Functional enzymes in nonaqueous environment: the case of photosynthetic reaction centers in deep eutectic solvents

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ABSTRACT. Deep eutectic solvents (DESs) are emerging as a new class of green solvents with the potential to replace organic solvents in several fundamental and applied processes. In this

work, we offer an unprecedented characterization of the behavior of the bacterial photosynthetic reaction center (RC) from *Rhodobacter sphaeroides* in a series of choline-chloride-based DESs. RC is a membrane-spanning three-subunit pigment-protein complex that, upon illumination, is capable to produce a stable charge-separated state. Thus, it represents the ideal model for carrying out basic studies of protein-solvent interactions. Herein, we first report that, in many DES mixtures investigated, RC (a) is stable, (b) is capable to generate the charge-separated state, and (c) even to perform its natural photocycle. It proved, indeed, to be effective in reducing quinone molecules to quinol by withdrawing electrons from cytochrome c. As an example of biotechnological application, a photoelectrochemical cell based on DES-dissolved RC has also been designed and successfully employed to generate photocurrents arising from the reduction of the electron-donor ferrocenemethanol.

KEYWORDS. Deep eutectic solvents; choline chloride; membrane protein; reaction centers, photochemistry.

INTRODUCTION

Nowadays, biocatalysis has been increasingly playing a key role in the development of a sustainable asymmetric synthesis, and is also central to the pharmaceutical industry for the production of chiral, nonracemic drugs.[1–3] After the pioneering work by Klibanov in 1984,[4] it is now well known that enzymes are able to function in organic solvents also in the absence of added water.[5–7] Exploiting enzymatic activity in an entirely nonaqueous or quasi nonaqueous environment not only yields potential benefits by increasing the low-water solubility of organic substrates and by making it easier product recovery from low-boiling organic solvents, but is

also an important and steadily growing topic because of its relevance to the full understanding of the catalytic mechanism as well as to the potential applications in the biotechnological industry.[8] Being the solvent itself mainly responsible for most waste generated in the chemical industries and laboratories, the replacement of conventional hazardous volatile organic compounds (VOCs) in favor of safe and biorenewable reaction media while preserving or improving overall catalytic performance would be an extraordinary step forward in the field of biocatalysis.[9] A major driver towards this goal was represented by the introduction of biocompatible, biodegradable, and economically attractive neoteric solvents (e.g., second-generation ionic liquids, the so-called deep eutectic solvents, etc.), derived from primary metabolites and inexpensive raw materials, as new environmentally friendly reaction media for biocatalytic processes.[10] Since then, several examples have been reported throughout the years showing that macromolecules designed by Nature to work in aqueous environment can be, indeed, forced to perform their catalytic reactions also in unconventional environments with somewhat surprising results in terms of reaction rate, chemical stability and/or selectivity.[11–25] To the best of our knowledge, however, no reports are currently available in nonaqueous environment for enzymes whose catalytic site is buried within the membrane of a cell and/or have active sites interfacing within the membrane as well as exposed to water. This is not surprising as these enzymes are already difficult to handle in isolated form in aqueous solution, where the presence of opportune detergents is often required to form homogeneous solutions.

Photosynthetic enzymes, the so-called reaction centers (RCs), are responsible for the conversion of sunlight in energy forms readily available for any living organisms of planet Earth. These photoconverters are present in all photosynthetic organisms, with an increasing degree of complexity passing from photosynthetic bacteria to algae and to plants. The RC extracted from

the purple photosynthetic bacterium *Rhodobacter* (*R.*) *sphaeroides* is widely accepted as model system in the photosynthesis community. This enzyme (descripted in ESI) catalyzes the energetic uphill electron transfer reaction from cytochrome to quinone. In isolated proteins, instead, an electron-hole couple is formed as consequence of light absorption (Figure 1) making the RC a biological analogue of a semiconductor system possessing a conversion yield close to unity. These photoactive biomolecules are hence very interesting for synthetic biology[26] and material science for energy conversion,[27,28] photocatalysis,[29] and biosensing.[30,31] RC is an integral membrane protein having the entire sequence of electron transfer reactions localized in the hydrophobic core of the protein and the two active sites localized one at the water interface, and the other in correspondence of the membrane bilayer. This dichotomy imposes some limitations in their applications, which could be by-passed in a solvent able to solubilize the protein, thereby allowing its activity in a homogeneous environment. In this framework, it appears very intriguing and challenging the possibility that an unconventional reaction medium may solubilize the RC, at the same time preserving its catalytic activity.

