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# Photochemical activity of the bacterial reaction center in polymer-like phospholipids reverse micelles

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**Absract** An integral membrane protein, the photosynthetic bacterial reaction center (RC), has been incorporated in reverse micelle viscoelastic gels made of phosphatidylcholine and phosphatidylserine. Due to the dynamic nature of the gels, the use of a technique which shares the same timescale of the charge recombination is advised, in order to correlate the kinetic behaviour of the RC to the hosting-system properties. Self-diffusion and conductivity measurements have been used to investigate the properties of the model system lecithin/cyclohexane/water. The results indicate that such techniques can describe the properties of the system on a long characteristic time-scale. As a consequence, the kinetic behaviour of the RC has been studied by means of flash-spectrophotometry and related to the structural properties of the hosting gel, investigated by means of conductivity. The conductivity data are consistent with a water-induced sphere-to-rod transition of the phospholipid aggregates. Furthermore, increasing the ratio [water]/[lipid], a maximum in the hydrodynamic dimension of the giant worm-like reverse micelles is found. The experimental $P^+$ decay has been resolved into three exponential components which are strongly affected by the system composition. The functionality of the binding site $Q_B$ is dependent on the ratio [water]/[lipid] supporting the hypothesis of a water role in the binding process.

**Key words** Charge-recombination – organogels – self-diffusion – membrane model

## Introduction

Reverse micelles at moderately high values of surfactant concentration and mole ratio of water to surfactant ($W_0$) are generally believed to have a droplet-like structure.

The addition of small water amount to reverse micellar solutions usually induces a spherical growth of the aggregates, with no significant changes of the macroscopic solution properties, such as viscosity. However, Scartazzini and Luisi reported in 1988 a completely different behaviour for lecithin reverse micelles in a number of organic solvents [1]. These solutions can be transformed into transparent, highly viscous, thermodynamically stable, viscoelastic systems (organogels) by the addition of tiny amounts of water. In later years, widespread investigations, mainly due to Schurtenberger and coworkers, have firmly established the close analogy between the behaviour of these lecithin reverse micellar solutions and the classical polymer solution and have explained such polymer-like properties with a water-induced one-dimensional growth

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of the micellar aggregates into very long and flexible cylindrical reverse micelles [2–4].

Lecithin microemulsion gel has been used as model system which describes the dynamic properties of "equilibrium polymer" [3] and (when made of a biocompatible oil) as transdermal drug delivery system [5]. Furthermore, polymer-like phospholipid reverse micelles can be successfully used as membrane model, supplying a mimetic system of the hydrophobic core of the plasmatic membrane. From this point of view, the introduction of an integral membrane protein could be significant in order to investigate the relevance of the lipid–protein interaction on the protein properties. The high viscosity of the phospholipid based gels is a disadvantage in studying chemical reactions catalyzed by enzymes, the mixing of the reactants (both enzyme and substrate/s) being extremely difficult. Conversely, the organogels seem to be the ideal media for the study of photochemically-active proteins, such as bacterial photosynthetic reaction centers, due to their excellent optical transparency. The bacterial reaction center (RC) from the purple bacterium *Rhodobacter sphaeroides* is a transmembrane protein complex which contains three subunits (L, M, and H) and several cofactors, namely four bacteriochlorophylls (*bchl*), two bacteriopheophytins (I), two quinones (Q) and one non-haeme high-spin iron atom ($Fe^{2+}$) [6]. Two *bchl* form a dimer (P), which acts as the light-driven primary electron donor. The absorption of a photon promotes P to its excited state (P*). An electron is consequently transferred through I to the first quinone acceptor ($Q_A$ which is located in a hydrophobic pocket of the protein) and subsequently to a second quinone molecule ($Q_B$) [7]. The radical anion $Q_B^-$ is tightly bound to the protein; in contrast, $Q_B$ is loosely bound so that the free exchange of quinone between the $Q_B$ binding site of RC and the membrane pool is possible. In the absence of any exogenous electron donor to $P^+$, the charge recombination between $P^+$ and $Q_B^-$ is observed. If, otherwise, the $Q_B$ binding site is empty or in the presence of an inhibitor of the electron transfer between $Q_A^-$ and $Q_B$, the light-induced charge separation and the successive recombination are limited to $P^+Q_A^-$. Since the negative charge is more stabilized when it resides on $Q_B$, the experimentally observed kinetics of $P^+$ decay depend on the parameters ruling the quinone exchange [8].

