rate of primary photochemistry in reverse micelles. We show that water affects the rate of primary photochemistry of *R. rubrum*. The NMR studies indicate that the photosynthetic activity is affected by the availability of water in reverse micelles. We also demonstrate that bacterial cells remain viable for many hours in HTS-reverse micelles in the presence of very small amounts of water. The availability of stable bacterial systems in reverse micelles opens interesting new perspectives in biotechnology.

Materials and methods

Materials: Span 80 and L- α -lecithin (type II-S) from soybean were obtained from Sigma (Saint Louis, MO, USA). Tween-85, hexadecane, and isoctane were from Fluka (Buchs, Switzerland). L- α -lecithin was partially purified according to Kagawa and Racker (1971). The rest of the reagents were of the highest quality available.

Wild type cells of *R. rubrum* were grown anaerobically in the medium of Sistrom (1960), photo-heterotrophically with succinate as carbon source, as described in Ghosh *et al.* (1994). During their optimum growth period, about 25 cm³ of cell suspension was centrifuged and the cells were collected as a pellet. The pellet was resuspended in 200 mm³ of M-medium. An aliquot of 5 mm³ of cell suspension was used for each measurement.

HTS reverse micelles: This micellar system 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 bacterial Sistrom-medium, the same medium that was used to grow the cells.

Phospholipid reverse micelles: 10 mg of the partially purified soybean phospholipids was dissolved in 1 cm³ of isoctane. Cells, equivalent to about 1 mg protein in ~70 mm³, were added to 1 cm³ phospholipid reverse micelles. The mixture was sonicated for 3-5 min in a water bath sonicator (Bandelin Sonorex Super RK-102H, Berlin, Germany) at ~3-4 °C. After sonication, 100 mm³ of ice cold 1 M MgCl₂ was added to the emulsion, which was then further vortexed for 1-2 min. The preparation

was centrifuged for 3-5 min at 66.7 rps (*Universal/K2S, Whistish*, Tuttlingen, Germany), and the organic fraction containing the protein-lipid-complexes (PLCs) was collected from the top of the tube.

Fluorescence measurements: BChl fluorescence induction kinetics was measured with a fluorometer, Plant Efficiency Analyzer (PEA, *Hansatech Instruments*, King's Lynn, Norfolk, England) with 650 nm irradiance of 600 W m⁻². Homogeneous irradiation over the exposed area was achieved by an array of 6 LED (peak at 650 nm), focused on the sample surface. BChl fluorescence signals were detected using a PIN photocell after passing through a long pass filter (>890 nm). All the experiments were done with 500 mm³ aliquots of cell suspension in 1-cm vials. The optical thickness of the sample was 5 mm and the diameter of the sample area irradiated was 4 mm.

Absorption change measurements: Irradiation-induced absorbance changes around 820 nm were measured by combining the *Hansatech P700*⁺ measuring system with the PEA-fluorometer. One of the red LEDs (out of 6) from the PEA head was replaced 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 radiation was monitored at the opposite side of the sample by a photodiode screened as well by an 820 nm interference filter. Actinic irradiation was provided by red LEDs through the PEA fluorometer. Rectangular spectroscopic vial (0.1×1×6 cm) was filled with the cell

suspension. The optical thickness of the cell sample was 0.1 cm, and the total exposed sample area was about 4 mm. The signal from the detector was simultaneously plotted with a chart recorder, digitised, and stored with a 12-bit resolution in a computer.

Proton nuclear magnetic resonance: ^1H -NMR spectra were recorded on a 200 MHz apparatus (*Bruker AC 200 F*) of the University of Geneva, Department of Pharmaceutical Chemistry (Srivastava *et al.* 1999b).

All experiments were repeated 3-4 times. However, one representative experiment is shown in each figure.

Results and discussion

Fast fluorescence induction kinetics of BChl was used to evaluate the photosynthetic activity of cells and organelles transferred to various reverse-micellar systems. When dark-adapted bacterial samples are exposed to saturating irradiance, the BChl fluorescence transient starts from F_0 intensity and increases to a maximum peak (P or M). 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.

