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Altered protein–chromophore interaction in dicyclohexylcarbodiimide-modified purple membrane sheets

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Previous studies of *N,N'*-dicyclohexylcarbodiimide (DCCD)-modified bacteriorhodopsin (Renthal, R. et al. (1985) Biochemistry 24, 4275–4279) used reaction conditions (detergent micelles) that are not optimal for subsequent physical studies. The present work describes new conditions for reaction of bacteriorhodopsin with DCCD in intact purple membrane sheets in the presence of 4.5% (v/v) diethylether and light. Like the detergent reaction system, the reaction is light induced, incorporates approximately 1 mol [^{14}C]DCCD per mol bacteriorhodopsin, and results in a bleached chromophore. Peptide mapping indicates that the likely site of modification in intact membranes is identical to the site in the detergent reaction system: Asp 115. The retinal chromophore of DCCD-modified purple membrane has an absorbance maximum at 390 nm and very little induced circular dichroism. The retinal is easily extracted in hexane, yielding a 3:1 ratio of all-*trans* to 13-*cis* retinal. Borohydride reduces the retinal onto the protein within the 1–71 region of the amino acid sequence. These results suggest that Asp-115 is near the retinal binding cavity of bacteriorhodopsin. When DCCD reacts with Asp 115, retinal is displaced from its binding site.

Introduction

Carboxyl side chains of bacteriorhodopsin are clearly involved in the proton pump of the purple membrane of *Halobacterium halobium*. Spectroscopic evidence [1–3] indicates a change in protonation of carboxyl groups during the pump cycle. Chemical modification of carboxyl groups by water-soluble carbodiimides alters pump kinetics [4], while reactivity of Asp 115 toward the apolar carbodiimide dicyclohexyl carbodiimide (DCCD) was found to require light [5]. Genetic substitution of Asp 85, 96 or 115 diminishes or completely blocks proton pumping [6].

DCCD-modified bacteriorhodopsin may be useful in further studies of the purple membrane proton pump mechanism. Although modifications of proteins are generally more readily accomplished by genetic techniques [6,7], the DCCD reaction is important because it is selective for an intermediate state during the proton pump cycle and therefore provides mechanistic information. We would like to examine the detailed kinetics of this reaction, to identify the point during the pump cycle when Asp 115 becomes accessible to DCCD, and to correlate the reaction kinetics with spectroscopic data. In order to pursue these questions, it seemed necessary to improve he reaction

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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conditions we previously reported [5]. For example, the absorbance maximum of DCCD-modified bacteriorhodopsin could not be observed in our detergent reaction system because of the presence of excess retinal, which we found to be necessary for photochemical stability of the detergent-solubilized bacteriorhodopsin. Thus, we felt it would be worthwhile to try to find conditions where DCCD would undergo a light-induced reaction with native purple membrane sheets. The starting point for this work was the observation by Oesterhelt and Hess [8] that purple membrane sheets exposed to diethylether show reversible slowing of the photoreaction cycle.

Experimental methods

Materials. Purple membrane was prepared from *Halobacterium halobium* S9 by a method similar to that of Oesterhelt and Stoeckenius [9]. DCCD, all-*trans* retinal and chymotrypsin were obtained from Sigma. [^{14}C]DCCD was obtained from Research Products International. Diethylether, obtained from Malinckrodt, was washed three times with a solution of 8% $FeSO_4 \cdot 7\ H_2O$ in 0.13 M H_2SO_4 and then distilled. Triton X-100 and reduced Triton X-100 were obtained from Sigma and Aldrich, respectively.

Reaction of purple membrane sheets with DCCD in the presence of diethylether. A typical reaction mixture contained 0.325 ml purple membrane (0.1 mM bacteriorhodopsin in deionized water) and 2.6 ml 0.05 M Hepes buffer (pH 7.0). The reaction vessel was a 11 cm Wheaton microware threaded reflux condenser (1 cm inner diameter) with teflon-lined caps at each end. A 1.5 cm glass spacer filled the lower end of the tube so that the entire sample was in the jacketed part of the tube. A solution of DCCD was prepared (0.16 mg/ml) in freshly distilled diethyl ether, and 0.2 ml was added to the reaction vessel. The vessel was quickly capped and mixed by very gentle tilting from horizontal twice per second for 1.5 min. The vessel was placed 25 cm in front of a Tensor lamp. The light was filtered through a 250 ml beaker containing 1% $CuSO_4 \cdot 5\ H_2O$. The beaker acted as a cylindrical lens, focusing the light in an even rectangle on the sample. The intensity, measured with a Kettering model 65A radiometer, was 12.8 W/m^2. After 1.5 h the ether was removed under reduced pressure (water aspirator).

