The Effect of Carboxyl Group Modification on the Chromophore Regeneration of Archaeopsin-1 and Bacterioopsin[1]

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Carboxyl group modification with DCCD and NCD-4 was employed to investigate the chemical environment of the side chains of archaeopsin-1 (aO-1) and bacterioopsin (bO). Some differences were observed between aO-1 and bO. Although DCCD or NCD-4 did not modify aO-1 in bleached membrane, they modified bO in bleached membrane and in mixed DMPC/CHAPS/SDS micelles at neutral pH, thereby affecting the opsin shift and the photocycle of the regenerated chromophore. On the contrary, after solubilization with SDS, aO-1 and bO were modified by DCCD and NCD-4, which decreased the chromophore regeneration. In particular, the reaction of aO-1 in SDS with NCD-4 proceeded in a 1:1 ratio at neutral pH. The fluorescence and CD spectra indicated that the modified site was located in the hydrophobic, asymmetrical region. Lysyl-endopeptidase digestion of NCD-4 modified aO-1 produced a fluorescent fragment and amino acid sequence analysis showed that Asp85 or Asp96 in helix C is a probable candidate for the modified residue at present. Kinetic CD measurements revealed that the introduction of *N*-acylurea at an Asp residue in helix C did not affect the formation of the transient intermediate but inhibited the side chain packing during refolding.

Key words: archaerhodopsin, bacteriorhodopsin, carboxyl group modification, *in vitro* refolding, side chain packing.

Bacteriorhodopsin (bR) is the light-driven proton pump in *Halobacterium salinarum* purple membrane (*1*). Other light-driven proton pumps have been found in several halobacteria (*2*), but their physicochemical properties have been less characterized except for those of archaerhodopsins. Archaerhodopsin-1 (aR-1) is a retinal protein of *Halorubrum* (formerly *Halobacterium*) sp. aus-1 claret membrane (*3*), and the amino acid sequence of archaeopsin-1 (aO-1) is 59% identical to that of bacterioopsin (bO) (*4*). Archaerhodopsin-1 and bR provide a useful experimental system for studying the *in vitro* refolding of a membrane protein. When claret or purple membrane is solubilized with SDS, aR-1, or bR is denatured, and retinal and most endogenous lipids can be separated from the protein moiety by gel filtration chromatography in the presence of SDS (*5*). The SDS-solubilized and denatured aO-1 or bO, thus obtained, regenerates a 560 nm chromophore upon transfer from SDS to DMPC/CHAPS/SDS

mixed micelles in the presence of all-*trans* retinal (*5-7*). When retinal is absent, a stable apoprotein accumulates in DMPC/CHAPS/SDS, which can bind to retinal to regenerate the 560 nm chromophore. Therefore, the refolding process can be divided into two stages which can be studied separately, that is, the formation of the stable apoprotein, and then the retinal binding to the apoprotein and the regeneration of the 560 nm chromophore.

We previously demonstrated that the first stage of the refolding proceeded through essentially the same processes for both aO-1 and bO, although the tertiary structure and stability of the apoprotein somewhat differed between them (*8*). The properties of the stable apoprotein are thought to be similar to those of bleached membrane. Historically, bleached membrane has been used for studying the second stage of refolding. Upon the addition of all-*trans* retinal, it combines with bO in bleached membrane to regenerate the 560 nm chromophore as follows:

bleached membrane + retinal
$$\rightleftharpoons 400\,nm \rightleftharpoons 435/460\,nm \rightleftharpoons 560\,nm$$
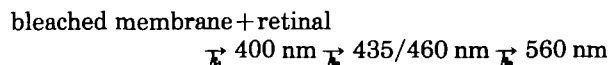
where the 400 nm intermediate formation is supposed to comprise fixation and ring-chain planarization of retinal in the binding site (*9*). The 435/460 nm intermediate is a chromophore noncovalently bound to the retinal binding pocket, which has been detected in bO at subzero temperature (*10, 11*). The 560 nm chromophore formation is one step of Schiff base formation. At 25°C and pH 7, the formation of the 435/460 nm intermediate seems to be the rate-limiting step for bO because the 560 nm chromophore was apparently regenerated without detection of the 435/

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Abbreviations: aO-1, archaeopsin-1; aR-1, archaerhodopsin-1; bO, bacterioopsin; bR, bacteriorhodopsin; CHAPS, 3-[(3-cholamidopropy)dimethylammonio]-1-propanesulfonate; DCCD, *N,N'*-dicyclohexylcarbodiimide; DMPC, dimyristoylphosphatidylcholine; NCD-4, *N*-cyclohexyl-*N'*-(4-dimethylamino-a-naphthyl)carbodiimide.