The polymer-like lecithin reverse micelles offer the opportunity to study the lipids mobility influence on the kinetic behaviour of the photoactive integral proteins. The successful attempt to solubilize the RC into lecithin microemulsion gels has been recently reported by our group [9, 10]. However, in order to preserve the photoactivity of the protein, the use of a mixture of phosphatidylserine (PS) and lecithin (PC) instead of pure PC has been necessary. In the present study, an attempt to correlate the

properties of both the hosting gel and the guest protein with the system composition (lipid concentration and $W_0$) is presented.

## Materials and methods

Soybean lecithin (epikuron 200) was a kind gift of Lucas-Meyer. Ubiquinone-10 (Q), phosphatidylethanolamine (PE), and phosphatidylserine (PS) (brain extract 85% of PS – sodium salt) were from Sigma. Cyclohexane and *n*-hexane were from Fluka. All the chemicals were of the highest purity available and were used without further purification.

RCs were isolated and purified from *Rhodobacter sphaeroides* R-26 strain as already described in the literature [11].

The phospholipids (PL) based organogels were prepared weighing the lipids in a screw cap glass vessel, dissolving them with the proper volume of organic solvents and then adding under stirring the water amount needed to obtain the desired $W_0$ [1].

The preparation of RC-containing phospholipid gels is described in detail elsewhere [9]; briefly, the RCs were first incorporated in lipid vesicles (PC/PE/PS 1:1:2) and then extracted in *n*-hexane in the presence of $Mg^{++}$. The water present in this RC/phospholipids organic solution was removed, incubating the extract for 6–8 h with anhydrous sodium sulphate (50 mg/mL), which was then removed by centrifugation.

In the experiments performed at increasing $W_0$, the desired amount of phospholipids (PC/PS 1:1 by weight) was first dissolved in a minimal amount of *n*-hexane and then added to the RC/phospholipids organic phase. Finally, the water was added to obtain the desired $W_0$'s.

In the experiments performed at increasing lipid concentration and fixed $W_0$ ($W_0 = 4$) the lipids, as *n*-hexane solution, were gradually added to the RC/phospholipids organic phase together with the water needed to keep constant the $W_0$.

All the kinetic measurements have been performed in the presence of an ubiquinone excess (6 mM – in the experiments, at different lipid concentrations, this value decreases due to dilution).

Self-diffusion coefficients measurements have been carried out as described elsewhere [12]. The accuracy of the self-diffusion coefficients was within 2%.

Conductivity measurements were made by means of a CDM-83 conductimeter (Radiometer) at 73 Hz, using a thermostatted immersion cell (Amel 192 K1, cell constant 1.06 $cm^{-1}$ at $T = 298 \pm 0.2$ K). The accuracy and reproducibility of the conductivity measurements were always better than 5%.

In the case of cyclohexane gels, in order to perform the conductivity measurements, the addition of KCl (0.25 M) has been necessary since lecithin carries no net charge. A fixed amount of KCl solution was added (to obtain a $W_0$ of 2) and further additions of water were performed for those sample with higher values of $W_0$. It has to be noticed that the presence of the KCl reduces the $W_0^{max}$ value to 8–10 at 298 K.

Conversely, in conductivity measurements on the PC/PS based organogels in $n$-hexane, no salt addition was needed since PS carries a negative net charge.