The stoichiometry of the reaction with DCCD was measured using [^{14}C]DCCD. In a 1 ml screw-cap vial, 0.1 ml purple membrane (0.1 mM bacteriorhodopsin in deionized water) was added to 0.8 ml ether-saturated buffer (0.02 M Hepes, pH 7.0). Immediately, 10 µl of an 18 mM solution of DCCD containing 20200 cpm [^{14}C]DCCD was added to the vial, which was then tightly capped and illuminated in the same manner as described for the nonradioactive reaction. After 1 h, the reaction was stopped by transferring to 0.2 ml 88% formic acid. The bacterioopsin was precipitated and washed with ammonia and acetone as described previously [5]. The protein was dissolved in 1% dodecyl sulfate and the radioactivity was measured by liquid scintillation counting. The radiolabeling experiment was scaled up to a 40 ml volume using a 2 × 16 cm glass-stoppered tube as a reaction vessel. The product was washed three times by extraction with 20 ml *n*-hexane in a 125 ml separatory funnel. The resulting emulsion was cleared by brief centrifugation and the membranes were washed twice by centrifugation and resuspension in deionized water. The DCCD-labeled product was subsequently cleaved with chymotrypsin and CNBr [10] and the resulting peptides separated by reversed phase HPLC [5,10].

Other methods. UV/vis spectra were measured on a Cary-14 spectrophotometer, fluorescence spectra on a Farrand MK1, and circular dichroism on a Jasco J-500C instrument. Reduction of the Schiff base [11], analysis of the extracted retinal by HPLC [12], gel electrophoresis [13], analysis of peptides [5,10], and measurement of light-induced pH changes [14] were by previously published methods.

Results

Regeneration of purple chromophore after exposure to diethyl ether

Under the conditions studied by Oesterhelt and Hess [8], i.e., 4 M NaCl and saturated diethylether, we did not observe any appreciable reaction of purple membrane with DCCD. When the salt concentration is diminished, the membrane loses its purple color and partially bleaches to a 480 nm

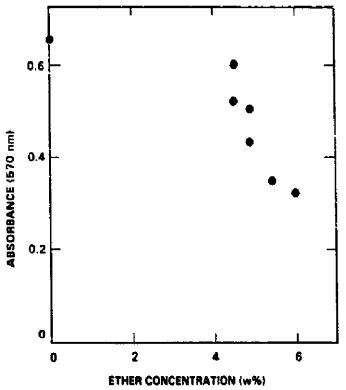

Fig. 1. Regeneration of purple chromophore after treatment of purple membrane with ether and light. Samples were prepared as described in the Experimental methods section for the DCCD reaction, except that no DCCD was added. Ether concentration given as % weight to volume. Illumination time: 1 h. Ether was removed by evaporation with water aspirator vacuum.

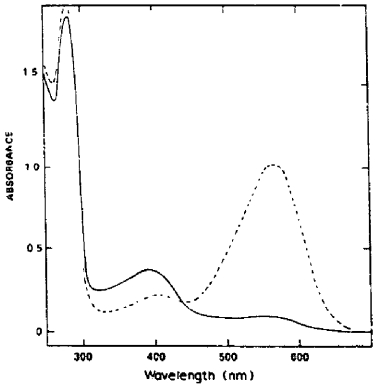

Fig. 2. UV/vis absorbance spectra of reaction product after addition of DCCD to purple membrane in 4.5% ether in light (solid line) or dark (dashed line). Reaction (and illumination, if present) for 1 h. Spectra were measured on reaction mixture (see Experimental methods) after removal of ether. Scattering baseline subtracted as described in text.

pigment. This form of the membrane is further bleached by light to an M-like state. The 570 nm native pigment can be regenerated by removing the ether under reduced pressure. The regeneration is incomplete at high ether concentrations (Fig. 1). For studies of the reaction of DCCD with ether-treated purple membrane, we chose an ether concentration of 4.5% (w/v). At this concentration, after illumination and removal of ether, regeneration of the 570 nm chromophore was usually greater than 80% in the absence of DCCD.

