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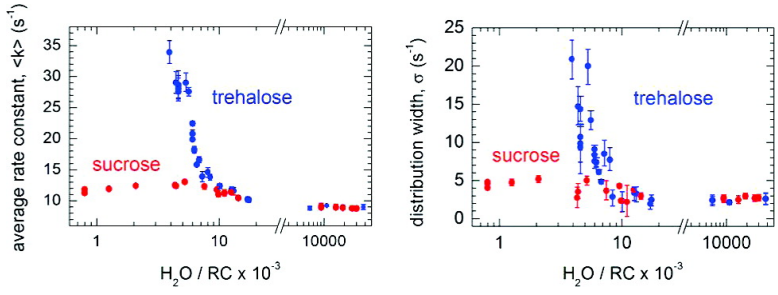

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Protein−Matrix Coupling/Uncoupling in "*Dry*" Systems of Photosynthetic Reaction Center Embedded in Trehalose/Sucrose: The Origin of Trehalose Peculiarity

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Abstract: Trehalose is a nonreducing disaccharide of glucose found in organisms, which can survive adverse conditions such as extreme drought and high temperatures. Furthermore, isolated structures, as enzymes or liposomes, embedded in trehalose are preserved against stressing conditions [see, e.g., Crowe, L. M. *Comp. Biochem. Physiol. A* **2002**, *131*, 505−513]. Among other hypotheses, such protective effect has been suggested to stem, in the case of proteins, from the formation of a water-mediated, hydrogen bond network, which anchors the protein surface to the water−sugar matrix, thus coupling the internal degrees of freedom of the biomolecule to those of the surroundings [Giuffrida, S.; et al. *J. Phys. Chem. B* **2003**, *107*, 13211−13217]. Analogous protective effect is also accomplished by other saccharides, although with a lower efficiency. Here, we studied the recombination kinetics of the primary, light-induced charge separated state ($P^+Q_A^-$) and the thermal stability of the photosynthetic reaction center (RC) of *Rhodobacter sphaeroides* in trehalose−water and in sucrose−water matrixes of decreasing water content. Our data show that, in sucrose, at variance with trehalose, the system undergoes a "*nanophase separation*" when the water/sugar mole fraction is lower than the threshold level ∼0.8. We rationalize this result assuming that the hydrogen bond network, which anchors the RC surface to its surrounding, is formed in trehalose but not in sucrose. We suggest that both the couplings, in the case of trehalose, and the nanophase separation, in the case of sucrose, start at low water content when the components of the system enter in competition for the residual water.

1. Introduction

Increasing attention has been recently devoted to the study of the intriguing properties of biomolecules embedded into sugar−water matrices. Glassy matrices of saccharides exhibit in fact an outstanding ability to protect against adverse environmental conditions, such as potentially detrimental freezing, heating, and dehydration.[1,2] Saccharide glasses are employed by several organisms that can long survive under extreme drought and high temperature, entering a state of suspended metabolism (anydrobiosis), which is preceded by a massive synthesis of specific carbohydrates.[3,4] Among sugars, the disaccharide trehalose appears to be the most effective protectant.[5−8] Its ability to preserve the structural and functional integrity of biostructures is largely employed in food, pharmaceutical, and biotechnology sciences to optimize long-term storage of biological samples (see, e.g., refs 9–12). Notwithstanding the large efforts devoted, neither the molecular mechanisms through which trehalose (or other saccharides) prevent biomolecules from denaturation nor the origin of the higher effectiveness of trehalose as bioprotectant have, to now, been fully understood.[13−16]

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The dynamics of proteins embedded in a trehalose glass have been first characterized by Eaton and co-workers who studied the CO recombination after flash photolysis in carboxy myoglobin (MbCO) and reported that, under such conditions, some part of the protein dynamics is strongly inhibited.[17,18] Furthermore, these authors also suggested the use of trehalose as a storage medium for hemoglobin-based blood substitutes.[19] Analogous hindering of protein dynamics was reported by Friedman and co-workers in a flash photolysis study on carboxy hemoglobin.[20] In full agreement, Mössbauer and elastic neutron scattering measurement[21,22] together with molecular dynamics (MD) simulations[23] showed that, for MbCO embedded in trehalose glasses, the large amplitude nonharmonic motions, coupled with the interconversion among high tier conformational substates,[24] are reduced with respect to hydrated systems. Furthermore, FTIR and flash photolysis measurements, together with MD simulations, pointed out that, in such systems, the dynamics of the water−sugar matrix and of the protein are both progressively reduced when the sample water content is decreased.[25–28] In particular, MD simulation[27] indicated that the number of hydrogen bonds (HB) in which water molecules are involved increases by decreasing the water content. Because the motional freedom of each water molecule decreases by increasing its number of HBs, the above result implies a decrease of the dynamics of the trehalose−water "solvent" at constant temperature, when the content of residual water is reduced. In turn, on the basis of the coupling between the structural relaxation of a protein and the relaxation of the surrounding HB network,[29] the decreased dynamics in MbCO−trehalose−water systems of very low water content was rationalized.[21,22,26] In particular, also based on FTIR results,[26] it was inferred that in such systems the protein is confined within a water-mediated network of hydrogen bonds, in which water molecules connect the protein surface to trehalose molecules irrespective of the interactions of the sugar molecules with the protein, thus anchoring the protein surface to the trehalose−water matrix and coupling the internal degrees of freedom of the protein to those of the external matrix[26,28,30] (see Supporting Information).

