Solubility diagram of the *Rhodobacter sphaeroides* reaction center as a function of PEG concentration

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Abstract In order to quantify the effect of polyethylene glycol 4000 (PEG) on the solubility of an integral membrane protein, we have crystallized the photochemical reaction center from *Rhodobacter sphaeroides* Y by batch method on a large range of PEG. The measurement of the solubility diagram display a semi-logarithmic dependence of solubility versus PEG concentration. Comparison of our results with previously published ones [Odahara, T., Ataka, M. and Katsura, M. (1994) Acta Cryst. D50, 639–642] suggests a notable effect of additional 1,2,3-heptane-triol and/or temperature on photochemical reaction center solubility.

Key words: β-Octylglucoside; Crystallization; Solubility; Membrane protein; Photochemical reaction center; Polyethylene glycol

1. Introduction

Solubility of a protein is an important thermodynamic parameter which reflects intermolecular interactions occurring between the macromolecules and the solvent constituents [1]. Solubility (S) refers to the concentration of soluble protein in equilibrium with crystallized protein at given values of pH, temperature and solvent components. Over the last decade, about 30 measurements for soluble proteins allowed to better appreciate effects of crystallization parameters.

Until 1980, it was considered as unlikely that membrane protein could crystallized; however, successful crystallizations opened the way [2,3]. Nevertheless progress in crystallization of membrane proteins was slow and the 3D structure determinations of membrane proteins are still rare [4–13]. The numerous difficulties in crystallization of membrane protein have encouraged crystallogenesis studies on this topic, especially on detergents effects. However solubility data for membrane proteins were lacking until recently [14]; furthermore the amphiphilic nature of these proteins, the necessity of detergent for their solubilization and for the crystallization might complicate the analysis of their solubility diagrams.

Most integral membrane proteins isolated in detergent solution can be roughly described as composed of a hydrophobic core surrounded by a detergent ring, flanked by two hydrophilic regions devoid of detergent. Both the solubility of the protein and the micellar properties of the detergent are affected by the crystallization parameters. Above the solubility curve, aggregation of the protein is energetically favored. In the same way, aggregation of detergent micelles occurs above the consolution boundary, leading to phase separation and partition of the membrane protein in the detergent-rich phase [15–17]. Nevertheless, crystallization may occur as well after phase separation, as in its absence. The knowledge of solubility diagrams of membrane proteins is a prerequisite to quantify how various parameters influence their crystallization.

In this study the solubility was measured for *Rb. sphaeroides* Y RC. This pigment–protein complex performs the initial step of energy conversion, a light-induced transmembrane charge separation. It consists of three polypeptides L (281 amino acids), M (307 amino acids) and H (260 amino acids) and several pigments and cofactors associated with L and M (total molecular mass 96 300 Da). It was crystallized in an orthorhombic form using β-OG as detergent, PEG 4000 and NaCl as crystallizing agents [18]. The X-ray structure has been determined at 3 Å resolution [11]. Studies by neutron crystallography [19] showed that in these crystals the order of magnitude of the volumic fraction of β-OG is 0.4.

Here we report the solubility for *Rb. sphaeroides* Y RC, solubilized with β-OG, as a function of PEG (the crystallizing agent) whereas all other parameters (temperature, detergent in solution and salt concentrations) are kept constant. The results are compared with the solubility diagram [14] obtained for RC also crystallized in the orthorhombic form with PEG, but at a different temperature and in the presence of 1,2,3-heptane-triol, an additive commonly used for crystallizing membrane proteins [20].

2. Materials and methods

2.1. Chemicals and materials

The chemicals were purchased from indicated source: EDTA (Sigma), LDAO (Fluka), β-OG (Bachem), PEG (Merck, purified according to [21]), NaN_3, NaCl and Tris (Merck).

The purity and concentration of RC solution were checked by measuring the absorbance at 280 nm and 802 nm with a Cary 2300 spectrophotometer in 100–1000 μl quartz cells (Hellma). The absorption coefficient of native RC at 802 nm is 288 ± 14 mM^{-1} cm^{-1} [22] and the ratio A^{280nm}/A^{802nm} is expected to be equal to 1.2. The conductivity and the refractive index were measured at 18°C with a radiometer conductivimeter and a refractometer, respectively.

