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Hydrodynamic Properties of Human Erythrocyte Band 3 Solubilized in Reduced Triton X-100

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ABSTRACT The oligomeric state and function of band 3, purified by sulfhydryl affinity chromatography in reduced Triton X-100, was investigated. Size exclusion high-performance liquid chromatography showed that a homogeneous population of band 3 dimers could be purified from whole erythrocyte membranes. The elution profile of band 3 purified from membranes that had been stripped of its cytoskeleton before solubilization was a broad single peak describing a heterogeneous population of oligomers with a mean Stokes radius of 100 Å. Sedimentation velocity ultracentrifugation analysis confirmed particle heterogeneity and further showed monomer/dimer/tetramer equilibrium self-association. Whether the conversion of dimer to the form described by a Stokes radius of 100 Å was initiated by removal of cytoskeletal components, alkali-induced changes in band 3 conformation, or alkali-induced loss of copurifying ligands remains unclear. After incubation at 20°C for 24 h, both preparations of band 3 converted to a common form characterized by a mean Stokes radius of 114 Å. This form of the protein, examined by equilibrium sedimentation ultracentrifugation, is able to self-associate reversibly, and the self-association can be described by a dimer/tetramer/hexamer model, although the presence of higher oligomers cannot be discounted. The ability of the different forms of the protein to bind stilbene disulfonates revealed that the dimer had the highest inhibitor binding affinity, and the form characterized by a mean Stokes radius of 114 Å to have the lowest.

INTRODUCTION

The human erythrocyte band 3 is a polytopic membrane glycoprotein (911 residues) that facilitates the one-for-one exchange of chloride for bicarbonate (Low, 1986; Passow, 1986; Tanner et al., 1989; Tanner, 1993). Although the functional characteristics of band 3 have been extensively studied (Knauf and Brahm, 1989; Liu et al., 1996), the smallest oligomeric form of the protein required for anion transport has not yet been resolved. Determination of the minimum oligomer required for functionality is critical to an understanding of the mechanism of band 3-mediated anion transport. Furthermore, higher oligomers may be important in regulating anion transport activity (Klingenberg, 1981; Salhany, 1992, 1996) in the transport of amino acids (Goldstein et al., 1996), in the binding of cytosolic proteins (Low et al., 1989), and in acting as senescence antigens (Low et al., 1985; Turrini et al., 1991).

In situ studies of band 3 by freeze-fracture electron microscopy (Margaritis et al., 1977; Weinstein et al., 1980), flash-induced transient dichroism (Nigg and Cherry, 1980; Mühlebach and Cherry, 1985), cross-linking studies (Steck, 1972; Wang and Richards, 1975), and radiation inactivation (Cuppoletti et al., 1985) have indicated that the predominant oligomeric species is the dimer with some band 3 tetramer present. Although these studies imply that the smallest oligomer of band 3 found in the native membrane is the dimer, it has been suggested that reconstituted monomeric band 3 is able to transport anions (Lindenthal and Schubert, 1991). However, Casey and Reithmeier (1991) were unable to confirm the presence of monomeric band 3 in detergent solution by using size exclusion high-performance chromatography (SE-HPLC). Furthermore, image reconstruction of two-dimensional arrays of the transmembrane domain of band 3 indicated that a putative anion channel could lie at the interface between adjacent monomers (Wang et al., 1994). Monomeric band 3 has also been shown to be unable to bind stilbene disulfonates, suggesting a conformational perturbation of the monomer that does not occur in monomers that are self-associated into a dimer (Boodhoo and Reithmeier, 1984).

There are conflicting reports about the oligomeric state of purified band 3 in detergent solution. The protein has been characterized as stable dimers (Clarke, 1975; Pinder et al., 1995), a self-associating mixture of monomers-dimers-tetramers (Pappert and Schubert, 1983; Schubert et al., 1983), and a polydisperse mixture of either monomers and dimers (Lukacovic et al., 1981), dimers and tetramers (Nakashima and Makino, 1980; Nakashima et al., 1981; Casey and Reithmeier, 1991; Schopfer and Salhany, 1992), or dimers and hexamers (Wong, 1993). The different hydrodynamic characteristics associated with different band 3 preparations indicate that the exact experimental conditions under which

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the protein is purified and examined are critical. Some purification protocols employ a high-pH wash to remove membrane cytoskeleton before band 3 solubilization (Casey et al., 1989). However, the conformations of the cytoplasmic domain and the membrane domain are perturbed by changes in pH (Low, 1986; Kang et al., 1992), and removal of the cytoskeleton by alkaline treatment may induce changes in band 3 conformation and/or oligomeric structure.

The concentration of purified band 3 in detergent solution, a parameter that will vary with purification protocol, affects the oligomeric state of the protein (Schopfer and Salhany, 1992). Dilution of solubilized band 3 initiated irreversible aggregation, a process that could be slowed by the addition of substrate anions, stilbene disulfonates, and certain types of phospholipid. A possible role of copurifying ligands in stabilizing band 3 dimers was proposed. In addition to dilution, increasing the temperature of purified band 3 in detergent solution may also induce self-association (Vince et al., 1997). At 37°C the half-life for aggregation of band 3 was ~1 h, whereas the membrane domain was more stable under identical conditions, indicating a role of the cytoplasmic domain in protein aggregation (Salhany et al., 1997; Vince et al., 1997). The whole protein may be protected against temperature-induced aggregation by the addition of dimyristoylphosphatidylcholine (Vince et al., 1997), again implying that copurifying ligands may be important for maintenance of the oligomeric state of band 3 in detergent solution.