Besides the last hypothesis, to which we shall refer as "anchorage hypothesis", other hypotheses have to now been proposed to explain the peculiar bioprotective effect of trehalose. These include the following:

1. The *water-replacement hypothesis*, according to which stabilization occurs via hydrogen bond (HBs) formation between the sugar and the biostructure;[13,14]

2. The *water-entrapment hypothesis*, according to which, in the dry state, trehalose rather than directly binding to proteins traps the residual water at the biomolecule−sugar interface by glass formation;[15]

3. The *high viscosity hypothesis*, according to which viscosity effects cause motional inhibition[16] and hindering of processes leading to loss of structure and denaturation. Furthermore, Green and Angell[31] suggested the peculiarity of trehalose to be related to its rather high glass transition temperature with respect to other glass-forming sugars.

The anchorage hypothesis, which is in line with the water-entrapment hypothesis, appears to hold true for proteins embedded in trehalose matrixes, while the water-replacement hypothesis seems to better describe trehalose−membrane interactions.[14,32–36]

The existence of protein−water−trehalose structures, whose rigidity increases by lowering the hydration level (protein−water−trehalose substates), foreseen by the anchorage hypothesis, has recently been confirmed in a study in which the nanoenvironments of single-GFP-mut2 proteins embedded in thin trehalose−water films were characterized through the protein's fluorescence brightness, bleaching dynamics, excited-state lifetime, and fluorescence polarization[37] (see also the Supporting Information).

Studies on MbCO embedded in sucrose, maltose, raffinose, and glucose showed that the protein−matrix coupling is the tightest in the case of trehalose.[38]

The photosynthetic reaction center (RC) from the purple bacterium *Rhodobacter sphaeroides* is a 100 kDa protein−pigment complex. Photon absorption by the primary electron donor P (a bacteriochlorophyll special pair) catalyzes a sequential electron transfer which leads in 200 ps to reduction of the primary quinone acceptor Q_A, located ∼25 Å away from P. From Q_A^-, the electron is subsequently delivered to a second ubiquinone molecule, bound at the Q_B site of the protein. When the electron transfer to Q_B is prevented by quinone deprivation or binding of inhibitors at the Q_B site, recombination of the primary charge separated state $P^+Q_A^-$ occurs through electron tunnelling.[39]

The electric field generated by light-induced charge separation within the RC perturbs substantially the protein, giving rise to a conformational relaxation, which stabilizes the $P^+Q_A^-$ state.[40–44] Accordingly, the RC can be trapped at cryogenic

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temperatures in a *dark-adapted* or in a *light-adapted* conformation, which differ substantially in the lifetime of the $P^+Q_A^-$ state.[41,42] These conformations consist of distributions of substates, as inferred from the broad, continuous spectrum of rate constants observed for charge recombination of the $P^+Q_A^-$ state at cryogenic temperatures. At room temperature, following primary charge separation, the RC rapidly relaxes from the *dark-adapted* to the stabilized, *light-adapted* conformation. Moreover, the available conformational substates are, at room temperature, rapidly sampled and averaged on the time scale of charge recombination, giving rise to almost exponential charge recombination kinetics. At variance, at cryogenic temperatures, the lack of both relaxation to the *light-adapted* conformation and substates interconversion results in acceleration of $P^+Q_A^-$ recombination and broadening of rate coefficients distribution.[41,42]

Embedding RC into trehalose−water glasses of decreasing water content progressively hinders the dynamics coupled to electron transfer;[45–47] in particular, in samples led to extreme drought, the after-flash kinetics of $P^+Q_A^-$ recombination abruptly accelerates, becoming broadly distributed and behaving, at room temperature, as at cryogenic temperatures in a glycerol−water mixture.[45] This similarity indicates that, in dehydrated trehalose−water matrixes, the thermal fluctuations between conformational substates, as well as relaxation from the *dark-adapted* to the *light-adapted* state, are sizably hindered even at room temperature over the time scale (10^{-1} s) of charge recombination. Furthermore, the structure and the photochemical activity of RCs embedded in dry trehalose matrixes are long preserved at room temperature. On the basis of the above observations, we have suggested that, as proposed for MbCO,[26] also for RC, a water-mediated network of hydrogen bonds locks the RC surface to the matrix, thus coupling the internal degrees of freedom of the protein to those of the water−sugar matrix.[28,46,47]

In order to compare the behavior of the RC in different sugar matrixes, we comparatively studied here the coupling of this protein complex to trehalose−water and to sucrose−water matrixes of decreasing hydration, by examining the kinetics of electron transfer and the protein structural stability.