2.2. RC preparation

The RC sample ($\approx$50 mg protein) was isolated and purified from *Rb. sphaeroides* membranes in presence of LDAO according to a procedure previously described [23,24]. This detergent was then exchanged for β-OG: the protein sample was adsorbed on a 3 ml DEAE-Sepharose (Pharmacia) mini-column equilibrated with TEA/β-OG buffer solution (15 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1% (w/v) NaN_3, 8 mg/ml β-OG), washed with 30 ml of the same solution and the RC was eluted by 1 M NaCl TEA/β-OG buffer. A Sephadex G25-column (PD10 Pharmacia), previously equilibrated with TEA/β-OG buffer, was then used to remove NaCl. The protein

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Abbreviations: RC, photochemical reaction center; β-OG, β-D-octylglucoside; LDAO, lauryldimethylamine N-oxide; *Rb., Rhodobacter*; PEG, polyethylene glycol 4000; C, conductivity; $n_D^{18°C}$, refractive index at 18°C

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was finally concentrated by ultrafiltration (Amicon membranes) to about 30 mg/ml and stored at 5°C in TEA/β-OG.

2.3. Crystallization methods and solubility measurements

Preliminary experiments set to screen RC crystallization when varying either PEG or NaCl concentration were done by microdialysis as already described [25].

Determination of solubility was done by the batch method [26]. A protein solution and a PEG solution were mixed at concentrations for which crystallization was expected to occur. For these experiments, three stock solutions were prepared in volumetric flasks, and characterized by their conductivity C and refractive index n_D at 18°C: (1) TEA/β-OG buffer, pH = 7.9 (see above): $n_D^{18°C} = 1.3360$, C = 1.6 $mOhm^{-1}$ cm^{-1}; (2) 30% (w/v) PEG in TEA/β-OG buffer, pH = 7.9: $n_D^{18°C} = 1.3760$, C = 0.63 $mOhm^{-1}$ cm^{-1}; and (3) 4.4 M NaCl in TEA/β-OG buffer, pH = 8.0: $n_D^{18°C} = 1.3750$, C = 120 $mOhm^{-1}$ cm^{-1}. Aliquots of these solutions were mixed in transparent plastic vials (Alltech) closed by water-tight silicon caps. Then the protein solution was added to this crystallizing solution (final volume of 30 μl or 50 μl) and mixed by Vortex. The vials were stored in a thermo-regulated chamber at 18°C and in the dark. Considering the volume of aliquots (≈ 10 μl) pipetted off the crystallization solution, and the reliability of the measurement (Hamilton syringes), the relative precision of initial concentrations was estimated as ±2% for PEG and NaCl, and as ±4% for RC.

Because of the large number of experiments and the choice of using RC purified from a single batch, the crystallization experiments had to be prepared with different volumes: for experiments at 6.5–7.5% (w/v) PEG, high initial protein concentrations were required and 30 μl aliquots were sufficient to measure the final protein concentration; for experiments at 8.1% (w/v) PEG and above, the initial protein concentrations were lower and 50 μl aliquots were necessary.

The solubility measurements were performed about 9 weeks after crystallization had started. Samples which had not crystallized or which presented either phase separation or protein precipitate were not taken into account. In the other ones, after centrifugation of the vials (1200×*g*, 5 min) the supernatant was filtered (Millipore Millex GV4 filters) to remove possible microcrystals, and its absorbance was measured at 802 nm for measuring the residual soluble protein concentration.

3. Results

3.1. Preliminary determination of the crystallization conditions

At the beginning of the study, conditions for obtaining X-ray quality crystals of the RC were already known. Starting from these conditions of crystallization (micro-dialysis of a 2 mg/ml protein solution against 10–14% (w/v) PEG, 220 mM NaCl and 8 mg/ml β-OG at 18°C [25]), a first series of micro-dialysis experiments were set to screen RC crystallization when varying either NaCl or PEG concentration. Although salts greatly change the physico-chemical properties of detergent and may act on membrane protein crystallization [27], crystallization was rather independent on NaCl concentration in the range 0.1–0.3 M NaCl; however, it did not occur for higher values of NaCl concentration (when PEG was kept at 10%, w/v). On the other hand, RC concentration for which crystallization occurred was strongly dependent on PEG final concentration (when 0.22 M NaCl was present). Consequently we chose to study RC solubility versus PEG concentration, keeping constant the other parameters: temperature (18°C), NaCl and β-OG concentrations in the buffer (0.22 M and 8 mg/ml, respectively).

For solubility determination, crystallization was carried out using the batch technique (see Section 2). Contrary to vapor diffusion or dialysis which involve an equilibration between the protein solution and a reservoir, the batch technique presents the advantage of an accurate knowledge of the composition of the solution. Prior to the solubility diagram determination, crystallization conditions were established in a large range of PEG (4 to 14% (w/v)) and of protein (1–30 mg/ml) concentrations as shown in Table 1. Crystals were monocrystalline prisms at high PEG concentrations or bush-like at lower PEG concentration. Their morphology was similar to the one observed by micro-dialysis method.

At 6.5 and 7.0% (w/v) PEG, crystallization was observed for the highest RC concentrations; it was less reproducible than for higher PEG concentrations, for which RC crystallization occurred in most trials. At 12 and 14% (w/v) PEG and for the highest RC concentrations, a phase separation appeared as a coexistence of brown droplets within a colorless solution. This phenomenon of phase separation has been previously described for RC [25] and also for other membrane proteins [28]. It was avoided in later experiments by lowering RC concentration.