Flash-induced redox changes of the primary electron donor of the RC were monitored at 600 nm (a minimum in light-dark spectrum of the RC) with a single beam spectrophotometer of local design. Actinic flashes were provided by a xenon lamp (3.25 J discharge energy), screened through two layers of Wratten 88A gelatin filter, giving a light pulse of 4 $\mu$s duration at half-maximal intensity. Rapid digitization and averaging of the amplifier output was done by a LeCroy TR 8818 transient recorder equipped with a 128 K memory module (MM 8105) and controlled by an Olivetti M280 PC. Deconvolution of the charge recombination kinetics into multiple exponential decays was performed by computer routines based on the Marquardt algorithm.

## Results and discussion

Although pure lecithin was found to be able to form gel in more than 50 different solvents [13], only two systems have been investigated in detail, namely PC/isooctane/water and PC/cyclohexane/water. In these systems the results coming from small-angle neutron scattering (SANS), static light scattering (SLS), and dynamic light scattering (DLS) experiments indicate a complex dependence of the micellar structure on both the lipid concentration and the $W_0$ [2]. At high lipid dilution a strong water-induced micellar growth, from relatively small spherical reverse micelles to giant worm-like aggregates, is observed. It was demonstrated by Schurtenberger and coworkers that these flexible giant reverse micelles are characterized by a contour length ($L$) which increases when $W_0$ is increased, keeping constant the values of both the cross-section diameter and the persistence length ($l_p$) of the aggregates. As soon as the concentration raises above a cross-over value $c^*$, these flexible, rod-like aggregates start to entangle, forming a transient network with static properties comparable to those of semidilute solutions of flexible polymers. A quantitative agreement between theoretical calculations performed on the basis of the renormalization group theory and the experimental data obtained for different static and dynamic properties is
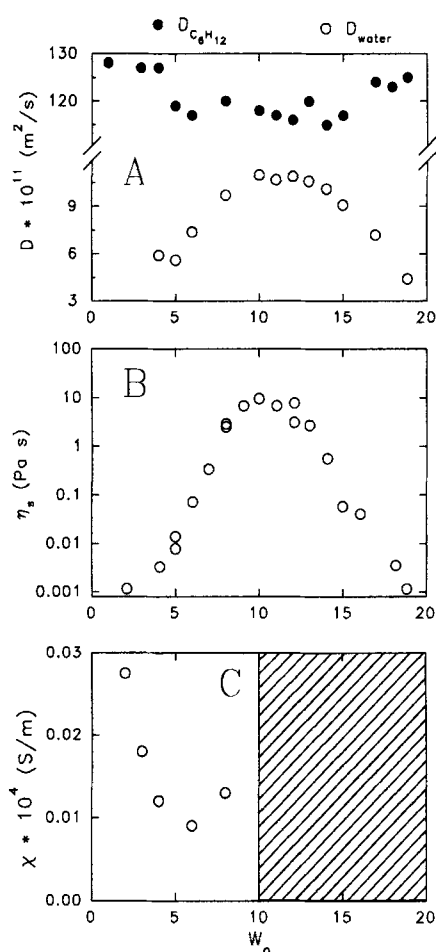


Fig. 1 Properties of the PC/cyclohexane gels. A Self-diffusion coefficients of water (hollow circle) and cyclohexane (filled circle) as function of $W_0$ ($C = 160$ mg/mL). B Zero Shear Viscosity as function of $W_0$ (data taken from ref. [2]). C Conductivity of KCl in a 160 mg/mL gels as function of $W_0$. The shaded area indicates the phase separation region

present in the literature [3]. On the other hand, as pointed out in ref. [3], this structural characterization of the system is restricted to static and/or dynamic properties observed on a very short characteristic timescale, where the finite lifetime of the aggregates can be neglected. A quite different behaviour has been reported using techniques characterized by longer timescales, such as the zero-shear viscosity ($\eta_s$). In this case, for both isooctane [14] and cyclohexane [2] based gels, $\eta_s$ ranges over several order of magnitude and shows a characteristic bell-shaped curve. A similar trend has been observed by measuring other dynamic properties, by means of techniques characterized by a long characteristic timescale. In Fig. 1A the values of both the solvent and the water self-diffusion coefficients (measured by means of PFGSE-NMR experiments) for the system PC/cyclohexane/water at different $W_0$ are shown. Figure 1B shows the dependence of $\eta_s$ on $W_0$ for the same