2. Experimental Section

2.1. RC Purification and Sample Preparation. The RCs were purified from *Rhodobacter sphaeroides* R-26 according to ref 48. Liquid samples were prepared by dissolving suitable amounts of trehalose or sucrose in aqueous buffer (10 mM Tris, pH = 8.0, 0.025% lauryl dimethyl amine-*N*-oxide (LDAO)); the RC was added to a final concentration of 2 μM. Measurements of $P^+Q_A^-$ recombination kinetics were performed in the presence of *o*-phenanthroline, a known inhibitor of Q_A^- to Q_B electron transfer, added to a final concentration of 10 mM to solutions of RCs which were not Q_B-reconstituted. Trehalose, from Hayashibara Shoij (Okayama, Japan), and sucrose, from Sigma (>99% purity), were used without further purification.

"Dry" samples, containing trehalose or sucrose in a fixed sugar/RC protein ratio equal to 10^4, were prepared according to the following procedures. RCs were diluted to 40 μM in 10 mM Tris, pH 8.0, 0.4 M trehalose (or sucrose), 0.025% LDAO, 10 mM *o*-phenanthroline. A thin layer (0.24 mL) of the liquid solutions, deposited on a 50 mm diameter optical glass window, was initially dried in a desiccator for ~8 h under N_2 flow, at room temperature. Further drying of the trehalose sample was obtained by leaving it under N_2 atmosphere at 30 °C for ~12 h and then under vacuum for approximately the same time; such treatments were alternatively performed for several days. A similar procedure was used for drying the sucrose samples, which, however, reached the maximal dehydration already after 2 days. To avoid water exchange with the environment during optical and NIR measurements, the optical window on which the sample was formed was inserted within a specifically designed, gastight, sample holder, filled with dry N_2.

2.2. Evaluation of the Water Content. The water content was evaluated by NIR spectroscopy from the area of the combination band of water in the 1930−1960 nm spectral region, using the RC absorption band at 802 nm as an internal standard (for details, see refs 45 and 47). As already reported,[45] in moderately dehydrated trehalose glassy matrixes, the combination band of water is centered at ~ 1950 nm; further drying makes the band peak to progressively shift toward higher wavelengths over a 10−15 nm range. Such shift is symptomatic of water molecules, which become progressively involved in strong hydrogen bonding.[49] Interestingly, in moderately dehydrated sucrose glassy matrixes, the combination band of water was centered at ~ 1935 nm, i.e. blue-shifted by ~ 15 nm as compared to what observed in trehalose matrixes of comparable water content. Moreover, in sucrose, the water band did not change position upon dehydration, except for a, few nm, blue-shift in the most dry samples. These spectral features are reminiscent of those observed in dehydrated RC-PVA films.[50]

2.3. Time-Resolved Visible Absorption Spectroscopy and Data Analysis. Charge recombination kinetics following flash photoexcitation were monitored at 422 nm[51] using a kinetic spectrophotometer of local design,[52] modified as follows. Excitation, at 90° with respect to the measuring beam, was provided by a frequency doubled Nd:YAG laser (Quanta System, Handy 710) delivering 200 mJ pulses of 7 ns width. The optical window on which saccharide matrixes were formed was placed at 45° with respect to the measuring beam in such an orientation to minimize laser light reflection toward the detector. The photomultiplier was protected from scattered excitation light by a 0.01% blocking, 10 nm bandwidth, interference filter, centered at 420 nm. Rapid digitization and averaging of the amplified photomultiplier signal was performed using a Le Croy 9410 digital oscilloscope controlled by an Olivetti M290 personal computer.

Fitting of P^+ decay to model kinetics was performed by least-squares minimization routines, based on a modified Marquardt algorithm.[53] Confidence intervals of fitting parameters were evaluated numerically as described in ref 45.

3. Results

3.1. Effects on the Kinetics of $P^+Q_A^-$ Recombination. Figure 1 compares the kinetics of $P^+Q_A^-$ recombination following a laser flash in RCs embedded in trehalose (panel A) or sucrose (panel B) at different hydration. As previously reported for trehalose,[45] also in the case of sucrose, the kinetics are nonexponential, already in water-rich systems; indeed, the survival probability of the $P^+Q_A^-$ state is described for all the

