Detergent Interaction with Band 3, a Model Polytopic Membrane Protein†

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ABSTRACT: The interaction of band 3, the 95-kDa anion-exchange protein of the human erythrocyte membrane, with a variety of nonionic detergents was studied. Band 3 dimers (Stokes radius = 76 Å) prepared in octaethylene glycol monododecyl ether ($C_{12}E_8$) could be exchanged into a variety of detergents by size-exclusion high-performance liquid chromatography (HPLC), with complete removal of $C_{12}E_8$ from band 3 being confirmed using radiolabeled detergent. Critical micellar concentration (cmc) values, determined for all detergents in the buffer used for HPLC analysis, ranged from 0.47 μM to 223 mM. Band 3 was found to aggregate in all detergents below their cmc, and concentrations of detergents 2–200 times the cmc were required to prevent aggregation. For detergents with a low cmc, it was important to ensure that the concentration of detergent micelles minimally equalled the concentration of protein. Hydrodynamic measurements and cross-linking studies showed that band 3 remained dimeric in most detergents above their cmc. Furthermore, circular dichroism and inhibitor binding studies supported the view that band 3 can retain its native structure after detergent exchange. Detergents with short alkyl chains (C_8) denature band 3, while detergents with longer alkyl chains (C_{12}) maintained the native structure of band 3. The ability to exchange band 3 into a variety of detergents with the maintenance of native structure is an essential prerequisite for crystallization trials. The results obtained in this study of band 3, a model polytopic (multispanning) membrane protein, may be generally applicable to other membrane proteins.

Band 3 is the 95-kDa integral membrane glycoprotein of the erythrocyte membrane, responsible for anion exchange (Jay & Cantley, 1986; Jennings, 1989). Anion exchange in erythrocytes is sensitive to inhibition by stilbenedisulfonates, and band 3 was first identified (Cabantchik & Rothstein, 1974) as the anion transporter by covalent labeling with the potent anion-exchange inhibitor 4,4′-diisothiocyanatostilbene-2,2′-disulfonate (DIDS).[1] Band 3 comprises approximately 50% by weight of the integral membrane protein of the erythrocyte, and the protein can be readily purified in the presence of detergents using standard chromatographic techniques (Casey et al., 1989). Mild trypsin treatment of ghost membranes divides band 3 into a carboxyl-terminal 52-kDa membrane domain and an amino-terminal 43-kDa cytoplasmic domain (Steck et al., 1976). Independent of the cytoplasmic domain, the 52-kDa domain mediates anion exchange (Grinstein et al., 1979; Kopito et al., 1989; Lepke et al., 1992). The 43-kDa domain binds hemoglobin and glycolytic enzymes and interacts with the cytoskeleton via ankyrin (Low, 1986). The amino acid sequences of mouse (Kopito & Lodish, 1985), rat (Kudrycki & Schull, 1989), chicken (Cox & Lazarides, 1988), rainbow trout (Hübner et al., 1992), and human (Tanner et al., 1988) erythrocyte band 3 proteins have been deduced by cDNA sequencing. The sequences are highly conserved, with the highest degree of similarity in the membrane domain which is predicted to span the membrane up to 14 times.

Band 3 exists as a mixture of dimers and tetramers in the membrane and in detergent solution (Jennings, 1984). Band 3 tetramers are thought to interact with the cytoskeleton, via binding to ankyrin (Low, 1986). Band 3 is able to equilibrate between oligomeric states in the plane of the membrane, but not in detergent solution (Casey & Reithmeier, 1991). However, another group has reported that band 3 in freshly isolated preparations, in TrX-100 solution, exists in a monomer–dimer–tetramer equilibrium (Schubert et al., 1983). Conditions were found in which homogeneous band 3 dimers could be isolated (Casey & Reithmeier, 1991). Although small crystals of band 3 have been formed using a heterogeneous mixture of band 3 oligomers (Reithmeier et al., 1989), homogeneous dimers may provide a superior starting preparation for protein crystallization trials. However, many different detergents need to be examined for their ability to support crystal growth. Band 3 must, therefore, either be purified in a series of detergents or be purified in one detergent and exchanged into others. In either case, the detergent used must maintain the native structure and function of band 3. Detergent exchange provides a more rapid means for preparing membrane proteins in a series of detergents. Gel filtration using an HPLC system is a suitable technique for detergent exchange since it is efficient, gentle, and rapid.

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[1] Abbreviations: ANSA, 8-anilinonaphthalene-1-sulfonic acid magnesium salt; BADS, 4-benzamido-4′-aminostilbene-2,2′-disulfonate; cmc, critical micellar concentration; C_8DAO, *N*,*N*-dimethyloctylamine *N*-oxide; C_{10}DAO, *N*,*N*-dimethyldecylamine *N*-oxide; C_{12}DAO, *N*,*N*-dimethyldodecylamine *N*-oxide; C_{12}DAPS, *N*-dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate; C_8E_5, octaethylene glycol monododecyl ether; ether; $C_{10}E_6$, hexaethylene glycol monodecyl ether; $C_{12}E_6$, hexaethylene glycol monododecyl ether; $C_{12}E_8$, octaethylene glycol monodecyl ether; $C_{12}E_9$, nonaethylene glycol monododecyl ether; $C_{14}E_8$, octaethylene glycol monotetradecyl ether; $C_{16}E_8$, octaethylene glycol monohexadecyl ether; C_8glucoside, octyl β-D-pyranoside; C_{10}glucoside, decyl β-D-pyranoside; C_{12}glucoside, dodecyl β-D-pyranoside; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; C_{10}maltoside, decyl β-D-maltopyranoside; C_{12}maltoside, dodecyl β-D-maltopyranoside; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonate; DTSSP, 3,3′-dithiobis(sulfosuccinimidyl propionate); EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; pCMB, *p*-(chloromercuri)benzoate; SE, size exclusion; TrX-100, Triton X-100; TrX-100_{red}, reduced Triton X-100.

