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LIGHT-INDUCED REACTION OF DICYCLOHEXYLCARBODIIMIDE WITH BACTERIORHODOPSIN

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SUMMARY

Bacteriorhodopsin solubilized in Triton X-100 micelles undergoes a light-induced reaction with [^{14}C]-dicyclohexylcarbodiimide at pH 7.5. The reaction has three consequences: 1) incorporation of radioactivity into bacteriorhodopsin, 2) irreversible bleaching of the retinal chromophore, and 3) alteration of the CNBr cleavage pattern. Reverse phase chromatography of the CNBr fragments from the modified bacteriorhodopsin reveals two new peptide fractions. The major fraction (91%) consists of two peptides of apparent molecular weights 10,700 and 5,400. N-terminal analysis suggests these peptides are bacteriorhodopsin residues 119-209 and 69-118, respectively. The modifications of these peptides are at sites that are exposed or activated by light, a property expected of groups involved in moving H^+ across the purple membrane during proton pumping.

The purple membrane from Halobacterium halobium contains a light-activated proton pump (1) which can generate a chemiosmotic proton gradient sufficient for ATP synthesis (2). The only protein in the purple membrane is bacteriorhodopsin (BR)[1], which has a molecular weight of 26,000 and contains the chromophoric group all-trans retinal (3). Recent structural studies on BR have provided a low resolution crystal structure (4) and the complete amino acid sequence (5,6). At the present time, little is known about the molecular mechanism of proton translocation. Several laboratories have been exploring the role in proton pump activity of various BR amino acid side chains by chemical modification (7-13). In view of the well-known modification by dicyclohexylcarbodiimide (DCC) of carboxyl groups in bacterial, mitochondrial and chloroplast ATPase (14), we began the present study to seek an analogous reaction with bacteriorhodopsin.

MATERIALS AND METHODS

Purple membrane was isolated from H. halobium S9 (15). [^{14}C]-DCC was obtained from Research Products International and diluted with non-radioactive DCC (Sigma) as described below.

1. Abbreviations: BR, bacteriorhodopsin; DCC, N,N'-dicyclohexylcarbodiimide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BBOT, 2,5-bis(5'-tert-butyl-2-benzoxazolyl)thiophene

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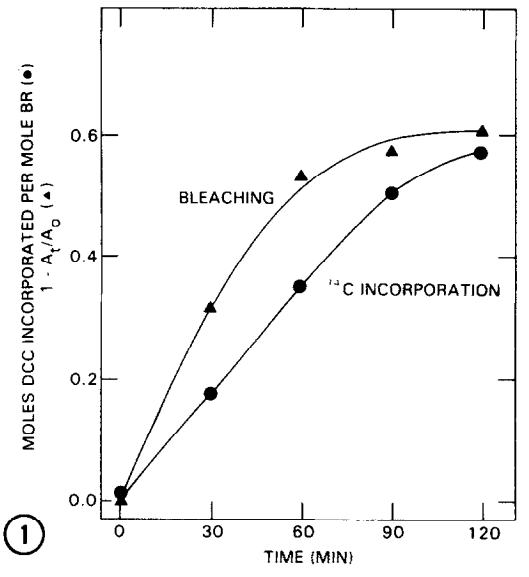
Measurement of reaction rate. Purple membrane (1.25 ml, 0.1 mM BR) was dissolved in Triton X-100 (1.25 ml, 0.8% w/v) and incubated at 23° C in the dark for approximately one day. To this solubilized membrane sample, the following additions were made: 0.1 M HEPES buffer pH 7.5 (0.25 ml), 0.4% Triton X-100 (2.5 ml), all-trans retinal (6 moles per mole BR in approximately 0.13 ml ethanol), and DCC (0.05 ml of 25 mM DCC in ethanol plus 15 µl of [^{14}C]-DCC stock solution; the [^{14}C]-DCC stock solution was prepared by adding 0.05 ml of [^{14}C]-DCC (50 Ci/mol in ether) to 0.3 ml of 0.4% Triton X-100). Reactions were done both in the dark and under continuous illumination. The light source was an ELE movie projector lamp filtered through 5 cm of 1% $CuSO_4$ and an Oriel 500 nm long pass filter to an intensity of 540 W/m^2. Aliquots of 1.0 ml were taken at 0, 30, 60, 90 and 120 minutes after addition of DCC. The absorbance at 550 nm was measured, and each aliquot was immediately added to 0.2 ml of 88% formic acid to stop the reaction. The protein was then precipitated by mixing with 5 ml acetone, 0.8 ml 30% ammonia (or dropwise until two distinct layers were observed), and 1.25 ml ethanol. The precipitated samples were centrifuged at 40,000 X g for 10 min at 4°. After the supernatants were removed, the pellets were resuspended in 5 ml acetone by brief sonication, and centrifuged again. The pellets were dried at room temperature and then resuspended in 1% sodium dodecyl sulfate, 0.01 M phosphate, pH 7. The ^{14}C radioactivity in the solubilized pellets was measured by liquid scintillation counting.