The presence of differing amounts of copurifying ligands may explain the inconsistencies in the hydrodynamic properties of purified band 3 in detergent solution. It is therefore important to determine the hydrodynamic characteristics of band 3 prepared by different chromatographic procedures. The present investigation characterizes band 3 prepared by sulfhydryl affinity chromatography. The protein is solubilized from either whole ghosts or ghosts stripped of cytoskeletal components and characterized using both SE-HPLC and analytical ultracentrifugation. We found that the choice of purification procedure, concentration of the protein, and subsequent incubation affected the hydrodynamic characteristics of purified band 3 in detergent solution. Possible in vivo roles of the different isolated forms of the protein are discussed.

MATERIALS AND METHODS

Materials

Recently outdated blood was obtained from the Regional Transfusion Centre (Birmingham, England). Triton X-100 (TX-100), specially purified for membrane research, was purchased from Boehringer Mannheim (Mannheim, Germany). Reduced Triton-100 (rTX-100) was obtained from Sigma Chemical Company (Poole, England). Sepharose CL-4B was obtained from Pharmacia (Uppsala, Sweden), and [[*p*-(chloromercuri)benzamido]ethyl]-Sepharose 4B (*p*-CMB) resin was synthesized from this according to the method of Lukacovic et al. (1981). Diethylaminoethyl (DEAE) cellulose was obtained from Whatman International (Maidstone, England). 4-Benzamido-4′-aminostilbene-2,2′-disulfonate (BADS) was synthesized according to the method of Kotaki et al. (1971). All other materials were reagent grade or better.

Isolation of band 3

All procedures were performed at 4°C unless otherwise stated. Erythrocyte ghost membrane was prepared by hemolytic lysis of red cells in 5 mM sodium phosphate, 0.5 mM EDTA (pH 8), containing 20 μg/ml phenylmethylsulfonyl fluoride (PMSF) (Dodge et al., 1963). Two protocols were used for the purification of band 3 protein. In the first, band 3 was solubilized from erythrocyte membrane after removal of cytoskeletal proteins, a procedure that solubilizes band 3 quantitatively (Casey et al., 1989). In the second protocol, band 3 was solubilized from the membrane without removal of cytoskeletal components (Casey and Reithmeier, 1993). This is thought to solubilize selectively band 3 not associated with the cytoskeleton.

Method 1

Erythrocyte ghost membranes were stripped of cytoskeleton by extraction with 10 volumes of ice-cold 2 mM EDTA (pH 12). Immediately after dilution of the ghosts, the membranes are pelleted by centrifugation (30,000 $\times$ *g* for 30 min) and subsequently washed three times with ice-cold 5 mM phosphate (pH 8). Band 3 was solubilized by the addition of 5 volumes of 1% (w/v) TX-100 in 5 mM phosphate (pH 8) for 1 h. After centrifugation (30,000 $\times$ *g* for 30 min), the supernatant was applied directly to a *p*-CMB affinity column at a flow rate of 0.5 ml/min (Lukacovic et al., 1981). The column was washed with 5 column volumes of 0.1% (w/v) TX-100 in 150 mM NaCl, 150 mM phosphate (pH 8). For SE-HPLC studies, the column was further washed with 5 column volumes of 0.1% (w/v) rTX-100, 100 mM NaCl, 5 mM phosphate (pH 7.2), and band 3 was eluted in the same buffer containing 25 mM β-mercaptoethanol. For analytical ultracentrifugation studies, band 3 was eluted in 0.1% (w/v) rTX-100, 5 mM phosphate (pH 8), containing 25 mM β-mercaptoethanol. Detergent exchange of TX-100 for rTX-100 was monitored with an on-line UV detector (Pharmacia, Uppsala) at 280 nm.

Method 2

Erythrocyte membranes were depleted of band 6 by two incubations (on ice for 20 min) with 150 mM NaCl, 5 mM phosphate (pH 7.4). After band 3 solubilization in 5 volumes of 1% (w/v) TX-100, 5 mM phosphate (pH 8), and centrifugation (30,000 $\times$ *g* for 30 min), the phosphate concentration of the supernatant was adjusted to 36 mM. The supernatant was applied to a DEAE-cellulose column and washed with 5 column volumes of 0.1% (w/v) TX-100, 36 mM phosphate (pH 7.4). Band 3, together with mainly glycophorin and band 4.2, was eluted with 0.1% (w/v) TX-100, 150 mM NaCl, 150 mM phosphate (pH 7.4). This protein solution was applied directly to a *p*-CMB column and treated as in method 1.

Size exclusion high-performance liquid chromatography

Size exclusion HPLC (SE-HPLC) experiments were performed at ambient temperature with a 7.5$\times$ 300 mM TSK 4000 SW column (Casey and Reithmeier, 1991). A Gilson 25SC pump was used at a flow rate of 0.5 ml/min. Purified band 3 (typically 0–2 mg/ml) was applied to the column with a 100-μl injection loop. Sample volumes were typically 30 μl, and the elution buffer was 100 mM NaCl, 5 mM phosphate (pH 7.2), containing 0.1% (w/v) rTX-100. Protein elution was monitored at 225 nm with a Gilson 119 UV/VIS absorbance detector. Chromatograms were analyzed with a Gilson 715 HPLC system software controller. Elution volumes were standardized according to the method of Ackers (1967). The column was calibrated according to the method of Casey and Reithmeier (1991).

Stilbene disulfonate binding assay

The binding of BADS to band 3 oligomers was measured by fluorescence enhancement in a Perkin-Elmer LS 50B fluorimeter, using an excitation wavelength of 280 nm and emission of 450 nm, according to the method of Casey et al. (1989).

