Analysis of the Oligomeric State of Band 3, the Anion Transport Protein of the Human Erythrocyte Membrane, by Size Exclusion High Performance Liquid Chromatography

OLIGOMERIC STABILITY AND ORIGIN OF HETEROGENEITY*

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Joseph R. Casey† and Reinhart A. F. Reithmeier‡

From the Medical Research Council Group in Membrane Biology, Departments of Medicine and Biochemistry, University of Toronto, Toronto, Ontario M5S 1A8, Canada

The oligomeric state of human Band 3 ($M_r = 95,000$), the erythrocyte membrane anion exchanger, was examined by size exclusion high performance liquid chromatography in solutions containing the nonionic detergent C$_{12}$E$_8$ (octaethylene glycol n-dodecyl monoether). Band 3 was heterogeneous with respect to oligomeric composition, the predominant (70%) species being a dimer that bound 0.57 mg of C$_{12}$E$_8$/mg of protein (Stokes radius = 78 Å, $s_{20,w} = 6.9$ S). Variable amounts of larger oligomers were also present; however, no evidence for equilibration between oligomeric species was observed in detergent solution. Analytical and large zone size exclusion chromatography showed that Band 3 could not be dissociated to monomers, other than by protein denaturation. The membrane domain of Band 3 ($M_r = 52,000$) was also dimeric, but without evidence for higher oligomeric forms, which implies that the interactions responsible for higher associations involve the cytoplasmic domain. Prelabeling of Band 3 with the anion exchange inhibitor 4,4'-disothiocyanostilbene-2,2'-disulfonate had no effect upon the oligomeric state of either intact Band 3 or its 52-kDa membrane domain. Band 3 oligomeric state could be reversibly changed in the membrane by altering the pH of the solution. The fraction of Band 3 not associated with the cytoskeleton was almost entirely dimeric. Band 3 purified from erythrocytes separated by density gradient centrifugation revealed that older red cells contained a larger proportion of higher oligomers than did younger cells. We conclude that Band 3, in the membrane and in C$_{12}$E$_8$ solution, exists as a mixture of dimers and larger oligomers. The higher oligomers interact with the cytoskeleton, increase in amount with cell age, and are held together by interactions of the cytoplasmic domain.

**The abbreviations used are: DIDS, 4,4'-disothiocyanostilbene-2,2'-disulfonate; BADS, 4-benzamido-4'-aminostilbene-2,2'-disulfonate; CMC, critical micellar concentration; BIDS, 4-henzamido-4'-isothiocyanostilbene-2,2'-disulfonate; DMMA, 2,3-dimethylmaleic anhydride; NDNS, 4,4'-dinitrostilbene-2,2'-disulfonate; $H_2$DIDS, 4,4'-dithiooctanodihydrostilbene-2,2'-disulfonic acid; HPLC, high performance liquid chromatography.

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§ To whom correspondence should be addressed: MRC Group in Membrane Biology, Rm. 7307, Medical Sciences Bldg., Dept. of Medicine, University of Toronto, Toronto, Ontario M5S 1A8, Canada. Tel.: 416-978-7739.
have been linked to formation of red cell senescence antigen (Lutz and Stringer-Wipf, 1983; Low et al., 1985). It has recently been proposed that ankyrin binds to Band 3 tetramers (Thevinin and Low, 1990; Mulzer et al., 1990).

Band 3 was first shown to be oligomeric by its ability to be cross-linked in membranes to dimers via cysteine residues (Steck, 1972). Other cross-linking studies have confirmed the oligomeric nature of Band 3, with most reagents showing dimeric or tetrameric structures (Jennings, 1984). Studies of intramembranous particles found in electron micrographs of freeze-fractured erythrocyte membranes suggest that there are enough particles to account for two to three Band 3 molecules/particle (Yu and Branton, 1976). Rotary shadow-cast micrographs show heterogeneity in the size of the particles, suggesting that some could be Band 3 dimers and others tetramers (Jennings, 1984). Hydrodynamic studies of detergent-solubilized Band 3 have indicated that Band 3 is dimeric (Yu and Steck, 1975; Clarke, 1975), a mixture of monomers and dimers (Luevakov et al., 1981), or a mixture of dimers and tetramers (Nakashima and Makino, 1980b) or in a monomeric equilibrium (Yu and Steck, 1983; Schubert et al., 1983). The oligomeric state of Band 3 in detergent solutions and, more important, in the membrane is still unresolved (Jennings, 1984).

To clarify the issue of Band 3 oligomeric state and its dynamics, Band 3 was characterized using a novel system, size exclusion HPLC. The system provides a rapid, sensitive, high resolution technique for separation of Band 3 oligomers suitable for routine analysis. The speed and sensitivity of the technique allowed analysis of Band 3 oligomeric structure under a wide range of conditions. Our results provide further evidence on the oligomeric state of Band 3 both in detergent solution and in the membrane. Furthermore, our studies investigate whether the oligomers equilibrate, how Band 3 oligomers are held together, and the functional significance of the different oligomers. An understanding of Band 3 oligomeric state is important to reduce the heterogeneity of solubilized Band 3 preparations as part of ongoing efforts to crystallize the protein.

**EXPERIMENTAL PROCEDURES**

*MATERIALS—[3H]H2DIDS was purchased from the Research Development Corp. (Hospital for Sick Children, Toronto, Ontario), and the unlabeled compound was purchased from Molecular Probes (Eugene, OR). DIDS was obtained from U. S. Biochemical Corp. 4,4'-Dinitrostilbene-2,2'-disulfonate (DND) was purchased from Aldrich. C8E8 (octaethylene glycol n-dodecyl monoether) was from Nikko Chemical Co. (Tokyo). [35S]C8E8 was purchased from Research Products International Corp. (Mount Prospect, IL). 2,3-Dimethylmaleic anhydride (DMMA) and 1,10-o-phenanthroline were purchased from Sigma. 3,3'-Dithiobis(sulfosuccinimidyl propionate) was from Pierce Chemical Co. Percoll was obtained from Pharmacia LKB Biotechnology Inc. All other chemicals were reagent grade or better.

*Isolation of Band 3 and Membrane Domain—Two different types of extraction protocols were used in the purification of Band 3 using, in most cases, red blood cells obtained from the Canadian Red Cross Society and also freshly drawn blood (Casey et al., 1989). In the first protocol, the cytoskeletal proteins are extracted from ghost membranes, and the membrane is then solubilized by detergent. This procedure quantitatively solubilizes Band 3 and is therefore representative of the total Band 3 population. Briefly, red cells were washed; ghosts were prepared by osmotic hemolysis, and the membranes were stripped of peripheral proteins with ice-cold 2 mM EDTA, pH 7.5, centrifugation at 100,000 g for 30 min at 4 °C. The extract was applied to aminoethyl-Sepharose 4B (0.2-0.5 ml of resin/mg of protein) and eluted with a 0-0.25 M sodium chloride linear gradient in 0.1% (v/v) C8E8, 5 mM sodium phosphate, pH 8.0. In some preparations, 1 mM dithiothreitol was present at all steps from cell lysis onward. In the second protocol, the intrinsic membrane proteins are solubilized directly from ghost membranes, leaving the cytoskeleton intact (Casey et al., 1989). Band 3 associated with the cytoskeleton is not solubilized under these conditions. 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; and the cytoskeleton was pelleted by centrifugation. Solubilized samples were dialyzed overnight against 5 mM sodium phosphate, pH 8.0, containing 0.2 mM dithiothreitol and 0.1% (v/v) C8E8. Band 3 was purified by chromatography on aminopropyl-Sepharose CL-4B as described above. The 52-kDa membrane domain of Band 3 was prepared by trypsin treatment of red cell ghosts, followed by alkali stripping, solubilization in C8E8, and DEAE-Sepharose CL-6B chromatography (Casey et al., 1989).