460 nm intermediate $(k_2 \ll k_3)$. In contrast, the relatively stable 435/460 nm chromophore was detected for aO-1 in bleached membrane and in DMPC/CHAPS/SDS. Therefore, the Schiff base formation is the rate-limiting step for aO-1 $(k_2 \gg k_3)$ (7). These findings suggested that some of the chemical nature of the retinal binding pocket differed between aO-1 and bO in bleached membrane.

In this study we found another difference between aO-1 and bO in their chemical reactivity to DCCD and its fluorescent analog, NCD-4. At neutral pH, DCCD and NCD-4 modified bO but not aO-1 in bleached membrane and DMPC/CHAPS/SDS. The 520 nm chromophore, whose photocycle was slowed, was regenerated with the modified bO. In contrast, aO-1 and bO were modified by DCCD and NCD-4 in SDS and the chromophore regeneration was decreased. The modified carboxyl residue was supposed to be different between aO-1 and bO in SDS.

EXPERIMENTAL PROCEDURES

Materials—CHAPS was purchased from Wako Pure Chemicals. DMPC and all-*trans* retinal were from Sigma Chemicals. NCD-4 was from Molecular Probes. DCCD was from Peptide Research. Ultrogel ACA 34 was from Bio-Sepra.

Preparation of aO-1 and bO—Archaerhodopsin-1 and bR were isolated as claret and purple membranes from *Halorubrum* sp. aus-1 and *H. salinarum* R_1M_1, respectively, according to the method described in Ref. *11*. Bleached membrane was prepared by irradiating a claret and purple membrane suspension in 1 M NH_2OH-HCl (pH 7.0) for several h at 25°C with orange light. Bleached membrane was collected by centrifugation $(100,000 \times g)$ for 30 min at 4°C and then washed several times with 10 mM sodium phosphate (pH 7.0). The SDS-solubilized and denatured aO-1 and bO were prepared from claret and purple membranes, respectively, as described in Ref. *8*. The claret and purple membranes (10 mg of each protein) were solubilized in 2% (w/v) SDS for 15 h at room temperature. The solubilized protein was chromatographed on an Ultrogel ACA 34 column (1.8×90 cm) equilibrated with 0.1 M NaCl, 0.2% (w/v) SDS, 20 mM sodium phosphate (pH 7.0), and 0.01% (w/v) NaN_3 at room temperature. Fractions of 2.5 ml were collected and the absorbance at 280 nm was measured. Protein fractions were concentrated by ultrafiltration (UP-20; Advantec Toyo), and then dialyzed against 500 volumes of 0.2% SDS and 0.01% NaN_3 for 3 days with two buffer changes at room temperature. Protein was determined by the Lowry method.

DCCD and NCD-4 Modification of aO-1 and bO—DCCD and NCD-4 were dissolved in ethanol and stored at −20°C before use. The concentration of NCD-4 was determined using an extinction coefficient of 10.25×10^3 $M^{-1} \cdot cm^{-1}$ at 335 nm (*12*). The final ethanol concentration in the incubation mixture was less than 0.5% (v/v).

Bleached membrane (100 μg protein/ml) was incubated with DCCD in 25 mM sodium phosphate at room temperature. The pH was adjusted with NaOH or HCl. The modified membrane was collected by centrifugation and then washed with 25 mM sodium phosphate (pH 7.0). Chromophore regeneration was started by adding all-*trans* retinal at 25°C and pH 7.0, as described previously (*7*). The SDS-solubilized and denatured aO-1 and bO (100 μg protein/ml)

were incubated with DCCD or NCD-4 at room temperature in 0.1% (w/v) SDS and 20 mM sodium phosphate or in 0.2% (w/v) DMPC/0.2% (w/v) CHAPS/0.08% (w/v) SDS and 20 mM sodium phosphate (pH 7.0). Chromophore regeneration was started by adding all-*trans* retinal to the bacterial opsin in 0.2% DMPC/0.2% CHAPS/0.08% SDS and 20 mM sodium phosphate (pH 7.0). The absorbance increase at 560 nm was measured at 20°C for aO-1 and bO as described previously (*8*).