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In this study, the oligomeric states of homogeneous preparations of dimeric band 3, isolated in $C_{12}E_8$ detergent, have been analyzed by size-exclusion HPLC in a variety of detergents. Since the cmc of detergents may vary with pH and ionic strength, cmc values for the detergents employed were determined under the conditions in which the band 3 oligomeric state was analyzed. As a member of a family of polytopic (multispanning) membrane proteins, band 3 is a good model to examine the detergent needed to support a homogeneous, dispersed oligomeric state.

EXPERIMENTAL PROCEDURES

Materials. Octaethylene glycol monododecyl ether ($C_{12}E_8$) was from Nikko Chemical Co., Tokyo, Japan. Pentaethylene glycol monooctyl ether (C_8E_5), hexaethylene glycol monododecyl ether ($C_{12}E_6$), octaethylene glycol monotetradecyl ether ($C_{14}E_8$), octaethylene glycol monodecyl ether ($C_{10}E_8$), hexaethylene glycol monodecyl ether ($C_{10}E_6$), octaethylene glycol monohexadecyl ether ($C_{16}E_8$), *N*,*N*-dimethyloctylamine *N*-oxide (C_8DAO), *N*,*N*-dimethyldecylamine *N*-oxide (C_{10}DAO), *N*,*N*-dimethyldodecylamine *N*-oxide (C_{12}DAO), and reduced Triton X-100 ($TrX\text{-}100_{red}$) were purchased from Fluka Chemicals. Triton X-100 (TrX-100), dodecyl β-D-maltopyranoside (C_{12}maltoside), octyl β-D-pyranoside (C_8-glucoside), dodecyl β-D-pyranoside (C_{12}glucoside), and *N*-dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate (C_{12}DAPS) were from Boehringer Mannheim. Decyl β-D-pyranoside (C_{10}-glucoside), decyl β-D-maltopyranoside (C_{10}maltoside), and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) were from Calbiochem. Sodium dodecyl sulfate (SDS) was from ICN. [^{14}C]$C_{12}E_8$ was purchased from Research Products International, Mount Prospect, IL. Perylene was purchased from Sigma Chemical Co. 3,3′-Dithiobis(sulfosuccinimidyl propionate) (DTSSP) was from Pierce Chemical Co. 8-Anilinonaphthalene-1-sulfonic acid magnesium salt (ANSA) was from Eastman-Kodak Co. All other chemicals were reagent grade or better. 4-Benzamido-4′-aminostilbene-2,2′-disulfonate (BADS) was synthesized as described (Casey et al., 1989).

Isolation of Band 3. Dimeric band 3 was purified from red blood cells obtained from the Canadian Red Cross Society, by modification of previously described protocols (Casey et al., 1989; Casey & Reithmeier, 1991). Briefly, red cells were washed, and ghosts were prepared by osmotic hemolysis in 5 mM sodium phosphate, pH 8.0. Ghost membranes were depleted of band 6 by two incubations of 20 min, on ice, in 150 mM NaCl/5 mM sodium phosphate, pH 7.4, containing 0.2 mM dithiothreitol. Band 3 was extracted from pelleted ghosts with 1 volume of 1% (v/v) $C_{12}E_8$/5 mM sodium phosphate, pH 8.0, containing 0.2 mM dithiothreitol, and the cytoskeleton was pelleted by centrifugation at 100000*g* for 30 min at 4 °C. This procedure solubilizes a homogeneous population of band 3 dimers, leaving the cytoskeleton and any associated band 3 intact (Casey & Reithmeier, 1991). The supernatant was applied to aminoethyl-Sepharose 4B (0.2–0.5 mL of resin/mg of protein) and eluted with a 0–0.25 M linear sodium chloride gradient in 0.1% $C_{12}E_8$ (v/v)/5 mM sodium phosphate, pH 8.0. Band 3 was also prepared in C_{12}-maltoside or TrX-100, using the same extraction and purification procedure.

Cross-Linking of Band 3. Red cells were treated with the membrane-impermeant bifunctional cross-linker 3,3′-dithiobis(sulfosuccinimidyl propionate) (DTSSP) to cross-link band 3 to covalent dimers (Salhany & Sloan, 1989).

High-Performance Liquid Chromatography (HPLC). SE experiments were performed at room temperature using either a 7.5 × 300 mm TSK 4000 SW column or SEC 4000 columns. A Spectra-Physics SP8800 HPLC pump was used, at a flow rate of 0.5 mL/min. Purified band 3 (1–2 mg of protein/mL) was injected onto the column using either a 20-μL or a 500-μL injection loop. Protein elution was monitored at 215 nm using a Spectroflow 757 Flowthrough absorbance detector (ABI Analytical Co.). Chromatograms were recorded and peaks integrated using a Rainin Macintegrator. The standard elution buffer contained various concentrations of detergents, 100 mM NaCl, and 5 mM sodium phosphate, pH 7.0. The column was equilibrated with at least 2 bed volumes of elution buffer before application of samples. The column was calibrated with suitable protein standards which do not bind detergent (LeMaire et al., 1986).

Exchange of detergent by SE chromatography was followed using [^{14}C]$C_{12}E_8$. Band 3 (50 μL, 1–2 mg of protein/mL) in 0.1% (v/v) $C_{12}E_8$, 0.1 M sodium chloride, and 5 mM sodium phosphate, pH 8.0, was incubated overnight at 4 °C after addition of 1 μL of 0.04% (v/v) $C_{12}E_8$ containing [^{14}C]$C_{12}E_8$ (56.4 mCi/mmol). An aliquot (5 μL) was then subjected to SE HPLC, and fractions (0.5 mL) of the eluant were collected and subjected to scintillation counting.