Deep eutectic solvents (DESs) represent an emerging class of neoteric fluids resulting by the correct combination of two or three environmentally safe and inexpensive components, which are able to engage in hydrogen-bond interactions with each other thereby forming an eutectic mixture with a melting point much lower than that of either of the individual components. Thanks to their shallow ecological footprint, biodegradability, attractive low price, tunable physico-chemical properties, and the easiness of preparation by simple gentle thermal mixing, DESs are increasingly being used in synthetic organic chemistry as well as in process technology particularly for their unusual solvent properties.[32–42] Breakthrough applications have recently emerged in the fields of organocatalysis,[43,44] organometallic chemistry,[45–49] metal-catalyzed

reactions,[50,51] and solar technology.[52] The recent exiting ascertainment in the biotechnological field that high-ordered G-quadruplex DNA structures are also able to exist in neat DESs, with an increased thermal and pH stability than in aqueous media, could have a profound impact on the future advances of chiral DNA-based hybrid catalysts with unprecedented applications in asymmetric synthesis.[53]

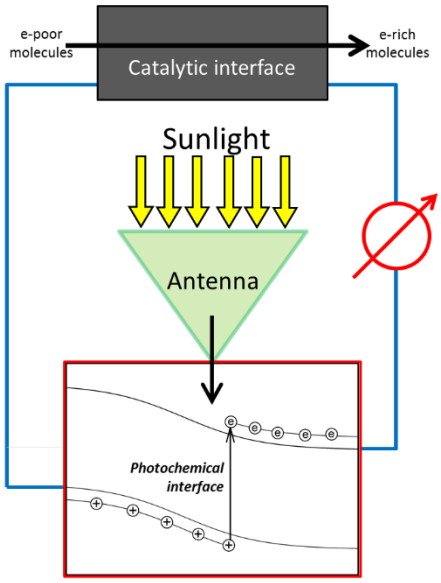


Figure 1. Schematic representation of a device able to convert sunlight into chemical energy. The photosynthetic apparatus is equivalent to such device, with the antenna represented by the Light Harvesting Complex, the photochemical interface represented by the Reaction Centre, the external circuitry represented by the non-photochemical metabolism, and finally, the catalytic interface of the biological apparatus is represented by the additional enzymes components involved in the dark reactions taking place after the photochemical events.

In this work, a new paradigm is presented: isolated integral membrane photosynthetic enzymes are able to perform their photoinduced catalytic reactions also in homogenous reaction media represented by custom-tailored DESs. This pioneering study opens up a new vista for

technological applications potentially involving any photosynthetic enzyme, including the so-called photosystem II, which is notoriously responsible of oxygen evolution in the photosynthetic apparatus of algae and plants.

EXPERIMENTAL

Chemicals. All chemicals were purchased at the highest purity available and were used without further purification. Decylubiquinone (dQ), ubiquinone-0 (UQ_0), ubiquinone-10 (UQ_{10}), ferrocenemethanol (FcnMeOH), triton X-100 (TX), potassium ferrocyanide ($Fe^{2+}CN$), sodium ascorbate, horse heart cytochrome c (cyt c), tris(hydroxymethyl)aminomethane (Tris), ethylenediaminetetraacetic acid (EDTA), choline chloride (ChCl), glycerol (Gly), ethylene glycol (EG), fructose (F) and urea (U), glutamic acid (Glu), proline (Pro), lactic acid (Lac) (90% aq. solution) and malonic acid (Mal) were purchased from Sigma. Lauryl dimethyl amino N-oxide (LDAO) was from Fluka. Bi-distilled water (W) was used in all experiments.

RC protein purification. RCs from *R. sphaeroides* carotenoidless mutant R26 were isolated as previously described.[54] Protein purity was checked using the optical absorbance ratio A_{280}/A_{802}, which was kept below 1.4. RCs final stocks were ~50 μM (determined spectrophotometrically using ε_{802} = 288 $mM^{-1}cm^{-1}$)[55] in Tris 20 mM, LDAO 0.025%, EDTA 1 mM, pH 8.0 (TLE) and stored at -20°C for later use.

DESs preparation. The employed DESs (ChCl/Gly 1:2, ChCl/U 1:2, ChCl/EG 1:3, ChCl/F/W 1:1:1, ChCl/W 1:2, ChCl/Glu/W 1:2:2.7, Gly/Pro 5:2, ChCl/Mal 1:1, ChCl/Lac 2:1 molar ratios) were prepared by gentle heating under stirring at 70 °C for 15 min the corresponding individual components in the appropriate molar ratio until a clear solution was obtained. All above DESs are fluid at room temperature, and show viscosities from 37 mPA (293.15 K) (ChCl/EG 1:3) to

1124 (298.15 K) (1ChCl/1Mal).[35,40] Hydrophilic DESs have a tendency to water absorption closely dependent on the duration time of exposure in open air. A survey of the hygroscopic properties of choline-based DESs using the Karl-Fischer titration method revealed a water content from 0.25 wt% (ChCl/Urea 1:2) to 0.52 wt% (ChCl/Gly 1:2) after a drying process conducted under high vacuum.[35,56,57] An on-site quantitative FT-IR analysis of residual water content in ChCl/Gly (1:2) eutectic mixture soon after its preparation was consistent with up to 2.2 wt% (1.6 M) water (see ESI for details).