Analytical ultracentrifugation

All sedimentation velocity and sedimentation equilibrium experiments were performed in a Beckman (Palo Alto, CA) Optima XL-A analytical ultracentrifuge equipped with automatic scanning absorption optics, in combination with an An-60 Ti rotor. Protein samples were analyzed immediately after purification.

Sedimentation velocity runs, which typically took 2–3 h, used 12-mm KELF standard double-sector centerpieces and were performed at 35,000 rpm, 20°C. Sample volumes were 450 μl, and the buffer composition of the reference sector was identical to that of the sample containing band 3. In particular, β-mercaptoethanol was added to both the reference sector solution and the buffer used for protein elution at the same time, because it is well known that the absorbance of this sulfydryl reagent at 280 nm is time dependent. Similar precautions were taken in preparing samples for sedimentation equilibrium analysis. Protein migration was detected at 280 nm, and data were analyzed by the transport method (Schachman, 1959). The molecular weight (weight average) of the band 3–detergent complex was calculated from a modified form of the Svedberg equation (Svedberg and Pederson, 1940):

$$M_w = \frac{6\pi N_A \eta^0_{20,w} R_S s^0_{20,w}}{(1 - \bar{\nu}\rho_{20,w})} \quad (1)$$

where N_A is Avogadro's number, $\eta^0_{20,w}$ (poise) is the viscosity of water at 20°C, R_S is the Stokes radius (obtained from SE-HPLC analysis), $s^0_{20,w}$ is the sedimentation coefficient corrected to the density and viscosity of water at 20°C and extrapolated to zero concentration, $\bar{\nu}$ (ml/g) is the partial specific volume of the protein-detergent complex, and $\rho_{20,w}$ (g/ml) is the density of water at 20°C. The frictional ratio (f/f_0) was calculated from experimentally obtained values of R_S or $s^0_{20,w}$ (Tanford, 1961):

$$\frac{f}{f_0} = R_S\left(\frac{4\pi N_A}{3M_W}\right)^{1/3} \quad (2)$$

The shape parameter P is given by (Tanford, 1961)

$$P = \frac{f}{f_0}\left(1 + \frac{\delta}{\bar{\nu}\rho_{20,w}}\right)^{-1/3} \quad (3)$$

where the shape contribution to the frictional ratio is denoted as P, following Perrin (1936), and the hydration of the protein-detergent complex is given by δ, which is the number of grams of solvent associated with 1 g of unsolvated protein-detergent complex. A value of $\delta = 0.2$ g/g, typical of soluble proteins, was used in the present investigation (Tanford, 1961). From P the sedimenting species can be modeled as a prolate ellipsoid of semiaxes a, b, and axial ratio a/b. A polynomial inversion procedure (ELLIPS1 of Harding and Cölfen, 1995) for accurately predicting a/b from the Perrin function P was employed.

Sedimentation equilibrium experiments used a six-channel centerpiece (three solution/solvent pairs) and were performed at 5000 rpm, 20°C. Sample volumes were 125 μl, and the buffer composition of the reference sector was identical to that of the sample containing band 3. Equilibrium was attained after 24 h, and there was no further change in the pattern over a period of a further 12 h, which indicates that there were no problems due to differential oxidation by the 25 mM β-mercaptoethanol present in both the solvent and solution channels. Furthermore, control experiments indicated that the absorbance at 280 nm of 25 mM β-mercaptoethanol in buffer did not increase sufficiently to invalidate equilibrium absorbance measurements obtained near the centrifuge cell bottom. Protein concentration was monitored at 280 nm, and the baseline was experimentally determined by increasing the rotor speed to 45,000 rpm after completion of the equilibrium sedimentation run. Data were analyzed by the M* method of Creeth and Harding (1982), using the QUICKBASIC routine MSTARA as described by Harding et al. (1992). The following two features of MSTARA were utilized: 1) estimation of the weight-averaged molecular weight over the entire distribution in the ultracentrifuge cell and 2) evaluation of point weight-averaged apparent molecular weights $M_{w,app}(r)$ as a function of local protein concentration $c(r)$ (or the equivalent concentration $A(r)$ in UV absorbance units) in the ultracentrifuge cell.

Differentiation between polydispersity and self-association was achieved by analysis using the omega function, $\Omega(r)$ (Milthorpe et al., 1975):

$$\Omega(r) = \frac{c(r) \cdot \exp^{[\phi_1 M_{w,app}(r_F^2 - r^2)]}}{c(r_F)} \quad (4)$$

where $c(r)$ is the total solute concentration at radial distance r. $M_{w,app}$ is the molar mass of the smallest sedimenting oligomer, $\phi_1 = (1 - \bar{\nu}\rho) \cdot \omega^2/2RT$, where $\bar{\nu}$ is the smallest sedimenting sedimenting species, ω is the angular velocity of the rotor (rad/s), and r_F is an arbitrary reference position in the cell. A group of programs named SEDPROG for Omega analysis was used (which is available from the RASMB ftp server, bbri.harvard.edu). The Omega function was fitted with reaction models to estimate equilibrium constants for association reactions and the degree of nonideality.