Determination of Critical Micellar Concentration. The critical micellar concentrations of nonionic detergents were determined by following the rise in fluorescence of the hydrophobic dye ANSA associated with micelle formation (Mast & Haynes, 1975). Standard elution buffer (2.5 mL), containing 16 μM ANSA, was titrated in a 1 × 1 cm fluorescence cuvette with consecutive additions of detergent stock solutions made up in elution buffer. Fluorescence was measured at room temperature in a stirred cell after each addition of detergent. Fluorescence (excitation = 345 nm, emission = 490 nm), detected with a Spex Fluorolog fluorometer, was corrected for dilution. ANSA is charged and cannot be used with some detergents. Therefore, cmc values for ionic and amine oxide detergents were similarly determined as follows using the hydrophobic dye perylene in a modification of a published protocol (Mast & Haynes, 1975). Perylene solution (25 μL) (0.1 mg/mL in chloroform) was placed onto the bottom of a dry 1 × 1 cm cuvette. The chloroform was removed under a stream of air, and 2.5 mL of standard buffer was added. Fluorescence (excitation = 250 nm, emission = 450 nm) was monitored upon consecutive addition of detergent stock solutions. In experiments with ANSA or perylene, cmc values were determined from the fluorescence "breakpoint" in plots of fluorescence versus concentration detergent, the breakpoint being defined as the intersection of two best-fit lines. The first of these lines was through the points at low detergent detergent, before the rapid fluorescence increase, and the second was through the region of rapid increase (Mast & Haynes, 1975).

BADS Binding Assay. Band 3 in detergents other than $C_{12}E_8$ or C_{12}maltoside was prepared by detergent exchange. Samples (100 μL) of band 3 at 1–2 mg/mL in $C_{12}E_8$ were applied to the SEC 4000 column and eluted with the appropriate detergent in 0.1 M sodium chloride/5 mM sodium phosphate, pH 7.0, and the band 3 peak was collected. Resolution of $C_{12}E_8$ from band 3, on this preparative scale, was determined using [^{14}C]$C_{12}E_8$. Detergent concentrations used in BADS binding experiments were 77 μM $C_{16}E_8$, 40 mM C_{12}DAO, 26 mM C_8E_5, 1.7 mM $C_{12}E_8$, and 1.7 mM C_{12}maltoside. In BADS binding assays, 2.0 mL of each sample (20–50 μg of band 3/mL of eution buffer) was placed in a 3-mL stirred fluorescence cell. Concentrated BADS was added sequentially up to a final concentration of 100 μM. The

Table I: Properties of Detergents

detergent	mol wt	aggregation[a] no.	micelle[b] MW	cmc[c] (mM)
C_8E_5	351	32	11000	6.0 (A)
$C_{10}E_6$	423	76	32000	0.46 (A)
$C_{10}E_8$	511			0.28 (A)
$C_{12}E_6$	481	105	50000	0.065 (A)
$C_{12}E_8$	539	120	65000	0.056 (A)
$C_{14}E_8$	567			0.0052 (A)
$C_{16}E_8$	595			0.00047 (A)
$TrX\text{-}100_{red}$	631	140	90000	0.21 (A)
C_8DAO	176			175 (A), 223 (P)
$C_{10}DAO$	201			9.1 (A), 6.0 (P)
$C_{12}DAO$	229	76	17300	0.48 (A), 0.23 (P)
C_{10}maltoside	483			1.4 (A)
C_{12}maltoside	511	98	50100	0.14 (A)
C_8glucoside	292	84	25000	25.4 (A)
C_{10}glucoside	322			4.2 (A)
C_{12}glucoside	348			0.14 (A)
$C_{12}DAPS$	336			2.1 (P)
SDS	288	100	28800	0.58 (P)
CHAPS	615	4–14	6000	6.2 (P)

[a] Aggregation number is a measure of the average number of detergent molecules in a micelle. [b] Micelle molecular weights determined by multiplying aggregation number by detergent molecular weight. Aggregation numbers from Neugebauer (1987). [c] Cmc determined using ANSA (A) or perylene (P) as probe.

binding of BADS to band 3 was measured by fluorescence enhancement (excitation = 280 nm, emission = 450 nm) at room temperature, in a Spex Fluorolog fluorometer. Fluorescence was corrected for dilution, self-quenching of the probe, and the background fluorescence of the sample and the probe.

Circular Dichroism Spectroscopy. Band 3, prepared in either 0.1% (w/v) (1.9 mM) $C_{12}E_8$ or 0.1% (w/v) (1.9 mM) C_{12}maltoside, was subjected to circular dichroism spectroscopy. Band 3 in 0.1 M sodium chloride, 5 mM sodium phosphate, pH 7.0, and the appropriate detergent was diluted 12-fold into distilled water, to a final protein concentration of approximately 40 μg/mL. Protein determinations were performed according to Lowry et al. (1951) and corrected to molar concentration (Casey et al., 1989). Far-ultraviolet circular dichroism spectra of band 3 were measured at room temperature using a Jasco J-720 spectropolarimeter and a quartz cell with a 0.1-cm path length. Spectra for buffer alone were subtracted from each spectrum. Secondary structure content (α-helix, β-sheet, random coil) was estimated by best-fit over the whole spectrum, using Jasco software and reference spectra from Yang et al. (1986).

Analytical Techniques. Protein assay was according to Lowry et al. (1951), using bovine serum albumin as standard.

RESULTS

Cmc Determinations. The interaction of band 3 with a variety of detergents above and below their cmc was studied after detergent exchange by size-exclusion HPLC. Detergents vary greatly in their molecular weights and aggregation numbers. Detergent properties also vary greatly depending on whether the detergent is in monomeric or micellar form. Therefore, a meaningful way to compare detergents and their interactions with membrane proteins is relative to their cmc. Table I lists the properties of the detergents used to examine band 3 oligomeric structure. To be rigorous, cmc values were determined under the conditions for SE chromatography. The table also lists aggregation numbers for some of the detergents (Neugebauer, 1987).

The cmc values range broadly, from 470 nM to 223 mM. As a general rule, the cmc decreases by an order of magnitude for every two carbons added to the detergent's alkyl chain. Comparison of detergents with C_{10} and C_{12} alkyl chains indicates that the order of cmc values is octaethylene glycol < hexaethylene glycol < glucoside < maltoside < dimethylamine oxide. That is, the amine oxides have the most soluble headgroup, which gives them the highest cmc.

