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Evidence for direct binding of glycerol to photosystem I

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ABSTRACT

The interaction between glycerol and photosystem I (PSI) was investigated using low temperature single-molecule spectroscopy. PSI complexes were dissolved in three different solutions: in buffer solution, in 66% glycerol/buffer solution, and in 66% glycerol/buffer solution that was afterwards diluted by buffer; the final glycerol concentration was <1‰. Mean fluorescence spectra and inter-complex heterogeneity of PSI complexes in 66% glycerol/buffer solution and in the re-diluted solution show high similarity, but differ from complexes in buffer solution indicating that the glycerol concentration is not the determining factor modifying the spectral properties. However, the exposure of PSI to a high glycerol concentration during sample preparation affects PSI and the effect is maintained if glycerol is removed from the solution.

1. Introduction

Glycerol is used in large amount for preparation and stabilization of proteins, tissues storage at low temperatures, protein crystallization as well as in cosmetics and pharmaceuticals [1,2]. It is not yet fully understood how glycerol – a polyol belonging to the class of non-ionic co-solvents – and proteins interact. There are a number of working hypotheses concerning all polyols not only glycerol. These hypotheses can be summarized into two classes: one class favors the direct interaction of the polyol with the protein surface and the other favors an indirect interaction [2–8]. For the direct interaction model a binding of the polyol to proteins via hydrogen bonds is assumed; this interaction stabilizes the native state. The direct interaction yields the creation or deletion of glycerol binding sites at the protein surface by specific reorientation of the proteins' N and O atoms favoring the formation of multiple H-bonds with glycerol. These reorientations induce specific changes in the structure of proteins [2]. The indirect interaction model, in contrast assumes an indirect action of the polyols by changing the structure and the dynamics of water molecules located close to the surface of proteins (hydration layer) [2–5,7,8]. In a recent study, the preferential hydration of the surface of proteins by a hydration layer with a thickness of ≈2 Å was shown. The polyols were depleted in this region, but at a distance of ≈4 Å a layer with an increased density of polyols was found that show the tendency to form clusters [7]. Based on these experiments the protein together with its hydration layer seems to be encapsulated by a shell of co-solvent molecules. The encapsulation yields a more ordered structure of the water molecules within the hydration layer and accordingly proteins are more shielded from influences that tend to destabilize them. Although the depletion of co-solvent molecules from the hydration layer seems to be generally accepted, many questions concerning the effects of polyols on proteins remain.

The experiments were carried out on photosystem I (PSI) – a key member of the photosynthetic apparatus. The main function of PSI is to capture and convert sunlight into electrical energy. In one PSI monomer from the cyanobacterium *Thermosynechococcus elongatus*, ~100 chlorophyll molecules are involved in efficient light-harvesting and excitation energy transfer leading to light-induced charge separation in the reaction center [9,10]. At low temperatures, specific chlorophyll molecules act as traps for the excitation energy, whereof a portion of this energy is released by fluorescence emission [11]. The fluorescence emission of these traps can be observed using low temperature single-molecule spectroscopy [12]. The combination of an antenna system with a highly efficient energy transfer chain terminated with fluorescent traps makes PSI a perfect system for studies with high spectral resolution at the single-molecule level. The high photo-stability of chromophores at low temperatures allows for the possibility of acquiring spectroscopic data for a large number of individual complexes. Specific properties e.g. sample heterogeneity, can be easily

Abbreviations: PSI, photosystem I

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accessed by reducing the amount of spectroscopic data to a low number of parameters most relevant for the actual problem. Hereby it becomes possible to compare the outcome of different sample preparations as recently shown by our group [13]. Using this approach it was possible to evaluate the heterogeneity induced by sample treatment on PSI, e.g. it turned out that PSI complexes embedded in a polyvinyl alcohol (PVA) matrix show a much larger heterogeneity than PSI complexes dissolved in glycerol/buffer mixtures [13]. Unfortunately, in our prior experiments it was not possible to judge whether or not glycerol interacts directly with PSI or not. Here we report the fluorescence emission of single PSI complexes that were exposed to a high glycerol concentration before they were highly diluted in buffer solution. Based on these results it is now possible to suggest a direct binding of glycerol to PSI.

