The Na/Ca-K Exchanger of Rod Photoreceptor Exists as Dimer in the Plasma Membrane†

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Received May 1, 1997; Revised Manuscript Received August 6, 1997⊗

ABSTRACT: The oligomeric state of the Na/Ca-K exchanger in the plasma membrane of bovine photoreceptors was investigated using chemical cross-linking techniques. In the natural membrane, virtually all Na/Ca-K exchanger could be cross-linked mainly to a complex having an apparent molecular mass of 490 kDa by cupric phenanthroline catalyzed disulfide bonding as evidenced by Western blotting. Stable cross-links of the exchanger were also obtained with the thiol-specific reagent *N*,*N*′-*p*-phenylidenedimaleimide. Neuraminidase treatment reduced the apparent molecular mass of the highly glycosylated Na/Ca-K exchanger and of the 490 kDa cross-link product by 50 and 85 kDa, respectively. DL-1,4-Bismaleimido-2,3-butanediol (BMBD), a novel cleavable dimaleimide, was synthesized in order to produce cross-links that were stable to reductive conditions. Purification of the BMBD cross-linked exchanger followed by two-dimensional SDS polyacrylamide electrophoresis identified the cross-linked homodimers of the exchanger. There was no indication of higher oligomers, suggesting that the exchanger exists as a dimer in the plasma membrane. Hydrodynamic properties of the detergent-solubilized exchanger were determined by velocity sedimentation and gel filtration chromatography. The Triton X-100-solubilized exchanger ran as a single species having a Stokes radius of 10.0 nm, a sedimentation coefficient of 5.4 S, and a partial specific volume of 0.74 mL/g in Triton X-100. Similar results were obtained for the CHAPS-solubilized exchanger. A molecular mass of 236 and 205 kDa was calculated for the exchanger−detergent complex and the detergent-free protein, respectively. Neuraminidase treatment further reduced the molecular mass of the exchanger indicating that glycosylation contributes significantly to the mass of the exchanger. Cross-links of the exchanger were not detected if cross-linking was attempted after solubilization in 10 mM CHAPS. However, after reconstitution of the purified exchanger into soybean phosphatidylcholine vesicles, chemical cross-linking yielded again dimers. On the basis of these cross-linking and hydrodynamic studies, we conclude that the exchanger exists as a homodimer in the rod outer segment plasma membrane but dissociates into a monomer when solubilized in detergent.

Calcium (Ca) plays an important role in intracellular signaling. Therefore, cells maintain a low cytoplasmic Ca concentration which is achieved either by Ca-ATPases or Na/Ca exchange. In vertebrate photoreceptors, Ca entering the cell through the cGMP-gated channel basically controls light adaptation (Yau, 1994; Lagnado & Baylor, 1994; Koch, 1994). These cells extrude Ca efficiently *via* Na/Ca-K exchange. Functionally, the retinal Na/Ca-K exchanger is comparable to the more widespread cardiac-type Na/Ca exchanger: (a) both transporter proteins use the inward Na gradient as a driving force to extrude intracellular Ca (Perry & McNaughton, 1993; Cervetto et al., 1989; Schnetkamp et al., 1989); (b) in both proteins Na/Ca exchange is an electrogenic process because one excess electrical charge is transported opposite to Ca (Lagnado & McNaughton, 1990).

Despite of these similarities, there are distinct functional and structural differences between the two transport proteins: (a) the Ca transport rate of the cardiac Na/Ca exchanger is more than an order of magnitude greater than the transport rate of the retinal Na/Ca-K exchanger (Hilgemann, 1996; Niggli & Lederer, 1991; Hilgemann et al., 1991; Huppertz & Bauer, 1994; Hodgkin et al., 1987); (b) the retinal Na/Ca-K exchanger uses the outward K-gradient as driving force in addition to the inward Na-gradient to extrude Ca (Perry & McNaughton, 1993; Nicoll et al., 1991; Friedel et al., 1991; Schnetkamp & Szerencsei, 1991; Cervetto et al., 1989; Schnetkamp et al., 1989) thus shifting the steady state equilibrium for Ca below 1 nM (Lagnado & McNaughton, 1990; Cervetto et al., 1989); (c) as opposed to the cardiac Na/Ca-exchanger, the retinal Na/Ca-K exchanger is heavily glycosylated (Cook & Kaupp, 1988; Reid et al., 1990); and (d) there is only little sequence homology between the two transporter proteins (Nicoll et al., 1990; Reiländer et al., 1992).

The molecular organization of these proteins has rarely been addressed experimentally. Previously, we have reported evidence that Na/Ca-K exchanger and cGMP-gated channels are associated in the plasma membrane (Bauer & Drechsler,

† Supported by the Deutsche Forschungsgemeinschaft (Ba 721/1-1) and a grant from the National Institutes of Health (EY 02422).

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⊗ Abstract published in *Advance ACS Abstracts,* October 15, 1997.

S0006-2960(97)01023-4 CCC: $14.00

1992). We report here that, moreover, the retinal Na/Ca-K exchanger exists as homodimer in the ROS plasma membrane.

Preliminary accounts of part of this work have been given to the Biophysical Society (Schwarzer et al., 1997; Bauer, 1995).

MATERIALS AND METHODS

Preparation of Rod Outer Segment (ROS) Membranes. Bovine ROS[1] were purified by discontinuous sucrose flotation (Bauer, 1988) or continuous sucrose gradient centrifugation (Molday & Molday, 1987). Aliquots of purified ROS containing 5 mg of rhodopsin were pelleted in 600 mM sucrose by centrifugation for 30 min at 15000*g* and 4 °C (buffer: 10 mM HEPES/KOH, pH 7.0, 300 mM KCl, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.1 mM EDTA, 0.5 mM DTT), shock-frozen in liquid nitrogen, and stored at −80 °C until use.

ROS membranes were obtained by hypotonic lysis of the ROS in 0.5 mM TRIS, 0.5 mM HEPES, pH 7.4, 1 mM EDTA, and the protease inhibitors 5 μg/mL aprotinin, 5 μg/mL leupeptin, 2.2 μg/mL E64, and 1 mM DTT (wash buffer) followed by centrifugation at 48300*g* for 15 min at 4 °C. The membranes were washed once in the same buffer and twice in the absence of DTT.

Synthesis of DL-1,4-Bismaleimido-2,3-butanediol (BMBD). DL-1,4-Diamino-2,3-butanediol dihydrobromide (846 mg, 3 mmol), prepared by the reaction of DL-1,4-dibromo-2,3-butanediol (Aldrich) with potassium phthalimide (Aldrich), followed by heating of the resulting DL-1,4-bisphthalimido-2,3-butanediol in 48% HBr according to a known procedure (Feit & Nielsen, 1967), was added to a well-stirred mixture of 1.0 g (12 mmol) of $NaHCO_3$ in 15 mL of tetrahydrofuran and 6 mL of water at 0 °C. The reaction mixture was stirred at 0 °C for 2 min, and 1.117 g (7.2 mmol) of *N*-methoxycarbonylmaleimide (Sigma) was added portionwise. After 10 min, 252 mg (3 mmol) of $NaHCO_3$ was added, and the mixture was stirred at room temperature for 3 h. The product was then isolated by extraction into ethyl acetate (5 × 50 mL). The combined ethyl acetate extracts were washed with water and brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. Addition of 10 mL of diethyl ether and filtration gave colorless crystals in 19.5% (164 mg) yield, m.p. 202−204 °C (decomp.). ^{1}H NMR (DMSO-d_6) δ 3.41 (m, 2H, CH), 3.64 (m, 4H, CH_2), 4.87 (d, 2H, OH), 7.05 (s, 4H, =CH); mass spectrum 281.1 $[M+H]^+$. Anal. Calcd for $C_{12}H_{12}O_6N_2$ (280.24): C, 51.43; H, 4.32; N, 10.00. Found: C, 51.64; H, 4.51; N, 9.43.