Solvent densities, viscosities, and partial specific volume

Solvent densities were determined at (20.00 ± 0.01)°C with an Anton-Paar model DMA 02C precision density meter. Solvent viscosities at (20.00 ± 0.01)°C were determined with a Schott-Geräte AVS-310 automatic viscometer. A partial specific volume for band 3 of (0.740 ± 0.007) ml/g (Dorst and Schubert, 1979) and a detergent/protein binding ratio of 0.77 g/g (Clarke, 1975) were taken from the literature. Using a measured value of (0.9734 ± 0.0003) ml/g for r-TX-100, and assuming that detergent/protein binding ratio is the same for rTX-100 as it is for TX-100, the partial specific volume of rTX-100–band 3 complex was calculated to be (0.842 ± 0.004) ml/g at 20.00°C.

Other methods

Protein concentrations were determined using a Coomassie-binding assay (Pierce, Chester, England). Sodium dodecyl sulfate gel electrophoresis was performed according to the method of Laemmli (1970).

RESULTS

Band 3 was purified using two different strategies, one of which stripped cytoskeleton from the membrane before solubilization, and the other of which solubilized band 3 without prior stripping of the cytoskeleton. From either method, sample purity was found to be greater than 95%, as determined by densitometry measurements of SDS-PAGE gels stained with Coomassie Blue.

SE-HPLC analysis

Fig. 1 *a* shows the elution profiles obtained by SE-HPLC analysis of pure band 3 produced from the two purification methods. Protein purified from method 1 elutes with a Stokes radius of (100 ± 2) Å. In contrast, band 3 purified

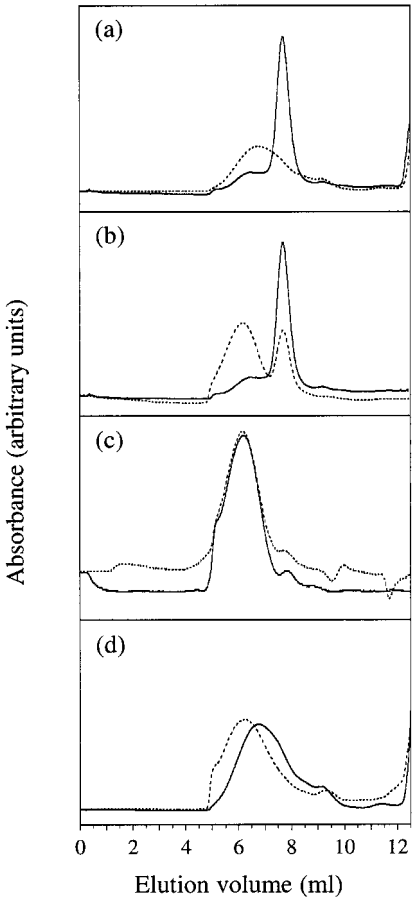


FIGURE 1 Size exclusion HPLC (SE-HPLC) analysis of purified band 3 and its oligomers applied to a 7.5× 300-mm TSK 4000 SW column, monitored at 225 nm. (*a*) Band 3 was purified from membrane that had been stripped of its cytoskeleton by 2 mM EDTA (pH 12) before purification (purification method 1; – – –), and band 3 was purified from membrane that had been depleted of band 6 but had not been stripped of cytoskeleton (purification method 2; ——). (*b*) SE-HPLC analysis of band 3 dimer prepared according to method 2, isolated by SE-HPLC chromatography, held at 4°C for 24 h, and rechromatographed (——), and dimer prepared according to method 2, isolated by SE-HPLC chromatography, held at 20°C for 24 h, and rechromatographed (– – –). (*c*) As in *b*, except that the higher oligomer prepared according to method 2 was isolated and rechromatographed. (*d*) As in *b*, except that band 3 was prepared according to method 1. The column was calibrated with standard proteins: thyroglobulin (R_S = 86 Å), ferritin (R_S = 63 Å), catalase (R_S = 52 Å), and aldolase (R_S = 46 Å). The void (V_0) and total volumes (V_t) were determined by the elution positions of blue dextran and β-mercaptoethanol, respectively. The elution buffer was 100 mM NaCl, 5 mM phosphate (pH 7.0), containing 0.1% (w/v) rTX-100. The peak eluting at 12.5 ml is β-mercaptoethanol, which is present in the protein sample.

from method 2 has a Stokes radius of (76 ± 2) Å. Casey and Reithmeier (1991) have shown the peak at 76 Å to be the dimer and tentatively assigned the species eluting with a Stokes radius of 100 Å as the tetramer. The oligomeric state of band 3 isolated using method 2 was found to depend on the concentration of purified protein. At concentrations of band 3 greater than 1.3 mg/ml in 0.1% (w/v) rTX-100, a mixture of both dimer and a higher oligomer, characterized by a Stokes radius of (114 ± 2) Å, was produced.

Fig. 1, *b* and *c*, shows SE-HPLC analysis of the two oligomers prepared according to method 2 (prepared at a protein concentration at which both oligomers were formed) both before and after incubation for 24 h at 20°C. After purification the oligomers were separated by SE-HPLC and rechromatographed immediately (*continuous lines*). These two oligomers were then incubated and reanalyzed by SE-HPLC (*dashed lines*). The sample separated as the pure dimer partly aggregated after incubation. Dimer held at 4°C for the same length of time showed no detectable change in elution profile. The higher oligomer did not aggregate; nor did it form any dimer. This suggests that there is no equilibrium between the two distinct oligomeric species under the conditions of this analysis and that the dimer is not stable at 20°C, but aggregates irreversibly to the higher oligomeric state. The rate of aggregation of the dimer was monitored by SE-HPLC during incubation at 20°C, and the half-life was estimated to be ~5 h (determined from integration of chromatograms, assuming a Gaussian distribution).