2. Materials and methods

PSI trimers from *T. elongatus* have been isolated as described in Ref. [14]. The samples were prepared starting from a solution of re-solubilized single crystals of PSI with a chlorophyll concentration of 3 mM. The samples were diluted in buffer (containing 20 mM Tricine, 25 mM $MgCl_2$ 4 mM β-DM, and 5 mM sodium ascorbate) or 66% glycerol/buffer mixture (w/w) in three steps to a final PSI trimer concentration of approximately 3 pM PSI. The β-DM concentration was kept constant (4 mM) during all dilution steps to avoid aggregation of PSI. The details of the sample preparation are shown in Fig. 1. In the first step 1 μl of the protein solution was dissolved in 250 μl of buffer (c) or 66% glycerol/buffer mixture (a, b). In the next two steps 4 μl were diluted in 250 μl buffer (b, c) or 66% glycerol/buffer mixture (a). Accordingly, the glycerol concentration in the three different samples is: 66% (a – glycerol/buffer mixture), ~0.2‰ (b – re-diluted buffer) and 0% (c – buffer). Finally, 1 μl of the highly dissolved protein solution was placed between two cover slips and transferred directly into the cryostat filled with liquid helium. Sample preparation and mounting were carried out under indirect daylight. The fluorescence measurements of individual PSI complexes were carried out using a home-built confocal microscope operating at 1.3 to 1.4 K as described in Ref. [13].

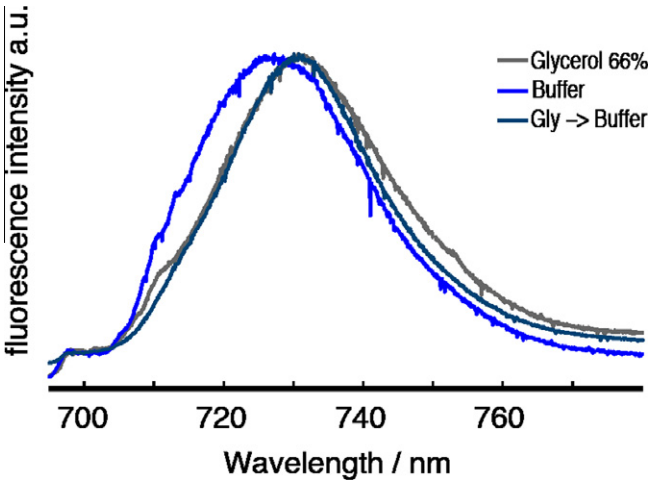

Fig. 2. Mean fluorescence emission spectra obtained by summation of all spectra collected from single PSI complexes for three different sample preparations. The spectra were scaled to similar magnitude.

3. Results

Spectral information for 137 single PSI complexes in buffer, 108 in 66% glycerol/buffer mixtures and 133 complexes in re-diluted buffer were recorded. In Fig. 2 the mean fluorescence spectra for the three samples are given. They show a single broadened band. Their maxima and respective widths (full width at half maximum – fwhm) were found at 726.5 ± 0.5 nm/31.8 ± 0.5 nm (buffer), 731.0 ± 0.5 nm/31.5 ± 0.5 nm (66% glycerol/buffer mixture), and 731.0 ± 0.5 nm/27.0 ± 0.5 nm (re-diluted buffer). The emission maxima reported for low temperature measurements at the ensemble level (with ⩾50% glycerol concentration) vary between 730 and 732 nm [15–17]. The ensemble values agree well with the fluorescence maximum determined for the average spectra in 66% glycerol/buffer mixture and in re-diluted buffer, whereas for buffer solution a blue-shift of several nanometers is observed, dis-