Spectroscopy—Fluorescence spectra were obtained with a Hitachi F-4010 spectrofluorimeter at 20°C as described previously (*8*). The tryptophan excitation wavelength was 290 nm and emission was monitored at 333 nm. The *N*-acylurea excitation wavelength was 335 nm and emission was monitored from 350 nm to 500 nm. CD was measured with a Jasco J-720 spectropolarimeter at 20°C. Far-UV and near-UV CD spectra were recorded at a scanning speed of 10 nm/min. The path lengths were 0.1 cm and 1 cm for the far-UV and near-UV regions, respectively. The protein concentrations were 100 and 300 μg/ml for the far-UV and near-UV regions, respectively. Base line correction for the respective solvents and signal averaging of CD spectra were performed with an NEC 98 computer. Laser-flash induced absorbance changes were measured with a time resolved spectrophotometer (TPS-601, UNISOKU) at 20°C, as described in Ref. *13*.

RESULTS

The Effects of DCCD and NCD-4 on the Chromophore Regeneration of aO-1 and bO in Bleached Membrane and DMPC/CHAPS/SDS—It was shown that DCCD modifies Asp115 and irreversibly bleaches bR in Triton X-100 solubilized purple membrane in the light (*14*, *15*). However, the effect of DCCD on NH_2OH-bleached membrane has not been reported. When aO-1 in bleached membrane was incubated with 100 μM DCCD at pH 7 for 12 h, a 560 nm chromophore was regenerated, as in the case of the control. In contrast to aO-1, when bO in bleached membrane was incubated with 60 μM DCCD at pH 7 for 12 h in the dark, a 520 nm chromophore was regenerated upon the addition of retinal, as compared with the control bO (Fig. 1, A and C). The laser-flash induced absorbance changes indicated that the increase of the M intermediate in the 520 nm chromophore was almost the same as that in the control, but the decay of the M intermediate was ten times slower as compared with that in the control (Fig. 1, B and D). NaN_3 had no effect on the M decay of the 520 nm chromophore. These results indicated that DCCD modified the carboxyl residue(s) of bO in the bleached membrane at neutral pH, and affected both the opsin shift and the photocycle kinetics of the regenerated chromophore. Visible CD spectra of the 520 nm chromophore showed positive CD at 510 nm (not shown). Therefore, DCCD modification inhibited the lattice reformation of the bleached membrane.

We observed that aO-1 and bO in DMPC/CHAPS/SDS showed the same behavior on DCCD modification as those in bleached membrane did (not shown), suggesting that the folding of the stable apoprotein in DMPC/CHAPS/SDS is similar to that in bleached membrane at neutral pH. In order to characterize the modification reaction, we used NCD-4 in the following experiments in addition to DCCD. NCD-4 is not fluorescent but after its reaction with a

protonated carboxyl group, the product, N-acylurea, is strongly fluorescent (*12*).

The Effects of DCCD and NCD-4 Modification in SDS on the Chromophore Regeneration of aO-1 and bO—When aO-1 in 0.1% SDS was incubated with DCCD or NCD-4 at pH 7.0 for 12 h, the chromophore regeneration decreased in DMPC/CHAPS/SDS at pH 7.0. This concentration dependency indicated that DCCD was less effective than NCD-4 (Fig. 2A). When 4.1 μM aO-1 in SDS was modified with various concentrations of NCD-4 at pH 7.0, the extent of the chromophore regeneration linearly decreased with an increase in the NCD-4 concentration. Extrapolation of the linear portion to the abscissa gave the concentration of NCD-4 which was necessary to abolish the chromophore regeneration, that is, 4.3 μM (Fig. 2B). In contrast to aO-1, the chromophore regeneration of bO did not linearly decrease with an increase in the NCD-4 concentration (Fig. 2B).

Fluorescence Properties of NCD-4 Modified aO-1 and bO—It has been reported that the emission maxima of N-acetylurea are 398 and 425 nm in hexane and ethanol, respectively (*12*). When aO-1 in 0.1% SDS was incubated with NCD-4 [aO-1/NCD-4 (mol/mol)<1] at pH 7.0, it showed a fluorescence spectrum with an emission maximum at 410 nm when excited at 335 nm (Fig. 3A, traces a and b). The fluorescence intensity increased with an in-

crease in the NCD-4 concentration. When the mole ratio of NCD-4/aO-1 exceeded one, the fluorescence spectra were slightly red-shifted with an apparent emission maximum at 420 nm (Fig. 3A, traces d and e). The inhibition of chromophore regeneration with aO-1 (up to 90% inhibition) was proportional to the fluorescence intensity of N-acylurea at 410 nm (Fig. 3C). This and Fig. 2B implied that NCD-4 reacted with one carboxyl group of aO-1 in SDS at pH 7.0 and inhibited the *in vitro* chromophore regeneration.