Fig. 1 *d* shows the effect of incubation for 24 h at 20°C on the aggregation state of band 3 prepared according to method 1. The elution position of this oligomer shifted from 6.75 to 6.18 ml. This corresponds to a change in the R_S from 100 to 114 Å. The Stokes radius of this species after incubation is identical to the Stokes radius of the higher oligomer prepared according to method 2.

The half-width at half-height of the three peaks describing the three different forms of band 3 was determined by fitting a Gaussian distribution to the elution profiles. For the peak characterizing protein with an R_S = 76 Å, the half-width at half-height was found to be (0.50 ± 0.04) ml; the corresponding values for the peaks characterizing protein with an R_S = 100 Å and R_S = 114 Å were (1.8 ± 0.4) and (1.9 ± 0.3) ml, respectively. The almost fourfold increase in peak widths for the two faster eluting forms of the protein, compared with the slow eluting form, which has previously been shown to be dimer only (Casey and Reithmeier, 1991), indicate possible multiple oligomers and/or a heterogeneous population of conformers.

Binding of BADS to the different oligomers

Fig. 2 presents the binding of BADS to different isolated forms of band 3 prepared from both methods and the effect of incubation for 24 h at 20°C on the ability of BADS to complex to these forms. The mean Stokes radius of the preparations was characterized by SE-HPLC both before and after incubation. It was found that pure dimeric band 3 prepared according to method 2 bound BADS with a K_d = 0.8 μM, similar to that found by Casey and Reithmeier (1991). After incubation the dimer and form characterized by R_S = 114 Å were separated by SE-HPLC. The dimer retained its affinity for BADS, whereas the faster eluting species exhibited reduced affinity for BADS binding. Band 3 prepared according to method 1 showed a lower affinity for BADS compared with the dimeric protein. The affinity

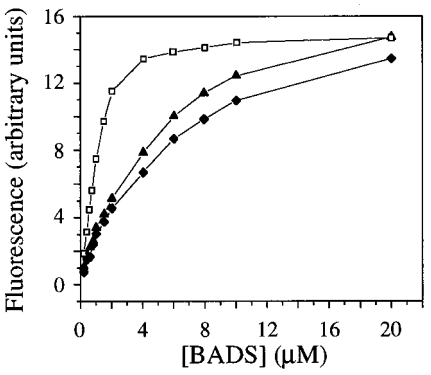


FIGURE 2 Concentration of BADS binding to isolated oligomers of band 3. Dimeric band 3 was prepared according to method 2 (R_S = 76 Å) (□). Band 3 was prepared according to method 1 (R_S = 100 Å) (▲). Band 3 was prepared according to either method 1 or method 2 after 24 h of incubation at 20°C (R_S = 114 Å) (◆). Oligomers were separated before fluorescence analysis by SE-HPLC.

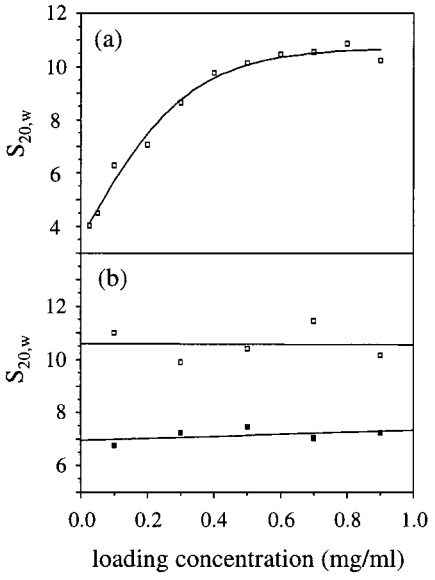


FIGURE 3 Sedimentation velocity analysis of band 3 prepared according to method 1 (*a*) and band 3 prepared according to method 2, where □ represents the measured $s_{20,w}$ value for dimeric band 3 (R_S = 76 Å) and ■ represents the measured $s_{20,w}$ value for the oligomer with R_S = 114 Å (*b*). Absorbances were measured at 280 nm, except for 0.025 and 0.05 mg/ml solutions, which were measured at 225 nm. The rotor speed was 35,000 rpm, and the temperature was 20°C. Band 3 was purified in 0.1% (w/v) rTX-100, 5 mM phosphate (pH 8), containing 25 mM β-mercaptoethanol.

of this form for the stilbene disulfonate was further reduced after incubation to a level comparable with that observed for the protein characterized by a Stokes radius of 114 Å. These observations support the hypothesis that band 3 prepared according to either method 1 or method 2 forms an "end state" with a common quaternary structure after incubation.

Sedimentation velocity

For centrifugation analysis, low ionic strength buffer was chosen because it has previously been argued that this condition better mimics possible in situ interactions mediated by the cytoplasmic domain (Liu and Palek, 1984; Walder et al., 1984; Kliman and Steck, 1980; see also Discussion). A critical role of the cytoplasmic domain in band 3 interactions in vitro has previously been suggested (Vince et al., 1997).

Fig. 3 *a* shows that the sedimentation coefficient of band 3 purified from method 1 increased with increasing protein concentration. The lowest value observed was at ~4S, the highest at 11S. The minimum sedimentation coefficient was previously assigned to monomeric band 3 and the highest to the tetramer (Dorst and Schubert, 1979). Recentrifugation of the same sample gave $s_{20,w}$ values that were identical to those obtained from the initial run. This behavior is common for equilibrium reactions in which the rate of self-association is fast or intermediate on the time scale of the experiment (each velocity experiment took ~3 h) (Schachman, 1959; Cann, 1970; Fujita, 1975). These observations contrast with the SE-HPLC analysis, which showed that the protein preparation was characterized by a mean R_S of 100 Å and assigned as tetramer (Casey and Reithmeier, 1991). The peak width of this eluting species was, however, much broader than was observed for the dimer, indicating protein heterogeneity. Furthermore, because increasing protein concentration favors the tetramer (Fig. 3 *a*), the SE-HPLC analysis may have been performed at a protein concentration at which tetramer is the prominent band 3 oligomer present. Alternatively, the low ionic strength used in sedimentation velocity analysis may have favored band 3 self-association mediated by the cytoplasmic domain (Vince et al., 1997).