Cross-Linking of Thiol Groups and NEM Modification of ROS Membranes. ROS membrane suspensions (0.15−0.2 mg of rhodopsin/mL) were cross-linked with dimaleimides (pPDM or BMBD) in 10 mM HEPES/NaOH, pH 6.5, 100 mM NaCl, 1 mM EDTA, 1 μM DTT, protease inhibitors (see above) at room temperature under dim red light. The reaction was started by adding 10−100 μM (normally 12 μM) reagent from a freshly prepared 10 mM stock solution in dimethyl sulfoxide (DMSO) and terminated with 10 mM DTT (final concentration). NEM modification of SH groups was carried out at 1 mM final concentration of reagent in the same buffer (stock solution: 100 mM NEM in DMSO, freshly prepared). The DMSO concentration in the ROS membrane suspension never exceeded 1% (v/v).

Catalyzed oxidation of adjacent SH groups to disulfide bonds was carried out at room temperature with 1,10-phenanthroline-complexed copper (CuPhe) in 30 mM HEPES/KOH, pH 7.4, containing protease inhibitors. A CuPhe stock solution of 2 mM $CuCl_2$ and 10 mM 1,10-phenanthroline was prepared in this buffer (1,10-phenanthroline stock solution: 500 mM in DMSO); all solutions were freshly prepared before use. The reaction was started by adding an equal volume of CuPhe-stock solution to the ROS membrane suspension (in 30 mM HEPES/KOH, pH 7.4), and terminated after 30 min by complexation of Cu(II) with a final concentration of 10 mM EDTA.

Cleavage of the Cross-Links. Disulfide cross-linked proteins separated by non-reductive SDS−PAGE were cleaved by incubation of the gel for 20 min at room temperature in a reductive sample buffer for SDS electrophoresis, i.e. in 5% (v/v) BME, 50 mM TRIS/HCl, pH 7.6, 1.6% (w/v) SDS, 12.5% (w/v) glycerol, 0.005% (w/v) bromophenol blue.

In ROS membrane suspensions, BMBD cross-links were cleaved at room temperature by incubation for 20 min in 30 mM $NaIO_4$ buffered with 20 mM triethanolamine/HCl, pH 7.5. To cleave BMBD cross-links in the gel, TRIS buffer had to be thoroughly replaced with 20 mM triethanolamine/HCl, pH 7.5, 0.1% (w/v) SDS by gentle shaking for 2 h with buffer changes every 15 min, because TRIS interferes with periodate. BMBD cross-links were then cleaved by incubation of the gel for 30 min in 30 mM $NaIO_4$. (It should be noted that potassium ions must not be present because these ions precipitate periodate.) The gel was then exposed for 15 min at room temperature to 300 mM $NH_2OH{\cdot}HCl$ with 0.1% (w/v) SDS adjusted to pH 7.5 with NaOH to inactivate the aldehyde groups generated by oxidative cleavage. Finally, the gel was incubated for 5 min in 1.6% (w/v) SDS, 50 mM TRIS/HCl, pH 7.6, 12.5% (w/v) glycerol, 0.005% (w/v) bromophenol blue before it was mounted onto the analytical gel (see below).

Neuraminidase Treatment of Cross-Linked ROS. An aliquot of purified ROS (containing 5 mg of rhodopsin) was resuspended in 30 mL of 10 mM HEPES/NaOH, pH 7.4, 100 mM NaCl, 540 mM sucrose, protease inhibitors (see above) and pelleted for 15 min at 7700*g*, 4 °C. The pellet was resuspended in 10 mM HEPES/NaOH, pH 6.5, 100 mM NaCl, 540 mM sucrose, 1 mM EDTA to a final concentration of 0.15 mg of rhodopsin/mL. Two aliquots of 12 mL (each containing 1.8 mg of rhodopsin) were taken out, one was cross-linked as follows, and the second served as a control. Cross-linking was started by adding 100 μM BMBD at room temperature and was stopped after 30 min by adding 10 mM DTT (final concentrations). Both aliquots were pelleted as before and washed twice in 10 mM HEPES/NaOH, pH 7.4, 100 mM NaCl, 540 mM sucrose. For neuraminidase treatment (Molday & Molday, 1987), the pellets were resuspended each in 500 μL of 20 mM TRIS/acetate, pH 7.2, 2 mM $MgCl_2$, 10 mM glucose, 5 mM taurine, 580 mM

[1] Abbreviations: BME, β-mercaptoethanol; BMBD, DL-1,4-bismaleimido-2,3-butanediol; CHAPS, 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; E64, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; NEM, *N*-ethylmaleimide; PC, L-α-phosphatidylcholine; pPDM, *N*,*N*′-*p*-phenylidenedimaleimide; ROS, rod outer segment; SDS−PAGE, sodium dodecyl sulfate polyacrylamide electrophoresis; TRIS, tris(hydroxymethyl)aminomethane.

sucrose. One half of each aliquot was preserved as a control. The second half of each aliquot was incubated for 2 h at 4 °C in 0.04 units of *Anthrobacter ureafaciens* neuraminidase per mg of rhodopsin. The aliquots were then pelleted and washed once hypotonically in 1 mM DTT as described above.

pPDM Treatment of Solubilized and of Reconstituted ROS Membrane Proteins. Solubilization and reconstitution of ROS membrane proteins were carried out as described previously (Bauer & Drechsler, 1992) with the following modification: the buffer was 10 mM HEPES/KOH, pH 6.5, 100 mM KCl, 1 mM EDTA, and protease inhibitors (see above), without DTT.

To treat solubilized ROS membrane proteins with pPDM, the ROS membranes were solubilized at 4 °C in 6 mM CHAPS with 2 mg of soybean PC/mL and then centrifuged for 10 min at 48300*g*. 100 μM pPDM was added to the supernatant at room temperature; after 10 min the reaction was stopped with 100 mM DTT. Reconstitution was performed by dialysis for 2 h at 4 °C in a commercially available setup (Liposmat, Diachema AG, Munich, Germany).

To cross-link reconstituted ROS membrane proteins with pPDM, the ROS membranes were solubilized in 10 mM CHAPS with 4 mg of soybean PC/mL as described before and dialyzed for 2 h. Cross-linking in 100 μM pPDM at room temperature was terminated after 10 min with 100 mM DTT. In both instances, the reconstituted vesicles were pelleted at 540000*g*, 4 °C for 30 min (Beckman TL-100, rotor TLA-100.3) and analyzed by SDS−PAGE.