Steady state and transient optical spectroscopy. Steady state optical spectra were recorded by a Cary 5000 (Agilent) UV-visible-NIR spectrophotometer. Light induced absorbance changes were recorded using a kinetic spectrometer of local design described elsewhere.[58] For single flash excitation, a Hamamatsu 15 W L4634-01 xenon lamp was employed, whilst for continuous light excitation a 250 W QTH lamp filtered with a low pass filter with $\lambda<700$ nm was used.

Electrochemical measurements. Electrochemical experiments were carried out by using a μStat400 workstation (DropSens, Spain). Planar screen-printed electrochemical cells (SPECs) Au 220AT and relevant connectors were also from DropSens. The SPEC ceramic support was L33 × W10 x H0.5 mm; the gold round shaped working electrode (WE) had a 4 mm diameter and was surrounded by an Ag quasi-reference electrode and a gold counter electrode. The SPEC was placed in horizontal position and the electrodes were covered with a 100 μL drop of DES containing the RC and the mediators. The illumination of the electrode surface was achieved by a 2.1 W led lamp with peak wavelength at 860 nm (Osram model SFH4783) placed in close proximity of the WE.

RESULTS AND DISCUSSION

Is the integrity of the RC preserved in DESs?

RCs were dissolved in nine different DESs, eight of which ChCl-based. The UV-Vis-NIR spectra of such solutions are shown in Figure 2 and Figure S3, and have been compared to the protein spectrum obtained in TLE buffer. Interestingly, all acid-based DESs, namely ChCl/Glu/W (trace H in Figure 2), ChCl/Lac, and ChCl/Mal, show a typical spectrum of a fully denaturated RC (see ESI for details). In all the other DESs, the optical features of the protein are preserved (traces B-G of Figure 2). Focusing on the NIR portion of the spectrum, some changes in the position of the peak at 865 nm (the right-most dashed line in Figure 2) are summarized in Table 1. The ratio between the absorptions peaks at 760, 802 and 865 is 1:2:1 in the native protein, but changes in the solubilizing environment modify these ratios.

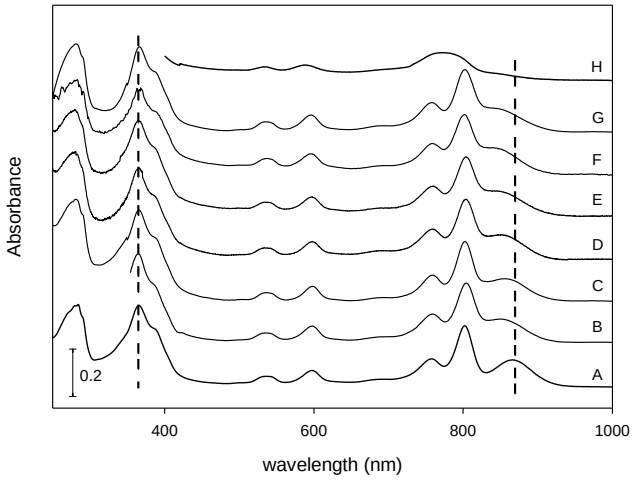


Figure 2. Steady state absorption spectra of 1 μM RCs in (A) TLE, (B) ChCl/F/W 1:1:1, (C) ChCl/Gly 1:2, (D) ChCl/W 1:2, (E) ChCl/U 1:2, (F) ChCl/EG 1:3, (G) Gly/Pro 5:2, (H) ChCl/Glu/W 1:2:2.7. Two dashed lines are drawn in correspondence of 365 and 865 nm.

The peak centered at around 865 nm originates from the bacteriochlorophyll dimer (D) and its position at room temperature is rather sensitive to the protein environment,[59,60] to the occupation

of the Q_B-binding site[61] and to the water content of the solubilizing environment.[62-64] In the latter case, the peak of the dimer undergoes to an hypsochromic shift up to 850 nm. The water content of ChCl/Gly (Figure 2 trace C) was assessed by means of Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) spectroscopy, looking at the spectral region between 1700 and 1600 cm^{-1} where the band due to the scissoring vibration mode of H_2O falls. The water concentration in ChCl/Gly was found to be 1.6±0.2 M. The preparation of RC-DES solutions requires the addition of water to deliver the protein from the stock solution up to a maximum concentration of 1.2 M. The final water concentration was always below 3 M and, based on FTIR spectral features, it cannot be considered "free-water" but rather water structurally belonging to the DES network (see ESI for more details). Although each RC has an average of at least 10^6 water molecules in the DES solution, the shift in the dimer peak position suggests that such water molecules tend to stay within the solvent network rather than "hydrate" the protein. A similar consideration can be drawn from the behavior of the DES ChCl/U. Indeed, concentrations of urea in buffer solution higher than 6 M do have a dramatic denaturating effect on the RC, determining the disappearing of the peak at 865 nm and major changes in the entire NIR portion of the spectrum with a rough first order kinetics having a half-time of 1 hour.[65] In the ChCl/U, the concentration of urea reaches the value of 13 M and yet no denaturation effect is recorded over one entire day. This behavior strongly suggests that molecules framed in DES network hardly interact with the protein solute as individual molecules, in line with the hypothesis that water present in DESs (where the 865 nm peak is shifted) cannot be considered "free water".

