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Light Activates the Reaction of Bacteriorhodopsin Aspartic Acid-115 with Dicyclohexylcarbodiimide†

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Received April 17, 1985

ABSTRACT: Conditions for a light-induced reaction between the carboxyl-modifying reagent *N,N'*-dicyclohexylcarbodiimide (DCCD) and bacteriorhodopsin in Triton X-100 micelles were previously reported [Renthal, R., Dawson, N., & Villarreal, L. (1981) *Biochem. Biophys. Res. Commun. 101*, 653–657]. We have now located the DCCD site in the bacteriorhodopsin amino acid sequence. [^{14}C]DCCD–bacteriorhodopsin (0.67 mol/mol of bacteriorhodopsin) was cleaved with CNBr. The resulting peptides were purified by gel filtration and reverse-phase high-performance liquid chromatography (HPLC). One major ^{14}C peptide (50%) and two minor fractions were obtained. The modified peptides were completely absent in the absence of DCCD, and 10 times less was obtained when the reaction was run in the dark. Amino acid analysis and sequence analysis showed that the major fraction contained residues 69–118. This region includes six carboxyl side chains. Quantitative sequence analysis ruled out significant amounts of DCCD at Glu-74, Asp-85, Asp-96, Asp-102, and Asp-104. The major ^{14}C peptide was also subjected to pepsin hydrolysis. HPLC analysis of the product gave only a single major radioactive subfragment. Amino acid analysis of the peptic peptide showed that it contained residues 110–118. The only carboxyl side chain in this region is Asp-115. Thus, we conclude that Asp-115 is the major DCCD site. The light sensitivity of this reaction suggests that Asp-115 becomes more exposed or that its environment becomes more acidic during proton pumping. The DCCD reaction blue-shifts the retinal chromophore. Such a result would be expected if Asp-115 is the negative point charge predicted to be near the cyclohexene ring of retinal.

Bacteriorhodopsin, the only protein in the purple membrane from *Halobacterium halobium*, is a light-activated proton pump (Stoeckenius & Bogomolini, 1982). The crystal structure (Henderson & Unwin, 1975) and the amino acid sequence (Ovchinnikov et al., 1979; Khorana et al., 1979) are known, providing the opportunity for understanding the molecular mechanism of a simple ion pump. Many proton pumps have been studied by chemical modification methods, and a general phenomenon seems to be inhibition by blocking of carboxyl groups with carbodiimides (Sebald et al., 1980; Esch et al., 1981; Prochaska et al., 1981; Pennington & Fisher, 1981; Phelps & Hatefi, 1981; Beattie & Villalobo, 1982; Sussman & Slayman, 1983). We previously reported that bacteriorhodopsin reacts with water-soluble (Renthal et al., 1979) and water-insoluble (Renthal et al., 1981) carbodiimides, resulting in alteration of H^+ pump function. These studies suggest an important role for carboxyl groups in the bacteriorhodopsin proton pump. In this paper we report the characterization of the product of the light-activated reaction of bacteriorhodopsin with *N,N'*-dicyclohexylcarbodiimide (DCCD)[1] and have identified the major site modified within its primary structure.

Experimental Procedures

Purple Membrane. The method of Oesterhelt & Stoeckenius (1974) was used to obtain purple membrane sheets from *H. halobium* S9. Stock solutions were stored at 10^{-4} M bacteriorhodopsin in 0.025% NaN_3 and washed free of azide prior to use by repeated centrifugation and resuspension in water.

Reaction of Purple Membrane with DCCD. Purple membrane was reacted with [^{14}C]DCCD by the method previously described (Renthal et al., 1981), except that the reaction volume was scaled up. A 12.5-mL suspension of purple membrane sheets (10^{-4} M bacteriorhodopsin) was added to

†Supported by grants from the National Institutes of Health (GM 25483 and RR 08194) and the Robert A. Welch Foundation (AX-736).
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[1] Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; PTH, phenylthiohydantoin; CNBr 10a, cyanogen bromide fragment of bacteriorhodopsin containing residues 69–118; SPITC, 4-sulfophenyl isothiocyanate; HPLC, high-performance liquid chromatography.