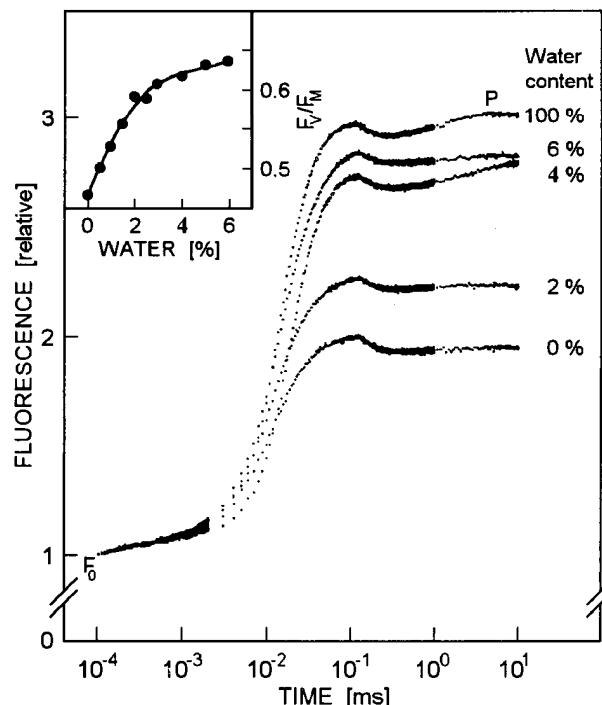


Fig. 1. Water content influences the fast BChl fluorescence induction kinetics of *R. rubrum* in the HTS-reverse micelles. After 1 h of incubation either in aqueous media (100 %) or in HTS micelles containing 6, 4, 2, and 0 % water, the cells were exposed to 600 W m^{-2} red actinic radiation. *Insert:* the effect of water on yield of primary photochemistry (F_V/F_M) after 1 h of incubation.

Effect of water on photosynthetic activity of *R. rubrum* in HTS-reverse micelles: It is surprising that complex structures as large as cells can be housed in

reverse micellar systems. It is thought that medium sized soluble proteins (5-10 nm) are hosted in the water pool of reverse micelles, which have similar dimensions (Tuena de Gómez-Puyou and Gómez-Puyou 1998). However, it is difficult to envisage how cells and organelles that have diameters in the range of μm , are accommodated in reverse micelles (Pfammatter *et al.* 1992). 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 used the HTS micellar system, perhaps the most successful in housing functional cells, probably due to its hydrophobic-hydrophilic balance (Pfammatter *et al.* 1989, 1992). In this system the water concentration can be varied from 0 to 6 %. Fig. 1 shows the BChl fluorescence induction transient of *R. rubrum* cells incubated either in water (100 %) or in HTS reverse micelles containing 0, 2, 4, or 6 % water. Cells were irradiated after 1 h of incubation in the respective media. The shapes of the transient of BChl fluorescence differed widely, depending on the physiological state and the growth conditions of the cells (Strasser and Ghosh 1995). Cells incubated in HTS-reverse micelles without water (0 %) lost a significant fraction (>50 %) of the BChl fluorescence intensity. Addition of 2 % water to HTS-reverse micelles increased the fluorescence intensity by ~30-55 % in comparison to the cells incubated in 0 % water. The cells incubated in HTS-reverse micelles with 4 and 6 % water behaved almost like cells in aqueous media, loosing only a very small fraction of their variable fluorescence (Fig. 1). The insert shows a clear increase in the quantum yield of primary photochemistry, measured as F_V/F_M , as a function of water concentration in the reverse micelles.

The effect of water on the maximum yield of primary photochemistry or the excitation energy trapping ($F_V/F_M = \phi_{P_0} = \text{TR}_0/\text{ABS}$) was even more pronounced after incubating the cells for longer times in HTS-reverse micelles (Fig. 2A). Cells incubated in the HTS system without water lost most of the BChl fluorescence in 24 h. However, insignificant differences in the fluorescence intensity were observed after 24 h of transferring the cells to HTS-reverse micelles containing 6 % water. The photo-oxidation of reaction