*Self-association of Band 3 in Membrane—Red cell ghosts, stripped with 2 mM EDTA, pH 12, were prepared as described above. The ghosts were divided into three batches and incubated either with 5 mM sodium phosphate, 0.5 mM dithiothreitol, pH 8.0 (control sample), or with 66 mM sodium citrate, 0.5 mM dithiothreitol, pH 5.5 (two samples), for 10 min at room temperature. After 10 min, one sample of pH 5.5-treated ghosts and the control ghosts were pelleted by centrifugation and rapidly solubilized by vortexing with 5 volumes of 1% C8E8 in 5 mM sodium phosphate, pH 8.0. The second sample of pH 5.5-treated ghosts was pelleted by centrifugation after the 10-minute incubation and then resuspended in 5 mM sodium dithiothreitol, pH 8.0, for a second incubation for 10 min at room temperature. The ghosts were then pelleted and solubilized as described for the other two samples. Band 3 was purified in parallel from the three samples by aminopropyl-Sepharose chromatography as described above.

*Separation of Old and Young Red Cells—Red cells were collected from a healthy donor and white cells were removed by washing three times with 0.9% NaCl. Cells were separated by density gradient centrifugation using Percoll (Bennett and Kay, 1981). Onto 30 ml of 80% Percoll, made up to 150 mM NaCl, 5 mM sodium phosphate, pH 7.4, 7.5 ml of washed packed cells diluted to 150 mM NaCl, 5 mM sodium phosphate, pH 7.4. The cells were separated into a series of bands by centrifugation at 16,000 rpm for 30 min at 4 °C in a Beckman JA-20 rotor. The top 20% (v/v) of the red cells were pooled as young cells, and the bottom 5% (v/v) as old cells. The pooled cells were washed free of Percoll with 0.9% NaCl, and Band 3 was then prepared from each pool of cells as described above.

*Chemical Modification of Band 3—Band 3 labeled with DIDS or H2DIDS was prepared by reacting red cells at 25% hematocrit with 10 μM DIDS or H2DIDS (Ramjeesingh et al., 1980) for 1 h at 37 °C in 150 mM NaCl, 5 mM sodium phosphate, pH 7.4. The second junction side of the gel was stained with Coomassie blue. Band 3 could be cross-linked to covalent dimers using 3,3'-dithiobis(sulfosuccinimidyl propionate) or Cu2+/O-phenanthroline. Red cells were treated with the impermeant bifunctional cross-linker 3,3'-dithiobis(sulfosuccinimidyl propionate) (Staros, 1982) following the method used for bis(sulfosuccinimidyl) suberate (Saltis and Sloan, 1989). Band 3 was purified as described above. Purified Band 3 was cross-linked by incubation with 40 μM CuSO4, 200 mM o-phenanthroline at 37 °C for 15 min (Casey et al., 1989). The reaction was stopped by addition of EDTA to a final concentration of 50 μM. HPLC—Small zone size exclusion experiments were performed at room temperature using a 7.5 × 300-mm TSK 4000 SW column. No improvement in resolution was seen by using a 7.5 × 600-mm column or by performing chromatography at 0 °C. Chromatography at 37 °C produced broad poorly resolved peaks, likely due to aggregation of detergent micelles. A Spectra-Physics 3300 HPLC pump was used at a flow rate of 0.5 ml/min. A pulse of Band 3 (10 μl) was injected onto the column using either a 200- or 500-μl injection loop. Sample volumes of 2–500 μl were used; however, volumes >10 μl resulted in decreased resolution. Protein elution was monitored at 215 nm. Using a waveguide detector (ABI Analytical Co.). Using a wavelength of 215 nm, as little as 1 pg of applied Band 3 protein could be readily detected. The standard elution buffer contained 0.1% (v/v) C8E8, 100 mM NaCl in
5 mM sodium phosphate, pH 7.0. A column could be used for ~300 runs in this solvent system before a decrease in resolution was noticeable. The column was calibrated with suitable protein standards that do not bind detergent (Le Maire et al., 1986).

Large zone experiments (Valdes and Ackers, 1979) were conducted using a TSK SW-pre column (7.5 x 75 mm) at room temperature at a flow rate of 0.1 ml/min. Purified Band 3 (0.45-450 µg of protein/ml) was injected through a filled 5-ml loop. The column buffer was the same as described above. Centroid boundary positions were determined from the midpoint between zero protein and the plateau protein level. Band 3 dimer and monomer elution positions were determined by chromatographing small aliquots (2 µl) of isolated Band 3 dimers and DMMA-treated Band 3 monomers, respectively.

**Analytical Ultracentrifugation**—sedimentation velocity studies were performed in a Beckman model E analytical ultracentrifuge at 68,000 rpm and 20 °C using a TSK 4000 SW HPLC column until

Equation 1:

\[
M_s = \frac{6\pi N\eta_w R_s \rho_m}{1 - \bar{v}_p \bar{v}_m}
\]

where \(N\) is Avogadro's number, \(\eta_w\) is the viscosity of water at 20 °C, \(R_s\) is the Stokes radius determined by size exclusion chromatography, \(s_{20,w}\) is the sedimentation coefficient at 20 °C in water, \(\bar{v}\) is the partial specific volume of the protein-detergent complex, and \(\rho_m\) is the density of water at 20 °C.

**Binding of C13E4 to Band 3**—Purified Band 3 in 0.1% C13E4 was applied to a 1-ml p-chloromercuribenzoate-Sepharose column until the resin was saturated. The [5% resin] (mg of resin) (Casey et al., 1989). The column was then equilibrated with buffer containing 5 mM sodium phosphate, pH 8.0, and the appropriate concentration of C13E4 containing tracer [14C]C13E4 until the radioactivity of column eluant reached a plateau equal to that applied to the column (10-20 column volumes). Band 3 was released from the matrix by including 0.1% 2-mercaptoethanol in the radioactive column buffer. Fractions (0.5 ml) were collected, and duplicate aliquots (25 µl) were assayed for radioactivity and protein content. C13E4 binding was calculated from the peak in radioactivity above background level associated with the eluted Band 3 protein.

**BADS Binding Assay**—BADS was synthesized as described (Casey et al., 1989). Purified Band 3 (500 µl) was applied to the 30-cm HPLC column eluted with standard elution buffer containing 0.05% C13E4. Fractions corresponding to Band 3 dimer and higher oligomers were collected separately. As described (Casey et al., 1989), 2 ml of 28.5 mM sodium citrate buffer, pH 7, was placed in a 5-ml fluorescence cell, and 200 µl of Band 3 (0.35 mg of protein/ml) was added. Concentrated BADS in citrate buffer was added sequentially up to a final concentration of 100 µM. The binding of BADS to Band 3 was measured by fluorescence enhancement in an Spex Fluorolog fluorometer. Excitation wavelength was 280 nm, and emission was 450 nm. Fluorescence was corrected for dilution, self-quenching of the probe, and the background fluorescence of the sample and the probe.