3.2. Equilibration kinetics

Crystals generally appear faster using the batch method than the dialysis one because supersaturation is reached immediately and not after equilibration of the solutions through the membrane. Indeed, crystallization occurred within 8 days

Table 1
Crystallization conditions used to determine the solubility of RC (in the presence of 220 mM NaCl), final concentrations of soluble protein and estimated supersaturations

PEG concentration (%w/v)	Sample volume (μl)	RC concentration (mg/ml)		Solubility (number of tests used for solubility determination)	Estimation of initial supersaturation
		Initial	Final		
6.5 ±0.1	30	42.3/35.9/31.2[a]	12.8 ±0.4	12.8 ±0.4 (5)	3.3/ 2.8/ 2.4[a]
6.75±0.1	30	35.6/31.2/24.5[a]	≥14.5	(0)	3.3[c]/ 2.9[c]/ 2.3[c]
7.0 ±0.1	30	26.7/22.4/18.4[a]	9.4 ±0.2	9.4 ±0.2 (3)	2.8[c]/ 2.4[c]/ 2.0[ac]
7.5 ±0.1	30	24.5/18.4/14.3[a]	≥10.1	(0)	3.8[c]/ 2.8[c]/ 2.2[ac]
8.1 ±0.1	50	13.5/11.0/ 9.2[a]	≥ 6.5	(0)	3.0[c]/ 2.4[c]/ 2.0[ac]
9.0 ±0.1	50	8.6/ 7.4/ 6.0	2.6 ±0.2	2.6 ±0.2 (2)	3.3/ 2.8/ 2.3
9.9 ±0.1	50	7.4/ 6.1/ 5.4	1.28±0.14	1.28±0.14 (4)	5.8/ 4.8/ 4.2
12.0 ±0.2	50	4.4/ 3.3/ 2.1	0.25±0.04	0.25±0.04 (3)	17.6/13.2/ 8.4
14.1 ±0.2	50	3.7[b]/ 2.7/ 1.5	0.09±0.01	0.09±0.01 (5)	38.5[b]/28.1/17.6

Underlined values indicate conditions for which precipitations, often in the presence of crystals, were observed after 9 weeks and which were not taken into account for the solubility calculation because the final RC concentration was then an overestimation of solubility.
[a]Each trial having not crystallized.
[b]Phase separation.
[c]Supersaturation calculated with interpolated values of solubility.

in all trials, and often within 1 day, whereas crystals were obtained by the dialysis method after a few weeks [25]. Fig. 1 shows that, after nucleation, soluble protein concentrations decreased quickly. After 3 weeks, protein concentrations remained already constant indicating that the equilibrium was reached. This is noticeably faster than for soluble proteins [29]. For each PEG concentration, it was checked that solutions of different initial RC concentrations had evolved to the same final concentration of soluble protein. Measurement of the solubility was thus performed about 9 weeks after the beginning of crystallization.

3.3. Phase diagram and initial supersaturation

A set of 49 experiments, differing by PEG and/or protein concentrations, was used to establish the phase diagram (Table 1). Among them, 22 fulfilled the required conditions and were retained to determine the solubility values. Several samples at 6.75, 7.5 and 8.1% (w/v) PEG were discarded because they had not reached equilibrium. This seemed to be due to crystals coexisting with some precipitated protein.

As shown in Fig. 2, the solubility fits with a logarithmic variation as a function of PEG concentration, within the range of tested conditions:

$$\log S = 3.0 - 0.29\ [\mathrm{PEG}]$$

where S is the solubility of the RC (mg/ml) and [PEG] the PEG concentration (% w/v). The correlation coefficient of this empirical function is 0.998.

Once this solubility curve was known, it was possible to calculate the initial supersaturation a posteriori in the various samples, by dividing their initial RC concentration by the solubility value measured at their PEG concentration (Table 1). The solubility curve was necessary in particular to calculate the supersaturation of the samples for which precipitation were observed (e.g. 6.75, 7.5 and 8.1% PEG). We observed that the minimal supersaturation for which crystals grew was in the range 2.3–2.9. On the other hand, for supersaturation higher than 30, phase separation was often observed.

4. Discussion

We succeeded in crystallizing *Rb. sphaeroides* RC in a range of PEG for which the solubility of the RC varied by two orders of magnitude, and on a range of supersaturation varying from 2 to 40. Exploring such a wide range of supersaturation and precipitant concentrations might be useful when optimizing crystallization conditions.