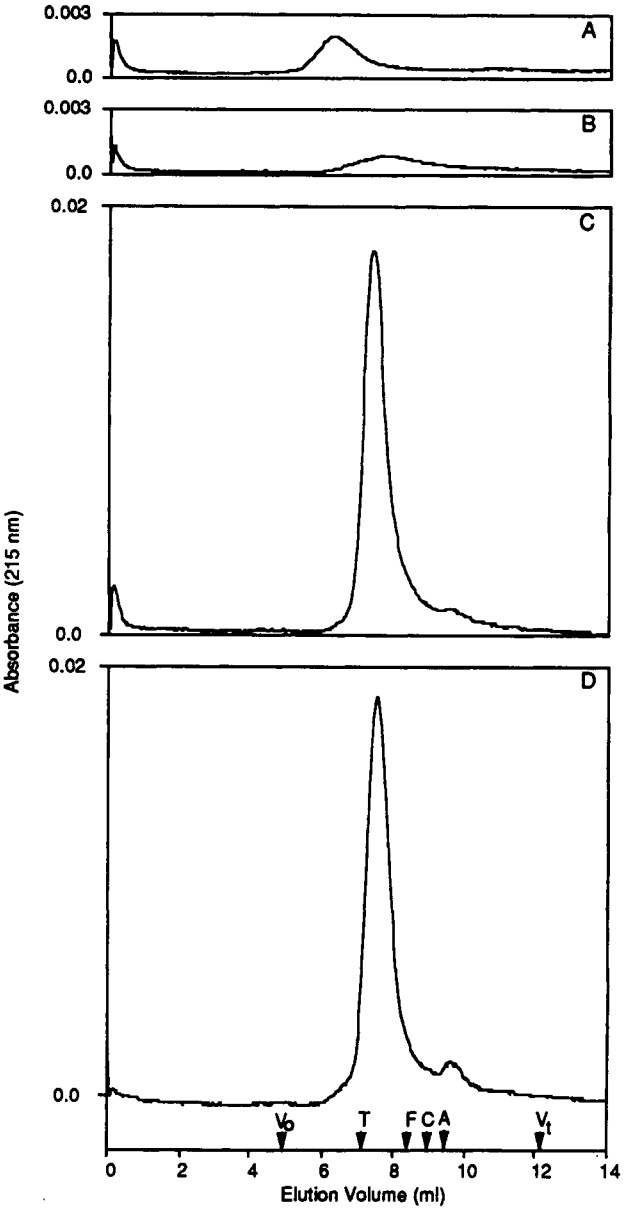

FIGURE 1: Detergent concentration dependence of the band 3 oligomeric state. Band 3 (6 μg) was chromatographed on a 30 × 0.75 cm SEC 4000 HPLC column in elution buffer containing 0.1 M sodium chloride, 5 mM sodium phosphate, pH 7.0, and (A) 0.028 mM $C_{12}E_8$, (B) 0.056 mM $C_{12}E_8$, (C) 0.112 mM $C_{12}E_8$, and (D) 1.85 mM $C_{12}E_8$.

Three detergents could not readily be used for studies of membrane proteins. C_{10}Glucoside and C_{12}glucoside were insufficiently soluble for use. At their cmc, the C_{10}glucoside detergent solution was cloudy while C_{12}glucoside would not dissolve completely. C_8DAO, while soluble, has an extremely high cmc (223 mM), so that very high detergent concentrations must be used for micelle formation.

Concentration Dependence for Band 3 Dispersion. Elution profiles of purified band 3 in various concentrations of $C_{12}E_8$ are shown in Figure 1. The major peak (R_s = 76 Å) seen in panel D has previously been identified as dimeric band 3 (Casey & Reithmeier, 1991). Below the cmc of $C_{12}E_8$ (panel A),

very little band 3 eluted from the column, indicating that band 3 is largely insoluble. Chromatography of band 3 at the cmc of $C_{12}E_8$ (0.056 mM) resulted in some disaggregation of the protein, so that the protein which eluted had a peak position close to that of dimeric band 3. Band 3 eluted at its dispersed dimeric position at 2 times the cmc (panel C). High concentrations of detergent did not result in dissociation of band 3 dimers. In panel D, the concentration of $C_{12}E_8$ was 30 times the cmc, yet the elution profile was unaltered from that at 2 times the cmc (panel C). The peak at 9.7 mL is due to micellar detergent and increases in amount with increasing detergent concentration.

Displacement of [^{14}C]$C_{12}E_8$ from Band 3. Band 3 binds 0.56 mg of $C_{12}E_8$ per milligram of of protein, equivalent to one micelle per band 3 monomer (Casey et al., 1992). Detergent exchange to replace the bound $C_{12}E_8$ was performed by SE chromatography, using an HPLC system. In each SE HPLC experiment, 5 μL of band 3, prepared in 1.9 mM $C_{12}E_8$, was applied to an HPLC column with a total volume of about 13 mL. Therefore, the amount of detergent applied to the column was small in comparison to the column volume. This system allowed a rapid, efficient exchange of detergent. The efficiency of exchange of the detergent by SE HPLC is shown in Figure 2. The band 3 protein peak in $C_{12}E_8$ eluted at 7.3 mL, corresponding to a Stokes radius of 76 Å. In a self-exchange experiment, [^{14}C]$C_{12}E_8$ associated with band 3 could be removed from the protein by a single passage on an SEC 4000 HPLC column (panel A). The radioactivity eluted close to but not at the total volume of the column, well removed from the protein peak. The recovery of radioactivity from the column was quantitative, showing that the detergent does not bind to the column packing. Approximately 30% of the $C_{12}E_8$ in the sample applied to the SEC column was calculated to be initially associated with band 3. The association of a single $C_{12}E_8$ per band 3 would give a peak of radioactivity of approximately 300 dpm. A maximum of 0.2 $C_{12}E_8$/band 3 was found after detergent exchange. Band 3 in $C_{12}E_8$ could also be effectively exchanged into C_{12}maltoside and C_{12}DAO (panels B and C), which have the same alkyl chain as $C_{12}E_8$, but different headgroups (Figure 2). Since [^{14}C]$C_{12}E_8$ is essentially removed from band 3 during passage down the column, the hydrodynamic properties observed for band 3 on the column must be due to a complex of band 3 and the new detergent.