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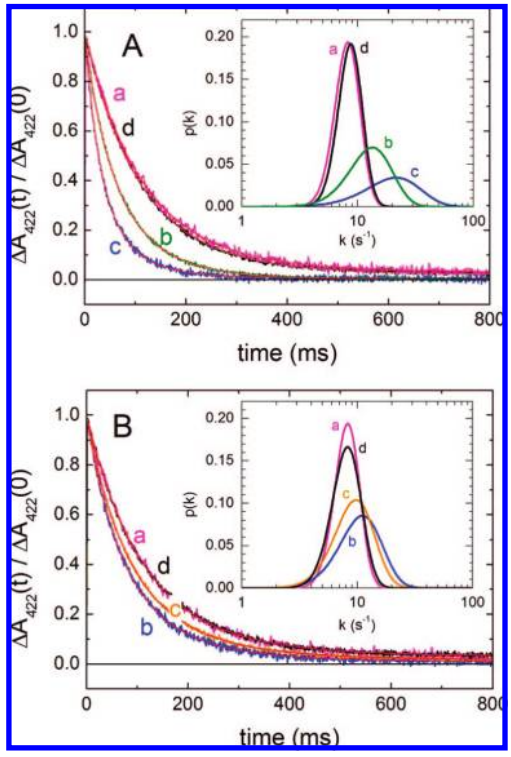

Figure 1. Kinetic analysis of $P^+Q_A^-$ recombination following flash excitation of RC in trehalose (A) and sucrose (B) matrixes. The decays of the absorbance change induced at 422 nm by a laser pulse have been normalized to the maximal change immediately after photoexcitation (t = 0). Best fits to eq 4 are shown as red curves. The insets show the corresponding rate distributions $p(k)$, defined by eqs 2 and 3. The uncertainties in best fitting $\langle k \rangle$ and σ values are reported in brackets, as the extremes of the confidence interval within two standard deviations. Panel A: trace a (magenta), solution in the absence of disaccharides ($\langle k \rangle$ = 8.8 (8.5, 9.1) s^{-1}, σ = 2.1 (1.1, 2.9) s^{-1}); trace b (green), dehydrated trehalose matrix, 6500 H_2O molecules per RC ($\langle k \rangle$ = 15.8 (15.6, 16.0) s^{-1}, σ = 6.1 (5.8, 6.4) s^{-1}); trace c (blue), further dehydrated trehalose matrix, 5600 H_2O molecules per RC ($\langle k \rangle$ = 27.6 (26.9, 28.3) s^{-1}, σ = 12.9 (11.7, 14.2) s^{-1}); trace d (black), trehalose solution obtained following resolubilization of the glass, in which traces b and c had been measured ($\langle k \rangle$ = 9.2 (9.1, 9.4) s^{-1}, σ = 2.1 (1.8, 2.4) s^{-1}). Panel B: trace a (magenta), same as trace a of panel A; trace b (blue), dehydrated sucrose matrix, 5200 H_2O molecules per RC ($\langle k \rangle$ = 13.0 (12.7, 13.4) s^{-1}, σ = 5.0 (4.4, 5.6) s^{-1}); trace c (orange), extensively dehydrated sucrose matrix, 800 H_2O molecules per RC ($\langle k \rangle$ = 11.2 (11.1, 11.4) s^{-1}, σ = 4.1 (3.8, 4.3) s^{-1}); trace d (black), sucrose solution obtained by redissolving the extensively dehydrated matrix ($\langle k \rangle$ = 8.9 (8.8, 9.1) s^{-1}, σ = 2.5 (1.9, 2.9) s^{-1}).

traces in the figures, by a continuous distribution p of rate constants k:

$$\frac{P^+Q_A^-(t)}{P^+Q_A^-(0)} = \frac{\Delta A_{422}(t)}{\Delta A_{422}(0)} = \int_0^\infty p(k)\, e^{-kt}\, \mathrm{d}k \qquad (1)$$

where $\Delta A_{422}(t)$ is the absorbance change measured at 422 nm at time t, with $t = 0$ for the laser pulse. For both trehalose and sucrose, the kinetics can be accounted for by assuming that $p(k)$ is a Γ distribution function:

$$p(k) = \frac{k^{n-1}}{k_0^n} \frac{\exp(-k/k_0)}{\Gamma(n)} \qquad (2)$$

where $\Gamma(n)$ is the gamma function and k_0 and n are related to the average rate constant, $\langle k \rangle$ and to the variance, σ^2, of the distribution by