In contrast to the concentration-dependent sedimentation coefficient observed for band 3 prepared from method 1, band 3 prepared from method 2 gave two distinct sedimenting species (Fig. 3 *b*). The slow moving boundary was calculated to have an $s^{o}_{20,w}$ value of (6.9 ± 0.1) S, and the faster moving boundary was characterized by a value of (10.6 ± 0.7) S. These sedimentation coefficients, previously assigned to the dimer and tetramer (Dorst and Schubert, 1979; Pappert and Schubert, 1983), were found to be independent of protein concentration in the range examined. This implies that the two forms do not participate in self-association reactions, at least not during the time course of the experiment, and that the system is behaving in a thermodynamically ideal manner. Control experiments with rTX-100 buffer showed no sedimenting boundaries.

Table 1 shows the hydrodynamic parameters calculated for band 3 prepared according to method 2 from both SE-HPLC and sedimentation velocity analysis. The molecular weight (calculated from Eq. 1) and oligomeric state are consistent with the slower sedimenting species representing a dimer-detergent complex. Previous studies have unambiguously shown that this slower sedimenting species with a Stokes radius of 76 Å is indeed dimeric (Casey and Reithmeier, 1991). Molecular weight determination using Eq. 1

TABLE 1 SE-HPLC and sedimentation velocity ultracentrifugation analysis of the hydrodynamic properties of band 3 oligomers purified from membrane that had been depleted of band 6 but had not been stripped of cytoskeleton

Parameter	Mean ± SD (n = 3)	
	Slow sedimenting species	Fast sedimenting species
Stokes radius (Å)	76 ± 2	114 ± 2
$s^{o}_{20,w}$	6.9 ± 0.1	10.6 ± 0.7
$M^{*}_{w,app}$	377,000 ± 16,000	868,000 ± 58,000
oligomeric state[#]	1.95 ± 0.1	4.4 ± 0.3
f/f_o[§]	1.50 ± 0.06	1.79 ± 0.06
f/f_o[¶]	1.55 ± 0.10	1.68 ± 0.28
P[‖]	1.39 ± 0.06	1.66 ± 0.05
P**	1.44 ± 0.08	1.49 ± 0.21
a/b[##]	7.0 ± 0.7	10.5 ± 0.9

*Determined from Eq. 1.

[#]Calculated assuming a detergent/protein binding ratio of 0.77 g/g (Clarke et al., 1975), a monomer molecular weight of 101.8 kDa, and an additional 7% contribution from sugars (Lux et al., 1989).

[§]Calculated from Eq. 2, using the measured Stokes radius obtained from SE-HPLC analysis.

[¶]Calculated from Eq. 2, using the measured sedimentation coefficient obtained from ultracentrifugation analysis.

[‖]Calculated using Eq. 3 from f/f_o determined using the measured Stokes radius and assuming a hydration value, δ, of 0.2 g/g (Tanford, 1961).

**Calculated using Eq. 3 from f/f_o determined using the measured sedimentation coefficient and assuming a hydration value, δ, of 0.2 g/g (Tanford, 1961).

[##]Axial ratio calculated from an average of the two Perrin shape parameters obtained for each sedimenting species.

requires an accurate measure of the Stokes radius of the protein-detergent complex. The present study used gel filtration to determine the Stokes radius, which gives reliable values for reasonably symmetrical particles (Nozaki et al., 1976). Highly asymmetrical particles are retarded, because of end-on insertion of the particle into the gel pores, leading to an anomalously low value for R_S and a concomitant underestimate of particle molecular weight. As the molecular weight determined for the slower sedimenting particle (with a measured Stokes radius of 76 Å) is consistent with a dimeric band 3–detergent complex, it may be inferred that the band 3–detergent complex is not retarded due to end-on insertion of the particle into gel pores.

The frictional ratios quoted in Table 1 may be used to estimate particle shape, but only if the hydration level of the complex is known. Thus the frictional ratio of 1.5 for the slower sedimenting species does not necessarily imply particle asymmetry. In Table 1 an arbitrary hydration value of 0.2 g/g was used to determine the axial ratio of the protein-detergent complex based on a simple prolate ellipsoid of revolution (Tanford, 1961). However, it should be stressed that the presence of detergent may increase the hydration of the complex, and indeed, it has been shown that the hydration of TX-100 micelles may be as high as 1.4 g/g (Yedgar et al., 1974). So, as another extreme, if it is assumed instead that the dimer–rTX-100 complex is spherical, a value of 2.0 g/g for the hydration may be derived from Eq. 3, using the measured frictional ratio of 1.5. In reality, of course, a situation between these extremes is highly likely, although an unequivocal picture requires additional experimental techniques. Any attempt to make further inferences about particle asymmetry requires more hydrodynamic measurements (viscosity, rotational diffusion, radius of gyration) beyond the scope of the current investigation. It is worth noting, however, that a more reliable (because it is detergent-free) frictional ratio determined for the band 3 cytoplasmic domain alone shows it to be asymmetrical, with a value of 1.6 (Appell and Low, 1981; Cölfen et al., 1996). Furthermore, the frictional ratios for both band 3–TX-100 and the transmembrane domain–TX-100 complexes have been reported as 1.7 and 1.47, respectively (Reithmeier, 1979).