Although the fluorescence intensity of N-acylurea of bO increased with an increase in the NCD-4 concentration and the emission maximum wavelength changed from 415 to 420 nm depending on the concentration of NCD-4 (Fig. 3B), the inhibition of chromophore regeneration of bO was not directly proportional to the fluorescence intensity at 410 nm (Fig. 3C). These observations thus indicated that even low concentrations of NCD-4 modified nonspecific site(s) of bO in addition to the site relevant to chromophore regeneration.

Isolation of the Fragment Containing the NCD-4 Modified Residue—Archaeopsin-1 was modified with NCD-4 (1:1, mol/mol) overnight in SDS at pH 7 and then digested with lysyl endopeptidase (LEP) at pH 8.5. The resulting fragments were separated by SDS-PAGE and
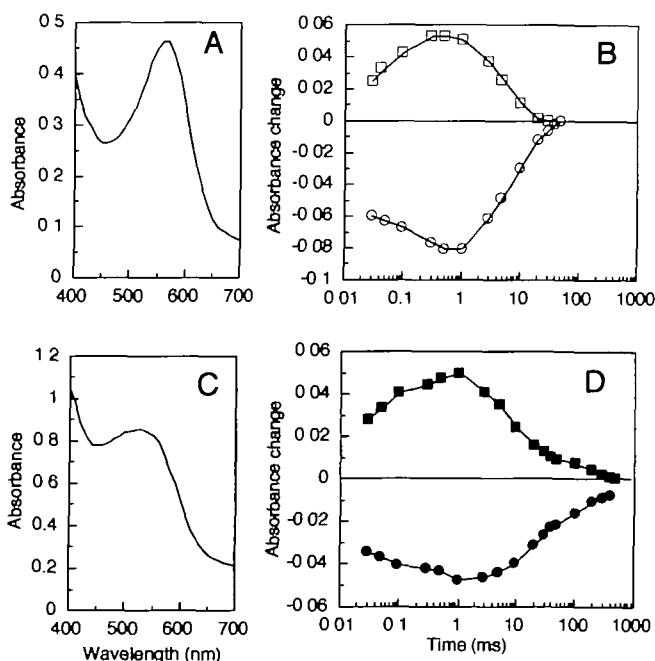

Fig. 1. **Effects of DCCD on the chromophore regeneration by bO in bleached membrane and photocycle kinetics.** Bacterioopsin in bleached membrane was incubated with 60 μM DCCD in 20 mM sodium phosphate (pH 7) for 12 h in the dark at 20°C. Then retinal was added to regenerate the chromophore. The membranes were collected by centrifugation and suspended in 20 mM sodium phosphate (pH 7). An absorbance spectrum (C), and the laser-flash induced absorbance changes (D) were measured at 410 nm (■) and 510 nm (●) as described under "EXPERIMENTAL PROCEDURES." As a control, an absorbance spectrum (A) and the laser-flash induced absorbance changes (B) were measured at 410 nm (□) and 570 nm (○) for the regenerated bR. The absorbance spectra were not corrected for the light scattering.
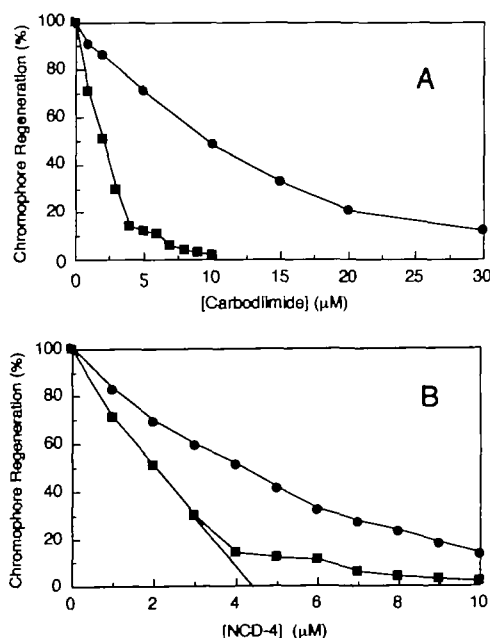