0006-2960/85/0424-4275$01.50/0

12.5 mL of 0.8% Triton X-100 (Sigma) and allowed to dissolve 24 h in the dark at room temperature. *all-trans*-Retinal (Sigma) was dissolved in ethanol, and 7.5 μmol was dried under N_2 in a small test tube. This was redissolved in 12.5 mL of 0.8% Triton X-100, then diluted with 12.5 mL of water, and added to the Triton X-100 solubilized purple membrane. To this was added 2.5 mL of 0.1 M *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid (Sigma), pH 7.5. The absorbance of this solution at 555 nm was ~0.9. A 25 mM stock solution of nonradioactive DCCD (Sigma) was prepared in ethanol. The desired amount of [^{14}C]DCCD (Research Products International) in pentane was pipetted into a small test tube and dried under N_2. Then 0.5 mL of the nonradioactive DCCD stock solution was added, thoroughly mixed with the [^{14}C]DCCD residue, and pipetted into the buffered, solubilized purple membrane–retinal solution. The reaction mixture was immediately transferred to the outer chamber of an Ace Glass Inc. 7878 photochemical reaction vessel. In the inner chamber was circulating a 0.2% solution of $Na_2Cr_2O_7 \cdot H_2O$ through a Sears evaporative cooler pump set in an open reservoir. To start the reaction, an Osram 100-W tungsten halogen lamp was placed in the central well of the reaction vessel and operated at 9 V, 8 A dc. The intensity, measured with a Kettering 65-A radiometer immediately outside the glass wall of the empty reaction vessel opposite the center of the beam, was 1400 W/m^2. The lamp was cooled with an Oster Model 202 drier, and the dichromate reservoir was set in ice water as needed to keep the temperature between 22 and 28 °C. The outside of the reaction vessel was wrapped in aluminum foil. The reaction was run for 90 min. The final absorbance at 555 nm was ~0.2. (In the absence of DCCD, an otherwise identical reaction mixture gave an absorbance of 0.88 after 90 min of illumination.)

After 90 min the reaction mixture was transferred to a 500-mL flask containing 10 mL of ice-cold 88% formic acid to destroy the unreacted DCCD. Purple membrane lipids, Triton X-100, and DCCD breakdown products were separated from the protein by addition of 285 mL of ice-cold acetone, 26 mL of 30% ammonia, and 52 mL of ethanol. The flask was placed on ice for 15 min to allow the protein to precipitate. Approximately 175 mL of supernatant was then decanted, and an additional 65 mL of acetone, 15 mL of NH_3, and 13 mL of ethanol were added. The protein was recovered by centrifugation in glass tubes, and the pellets were washed twice with cold acetone. During this step, a considerable amount of ammonium formate crystallizes on the sides of the centrifuge tubes. In some experiments this was removed with an additional wash of 1:1 acetone–water. Residual acetone and water were removed from the pellets by vacuum drying for several hours.

Purification of DCCD-Modified Peptides. The DCCD-modified bacteriorhodopsin was dissolved in 2.5 mL of 88% formic acid, diluted with 2.5 mL of heptafluorobutyric acid (Pierce Chemical Co.), and added to 1.6 g of CNBr (Eastman). The cleavage was performed for 24 h at room temperature in the dark. The product was concentrated to about 3 mL under a stream of N_2, transferred to a 300-mL flask, diluted with about 5 mL of water, and freeze-dried. The large flask was useful because the sample tends to melt and bump vigorously under reduced pressure. After about 12 h, no trace of CNBr could be detected. The residue in the flask (not completely dry) was diluted with 3.75 mL of 88% formic acid and 8.75 mL of ethanol and applied to a column of LH-60 (Gerber et al., 1979). Peptides were identified by a Coomassie dye binding assay (Peterson, 1983) and by liquid scintillation counting (Aquasol 2). The radioactive fractions were pooled, dried under reduced pressure, and applied to a high-performance liquid chromatograph as previously described (Gerber et al., 1979; Renthal et al., 1981) except a Brownlee RP-300 column (C_8) was used. Radioactive HPLC fractions were pooled and dried. Peptides resulting from clostripain cleavage (Gerber & Khorana, 1982) were separated on a column of LH-20. The small amino-terminal peptide was quantitated by acid hydrolysis and reaction with *o*-phthalaldehyde.

Characterization of DCCD-Modified Peptides. Acid hydrolysis of DCCD-modified peptides was performed in 6 N HCl, 110 °C for 24 h, or in 2:1 concentrated HCl–trifluoroacetic acid at 150 °C for 2 h (Tsugita & Scheffler, 1982). Amino acid analysis was performed on a Durrum 500 instrument or by a phenyl isothiocyanate method similar to that of Heinrickson & Meredith (1984).