system (data taken from ref. [2]). The PFGSE-NMR results suggest a different mechanism of diffusion for the oil and the water molecules, in agreement with the structural model of giant worm-like reverse micelles. The $D_{oil}$ values are consistent with a solvent diffusion within a network characterized by an average mesh size which is function of $W_0$ [15]. The minimum in $D_{oil}$ corresponds to the maximum $L$; for higher values of $W_0$ a decrease in the contour length is observed, as proposed in the case of rheological measurements [14]. The water behaviour is quite different. $D_{water}$ values show a maximum of mobility in correspondence of the maximum in $\eta_s$. Furthermore, the NMR echo decays are strictly mono-exponential, ruling out the hypothesis of a restricted diffusion. The $H_2O$ solubility in cyclohexane being negligible, the water diffusion should be due to the motion of the $H_2O$ molecules along a tortuous curvilinear path inside the worm-like giant reverse micelles. The maximum in $D_{water}$ should correspond to a maximum in the average contour length of the aggregates. Another strong indication that (on a long timescale) the dimension of the micelles passes through a maximum when $W_0$ is increased, comes from the conductivity measurements. The conductivity ($\chi$) of cyclohexane based gels, prepared with aqueous KCl solution, has been measured. The $\chi$ values, shown in Fig. 1C, are extremely low and show a minimum when plotted as a function of $W_0$. This indicates that the system is below the percolation threshold and suggests that the charge carriers are the aggregates moving in a non-conductive oil. The conductivity behaviour of water in oil microemulsion at low water content has been explained on the basis of the charge fluctuation model [16, 17, 12], considering the energy needed to charge a body in a dielectric medium. A complete calculation, however, is present only in the case of a spherical geometry [16, 17] and cannot be used quantitatively in the case of polymer-like reverse micelles. Nevertheless, from a qualitative point of view, $\chi$ for tubular reverse micelles arranged in a random coil should be inversely proportional to the hydrodynamic radius of the aggregates. Consequently, the minimum in the plot of $\chi$ vs. $W_0$ should indicate a maximum in the dimension of the coil. It should be pointed out that this minimum matches neither with the maximum observed in the viscosity curve of pure lecithin gels (Fig. 1B), nor with the maximum observed in the $D_{water}$ vs. $W_0$ plot (Fig. 1A). Moreover, the phase separation occurs at a lower $W_0$ than the unperturbed system (i.e.: without KCl) and the $W_0$ where the maximum viscosity is observed (as judged by eye) is around 5–6 instead of 10–12. These experimental evidences could be explained taking into account the effects exerted by KCl on the micellar properties. As found by Biocelli and coworkers on an analogous system (lecithin reverse micelles in benzene) by means of IR and $^1H$-$T_1$

NMR measurements [18, 19], the presence of relatively high concentrations of KCl (0.1–1 M) induces a conformational modification of the phosphocoline moiety of the lecithin (from perpendicular to parallel to the long axis of the molecule) and an increase in the amount of water bound to the surfactant polar head. Since for lecithin organogels both these effects have been proved to be a crucial step in the gel formation [20, 21], the presence of the KCl would shift to lower $W_0$ all the gel features, phase separation and maximum viscosity included.