Detergent Exchange of Band 3. To examine both which detergents would disperse band 3 and the amount of detergent required to do so, band 3 purified in $C_{12}E_8$ was subjected to SE HPLC in a series of other detergents that varied greatly in cmc. The behavior of band 3 was examined for each detergent at concentrations below, equal to, and at multiples of 2, 5, 10, or higher than the cmc, until band 3 eluted as a dispersed peak. In every nonionic detergent examined (see Table I), little, if any, band 3 eluted from the column at detergent concentrations at or below the cmc for the detergent. The behavior of band 3 in $C_{12}E_8$ is typical, in that a concentration of detergent twice the cmc is sufficient to maintain the dimeric state of the protein, as seen in Figure 1. Once a purely dimeric state is reached, increases of detergent concentration had no effect upon the protein's oligomeric state. As seen in Table II, however, concentrations of detergent greater than twice the cmc were sometimes required to disperse the protein. For example, to disperse band 3, detergent concentrations 100 times and 200 times the cmc were required in $C_{16}E_8$ and C_{12}DAO, respectively.

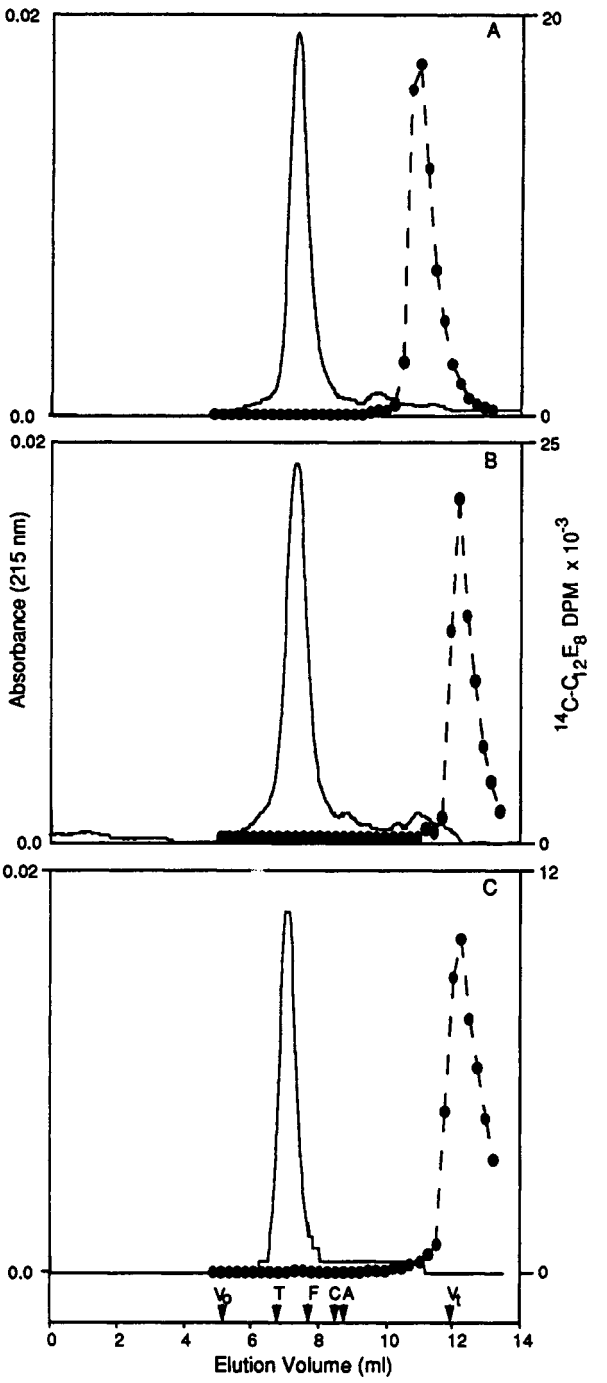

FIGURE 2: Exchange of detergent by SE HPLC. Band 3 was equilibrated overnight with [^{14}C]$C_{12}E_8$, and an aliquot (6 μg) was subjected to chromatography on a 30 × 0.75 cm SEC 4000 HPLC column. Elution buffer was 0.1 M sodium chloride/5 mM sodium phosphate, pH 7.0, containing (A) 1.9 mM $C_{12}E_8$, (B) 1.4 mM C_{12}-DAO, or (C) 0.7 mM C_{12} maltoside. Fractions (0.5 mL) of eluant were collected, and radioactivity was determined by scintillation counting. The solid line shows the absorbance at 215 nm. The dashed line is the elution of [^{14}C]$C_{12}E_8$. Shown at the bottom are the elution positions for the standard proteins (T) thyroglobulin (R_S = 86 Å), (F) ferritin (63 Å), (C) catalase (52 Å), and (A) aldolase (46 Å). The void volume, V_0, was determined from the elution position of Blue Dextran 2000 (average molecular weight 2 × 10^6), and the total volume, V_t, was determined from the elution position of 2-mercaptoethanol.

Band 3 was primarily dimeric in all of the nonionic detergents examined. This was shown by the fact that the Stokes radius for band 3 in all these detergents was very similar to that found in $C_{12}E_8$, in which it is dimeric (Casey & Reithmeier, 1991). Also, band 3 which had been cross-linked

Table II: Oligomeric Properties of Band 3 in Various Detergents

detergent	cmc (mM)	min[c] [det] (mM)	R_s (Å)	comments[a]	BADS K_d (μM)
C_8E_5	6.0	30	74	D	7.1
$C_{10}E_6$	0.46	0.92	77	D	
$C_{10}E_8$	0.28	2.8	77	D	
$C_{12}E_6$	0.065	0.33	74	D	
$C_{12}E_8$	0.056	0.112	76	D	1.7
$C_{14}E_8$	0.0052	0.52	79	D	
$C_{16}E_8$	0.00047	0.047	83	D	1.0
TrX-100$_{red}$	0.21	1.4	78	D	
C_8DAO	223	446[b]	80	major peak, S	
C_{10}DAO	6.0	60	73	D, S	
C_{12}DAO	0.23	46	76	D, S	1.4
C_{10}maltoside	1.4	14	71	D, S	1.2
C_{12}maltoside	0.14	0.28	75	D	
C_8glucoside	25.4	127[b]	69	D, S	
C_{10}glucoside	4.2	8.4[b]	83	broad; det poorly soluble	
C_{12}DAPS	2.1	10	72	D, some at void	
SDS	0.58	5.8	78	M	
CHAPS	6.2	6.2[b]	(78)	very broad	

[a] D, monodisperse dimeric elution; M, monomer; S, shoulder on the peak. [b] The detergent concentration listed was the highest that could be practically tested. [c] Minimum [det] is the concentration of detergent minimally required to maintain dispersed, dimeric elution from the SEC column.

on its extracellular face with DTSSP, to form covalent dimers, was analyzed in $C_{12}E_8$, C_8E_5, $C_{12}E_6$, $C_{10}E_6$, $C_{10}E_8$, $C_{14}E_8$, and $C_{16}E_8$. Covalent cross-linking of band 3 did not alter the elution position of the protein relative to the un-cross-linked protein (data not shown).