Analysis of reaction product. A large scale (121 ml) reaction of BR with [^{14}C]-DCC was done under the conditions described above, except the DCC:BR ratio was 14 moles/mole, the excess retinal was omitted, and the BR concentration was 8 µM after all additions were made. The [^{14}C]-DCC specific radioactivity was 2.4 X 10^{11} cpm/mol in the reaction. After 45 min of illumination by four projector lamps, the reaction was stopped with 21.5 ml 88% formic acid. The protein was precipitated by addition of 655 ml acetone, 60.5 ml 30% ammonia, and 131 ml ethanol. After 400 ml of the upper phase was decanted, an additional 150 ml acetone, 35 ml ammonia and 30 ml ethanol were added to produce a single phase. The precipitated protein was collected by centrifugation and washed with 100 ml acetone. The pellet was cleaved with CNBr and chromatographed by gel filtration (LH-60) and reverse phase high performance liquid chromatography as described by Khorana and coworkers (6,16). Electrophoresis was performed by the method of Swank and Munkres (17). The dansyl chloride method of Gray (18) was used for N-terminal analysis on polyamide layers.

RESULTS

Purple membrane solubilized in Triton X-100 detergent at pH 7.5 undergoes a light-induced reaction of BR with DCC (fig. 1). Two products were initially detected at a 10:1 molar ratio of DCC:BR: 1) formation of a covalent DCC-BR adduct, as indicated by incorporation of radioactivity when [^{14}C]-DCC was used; and 2) bleaching of the retinal chromophore of BR. Since Triton X-100 itself causes light-induced bleaching of BR at neutral pH, we developed a procedure for stabilizing the pigment in detergent so that the bleaching we observed was entirely due to DCC. Addition of 6 moles of retinal per mole of BR to the detergent-solubilized membrane prior to illumination protects BR almost completely from bleaching at pH 7.5 in the absence of DCC. Excess retinal does not substantially affect the amount of incorporation of [^{14}C]-DCC. In the dark, bleaching and radioactivity incorporation were not significant.

The rate of incorporation of radioactivity into BR appears to lag slightly behind the rate of bleaching. This result suggests the bleaching and incorporation reactions occur at different sites.



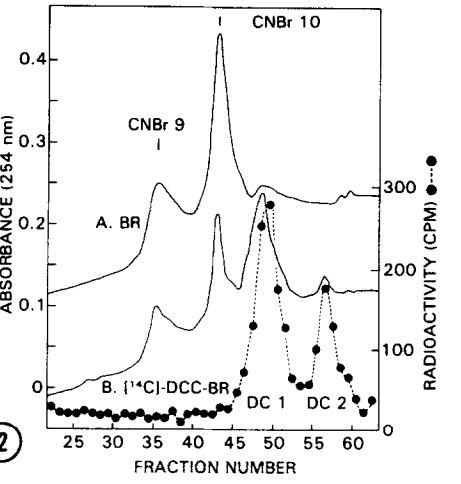


Figure 1. Light-induced reaction of DCC with BR in Triton X-100. Incorporation of [^{14}C]-DCC radioactivity, expressed as moles of DCC bound per mole of BR (●) is compared with the simultaneous bleaching of the retinal chromophore, expressed as the fractional loss of absorbance (A) at 550 nm (1 - A_t/A_o, where subscript o is initial time and t is reaction time) (▲). Incorporation of radioactivity is corrected for a small amount of [^{14}C]-DCC that reacts with BR in the dark. For example, at 120 min, the DCC incorporation is 0.74 moles/mole in the light and 0.17 moles/mole in the dark.