Table 1. Spectral features of RC in DESs.

DES	D peak (nm)	A_{802}/A_D	A_{802}/A_{760}
ChCl/F/W 1:1:1	851	2.4	2.1
ChCl/Gly 1:2	855	2.57	2.3
ChCl/W 1:2	853	2.4	2.0
ChCl/EG 1:3	848	2.2	2.0
ChCl/U 1:2	848	2.3	2.2
Gly/Pro 5:2	846	2.6	2.1

The noteworthy stability of RC in many DESs agrees with the findings of a recent molecular dynamics simulation study,[66] which identifies the strong intermolecular H-bonding network between DES components as responsible for preventing the solvent diffusion into the protein core. It cannot be ruled out, however, that hydrogen bonds between DESs and amino acids residues of the protein may contribute to increase enzyme stability. The solvation ability of DESs, however, differs from water and the solvation mechanism remains unclear. Some hypotheses about DES influence on protein conformation have been developed in the case of soluble proteins.[67] The photosynthetic RC is a membrane protein with a substantially different protein-solvent interface due to the presence of detergent molecules surrounding the hydrophobic portion of the enzyme. Currently, no literature on such proteins class in DESs is available for comparison.

Does RC maintain its photoactivity in DESs?

Following light absorption, an electron is transferred from the singlet excited state D* to the first electron acceptor Q_A, and subsequently to the final electron acceptor Q_B, forming the charge separated state $D^+Q_B^-$. The fate of this electron-hole pair is a charge recombination (CR) reaction that can be monitored spectrophotometrically following the recovery of light absorption at 860

nm after single flash excitation. The light-minus-dark spectrum (Figure S1 lower panel, ESI) presents indeed an intense negative signal at this wavelength associated to the dimer photo-oxidation. When the Q_B site is either empty or inhibited, the CR reaction follows a first order kinetics with a decay constant of about 10 s^{-1}.[68] This constant, relevant to $D^+Q_A^-$ recombination, is substantially independent upon temperature.[69] Empty Q_B sites can be repopulated by adding exogenous quinone, resulting in a CR reaction with a rate one order of magnitude slower, strongly dependent upon temperature and solubilizing environment. In intermediate conditions, *i.e.* when the Q_B sites are partially occupied, the first order CR reactions of both $D^+Q_A^-$ and $D^+Q_B^-$ occur, and the kinetics follows a biexponential decay. The relative amplitude of the slow phase thus represents a measure of the RC fraction with occupied Q_B-site.

Figure 3 shows the CR kinetics of the RCs in aqueous solution and in five DESs (black traces). The RC was found fully active in DESs corresponding to spectra B-F in Figure 2. Although not denatured in Gly/Pro, the photoactive portion of the proteins account for less than 10% of the analytical concentration; thus, it was not further investigated. The fitting parameters obtained for the biexponential decay of the recorded traces are summarized in Table 2. The lifetime of the fast decay component in all systems was found in good agreement with the known values in aqueous buffer in the interval of 7-10 s^{-1}.[68] In the aqueous buffer used in this experiment (Tris 20 mM, TX 0.03% (w/v), EDTA 1 mM (TTE)), the fraction of RCs with the Q_B-site occupied is 61%. In all DESs, except ChCl/EG, the decays show a fast component larger than that detected in TTE, indicating that these solvents promote the quinone extraction from the Q_B-pocket. The extreme case occurs in ChCl/W where quinones are fully extracted. On the contrary, in ChCl/EG, 83% of the Q_B-sites host the quinone, a level even higher than that found in TTE.

