FORMATION AND PROPERTIES OF BACTERIORHODOPSIN MONOMERS IN THE NON-IONIC DETERGENTS OCTYL-β-D-GLUCOSIDE AND TRITON X-100

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1. Introduction

Since the bacteriorhodopsin molecules of the purple membrane of *Halobacterium halobium* are arranged in a two-dimensional hexagonal lattice of trimers, protein–protein interactions may well affect its structure and function (e.g., the pumping rate). It is therefore of interest to reversibly disaggregate bacteriorhodopsin in order to compare its properties in the aggregated and in the monomeric state.

It has been shown that the exciton coupling effects in the visible circular dichroism (CD) spectra can be used to monitor the state of aggregation of bacteriorhodopsin and to distinguish between monomeric and aggregated bacteriorhodopsin [1–5]. The exciton CD couplet consists of a positive band with maximum at about 535 nm and a negative band with extremum at about 600 nm and occurs only if specific bacteriorhodopsin aggregates are present. In bacteriorhodopsin containing phosphatidylcholine vesicles, reversible aggregation of bacteriorhodopsin can be induced by lowering the temperature [5]. Whereas the hexagonally aggregated state of the protein is characterized by an exciton CD spectrum, the monomeric state is characterized by a positive CD band centered at the absorption maximum [5]. A similar 'monomer' or intrinsic CD band is also observed with monomeric rhodopsin [6].

It is shown on the basis of the disappearance of the exciton CD bands that solubilization of bacteriorhodopsin in Triton X-100 and octyl-β-D-glucoside leads to the formation of protein monomers. These observations are supported by centrifugation experiments and by measurements of the rotational correlation time and of the Stokes radius of the bacteriorhodopsin–detergent micelle. Data are presented on the kinetics and on the pH and ionic strength dependence of the solubilization process.

The following characteristics are desirable for a detergent: high critical micelle concentration, nondenaturing, chemically well-defined and suitable for spectroscopy. In terms of these criteria octylglucoside offers a number of significant advantages over Triton X-100.

The absorption spectrum, secondary structure, light–dark adaptation and photochemical cycle of bacteriorhodopsin differ very little in the solubilized and in the native state, indicating that protein–protein interactions affect these properties only in a minor way. Upon detergent dialysis, protein reassembly occurs and most of the changes induced by solubilization are reversed.

2. Materials and methods

Triton X-100 was purchased from Packard Instrument Co. Inc. Octyl-β-D-glucoside was synthesized according to standard procedures [7,8]. All results described here were obtained with this product. Preliminary measurements showed that solubilization with octylglucoside from Calbiochem is about twice as slow as with the product used here. CD measurements and flash experiments were performed as in [1,9]. Fluorescence measurements were carried out with an RRS 1000 direct recording corrected spectrofluorometer (Schoeffel Instrument Corp.). Gel filtration chromatography was performed with controlled

pore glass CPG-10. Halobacteria were grown and the purple membrane isolated as in [2].

3. Results and discussion

3.1. *Solubilization in Triton X-100*

In fig.1 we present data on the kinetics of solubilization of dark-adapted purple membrane in Triton X-100. The centrifuge data (■) show that at pH 6.88 and at 20°C complete solubilization is reached in about 20 h. Immediately after the addition of Triton X-100, before any significant solubilization occurs, the A_{max} decreases in amplitude (fig.1, ▲) and shifts towards the blue. The observed changes are most likely due to a detergent-induced change in the environment of the chromophore. After a 19%