Purification of Na/Ca-K Exchanger. The Na/Ca-K exchanger was purified following the procedure of Cook and Kaupp (1988), except that potassium ions were replaced in all buffers with sodium ions and that the protease inhibitors aprotinin (5 μg/mL), leupeptin (5 μg/mL), and E64 (2.2 μg/mL) were used. Washed ROS membranes (with or without prior cross-linking) containing 25 mg of rhodopsin were solubilized (1 mg of rhodopsin/mL) in 18 mM CHAPS, 2 mg of soybean PC/mL, 10 mM HEPES/NaOH, pH 7.4, 150 mM NaCl, 10 mM $CaCl_2$, 1 mM DTT, and the above protease inhibitors, as described in the previous paragraph. The solubilized proteins were applied to a DEAE cellulose column (1 × 3 cm), and the bound fraction eluted with the same buffer system but 750 mM NaCl and 15 mM CHAPS. This eluate was cycled for 90 min on an AF-red Fractogel-TSK (Merck) column (1 × 3 cm) at 0.25 mL/min to remove the cGMP-gated channel protein (Cook & Kaupp, 1988). The nonbound fraction was applied to a second AF-red Fractogel-TSK column to remove residual channel protein. After addition of 1 mM $MgCl_2$ and 1 mM $MnCl_2$ (final concentrations), the eluate was applied to a Con-A-Sepharose 4B column (1 × 3 cm). The bound fraction was eluted with 100 mM α-methyl-D-mannoside buffered in 15 mM CHAPS, 2 mg of soybean PC/mL, 10 mM HEPES/NaOH, pH 7.4, 100 mM NaCl, 10 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM DTT, protease inhibitors. To reconstitute the eluted protein into vesicles, the eluate was dialyzed against 10 mM HEPES/NaOH, pH 7.4, 100 mM NaCl. All purification and reconstitution procedures were carried out at 4 °C.

Electrophoresis and Protein Assay. Unless noted otherwise, SDS−PAGE was carried out on Laemmli-type gels (Laemmli, 1970). The stacking gel contained 3% acrylamide, and the analytical gel contained a linear gradient of 3.5%−7.5% acrylamide. Slab gels of 7.3 cm × 8.3 cm and 1 mm thickness (Biometra) were used for electrophoresis (run at 20 mA). For a consecutive analysis of the proteins in the second dimension after cleavage of cross-links, a 1.5 mm thick analytical 3.5%−7.5% gradient slab gel of the same size was prepared, and the gel lane to be analyzed was fixed with 1% (w/v) agarose on top of this gel, which was then run at 25 mA. The polyacrylamide gels were run for 15 min at 20 mA before the samples were applied. Samples were denatured at room temperature for 30 min in 1.6% (w/v) SDS, 50 mM TRIS/HCl, pH 7.6, 12.5% (w/v) glycerol, 0.005% (w/v) bromophenol blue, with or without 5% (v/v) BME for reductive or non-reductive electrophoresis of the proteins, respectively. Molecular masses were determined based on the marker proteins (HMW-SDS kit, Pharmacia): myosin (212 kDa), α_2-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa), glutamic dehydrogenase (53 kDa). Protein content was determined following the Amido Black procedure (Kaplan & Pedersen, 1985) using Millipore HA filters of 0.45 μm pore size.

In some experiments, molecular masses were also determined by SDS−PAGE according to Weber and Osborn (1969) using as protein standards cross-linked rabbit phosphorylase *b* (Sigma P8906; molecular masses of the monomer up to the pentamer were 97.4, 194.8, 292, 389.6, and 487 kDa) and the HMW-SDS kit of Pharmacia (see before). The polyacrylamide gradient gels had the same size as described above (7.3 cm × 8.3 cm, 1 mm thick; gradient 3.5%−7.5%). The electrode buffer was 28 mM NaH_2PO_4 and 72 mM Na_2HPO_4 (pH 7.2) containing 0.1% (w/v) SDS. Samples were denatured for 30 min at 40 °C in 1% (w/v) SDS, 2% (v/v) BME, 6 M urea, 0.015% (w/v) bromophenol blue in the same phosphate buffer. The electrophoresis was run for 14 h at 12 mA.

Western Blotting. Proteins separated by SDS−PAGE were electrophoretically transferred onto a polyvinylidene difluoride membrane (Immobilon P, Millipore) using the Biometra Fast Blot B33 setup. The transfer buffer was 192 mM glycine, 25 mM TRIS/HCl, pH 8.3, 10% (v/v) methanol. Five layers of filter paper, soaked with transfer buffer, were placed on each side of the Immobilon P membrane-gel sandwich. Blotting conditions were 2 h and 2.8 mA/cm^2. The blotted membrane was dried and stored at 4 °C until labeled with antibodies.

Before labeling, the blotted membrane was blocked with 1% (w/v) nonfat milk in phosphate-buffered saline (136 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) for 30 min and then incubated for 1 h with the primary antibody in phosphate buffer containing 0.02% (w/v) Tween 20. After three 10 min washes in the same buffer, the blot was incubated for 30 min in horseradish peroxidase conjugated secondary antibody, followed by another three 10 min washes in phosphate-buffered saline containing 0.02% (w/v) Tween 20 before being washed in phosphate-buffered saline without Tween 20 for at least 30 min. All procedures were carried out at room temperature. Antibody labeling was detected using ECL reagents (Amersham) following the instructions of the manufacturer. Finally, the antibodies were removed from the membrane by incubation in 2% (v/v) BME, 2% (w/v) SDS, 62 mM TRIS/HCl, pH 6.8, at 65 °C for 45 min, followed by another three washes in phosphate-buffered saline. The latter protocol removed the antibodies completely, thus allowing relabeling of the same blotted membrane with another antibody.

Monoclonal antibodies directed against epitopes of the rod Na/Ca-K exchanger exposed on the cytoplasmic (PMe 1B3) and extracellular (PMe 2D9) surface of bovine ROS plasma membranes have been previously described (Reid et al., 1990a,b).

Velocity Sedimentation and Gel Filtration Chromatography. Untreated, neuramindase-treated, and pPDM cross-linked ROS membranes were solubilized at a final protein concentration of 1 mg/mL in Buffer A (10 mM HEPES, pH 7.4, 150 mM KCl, 2 mM $CaCl_2$, 1 mM DTT) containing either 1.0% Triton X-100 or 16 mM CHAPS. For velocity sedimentation analysis, 100 μL of solubilized membrane containing marker enzymes (malate dehydrogenase, aldolase, and catalase) were layered on a 4.8 mL 5%−20% linear sucrose gradient in H_2O or D_2O made with Buffer A containing 0.2% Triton X-100 or 12 mM CHAPS, and centrifugation was carried out for 6.5 h (H_2O) and 16 h (D_2O) at 49 000 rpm in a Beckman SW 50.1 rotor at 4 °C. After centrifugation, the tubes were punctured and 8 drop fractions were collected and assayed for the Na/Ca-K exchanger by Western blotting and laser densitometry and for marker enzyme activity as previously described (Goldberg & Molday, 1996).