$$\langle k \rangle = nk_0 \quad \sigma^2 = nk_0^2 \qquad (3)$$

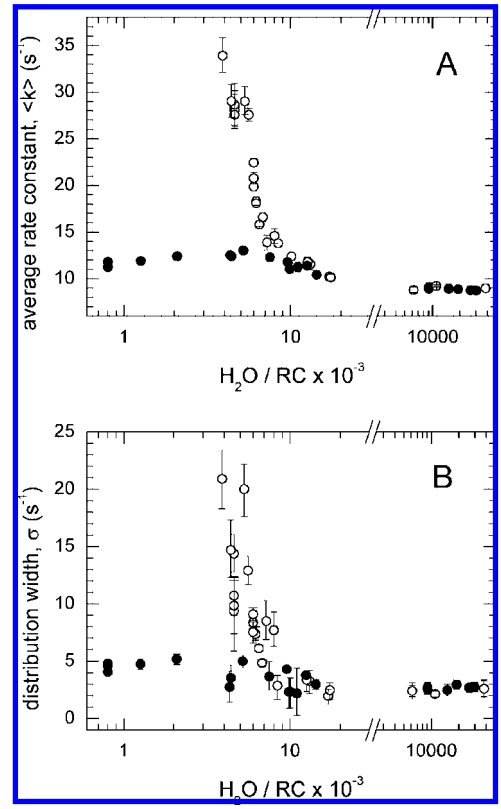

Figure 2. Dependence of the kinetics of $P^+Q_A^-$ recombination upon the H_2O/RC ratio in sucrose (closed circles) and trehalose (open circles) solutions and amorphous matrixes. Kinetic analysis was performed as illustrated in Figure 1. Panels A and B show the values of the average rate constant, $\langle k \rangle$, and of the rate distribution width, σ, respectively. For both disaccharides, the experimental points on the right of the break in the abscissa scale were obtained in solutions, while those on the left in glassy matrixes. Vertical bars indicate confidence intervals within two standard deviations.

By taking the Laplace transform of $p(k)$ and using eq 3, one obtains for the charge recombination kinetics a power law of the form

$$\frac{\Delta A_{422}(t)}{\Delta A_{422}(0)} = \left(1 + \frac{\sigma^2}{\langle k \rangle} \cdot t\right)^{-\langle k \rangle^2/\sigma^2} \qquad (4)$$

which we fitted to the traces of Figure 1, leaving $\langle k \rangle$ and σ as free parameters.

Dehydration of the trehalose systems results in progressive and continuous acceleration of the recombination kinetics (Figure 1A) and broadening of the rate distribution (Figure 1A, inset) (cf. ref 45). Such effects are very large at the water/trehalose mole fraction ~0.4, which is the lowest water/trehalose ratio obtained by drying the sample under vacuum, for several days, at room temperature. At variance, for RCs embedded in sucrose (Figure 1B), almost no effects are observed even at much lower water/sugar ratios. At a comparable content of residual water, a rate constant $\langle k \rangle$ equal to 28 s^{-1} (Figure 1A, trace c) and 13 s^{-1} (Figure 1B, trace b) is obtained in trehalose and sucrose, respectively. The corresponding values of the distribution width σ are 13 and 5 s^{-1} (see Figure 1, insets).

The difference of recombination kinetics in the two sugars appears in the plot of $\langle k \rangle$ and σ versus water content, shown in Figure 2. By decreasing the content of residual water from ~2 to ~0.8 water molecules per sugar (corresponding to a water

mass ratio to dry sample mass, i.e., (H_2O mass)/(sugar mass + RC mass), of ~0.102 and ~0.0409, respectively, or to ~20×10^3 and ~8×10^3 H_2O molecules per RC), the average rate constant and the distribution width increase slightly, assuming essentially coincident values in both sugars. By further dehydration, in trehalose, both $<k>$ and σ undergo a sudden, dramatic increase, reaching values close to 35 and 20 s^{-1}, respectively (cf. refs 45 and 47), while in sucrose, they slightly decrease, reaching the values obtained when the RC is dried in the absence of sugars.[45] Noteworthy, in trehalose, the content of residual water can barely be reduced below ~0.5 water per trehalose molecule (corresponding to ~5×10^3 H_2O/RC), while in sucrose, it can be easily decreased to the NIR detection limit, that is, ~800 H_2O/RC (corresponding to ~0.08 water per sucrose molecules). This indicates that in the trehalose sample the binding energy of residual water is much larger than that in sucrose.

Data in Figures 1 and 2 evidence that, at variance with trehalose, and in spite of the very low water content, the RC dynamics are barely reduced in dry sucrose.

We suggest that the sudden, large increase of both $<k>$ and σ, which starts at water/trehalose ratios lower than ~0.8, indicates that the competition for residual water, among the various components, leads to the formation of a solid, continuous water−trehalose matrix and of a stable, water-mediated HB network, which connects the RC to the matrix. In particular, the rather high binding energy of the residual water molecules, evidenced by the very low evaporation rate, plausibly stems from the water molecules being all, at extreme dehydration, bonded via four hydrogen bonds,[26,27] thus leading, at room temperature, to a situation analogous to the "*soft protein solid solvent*" simulated by Karplus and co-workers.[54]

At variance, in sucrose, upon decreasing the water content, both $<k>$ and σ, after a slight increase which parallels the one in trehalose, exhibit, at water/sugar below ~0.8, a progressive decrease which makes the values most similar to the ones observed in solution or when the complex is dried in the absence of sugars.[45] The observed different behavior between trehalose and sucrose can be rationalized based on MD simulations of binary saccharide/water systems; these simulations[55,56] showed that two intramolecular hydrogen bonds can be formed in sucrose, while only a single internal HB can be formed in trehalose. This makes sucrose, on average, more rigid and having fewer sites disposable to form hydrogen bonds with its surroundings under conditions of low water. One can, therefore, infer that, when by drying, competition for residual water among the various components would increase the number of intermolecular HB bonds in which water and sugar molecules are involved, the formation of internal HB causes in sucrose the lack of suitable *connectivity* between the RC surface and its surroundings. This causes uncoupling of the protein complex from its environment (nanophase separation). The slight increase of both $<k>$ and σ observed in Figure 2 in sucrose suggests the onset of a loose hydrogen bond network, coupling the RC to its surroundings, which is started at the same hydration levels as that for trehalose, but collapses by further decreasing the water content. Lack of protein−matrix coupling in sucrose is confirmed by a study of single-GFP-mut2 proteins embedded in thin sucrose−water films; indeed, at variance with trehalose,[37] no protein−water−sugar nanostructures were detected in sucrose (G. Chirico, private communication).