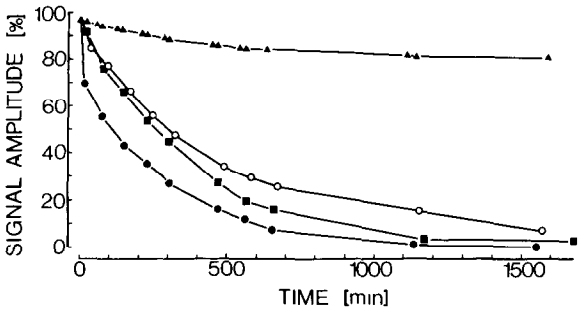

Fig.1. Kinetics of the solubilization of purple membrane in Triton X-100. Bacteriorhodopsin 12 μM. Triton X-100 0.2% (w/w). Phosphate buffer, 25 mM (pH 6.88) 20°C. All signals are normalized with respect to their value before solubilization. Solubilization starts at time zero. (■) Percentage non-solubilized material. A purple membrane sample was considered to be solubilized if it failed to sediment when subjected to 200 000 × *g* for 45 min. The percentage solubilization was determined by dividing the absorbance of the supernatant after centrifugation by the absorbance before centrifugation. (▲) Absorbance at the maximum in the longest wavelength band, normalized by the absorbance before solubilization. The data are corrected for the decrease in light scattering upon solubilization. The 19% change in extinction coefficient only affects the calculation of the percentage solubilization in a minor way. (○) Ellipticity at 318 nm. (●) CD exciton band amplitude, difference in ellipticity at the positive and negative extremum divided by the difference before solubilization. Upon complete solubilization a small positive CD monomer band centered at about 560 nm remained ([θ] at the maximum ~8000 deg cm²/dmol). This small component has been subtracted in the calculation of the relative exciton amplitude.

decrease in absorbance, a constant time independent value is reached (fig.1, ▲). This is not accompanied by the appearance of a band at 380 nm, indicating that chromophore loss does not occur. When solubilization is complete, dark-adapted bacteriorhodopsin has an A_{546} max. The two extrema of the CD exciton spectrum shift towards the blue upon the addition of Triton X-100, reflecting the corresponding shift in the absorption spectrum. The exciton CD amplitude (fig.1, ●) closely follows the time course of solubilization (■) and is zero when solubilization is complete. From this we conclude that solubilization to the stage of monomers occurs. This conclusion was confirmed by a determination of the molecular weight of bacteriorhodopsin in the mixed Triton–lipid–bacteriorhodopsin micelles [10]. It is interesting to note that the decrease in the optical activity of the electric-dipole forbidden 318 nm CD band (fig.1, ○) also closely follows the time course of solubilization. Since this band is due to retinal–protein interactions [11], these interactions are changed during solubilization.

The kinetics of solubilization is strongly pH dependent. Whereas at pH 6.88 and 20°C complete solubilization is reached in about 20 h, at pH 5 it takes 48 h to reach about 60%. The solubilized bacteriorhodopsin preparations were stable for several days when stored in the dark at room temperature and showed no evidence for chromophore loss.

In terms of a number of criteria, the state of bacteriorhodopsin in the solubilized form differs only little from that in the native membrane. The phenomenon of light–dark adaptation can still be observed in the monomeric state. The A_{max} occurs at 552 nm in the light-adapted state, shifting to 546 nm upon dark adaptation. The function of the solubilized bacteriorhodpsin was further tested by investigation of the photocycle. Whereas the formation of the M_{412} intermediate for Triton-solubilized bacteriorhodopsin was about 3-times faster than for bacteriorhodopsin in the purple membrane under the same conditions, the half-life of the decay remained the same.

Since solubilized bacteriorhodopsin is monomeric, light-scattering artefacts in the far ultraviolet CD spectra are much reduced allowing an estimate of the percentage α-helix to be made. Based on the ellipticity at 222 nm and assuming an extinction coefficient of 62 700 M^{-1} cm^{-1} for the light-adapted chromophore

in the purple membrane (corrected for light scattering; M. Rehorek and M. P. H., unpublished results) and 243 amino acids/bacteriorhodopsin, we calculate 72% α-helix. A value of 70% was obtained in Triton X-100 [10]. For bacteriorhodopsin in the purple membrane an α-helix content of 73% was calculated from CD spectra corrected for light scattering and flattening distortions [12]. Based on electron diffraction measurements, the bacteriorhodopsin in the native purple membrane was estimated to have an α-helix content of 70–80% [13]. We may thus conclude that solubilization does not lead to a major change in secondary structure.