**Circular Dichroism**—Circular dichroism measurements were recorded on a Jasco 4-141A spectropolarimeter at room temperature using cells with 1-mm path length. Fractions from the aminothiol column containing either dimers or larger oligomers were diluted to 100 µg of protein/ml using 0.05% C13E4, 5 mM sodium phosphate, pH 8.0. Mean residual ellipticities were calculated using the mean residue weight of Band 3 of 113, based on the sequence of human Band 3 (Tanner et al., 1988). Estimates of the fraction of α-helix were calculated from Equation 2 (Chen et al., 1972):

\[
\theta = (\theta_0^2 + 2340) / -30,300
\]

where [θ]222 is the mean residue ellipticity at 222 nm.

**Analytical Techniques**—Protein assay was according to Lowry et al. (1951). Samples containing dithiothreitol were analyzed using the protein assay of Bradford (1976). Sodium dodecyl sulfate gel electrophoresis was performed according to Laemmli (1970). Protein bands were stained with Coomassie Blue and scanned with a Hoefer GS300 scanner. The concentration of oxidants in detergent samples was determined by the method of Chang and Bock (1980).

**RESULTS**

**Chromatographic Behavior of Band 3 and Identification of Major Band 3 Species**—Band 3 was purified from 2 mM EDTA, pH 12-stripped human erythrocyte ghosts by solubilization with the nonionic detergent C13E4 and chromatography using aminothiol-Sepharose. A scan of the sodium dodecyl sulfate gel in Fig. 1A (inset) shows that the preparation contained >95% Band 3. Some preparations contained small amounts of spectrin and Band 3 degradation products.

A typical chromatogram for Band 3 in 0.1% C13E4 solution resolved by size exclusion chromatography using a TSK 4000 SW HPLC column (7.5 x 300-mm) monitored at 215 nm (panel A) and 280 nm (panel B). For most experiments, the detection wavelength was set at 215 nm because of the 20-fold increase in sensitivity relative to 280 nm. In the experiment illustrated in Fig. 1, Band 3 eluted as two major species: the predominant peak at 8.0 ml with a Stokes radius of 76 Å and a smaller peak at 6.9 ml with a Stokes radius of 97 Å. Average Stokes radii of Band 3 oligomers from several preparations are summarized in Table I. There are oligomers larger than these two species, and some aggregated Band 3 elutes at the void volume. A small peak eluting at the position of catalase (Fig. 1B, arrowhead C) was seen in some preparations. Routinely, red cell ghosts used in Band 3 preparations were stripped of peripheral proteins by rapid treatment with ice-cold 2 mM EDTA, pH 12. As a control for any effect this may have on the oligomeric state of Band 3, Band 3 was also prepared from ghosts stripped with 2 mM EDTA, pH 7.5, and 1 M KI. This preparation showed the same predominance of dimer as in Fig. 1. Similarly, Band 3 was prepared in parallel from both freshly collected and outdated blood bank blood, and no significant difference in the chromatographic profile was observed.

**FIG. 1.** Size exclusion HPLC analysis of Band 3 (7 µg in A and 35 µg in B), applied to 7.5 x 300-mm TSK 4000 SW column, monitored at 215 nm (A) and 280 nm (B). Shown at the bottom are the elution positions for the standard proteins: T, thyroglobulin (M, = 88 kDa); F, ferritin (63 kDa); C, catalase (52 kDa); and A, aldolase (46 kDa). The void volume (V) was determined from the elution position of blue dextran 2000 (average M, = 2 x 10^6), and the total volume (V) was determined from the elution position of 2-mercaptopethanol. Inset (A), 10% polyacrylamide gel showing the purity of the Band 3 sample.
The major peak in Fig. 1 was identified as Band 3 dimer by the following criteria. Cross-linking of Band 3 to a covalent dimer via oxidation of sulfhydryl groups in the cytoplasmic domain by Cu²⁺/o-phenanthroline or by exofacial treatment of intact erythrocytes with the lysine-directed reagent 3,3’-dithiobis(sulfosuccinimidyl propionate) had no effect on the HPLC profile (data not shown). Sodium dodecyl sulfate-polyacrylamide gels run in the presence and absence of reducing agent (1% 2-mercaptoethanol) confirmed that the bulk of Band 3 had been cross-linked to dimers by either procedure.

To confirm that the major peak seen on HPLC analysis corresponds to Band 3 dimers, the mass of the polypeptide in the polypeptide-detergent complex was determined. To calculate the mass of polypeptide in the protein-detergent complex, the amount of detergent bound must be known. The concentration dependence of [¹⁴C]C₁₂E₈ binding to Band 3 was determined, and the results are shown in Fig. 2. Band 3 bound a maximum of 0.57 mg of C₁₂E₈/mg of protein, with decreased binding below the CMC of the detergent (0.005%). This value agrees with an indirect estimate of detergent concentration dependence of [¹⁴C]C₁₂E₈ binding to Band 3 (Helinius et al., 1983). The sample also contained minor species with sedimentation coefficient (s₂₀,₅₀ = 6.9), the molecular mass for the major Band 3 species was calculated to be 330 kDa, following Equation 1. Subtracting the detergent component (0.57 mg of detergent/mg of Band 3), the protein component constitutes a mass of 210 kDa. Given that the monomer molecular weight for the Band 3 polypeptide is 102,000, the peak with a Stokes radius of 78 Å is therefore Band 3 dimer.

As shown in Fig. 1 and indicated by the sedimentation velocity results, Band 3 preparations are heterogeneous with respect to oligomeric composition. The shoulder on the dimer peak at smaller elution volume is a higher oligomeric form of Band 3 (Table 1) since sodium dodecyl sulfate-polyacrylamide gel analysis of the protein in these fractions showed only the presence of Band 3. To estimate the fraction of Band 3 dimers in a preparation, all Band 3-containing fractions eluted from the aminoethyl-Sepharose column were pooled and subjected to size exclusion chromatography. Band 3 dimer fraction was estimated by assuming a gaussian distribution, which is validated by the appearance of the Band 3 dimer peak using preparations containing only dimers (see below). Three different preparations of Band 3 had an average of 70% dimers and 30% higher oligomers. If one assumes that the higher oligomers are primarily tetramers, then these values imply that the average state of association of Band 3 in C₁₂E₈ solution is 2.6.

The peak of absorbance at 10.4 ml in Fig. 1 has been identified as mixed micelles of C₁₂E₈ and lipid (Table 1) as follows. Detergent micelles elute at this volume as shown by monitoring eluant radioactivity after applying a sample of 1% [¹⁴C]C₁₂E₈. Phosphatidylcholine (1 mg/ml) dissolved in 1% C₁₂E₈ also eluted at this position. As shown in Fig. 1, upon change of the detection wavelength from 215 to 280 nm, the size of the micelle peak decreased disproportionately relative to the Band 3 peak. On sodium dodecyl sulfate-polyacrylamide gels, no protein is seen in fractions corresponding to the micelle peak. Finally, in studies of the Ca²⁺-ATPase in C₁₂E₈ from sarcoplasmic reticulum, a peak of detergent was also seen at this position (Andersen et al., 1986). The small peak occasionally seen between the dimer peak and the micelle peak may represent Band 3 monomers or degradation products of Band 3.