Sedimentation equilibrium

Band 3 prepared according to either method 1 or method 2 gave identical results when analyzed by equilibrium sedimentation at a rotor speed of 5000 rpm and a temperature of 20°C. As demonstrated by SE-HPLC, both the dimer and the form characterized by a Stokes radius of 100 Å are not stable at 20°C, associating to the higher oligomer described by R_S = 114 Å (Fig. 1 *b* and *d*). SE-HPLC analysis confirmed that the conversion of both of these oligomers to the 114 Å form of the protein had occurred during equilibrium sedimentation. Centrifugation at 4°C retarded but did not eliminate this conversion, leading to a mixture of different band 3 forms that was deemed too complex to analyze. Because SE-HPLC analysis showed that both the dimer and the 100 Å forms are stable at 4°C over a time period similar to that of the centrifugation equilibrium experiment (Fig. 1 *b* and *d*), dilution of the purified protein before centrifugation may have promoted protein aggregation over this 24-h time period. In a typical experiment band 3 was purified at a concentration of 4 mg/ml (from either method of preparation) and subsequently diluted at least fourfold before centrifugation. A similar dilution-induced aggregation has been observed by Schopfer and Salhany (1992).

To determine whether the form of band 3 characterized by a mean Stokes radius of 114 Å is in a single oligomeric state or a mixture of different oligomers, equilibrium plots of ln c against r^2 were first examined. Linearity of such a curve is consistent with a single macromolecular species (but not unequivocal proof, because of opposing effects of heterogeneity and nonideality). In fact, analysis of the sedimentation equilibrium profiles indicated deviation from linearity, implying significant redistribution of solute species of different molecular weights within the solution column of the ultracentrifuge cell. These observations were independent of the initial loading concentration of the protein in the centrifuge cell. Control experiments showed that detergent alone did not reequilibrate; therefore, aggregation of the protein caused by detergent redistribution was discounted as a reason for sample heterogeneity.

Fig. 4 *a* shows the variation of M^* as a function of ξ, where ξ is the radial displacement squared parameter (ξ =

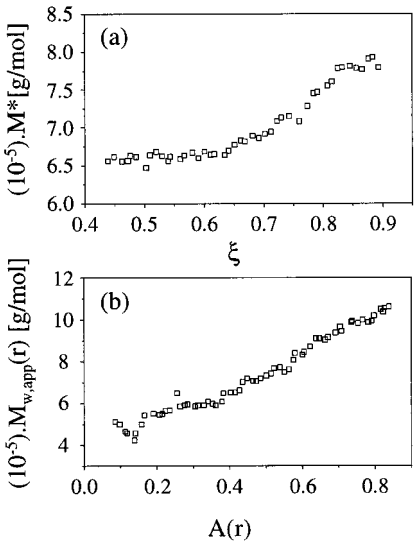


FIGURE 4 Sedimentation equilibrium analysis of band 3 form previously characterized by R_S = 114 Å. (*a*) M^* is plotted versus ξ, the radial displacement squared parameter, at 5000 rpm. (*b*) The same sample analyzed by conventional point-averaged molecular weight determination. The initial loading concentration was 0.5 mg/ml, the absorbance was measured at 280 nm, and the temperature was 20°C. Band 3 was purified in 0.1% (w/v) rTX-100, 5 mM phosphate, pH 8, containing 25 mM β-mercaptoethanol.

$(r^2 - a^2)/(b^2 - a^2)$; a and b are the radial positions of the meniscus and cell bottom, respectively). From the M^* analysis and the identity that $(M^*(\xi - 1) = M_{w,app}$, the average molecular weight over the whole cell, the latter parameter of the protein-detergent complex was estimated to be (820,000 ± 60,000) Da, corresponding to an average oligomeric state of (4.2 ± 0.4). The mean Stokes radius of this form of the protein was 114 Å, also slightly larger than the putative tetramer (Casey and Reithmeier, 1991), indicating agreement between the two methods of analysis. Furthermore, from SE-HPLC analysis, oligomeric heterogeneity was suggested by the large peak width of this form of the protein, compared to the dimer, which concurs with sedimentation equilibrium analysis.

Fig. 4 *b* presents $M_{w,app}(r)$, the local point-averaged molecular weight (calculated from local gradients of ln $A(r)$ against r^2), plotted against $A(r)$, the absorbance at distance r along the cell, for the same data as in Fig. 4 *a*. There is clearly considerable redistribution of solute, implying sample size heterogeneity. By extrapolation of $M_{w,app}(r)$ back to zero protein concentration, a weight-averaged molecular weight of ~420,000 was estimated for the smallest band 3–detergent complex present, implying dimer. Sedimentation equilibrium at protein concentrations down to 0.1 mg/ml confirmed the presence of a dimeric band 3–detergent complex (data not shown). Although these observations do not rule out the possible presence of monomer, there is no evidence for it here. At high protein concentration, found near the cell bottom, where protein association is promoted, values of ~1.0–1.2 million are seen, indicating a protein-detergent complex to at least the level of hexamer, although significant amounts of higher order oligomers cannot be ruled out.