Since, as already told, the RC solubilization in the organogels requires the presence of the negatively charged phospholipid PS, a characterization of the hosting system PC/PS appeared necessary. It has to be pointed out that the PC/PS organogels show features typical of a viscoelastic solution, such as the response to an angular deformation (Weissenberg effect [22]). This evidence, together with the already reported $^{31}P$-NMR spectra of the PC/PS organogels in $n$-hexane [10] allowed us to extend to this system the expression organogel. On the basis of this assumption, it was possible to determine the amount of water needed to obtain the maximum viscoealstic response as the $W_0$ value where maximum is the Weissenberg effect ($W_0 \approx 3$) and the $W_0$ value where phase separation occurs $W_0 \geq 7$). It should be remembered that in the RC the charge recombination occurs with a lifetime of the order of 1 s when the charge separation involves $Q_B$, and with a faster lifetime (0.1 s) in the presence of a $P^+Q_A^-$ recombination. Due to the dynamic nature of the gels, the use of a technique which shares the same timescale of the charge recombination is advised, in order to correlate the kinetic behaviour of the RC to the hosting-system properties. For this reason PC/PS in $n$-hexane gels have been investigated using conductivity. Since the PS is an anionic phospholipid, it is possible to measure the conductivity of the system over a wide range of surfactant concentrations ($C$) without adding any electrolytes. The results are summarized in Fig. 2. At high dilution and low $W_0$ ($W_0 = 0.5$ and 1), $\chi$ increases linearly with the PS concentration reflecting an increase in the aggregates concentration with constant dimensions. This is the behaviour expected for spherical or pseudo-spherical reverse micelles at constant $W_0$. However, for higher lipid concentration values a decrease in the slope of $\chi$ vs. $C$ curve is observed. For samples at $W_0 = 1.8$ the two effects are comparable, resulting in a maximum in the $\chi$ vs. $C$ plot at about 80 mg/ml. The increase of the $W_0$ to 2.8 levels-off the measured $\chi$ at high dilution, while for $C > 80$ mg/ml $\chi$ decreases when $C$ increases (Fig. 2A). Also, in the case of PC/PS organogels a plot of $\chi$ vs. $W_0$ at fixed $C$ shows a minimum in correspondence of the maximum of viscosity (Fig. 2B).

Although the conductivity behaviour of the $n$-hexane gels made of a mixture of PC and PS is not yet fully and

**Fig. 2** Conductivity behaviour of PC/PS (1:1 in weight) $n$-hexane gels. **A** Conductivity values as function of total lipid concentration at different $W_0$. **B** Conductivity of a 160 mg/mL PC/PS gels as function of $W_0$
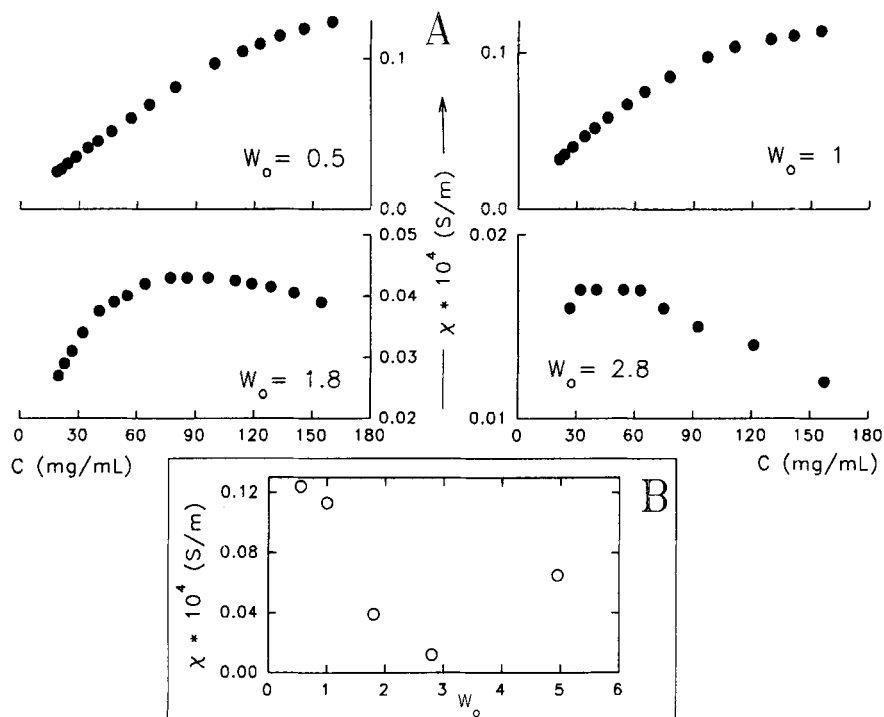
quantitatively understood, we believe that the above reported data suggests a water-induced sphere-to-rod transition of the micellar aggregates. Furthermore, it is evident that increasing $C$, at constant $W_0$, a decrease in the mobility of the aggregates, probably due to the formation of the transient network, is observed.