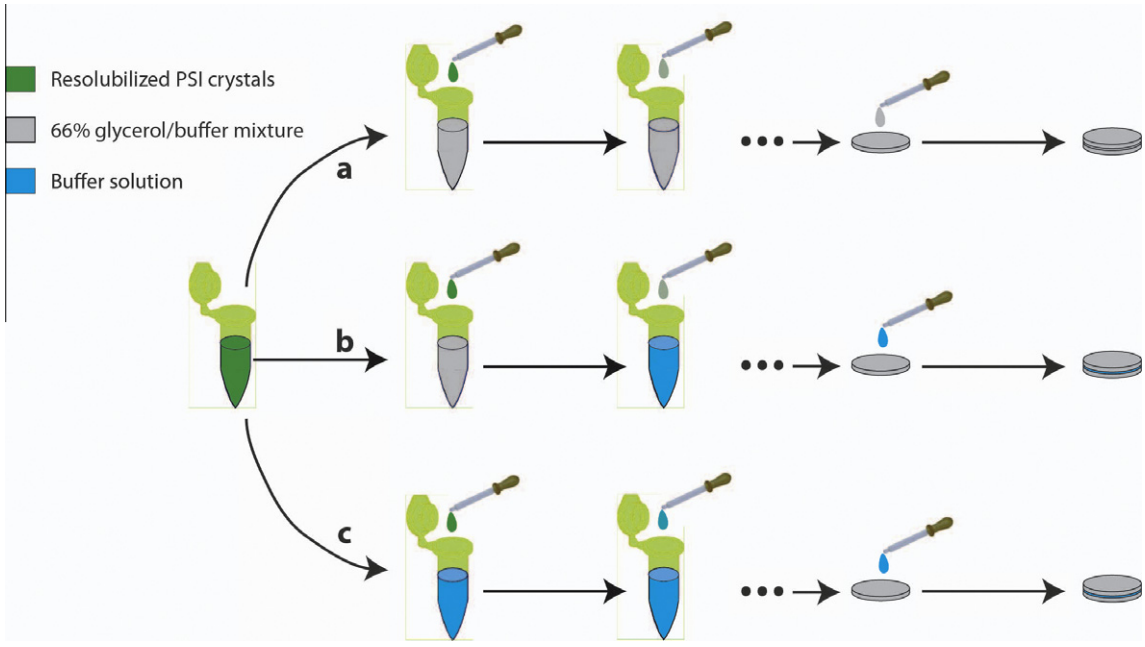

Fig. 1. Illustration of sample preparation: Three samples (a–c) were prepared starting from a solution of re-solubilized single crystals of PSI. The initial PSI solution was then diluted in buffer or 66% glycerol/buffer mixture in three steps as indicated. For details of the sample preparation see Section 2.