Stability of Band 3 Oligomers in C₁₂E₈ Solution—The ability of Band 3 dimers and higher oligomers to equilibrate in detergent solution was examined. Band 3 oligomers were separated by size exclusion HPLC, and fractions of the eluant were collected. Fractions corresponding to the dimer and higher oligomer positions were then reapplied to the HPLC column either immediately after or up to several days after collection, and their elution positions were determined. Fig. 3 shows the rechromatography of isolated dimers and tetramers less than 1 h after their original separation. The separated peaks rechromatographed to their original elution positions. A similar result was obtained after storage of the isolated dimer and tetramer fractions at 4 °C for 1–2 days. The dimer and higher oligomeric forms of Band 3 therefore are stable and do not equilibrate during chromatography in detergent solution. Size exclusion chromatography can therefore be used to purify defined oligomeric forms of Band 3.

Band 3 oligomers could also be separated by ion exchange chromatography (Fig. 4). Within a given Band 3 preparation, fractions eluted from the aminoethyl-Sepharose column at higher salt concentrations consistently contained more of the larger species than earlier fractions. The salt gradient, however, was not responsible for the formation of higher oligomers (see below). Fig. 4A shows a typical elution profile for Band 3.

### Table 1

Stokes radii of Band 3 species determined by size exclusion chromatography

| Band 3 species                        | Re Å |
|--------------------------------------|------|
| Dimer                               | 77.6 (20) |
| DMMA-treated monomer                | 59.9 (3) |
| Larger species (tetramer)           | 100 (10) |
| H₂DIDS-labeled dimer                | 78.3 (5) |
| 52-kDa membrane domain              | 62.5 (2) |
| H₂DIDS-labeled 52-kDa membrane domain | 62.5 (2) |
| Detergent micelle                   | 40 (2)  |
Fig. 3. Rechromatography of isolated Band 3 oligomers after resolution by size exclusion chromatography. Band 3 (500 ml, at 1.9 mg of protein/ml) was applied to a TSK 4000 SW column (A). Brackets in A indicate the Band 3 fractions that were reapplied to the same column, as seen in B and C. In B and C, 10 µl of the isolated oligomers (as seen in brackets) were chromatographed 1 h after their original isolation. See Fig. 1 legend for definitions of standard proteins.

3 purified by aminoethyl chromatography. The third peak contains Band 3. Fig. 4 (B–D) shows size exclusion HPLC chromatograms of three fractions, 24, 26, and 30, eluted from an aminoethyl-Sepharose column with a salt gradient. The earliest fraction contained primarily Band 3 dimer, whereas later fractions contained more of the higher oligomeric species of Band 3. The ability to resolve oligomers of Band 3 by anion exchange chromatography indicates that the oligomers are stable over the time course of the chromatographic procedure.

To examine whether the observed oligomeric state was an artifact of the Band 3 preparation technique, a number of control experiments were performed. It has been suggested that the production of stable Band 3 dimers is due to the activity of oxidizing impurities in detergents on Band 3 (Schubert et al., 1983). Crude grades of polyoxyethylene detergents have been shown to contain oxidizing free radical species (Chang and Bock, 1980). To determine if free radical-induced oxidation produced dimers of Band 3, the C12E8 stock used for the preparation of Band 3 was assayed for oxidant levels, and none were detected. Band 3 prepared using a C12E8 stock containing the antioxidants 1 mM EDTA and 0.1 mM butylated hydroxytoluene gave chromatograms no different than those seen in Fig. 1. As a further precaution against time-dependent oxidation, Band 3 was generally analyzed within 18 h of membrane solubilization. Size exclusion chromatography of Band 3 in the presence or absence of 1 mM dithiothreitol or 0.1% 2-mercaptoethanol gave chromatograms no different than those seen in Fig. 1. However, in the presence of dithiothreitol, at all stages of the preparation; yet no difference of the chromatographic profile was observed.

Some differences in the oxidation state of sulphydryl residues were observed in the dimer compared to the larger oligomer forms of Band 3. Sodium dodecyl sulfate-polyacrylamide gels of Band 3, prepared in the absence of dithiothreitol, were run under reducing versus nonreducing conditions. Dimeric Band 3 was found to contain little disulfide-cross-linked protein. In contrast, the tetramer and higher oligomeric forms of Band 3 contained a significant proportion (25%) of disulfide-cross-linked Band 3. Although the higher oligomers contained disulfide-linked Band 3, reduction of these bonds did not result in dissociation of the oligomers during HPLC.

Band 3 could be shifted toward higher oligomeric forms under some conditions. Long periods of incubation at 4 °C (1 week or longer) caused only a slight shift toward aggregation.
Similarly, overnight incubation at 22 °C caused little or no change in Band 3 oligomeric state. In contrast, incubation of Band 3 at 37 °C for several hours induced irreversible Band 3 aggregation, even in the presence of 0.1% 2-mercaptoethanol. Aggregation of Band 3 at 37 °C may be promoted by aggregation of detergent micelles that occurs at higher temperatures, near the detergent’s cloud point. Concentrating Band 3 to 15 mg of protein/ml in a centrifugal concentrator at 3000 \( \times g \) for 30 min caused a slight shift toward higher oligomeric state. Dilution of the concentrated pool with column buffer followed by size exclusion analysis showed that the aggregation process was irreversible.

Band 3 also aggregates irreversibly during ultracentrifugation (data not shown). Samples of Band 3 analyzed by size exclusion HPLC before and after analytical ultracentrifugation (156,000 \( \times g \), 2 h, 20 °C) showed that a portion of Band 3 had aggregated during the centrifugation run, whereas a control sample held at 20 °C for the same time did not change its oligomeric state. Centrifugation at high speed (163,000 \( \times g \), 2 h, 20 °C) in an ultracentrifuge also induced Band 3 to form irreversible high molecular weight aggregates, the bulk of which appeared at the void volume of the TSK 4000 SW column.

Attempts were made to vary the Band 3 oligomeric state by changes in the ionic strength and pH of the elution buffer. HPLC was performed with the elution buffer containing up to 1 M sodium chloride (data not shown). Although high salt concentrations changed the chromatographic properties of the TSK column slightly, the elution profile and the Stokes radius of the Band 3 dimer did not change significantly (\( R_s = 73 \, \text{Å} \) in 1 M NaCl versus 78 Å in 0.1 M NaCl). The effect of acidic elution buffers on the chromatographic profile of Band 3 was examined (data not shown). The effects of alkaline pH were not examined because of the lability of TSK column matrix above neutral pH. At pH 4.8, Band 3 was aggregated and did not elute from the column. At pH 5.8, Band 3 was less severely aggregated and eluted as a smear with the dimer peak height reduced by 80%. In contrast, Band 3 prelabeled with DIDS had an elution profile at pH 5.8, which is no different than that at pH 7.0. DIDS labeling of Band 3 increases the protein’s solubility at acidic pH by decreasing the protein’s isoelectric point.

The oligomeric state of sarcoplasmic reticulum Ca\(^{2+}\)-ATPase was observed to shift from dimer toward monomer at high C\(_{12}E_8\) concentrations (Andersen et al., 1986). The effect of C\(_{12}E_8\) on Band 3 was therefore examined over the range 0.001-1% in the column buffer. At all concentrations >0.01%, Band 3 chromatograms were indistinguishable from one another. No dissociation of Band 3 to monomer was noticed up to 1% C\(_{12}E_8\). In addition, treatment of the Band 3 sample with C\(_{12}E_8\) up to 10% followed by chromatography in buffer containing 0.1% C\(_{12}E_8\) did not change the elution pattern. At detergent concentrations <0.001%, Band 3 aggregated and eluted as a broad peak starting at the void volume. The CMC of C\(_{12}E_8\) is 0.005% (Helinius et al., 1979), which suggests that Band 3 aggregates below the CMC of the detergent.