Manual amino-terminal sequence analysis was done according to the method of Weiner et al. (1972) as modified by Nicholas (1984). Automated sequence analysis of 4-sulfophenyl isothiocyanate (SPITC) modified, clostripain-cleaved, DCCD-modified CNBr 10a (residues 69–118) was done as described by Gerber & Khorana (1982) on a Beckman 890C sequencer. Two separate runs were performed. One sample was lactonized and treated with ethylenediamine prior to reaction with SPITC, and essentially the 0.1 M Quadrol program of Gerber & Khorana (1982) was used (separate benzene and ethyl acetate washes). The second sample was not treated with ethylenediamine, and combined benzene and ethyl acetate washes were used. Anilinothiazolinones were converted in 1 M aqueous HCl, 80 °C. PTH-amino acids were separated on an Altex Ultrasphere ODS column. (Solvent A = 0.08 M acetic acid, pH 4.0; solvent B = methyl alcohol, 30-min linear gradient from 20% B to 60% B, followed by a 7-min hold.) Yields were estimated by peak heights, compared to a PTH-amino acid mixture that was back-hydrolyzed in HI (Perham, 1975), and quantitated by amino acid analysis. Radioactivity in aliquots of each butyl chloride wash was measured by liquid scintillation counting. The benzene or combined benzene–ethyl acetate washes were collected and counted, and the residue remaining in the sequenator cup after 22 rounds was removed with 88% formic acid or trifluoroacetic acid and counted.

Gel electrophoresis in urea and dodecyl sulfate was done according to the method of Swank & Munkres (1970).

Pepsin Hydrolysis of the Major DCCD-Modified Peptide. Approximately 30 nmol of dried DCCD-modified peptide was dissolved in 0.1 mL of 88% formic acid and then diluted with 0.6 mL of H_2O. To this was added 0.1 mL of pepsin [Worthington; 0.5 mg/mL in 0.01 M HCl; activity = 1300 units/mg, measured with denatured hemoglobin as the substrate, according to a method similar to that of Kassell & Meitner (1970)]. Proteolysis proceeded for 3 h at 25 °C, and the sample was then lyophilized. The peptic peptides were dissolved in 0.5 mL of 88% formic acid and immediately applied to a reverse-phase HPLC column (same method as for CNBr peptides described above). Pepsin emerged in the void volume. The amino acid composition of the major radioactive fragment of the DCCD peptide was determined as described above.

RESULTS

Localization of DCCD Modification Sites. We previously showed that light-induced modification of bacteriorhodopsin with [^{14}C]DCCD followed by reaction with CNBr produces a complex mixture of ^{14}C-labeled peptides, possibly due to incomplete CNBr cleavage. We now find that reaction with CNBr in 88% formic acid–heptafluorobutyric acid (1:1) (Ozols

Table I: Yield of DCCD-Modified Bacteriorhodopsin and Peptides

step[a]		cpm	DCCD (nmol)	bacteriorhodopsin (nmol)	mol of DCCD/mol of bacteriorhodopsin	% yield from previous step
1	reaction mixture	7.82×10^7	12500	1250	10	
2	DCCD–bacteriorhodopsin	4.05×10^6	646	971	0.67	78[b]
3	LH-60 eluent					
	total yield	2.66×10^6	424	ND[c]		66[d]
	HC-2	1.52×10^6	243	ND		
4	HC-2B	1.74×10^5	27	ND		
	HC-2C	4.77×10^5	76	ND		63[d]
	HC-2D	3.12×10^5	50	ND		
5	major pepsin peptide	8.82×10^4	14	15	0.9 ± 0.4	25[b,e]

[a] For step 1, see Experimental Procedures for details. In step 2, DCCD–bacteriorhodopsin is the precipitated protein obtained after the DCCD reaction. Step 3 refers to the chromatographic separation in Figure 1. Step 4 refers to the separation in Figure 2. Step 5 refers to the separation in Figure 4. [b] Based on peptide yield. [c] Not determined. [d] Based on radioactivity yield. [e] Different preparation from sample analyzed in steps 1–4. Total of four fractions from two separate experiments.