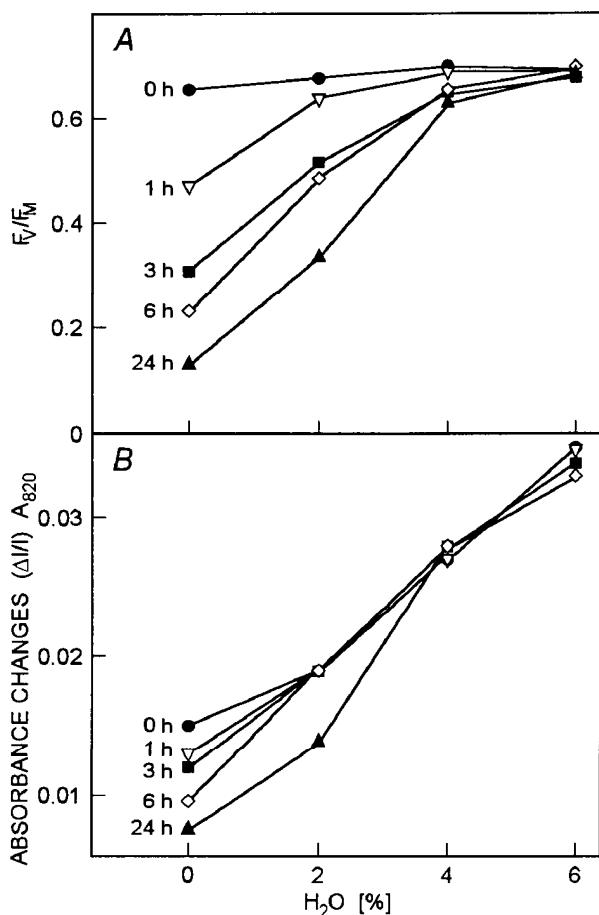


Fig. 2. Incubation time enhances the influence of water on primary photosynthetic activity of *R. rubrum* in the HTS-reverse micelles. Cells in HTS-reverse micelles containing various amounts of water were incubated for the indicated times and measurements were taken. *A*: Water-dependence of the F_v/F_M ratio. *B*: Changes in absorbance at 820 nm.

centres, measured as the absorbance changes at 820 nm, was also very sensitive to the presence of water in the HTS-reverse micelles (Fig. 2B). Cells incubated in HTS-reverse micelles without water (0 %) showed less than 50 % absorbance changes, immediately after incubation. Hence the lack of water had a negative influence on the stability of the cell absorption change. However, insignificant differences were observed when the cells were incubated in HTS-reverse micelles with 6 % water.

Does water affect photosynthetic activity and/or the transfer and stability of membrane-protein complexes in micelles? The quantum yield of primary photochemistry (F_v/F_M) and absorption changes at 820 nm appear to be water sensitive. Yet, increasing the water pool in the micellar system could be simply protecting the cells from deleterious detergent effects. To evaluate the contribution of these two possibilities, *R.*

rubrum cells were incubated in presence of 0 % water, allowed to stabilise for 10 min, and thereafter supplemented with 2 % of water. An increase in F_v/F_M was observed upon water addition (Fig. 3A). The cells were allowed to further stabilise at this stage for 10 min, and 4 % water was added again. A small increase in the photosynthetic activity was observed once more (Fig. 3A). Adding 4 and 6 % water after stabilising the cells in HTS-reverse micelles with 0 % water caused larger increases in photosynthetic activity, measured as F_v/F_M . The increases in activity were directly related to the amount of water added to HTS-reverse micelles. However, in all the cases, even after rising the HTS micellar water content up to 6 % (Fig. 3A, C), the F_v/F_M recovery did not reach that found in cells directly transferred to HTS with 6 % water (Fig. 3D). These results confirm that water influences the photosynthetic activity of the cells in the HTS-reverse micelles, but also show that this solvent affects the transfer process itself.

At low water concentrations, where the micelles are smaller and most of the water is bound, its behaviour is anomalous and its physical properties are somewhat different from those of bulk water (Luisi *et al.* 1988). Probably the HTS-reverse micelles with no added water are unable to prevent organic solvent and detergent contact with the cells. This is why under this condition, even after increasing the water concentration up to 6 %, the activity did not reach the level found when the cells were transferred directly into reverse micelles containing 6 % water. Increasing water content enlarges the size of the reverse micelles, perhaps helping to protect the cells from the organic solvent and detergents. These findings indicate that water in reverse-micelles affects the photosynthetic activity of the cells both intrinsically and altering the transfer of functional protein-complexes.