Reaction of ether-treated purple membrane sheets with DCCD

In the presence of ether and light, DCCD appears to cause irreversible bleaching of purple membrane in a manner similar to the light-induced DCCD reaction we previously observed with bacteriorhodopsin in Triton X-100 micelles [5]. In the dark, the bleaching caused by DCCD in ether-treated membrane occurs at a much slower rate (Fig. 2).

Incorporation of [^{14}C]DCCD into ether-treated purple membrane

After reaction of ether-treated purple membrane with [^{14}C]DCCD approx. 1 mol of DCCD was incorporated per mol bacteriorhodopsin (981 c.p.m. incorporated in 10 nmol bacteriorhodopsin/112 c.p.m. per nmol).

The radiolabeling experiment was scaled up to a 40 ml volume and the pattern of labeling on bacteriorhodopsin was examined. The HPLC separation of CNBr peptides (Fig. 3) closely matches the pattern of labeling previously found for the DCCD reaction in Triton X-100 [5].

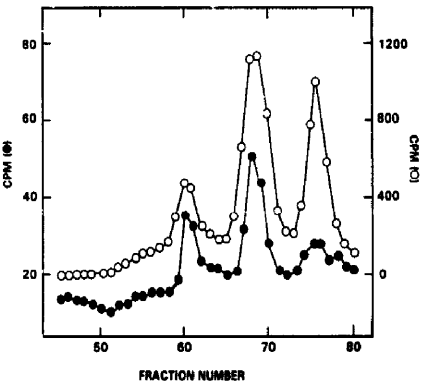

Fig. 3. HPLC separation of CNBr fragments of [^{14}C]DCCD-bacteriorhodopsin. C_8 reversed phase column, 5% formic acid, water/ethanol gradient. Open circles, reaction in Triton X-100 (from Renthal et al. [5]). Filled circles, reaction with purple membrane sheets in ether and light.

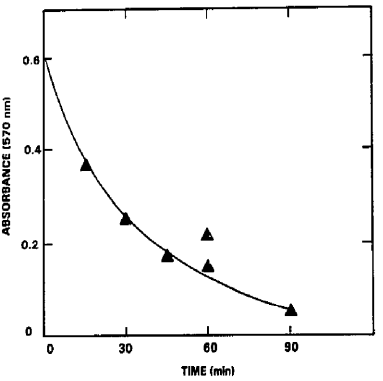

Fig. 4. Time dependence of the DCCD reaction with purple membrane sheets in 4.5% ether. Rate followed by measuring absorbance at 570 nm after removal of ether. Line fitted to single exponential.

Optimization of reaction conditions

The DCCD-induced bleaching of purple membrane was used as an indication of the extent of reaction. The reaction rate, DCCD concentration dependence, and temperature dependence were investigated.

The rate of bleaching proceeded with close to single exponential decay kinetics. The first-order rate constant was 35 min^{-1} for a 5:1 ratio of DCCD to bacteriorhodopsin at 25°C and 4.6% ether (Fig. 4). At a 6.6:1 mol ratio, the reaction appeared to be more than 80% complete after 1 h (Fig. 5). When the reaction was run at 15°C, only half as much bleaching was observed in 1 h.

DCCD-modified purple membrane samples for physical studies were prepared usually at a 5:1 mol ratio DCCD/bacteriorhodopsin, 25°C, 4.5% ether, and 1 h reaction time.