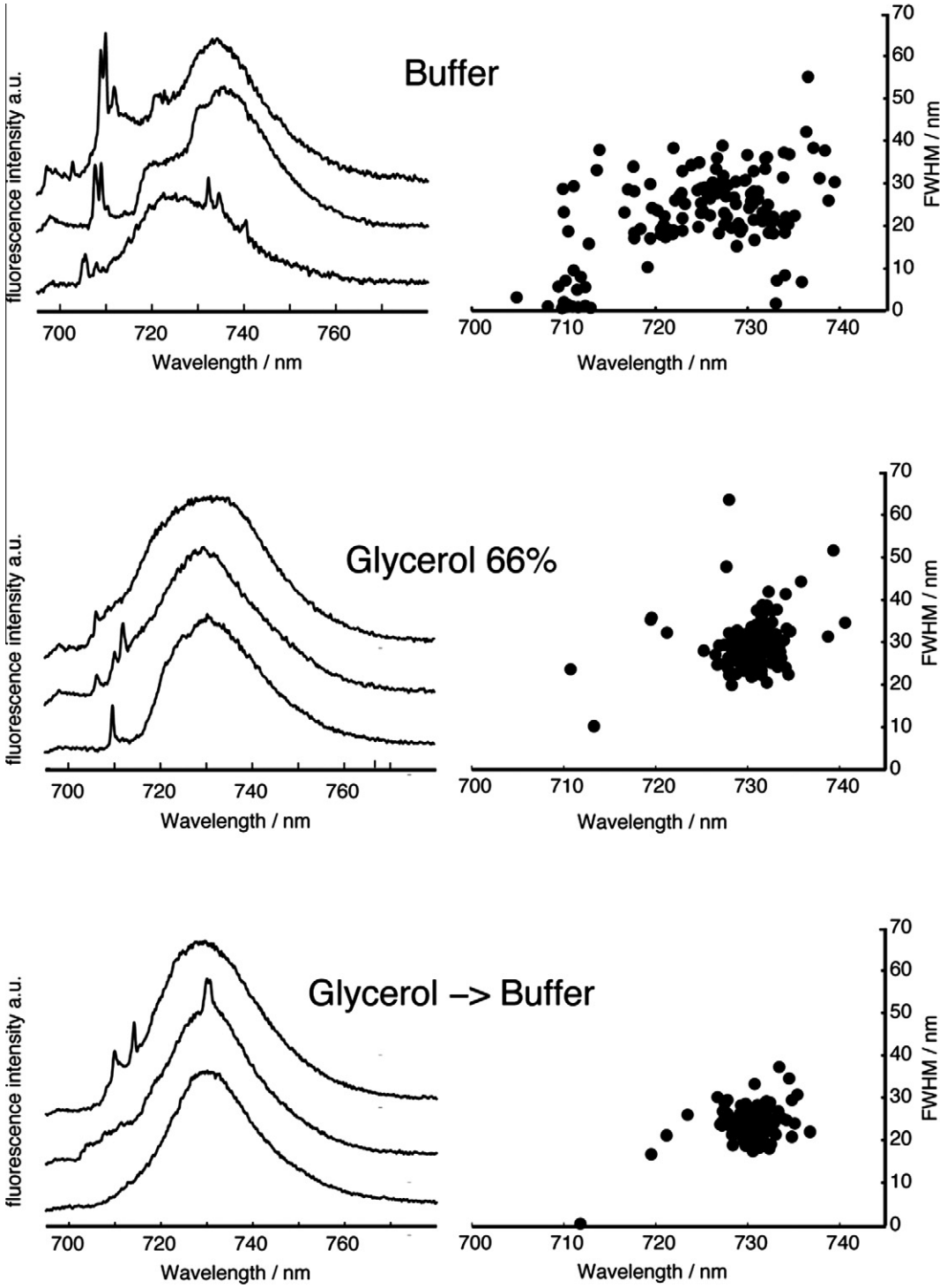

Fig. 3. Left side: Fluorescence emission spectra from three different individual complexes taken in the solutions as indicated; λ_{exc} = 680 nm, T = 1.4 K, $t_{acquisition}$ = 120 s, and I_{exc} = 100 μW. Right side: 2D-scatter plots obtained from the evaluation of the emission spectra from single PSI complexes for the different datasets. Evaluation of the wavelength position and fwhm was done with an algorithm (see text for details).

cussed in detail in Ref. [13]. In Fig. 3 on the left side, three representative spectra of single PSI complexes recorded in the different solutions are shown. The spectra are composed of two different contributions: zero-phonon lines and broad intensity distributions. In the spectra of single PSI complexes from *T. elongatus*, the zero-phonon lines are found predominately in the wavelength range <720 nm. The maxima of the broad intensity distributions are located in the wavelength range >720 nm [18]. The variation of line width of zero-phonon lines in the spectra is due to spectral diffusion [19]. The spectra recorded in buffer solution show a pronounced variation of the shape and the wavelength position of the broad intensity distributions, whereas the spectra taken in 66% glycerol/buffer solution and in re-diluted buffer show smaller variations. Aside from this, it is striking that the intensity and the number of the zero-phonon lines in 66% glycerol/buffer solution and in re-diluted buffer is lower than those seen in buffer solution.

To gain better insight into the variation of shape and intensity, we used an algorithm to determine wavelength position and full-width at half maximum of the contribution with the highest peak intensity in the spectra regardless of whether it is a zero-phonon line or a broad distribution. To accomplish this, the first 120 s of a series of fluorescence emission spectra were selected and averaged resulting in a S/N >60, then a constant offset due to the CCD camera is subtracted. Starting from the maximum position within the spectra the fwhm is determined. The maximum positions and the fwhm were collected for all spectra and presented in 2D-scatter plots shown in Fig. 3 on the right side [13]. The 2D-scatter plots give insight into the intercomplex heterogeneity present between the different PSI complexes in a sample. The overall appearance of these three plots indicates similarities between the heterogeneity of PSI complexes in 66% glycerol/buffer mixture and re-diluted buffer, but not with the PSI complexes in buffer solution. In buffer solution the most intense contributions found in the spectra of single PSI complexes are predominantly within λ_{max} = 716–734 nm and fwhm = 11–31 nm (the appropriate standard deviations are given). In addition, a number of narrow contributions (fwhm $\leqslant$ 10 nm) are present at around 710 nm and 733 nm. In 66% glycerol/buffer mixture and re-diluted buffer the contributions are more clustered. For 66% glycerol/buffer mixture they are found between λ_{max} = 726–734 nm and fwhm = 22–34 nm and λ_{max} = 728–733 nm and fwhm = 20–28 nm for re-diluted buffer, respectively. The clustering of the contributions visualizes a decrease of the intercomplex heterogeneity. This decrease is due to a more compact and homogeneous protein conformation with less structural variability induced by glycerol [1–3,13].