**Band 3 Monomers**—Since the literature contains references to the presence of monomeric Band 3 in detergent solution (Pappert and Schubert, 1983; Lucakovic et al., 1981), attempts were made to observe and to generate Band 3 monomers. Human Band 3 could be dissociated into monomeric state by treatment of solubilized Band 3 with DMMA as previously shown for bovine Band 3 (Nakashima and Makino, 1980b). Fig. 5 shows the elution profile of DMMA-treated Band 3, relative to a chromatogram for dimeric Band 3. The elution positions of the dimer and monomer are sufficiently different (Table I) that a significant amount of monomeric Band 3 in a preparation would appear as a shoulder on the dimer peak if it were not fully resolved. Band 3 could also be dissociated to monomers by treatment of the sample with 1% sodium dodecyl sulfate. HPLC analysis of the sample in 0.1% C\(_{12}E_8\) produced two protein peaks: a monomer with a Stokes radius of 60 Å and a reassociated dimer with a Stokes radius of 78 Å. The Stokes radii for the dissociated forms of Band 3 are likely larger than those for a “native” Band 3 monomer. The elution position of the monomeric form of the Ca\(^{2+}\)-ATPase, which has a subunit molecular weight similar to Band 3, may be a better marker for a native Band 3 monomer. The Ca\(^{2+}\)- ATPase monomer, produced in 1% C\(_{12}E_8\), was found to have a Stokes radius of 50 Å, compared to 56 Å found by Andersen et al. (1986). Native Band 3 monomers with a Stokes radius similar to the Ca\(^{2+}\)-ATPase monomer would appear as a separate peak between the peaks for Band 3 dimer and detergent micelle. The chromatogram illustrated in Fig. 1 does not contain a significant peak between the dimer position and the detergent micelle that may correspond to Band 3 monomers. However, the presence of a small amount of monomeric Band 3 cannot be excluded.

If Band 3 exists in a monomer/dimer equilibrium, dilution of the protein sample may promote the dissociation of Band 3 dimers. The effect of varying the concentration of Band 3 in the sample applied to the size exclusion column was examined over a broad protein range (18–2000 \( \mu \)g of protein/ml). Application of protein samples containing as low as 18 \( \mu \)g of protein/ml did not change the elution profile (data not shown). The peak protein concentration of dimeric Band 3 eluting from the column was as low as 0.1 \( \mu \)g/ml in these experiments, yet no dissociation was noted.

Another method to assay protein dissociation is large zone size exclusion chromatography (Valdes and Ackers, 1979). In large zone size exclusion chromatography experiments, a large bolus of protein is applied to a short column until a plateau is reached, which represents the concentration of the applied sample. The centroid elution volume is the weight average of the elution volumes of the various oligomeric species. The procedure is repeated over a range of protein concentrations to detect concentration-dependent changes of oligomeric state. Fig. 6 shows large zone size exclusion chromatography of Band 3 dimers and DMMA-generated Band 3 monomers. The elution positions of the dimer and monomer can be readily distinguished. Over the concentration range of 0.45–450 \( \mu \)g of protein/ml, the leading edge centroid position of the chromatogram is at the same elution volume (Table II), which suggests that the dimer does not shift toward the monomer position with decreasing Band 3 concentration. The Band 3 dimer is the major component in C\(_{12}E_8\), and this oligomeric...
form is stable over the 1000-fold range of protein concentrations tested.

Circular Dichroism Measurements of Oligomer Secondary Structure—Conformational changes in Band 3 may occur upon formation of higher oligomers or during dissociation to monomer. Structural differences between the different oligomeric forms of Band 3 were therefore examined using circular dichroism spectroscopy (Fig. 7). Fractions separated by ion exchange chromatography using aminoethyl-Sepharose shown to contain either Band 3 dimer or larger species were analyzed. Dimeric Band 3 showed a helix fraction of 0.45, whereas the higher oligomers had a slightly lower helix content of 0.42. Circular dichroism analysis of monomeric Band 3 prepared by DMMA treatment of ghost membranes showed that it has a helix fraction of 0.26, suggesting that it is highly denatured relative to control Band 3.

Stilbenedisulfonates and Band 3 Oligomeric State—Since anion transport can be measured only in vesicle preparations, inhibitor binding is a convenient functional assay for Band 3 in solution. BADS is a noncovalently binding stilbenedisulfonate that inhibits Band 3 anion exchange activity ($K_t = 0.1 \mu M$). BADS fluorescence is enhanced upon binding to Band 3. The concentration dependence of fluorescence enhancement is a measure of the affinity of binding of BADS by Band 3 (Lieberman and Reithmeier, 1983). Fig. 8 shows BADS titrations of Band 3 dimers and higher oligomers that were separated on the TSK 4000 SW column, similarly to those shown in Fig. 3. Both preparations contain a high affinity BADS-binding site. Plots of $1/[\text{BADS}]$ versus $1/\text{fluorescence}$ show that the high affinity binding has a $K_t$ of 0.6 $\mu M$. The double-reciprocal plot for dimeric Band 3 is linear, whereas the plot for tetramers is multiphasic, suggesting multiple binding classes in the preparation. At 10 $\mu M$ BADS, the dimeric population is saturated with BADS, but the tetrameric population is only 69% saturated. At high concentrations of BADS, tetrameric Band 3 can be saturated to close the level attained for the dimeric sample.

The noncovalently acting anion transport inhibitor DNDS has been reported to change the conformation of Band 3 to a state that can form tetramers by self-association of bis(sulfosuccinimidyl) suberate-cross-linked dimers (Salthany et al., 1990). Size exclusion HPLC was performed in the presence of a range of DNDS concentrations up to 250 $\mu M$ (data not shown). The absorbance reading of the flow-through spectrophotometer was zeroed to compensate for the high absorbance of DNDS. After base-line adjustment, chromatograms run in the presence of DNDS were no different than those in its absence. The effect of the irreversible inhibitor DIDS on the Band 3 oligomeric state was also determined. In this experiment, DIDS labeling of Band 3 was performed using intact cells. As seen in Fig. 9, Band 3 prepared from red cells labeled with DIDS had an elution pattern very similar to that of control Band 3, and the Stokes radii of the dimer forms were very similar (Table I). The binding of covalently or noncovalently acting stilbenedisulfonates to Band 3 did not have any influence on the oligomeric state of the protein in detergent solution.

Oligomeric State of Membrane Domain—The chromatographic properties of Band 3 and its membrane domain were compared in samples prepared from the same unit of blood. HPLC analysis of the 52-kDa fragment of Band 3 showed that the membrane domain elutes as a single species ($R_g = 60$ Å) (Table I), with none of the higher oligomeric shoulder seen for intact Band 3 (Fig. 9B). The peak following the major
peak corresponds to the position of detergent micelles (Table I). This single protein peak corresponds to the dimeric 52-kDa domain since it coelutes with 52-kDa domains covalently cross-linked to dimers with 3,3′-dithiobis(sulfosuccinimidyl propionate). The lack of higher oligomers seen in parallel preparations of intact Band 3 (Fig. 9A) suggests that the higher order associations seen in intact Band 3 are due to interactions between the cytoplasmic domain. The binding of DIDS has been reported to change the oligomeric state of the membrane domain of bovine Band 3 (Tomida et al., 1988). At neutral pH, the 52-kDa fragment labeled with DIDS elutes from the size exclusion column identically to the control 52-kDa fragment.