Absorption spectrum of DCCD-modified purple membrane sheets

The UV/vis absorption spectrum of DCCD-modified purple membrane sheets is shown in Fig. 2. The spectrum was corrected for light-scattering by subtracting an extrapolated long-wavelength baseline from a log–log plot. The baseline indicated an inverse square relationship between the scattering and wavelength, which is expected for scattering particles the size of purple membrane sheets (approx. 0.5 μm). The absorbance maximum appears to be near 390 nm, with an extinction coefficient of approx. 15000 $M^{-1} \cdot cm^{-1}$. A similar spectrum was obtained from bacteriorhodopsin modified by DCCD in Triton X-100 micelles that was purified by precipitation and regenerated according to the methods of Liao et al. [15] (data not shown).

The absorbance maximum at 390 nm is not far from the 360 nm absorbance found upon addition of excess all-*trans* retinal to purple membrane. Is retinal bound to DCCD-modified bacteriorhodopsin at all? Extraction with hexane removes the retinal from the modified membrane, in contrast to intact purple membrane. Analysis of the extract by HPLC showed a 13-*cis* to all-*trans* retinal ratio of 1 to 2.8.

However, the retinal appears bound as a Schiff base. Reduction of the DCCD-modified membrane with $NaBH_4$ results in a fluorescent product displaying an emission maximum at 500 nm, and a structured excitation spectrum containing sharp bands at 340 nm, 360 nm and 380 nm. The latter is characteristic of the reduced Schiff base of bacteriorhodopsin. Borohydride-reduced DCCD-modified purple membrane was cleaved with chymotrypsin and analyzed by gel electrophoresis. In contrast to control experiments using reduced, ether-treated membrane, the reduced, DCCD-treated membrane showed nearly all the retinyl fluorescence on the small chymotrypsin fragment, CI (M_r = 7900).

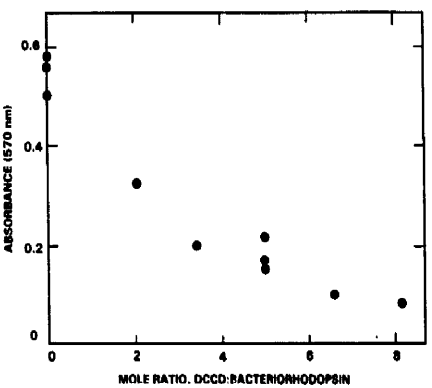

Fig. 5. DCCD concentration of reaction with purple membrane sheets in 4.5% ether. Absorbances at 570 nm measured after 1 h of reaction in light.

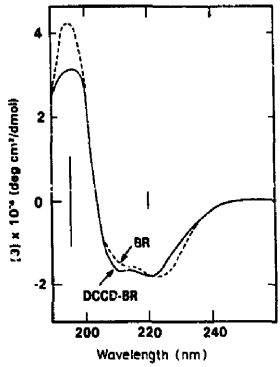

Fig. 6. Ultraviolet CD spectra of bacteriorhodopsin (BR) in 0.4% reduced Triton X-100. Solid line, after reaction with DCCD in the light. Dashed line, before reaction. Vertical bars show noise level.

Conformation of DCCD-modified purple membrane

The altered chromophore of the DCCD-modified membrane could result from the trivial circumstance of inducing gross denaturation of bacteriorhodopsin. That this is not the case may be seen in Fig. 6. The UV CD spectrum of DCCD-modified bacteriorhodopsin in reduced Triton X-100 retains a significant amount of apparent secondary structure, comparable to a sample not reacted with DCCD. However, the visible spectrum of membrane sheets (Fig. 7) shows a gross diminution of the induced CD of retinal in DCCD-modified membrane. Ether treatment decreases the intensity of the visible CD spectrum, but the general features are the same as native membrane, notably the cross-over due to an exciton interaction. In parallel with the loss of the CD spectrum, the DCCD-modified membrane fails to diffract X-rays (Heyn, M., personal communication). Thus, the modified membrane appears to be disordered.

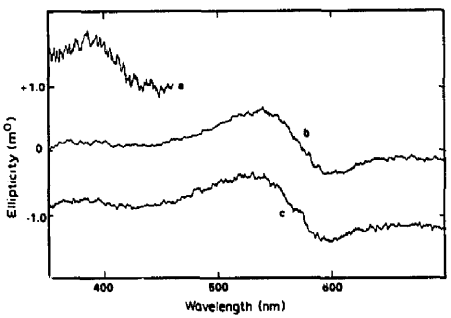

Fig. 7. Visible CD spectra of purple membrane sheets: (a) after reaction with DCCD in ether and light; (b) purple membrane unmodified; (c) purple membrane reacted with ether but no DCCD. Scale offset +1 m^0 for (a) and −1 m^+ for (c). Expansion (a) ×10, (b) ×1, (c) ×2. Conditions same as Fig. 2.