Fig. 2. **A: DCCD and NCD-4 modification of aO-1 in SDS.** aO-1 (90 μg) was incubated with various concentrations of DCCD (●) and NCD-4 (■) in 0.8 ml of 0.1% SDS and 20 mM sodium phosphate (pH 7.0) at room temperature for 12 h. Chromophore regeneration was started by adding 0.2 ml of 1% DMPC, 1% CHAPS, and 25 μM all-*trans* retinal in the dark. The absorbance increase at 560 nm after 12 h incubation was plotted as a percentage of the control (A_{560}=0.11). **B: Effects of NCD-4 modification on the chromophore regeneration of aO-1 (■) and bO (●).** The data for aO-1 are the same as in Fig. 2A. aO-1 or bO (90 μg protein each) was incubated with various concentrations of NCD-4 in 0.8 ml of 0.1% SDS and 20 mM sodium phosphate at pH 7.0 and room temperature for 12 h. Chromophore regeneration was started by adding 0.2 ml of 1% DMPC, 1% CHAPS, and 25 μM all-*trans* retinal in the dark. The absorbance increase at 560 nm after 12 h incubation was plotted as a percentage of the control. A_{560} of bO was 0.12.

then electrotransferred to a polyvinylidenedifluoride (Immobilon-P[SQ]; Millipore) membrane. The single fluorescent band detected under a UV lamp was cut out and the N-terminal amino acid sequence of the material in this band was determined to be EAREYYSITILVPGIAX-AAYLXMFF. Therefore, the modified residue is present in the LEP fragment [Glu38–Lys102] of aO-1 (4) [to avoid confusion, the residue numbers of aO-1 used are the same as those at the corresponding positions of bO (16)]. Thus, the candidate NCD-4 modified residues are Glu68, Glu72, Asp76, Asp85, and Asp96. Although we have not yet identified the modified residue, Asp85 or Asp96 in helix C is a probable candidate in the case of aO-1 at present because a lipophilic carbodiimide such as DCCD or NCD-4 reacts with a carboxylate anion located in a hydrophobic environment in an acid-catalyzed reaction (17).

In contrast to aO-1, when bO in SDS was incubated with NCD-4 (1:1, mol/mol), conditions for 50% inhibition of chromophore regeneration, the LEP fragments of the modified bO gave two main fluorescent bands with other
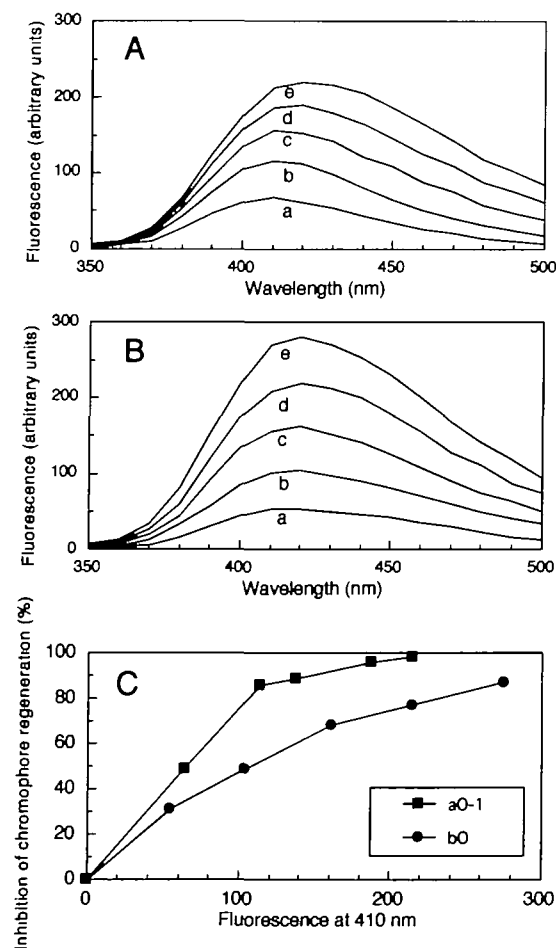
faint bands on an Immobilon-P[SQ] membrane, as speculated from Fig. 3C. Therefore, NCD-4 reacted with at least two carboxyl residues of bO in SDS under these conditions, but further analysis has not yet been performed.