Gel filtration chromatography was used to determine the Stokes radius of the solubilized Na/Ca-K exchanger by the method of Laurent and Killander (1964). Detergent-solubilized ROS membranes (150 μL) containing 3% glycerol were applied to a 33 × 0.7 cm Sepharose CL-4B or Sepharose CL-6B column equilibrated in Buffer A containing 0.2% Triton X-100 or 12 mM CHAPS. Fractions (125 μL) from the column collected at 4 °C were assayed for the Na/Ca-K exchanger by Western blotting and laser densitometry. The column was calibrated using marker proteins (aldolase, catalase, ferritin, and thyroglobulin) as previously described (Goldberg & Molday, 1996).

The sedimentation coefficient ($s_{20,w}$) and partial specific volume (ν) of the exchanger−detergent complex were calculated from the measured sedimentation coefficients in H_2O (s_H) and D_2O (s_D) according to the method of Clarke and Smigel (1989). The molecular weight of the exchanger−detergent complex was determined from the $s_{20,w}$, ν, and Stokes radius using the Svedberg equation, and the molecular weight of the protein portion of the exchanger−detergent complex was estimated from the partial specific volumes of the detergent, protein, and protein−detergent complex with the assumption that detergent binding is the same in H_2O and D_2O (Clarke & Smigel, 1989). Partial specific volumes used in the analysis are 0.908 mL/g for Triton X-100 (Tanford et al., 1974), 0.81 mL/g for CHAPS (Hjelmeland et al., 1983), and 0.714 mL/g for the Na/Ca-K exchanger [calculated from the amino acid composition (Zamyatnin, 1984)].

RESULTS

SH Cross-Linking of the Na/Ca-K Exchanger in the ROS Membrane. The retinal Na/Ca-K exchanger associates into oligomers in the rod outer segment (ROS) plasma membrane. If ROS membranes were hypotonically washed under non-reducing conditions and analyzed with discontinuous Laemmli-type SDS−PAGE in the absence of BME, a strong band at 250 kDa for the monomeric exchanger along with two minor bands at 190 and 340 kDa were detected by Western blot analysis using the monoclonal antibody PMe 2D9 (Figure 1, lane a). Catalyzed oxidation of adjacent thiol groups to disulfide bonds with 1,10-phenanthroline-complexed copper (CuPhe), in the absence of reducing reagents, yielded heavy labeling at 490 kDa along with bands at 420, 340, and 250 kDa (Figure 1, lane b). The monomer band at 250 kDa was very weak proving that most of the exchanger was in the oligomeric state.

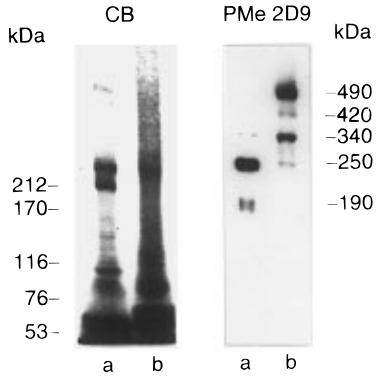

FIGURE 1: Formation of disulfide bridges of the retinal Na/Ca-K exchanger. ROS membranes prepared from purified ROS by hypotonic washes once in 1 mM DTT and twice in the absence of reducing agents. SDS−PAGE was performed in a gradient gel (3.5%−7.5% polyacrylamide) and in the absence of reducing agents. Shown are a Coomassie Blue stained gel (CB) and a Western blot labeled with the monoclonal antibody PMe 2D9 against the Na/Ca-K exchanger. Washed membranes (lane a), after catalyzed oxidation of adjacent thiol groups with phenanthroline-complexed copper (CuPhe; lane b).

Because cross-linked proteins sometimes migrate anomalously on Laemmli-type gels, molecular masses were also determined by continuous SDS−PAGE according to Weber and Osborn (1969). Using cross-linked phosphorylase *b* as protein standard (see Materials and Methods), we found again apparent molecular masses of 250 and 490 kDa for the monomeric and the cross-linked exchanger, whereas the minor bands ran at 290 and 390 kDa (data not shown). This result confirms that analysis of the monomeric and the cross-linked exchanger by discontinuous SDS−PAGE is adequate.

Noncleavable linkages between adjacent thiol groups were formed using the homo-bifunctional cross-linking reagent pPDM. SDS−PAGE in 5% BME and Western blotting of ROS membranes treated with pPDM showed three distinct additional bands at the apparent molecular masses of 340, 420, and 490 kDa in addition to the 250 kDa monomer exchanger band (Figure 2, lane b). No cross-linked bands were observed if the SH groups of the membrane proteins were modified with NEM before pPDM treatment, proving that pPDM specifically cross-linked adjacent cysteine residues (Figure 2, lane c). There was no labeling above 490 kDa, which is about twice the apparent molecular mass of the monomer exchanger molecule, suggesting that the retinal Na/Ca-K exchanger exists as a homodimer in the plasma membrane.

As opposed to cross-linking *via* disulfide bonds, there was generally a distinct band at 250 kDa due to monomeric exchanger after cross-linking with bismaleimides, suggesting that the cross-linking efficiency for bismaleimides was reduced. Several reasons may account for this observation. (a) Although pPDM presumably permeates the membrane, only about half of the exchanger molecules are easily

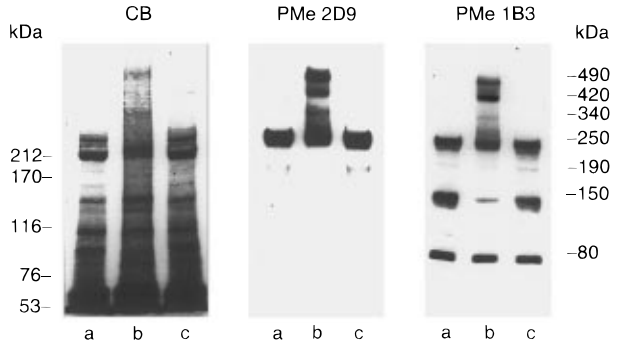

FIGURE 2: Thiol-specific reagent *p*-phenylidenedimaleimide (pPDM) cross-links the Na/Ca-K exchanger. ROS membranes hypotonically washed as in Figure 1 (lane a), after cross-linking with pPDM (lane b), and after NEM-blockage of cysteines followed by treatment with pPDM (lane c). Electrophoresis was carried out in 5% β-mercaptoethanol (BME) on a 3.5%−7.5% polyacrylamide gel. The gel was stained with Coomassie Blue (CB), and a second gel was electrotransferred onto an Immobilon membrane. The Western blot was consecutively labeled with two different monoclonal antibodies against the Na/Ca-K exchanger, *viz*. with PMe 2D9, and after removal of this antibody, with PMe 1B3 which recognizes a domain in the large cytoplasmic loop (Reiländer et al., 1992).

FIGURE 3: Cross-linking of adjacent SH groups with the cleavable reagent BMBD. Immunoblot of a polyacrylamide gradient gel after electrophoresis of washed ROS membranes in 5% BME, as in Figure 2. The blot was labeled with the monoclonal antibody PMe 2D9. Control membranes (lane a); membranes after cross-linking with BMBD (lane b); BMBD cross-linked membranes after oxidation with 30 mM $NaIO_4$ (20 min, room temperature, lane c); control membranes incubated for 50 min at room temperature in 30 mM $NaIO_4$ (lane d). Note, this blot was exposed longer than the blots in Figures 1 and 2 to verify that the BMBD cross-links were completely cleaved.

accessible for SH cross-linking due to the sidedness of membrane vesicles. (b) Disulfide bridges formed after hypotonic washes under non-reducing conditions (see above) prevent cross-linking. (c) Two molecules of the reagent may bind to adjacent SH groups thus preventing the formation of cross-links; this effect becomes apparent at higher concentrations of reagent.