Photosynthetic characteristics of BPC in PLC-reverse micellar system: Several membrane bound enzymes remain highly active in phospholipid reverse micelles. The transfer of lipid-protein complexes to phospholipid reverse micelles requires sonication and extraction into the apolar phase. These procedures break cells and thus membrane fragments and/or protein-lipid complexes are probably transferred to reverse micelles (Darszon and Shoshani 1992). We explored the characteristics of this micellar system to lodge functional photosynthetic complexes. BPCs in PLC-reverse micelles (prepared as described in Materials and methods) were directly used to measure photosynthetic activity. BPCs in PLC-reverse micelles showed a full BChl induction curve when exposed to actinic radiation (Fig. 4) and displayed the basic characteristics of the photosynthetic complex. For example, the rate of BChl fluorescence increase between 1 ms to the half rise time of the maximum fluorescence

($\tau_{1/2}$) was directly related with the amount of actinic radiation (Fig. 4, *upper insert*). A typical irradiance saturation curve for absorption changes at 820 nm was observed when these BPCs were exposed to different actinic irradiances (Fig. 4, *lower insert*).

Effect of water on BPC in PLC-reverse micelles: The water content in PLC-reverse micelles is about 0.5 to 1.0 % (Darszon 1982) but, unlike HTS-reverse micelles, it can not be increased by adding more water. However, this amount of water can be decreased evaporating the

organic solvent in the micellar system under a gentle stream of N_2 and then restoring the original volume with pure solvent (Ayala *et al.* 1986). BPCs in PLC-reverse micelles were bubbled with N_2 to reduce their original volume to 75, 50, 25, and 12 %. The yield of primary photochemistry, measured as the F_v/F_M ratio, diminished as the volume decreased (Fig. 5A). This result is consistent with the findings discussed above indicating that the water contents in reverse micelles influence photosynthetic activity.

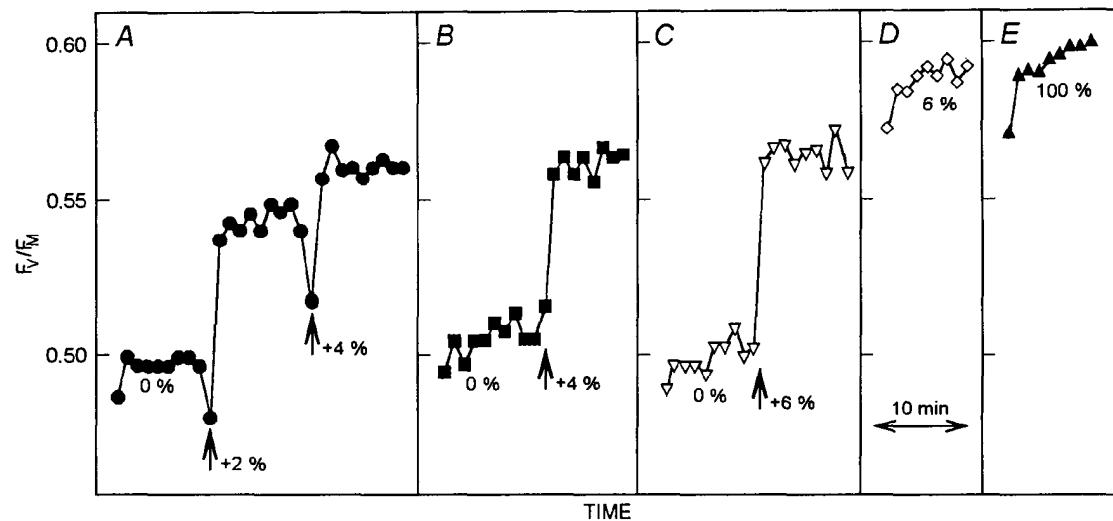


Fig. 3. Water influences mainly the primary photosynthetic activity of *R. rubrum* cells in HTS-reverse micelles but also the transfer process into the micellar system. *R. rubrum* cells were first transferred to HTS-reverse micelles with 0 % water and fluorescence kinetics measured during 10 min once every minute. Later on, 2 (A), 4 (B), or 6 (C) % water was added and measurements continued for another 10 min. In (D) similar measurements were carried out in cells directly transferred to HTS-reverse micelles containing 6 % water, or in cells in buffer (E). The figure shows changes in the F_v/F_M .

