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Determination of the number of detergent molecules associated with the reaction center protein isolated from the photosynthetic bacterium *Rhodopseudomonas viridis*

Effects of the amphiphilic molecule 1,2,3-heptanetriol

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Abstract

Detergent-free reaction center (RC) proteins from the photosynthetic bacterium *Rhodopseudomonas viridis* were obtained using Bio-Beads SM-2. With these RCs, the amount of detergent molecules associated with the protein was measured by determining the detergent concentration at which re-solubilization occurred as a function of the RC concentration. For *N,N*-dimethyl dodecylamine-*N*-oxide (LDAO), Triton X-100 and β-octylglucoside 260 ± 30,105 ± 10 and 360 ± 100 detergent molecules were necessary to dissolve the protein, respectively. With this technique we have studied the effect of the amphiphilic molecule 1,2,3-heptanetriol, which is essential in the crystallization process of these RCs. Addition of 5% 1,2,3-heptanetriol reduces the value for LDAO to 120 ± 20 LDAO/RC, supporting the notion that crystallization of the RCs is promoted by increasing the number of protein–protein contacts.

Key words: Photosynthesis; Membrane protein; Detergent; Heptanetriol; Bio-Beads

1. Introduction

In 1982 Michel [1] succeeded in crystallizing the membrane-bound reaction center (RC) protein from the photosynthetic bacterium *Rhodopseudomonas (Rps.) viridis*. Since then, several other RC-proteins have been successfully crystallized [2–7]. X-Ray analysis has revealed the complete polypeptide and chromophore structure of the RC with up to 2.3 Å resolution.The RC crystal structure forms the basis for several new concepts about the mechanism of photosynthetic energy conversion.

The choice of the detergent for solubilizing the protein plays a crucial role in the crystallization process; for *Rps. viridis* *N,N*-dimethyl dodecylamine-*N*-oxide (LDAO) was used, for *Rhodobacter (Rb.) sphaeroides* β-octylglucoside (β-OG). When LDAO was used, it was essential to add the amphiphilic molecule 1,2,3-heptanetriol to the solution for inducing crystallization. Except for a single molecule of LDAO [8], no molecules of LDAO or 1,2,3-heptanetriol were observed in the X-ray diffraction structure. It was suggested that 1,2,3-heptanetriol fine-tuned the micellar layer surrounding the protein, enabling protein–protein and protein–detergent interactions to construct the crystal. Recent measurements on the micellar radius of LDAO in solution have indeed shown a decrease of the radius upon addition of 1,2,3-heptanetriol [9].

Using neutron diffraction and H_2O/D_2O contrast variation, the size of the detergent ring surrounding the protein was determined. It was concluded that the RC was associated with about 110 LDAO molecules (*Rps. viridis*) [10,11] or with 205 ± 35 β-OG molecules (*Rb. sphaeroides*) [12] . These values apply for RCs in a solution suitable for crystallization (for *Rps. viridis*: about 1 M ammonium sulphate, pH 6–6.5 and 3–5% 1,2,3-heptanetriol) and may be different in a standard solution of 10 mM Tris, pH 8, 0.1% LDAO and 1 mM EDTA.

Here we report on a new and simple method to determine the detergent/RC ratio. For LDAO, Triton X-100 and β-octylglucoside we found that 260 ± 30, 105 ± 10 and 360 ± 100 detergent molecules are necessary to dissolve the protein, respectively. These values agree quite well with the values determined for *Rb. sphaeroides* RCs in a Triton X-100 and LDAO solution using ^{14}C labeled detergents, viz. 289 and 103 detergent molecules bound for LDAO and Triton X-100, respectively, in experiments published after completion of this present work

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[13]. We find that the solubilization ratio for LDAO is reduced more than 50% reduction when 5% 1,2,3-heptanetriol is added. These results demonstrate for the first time that the small amphiphilic 1,2,3-heptanetriol promotes crystallization by reducing the number of large detergent molecules bound to the RC protein, thus increasing protein–protein contacts.