Moreover, a weak band was marked at 190 kDa with PMe 2D9. Reprobing of the same Western blot with PMe 1B3, another monoclonal antibody against the exchanger (Reid et al., 1990a) which recognizes the cytoplasmic domain (Reiländer et al., 1992), yielded additional bands at 150 and 80 kDa which were not recognized by PMe 2D9. These bands which are blotted more efficiently than the monomer exchanger due to the lower molecular mass (Wang et al., 1989) suggest that a small proportion of the protein was degraded although the protease inhibitors aprotinin, leupeptin, and E64 were used throughout. Cross-linking of these fragments to themselves or to an exchanger may account for some of the cross-linked products intermediate between the 250 kDa monomer and 490 kDa dimer (see below).

Cross-Linking with a Cleavable SH-Specific Reagent. To identify the covalently bonded proteins, the cross-links had to be cleaved again. Cross-links formed with pPDM cannot be cleaved, however. Reductive cleavage is possible for disulfide bridges, but these bonds are not suited for purification procedures that require reductive conditions. Therefore, the cleavable bismaleimide BMBD was synthesized by a procedure similar to the method for the preparation of bismaleimidoalkanes (Cheronis et al., 1992). BMBD yielded the same cross-links as pPDM between adjacent thiol groups which were stable under reductive conditions but were readily cleavable at neutral pH by oxidation of the glycol group with sodium periodate (Figure 3). The advantage in using BMBD was that the cross-linked products could be purified before being analyzed after oxidative cleavage (see below).

Analysis of Cross-Linked Exchanger after Cleavage. Both BMBD-mediated cross-links and CuPhe-catalyzed disulfide bonds could be completely cleaved in a 3.5%−7.5% polyacrylamide gel matrix. BMBD cross-linked ROS membranes were subjected to SDS−PAGE in BME and were then oxidatively cleaved in the gel matrix and analyzed in a second gradient gel (3.5%−7.5% polyacrylamide). Western blot analysis of this gel labeled with PMe 1B3 exhibited distinct spots at 250 kDa (Figure 4). Proteins which were not cross-linked are localized along a diagonal line after electrophoresis in the second dimension. The band localized at 490 kDa in the first dimension gel yielded a strongly labeled spot at 250 kDa, whereas the band at 420 kDa gave spots at 250 and 150 kDa, suggesting that this band was due to a cross-link of an intact exchanger molecule with a partially degraded exchanger molecule. The second spot at 150 kDa is presumably due to dimerized 150 kDa protein. It should be stressed that there is no uncleaved material at 490 kDa in the Western blot, verifying that the cross-links were completely cleaved. These findings were further supported by similar experiments carried out on CuPhe treated ROS membranes. The 490 kDa cross-linked product observed after electrophoresis in the first dimension under nonreducing conditions was completely reduced to a 250 kDa product when electrophoresis was carried out under reducing conditions in the second dimension (data not shown).

Neuraminidase-Treated Membranes. The above findings suggest that either dimerization of the exchanger or association with some other retinal protein with molecular mass similar to the exchanger took place. Since there are several proteins in ROS membranes with molecular masses near 250 kDa, the molecular mass of the Na/Ca-K exchanger was intentionally reduced to discriminate between these two possibilities. Removal of sialic acid residues by neuraminidase as described (Molday & Molday, 1987; Reid et al., 1990) reduced the apparent molecular mass of the Na/Ca-K exchanger by about 50 kDa, i.e., to 200 kDa (Figure 5). Upon cross-linking with BMBD and consecutive neuraminidase treatment of ROS membranes, the highest cross-linked product of the exchanger was detected at 405 kDa, i.e., 85 kDa lower than in nontreated membranes (Figure 5, lane d). The difference of molecular masses between nontreated and neuraminidase-treated membranes was, therefore, almost

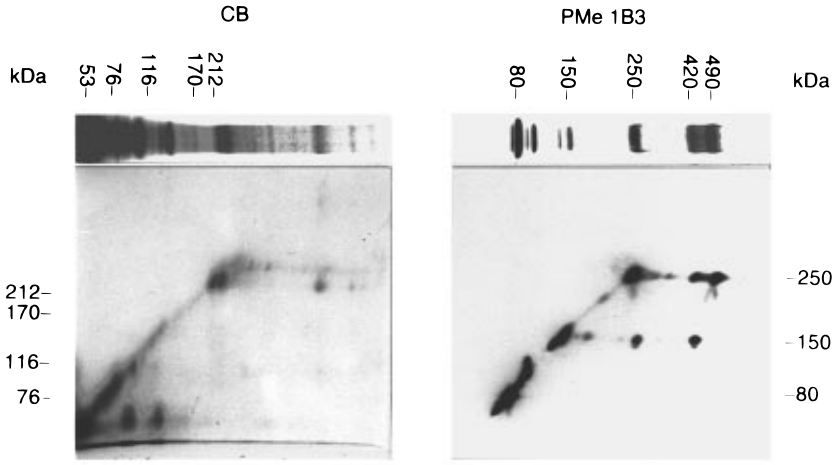

FIGURE 4: Cleavage and immunoblot analysis of BMBD cross-links of Na/Ca-K exchanger. Hypotonically washed ROS membranes were cross-linked with BMBD and subjected in 5% BME to SDS−PAGE, as in Figure 3. One lane of the gel was cut out, the cross-links oxidatively cleaved (see Materials and Methods), and analyzed by a second 3.5%−7.5% SDS−PAGE. After the second electrophoresis, proteins which were not cross-linked are located on a diagonal line; cross-linked proteins which were oxidatively cleaved are marked right from the diagonal line. For the sake of clarity, one lane of the first electrophoresis is shown on top of the second electrophoresis. The Coomassie Blue-stained gels (CB) are shown together with PMe 1B3 labeled Western blots of the first and the second electrophoresis gels.

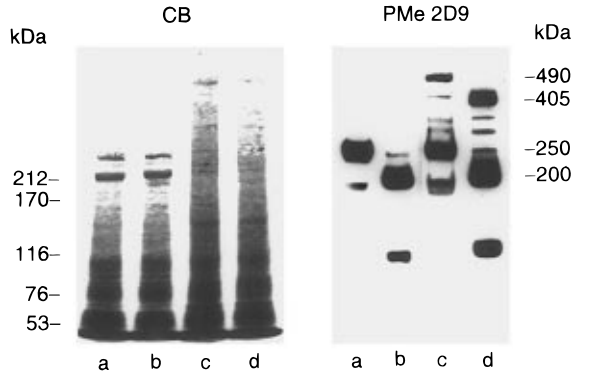

FIGURE 5: Influence of neuraminidase treatment on cross-links of the Na/Ca-K exchanger. Purified ROS were cross-linked with BMBD, and subsequently treated with neuraminidase (*A. ureafaciens*). Neuraminidase treatment was carried out as described (Molday & Molday, 1987). As a control, purified ROS without cross-linking were subjected to the neuraminidase treatment. ROS membranes were then prepared by a hypotonic wash in the presence of 1 mM DTT. SDS−PAGE and Western blotting was carried out as in Figure 2. Control membranes without cross-linking (lane a); neuraminidase-treated membranes without cross-linking (lane b); control membranes with BMBD cross-linking (lane c); neuraminidase-treated membranes with BMBD cross-linking (lane d). Shown are a Coomassie Blue-stained gel (CB) and a Western blot labeled with PMe 2D9.