**Reversible Association of Band 3 in Membrane**—Little evidence was seen for a change of the oligomeric state of Band 3 in C$_{12}$E$_{5}$ solution. Results therefore suggest that detergent solution may not be an ideal model for the study of membrane protein oligomeric dynamics. Whether Band 3 oligomeric state is dynamic in the membrane and whether a change in oligomeric state could be observed after detergent solubilization were examined. Band 3 will associate in the plane of the membrane at pH 5.5, near its isoelectric point. Transport is reversibly inhibited at this pH, and residual transport is fully phloretin-inhibitable (Gunn et al., 1975). Treatment of ghost membranes at pH 5.5 therefore does not result in irreversible denaturation of Band 3. Stripped red cell ghosts were incubated at either pH 8.0 or 5.5 for 10 min at room temperature. A sample of pH 5.5-treated ghosts was then incubated at pH 8.0 to reverse the Band 3 association. The ghost samples were then rapidly solubilized with C$_{12}$E$_{5}$ at pH 8.0, and Band 3 was purified from each ghost preparation and examined by size exclusion HPLC. Fig. 10 shows that Band 3 treated at low pH in the membrane (panel B) is much more associated than control Band 3 (panel A), which was never exposed to low pH. This associated state of Band 3 remains even after solubilization and purification of the protein. The state of Band 3 in detergent solution appears to parallel the state of Band 3 in the membrane at the moment of solubilization. Re-equilibration of pH 5.5-treated ghosts at pH 8.0 before solubilization produced a chromatographic profile (panel C) similar to control Band 3 (panel A). This suggests that the Band 3 association produced in the membrane at low pH is reversible. A similar low pH treatment of Band 3 in detergent solution resulted in irreversible aggregation of the protein (see above). Therefore, a reversible association of Band 3 induced by acidic pH can occur in the membrane, but not in detergent solution.

**Band 3 Oligomer-Cytoskeleton Interactions**—Band 3 exists in at least two forms in the red cell membrane. One form, containing fractions eluted from aminoethyl-Sepharose, Band 3 was prepared from ghosts incubated at pH 8.0 (A), at pH 5.5 (B), and at pH 5.5 and then at pH 8.0 (C). See Fig. 1 legend for definitions of standard proteins.

![Fig. 9. Effect of DIDS labeling on elution profiles of intact Band 3 and Band 3 membrane domain (M, = 52,000). A, upper, 2 µg of intact Band 3; lower, 1.6 µg of Band 3 labeled with DIDS. B, upper, 1.4 µg of the 52-kDa membrane domain; lower, 4.2 µg of the 52-kDa membrane domain labeled with DIDS. See Fig. 1 legend for definitions of standard proteins.](image)

![Fig. 10. Reversible association of Band 3 in membrane. Shown are size exclusion chromatograms of pools of all Band 3-containing fractions eluted from aminoethyl-Sepharose. Band 3 was prepared from ghosts incubated at pH 8.0 (A), at pH 5.5 (B), and at pH 5.5 and then at pH 8.0 (C). See Fig. 1 legend for definitions of standard proteins.](image)
**DISCUSSION**

**Major Band 3 Oligomer in C12E8 Solution Is Dimer**—Our results indicate that Band 3 isolated from stripped ghost membranes using C12E8 is heterogeneous with respect to oligomeric state. The major Band 3 oligomer observed is a protein-detergent complex with a Stokes radius of 78 Å, consisting of a Band 3 dimer that binds 0.57 mg of C12E8/mg of protein. This Stokes radius is in close agreement with previous values found by gel filtration for human Band 3 in Triton X-100 (73 Å) (Reithmeier, 1979) and bovine Band 3 in C12E8 (80 Å) (Nakashima and Makino, 1980b). Some of the broadness of the protein peaks could be due to heterogeneity of the mass of residual lipid bound by Band 3, which has been shown to influence the Stokes radius of the Ca2+-ATPase (Andersen, 1989). Whereas 70% of Band 3 existed as a dimer, the remaining 30% of Band 3 was associated as a tetramer and higher oligomeric forms. The tetramer has a Stokes radius of 100 Å, in agreement with the assignment made by Nakashima and Makino (1980b), who also saw a species of this size for bovine Band 3 in C12E8-polyacrylamide gels. If one assumes that the majority of the oligomers higher than dimers are tetramers, then the average associated state of Band 3 in C12E8 solution is therefore 2.6. This is in agreement with the estimate from electron microscopy that suggested that the average intramembranous particle contained two to three molecules of Band 3 (Yu and Branton, 1976).

The suggestion was made that stable Band 3 dimers are an artifact resulting from time-dependent, detergent-mediated oxidation of Band 3 (Pappert and Schubert, 1983; Schubert et al., 1983). To prevent oxidation-induced changes of Band 3 oligomeric state, highly purified detergent was used, oxidation inhibitors were included in preparations, fresh blood was used, alternate membrane-stripping procedures were employed, and Band 3 was analyzed as soon as possible after membrane solubilization. Yet, the predominant oligomeric species found was the dimer, with some tetramer. No time-dependent conversion of Band 3 to dimers was observed. Band 3 dimers do not appear to be an artifact.

**Band 3 Oligomeric Forms Do not Equilibrate in C12E8 Solution**—Evidence was presented that demonstrates that Band 3 dimers and larger oligomers are not in equilibrium in C12E8 solutions. No shift of leading edge centroid elution position of Band 3 was observed over a wide range of protein concentrations in large zone size exclusion chromatography experiments. Band 3 oligomers could be resolved by size exclusion or ion exchange chromatography. Isolated oligomeric populations were stable for days without shifts toward mixtures of oligomers. Our results are consistent with those observed on C12E8-polyacrylamide gels, which showed that dimers and a larger species could be fully resolved, without band smearing, which suggests that equilibration of the species did not occur during electrophoresis (Nakashima and Makino, 1980b). Also, Macara and Cantley (1983) cite experiments in which two
populations of Band 3 were mixed together (one labeled with BIDS and the other with eosin maleimide), and mixed oligomers were not observed to form.

Pappert and Schubert (1983) have studied the oligomeric state of human Band 3 in C\textsubscript{6}E\textsubscript{6} by sedimentation analysis. Freshly isolated Band 3 was reported to exist in a monomer/dimer/tetramer equilibrium; but upon storage for several hours, the protein converted to a stable dimer. In our experiments, we have not been able to detect such an equilibrium, even immediately after purification of the protein. Pappert and Schubert (1983) interpreted the observed curvilinear sedimentation velocity results as evidence for equilibrium between oligomeric species during the time course of centrifugation. The observation that Band 3 aggregates during analytical ultracentrifugation raises questions about oligomeric state determinations using this technique. Structural changes in membrane proteins may be caused by high hydrostatic pressure that occurs in the analytical ultracentrifuge (Andersen, 1989).