Table II: Amino Acid Compositions of Major CNBr and Pepsin Radiolabeled Peptides

amino acid	found (nmol) for HC-2C	ratio[a]	expected (residues 69–118)	found (nmol) for peptic peptide	ratio[b]	expected (residues 110–118)
Asp	8.6	5.8	6	5.6	0.9	1
Glu	4.4	3.0	3	0.6	0.1	0
Gly	8.4	5.8	5	17.8	2.9	2
Ser	0.8	0.5	0	0.6	0.1	0
Thr	3.5	2.4	3	0.2	0	0
Ala	8.8	5.9	6	8.7	1.4	2
Arg	1.8	1.2	1	0	0	0
Pro	5.4	3.6	3	0	0	0
Tyr	1.8	1.2	2	0	0	0
Val	5.7	3.8	3	5.3	0.9	1
Ile	4.4	3.0	3	5.5	0.9	1
Leu	16.3	11.0	10	7.5	1.2	1
Phe	2.2	1.5	2	0	0	0
Lys	0.6	0.4	0	0	0	0

[a] Calculated from analysis assuming 1.48 nmol of peptide. [b] Calculated from analysis assuming 6.19 nmol of peptide.

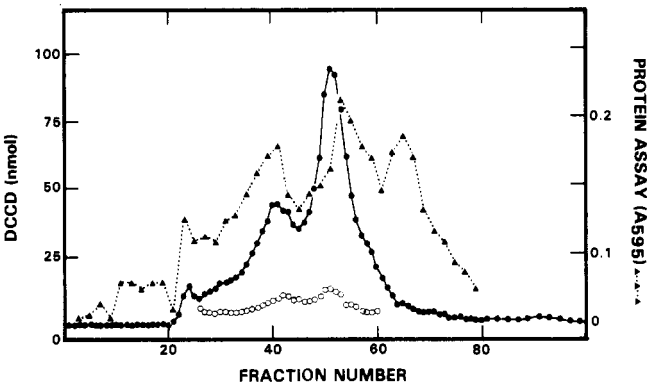

FIGURE 1: LH-60 chromatography of CNBr-cleaved [^{14}C]DCCD–bacteriorhodopsin. Peptides were eluted in 88% formic acid–ethanol (3:7) from a 2.5 × 90 cm column. Fractions of 4.1 mL were collected at a rate of four fractions per hour. Radioactivity was sampled (20 μL) from each fraction and converted to nanomoles of DCCD (circles). An aliquot was also used in a peptide dye-binding assay (triangles). Filled circles are the product of reaction in the light; open circles are the product of reaction in the dark. Fractions 45–56 (HC-2) were subjected to further analysis.

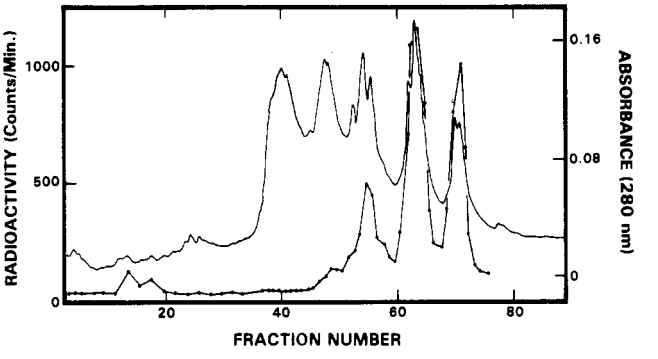

FIGURE 2: High-performance liquid chromatography of CNBr fragments of [^{14}C]DCCD–bacteriorhodopsin. Fraction HC-2 (Figure 1) was further separated by reverse-phase chromatography on a Brownlee RP-300 column (4.6 × 250 mm) and eluted with a linear water–ethanol gradient from 40% to 80% ethanol. Both solvents contained 5% formic acid. The eluent was monitored at 280 nm (continuous line). The flow rate was 1.0 mL/min, and 1.0-mL fractions were collected. Aliquots of 20 μL were removed for measuring radioactivity (filled circles). Fractions 37–50 were pooled as HC-2A, fractions 52–57 as HC-2B, fractions 60–65 as HC-2C, and fractions 68–72 as HC-2D.

& Gerard, 1977) gives significantly higher yields of cleaved products. All of the radioactive material eluted in the high molecular weight region after LH-60 chromatography of the CNBr fragments (Figure 1 and Table I). When [^{14}C]DCCD was reacted with bacteriorhodopsin in the dark, only about 10% of the level of labeling in the light was obtained (Figure 1). The major radioactive peak (fractions 45–56; HC-2) was further resolved by reverse-phase HPLC (Figure 2). Only three significant radioactive fractions were obtained from this separation: HC-2B, HC-2C, and HC-2D. Amino acid analysis and amino-terminal sequence analysis showed that the nonradioactive HC-2A contained residues 164–209 and 69–118. If DCCD was omitted from the reaction miture, only HC-2A was obtained. This result suggests that HC-2B, HC-2C, and HC-2D are derived from HC-2A by modification with the nonpolar DCCD moiety. This was confirmed by amino acid analysis (Table II) and amino-terminal sequence analysis, which showed that the major radioactive peak, HC-2C, contained residues 69-118. The amino acid composition of HC-2D was similar to HC-2C. All four fractions had apparent molecular weights of 5300, as measured by calibrated urea–dodecyl sulfate–polyacrylamide gel electrophoresis. By comparison of the absorbance or amino acid analysis to the