By prolonged dialysis for 7 days at 4°C, most of the Triton X-100 could be removed. The low critical micelle concentration (0.24 mM) is a disadvantage in this procedure. During dialysis, reassembly occurs and large hexagonal crystalline domains are formed again [5]. In the course of this reassembly process the absorption maximum in the light-adapted state shifts back to 568 nm, the exciton CD bands reappear and the CD at 318 nm resumes its original value. The formation of the M_{412} intermediate is still faster in this reassembled state than in the purple membrane, whereas the half-life of the decay is about the same.

3.2. *Solubilization in octyl-β-D-glucoside*

The non-ionic detergent octyl-β-D-glucoside has been successfully used in solubilization and reconstitution studies [7,8,14,15]. Figure 2 shows the CD spectrum of dark-adapted purple membrane after the addition of octylglucoside as a function of time. As in the case of Triton X-100, the exciton bands disappear. No positive 'monomer' band could be detected however when solubilization was complete. It is well known in the case of solubilized rhodopsin that the amplitude of the intrinsic CD varies with different detergents [6]. From the disappearance of the exciton coupling effects we conclude that solubilization into monomers occurs. This conclusion was confirmed by a determination of the Stokes radius of the bacteriorhodopsin–lipid–octylglucoside micelle. Gel filtration experiments gave a value of 28 ± 5 Å, which virtually excludes the possibility that the micelles contain more than one bacteriorhodopsin molecule. In agreement with these observations no transient linear dichroism could be detected anymore when purple membrane was solubilized in octylglucoside, indicating

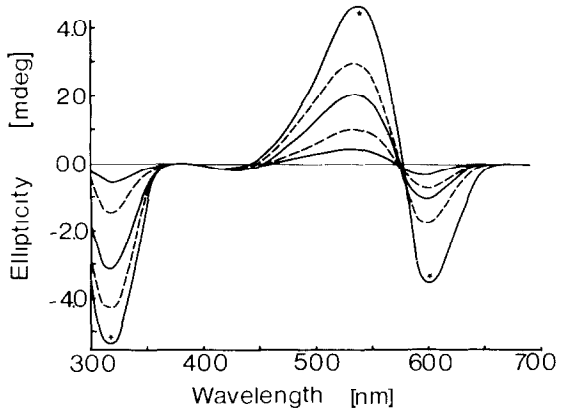

Fig.2. CD spectra of bacteriorhodopsin at various times during the solubilization process in octyl-β-D-glucoside. Bacteriorhodopsin 11 μM. Octylglucoside 41 mM. Phosphate buffer, 25 mM (pH 6.88) 20°C. The spectrum with the largest amplitude at each wavelength was taken before addition of octylglucoside (star). Subsequent CD spectra were taken at 17, 177, 591 and 1107 min after the addition of octylglucoside.

that the solubilized particles are smaller than 300 Å (R. J. Cherry, personal communication). Since the density of octylglucoside is ~1.26 g/cm^3 (unpublished results), the determination of the bacteriorhodopsin molecular weight in the octylglucoside micelle, by the method in [10] cannot be performed.

The time dependence of ΔA, CD and fluorescence accompanying solubilization of bacteriorhodopsin in octylglucoside are plotted in fig.3. The A_{max} decreases in a similar way as with Triton X-100. An immediate blue shift and a drop in absorbance occur upon the addition of the detergent. At the end of the time scale of fig.3 the centrifugation test confirmed that no sedimentable material remained. Further experiments (data not shown) demonstrated that, as in the case of Triton X-100, the decrease in the exciton CD amplitude and in the CD at 318 nm closely follow the decrease in the percentage sedimentable material. Bacteriorhodopsin solubilized in octylglucoside under the conditions of fig.2, was stable at 4°C for several days. At room temperature, however, progressive chromophore loss occurred after about two days.