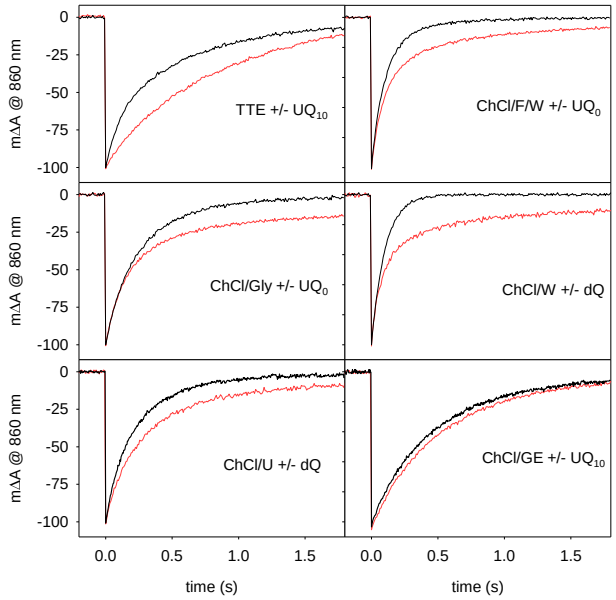


Figure 3. CR in various environments recorded at 860 nm after a saturating flash light excitation. Black traces are detected with pristine protein and red traces are detected after the addition of the indicated quinone.

Different quinones in suitable amounts were added to the solutions in the attempt to restore the Q_B functionality. The relevant CR traces are shown in red in Figure 3 and the fitting parameters are reported in Table 2.

It should be mentioned that the tested quinones have shown different ability in repopulating the Q_B sites in the various DESs. For example, in ChCl/W, the addition of UQ_{10} has no effect, whereas the addition of dQ restores 30% of the Q_B functionality. Therefore, only the results relevant to the quinone giving the highest amplitude of the slow phase for each DES are reported.

Table 2. Results of the deconvolution analysis of the charge recombination experiments shown in Figure 3.

Samples	no quinone added		quinone added	
	Amplit. (%)	k (s^{-1})	Amplit. (%)	k (s^{-1})
TTE buffer[a]	39±1	7.9±0.2	-	-
	61±1	1.32±0.02	100.0±0.5	1.30±0.01
ChCl/F/W[b]	80±2	10.0±0.2	66±1	11.5±0.15
	20±1	3.2±0.3	34±1	1.88±0.04
ChCl/Gly[b]	36±3	8.0±0.3	70±1	6.5±0.1
	64±3	2.8±0.1	30±1	1.00±0.02
ChCl/U[c]	37±1	10.0±0.2	44±1	8.0±0.2
	63±1	3.1±0.1	56±1	1.80±0.05
ChCl/W[c,*]	100±1	9.8±0.2	71±1	11.4±0.3
	-	-	29±1	1.10±0.03
ChCl/EG[d]	17±1	8.0±0.5	15±1	8.0±0.4
	83±1	1.80±0.04	85±1	1.70±0.03

a) UQ_{10} 40 μM; b) UQ_0 1 mM; c) dQ 250 μM; *adding UQ_{10} has no effect; d) UQ_{10} 1 mM

Can the RC photocycle be reproduced in DESs?

Once assessed the RC ability to generate the $D^+Q_B^-$ state, the question arising is whether the photoenzyme is still able to perform its photocycle (Figure S1, left bottom panel, ESI) if a second protein, *i.e.* the water-soluble cyt c, is involved. According to Figure S1, left bottom panel, the protein performs *in vivo* a photocycle in which the UQ_{10} sitting in Q_B is reduced to ubiquinol in a two-step reaction with the simultaneous oxidation of two cyt c^{2+} proteins. The formation of ubiquinol, in turn, requires the uptake from the solution of two H^+ ions. The proceeding of the cycle can be followed spectrophotometrically by recording, under continuous light excitation, the absorption decrease at 550 nm due to cyt c^{2+} oxidation. The normal behavior

in aqueous environment is shown in Figure 4 (left panel, green trace): when RC 1 μM is dissolved in TLE in the presence of 250 μM dQ, a very rapid oxidation of cyt c^{2+} (10 μM) is promoted upon illumination with a red-filtered 250 W QTH lamp.

A preliminary study of the behavior of this protein in DESs is hence required, which was limited to the two less viscous DESs, namely ChCl/W and ChCl/EG. The photocycle indeed requires suitable diffusion rate of the different molecules involved to be sustained. In ChCl/W, the optical spectrum of cytochrome shows no denaturation of the protein. Its redox activity is retained, since the reduction of cyt c^{3+} to cyt c^{2+} is observed upon addition of ferrocyanide ($Fe^{2+}CN$) or ascorbate (see Figure 4, right panel). Conversely, no reduction of cyt c could be detected in ChCl/EG due to the insolubility of both $Fe^{2+}CN$ and ascorbate. Moreover, the rapid oxidation of pre-reduced cyt c is observed in ChCl/EG and ChCl/U. In summary, our results indicate that the DES potentially useful for hosting the RC photocycle is ChCl/W. We dissolved RC 1 μM in ChCl/W (1 mL) in the presence of 10 μM cyt c^{2+} and 250 μM dQ. This synthetic hydrophobic ubiquinone, having a 10-carbon saturated side chain, proved to be very effective in promoting the RC photocycle compared to other ubiquinone analogs.[70] In these conditions, however, we could not detect any absorbance change upon illumination (black trace in Figure 4). This means that the photocycle is unpaired either at the donor side (the cyt c^{2+} cannot reduce D^+) or at the acceptor side (ubiquinol is not formed or the quinone/quinol exchange is blocked). The small absorbance changes observed as the light is switched on and off (black arrows in Figure 4), are due to the formation of D^+ and its subsequent disappearing.