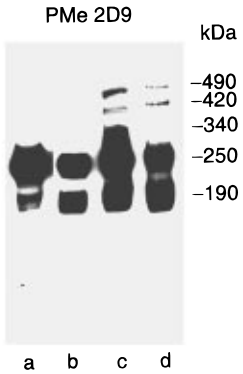

FIGURE 6: Cross-linking of solubilized and of reconstituted ROS membrane proteins. Comparison between the cross-linking efficiency of the exchanger in solubilized ROS membranes and in ROS membrane proteins which were reconstituted in soybean phosphatidylcholine (PC) vesicles. Solubilization and reconstitution of ROS membrane proteins was performed as described (Bauer & Drechsler, 1992). ROS membranes solubilized in 6 mM CHAPS containing 2 mg of soybean PC per mg of rhodopsin were treated with pPDM and then reconstituted by dialysis (lane a). In a different experiment, ROS membranes were solubilized in 10 mM CHAPS containing 4 mg of soybean PC per mg of rhodopsin and reconstituted by dialysis (lane b) and then cross-linked with pPDM (lane c). Finally, Na/Ca-K exchanger was purified, reconstituted into PC vesicles, and cross-linked with pPDM (lane d). Lanes a−c contain 20 μg of rhodopsin, and lane d contains 5 μg of protein. The SDS−PAGE was carried out as above. The Western blot was labeled with PMe 2D9.

twice as great for the highest cross-linked bands as for the monomer exchanger. After cleavage of the BMBD cross-links, the 405 kDa band yielded only a single spot at 200 kDa in the immunoblot of the two-dimensional SDS−PAGE, confirming that this band is due to a homodimer (not shown).

Cross-Linking of Solubilized, Reconstituted, and Purified Na/Ca-K Exchanger. If cross-linking with pPDM was attempted with ROS membrane proteins solubilized in 6 mM CHAPS, i.e. above the critical micellar concentration of the detergent, no cross-linked bands of the exchanger were detectable (Figure 6, lane a). If, however, solubilized ROS membrane proteins were reconstituted into soybean PC vesicles, a small fraction of Na/Ca-K exchanger was cross-linked with pPDM (Figure 6, lane c). Similarly, a small fraction of cross-linked exchanger was detected when the exchanger was cross-linked after purification by DEAE ion exchange and AF red Fractogel chromatography and reconstitution into soybean PC vesicles (Figure 6, lane d). These findings suggest that there is no dimerization of the exchanger when solubilized in CHAPS but partial dimerization takes place upon reconstitution into lipid vesicles.

Purification and Analysis of the Cross-Linked Exchanger. It has been inferred from Ca-flux studies that the retinal Na/Ca-K exchanger and the cGMP-gated channel are associated (Bauer & Drechsler, 1992). To examine if there are cross-links between both proteins, ROS membranes were cross-linked with BMBD, and the exchanger and its cross-linked products were purified following mainly a standard protocol (Cook & Kaupp, 1988). After oxidative cleavage of the 490 kDa, and the 420 kDa cross-links in the gel matrix, two-dimensional SDS−PAGE and immunoblot analysis with

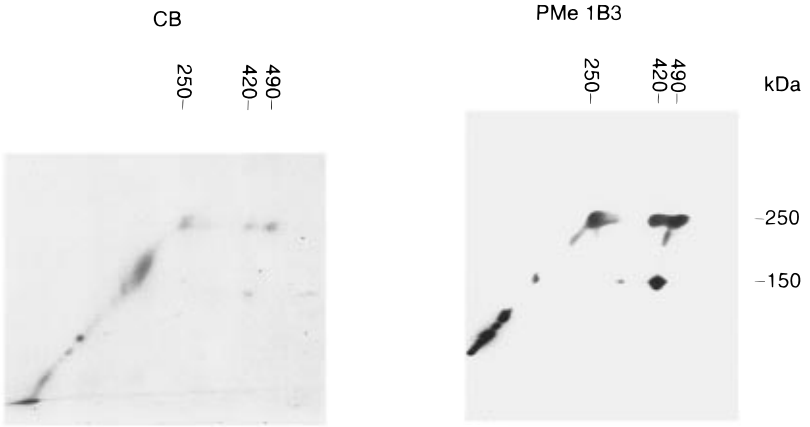

FIGURE 7: Purification and two-dimensional immunoblot analysis of cross-linked Na/Ca-K exchanger. Hypotonically washed ROS membranes were cross-linked with BMBD. The Na/Ca-K exchanger was purified from ROS membranes solubilized in 18 mM CHAPS containing 2 mg of soybean PC/mL. The purification protocol comprised consecutive chromatography through DEAE-, AF-red-, and Con-A-Sepharose columns (see Materials and Methods). The purified protein was reconstituted by dialysis. Protein analysis was performed as in Figure 4: cross-linked and not cross-linked protein (3.4 μg) were separated by SDS−PAGE, and the cross-links cleaved oxidatively with $NaIO_4$, and analyzed by a second SDS−PAGE. A Coomassie Blue-stained gel (CB) and a Western blot labeled with the monoclonal antibody PMe 1B3 are shown.

PMe 1B3 revealed distinct spots at 250 kDa (Figure 7), proving that these cross-links contained an intact exchanger molecule. The 420 kDa cross-linked product yielded after cleavage a second spot at 150 kDa suggesting that this band was a cross-link of an intact and a degraded exchanger molecule. Reprobing of the same blot with the monoclonal antibody PMs 5E11, directed against the β-subunit of the cGMP-gated channel (Chen et al., 1994; Molday et al., 1990), yielded no labeling at 490 kDa (not shown). Therefore, the 490 kDa band was not due to a cross-link between exchanger and β-subunit of the cGMP-gated channel. Furthermore, reprobing of the same blot with PMc 2G11, a monoclonal antibody against the α-subunit of the channel (Molday et al., 1992), yielded no labeling, confirming that the cGMP-gated channel was not present (not shown).