2. Materials and methods

RCs from *Rps. viridis* were isolated as in [14] and suspended in 0.1% LDAO, 10 mM Tris and 1 mM EDTA. The detergent was removed by adding about 10 g of Bio-Beads SM-2 (Bio-Rad Laboratories) [15,16] to a 20 ml solution RCs at a concentration of about 30 μM protein, and incubating the solution overnight on a shaker at 4°C. The strongly turbid solution was then allowed to stand for a few minutes in order to let the Bio-Beads precipitate. The supernatant was removed and the Bio-Beads were washed twice with 50 mM Tris, pH 8.0. The collected supernatant was spun for 10 min at about 3000 rpm in a table-top centrifuge. The pellet of detergent-free RCs was washed twice with 50 mM Tris, pH 8.0, resuspended in about 4 ml Tris buffer and kept at 4°C. The efficiency of the detergent removal procedure was checked with CP/MAS NMR spectroscopy. The remaining LDAO was below the CP/MAS NMR detection limit, i.e. more than 95% of the LDAO was removed (Van Liemt et al., to be published). Such virtually complete removal of detergent was only obtained when the RCs were mixed together with the Bio-Beads. When RCs were dialyzed for 1–2 days vs. a solution containing Bio-Beads, the RCs did precipitate but some LDAO (< 20%) remained in the precipitate.

The level of scattering of a RC solution was determined by measuring the absorption at 500 nm in a 1mm cuvette with a Zeiss QII absorption photospectrometer. At this wavelength the absorption spectrum of RCs of *Rps. viridis* has a minimum, and the relative effect of scattering is most easily measured. The apparent absorption at 500 nm will be labeled 'level of scattering' throughout this article. By measuring the absorption at 500 nm as a function of the amount of detergent needed to solubilize different concentrations of RCs, the detergent/RC ratio can be determined.

Prior to each scattering measurement, the cloudy RC solution was shaken once and sonicated in a bath sonicator for 10 s. After each experiment, the true absorption spectrum of the completely resolubilized RCs was obtained with a Shimadzu UV-360 photospectrometer and the RC concentration determined, using $e^{830} = 3 \times 10^5\ M^{-1} \cdot cm^{-1}$ [17]. All measurements were performed at room temperature. LDAO (30% solution) and high and low-melting 1,2,3-heptanetriol isomers were obtained from Fluka; β-OG and 1,2,3-heptanetriol were obtained from Sigma Chemical Co; Triton X-100 was obtained from Merck.

3. Results

Fig. 1 shows the level of scattering measured at 500 nm in a solution of detergent-free RCs from *Rps. viridis* as a function of the amount of LDAO added to the sample. The scattering was measured 20–30 s after adding the detergent. At low concentrations of LDAO, the solution is very turbid and the scattering is high. Often a slight increase in the scattering is observed when the detergent concentration is increased. This is probably due to the dispersion of large RC-clusters into smaller ones. This increase is followed by a sharp decrease of the scattering, which indicates the solubilization of the RC. In this range, the scattering stabilized in about 30 s after detergent addition. The inset of Fig. 1 shows the absorption spectrum of the RC solution before and after sufficient detergent has been added. It shows that after addition of the detergent virtually no scattering is observed, and that no degradation of the protein has occurred.

Fig. 2A shows the detergent concentration at which the scattering suddenly decreases as a function of the concentration of RCs for three different detergents: LDAO, Triton X-100 and β-OG. The data points for each detergent in Fig. 2A were fitted to a straight line. Extrapolating these lines to zero protein concentration gives the CMC value, while the slope of the straight line determines the amount of detergent molecules needed to dissolve the RC. The results are summarized in Table 1. The detergent/RC value for β-OG is less accurate than that of LDAO due to the larger CMC value of β-OG, which makes it less sensitive to the RC concentration.

We have tried to determine the effect of 1,2,3-heptanetriol on the detergent/RC ratio under conditions where crystallization occurs. However, when detergent-free RCs were dissolved in a solution with pH < 7.4, the RCs did not completely resolubilize, not even after increasing the LDAO concentration to 4 mM in a 5 μM protein solution (data not shown). If the RCs were first solubilized with LDAO, however, and then transferred to a buffer solution with pH < 7.4, no precipitation occurred. Thus, it appears that 'naked' RCs are more sensitive to pH than RCs protected by detergent. To avoid the problem of incomplete solubilization in the crystallization solution we have measured the effect of 1,2,3-