Of the detergents investigated, C_8DAO, C_{12}DAO, and C_8-glucoside required the highest concentration of detergent to disperse band 3. All of the polyoxyethylene detergents fully dispersed band 3, but the minimum detergent concentration varied greatly depending upon the cmc. $C_{16}E_8$, which has a very low cmc, was able to maintain the oligomeric structure of band 3 at micromolar concentration, while 30 mM C_8E_5 was required. C_8DAO was an extreme case since a concentration greater than 446 mM was required to disperse band 3. At 446 mM, the detergent had an absorbance at 215 nm greater than 1 which precludes using higher detergent concentrations for SE HPLC analysis. C_8DAO may therefore not be useful for the chromatographic analysis of membrane proteins.

Stokes radii for various band 3/detergent complexes are summarized in Table II. Also given in Table II are the detergent concentrations at which the Stokes radius was determined. In general, the Stokes radii (R_s) are directly related to the molecular weight of the detergent monomer. For example, the complex in $C_{10}E_6$ has R_s = 77 Å, while in $C_{16}E_8$ it has R_s = 83 Å. The primary determinant of the R_s of the protein/detergent complex is the length of the alkyl chain of the detergent. That is, for a given alkyl chain length, the R_s values differ little between detergents (Table II).

Band 3 was also extracted from ghosts and purified in TrX-100, C_{12}maltoside, and C_8glucoside. Band 3 purified in the first two detergents, when resolved by SE HPLC in 0.1% $C_{12}E_8$, gave an elution profile identical to the protein purified in $C_{12}E_8$. Therefore, detergent exchange leaves band 3 in the same state that it would be in if the detergent of exchange were used to prepare band 3. Chromatography of the TrX-100 and C_{12}maltoside preparations in the same detergent (TrX-100$_{red}$ was used for HPLC analysis of the TrX-100 preparation) used in purification yielded the same result as band 3 purified in $C_{12}E_8$. In contrast, band 3 solubilized in 1.0% C_8glucoside and purified in 0.1% C_8glucoside was highly aggregated when resolved SE HPLC in either 0.1% $C_{12}E_8$ or 0.1% C_8glucoside (data not shown). A detergent concentration of 0.1% C_8-glucoside is below the detergent's cmc which results in irreversible aggregation of the protein.

Ionic Detergents. In the ionic detergent SDS, some band 3 eluted at or below the detergent's cmc. Band 3 is monomeric in SDS (Moriyama & Makino, 1985), yet the detergent–protein complex had a Stokes radius of 78 Å, similar to the complexes with nonionic detergents. The high Stokes radius is due to the asymmetric nature of the SDS–protein complex (Reynolds & Tanford, 1970). Unlike SDS, very high concentrations of C_{12}DAPS were required for dispersed elution of band 3. CHAPS, a zwitterionic detergent, did not disperse band 3 very well. At the detergent's cmc (3.1 mM), a low amount of the protein eluted from the SE column at the column's void volume. At 6.2 mM CHAPS, band 3 eluted as a broad peak. Higher concentrations of CHAPS could not be used because of the high absorbance of the detergent.

Inhibitor Binding and Circular Dichroism. The affinity with which band 3 binds stilbenedisulfonate anion-exchange inhibitors is a sensitive indicator of the native conformation of band 3. To determine whether the detergents which maintain the dispersed, dimeric state of band 3 also support a native band 3 conformation, the affinity of band 3 for binding to BADS was determined after detergent exchange. The K_d values for BADS binding to band 3 are shown in Table II. The binding constants for BADS indicate that band 3 is native in the five, except C_8E_5.

Of the detergents examined, C_{12}maltoside appeared to be the best candidate for use in future crystallization trials; at low concentrations, it dispersed band 3, and it supported high-affinity BADS binding. Therefore, the secondary structure for band 3 prepared in C_{12}maltoside was compared with band 3 prepared in $C_{12}E_8$. The two CD spectra were very similar, and estimation of secondary structure showed that band 3 in 0.16 mM $C_{12}E_8$ was composed of 41% α-helix while band 3 prepared in 0.16 mM C_{12}maltoside was 49% α-helix.

DISCUSSION

Detergent Exchange. The results presented in this paper show that the detergent associated with a membrane protein can be exchanged for another detergent with retention of native structure and functional state. Since the detergent surrounding the membrane domain of band 3 can be readily exchanged, it is clear that the detergent is not tightly or irreversibly bound to the protein. Figure 3 is a model for the interaction between detergent and a protein during the exchange of detergents. Initially, the hydrophobic regions of the protein are surrounded by a micellar array of detergent, as is observed for the photoreaction center (Roth et al., 1989, 1991). The detergent surrounding the protein can be completely exchanged for a new detergent provided that the concentration of new detergent is above its cmc and at sufficiently high concentration to supply micellar amounts of detergent. As illustrated in Figure 3, detergent exchange likely occurs by replacement of individual monomers of the new detergent free in solution. As detergent exchange proceeds, an intermediate stage occurs in which the protein is surrounded by a mixed micellar array of the initial and final detergents. Finally, the hydrophobic zone of the membrane protein is surrounded by a micellar array of the new detergent. At each stage, detergent monomers are in equilibrium with both micelles of detergent surrounding the protein, and free detergent.