4. Discussion

The spectral properties and the heterogeneity of PSI in the different samples are shown by mean fluorescence spectra (Fig. 2), fluorescence spectra of single complexes, and 2D-scatter plots (Fig. 3). Comparing these properties shows pronounced similarities between PSI in 66% glycerol/buffer mixture and in re-diluted buffer. Those similarities are not observed with the data of PSI complexes in buffer solution that serve here as control experiment where no glycerol was ever added. Although this control experiment and the re-diluted solution contain almost no glycerol – the solutions are almost chemically identical – the spectral properties of the dissolved PSI complexes deviate remarkably. This observation stands in contrast to an assumption that a certain glycerol concentration in the solution is the determining factor for the homogenization of proteins. However, the similarities observed between the PSI complexes in 66% glycerol/buffer mixture and re-diluted buffer show that glycerol must be – somehow – involved in the homogenization process. In our experiments, the only reasonable explanation for this is that the exposure of PSI to glycerol for a certain amount of time (during the sample preparation) induces the homogenization of PSI. The way in which this short-time exposure affects PSI is comparable to the effects induced by a constant, high glycerol concentration (see Fig. 3). Therefore, the reason for the observed similarities and/or the discrepancies in the different sample types seems to depend on an interaction between glycerol and PSI that is established during the exposure of PSI to a high glycerol concentration. The effect of glycerol on the protein conformation is even maintained if the initial solution containing a high glycerol concentration is diluted two times by buffer (see Fig. 1b); hence the interaction between glycerol and PSI is lasting and for at least several minutes independent from the concentration in the solution.

The collection of the presented data enables us now to discern between a direct and an in-direct interaction model of polyols with proteins [2–5,7,8]. As mentioned before, experiments on PSI in 66% glycerol/buffer mixture show a remarkable homogenization of PSI induced by glycerol, but the high glycerol concentration can account for both models [13]. In re-diluted buffer this situation is changed. The residual amount of glycerol present in the final solution (0.2‰) is too small to form stable clusters of glycerol molecules [20] that can encapsulate PSI with its hydration layer completely, which is a prerequisite for the indirect interaction model. Therefore this interaction model cannot explain the observed similarities between PSI complexes in 66% glycerol/buffer mixture and re-diluted buffer [7]. The influence of glycerol on PSI seems to rely on an interaction that is established at a high glycerol concentration between glycerol and PSI. If glycerol is removed from the solution (during the dilution steps), the interaction is maintained for at least several minutes. Consequently, a direct binding of glycerol to PSI is most probable. These observations are in agreement with the direct interaction model of polyols to the surface of proteins. Based on the model, the reduction of heterogeneity in the presented data (Fig. 3), is due to a more compact protein conformation induced by reorientations of the protein after binding of glycerol [1,2]. In addition, the presented approach opens the possibility to quantify the threshold value, at which an interaction between the proteins and glycerol is established and the appropriate time-constants.

In conclusion, single-molecule spectroscopy performed solely on PSI complexes dissolved in solutions with high glycerol concentrations show a homogenization of PSI, but proved insufficient to determine whether the direct or indirect interaction model between glycerol and the surface of proteins is valid. In combination with the spectroscopic results on re-diluted PSI complexes presented here this drawback can be overcome and a direct binding of glycerol to the surface of PSI can be visualized by the remaining homogenization of the complexes induced by glycerol.

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