In Vitro Refolding Kinetics of NCD-4 Modified aO-1— We analyzed the effect of NCD-4 modification on the first stage of the refolding of aO-1 by means of CD measurements. The far-UV CD spectrum of the modified aO-1 in SDS was indistinguishable from that of the unmodified aO-1 (Fig. 4A). Upon transfer from SDS to CHAPS/SDS, $-[\theta]_{222}$ of the modified aO-1 increased by 6% within 30 s and then decreased (Fig. 4B, trace b). Upon transfer to DMPC/CHAPS/SDS (Fig. 4B, trace c), the fast increase in $-[\theta]_{222}$ of the modified aO-1 occurred but the following decrease was slower as compared with in the case of transfer to CHAPS/SDS. Similar CD changes were observed for the unmodified aO-1 (8). The near-UV CD spectrum of the modified aO-1 in SDS was essentially the same as that of the unmodified aO-1 except that negative CD around 330 nm was observed for the former (Fig. 5A). This negative CD band indicated that the N-acylurea conjugate was located in an asymmetrical environment in the aO-1 polypeptide in SDS. Upon transfer from SDS to DMPC/CHAPS/SDS, the near-UV CD spectrum of the modified aO-1 did not change so much (Fig. 5B), while large CD changes occurred for the unmodified aO-1, as reported previously (8). Similar results were obtained for the DCCD modified aO-1. These observations indicated that the
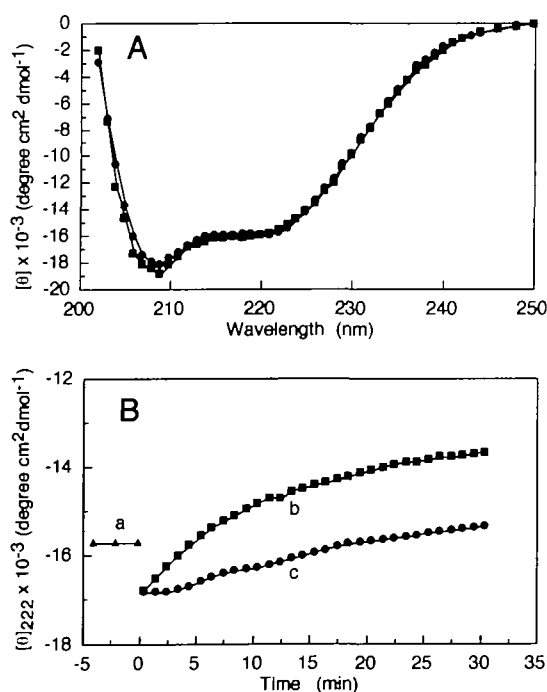

Fig. 3. **Fluorescence spectra of the *N*-acylurea conjugates of aO-1 (A) and bO (B).** NCD-4 modification was carried out as in Fig. 2. Fluorescence spectra were measured as described under "EXPERIMENTAL PROCEDURES." The protein concentration was 110 μg/ml. NCD-4 concentrations: a: 2, b: 4, c: 6, d: 8, e: 10 μM. (C) **Relationship between the inhibition of chromophore regeneration and the fluorescence intensity of *N*-acylurea at 410 nm.** The data in Figs. 2B, 3A, and 3B were replotted.

Fig. 4. **Far-UV CD spectra of NCD-4 modified aO-1, and the CD changes upon transfer from SDS to CHAPS/SDS and DMPC/CHAPS/SDS.** aO-1 was modified with NCD-4 (1:1.5, mol/mol) in 0.1% SDS and 20 mM sodium phosphate (pH 7.0). A: Far-UV CD spectra were measured in 0.1% SDS and 20 mM sodium phosphate (pH 7.0). B: The ellipticity at 222 nm of NCD-4 modified aO-1 in SDS is shown as trace a. An aliquot (0.8 ml, 100 μg/ml) was mixed with 0.2 ml of 1% CHAPS (■, trace b) or 1% DMPC and 1% CHAPS (●, trace c) at time zero. After 30 s, the ellipticity at 222 nm was recorded for 30 min.
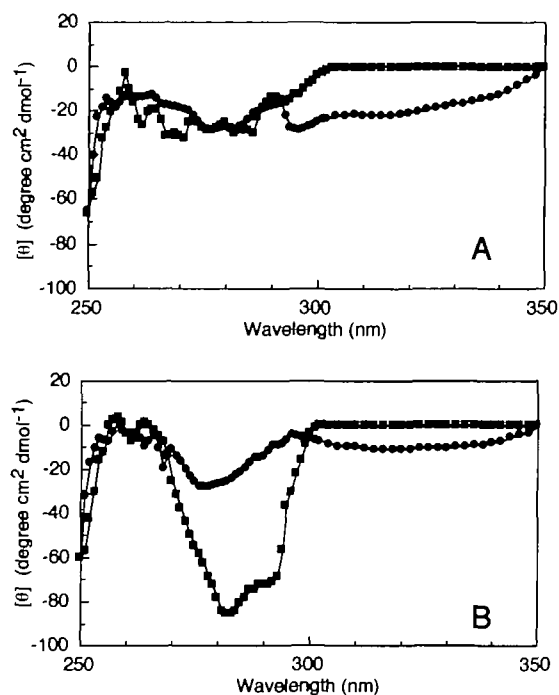
Fig. 5. **The effects of NCD-4 modification on the near-UV CD spectra of aO-1.** A: CD spectra of aO-1 (■) and NCD-4 modified aO-1 (●) in SDS. B: CD spectra of aO-1 (■) and NCD-4 modified aO-1 (●) in DMPC/CHAPS/SDS. aO-1 was modified with NCD-4 (1:1.5, mol/mol) in 0.1% SDS and 20 mM sodium phosphate (pH 7.0). An aliquot (1.6 ml, 350 μg/ml) was mixed with 0.4 ml of distilled water or 1% DMPC and 1% CHAPS at 20°C. After 30 min, CD spectra were measured. Each spectrum is the average of 6 scans.