Table III: Sequence Analysis of Major DCCD–Bacteriorhodopsin CNBr Peptide[a]

	PTH-amino acids observed (nmol)			DCCD recovered (nmol)	
sequence round	69–118	70–118	83–118	chlorobutane wash	benzene–ethyl acetate wash
1	V, 1.2	P, 4.3	Y, 2.8	0.02	0.03
2	P, 5.3	F, 1.3	A, 3.4	0.03	0.06
3	F, 1.1	G, 4.3	D, 0.9	0.08	0.12
4	G, 9.0	G, 9.0		0.04	
5	G, 4.7	E, 6.0	L, 3.0	0.04	
6	E, 7.0	Q, 1.7	F, 2.0	0.07	
7	Q, 2.1	N, 2.8		0.12	
8	N, 3.1	P, 1.1		0.08	1.55[b]
9	P, 1.5	I, 2.0		0.09	
10	I, 2.6	Y, 2.7	L, 1.4	0.09	
11	Y, 3.7		L, 1.5	0.06	
12		A, 1.3	L, 1.4	0.09	
13	A, 2.8	R, 2.6	L, 1.3	0.09	
14	R, 5.0	Y, 1.8	D, 0.1	0.09	0.15
15	Y, 1.8	A, 1.2	L, 0.6	0.09	
16	A, 1.8	D, 0.4		0.13	
17	D, 0.6		L, 0.9	0.17	
18				0.13	1.09[c]
19				0.11	
20				0.10	
21				0.10	
22				0.08	
remaining[d]					4.60

[a] The total amount of peptide applied (in terms of DCCD) was 27.6 nmol. [b] Rounds 4–13 pooled. [c] Rounds 15–22 pooled. [d] After round 22, the cup was extracted 4 times with 0.6 mL of trifluoroacetic acid. The total apparent yield of radioactivity was 35%.

radioactivity, HC-2D appears to contain the same stoichiometry of DCCD as HC-2C. Thus, it is unclear why it would have a longer retention time. It may be identical with HC-2C except for the C-terminus (for example, lactone vs. carboxyl) or for different extents of Trp oxidation, Tyr bromination, Gln/Asn deamidation, or Ser/Thr formylation. When the DCCD reaction product was washed with acetone–water to remove ammonium formate prior to the CNBr reaction, only a single radioactive peak was found, which corresponded to HC-2C. This indicates that NH_4HCO_2, in the presence of acidic CNBr, partially converts HC-2C to HC-2D. Thus, the major light-induced DCCD-modified sites appear to be on a peptide containing residues 69–118, CNBr 10a. [Gerber & Khorana (1982) have referred to this peptide as CNBr 9a.]

Sequence Analysis of [^{14}C]DCCD CNBr 10a. There are six possible sites on CNBr 10a for reaction with DCCD: Glu-74, Asp-85, Asp-96, Asp-102, Asp-104, and Asp-115 (Figure 3). We further localized the modification by amino acid sequence analysis. [^{14}C]DCCD CNBr 10a was modified with SPITC and partially cleaved with clostripain. The clostripain peptide that was removed from the amino terminus contains Glu-74, but no ^{14}C was removed by this treatment, ruling out Glu-74 as the DCCD site. This conclusion was confirmed by sequence analysis. The sequence run also passed Asp-85, Asp-96, Asp-102, and Asp-104 without encountering the DCCD site (Table III).

Pepsin Hydrolysis of HC-2C. Partial cleavage of HC-2C with pepsin produced one major subfragment, several minor subfragments, and some uncleaved starting material (Figure 4). Amino acid analysis suggested that the major fragment contained residues 110–118 (Table II and Figure 3). This assignment was confirmed by fast atom bombardment mass spectrometry (R. Renthal, M. Bernard, and S. Weintraub, unpublished experiment). This peptide includes only a single carboxyl side chain, Asp-115, which therefore must be the major DCCD site. The stoichiometry of labeling was near the expected 1.0 (Table I).