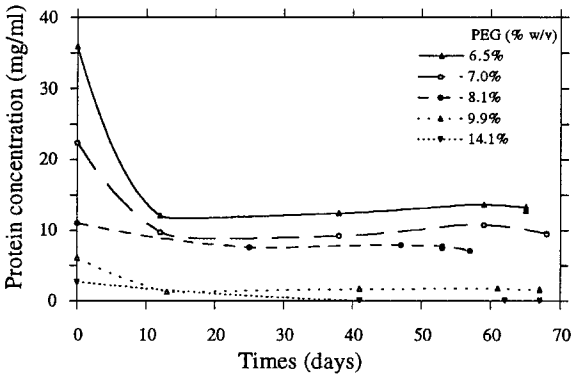

Fig. 1. Kinetics of equilibration.

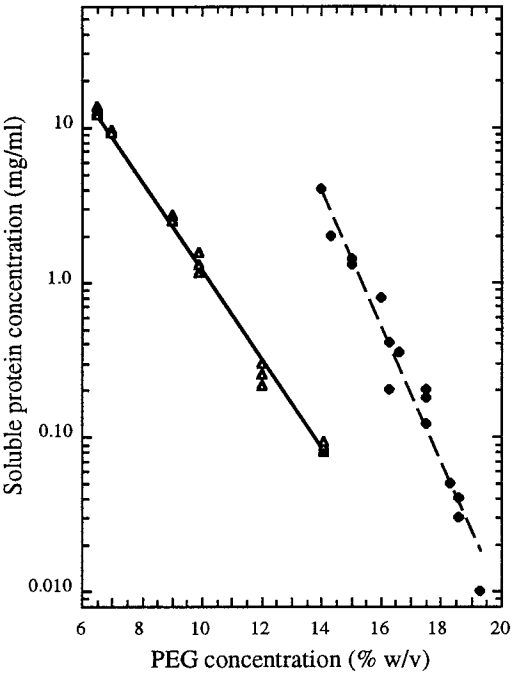

Fig. 2. Phase diagrams of RC in presence of 8 mg/ml β-OG and 0.22 M NaCl in TEA buffer (plain line, this work), and in identical conditions except for the presence of 1% (w/v) 1,2,3 heptane-triol (dotted line, data replotted from [14]).

A logarithmic dependence of the solubility of the protein/detergent complex versus PEG concentration was observed. This variation bears a formal resemblance with that observed for a number of soluble proteins in presence of salts (in the so-called 'salting out' region) [30]. However, the underlying mechanisms are probably different because of the absence of electrostatic effects with PEG; they are also less documented for PEG than for salts [1]. A similar dependence of protein solubility versus PEG has been reported for some soluble proteins, although in that case protein concentration of supernatant was measured after precipitation (and not crystallization) [31]. For a membrane protein which is partly shielded from the aqueous solvent by its bound detergent, crystallization is governed by the interactions of both protein and detergent interfaces with solvent. Interactions at the hydrophilic surfaces of the membrane protein might be modified by PEG in the same way as for soluble proteins. Interactions at the interfaces between protein-bound detergent and solvent and their modifications by PEG are still not clear. It is known from the phase diagram of β-octylglucoside in pure water or in presence of NaCl [32,33] that PEG lowers the consolution boundary between the micellar solution and the two-phase domain; a similar event can be expected in more complex micellar solutions containing membrane proteins. Such an effect of PEG might play a role in promoting nucleation; indeed approaching the consolution boundary has been shown to promote crystallization of several membrane proteins [33].

The RC solubility determined in this work is significantly different from results previously published by Odahara et al. [14] for the same protein. These authors used a batch method under conditions similar to our experiments, except for the

presence of a small amphiphile (1% (w/v) 1,2,3-heptane-triol) and for the temperature (25°C). Some of the protein precipitated right after mixing the batch components and was eliminated before crystallization could proceed. In our case, such a precipitation was not observed in most samples and when it occurred, these samples were not used. However, both solubility curves follow a logarithmic variation versus PEG concentration but do not have the same slope and differ in the PEG and protein ranges (see Fig. 2). In particular no crystallization was observed by Odahara et al. at PEG concentrations lower than 14% (w/v). Such a shift of the solubility curves can be related to the difference of temperature and/or to the presence of 1,2,3-heptane-triol. Both parameters may affect the solubility of RC as well as the consolution boundary of the detergent. The small amphiphile has been introduced as a crystallization 'additive' (usually present at a quite high concentration) for a number of membrane protein crystallization [15]. It was shown to modify size and shape of the micelles of another non-ionic detergent, LDAO, in aqueous solution [34] and to shift the consolution boundary of this detergent in presence of PEG [35]. Similarly the phase diagram of β-octylglucoside presents a temperature dependant consolution boundary when PEG is added [36]. Unfortunately no experimental data exist on the phase diagram of the β-octylglucoside/PEG/heptane-triol/temperature system to assess the contribution of the two latter parameters.

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