To confirm this water dependence, the PLC-reverse micelles containing BPCs were evaporated to 25 % of their original volume. Then they were supplemented with (1) PLC-reverse micelles prepared without cells with their usual water content (hydrated, without bubbling N_2), or (2) PLC-reverse micelles prepared without cells. Their original volume was reduced to 25 % with N_2 bubbling, and then the original volume was restored with pure solvent (dehydrated) to keep the lipid concentration constant (dehydrated). Increasing the water content of the dehydrated BPCs in PLC-reverse micelles in this manner stimulated their photosynthetic activity (Fig. 5B). In contrast, no stimulation in the photosynthetic activity was observed when dehydrated PLC-reverse micelles were used (Fig. 5B).

To further establish the influence of water on photosynthetic activity, dehydrated (25 % of the original volume) BPCs in PLC-reverse micelles were mixed with HTS reverse micelles where water can be controlled

more easily (Fig. 6). Activity was first recorded after mixing dehydrated BPCs and HTS-reverse micelles with 0 % water. Addition of 2 % water to the system enhanced F_v/F_M by 12 %. Another 2 % water addition caused activity stimulation again; at 6 % water content the F_v/F_M ratio increased by more than 21 % with respect to the initial condition of 0 % water.

Behaviour of water in HTS-reverse micelles and its effect on photosynthetic activity: Water can be easily quantified using NMR technique, which also allows an estimation of the bound-to-free water ratio (Maitra 1984). The integral of water peak area gives the quantity of water in the system. However, the water resonance shift (measured from the main $-CH_3$ at $1.17\text{ cm}^3\text{ m}^{-3}$) gives a general idea about the behaviour of water in the reverse micellar system. The bound to free water ratio in reverse micelles changes as a function of the total amount of water in the system (Maitra 1984, Kernen *et*

al. 1997). We measured the resonance shifts of water protons in HTS-reverse micelles in the presence of different amounts of water. The water resonance increased from about 3.4 to 3.8 as the water concentration was raised up to 6 % (v/v) (Fig. 7). The

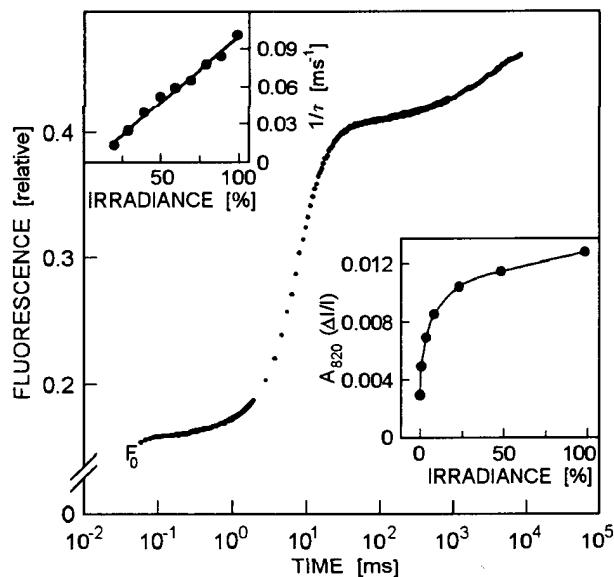


Fig. 4. Functional *R. rubrum* BPCs can also be transferred to PLC-reverse micelles. A BChl fluorescence induction transient of *R. rubrum* BPCs in this micellar system is displayed in the central part of this figure. PLC-reverse micelles were formed and *R. rubrum* BPCs transferred to them as described in Materials and methods. The upper and lower inserts show the rate of fluorescence increase between 1 ms and the half rise time of the maximum fluorescence ($\tau_{1/2}$) and the absorption changes at 820 nm of BPCs in PLC-reverse micelles as a function of irradiance. Fluorescence measurements were done with 500 mm³ aliquots of PLC suspension in 1-cm vials. The optical thickness of the sample was 5 mm and the diameter of the sample area irradiated was 4 mm. For the absorption changes the PLC was filled in rectangular spectroscopic vial (0.1×1×6 cm), so the optical thickness of the sample was 0.1 cm, and the total exposed sample area was about 4 mm.

insert in Fig. 7 illustrates that all the water added to the HTS-reverse micelles was accounted for in the NMR measurements as a linear increase in the integral area of the water peak. The first point (in presence of 0.5 % of water) was always a bit away from the main line due to the merger of the water peak with some unidentified peak.