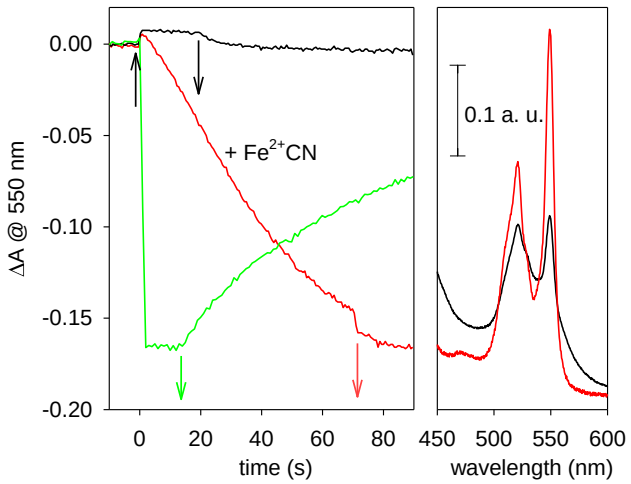
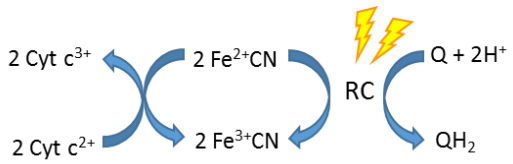


Figure 4. Left panel: kinetics of cytochrome oxidation followed by absorbance change at 550 nm. Conditions: ChCl/W with RC 1 μM, cyt^{2+} 10 μM, dQ 250 μM. In the experiment relevant to the red trace, $Fe^{2+}CN$ in grains was also added. The green trace is recorded in TLE buffer, with same conditions, but without the $Fe^{2+}CN$. Right panel: optical spectra of 10 μM cyt c in ChCl/W in oxidized (black line) and reduced (red line) states. Reduction was performed by the addition of ascorbate in grains. Upwards and downwards arrows indicate the time of exciting light switching on and off, respectively.

The result dramatically changes if a few grains of $Fe^{2+}CN$ are added to the ChCl/W solution (red trace of Figure 4). In this case, upon illumination, the sudden upwards absorption change is followed by a slow but constant absorbance decrease, indicating that cyt c^{2+} is being oxidized. When the light is switched off, after 70 s, a sudden further absorbance decrease is observed (D^+ disappearing) and then the signal remains constant. The total absorbance change (150 mOD) indicates that almost all added cyt c^{2+} has been oxidized to cyt c^{3+}, as confirmed by the final optical spectrum (not shown). These findings clearly demonstrate that $Fe^{2+}CN$ is able to sustain the photocycle. Being a reduced molecule, $Fe^{2+}CN$ cannot act at the acceptor side withdrawing

electrons from the quinones. Therefore, it is possible to hypothesize that $Fe^{2+}CN$, undergoing oxidation at the RC donor side, and reduction at the cyt c side, acts as a mediator between the two proteins (Scheme 1).



Scheme 1. Representation of photocycle in ChCl/W. Two photons promote the conversion of one quinone molecule to quinol with the simultaneous oxidation of two cyt c^{2+} proteins. $Fe^{2+}CN$ shuttles electrons from cyt c to RC.

Cytochrome c and reaction centre exchange the electron by a reaction that requires an initial docking of the heme protein to the periplasmic side of the RC. This, in turn, requires that two proteins come into contact with each other. Once the docking has taken place, the electron transfer reaction is not influenced by external factors.[71-73] The docking step in DESs may be impaired by the low diffusion of the proteins in a viscous fluid (e.g. the ChCl/EG DES has a viscosity roughly 20 times larger than water[35]). Finally, according to Table 2, only 30% of the RCs have the Q_B-site populated in the presence of 250 μM dQ and is actually involved in the photocycle, contributing to make the cyt c oxidation slower in DES as compared to the TLE buffer. On the other hand, the observed photo-oxidation of cyt c, promoted by the $Fe^{2+}CN$/RC system, implies the full functionality of the RC acceptor side, meaning that the quinone substrate is able to uptake protons from the DES surrounding environment. This non-trivial result demonstrates indeed that, despite the new network of interactions established with ChCl, the

water present in the ChCl/W DES here used can still effectively act as a Brønsted acid releasing protons to the quinone substrate.