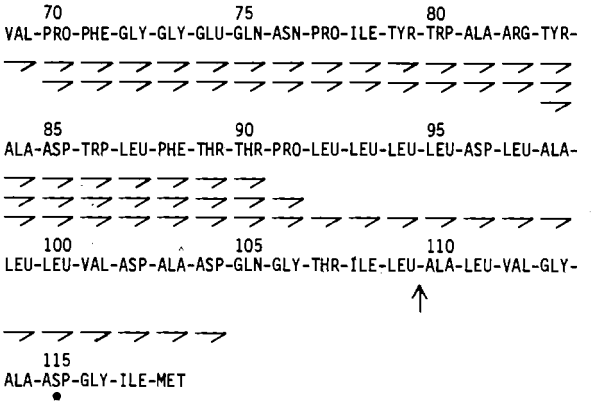

FIGURE 3: Amino acid sequence of the major DCCD-modified peptide (HC-2C). The sequence shown is from Khorana et al. (1979). Horizontal arrows show automated Edman degradation (Table III) of a mixture of residues 69–118, 70–118, and 83–118. The vertical arrow shows the pepsin cleavage site that produces the major radioactive subfragment of HC-2C (Figure 4 and Table II). The DCCD-reactive site is identified as Asp-115.

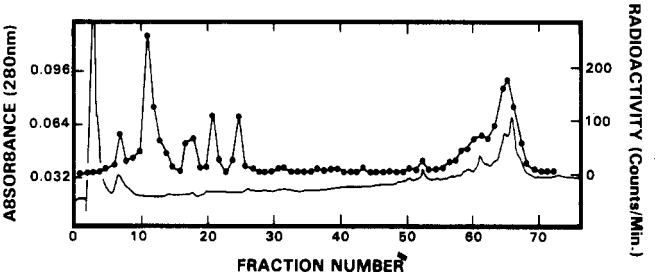

FIGURE 4: High-performance liquid chromatography of the pepsin digest of the major DCCD-modified peptide (HC-2C). Peptic peptides from cleavage of fractions 60–65 (Figure 2) were separated on a Brownlee RP-300 column (same conditions as in Figure 2). Fractions 11 and 12 were hydrolyzed in 6 N HCl, and the amino acid composition was determined (Table II). These fractions contained 25% of the total eluting radioactivity. Uncleaved starting material amounted to 38%.

Discussion

The light-induced reaction of bacteriorhodopsin with the liposoluble carbodiimide DCCD modifies Asp-115 and also bleaches the purple chromophore. Most of the suggested folding patterns for bacteriorhodopsin (Steitz et al., 1982; Kyte & Doolittle, 1982; Ovchinnikov, 1982; Huang et al., 1982)

predict a membrane interior location for Asp-115. Carbodiimide reactions with carboxyl groups are acid catalyzed (Khorana, 1953; Carraway & Koshland, 1972). Since Asp-115 is not very reactive toward DCCD in the dark, either this residue becomes more exposed or its environment becomes more acidic during proton pumping.

Interaction of the retinal protonated Schiff base with two negative point charges was invoked by Nakanishi et al. (1980) to explain the chromophoric properties of purple membrane. One perturbing group is postulated to be the protonated Schiff base counterion, and the other is in the vicinity of the cyclohexene ring. When the Schiff base counterion is protonated, a red shift is predicted for retinal, suggesting a mechanism for formation of the acid-induced blue pigment. Protonation of the cyclohexene ring site would produce a blue shift. Since we find that modification of Asp-115 with DCCD blue-shifts the purple chromophore, a possible explanation of this effect is that DCCD abolishes the point charge near the cyclohexene ring. Alternatively, the bulky cyclohexyl groups of DCCD might displace the cyclohexene ring from its interaction with the negative point charge, either directly or indirectly. Infrared spectroscopy has revealed light-induced changes in carboxyl protonation (Engelhard et al., 1985; Rothschild et al., 1981; Bagley et al., 1982). Our results make Asp-115 a candidate for one of the groups involved in these spectroscopic changes.

If crystalline purple membrane sheets can be re-formed from the Triton X-100 solubilized DCCD reaction product, diffraction studies of the location of Asp-115 in the three-dimensional structure of purple membrane might be attempted. It would also be interesting to study the photochemistry (if any) of the DCCD-modified bacteriorhodopsin.

Registry No. DCCD, 538-75-0; Asp, 56-84-8.

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