The recombination kinetics measured in solution upon redissolving RC immediately after the run of measurements is essentially restored for both the sugar systems (Figure 1). At variance, redissolving the system long after the runs results in a progressive loss of function in sucrose but not in trehalose.

3.2. Thermal Stability of the RC in Dehydrated Trehalose and Sucrose Matrixes. A remarkable superiority of trehalose in preserving the structural and functional activity at low water content and elevated temperatures has been demonstrated for a number of soluble proteins, including DNA restriction and modifying enzymes,[6,7,57] glucose-6-phosphate dehydrogenase,[8] and trypsin.[58] One therefore expects, in line with the results reported in the preceding section, RCs to exhibit a better thermal stability when embedded in dry trehalose than in dry sucrose matrixes. To test this hypothesis, we compared the thermal stability of RCs embedded in trehalose and in sucrose matrixes, upon incubation at 37 °C for 6 days, under dry nitrogen atmosphere, of samples of initial water content ~5000 H_2O/RC. We checked the RC thermal stability by recording at selected time intervals the NIR spectrum between 690 and 950 nm,[59] which is characterized by four absorption bands (Figure 3). The exact position of the bands, particularly of the special pair absorption peak (at ~860 nm), varies somewhat in response to changes in detergent type and concentration,[60,61] dehydration both in the presence and in the absence of sugar,[45] and phase segregation of RCs.[62]

As shown in Figure 3A, the spectrum of the RC embedded in trehalose did not change appreciably even after 6 days of incubation (cf. spectra a and d). Furthermore, when redissolving the sample, the amount of primary donor photo-oxidized by a laser pulse was found to be unaffected as compared to what was measured in solution before dehydration (not shown). On the contrary, the spectrum of the RC embedded into the sucrose matrix underwent drastic changes, as shown by the temporal sequence (a′−d′) in Figure 3B. A progressive amplitude decrease of the bands at 860 and 800 nm (already pronounced after 20 h incubation) was accompanied by a concomitant increase of the band at 760 nm. Because both bacteriochlorophyll and bacteriopheophytin exhibit in organic solvent the absorption maximum in the spectral range of 750−770 nm,[63] one can infer that BChl (and possibly BPheo) molecules of the RC have lost progressively their native protein environment. To follow the kinetics of the loss of native state, we fitted the spectra recorded during incubation in terms of three Gaussian bands centered at ~760 nm (band 1), ~800 nm (band 2), and ~860 nm (band 3) (see Figure 4A) and normalized the area of each Gaussian to their sum. In order to have unitary excursion for the decays of all the sub-bands analyzed, each normalized area has been further normalized to its whole variation during the incubation time. The results indicate that the decrease observed in the spectral contributions of bands 2 and 3 roughly balances the

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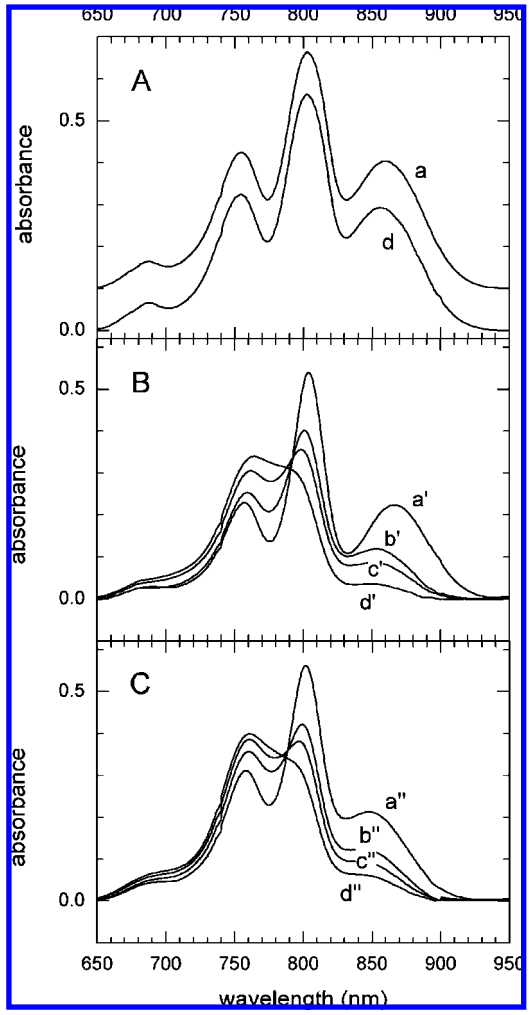