The kinetic behaviour of the RC hosted in the gel was investigated at different values of $W_0$ and $C$ and reveals several interesting features. First of all, three exponentials are required to fit the decay of $P^+$: a fast phase with half-times $(t_{1/2})$ shorter than 100 ms, an intermediate phase characterized by $t_{1/2}$ values ranging from 150 ms to 700 ms, and a slow phase with $t_{1/2}$ longer than 1 s. The $t_{1/2}$ values obtained for the fast phase are in good agreement with those found in aqueous system [8]; furthermore, in the gels the addition of terbutryne, an inhibitor of the electron transfer from $Q_A^-$ to $Q_B$, results in a monoexponential decay of $P^+$ with the same $t_{1/2}$ values (data not shown). On the basis of these considerations the fast phase can be attributed to the charge recombination occurring between $P^+$ and $Q_A^-$ in RC lacking quinone at $Q_B$ site. As a consequence, the intermediate and slow phases can be attributed to the $P^+Q_B^-$ recombination. A detailed discussion of the origin of the biexponential behaviour of the $P^+Q_B^-$-recombination of RC in organic environments is not the aim of this paper and can be found elsewhere [9, 10].

Both the relative amplitudes and the half-times of the three exponential phases are influenced by the $W_0$. In-

creasing the $W_0$ (at $C = 160$ mg/mL) the slowing down of the overall charge recombination rate is observed (see Fig. 3A). Figure 3B shows the relative amplitude of the fast, intermediate, and slow phases ($A_f$, $A_i$, and $A_s$ respectively) as a function of $W_0$. It is clear that the increase of $W_0$ induces a linear decrease of $A_f$ and a correspondent increase of $A_s$, suggesting an increase of the number of the $Q_B$ sites accessible to the quinone dissolved in the organic bulk. Otherwise, the analysis of the half-times dependence on $W_0$ of the three exponential phases (as shown in Fig. 4) reveals a maximum in the $t_{1/2}$ in correspondence of $W_0 = 3$, indicating that the charge separated states are more stabilized under this condition. In particular, a comparison between the half-times of the fast phase at different $W_0$'s reported in Fig. 4A and the conductivity data of Fig. 2B, suggests a correlation between the characteristic of the hosting system and the stabilization of the $P^+Q_A^-$ state. Recently, it has been reported that the rate constant for the charge recombination between $P^+$ and $Q_A^-$ is increased by a factor of 2 or 3 in dehydrated phospholipid reverse micelles in $n$-hexane [23]. The same feature has been previously found in dried films of the native protein [24]. In order to test the existence of any correlation among the changes in both the stabilization of the charge separated states $(t_{1/2})$ and the functionality of the $Q_B$ binding site $(A_f)$ with the total amount of water and/or the $W_0$, a set of experiments at constant $W_0$ and different lipid concentration has been performed (Fig. 5).