Hydrodynamic Properties and the Molecular Weight of the Detergent-Solubilized Exchanger. Gel filtration chromatography and velocity sedimentation were carried out to determine the hydrodynamic properties and the size of the detergent-solubilized exchanger. As shown in Figure 8, the Triton X-100-solubilized exchanger eluted from a calibrated Sepharose CL 4B column as a single component having a Stokes radius of 10.0 nm. Centrifugation on sucrose gradients prepared in H_2O and D_2O also yielded single species of the exchanger having sedimentation coefficients of $s_H = 3.27$ S in H_2O and $s_D = 1.65$ S in D_2O (Figure 9). The small change in the position of the exchanger relative to the marker enzymes in H_2O and D_2O indicates that the exchanger binds a comparatively small amount of detergent. A value of 0.148 g of Triton X-100 bound per g of protein is calculated from a partial specific volume (= 0.739 mL/g) of the exchanger−detergent complex. Relatively low detergent binding to the exchanger suggested by this technique is likely due in part to extensive glycosylation of the exchanger (Reid et al., 1990a). Indeed, removal of terminal sialic acid residues with neuraminidase resulted in an increase in the partial specific volume of the exchanger−detergent complex to 0.754 mL/g and a corresponding apparent increase in Triton X-100 binding to 0.265 g of detergent bound/g of protein (Table 1).

From the sedimentation coefficient ($s_{20,w} = 5.42$ S), the partial specific volume (= 0.739 mL/g) and the Stokes radius

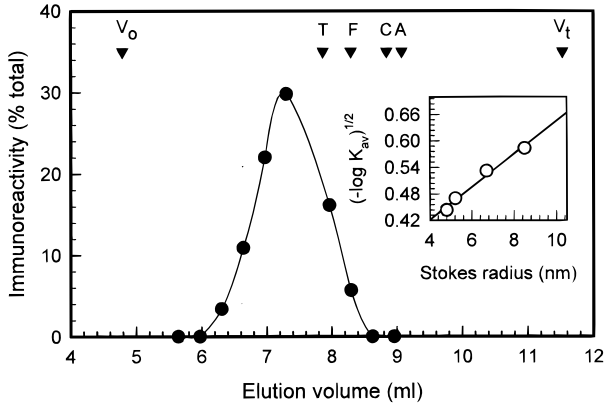

FIGURE 8: Gel filtration chromatography of the Triton X-100-solubilized Na/Ca-K exchanger. ROS membranes (150 μg) solubilized in 1% Triton X-100 were applied to a Sepharose CL-4B column and fractions were assayed for the exchanger on Western blots labeled with the PMe 2D9 antibody and quantified by laser densitometry. The positions of the void volume (V_o), total volume (V_t) and marker proteins thyroglobulin (T), ferritin (F), catalase (C), and aldolase (A) are indicated. The inset illustrates the calibration plot of the marker proteins of known Stokes radii. The distribution coefficient $K_{av} = (V_e - V_o)/(V_t - V_o)$, where V_e is the elution volume of the proteins.

($a = 10$ nm), a molecular mass of 236 kDa was calculated for the exchanger−Triton X-100 complex. A molecular mass of 205 kDa is obtained for the exchanger when the amount of detergent bound is taken into account. A comparable value was obtained for the CHAPS solubilized exchanger (Table 1). The calculated molecular mass of the exchanger represents an upper limit since the contribution of the carbohydrate to the partial specific volume of the exchanger is unknown. A reduced molecular mass (174 kDa) calculated for the neuraminidase-treated, detergent-free exchanger indicates that carbohydrates contribute significantly to the mass of the exchanger, a result consistent with SDS−PAGE analysis of the exchanger (Reid et al., 1990a). From these results, we conclude that the Na/Ca-K exchanger solubilized in either Triton X-100 or CHAPS exists primarily as a monomer. The values for the hydrodynamic properties and molecular mass of the exchanger are summarized in Table 1.

Table 1: Hydrodynamic Properties of the Bovine Rod Photoreceptor Na/Ca-K Exchanger and the Neuraminidase-Treated Exchanger[a]

parameter	Triton X-100[b]	CHAPS[c]
sedimentation coefficient ($s_{20,w}$)	5.42 ± 0.32 S (4.95 S)	5.87 S
partial specific volume (ν)	0.739 ± 0.026 mL/g (0.754 mL/g)	0.720 mL/g
Stokes radius	10.0 ± 0.2 nm (9.6 ± 0.2 nm)	9.4 ± 0.2 nm
mol. mass of exchanger−detergent complex	236 ± 27 (219 kDa)	229
detergent binding (g/g of protein)[d]	0.148 ± 0.008 (0.265)	0.067
mol. mass of exchanger (free of detergent)	205 ± 33 (174 kDa)	214

[a] Numbers represent the mean value and when indicated ± standard deviation calculated at the 90% probability level. [b] Calculated from velocity sedimentation experiments in H_2O ($n = 5$) and D_2O ($n = 4$) sucrose gradients and three trials of Sepharose CL-4B chromatography. [c] Calculated from velocity sedimentation experiments in H_2O ($n = 2$) and D_2O ($n = 2$) sucrose gradients and three trials of Sepharose CL-6B chromatography. Values in parentheses represent the hydrodynamic characteristics of the neuraminidase-treated exchanger from velocity sedimentation experiments in H_2O ($n = 2$) and D_2O ($n = 2$) sucrose gradients and three trials of CL-4B chromatography. [d] Calculated using $\nu = 0.714$ mL/g for the exchanger protein, $\nu = 0.908$ mL/g for Triton X-100, and $\nu = 0.81$ mL/g for CHAPS.

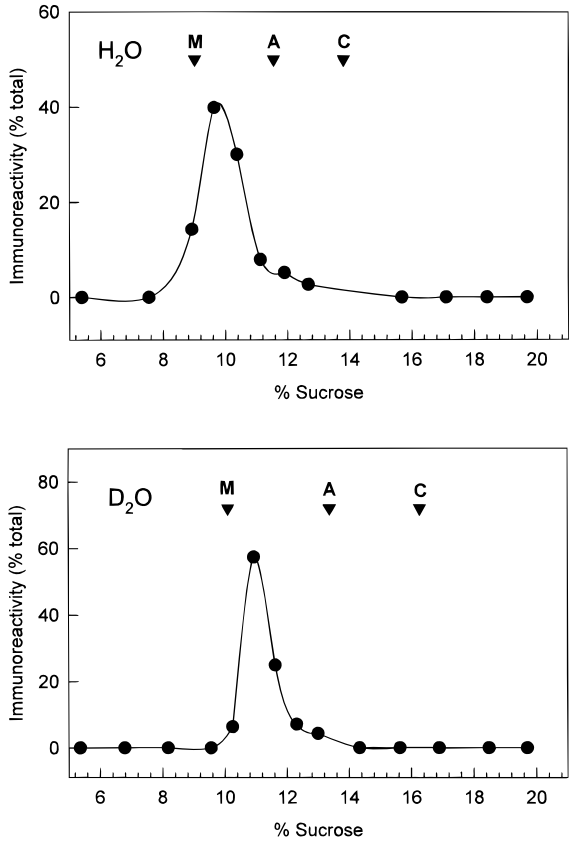

FIGURE 9: Velocity sedimentation of the Triton X-100-solubilized Na/Ca-K exchanger. ROS membranes solubilized in 1% Triton X-100 were sedimented through a 5%−20% sucrose gradient prepared in H_2O (upper panel) or D_2O (lower panel) together with marker enzymes. Fractions were assayed for exchanger on Western blots labeled with the PMe 2D9 antibody and quantified by laser densitometry. Marker enzymes malate dehydrogenase (M), aldolase (A), and catalase (C) sedimented to the indicated positions.