Functional studies on DCCD-modified purple membrane

DCCD-modified purple membrane sheets were examined for light-induced steady-state changes in proton binding [14], using illumination with a projector lamp through a 400 nm broad band (20 nm bandwidth) interference filter. If the 390 nm pigment of DCCD-modified purple membrane is a trapped or stabilized M-like state, then it might be expected to display the blue light effect [16]. However, no light-induced pH changes were observed beyond the electrode light response.

Discussion

Treatment of purple membrane with low concentrations of diethyl ether (less than 4.5%, see Fig. 1) reversibly bleaches the chromophore to a 480 nm absorbing form. The 480 nm form undergoes reversible light-induced bleaching to an M-like 400 nm form [8]. We have used ether as a perturbant to introduce the carboxyl-modifying reagent dicyclohexylcarbodiimide (DCCD) into the interior of bacteriorhodopsin. We have found that DCCD modifies bacteriorhodopsin when the membrane is exposed to light, but essentially no reaction is observed in the dark (Fig. 2). These results are similar to our previous report of light-dependent modification by DCCD of Asp 115 in bacteriorhodopsin solubilized in detergent micelles. Although we have not directly determined the sequence position of the modified site in ether-treated membrane, the peptide map of [^{14}C]DCCD-modified, CNBr-cleaved fragments is quite similar to that observed for the detergent system (Fig. 3). Thus, we tentatively conclude that the same site, Asp 115, is modified in both sets of reaction conditions.

The UV/vis absorbance spectrum of the DCCD-modified bacteriorhodopsin shows very

little opsin shift (Fig. 2). This could be due to (1) gross unfolding of bacteriorhodopsin; (2) dissociation of the Schiff base; (3) displacement of retinal from its normal binding site; or (4) trapping of bacteriorhodopsin in the photointermediate M. Gross unfolding seems unlikely, in view of the low wavelength CD spectrum (Fig. 6) which shows the helix content unaffected by the DCCD modification. Proteolytic cleavage by chymotrypsin, which is also a good indicator of conformational integrity, appears unaffected by the DCCD modification.

Dissociation of the Schiff base may have occurred, since the retinal is easily extracted from the modified membrane. But reduction of retinal with borohydride onto the small chymotrypsin fragment suggests it is probably bound near lysine 30, 40 or 41. Furthermore, most of the molecules available for reduction must be bound as Schiff bases, since no evidence for retinol was observed in the fluorescence spectrum of the reduction product.

There is some similarity, suggesting the DCCD-modified membrane is like photointermediate M. The absorbance maximum near 400 nm (Fig. 2), the formation as a photoproduct, and the absence of crystallinity at room temperature are suggestive. However, the extracted chromophore is mostly all-*trans* retinal, whereas M is 13-*cis*. Moreover, the DCCD-modified membrane almost completely lacks induced CD in the region of the retinal absorbance (Fig. 7) in contrast to the CD spectrum of M [17]. The CD spectrum suggests the retinal is not bound preferentially in any particular fixed geometry after the DCCD reaction. Finally, there is no blue-light effect in DCCD-modified purple membrane.

Taken together, the results seem to indicate retinal has been displaced from its binding site by DCCD. Since it is likely that DCCD reacts with Asp-115 in native membrane, the simplest interpretation is that Asp-115 must be near the retinal binding cavity. This conclusion is supported by the wavelength shift observed by Mogi et al. [6] in genetically altered D115N bacteriorhodopsin. The absorbance maximum of DCCD-modified purple membrane is reminiscent of an intermediate formed when retinal is added to *apo*-membrane [18], suggesting that the non-crystalline, retinal-dislocated DCCD-purple membrane is similar to an intermediate in the pigment generation pathway.

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