Can DESs improve the RCs thermal stability?

The thermal stability of the RC in aqueous buffer has been widely studied by either recording circular dichroism (CD) or absorbance spectra at increasing temperatures,[74,75] or monitoring the time course of absorbance changes once reached the desired temperature.[74,76] These measurements yield to different results since as denaturation depends both on the temperature and the heat duration of the heat treatment. A further reliable approach consists of measuring a physical property of the sample relevant to the protein structural integrity while heating at constant rate, to provide a more accurate determination of the denaturation temperature T_d. Using CD spectra as observable, the T_d of RC in aqueous solution, containing either LDAO or octylglucoside as solubilizing detergents, was found equal to 52 °C and 63 °C, respectively.[77]

Since denatured RCs lose the ability to perform charge separation under light, the amplitude of the absorbance fall at 860 nm after a single flash due to D^+Q^- generation was measured at different temperatures, aiming to infer the T_d value. Different DESs were employed and the results were compared with RC behavior in TLE buffer. The temperature was raised from 25 to 74 °C with an equilibration time of 5 min, corresponding to a rate of roughly 1 °C/min. The curves depicting the activity decay of RCs with temperature are shown in Figure 5.

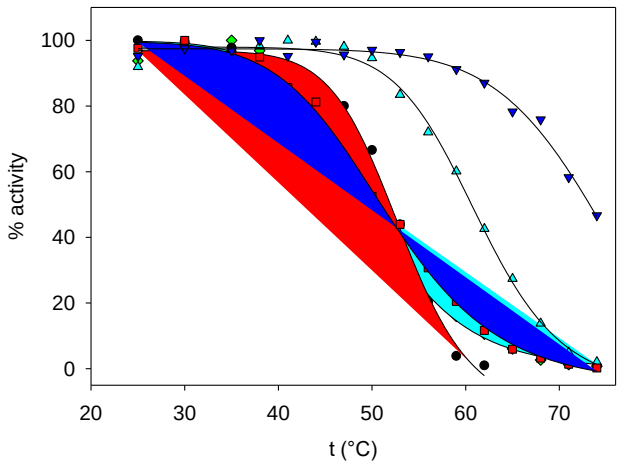


Figure 5. Thermal stability of RC in TLE (black circles), ChCl/EG (red squares), ChCl/W (green diamonds), ChCl/U (cyan up triangles) and ChCl/Gly (blue down triangles). The experimental data are fitted to a 4 parameter logistic curve (black lines).

The values of the T_d, determined by fitting the experimental points to a 4 parameter logistic curve, were 53.1±0.7 °C, 51.6±0.4 °C, 50±0.5, 61.0±0.5 and 77±8 for TLE, ChCl/EG, ChCl/W, ChCl/U and ChCl/Gly, respectively. Our T_d value in TLE is in perfect agreement with that found previously[77] using DSC with a similar temperature increase rate. In all investigated systems, the denaturation has been found irreversible upon protein cooling at room temperature. Remarkably, while the T_d values in ChCl/EG and ChCl/W are slightly lower than that in aqueous buffer, in ChCl/U and especially in ChCl/Gly, significantly higher T_d values have been detected, indicating an interesting protective role played by these eutectic mixtures.

Can RC photocurrents be generated in DESs?

Photocurrent generation in RC-based photoelectrochemical cells (PECs) occurs when RC photocycle is activated by light in the presence of suitable electron donors and acceptors. In the

classical three electrodes configuration, no current flows in dark conditions at the open circuit voltage (OCV). However, at this potential, a current appears upon illumination (photocurrent), due to the equilibrium displacement arising from the oxidation of the donor and the reduction of the acceptor. The current can be cathodic or anodic depending on whether the electrochemical reduction of the donor or the oxidation of the acceptor occurs at the working electrode. In the literature, there is a huge amount of work dealing with the use of photosynthetic biomolecules for solar energy conversion. A recent review[78] summarizes the performances achieved with protein-based devices, depending on light intensity, protein loading/coverage, electrode material, use of antennas and alternative electrolyte/mediators, and so forth. The reported current densities span from 0.02 to 120 μA/cm^2, pointing out that the specific experimental conditions greatly affect the obtained photoresponse. Thus, we compare our result in DES with a control experiment in which the only parameter changed was the solvent.

The physiological electron donor to the dimer is the water-soluble cytochrome c_2, having a midpoint potential of 345 mV.[79] Unfortunately, as previously shown, in DESs the direct electron transfer from cyt c to RC is unpaired, hence the organic electron donor FcnMeOH ($E_m \approx 400$ mV) was used in this work. On the other side, electron acceptors usually belong to the class of ubiquinones and, in particular, dQ gave the best results in our system. ChCl/Gly solvent was chosen to take advantage of its viscosity, being the employed geometry a simple 100 μL drop cast on the screen-printed PEC. The viscous ChCl/Gly drop is stable during the measuring time and shows no signs of solvent evaporation. For the aqueous electrolyte, the PEC was dipped in the solution contained in a cuvette. The same distance from the light source was, however, maintained.