Sedimentation Analysis of the Cross-Linked Exchanger. The sedimentation behavior of the exchanger which had been cross-linked in the ROS membrane with pPDM prior to solubilization in Triton X-100 was studied (Figure 10). Western blots of the fractions from the sucrose gradient revealed the presence of two species which sedimented at different rates. The slower component sedimented at the same rate as the uncross-linked exchanger and corresponded to the 250 kDa noncross-linked (monomeric) exchanger observed by SDS−PAGE; the faster component contains the 490 kDa cross-linked (dimeric) exchanger.

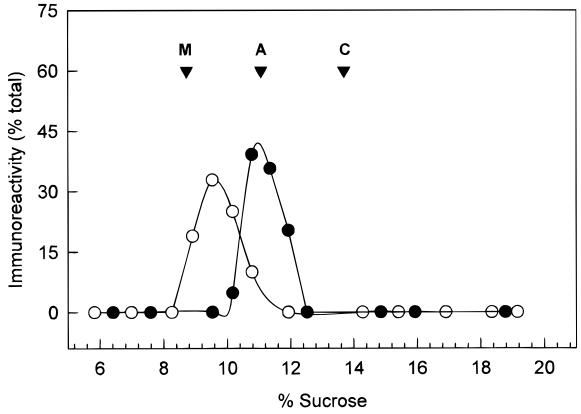

FIGURE 10: Velocity sedimentation of the pPDM-cross-linked Na/Ca-K exchanger. ROS membranes were cross-linked with pPDM, solubilized in Triton X-100, and sedimented through a 5%−20% sucrose gradient prepared in H_2O. Fractions were assayed for the noncross-linked (○) exchanger (apparent $M_r \sim 250$K by SDS−PAGE) and the cross-linked (●) exchanger ($M_r \sim 490$K by SDS−PAGE) on Western blots labeled with the PMe 2D9 antibody and quantified by laser densitometry. Positions of the marker enzymes malate dehydrogenase (M), aldolase (A), and catalase (C) are indicated.

DISCUSSION

Our results demonstrate for the first time that the Na/Ca-K exchanger exists as a homodimer in the plasma membrane of photoreceptor ROS. The self-association of the exchanger appears to be specific for the following reasons. (a) Although the Na/Ca-K exchanger comprises only a minor fraction of the ROS plasma membrane proteins (Cook & Kaupp, 1988), virtually all of the exchanger can be disulfide-bonded in the biological membrane to dimers but not to higher oligomers. (b) Although the exchanger contains six cysteines only a dimer band is labeled. (c) Partial dimerization must take place at lower detergent concentrations because dimers of the exchanger are detected after reconstitution by dialysis of the solubilized exchanger into soybean PC vesicles.

On the other hand, hydrodynamic studies indicate that the Triton X-100- and CHAPS-solubilized exchanger exists as a monomer. The calculated molecular mass of the exchanger is higher (~205 kDa) than that predicted from its amino acid sequence (~130 kDa). This difference appears to be due largely to the glycosylation of the exchanger since removal of terminal sialic acid residues by neuraminidase leads to a significant reduction in the apparent molecular mass of the exchanger. The inability of the detergent-solubilized exchanger to be cross-linked into a dimer further supports the view that the exchanger exists as a monomer upon solubilization in Triton X-100 or CHAPS.

Besides the 490 kDa dimer band of the exchanger, we find cross-links of the exchanger at 420 and 340 kDa upon

cross-linking of the exchanger in the ROS plasma membrane. The weak band at 420 kDa is probably due to an association of an intact exchanger with the 150 kDa exchanger fragment, because after cleavage of the cross-links and SDS−PAGE this fragment is labeled with PMe 1B3 and also with a polyclonal antibody against the exchanger[2] (data not shown). On the other hand, the origin of the distinct 340 kDa cross-link is not clear. It was only obtained in the natural ROS membrane and after reconstitution of solubilized ROS proteins into soybean PC, but it was not found if the purified and reconstituted exchanger was cross-linked.

Na/Ca exchangers belong to the more general family of transporter proteins which share the common structural feature of about 12 transmembrane domains (Reithmeier, 1994). Many members of this family exist *in situ* as oligomers, mostly dimers, including, *e.g.*, Na-driven transporters (Pessino et al., 1991; Fafournoux et al., 1994; Otsu et al., 1993; Fliegel et al., 1993) and the erythrocyte anion transporter (Casey & Reithmeier, 1991, 1993). The oligomeric states of several transporters have been determined from hydrodynamic measurements. For example, the anion exchanger of red blood cells has been reported to exist as a dimer in detergent (Casey & Reithmeier, 1993), whereas the sodium- and chloride-coupled glycine transporter has recently been shown to be a monomer in CHAPS (Lopez-Corcuera et al., 1993). Cross-linking studies taken together with hydrodynamic studies indicate that the Na/Ca-K exchanger exists as a homodimer in the ROS plasma membrane but dissociates into a monomer when solubilized in nondenaturing detergents.

As opposed to the Na/Ca-K exchanger, the more widespread cardiac-type Na/Ca exchanger has not yet been reported to be dimerized. However, given the fact that the Ca-ATPase of the plasma membrane, the major Ca-extruding protein of most cells, has also been found to be dimerized *in situ* (Vorherr et al., 1991), it appears likely that dimerization is a common feature of other Ca-extruding proteins, as well.

What is the functional significance of dimerization of the Na/Ca-K exchanger? Many transport proteins are found to form dimers but, in most cases, it is unclear if oligomerization influences these proteins functionally. For the Na/H exchanger of placental brush border membranes it was reported that oligomerization does not influence the transport properties of the protein (Fafournoux et al., 1994). Presumably, the retinal Na/Ca-K exchanger is also functionally active as a monomer because this study indicates that it is predominantly reconstituted in the monomeric state, and the reconstituted exchanger has previously been shown to be fully active (Cook & Kaupp, 1988; Bauer & Drechsler, 1992). Dimerization may also contribute to the observed cooperative dependence on Na of Ca transport (Huppertz & Bauer, 1994). Moreover, recalling the low exchange rate of this transport protein (Huppertz & Bauer, 1994; Hodgkin et al., 1987), the functional significance of dimerization may be the local doubling of the exchange rate.

In summary, we reported here immunoblot studies and hydrodynamic measurements which show that (a) the Na/Ca-K exchanger of ROS could be cross-linked with SH-specific reagents; (b) removal of sialic acid residues reduced the apparent molecular mass of both cross-linked and monomer exchanger, the reduction in molecular mass being almost twice as great for the cross-linked than for the monomeric protein; (c) purification of the cross-linked exchanger and subsequent cleavage of the cross-links revealed that the exchanger was cross-linked to a homodimer; (d) no cross-links of the exchanger were observed if cross-linking was attempted with the solubilized exchanger. Together with the hydrodynamic studies, these findings provide compelling evidence that the exchanger exists as a homodimer in the natural membrane and as a monomer in detergent.

[2] Gift of H. Reiländer.

ACKNOWLEDGMENT

We thank Dr. H. Reiländer for providing a polyclonal antibody against the Na/Ca-K exchanger. The technical assistance of H. Schauf is gratefully acknowledged.

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BI9710232