Fig. 6. **The Trp fluorescence change of DCCD-modified aO-1 upon transfer from SDS to CHAPS/SDS.** aO-1 was modified with DCCD (1:2, mol/mol) in 0.1% SDS and 20 mM sodium phosphate (pH 7.0). A: control aO-1, B: DCCD-modified aO-1. The fluorescence at 333 nm was monitored for 1.6 ml of sample (trace a), and 0.4 ml of distilled water (trace b) or 1% CHAPS (trace c) was added at time zero (arrow) under continuous stirring.

carbodiimide modification did not affect the secondary structure change but abolished the tertiary structure change during the first stage of the refolding of aO-1. Therefore, the inhibition of chromophore regeneration of aO-1 on the modification of a single carboxyl residue in SDS can be explained as follows. The *N*-acylurea introduced into helix C is so bulky that it inhibits the final step of the first stage of refolding, that is, the proper packing of the side chains in the regeneration medium (DMPC/CHAPS/SDS).

The refolding of the NCD-4 modified aO-1 could not be monitored as Trp fluorescence because fluorescence energy transfer occurred from Trp to *N*-acylurea in the NCD-4 modified aO-1 (Kameyama, K. *et al.*, in preparation). Thus, we measured the Trp fluorescence changes during refolding of the DCCD modified aO-1. Upon transfer from SDS to CHAPS/SDS, the intrinsic Trp fluorescence of the DCCD modified aO-1 (Fig. 6B) increased by 36% within 1 s, as in the case of the unmodified aO-1 (Fig. 6A). Furthermore, upon transfer from SDS to DMPC/CHAPS/SDS, the Trp fluorescence of the DCCD modified aO-1 increased by 62% with fast and slow phases (data not shown). The extents of the fast and slow phases were 36 and 26%, respectively, compared to the case of the unmodified aO-1 (*8*). Therefore, the carbodiimide modification did not affect the formation of the apolar environment around Trp residues upon transfer from SDS to CHAPS/SDS and DMPC/CHAPS/SDS (Fig. 6). These observations may support the idea that Trp fluorescence changes do not
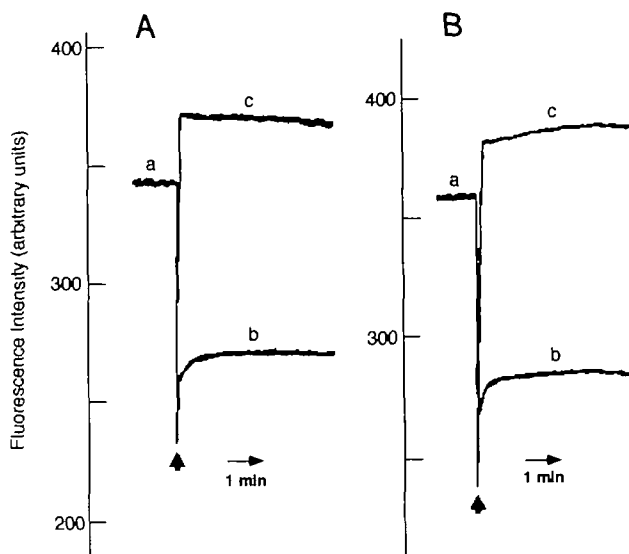
always follow the formation of the retinal binding pocket, as suggested in the previous study (*8*). Rather, the Trp fluorescence may change even when the proper packing of the side chains does not occur in the regeneration medium (DMPC/CHAPS/SDS).