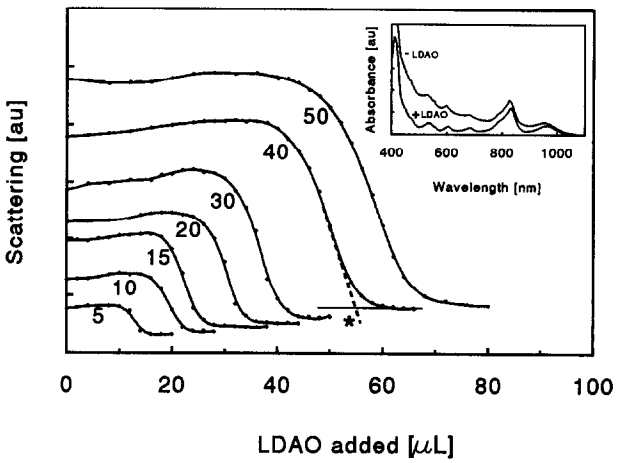

Fig. 1. The change in scattering measured at room temperature at 500 nm of initially detergent-free RCs from *Rps. viridis* as a function of the amount of LDAO (0.5% w/w) added. The curves represent different initial RC concentrations; the numbers give the amount of RCs added in μl of the stock dispersion of precipitated RCs. The sample volume in the 1 mm cuvette at the start of the experiment was 300 μl. Solubilization occurs at the intersection of the tangent of the solubilization curve at its inflection point and the baseline (indicated by an asterisk for the 40 μl curve). The RC concentration at the intersection was calculated from the OD of the completely solubilized sample, taking into account the volume of added detergent solution. Inset: the absorption spectrum of a RC solution before and after re-solubilization.

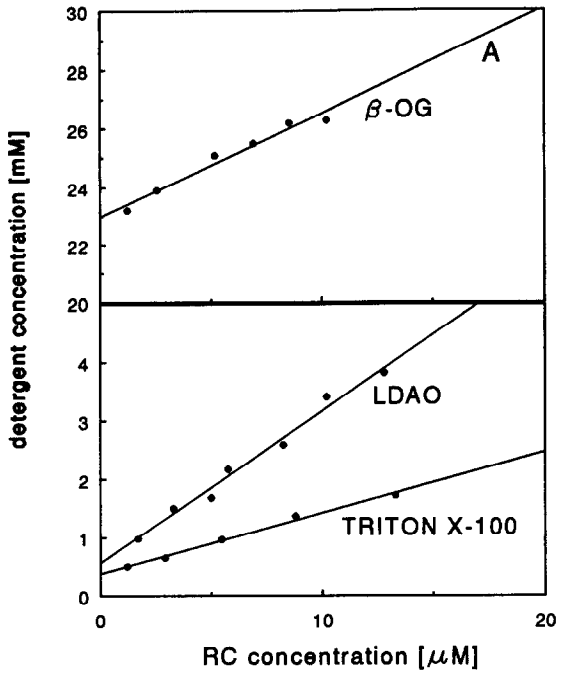

Fig. 2. (A) The amount of detergent needed for total solubilization of the RC as a function of the RC concentration for three different detergents. (B) Same as (A) with different amounts of 1,2,3-heptanetriol added to the sample, prior to the addition of LDAO.

heptanetriol on the LDAO/RC ratio and the CMC in a buffer of 50 mM Tris at pH 8. The results are shown in Figs. 2B and 3. No change in the CMC of LDAO was observed (Fig. 3A), but a striking decrease of the LDAO/RC ratio of more than 50% was found when increasing the 1,2,3-heptanetriol concentration from 0% to 5% (Fig. 3B). We did not find a clearly different behavior for the two isomers of 1,2,3-heptanetriol under our conditions of measurement. They both had similar effects on the LDAO/RC ratio at 5% heptanetriol (data not shown).

4. Discussion

We have demonstrated that the use of Bio-Beads offers a convenient way to measure detergent/RC ratios under various conditions. After the RCs have been precipitated, they can be fully resolubilized by adding detergent, as is shown in Fig. 1B. The solubilization takes place at a detergent concentration that is strongly dependent on the protein concentration. It takes almost 10 times more LDAO to solubilize a 10 μM than a 1 μM protein dispersion. This is due to the large number of detergents needed to solubilize the RC, compared to that at the CMC.

The values found for the number of detergent molecules associated with the RC for Triton X-100 and LDAO (105 and 260, resp.) are in excellent agreement with the recently determined values of 103 and 289 using ^{14}C-labeled detergent [13] and give further support to the validity of our method. Roth et al. [10] first measured the detergent/RC ratio in crystallized RCs of *Rps. viridis* and found about 110 LDAO/RC. This value is more than two times lower than the value of 260 LDAO/RC needed for solubilization (Table 1). This indicates that for crystallization more than half the detergent molecules associated with the RCs must be removed. With the Bio-Bead technique we have for the first time demonstrated that this is due to the effect of 1,2,3-heptanetriol on the LDAO/RC ratio. Adding 1,2,3-heptanetriol reduces the number of detergent molecules associated with the RC by more than 50% at 5% 1,2,3-heptanetriol. Apparently, by adding 1,2,3-heptanetriol part of the bound LDAO molecules surrounding the protein is replaced by the small triol, thus increasing the number of protein–protein interaction sites that are necessary for building the crystal. This triol-effect is similar to the one found in solution [9]. However, no effect on the CMC of LDAO was found when adding 1,2,3-heptanetriol as in [9]. The number found for the detergent/RC ratio at 5% 1,2,3-heptanetriol (120 ± 20) corresponds well with the one found in a crystal.