A sharp increase in the resonance shift was seen after adding 1 % of water (v/v) to reverse micelles and saturation occurred between 4 and 5 % water in the system. As discussed above, the maximum quantum yield of primary photochemical activity, measured as BChl fluorescence kinetics of bacterial cells (F_v/F_M),

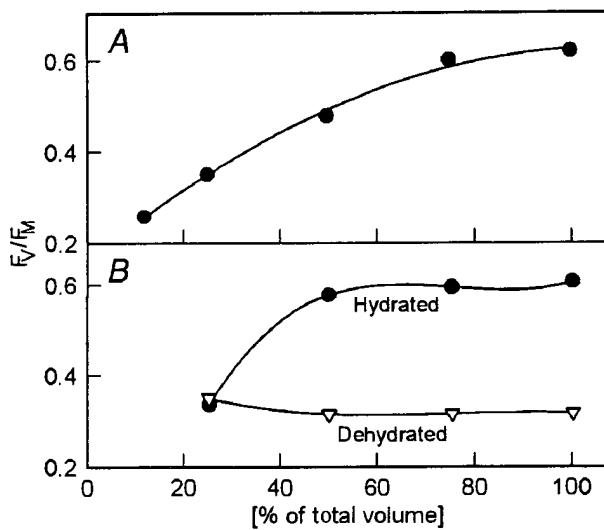


Fig. 5. Water also enhances the photosynthetic activity of BPCs in PLC-reverse micelles. The organic solvent plus water of PLC-reverse micelles containing BPCs were evaporated (azeotrope) under a gentle N₂ stream to 75, 50, 25, and 12 % of their original volume (2 cm³). The remaining concentrated PLC-reverse micelles were refilled with pure organic solvent to the original volume of 2 cm³ and the BChl fluorescence kinetics was measured (A). In B the BPCs in PLC-reverse micelles were evaporated up to 25 % of their original volume (2 cm³) and the volume restored gradually with dehydrated or hydrated PLC-micelles.

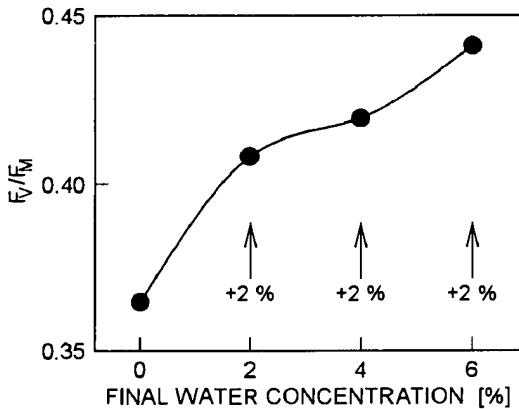


Fig. 6. Water-dependent increases in photosynthetic activity are revealed after transferring BPCs in PLC-reverse micelles with <<1 % water content to HTS-reverse micelles where water can be increased up to 6 %. The volume of the PLC-reverse micellar system containing BPCs was reduced to 25 % of its original volume. The remaining volume was then transferred into HTS-reverse micelles containing 0 % water and fluorescence kinetics measured. Thereafter, water was added and F_v/F_M measurements were performed after 1 min of incubation.

incubated in HTS-reverse micelles, displayed a very similar water dependence (Fig. 2). A direct correlation was observed between the water resonance shift and the

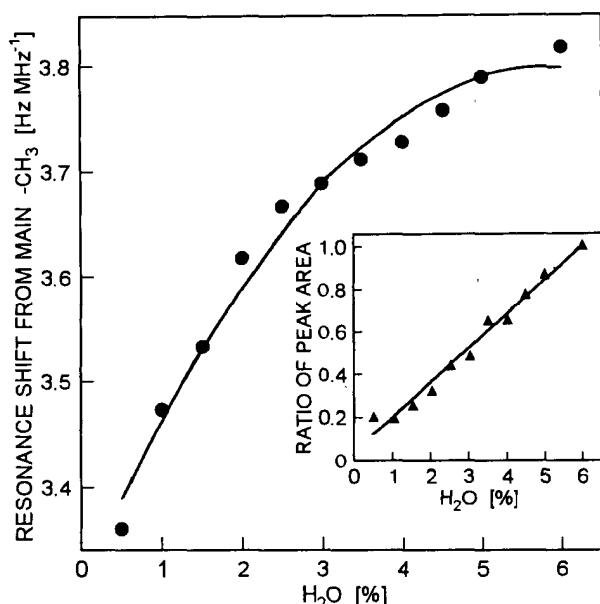


Fig. 7. ^1H -NMR water resonance shifts as a function of water concentration in HTS-reverse micelles containing *R. rubrum* cells. *Inset*: the peak area of water, in arbitrary units, as the water content in HTS-reverse micelles increases.

primary photosynthetic activity of cells. Though the total water content increases linearly as water is added to reverse micelles, the availability of free water is limited and does not appear linearly.