**Fig. 3** Kinetic behaviour of the bacterial reaction center in PC/PS $n$-hexane gels ($C = 160$ mg/mL). **A** Charge recombination kinetics of RC in gel at $W_0 = 4(1)$ and $W_0 = 0(2)$. The $P^+$ recovery has been fitted as a sum of three exponentials (solid lines). In the upper and lower part of the graph are shown the residuals. **B** Relative amplitudes of the exponential phases obtained by the deconvolution of the experimental traces, recorded in RC containing PC/PS $n$-hexane gels ($C = 160$ mg/mL)
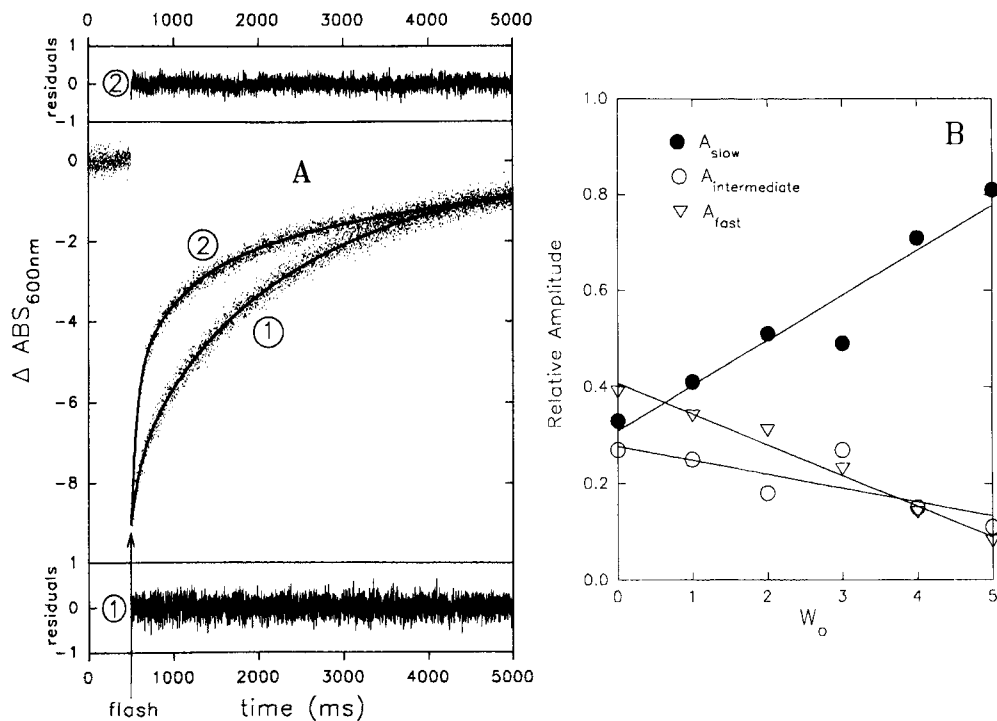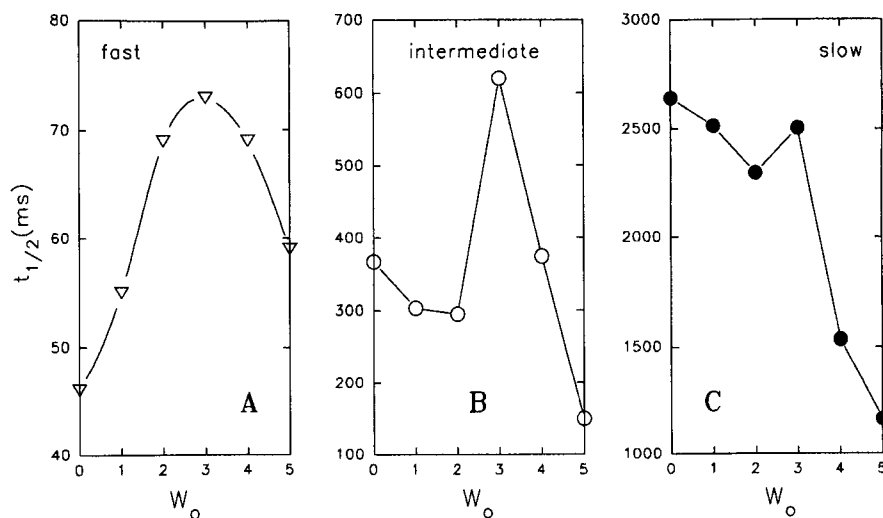


**Fig. 4** $W_0$ dependence of the half-times of the fast ($A$), intermediate ($B$), and slow ($C$) phases of the $P^+$ recovery in RC containing PC/PS $n$-hexane gels ($C = 160$ mg/mL)