Figure 3. Near-infrared absorbance spectra of RC−trehalose (A) and RC−sucrose (B) matrixes, and of a RC film dehydrated in the absence of sugar (C), following incubation at 37 °C over a period of 6 days. Both the sucrose and the trehalose systems were dried at room temperature to a final content of residual water corresponding to about 5×10^3 H_2O/RC. The RC film was dehydrated in the absence of sugars to NIR undetectable water contents. All samples were then thermostatted under a dry N_2 atmosphere at 37 °C ($t = 0$ h); spectra were recorded at selected times. Panel A: spectra of RC in trehalose, recorded at $t = 0$ h (spectrum a, offset at 0.1 absorbance for visual clarity) and following incubation for 6 days, i.e., at $t = 144$ h (spectrum d). Panel B: spectra a′, b′, c′, and d′ were measured in the sucrose matrix at $t = 0$ h, $t = 20.5$ h, $t = 45.5$ h, and $t = 147$ h, respectively. Panel C: spectra a″, b″, c″, and d″ were measured in the RC film at $t = 0$ h, $t = 21$ h, $t = 49$ h, and $t = 109$ h, respectively. All spectra were corrected for background scattering between 640 and 950 nm.

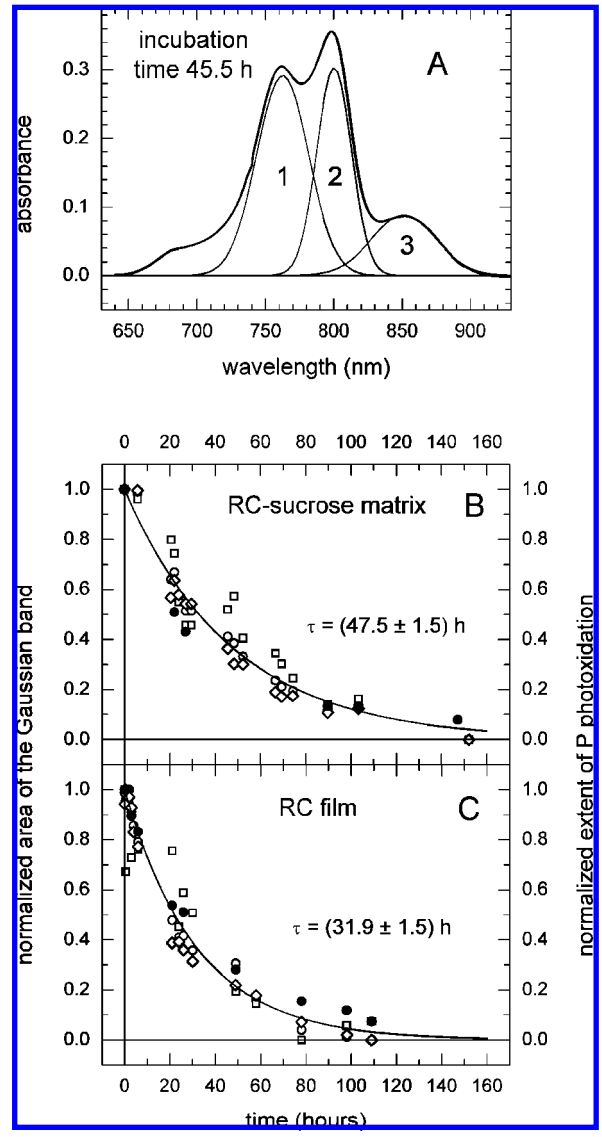

Figure 4. Typical spectrum recorded after long incubation at 37 °C in sucrose matrix (panel A); the sum of four Gaussian bands was fitted to each spectrum; individual Gaussian bands at 750 nm (band 1), 800 nm (band 2), and 850 nm (band 3) are plotted as thin lines; analogous features are exhibited by samples dehydrated in the absence of sugar. Time dependence of the NIR RC absorbance changes observed in sucrose (panel B) and in a film dehydrated in the absence of sugar (panel C), during incubation at 37 °C. The area of each sub-band was first normalized to the sum of the areas of the three sub-bands and, then, in order to have unitary excursion for the decays of all the sub-bands, it was further normalized to its overall variation; such normalized areas are plotted as a function of the incubation time. For band 1, whose area increases with time, the complement to one has been plotted (open circles). Open squares and diamonds correspond to band 2 and band 3, respectively. The extent of P photo-oxidized by a single laser pulse, normalized to the one at $t = 0$, is also shown (closed circles). A single exponential decay fitted all the data reported (continuous curves in panels A and B).

increase in those of band 1, giving rise to comparable kinetics (Figure 4B, open symbols). Furthermore, we monitored the RC primary photochemical activity during incubation by measuring the extent of photo-oxidation of the primary electron donor, P, induced by a single laser pulse. As expected, a progressive photochemical inactivation parallels the kinetics of the NIR absorbance changes (Figure 4B, closed symbols). Fitting simultaneously an exponential decay to the time dependence of the normalized areas of bands 1, 2, and 3 and the normalized extent of P photo-oxidation gave a characteristic time, τ, of about 48 h (see the continuous curve in Figure 4B).