The detergent/RC ratio found for Triton X-100 (105 ± 10) is 2–3 times smaller than found for LDAO (Table 1). This is probably due to the larger head-group of the Triton molecule [13]. The CMC values found for the three detergent are in reasonably good agreement

Table 1

CMC values and detergent/RC ratios determined for three detergents for RCs from *Rps. viridis*. The bracketed numbers represent reported CMC values.

Detergent	CMC [mM]	# Molecules/RC
LDAO	0.6 ± 0.2 (1.1)	260 ± 30
TRITON X-100	0.4 ± 0.2 (0.24)	105 ± 10
β-OG	23 ± 1 (22–23)	360 ± 100

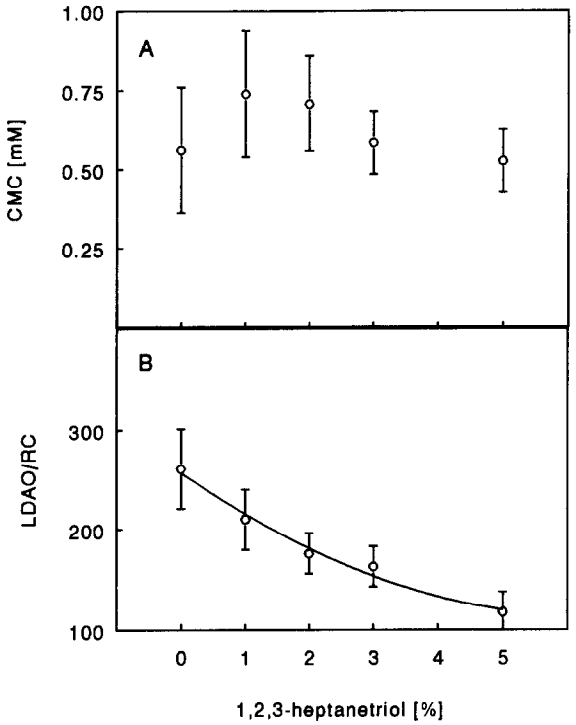

Fig. 3. (A) CMC values, obtained from Fig. 2B by extrapolating the straight line to zero protein concentration, as a function of 1,2,3-heptanetriol concentration. (B) LDAO/RC ratios determined by the slopes in Fig. 2B as a function of 1,2,3-heptanetriol concentration.

with reported values; notably the large difference found for LDAO and β-OG, agrees well with the literature. However, the measured CMC for LDAO (0.6 mM) is significantly lower than the reported value of 1.1 mM. This could indicate that RC solubilization occurs below the CMC of protein-free detergent [18].

We were not able to demonstrate a clear difference in the behavior of the two isomers of 1,2,3-heptanetriol under our conditions of measurement. They both had similar effects on the LDAO/RC ratio at 5% heptanetriol although only the high-melting isomer leads to crystallization. Possibly, under the different condition of crystallization (1 M ammonium sulfate; pH 6–6.5) the two isomers behave differently. We were not able to check this since the RCs could not be completely resolubilized at pH < 7.4.

This method presented here to determine the protein/detergent ratio can in principle be used for any membrane protein, on the condition that the protein does not denature upon precipitation. Note, however, that when using this method careful attention should be given to a few possible drawbacks when comparing to more direct techniques like radioactive labeling of detergents [13].

(i) If the protein binds additional detergent molecules after resolubilization, this will not be measured with this technique.

(ii) Two or more proteins could occupy one detergent micelle at low detergent concentrations; this would result in low detergent/protein ratios.

(iii) Redissolving the protein could, in principle, be slow and the amount of scattering should be measured after the resolubilization process has fully relaxed. However, with the proteins and detergents used in this study resolubilization was quite fast.

We note that the Bio-Beads may be used with advantage in other ways. For example, RCs may be concentrated simply in a low-speed centrifuge. Using a Beckman L5 75B ultracentrifuge we obtained samples of RCs from *Rb. sphaeroides* with an optical density of about 1000 per cm at 800 nm. Buffer and/or detergent can be changed without a DEAE column. Bio-Beads may also be used to separate membrane protein from water-soluble proteins.

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