Figure 2. Chromatography of high molecular weight CNBr fragments of BR (A.) and the product of the reaction of BR with [^{14}C]-DCC (B.). Column: Biosil ODS-10, 4 X 250 mm. Eluent: 5% formic acid, linear gradient of water/ethanol. Flow rate: 1 ml/min. Fraction size: 1 ml. Radioactivity (●) was measured in each fraction by withdrawing 0.4 ml and adding 9.6 ml of scintillation fluid prepared from 5 volumes of toluene containing 0.4% BBOT to 1 volume ethanol. Fractions are identified as CNBr 9 (residues 164-209) and CNBr 10 (residues 69-118) by comparison with reference (15). New fractions DC 1 and DC 2 are the only observed effect of the light-induced DCC reaction. DC 1 contains 91% of the 254 nm absorbance of the new peptides.

We analyzed the product of a large scale light-induced reaction of BR with [^{14}C]-DCC to attempt to determine the nature of the product. Extraction with acetone removed Triton and unreacted DCC, and this permitted CNBr cleavage of BR with about 50% yield. The CNBr fragments were purified by gel filtration (LH-60) and reverse phase high performance liquid chromatography (fig. 2). DCC causes an alteration in the chromatographic elution pattern of the CNBr fragments: the peptides corresponding to CNBr 9 (residues 164-209) and CNBr 10 (residues 69-118) were isolated in diminished yield (62% and 56% of control, respectively), and two new fractions were obtained (DC 1 and DC 2). The amount of DC 1 and DC 2 exactly accounts for the decreased yield of CNBr 9 and 10. Both new fractions contain radioactivity, but the cpm profile of DC 1 does not exactly coincide with

the 254 nm absorbance profile. Polyacrylamide gel electrophoresis in urea and sodium dodecyl sulfate revealed that DC 1 consists of two peptides: DC 1a and DC 1b. Integration of stained gels showed that the ratio of DC 1a : DC 1b was about 2:1. The apparent molecular weights were: DC 1a = 10,700; DC 1b = 5,400 (standards were BR, 26,000; BR residues 72-248, 18,200; BR residues 1-72, 7,800; CNBr 10, 5,500; and CNBr 9, 5,000). N-terminal analysis of DC 1 showed only valine and isoleucine, in approximately equal amounts, while DC 2 contained predominantly N-terminal valine.

DC 1a most likely results from incomplete CNBr cleavage at methionines 145 and 163. This would produce a peptide with a molecular weight of 10,100 (residues 119-209) and N-terminal isoleucine. DC 1b and DC 2 are probably DCC derivatives of CNBr 10 (molecular weight, 5,500; N-terminal valine.)

At higher DCC:BR ratios (greater than 20:1), or at pH 5.8, light-induced bleaching and radioactivity incorporation was also observed. At a ratio of 100:1, the bleaching reaction occurs rapidly in the dark. The products of the reaction at acid pH and at high DCC:BR ratios were analyzed by polyacrylamide gel electrophoresis. Extensive intermolecular cross-linking was found.

DISCUSSION

We have found that after detergent solubilization, BR undergoes a light-induced reaction with DCC. The number of reactive sites is small, and the results are consistent with as few as two sites being involved. BR incorporated in chloroplast liposomes does not appear to undergo a similar reaction (19).

There are three consequences of this reaction: 1) bleaching of the retinal chromophore; 2) incorporation of radioactivity into BR when [^{14}C]-DCC is used; and 3) alteration of the CNBr cleavage pattern. The incorporation most likely occurs at carboxyl groups through formation of the N-acyl urea of DCC. However, DCC could also react with lysine or tyrosine in a way that would lead to ^{14}C incorporation. The reaction also somehow inhibits CNBr cleavage at Met 145 and 163. CNBr cleavage of peptides is known to be inhibited by oxidation of methionine or by steric effects (20). A steric effect that could be caused by DCC is the formation of an intramolecular crosslink. The bleaching of the retinal chromophore does not appear to be caused by reaction directly with the Schiff base lysine at the retinal attachment site, since this position has been identified as Lys 41 (21) or Lys 216 (22), neither of which is present in the modified peptides.

The three dimensional structure of BR is probably not drastically altered by Triton solubilization: the retinal absorbance spectrum is only slightly altered (23-25), photocycling occurs (25) and light-induced proton release and uptake is found (26). The sites affected by the light-induced reaction of DCC with BR must undergo some change in exposure or reactivity during the BR photoreaction cycle.

Thus, it is probable that some or all of the DCC-modified groups are functionally important in proton pumping.

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