To assess the (limited) extent to which sucrose protects the RC complex, we performed the same set of measurements on a RC film extensively dried in the absence of sugar. Figure 3C shows the evolution of the NIR RC spectra recorded in the film dehydrated in the absence of sugar, during incubation at 37 °C. As evident, the denaturation kinetics and the loss of photoxidation appear only slightly faster in the absence of sugar; indeed, a simultaneous fitting gives a characteristic time, τ, close to 32 h (see Figure 4C). In summary, while in trehalose the RC structural and functional integrity are long preserved, in sucrose both the native state and photoactivity are irreversibly lost as if the RC would have been dried in the absence of sugar.

4. Discussion

The whole set of the reported results can be summarized as follows:

(i) In trehalose, upon decreasing the content of residual water below ~0.8 water molecules per sugar, both the average rate constant and the rate distribution width of primary charge recombination undergo a steep increase, reaching very high values at a water/sugar mole ratio of ~0.4, which represents the lowest value attainable. Such behavior is consistent with the formation of a water-mediated HB network, which anchors the protein to its surroundings, and in which water molecules are all four HBs bonded. At variance, in sucrose, both the average rate constant and the rate distribution width exhibit a slight increase when decreasing the water content to ~0.8 water molecules per sugar, but upon further dehydration, they fall down, becoming comparable to those measured in RC samples extensively dehydrated in the absence of sugar. Such sucrose samples can lead to extremely low contents of residual water, undetectable by NIR spectroscopy.[45] These observations make clear that, in the sucrose matrix, the conformational RC dynamics probed by the $P^+Q_A^-$ recombination kinetics are only marginally reduced as compared to those in solution, in spite of the extreme viscosity and hardness of the encompassing matrix.

(ii) While the RC structural integrity is progressively lost in sucrose, following storage at 37° over a period of a few days, it is very long maintained in trehalose under the same storage conditions; furthermore, the kinetics of loss of integrity, in sucrose, are most similar to that observed when drying RC in the absence of sugar.

The above points are fully consistent with the conclusion that, at variance from trehalose, no coupling takes place between the RC complex and the sucrose matrix and suggests protein−matrix "*nanophase separation*" to occur in sucrose, at suitably low water content. In this respect, it has been observed in an NMR study of lysozime mobility in protein−water−sugar amorphous systems that at low water the protein and the sugar "phase separate" in lactose but not in trehalose matrixes.[64]

We believe that the reported data support the notion that the peculiar efficacy of trehalose in restricting protein dynamics at very low water contents is mainly due to its ability to form water-mediated hydrogen bond networks which connect groups at the protein surface with the surrounding environment. At variance, it appears that, in spite of its structural similarity with trehalose, sucrose has a lower propensity to form such water-mediated HB networks.[65,66] This suggestion is in line with the following:

(i) MD simulations of sucrose− and trehalose−water systems, which showed that, due to formation of two intramolecular hydrogen bonds,[55] opposite to only one formed in trehalose,[56] sucrose is, on average, more rigid and has less sites disposable to form hydrogen bonds with its surroundings.

(ii) The observation (see Experimental Section) that the combination band of water is red-shifted by about 15 nm in moderately dehydrated trehalose with respect to sucrose samples of comparable water content. Such red shift, upon extensive dehydration, further increases in trehalose, by 10−15 nm at the largest dehydration attained. At variance, in sucrose, the band does not change position even at dehydration levels not attainable in trehalose. Since a red shift is diagnostic of involvement in hydrogen bonding,[49] these spectral features support the suggestion that residual water is less hydrogen bonded in the sucrose than in the trehalose, and that, in the latter, a large fraction of water molecules is four HBs bonded at the lowest water contents attainable.

Our results suggest that the better bioprotective effect of trehalose with respect to sucrose arises from the formation, in trehalose, under condition of drought, of hydrogen bond networks, which tightly couple the biomolecules to the surrounding hard matrix. This makes the appearance of structural alterations a more rare event in trehalose than in sucrose since it strongly involves alterations of the surrounding hard matrix.[32,38]

The reported results clarify, at the microscopic level, the origin of the trehalose peculiarity and enable one to conclude that while the *anchorage hypothesis* better accounts for the bioprotective effect of trehalose, the *high viscosity hypothesis* accounts for the (limited) bioprotective effect of sucrose. More general, the presently reported results enable one to understand why carbohydrates that have potential to hydrogen bond with the surroundings may exhibit largely different capability in preserving dry biomaterials.

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Supporting Information Available: Additional experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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