Conclusions: The influence of water in protein function, structure and organisation is undisputed. However, the mechanisms involved in how water influences these crucial parameters for life is not fully understood (Kauzman 1959, Rupley and Careri 1991, Makhadze and Privalov 1993). The use of reverse micellar systems in the study of enzyme catalysis, protein folding, and structure has gained attention in recent time since their water content can be controlled (Tuena de Gómez-Puyou and Gómez-Puyou 1999). In addition, variations in the water content of reverse micelles have revealed interesting photoactive intermediates (Darszon *et al.* 1979, Escamilla *et al.* 1989, Barrabin *et al.* 1993). For example, mitochondrial cytochrome oxidase can be reduced by ascorbate and cytochrome *c* in a reverse micellar system composed of toluene, phospholipids, and water (0.3 %), but its oxidation is highly impaired. This is because the catalytic cycle is arrested when the half-reduced state is reached ($\text{a}^{+2}\text{a}3^{+3}$). When the water content of the system is raised to 1.3 %, cytochrome oxidase can oxidise, but at an extremely low rate. This experiment suggested a water requirement in the catalytic cycle of cytochrome oxidase (Escamilla *et al.* 1989), which had been proposed (Kornblatt *et al.* 1988, Sassaroli *et al.* 1989) and later confirmed (Bona *et al.* 1990).

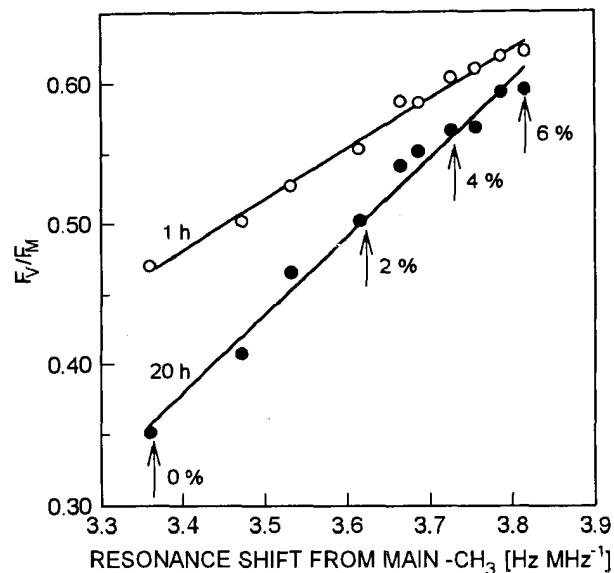


Fig. 8. Correlation between water NMR resonance shifts at various water concentrations in HTS-reverse micelles containing *R. rubrum* cells and the yield of primary photosynthetic activity (F_v/F_M). The water resonance shifts were measured from the main $-\text{CH}_3$. Cells were either incubated for 1 or 20 h in HTS-reverse micelles containing different amounts of water. Arrows indicate the % of water in HTS-reverse micelles for that particular point.

In purple bacteria, the reaction centres mediate the initial steps of a light-driven proton pump, coupling transfer of electron to proton uptake. Site directed mutagenesis of this reaction centre has indicated residues close to Q_B that are important for proton transfer. The structure of the reaction centre 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 stabilising the charge on the reduced quinone (reviewed in Okamura and Feher 1995).

In the present work we found that functional photosynthetic complexes from *R. rubrum* cells can be transferred to reverse micelles formed either from HTS or from phospholipids in isoctane. The amount and physical state of water influences the primary photosynthetic activity in both reverse-micellar systems (Fig. 8). In the case of HTS-reverse micelles whole bacteria are responsible for the photosynthetic activity while in PLC-reverse micelles the bacterial protein-lipid complexes are active. The combination of these two reverse-micellar systems represents an attractive tool to unravel the mechanisms by which water regulates and influences the organisation and function of these bacterial photoactive complexes.

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