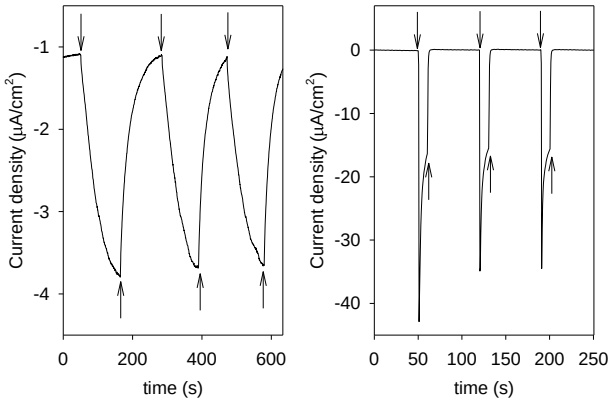


Figure 6. Photocurrents generated on Au working electrode in the PEC prepared with ChCl/Gly (left panel) or aqueous phosphate 90 mM, TX 0.03% pH 7.0 (right panel) as electrolytes, both containing RC 1 µM, FcnMeOH 300 µM, and dQ 150 µM. Downwards and upwards arrows indicate the time of light switching on and off, respectively.

Cathodic photocurrents were generated by illuminating with a LED light at 860 nm and applying a potential of -0.3V and -0.1 V (*vs* quasi reference Ag), for ChCl/Gly and the aqueous buffer, respectively. Both values are slightly lower than the OCV. The reaction promoted by the light is described by Eq. 1.

$$2\text{FcnMeOH} + \text{dQ} + 2\text{H}^+ \xrightarrow[h\nu]{\text{RC}} 2\text{FcnMeOH}^+ + \text{dQH}_2 \quad \text{Eq. 1}$$

On the other hand, the reaction responsible for the photocurrent at the working electrode is described by Eq. 2.

$$\text{FcnMeOH}^+ + e^- \rightarrow \text{FcnMeOH} \quad \text{Eq. 2}$$

In Chl/Gly (Figure 6, left panel), the signals were reversible and repeatable over many cycles. However, the photocurrent did not reach completely the plateau (~ 3 µA/cm^2) within the 100 s illumination time. A similar time is needed for the return to the baseline value. It is worthwhile

to note that the current density obtained ranks in the average of the results summarized in the above-mentioned review.[78] The behavior in the aqueous electrolyte (Figure 6, right panel) reflects its much lower viscosity and also the higher intrinsic photoactivity of the RC: a very high current peak (30-40 $\mu A/cm^2$) is reached immediately upon illumination, followed by a rapid decrease up to 15 $\mu A/cm^2$ at the end of the 10 s illumination time. The baseline value is finally reached after 1-2 s. Under these conditions, the photochemical reaction of the RC in aqueous electrolyte is so fast that the mediators are rapidly consumed and the photocurrent cannot be sustained at its peak value. In the case of ChCl/Gly, the photochemical and electrochemical reaction rates are better balanced and a stable plateau photocurrent could be obtained. Overall, these data show that the implementation of devices similar to the one sketched in Figure 1 is conceivable in nonaqueous solvents, paving the way to the use of DESs in the light energy conversion process.

CONCLUSIONS

Isolated photosynthetic RCs have been solubilized in nine different deep eutectic solvents of different composition. In five DESs, the photoenzyme retains its ability to generate a charge-separated state, proving that any structural modification which may have occurred in such systems is not relevant for the correct functioning of the protein. Even though underperforming in comparison with aqueous systems, DES embedded RCs were able to accomplish their photocycle leading to the production of quinol, thus efficiently mediating the transfer of both electrons and protons across a nonaqueous medium.

These intriguing results open the door to novel biotechnological applications of photosynthetic enzymes from solar energy conversion to photocatalysis. The noteworthy result from a basic

research point of view is the demonstration that a complex membrane protein, such as RC, can work properly in the nonphysiological environment represented by a eutectic mixture. Interestingly some physico-chemical properties, such as thermal stability, can even be improved. Further investigations on the performance of different membrane proteins in DESs (e. g., receptors, transporters, etc.) are ongoing in our laboratories and results will be reported in due course.

ASSOCIATED CONTENT

Supporting Information. The Supporting Information is available free of charge via the Internet at http://pubs.acs.org.

Description of the photosynthetic RC and RC optical spectra in additional DESs . Determination of water content in ChCl/Gly with FTIR spectroscopy. Characterization of RC solubilized in water/glycerol 1:2. (DOC)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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