The rate of solubilization increases with increasing pH. Furthermore, a marked dependence on ionic strength was observed. In 25 mM phosphate buffer (pH 6.88) solubilization occurred in the presence of

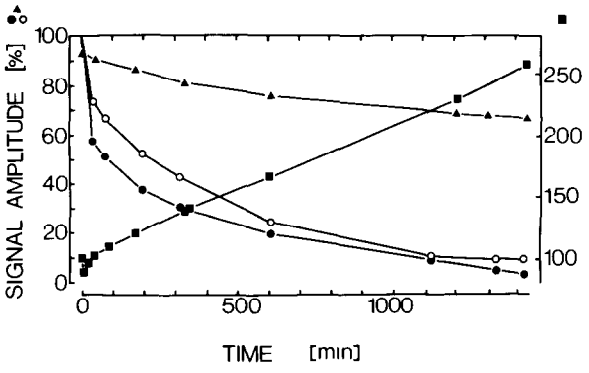

Fig.3. Time course of the absorbance, CD and fluorescence changes accompanying bacteriorhodopsin solubilization in octyl-β-D-glucoside. Bacteriorhodopsin 11 μM. Octylglucoside 41 mM. Phosphate buffer, 25 mM (pH 6.88) 20°C. All signals are normalized with respect to their value before solubilization. For the fluorescence data the scale of the signal amplitude is indicated on the right. (▲) Absorbance at the maximum of the longest wavelength band. (○) Ellipticity at 318 nm. (●) CD exciton band amplitude, difference in ellipticity at the positive and negative extremum. (■) Fluorescence emission at 328 nm (excitation at 280 nm).

15 mM added NaCl, but at a rate ~3-times smaller than without NaCl. No solubilization at all was found in samples containing in addition 2 M, 0.8 M or 0.14 M NaCl.

As with Triton X-100, many properties of bacteriorhodopsin differ only little in the monomeric and in the native state. Light–dark adaptation, for instance, is still observable in the solubilized form (the light-adapted form absorbs at 553 nm). The secondary structure of octylglucoside-solubilized bacteriorhodopsin, determined from the CD at 222 nm, contains about 69% α-helix and is thus quite similar to that in the purple membrane. No difference in secondary structure could be detected in the light- and dark-adapted state. The half-life of the decay of the M_{412} intermediate at 20°C was somewhat shorter than in the native purple membrane. Preliminary results indicate that the cycle is slightly different in octylglucoside-solubilized bacteriorhodopsin than in the purple membrane. Figure 3 shows that the tryptophan fluorescence at the emission maximum (324 nm for purple membrane in water) increases more than 2-fold during solubilization. Because of the interfering absorbance and emission of Triton X-100, it is not possible to determine whether such an increase also occurs during solubilization in that detergent.

Since the critical micelle concentration of octyl-glucoside is 25 mM, the detergent can be removed more rapidly than Triton X-100. Using the return of the amplitude of the exciton CD effect to its original value as a signal to monitor the detergent removal, equilibrium was reached in 40 h in octyl-glucoside as opposed to about 7 days with Triton X-100. As in the case of Triton X-100, most of the effects of solubilization are reversible upon slow dialysis: the A_{max} shifts back to 568 nm and the exciton CD bands reappear with the same specific ellipticity as in the native purple membrane. From the latter observation it may be concluded that as with Triton X-100, reassembly occurred.

In conclusion, the experiments reported here indicate that bacteriorhodopsin can be solubilized in monomeric form in both Triton X-100 and octyl-β-D-glucoside and that qualitatively the features of the kinetics of solubilization are the same. Both Triton X-100 and octylglucoside can be considered to be suitable detergents, since no gross structural and functional alterations occur during solubilization. Removal of the bulk of the detergent by slow dialysis leads to reassembly and reversal of most of the changes induced by solubilization. A small amount of detergent which remains bound after dialysis is probably responsible for the lack of reversibility in the tryptophan fluorescence change and for an increased temperature dependence of the exciton CD after reassembly. Octylglucoside is a chemically well-defined compound which is superior to Triton X-100 as a solubilizing agent with respect to ultraviolet spectroscopy. On the other hand, bacteriorhodopsin is more stable in Triton X-100.

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