Requirements for Micellar Detergent. We observed that in all nonionic detergents tested, band 3 was aggregated at or

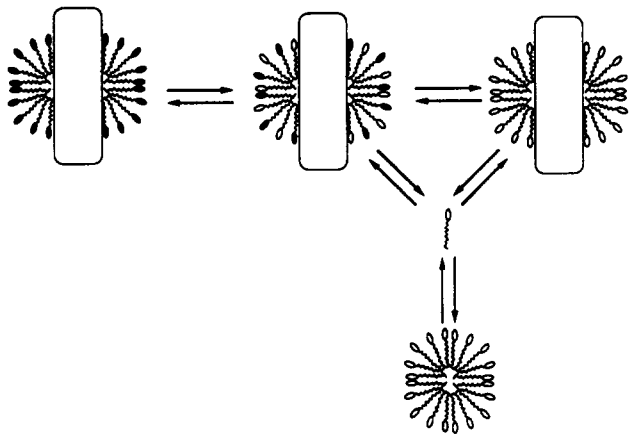

FIGURE 3: Model for the interaction of band 3 with detergents during detergent exchange. The membrane protein in cross section (rectangles) is surrounded by a micellar array of detergent (filled). Monomers of the second detergent (open) are in equilibrium with micelles of the detergent. During detergent exchange, the protein passes through an intermediate state in which it is surrounded by a combination of the two detergents. Finally, the protein is exchanged into the second detergent. Below the cmc of the detergent, the micelle belt surrounding the protein is unstable.

below the cmc of the detergent. Monomers are therefore incapable of maintaining band 3 in a dispersed state, regardless of the molar concentration of detergent. This is likely due to the inability of detergent monomers to form a stable micellar arrangement around the hydrophobic zone of band 3 below the cmc. Therefore, at all times from the moment of solubilization onward, a membrane protein must be kept in a detergent as a concentration above its cmc. This assertion is in accord with an earlier observation made by sucrose gradient ultracentrifugation that band 3, in the presence of varied concentrations of TrX-100, aggregated below the detergent's cmc, but was dimeric at concentrations just above the cmc (Clarke, 1975).

That the minimum detergent concentration for dispersion of band 3 in nonionic detergents is above the cmc as a rule suggests that band 3 binds micellar amounts of detergent. Measurements of the binding of detergent by band 3 show that the protein binds very close to a micelle/band 3 monomer of the polyoxyethylene detergents $C_{12}E_8$ (Casey & Reithmeier, 1991), $C_{12}E_9$ (Pappert & Schubert, 1983), and TrX-100 (Clarke, 1975). However, band 3 bound C_8glucoside with a maximum stoichiometry of 208:1, the equivalent of approximately 3 micelles/band 3 monomer (Werner & Reithmeier, 1985). The binding of multiple micelles may require micelle fusion to occur. The detergent binding curves for TrX-100 (Clarke, 1975) and $C_{12}E_8$ (Casey & Reithmeier, 1991) show that little or no detergent binds to band 3 below the detergent's cmc while some binding is seen below the cmc for C_8glucoside (Werner & Reithmeier, 1985). On the basis of our observations of the oligomeric state of band 3 in C_8glucoside, concentrations of this detergent lower than its cmc are unable to disperse the protein. Detergent binding below the cmc may be a consequence of C_8glucoside trapped within a protein aggregate. We cannot, however, rule out that C_8glucoside, unlike the polyoxyethylene detergents, is able to interact with band 3 below its cmc.

Binding of micellar amounts of detergent may be a general feature for membrane proteins with a size similar to band 3. The renal parathyroid hormone receptor is found in vivo as a dimer, predicted to span the bilayer 14 times (Karpf et al., 1988). Like band 3, the 14 transmembrane segments of the renal parathyroid receptor bind very close to a micelle of $C_{12}E_8$ (Karpf et al., 1988). Similarly, the cerebral γ-aminobutyric acid receptor, with seven transmembrane segments per monomer, binds close to a micelle of TrX-100/dimer (Mamalaki et al., 1989). The Ca^{2+}-ATPase of sarcoplasmic reticulum, which has a transmembrane structure similar to band 3 and is also a dimer, binds 122 molecules of $C_{12}E_8$/monomer (LeMaire et al., 1986). Interestingly, although the aggregation number of C_8glucoside is 84, the crystal structure for the reaction center of *Rhodobacter sphaeroides* shows that the protein binds 205 ± 35 molecules of C_8glucoside (Roth et al., 1991), which is very close to the amount bound by band 3. The 3 subunits of the photoreaction center contain a total of 11 transmembrane segments, similar to band 3.

Our results with band 3 in combination with those from other membrane proteins suggest that membrane proteins interact with micellar detergent. This may be due to direct binding of band 3 to a preformed detergent micelle or due to highly cooperative assembly of monomers around the hydrophobic zone of band 3. However, Tanford has pointed out that micellar binding cannot be distinguished from a highly cooperative interaction with monomers of detergent (Tanford, 1980). It is unlikely that detergent exchange occurs by displacement of an entire micelle by a micelle of new detergent. Rather, exchange occurs by replacement of individual monomers (Figure 3). If a free micelle and a micelle surrounding the hydrophobic domain of a protein have a similar organization and stability, then micelle formation around a protein will occur in a similar fashion to free micelle formation. For micelles that are more stable in the free state (C_8glucoside), high concentrations of detergent may be required to ensure full binding to the protein. For some detergents, a single micelle is sufficient, but detergents with short alkyl chains (C_8glucoside) may require multiple micelles to satisfy the hydrophobic zone of the protein. Micelle fusion may be required to allow full binding, and this occurs at much higher detergent concentrations than the minimum amount required for a stoichiometric amount for the protein.

Binding of micellar detergent implies that for membrane protein dispersion there is minimally a 1:1 stoichiometry of detergent micelles to protein. The general case equation to calculate the total concentration of detergent required for protein dispersion is therefore

$$[\text{total detergent}]_{min} = \text{cmc} + [\text{protein}](\text{detergent aggregation no.})$$

This relationship implies that for detergents with a low cmc, care must be taken to ensure that the detergent concentration is sufficient for the amount of protein in solution. An example of the equation is provided by band 3 at 1 mg/mL, which requires a minimum of 1.3 mM $C_{12}E_8$ (0.07% w/v), which is 20-fold above the cmc (0.056 mM). The requirement for concentrations of detergent well in excess of the cmc is particularly important in experiments where the protein concentration may become very high, or in the use of detergents with low cmc.