Resolution of Band 3 oligomeric species on anion exchange resin is probably a function of the increased number of negative charges on the higher oligomers, that is, for a fixed surface charge density, a larger particle will bind more strongly than a smaller particle because the larger has more bonds to the resin. Although it is less likely, it cannot be excluded that post-translational modifications of Band 3 alter the charge densities of dimers and tetramers. Carboxyl methylation (Freitag and Clarke, 1980) and phosphorylation (Waxman, 1979) of Band 3 are known to occur. Preferential carboxyl methylation of dimers or phosphorylation of tetramers could explain oligomeric resolution on anion exchange resin.

The ability to separate Band 3 oligomers by aminoethyl-Sepharose or size exclusion HPLC will allow a comparison of the structure and function of these two forms of Band 3. Differences between Band 3 dimers and tetramers were noted. The slight reduction of estimates of the helical content of higher Band 3 oligomers relative to dimeric Band 3 suggests that the secondary structure of the higher oligomers may be slightly altered. The small differences in the circular dichroism spectra of the two forms of Band 3 may, however, be a consequence of different degrees of association of protomers with identical secondary structure. The higher oligomers of Band 3 had a higher level of disulfide cross-linking. This suggests that they may have been oxidized in vivo or that they may be more readily oxidized after solubilization. Further structural differences between dimers and higher oligomers are suggested by the BADS binding data. The data indicate that some Band 3 molecules in higher oligomeric states have a decreased affinity for the inhibitor. Loss of BADS binding ability may correlate with loss of anion transport activity. Indeed, aged red cells, which we have shown to contain highly enriched membrane proteins may be caused by high hydrostatic pressure that occurs in the analytical ultracentrifuge (Andersen, 1989).

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DNDS promotes the noncovalent association of cross-linked Band 3 dimers in the membrane to form a tetramer that is stable to dissociation by sodium dodecyl sulfate up to 60 °C (Salhany et al., 1990). DIIDS labeling of Band 3 did not result in formation of these Band 3 tetramers, and DIIDS blocked the effect of DNDS. The Band 3 population not cross-linked by bis(sulfosuccinimidyl) suberate did not form sodium dodecyl sulfate-stable dimers or tetramers even in the presence of DNDS. Our results are in agreement with these observations, that is, without prior cross-linking, DIIDS prelabeling of Band 3 or saturating Band 3 with DNDS had no effect on the oligomeric state of the protein. The formation of tetramers as seen by Salhany et al. required the prior cross-linking of Band 3 to dimers. The Band 3 tetramers observed in sodium dodecyl sulfate by Salhany et al. may correspond to tetramers in the membrane.

**Band 3 Can Be Reversibly Associated in Membrane—**Previous workers (Mühelbach and Cherry, 1986; Dempsey et al., 1986) have suggested that temperature-dependent reversible changes of Band 3 oligomeric state occur both in red cells and reconstituted vesicles. Consistent with these reports, we have found that Band 3 oligomers can equilibrate in the membrane. The results show reversible pH-dependent association of Band 3 in the membrane, but not in detergent solution. The oligomeric state existing at the moment of solubilization may be preserved in detergent solution. A theoretical thermodynamic argument has been put forward (Grasberger et al., 1986) that membrane protein association is 106 times more favorable in the plane of the membrane than in solution due to restriction of mobility and orientation in the membrane. That Band 3 oligomers did not dissociate completely in detergent solution, in spite of the thermodynamic tendency to dissociate, indicates the strength of the inter-Band 3 interactions. The differences in oligomeric state observed between isolated cell populations suggest that Band 3 in detergent solution may accurately reflect the forms in the membrane, that is, detergent solubilization of Band 3 does not appear to disrupt intermolecular interactions such that oligomeric states are homogenized.

Equilibration of Band 3 oligomers in the plane of the membrane opens future avenues of research. Rapid solubilization of Band 3 from the membrane does not appear to alter dramatically Band 3 oligomeric form. Band 3 oligomeric state can be rapidly and easily determined, and oligomers can be quantitated by size exclusion HPLC. Taken together, these results suggest a ready method to examine indirectly Band 3 oligomeric state in the membrane. In the past, biophysical studies of Band 3 oligomeric state have referred to populations of Band 3 with different mobilities. Correlation of size exclusion HPLC results with electron spin resonance measurements of Band 3 mobility will allow assignment of mobility classes to specific oligomeric species. Oligomeric dynamics and perturbants of oligomeric state can also be examined using this approach.

**Band 3-Cytoskeleton Interactions—**Studies of Band 3 rotational mobility indicate that there are multiple classes of Band 3 varying in mobility (Nigg and Cherry, 1980; Tsuji et al., 1988). Between 10 and 20% of Band 3 is linked to the cytoskeleton via ankyrin (Bennett, 1982). The suggestion has been made that the immobilized fraction interacts with the cytoskeleton (Beth et al., 1986; Tsuji et al., 1988). It has been proposed that Band 3 tetramers selectively interact with ankyrin (Thevinin and Low, 1990; Mulzer et al., 1990). Here we have shown that the fraction of Band 3 not associated with the cytoskeleton is almost exclusively dimeric, suggesting that higher oligomers are indeed associated with the cytoskeleton. Using a similar method to extract Band 3 directly from the membrane, Pappert and Schubert (1983) also observed a single Band 3 oligomer (after several hours) that they identified as dimer. Yu and Steck (1975), using sucrose gradient centrifugation, detected only dimeric Band 3 after direct Band 3 extraction from ghosts. Band 3 mobility in red cell membranes measured by electron spin resonance spectroscopy has previously been shown to be heterogeneous and not to vary in the absence or presence of cytoskeletal proteins (Bittman et al., 1984). These results are consistent with our observation of both dimers and higher oligomers in Band 3 samples prepared from ghosts stripped of the cytoskeleton. Higher Band 3 oligomers interact with the cytoskeleton, but the cytoskeleton is not required to maintain the oligomeric state.

That the membrane domain of Band 3 is found exclusively as a dimer, with no higher or lower order oligomers, suggests that the membrane domain alone is sufficient to maintain dimeric structure and that higher order interactions are mediated by the cytoplasmic domain. This conclusion is consistent with studies using spin-labeled Band 3 in which Nigg and Cherry (1980) found that trypsin treatment of ghost membranes, which released the cytoplasmic domain, increased the rotational mobility of Band 3 more than did membrane stripping alone.

Since Band 3 preparations in the absence of the cytoskeleton yield ~70% dimers and 30% higher oligomers, one can calculate that of the 1.2 × 106 Band 3 molecules/cell, there are ~360,000 arranged in higher oligomeric states. Thevinin and Low (1990) estimate that there are 200,000 ankyrin-binding sites/red cell. Since effectively all higher oligomers were retained by the cytoskeleton, there are ~360,000 Band 3 molecules associated with the cytoskeleton. There are thus 1.8 immobilized Band 3 molecules/molecule of bound ankyrin. If one assumes that the higher oligomers are all tetramers, then this is close to two molecules of ankyrin/Band 3 tetramer. A single ankyrin molecule may interact with two Band 3 molecules, cross-bridging two Band 3 dimers. Band 3 tetramers are found in the absence of ankyrin; however, ankyrin may play a role in stabilization of tetramers in the membrane.