Since the kinetics in the organic solvents are strongly influenced by the quinone concentration [25] the analysis of the $t_{1/2}$ dependence on $C$ is complicated by the changes occurring in this parameter (see Materials and Methods). Nevertheless, Fig. 5A clearly shows that at fixed $W_0$ the relative amplitude of the fast, intermediate, and slow phases are unaffected by the lipid and water concentrations. This information together with the data of Fig. 3B, indicates that the degree of organization of the water may play a role in the RC binding affinity for the unbiquinone molecules. This conclusion is in agree-

ment with the X-ray structure of the RC from *Rhodobacter sphaeroides* which reveals several water molecules buried in the core of the protein, some of them being well positioned to play a role in the binding process of the secondary quinone molecule, $Q_B$ [6]. It should be mentioned that similar conclusions were recently proposed on the basis of investigations in aqueous systems at high osmolality [26].

A comparison of Fig. 4A and Fig. 5B indicates that the decrease in the stability of the $P^+ Q_A^-$ state is related to the $W_0$ and not merely to the total amount of water. From the
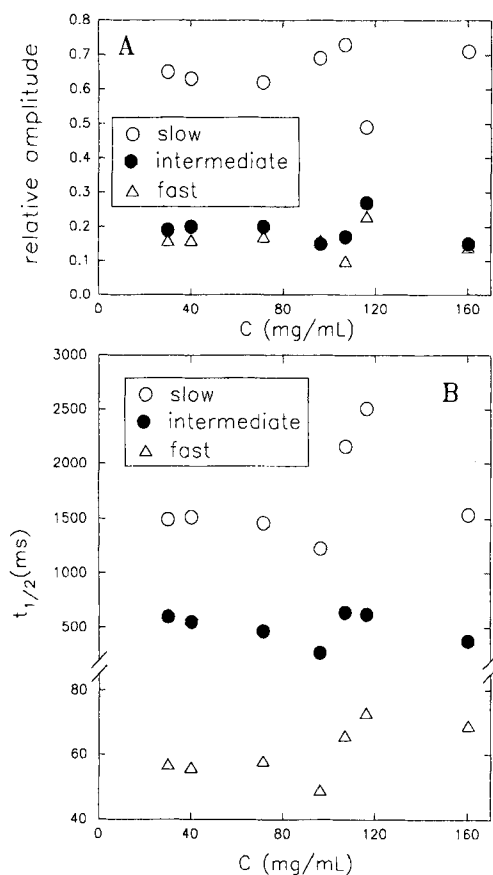
Fig. 5 Kinetic behaviour of RC in PC/PS $n$-hexane gels at fixed $W_0$ ($W_0 = 4$) and different total lipid concentration. **A** Relative amplitudes of the exponential components of $P^+$ reduction at different values of $C$. **B** Total concentration dependence of the half-times of the fast (hollow triangle), intermediate (filled circle) and slow (hollow circle) components of the $P^+$ recovery

x-ray structure of the RC there does not result any direct role for the water on the binding process of the ubiquinone at the $Q_A$ site; this feature could be reasonably assigned to a change in the protein conformation.

As illustrated in Fig. 5B the $t_{1/2}$ of the three phases shows a monotonic dependence on $C$ with an abrupt discontinuity at $C \approx 110$ mg/mL. This evidence suggests a structural transition of the hosting system, similar to that implied by the change in the slope of $\chi$ vs. $C$ plot at lower $W_0$. At this stage of the work it is unfortunately impossible to prove this statement, lacking any information on the conductivity behaviour of the PC/PS gels at $W_0 = 4$.

## Conclusions

Quite surprisingly, a close relation between the activity of a guest protein and the network properties of the host system has been found. Since some aspects of these interactions are not yet fully understood further measurements on both the system and the protein are required. The above reported data suggest that the polymer-like reverse micelles can be a powerful tool to investigate the lipid–protein interactions and to clarify the role played by the water and by lipid dynamics on membrane protein activity.

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