The concentration of nonionic detergent required to disperse band 3, or any other membrane protein, may be many times higher than the theoretical minimum of one micelle per protein molecule. Micelles of such detergents may bind membrane proteins poorly, or multiple micelles may be required. C_8-DAO, C_{12}DAO, and C_8glucoside fall into this category, as seen in Table II. Although band 3 was partially dispersed in C_8glucoside at a detergent concentraton 5 times the cmc, because of the high cmc of C_8glucoside (25.4 mM), the concentration of micelles (1.2 mM) is 1000-fold in excess of

the concentration of band 3 (1 μM). Those detergents which show a large gap between theoretical and actual detergent requirements may not interact well with band 3. A high concentration of micelles is required to drive detergent association with the protein.

Band 3 Retains Its Native Structure in a Variety of Nonionic Detergents. Results from the BADS binding experiments indicate that native band 3 structure is maintained in $C_{12}E_8$, $C_{16}E_8$, C_{12}maltoside, and C_{12}DAO. In another study, human band 3 in ghosts stabilized directly in 0.1% $C_{12}E_8$ and C_{12}-DAO had BADS binding constants of 0.9 and 1.3 μM, respectively (Lieberman & Reithmeier, 1983). A K_d of 1.3 μM for binding of BADS by bovine band 3 was reported in the presence of $C_{12}E_9$ (Moriyama & Makino, 1985). In addition to the BADS binding results, the similarity of the circular dichroism spectra for band 3 in $C_{12}E_8$ and C_{12}maltoside indicates that C_{12}maltoside maintains the native secondary structure observed for band 3 in $C_{12}E_8$.

Not all detergents are able to maintain the native structure of band 3. The BADS binding constant for band 3 in C_8E_5 is about 5-fold higher than that for the other detergents, indicating that band 3 is not native in C_8E_5. In a previous study, C_8glucoside was found to denature band 3; in 40 mM C_8glucoside, the K_d for BADS binding was 10 μM (Werner & Reithmeier, 1985). Similarly, bovine band 3 was found to be denatured by ionic (no BADS binding in SDS or sodium cholate) and zwitterionic detergents (no BADS binding in CHAPS and K_d for BADS binding in zwittergent 3-10 = 77 μM) (Moriyama & Makino, 1985). In addition to loss of BADS binding, we found that band 3 eluted from the SEC column as a polydisperse aggregate in CHAPS. Ionic and steroid-based detergents are, therefore, unsuitable for maintenance of band 3 structure. Our results indicate that the ability of a detergent to maintain the dispersed, dimeric state of band 3 does not assure its ability to maintain the protein's native conformation, as seen for C_8E_5. Studies of the Ca^{2+}-ATPase of sarcoplasmic reticulum also showed that not all detergents maintained the protein's structure. Native structure and function were maintained in polyoxyethylene detergents with alkyl chains 12 carbons or longer (Lund et al., 1989). The Ca^{2+}-ATPase was denatured in the C_8 detergent C_8E_4 (Lund et al., 1989).

Detergents which are suitable for use in crystallization trials must initially disperse the protein completely to a homogeneous state (Kuhlbrandt, 1988). As seen here, the homogeneous band 3 dimers can be prepared in a variety of detergents ($C_{10}E_6$, $C_{12}E_6$, $C_{12}E_8$, $C_{14}E_8$, $C_{16}E_8$, and C_{12}maltoside), but detergent conditions must be adjusted to maintain this state. C_{12}-Maltoside is especially attractive since it disperses band 3 at low concentrations and maintained band 3 structure and function as measured by circular dichroism and BADS binding affinity. Function of the Ca^{2+}-ATPase of sarcoplasmic reticulum was also enhanced slightly in C_{12}maltoside, compared to $C_{12}E_8$ (Lund et al., 1989).

$C_{16}E_8$ is an exceptional detergent in that it has an extremely low cmc (0.47 μM). It may be a good candidate for studies of band 3 and other eukaryotic membrane proteins where low monomeric concentrations of detergent are required. That the K_d for BADS binding to band 3 was normal in $C_{16}E_8$ also suggests that it can support native protein structure. Furthermore, recent work indicates that band 3 is stabilized by long-chain fatty acids (Gruber & Low, 1988; Maneri & Low, 1988) and that the lipids which copurify with band 3 are enriched in long-chain fatty acids (Maneri & Low, 1989). Therefore, if detergents interact with band 3 in a way similar to fatty acids, then a detergent with a long alkyl chain might provide an excellent environment for band 3. However, recent evidence from calorimetry studies of band 3 in polyoxyethylene detergents with chain lengths C_{10}–C_{16} shows that C_{12} provides the optimal alkyl chain length for thermal stability of band 3 (Sami et al., 1992).

C_8 detergents are not suitable for band 3. None of the C_8 detergents examined maintained a completely dispersed oligomeric state of band 3. Native structure is not maintained in C_8 detergents, based on the work presented here and previous work on band 3 (Werner & Reithmeier, 1985) and the Ca^{2+}-ATPase of sarcoplasmic reticulum (Lund et al., 1989). The short C_8 alkyl chain results in a high cmc for these detergents, thereby requiring large molar amounts of detergent to satisfy the requirement of membrane proteins for micellar amounts of detergent. The photoreaction center from *Rhodobacter sphaeroides* was successfully crystallized in C_8glucoside, which appears contradictory. However, this is a prokaryotic membrane protein, whose native membrane [25 Å thick (Roth et al., 1989)] is narrower than the eukaryotic plasma membrane [30 Å thick (Lewis & Engelman, 1983)]. The C_8 alkyl chain is therefore sufficient to satisfy the shorter hydrophobic zone of the protein.

The results presented here, taken along with previous studies, suggest that those detergents which support membrane protein structure must be present in sufficient quantity to provide micellar amounts for each protein molecule. The approach of detergent exchange by SE HPLC provides a ready means to assess detergent requirements for membrane protein dispersion. The approach may be useful to prepare proteins in other detergents and to expand the list of detergents which may be useful for membrane protein crystallization.

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