**Band 3 Oligomeric State and Senescence—**Since Band 3 isolated from older red cells contains more of the higher oligomeric forms of Band 3 than that from younger cells, the origin of higher oligomeric forms of Band 3 appears to be linked to red cell aging (Lutz and Stringaro-Wipf, 1983). The decrease of dimer fraction in aged red cells does not arise only from an increase in tetramers, but is largely due to an increase in even larger oligomers. The highest oligomeric forms that appear at the void volume may be directly involved in red cell senescence. This is consistent with the fact that Band 3 is known to be found in Heinz bodies, protein aggregates in the membranes of abnormal and old red cells (Low et al., 1985). Furthermore, some form of modified Band 3 has been implicated as the senescence antigen for red cell turnover (Kay, 1984). It is possible that Band 3 develops higher order associations as the red cell ages, and these may play some part in signaling cell age, or they could be incidental to cell aging. The cytoplasmic domain likely plays a key role in cell aging. Alterations in this domain result in a shift of Band 3 oligomeric state toward higher forms. This alteration in the cytoplasmic domain may be due to sulfhydryl oxidation or perhaps a change in the interaction with hemoglobin or the cytoskeleton. The clustering of Band 3 mediated by the cytoplasmic domain may act as a transmembrane signal to promote binding of antibodies to the external face of Band 3 in aged cells.

Recently, Band 3 sulfhydryl oxidation was implicated as a...
requirement for formation of red cell senescence antigen (Beppu et al., 1990). It was shown in this paper that disulfide linkages were not required to maintain the oligomeric structure of Band 3. However, the more highly associated oligomers were observed to be more disulfide-cross-linked than the dimers. The formation of higher order oligomers could therefore be a step that precedes sulfhydryl oxidation in the process of formation of senescence antigen (Lutz and Stringaro-Wipf, 1983). The tendency of the more associated Band 3 oligomers to form disulfide linkages could be due to the increased local concentration of Band 3 found in these oligomers relative to dimers. Also, the dimers may have a conformation that is not conducive to intermolecular disulfide linkages. The associations between dimers to form larger oligomers appear to be different than those that hold together monomers to form dimers in allowing disulfide linkages. Thus, it seems that Band 3 oligomers larger than dimers may be implicated in red cell senescence.

In summary, Band 3 oligomeric state in C2E8 solution consists predominantly of dimers and, to a lesser extent, tetramers and larger oligomers. Whereas Band 3 in detergent solution can be caused to aggregate to a very large size, no shift of Band 3 dimers/tetramer ratio could be induced. No evidence was found for a large amount of Band 3 monomers in detergent solution, but monomeric Band 3 could be produced by protein denaturation. Results suggest that the oligomeric state of Band 3 in C2E8 is stable and may represent a “snapshot” view of the oligomeric state of Band 3 existing at the moment of solubilization. Some of Band 3 heterogeneity can be explained by the shift of Band 3 to large oligomers as the cell ages. The role of Band 3 tetramers may be to interact with the cytoskeleton.

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REFERENCES

Andersen, J. P. (1989) Biochim. Biophys. Acta 988, 47-72
Andersen, J. P., Vilsen, B., and Møller, J. V. (1986) Biochemistry 25, 6439-6447
Bennett, Y. (1982) Biochim. Biophys. Acta 689, 470-484
Bennett, G. D., and Kay, M. M. B. (1981) Exp. Haematol. (Oak Ridge) 9, 297-307
Beppu, M., Mizukami, A., Nagoya, M., and Kikugawa, K. (1990) J. Biol. Chem. 265, 3228-3233
Beth, A. H., Conturo, T. E., Venkataramu, S. D., and Staros, J. V. (1986) Biochemistry 25, 3854-3862
Bittman, R., Sakaki, T., Tani, A., Devaux, P. F., and Nishini, S. I. (1984) Biochim. Biophys. Acta 769, 85-95
Bodhoo, A., and Reithmeier, R. A. F. (1984) J. Biol. Chem. 259, 785-790
Bosman, L. J. C. G. M., and Kay, M. M. B. (1980) Biochem. Cell Biol. 58, 1419-1424
Bredford, M. M. (1976) Anal. Biochem. 72, 248-254
Cabanchik, Z. I., and Lieberman, D. M. (1974) J. Membr. Biol. 15, 207-226
Casey, J. R., Lieberman, D. M., and Reithmeier, R. A. F. (1989) Methods Enzymol. 173, 454-462
Chang, H. W., and Bock, E. (1980) Anal. Biochem. 104, 112-117
Chen, Y. H., Yang, J. T., and Martinez, H. M. (1972) Biochemistry 11, 4120-4131
Clarke, S. (1975) J. Biol. Chem. 250, 5459-5469
Cox, J. V., and Lazarides, E. (1988) Mol. Cell. Biol. 8, 1327-1335
Cuppoletti, J., Goldfinger, J., Kang, B. J., Berenski, C., and Jung, C. Y. (1985) J. Biol. Chem. 260, 1917-1917
Deisenhofer, J., Epp, O., Miki, K., Huber, R., and Michel, H. (1985) Nature 318, 603-605
Dempsey, C. E., Ryba, N. J. P., and Watts, A. (1986) Biochemistry 25, 2180-2187
Dixon, H. B. F., and Perham, R. N. (1986) Biochim. Biophys. Acta 109, 312-313
Freitag, C., and Clarke, S. (1981) J. Biol. Chem. 256, 6102-6108
Grasberger, B., Minton, A. P., Delisi, C., and Metzger, H. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 6203-6209
Gristina, S., Ship, S., and Rothstein, A. (1978) Biochim. Biophys. Acta 507, 294-304
Gurny, B., Wieth, J. O., and Tosteson, D. C. (1975) J. Gen. Physiol. 65, 731-749
Helenius, A., McCauslin, D. R., Fries, E., and Tanford, C. (1979) Methods Enzymol. 56, 734-749
Jay, D., and Cantley, L. (1986) Annu. Rev. Biochem. 55, 511-533
Jennings, M. L. (1984) J. Membr. Biol. 80, 105-117
Jennings, M. L. (1989) Annu. Rev. Biochem. Biophys. Chem. 18, 397-430
Jennings, M. L., and Passow, H. (1979) Biochim. Biophys. Acta 554, 498-519
Kay, M. M. B. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 5755-5757
Klingenberg, M. (1981) Nature 290, 449-454
Kopoto, R. R., and Lodish, H. F. (1985) Nature 316, 234-238
Leonard, K. H. (1970) Nature 227, 680-685
Le Maire, M., Agerbeck, L. P., Monteliethe, C., Andersen, J. P., and Møller, J. V. (1986) Anal. Biochem. 153, 523-535
Lieberman, D. M., and Reithmeier, R. A. F. (1983) Biochemistry 22, 4028-4033
Low, P. S. (1986) Biochim. Biophys. Acta 864, 145-167
Low, P. S., Waugh, S. M., Zinke, K., and Drenckhahn, D. (1985) Science 227, 531-533
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
Lucakovic, M. F., Feinstein, M. B., Sha’afi, R. I., and Perrie, S. (1981) Biochemistry 20, 3145-3151
Lutz, H. (1987) Biomed. Biochim. Acta 46, 65-71
Lutz, H. U., and Stringaro-Wipf, G. (1983) Biomed. Biochim. Acta 42, 117-121
Macara, I. G., and Cantley, L. C. (1981) Biochemistry 20, 5095-5105
Makino, S. (1986) J. Biol. Chem. 261, 4755-4760
Makino, S., and Charlie Pirraglia for preparation

Yu, J., and Branton, D. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 3891-3895
Yu, J., and Stock, T. L. (1975) J. Biol. Chem. 250, 9167-9184