DISCUSSION

The Differences in Chemical Reactivity between aO-1 and bO in Bleached Membrane—The results of differential scanning calorimetry and FTIR spectroscopy suggested that the loop regions of bO in bleached membrane had a more open conformation with weak interhelical interactions (*18, 19*). This view was supported by the observation that loop BC was cleaved by chymotrypsin in bleached membrane but not in the native bR (*20, 21*). Furthermore, it was shown that the transmembrane α_{II} helical region acquires motional freedom after removal of retinal (*22*). Therefore, DCCD and NCD-4 could penetrate into the hydrophobic space and modify the site(s) of bO in bleached membrane at neutral pH. Since the modified bO can bind to retinal and the 520 nm chromophore is thereby regenerated, the modification would indirectly influence the local environment of the retinal binding pocket. In addition, it can be easily supposed that the introduction of a bulky cyclohexyl ring into the retinal binding pocket could reduce the access of retinal. Therefore, candidates for the modified site might not be internal Asp residues (D85, D96, D115, and D212) in the retinal binding pocket but Asp and Glu residues located near the surface. It has been reported that modification of a surface carboxyl residue (Glu74) of purple membrane with water-soluble carbodiimide affected the acid-induced purple-to-blue transition and caused inhibition of the chromophore regeneration rate, but did not induce a blue-shift of the purple chromophore (*23*). Thus

the DCCD and NCD-4 modified site(s) may not be Glu74. Further analysis of the modified site(s) is required. In contrast, the lack of effects of DCCD and NCD-4 would indicate that the loop and/or transmembrane of aO-1 in bleached membrane are less flexible than those of bO, and thus DCCD and NCD-4 can not penetrate into the hydrophobic region at neutral pH. Another possibility is that DCCD and NCD-4 modify sites of aO-1 which are not related to retinal binding or the opsin shift. In accordance with this interpretation, we observed that when bR/purple membrane was solubilized with 0.5% MEGA-9 instead of Triton X-100 and then incubated with DCCD overnight in the dark, λ_{max} of the chromophore shifted from 560 nm to 505 nm, whereas little blue shift occurred for MEGA-9 solubilized aR-1/claret membrane in the presence of DCCD. Thus, it can be concluded that the location and chemical reactivity of some carboxyl residues differ between aO-1 and bO in membranes. The comparison of the 3D structure of aR-1 with that of bR will provide valuable information about the side chains (X-ray crystallography of aR-1 is in progress in collaboration with Prof. Kouyama of Nagoya University).

The Differences in Chemical Reactivity between the D-State and N-State—In the previous study, we designated the bacterial opsins (aO-1 and bO) in SDS, CHAPS/SDS and DMPC/CHAPS/SDS as the D-state, D_2-state, and N-state, respectively (8). In contrast to the native bR and bleached membrane, little is known about the structures of aO-1 and bO in the D-state and D_2-state. That lysyl endopeptidase and V-8 protease attacked aO-1 in the D-state but not in the N-state (Y. Sugiyama, unpublished observation) indicated that the surface loop regions were exposed to an aqueous environment only in the D-state. We applied chemical modification to obtain structural information about the helical (hydrophobic) regions in the D-state. The finding that the inhibition of the chromophore regeneration by carboxyl group modification occurred only for the D-state of aO-1 and bO (Fig. 2) clearly showed differences in the chemical environments of carboxyl residues between the D-state and N-state. The spectroscopic properties of the N-acylurea conjugate indicated that the modified site, which would be Asp85 or Asp96 in the case of aO-1, was located in a highly hydrophobic (less than hexane but more than ethanol) (Fig. 3) and optically active environment in the D-state (Fig. 5). In addition, the hydrophobic regions around and near the internal carboxyl group(s) in the D-state are more flexible than those in the N-state, to which DCCD or NCD-4 can easily gain access and react with in an acid-catalyzed reaction at neutral pH. This interpretation would be supported by the observation that the protonated carboxylic groups of bR become ionized and the interactions between the helices are lost on SDS solubilization (24, 25).

The Differences between aO-1 and bO in the D-State— The present results would indicate that NCD-4 modified Asp85 or Asp96 of aO-1 in the D-state. The observation of regeneration of the chromophore on incubation of an Asp212→Asn mutant of bO with 10 μM NCD-4 (data not shown) suggested that the modified site relevant to chromophore regeneration was Asp212 in the case of bO in the D-state. In addition, a modification not relevant to chromophore regeneration occurred when bO was incubated with low concentrations of NCD-4. Thus, the chemical environments around the internal carboxyl residues and the spatial

arrangements of helical regions would differ between aO-1 and bO in the D-state. These differences might be related to the previous observation that aO-1 exhibited a lower affinity to DMPC in the chromophore regeneration medium than bO did (8). Further analysis of aO-1 and bO in the D-state by means of dynamic light scattering measurements will provide us with information on *in vitro* refolding (Kameyama, K. *et al.*, in preparation).

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