Analysis of equilibrium sedimentation data by the Omega function

The Omega function ($\Omega(r)$; Eq. 4) was used to differentiate between self-association and polydispersity in the sample, as it is more discriminating than the traditional plot of $M_{w,app}(r)$ versus $A(r)$ for several loading concentrations (Roark and Yphantis, 1969; Ralston and Morris, 1992). For a self-associating system, $\Omega(r)$ is a continuous function that increases with increasing protein concentration $c(r)$, and for different initial protein loading concentrations, plots of $\Omega(r)$ against concentration $c(r)$ will coincide at common point concentrations. Polydisperse systems will not show overlap at common point concentrations from different initial protein concentrations (Milthorpe et al., 1975; Ralston and Morris, 1992).

Fig. 5 shows a plot of $\Omega(r)$ against $A(r)$ (absorbance) for three data sets obtained at three different initial protein loading concentrations. The data sets show good overlap, indicative of a self-associating system. The Omega function was calculated assuming a smallest molecular weight sedimenting species of dimer-detergent complex, as deduced from $M_{w,app}$ analysis (Fig. 4 *b*). Results from the Omega analysis were modeled by monomer/dimer, monomer/dimer/tetramer, dimer/tetramer, and dimer/tetramer/hexamer self-association models. Of these, the dimer/tetramer/hexamer model is best described by the Omega analysis, as judged by a lack of systematic trends in the residual plot and by the sum of squared residuals for alternative models (Fig. 6). The sum of squared residuals for the monomer/dimer, monomer/dimer/tetramer, and dimer/tetramer models were

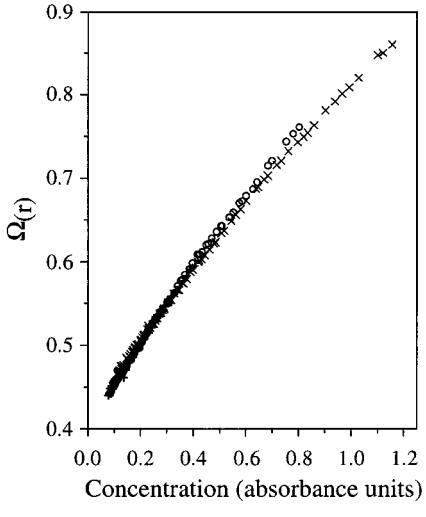


FIGURE 5 Omega analysis as a diagnostic test of self-association. Omega analysis of the protein with a rotor speed of 5000 rpm shows an overlap of samples with different loading concentrations of protein. The initial loading concentrations were 0.2 (+), 0.6 (○), and 0.9 (×) mg/ml. c_F = 0.3 mg/ml.

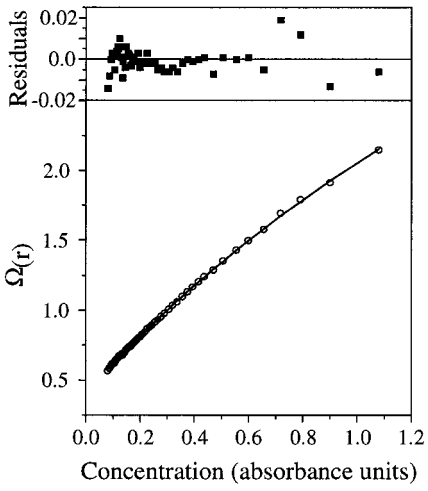


FIGURE 6 Fitting of data obtained from the Omega function analysis. The best fit for the sample at 5000 rpm was obtained from a dimer/tetramer/hexamer model of the pooled data from Fig. 5, with equilibrium constants $K_{12} = (1.6 \pm 0.5) \times 10^5\ M^{-1}$ and $K_{14} = (7.3 \pm 0.8) \times 10^{16}\ M^{-3}$, and the second virial coefficient, B, calculated as $(1.6 \pm 0.3) \times 10^{-7}$ L mol g^{-2}.

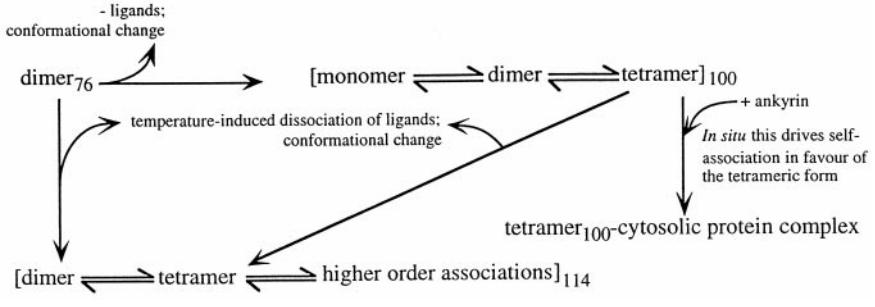
190, 7, and 7 times that of the dimer/tetramer/hexamer. It should be stressed that although the molecular weight at the bottom of the cell corresponds to a hexamer-detergent complex, higher oligomeric species, which would sediment to the cell bottom, may also be present. It seems more likely, based on the grounds of symmetry, that an indefinite association better describes this form of the protein. Attempts were made to characterize these putative higher oligomeric species by sedimentation equilibrium analysis at 3000 rpm. However, this low rotor speed was unable to induce sufficient redistribution of the protein oligomers for effective analysis. Identification of these higher oligomers was not pursued further, because their formation is a consequence of protein instability in detergent solution and is not physiologically relevant.

DISCUSSION

The present study characterizes the hydrodynamic properties of purified band 3 with and without prior stripping of the cytoskeleton in both near-physiological and low ionic strength buffer systems. SE-HPLC analysis of band 3 was performed in buffer containing 100 mM NaCl, so that nonspecific electrostatic interactions did not affect Stokes radius determinations. Band 3 purified from whole erythrocyte membranes was characterized by a Stokes radius of 76 Å, previously assigned as the dimer (Casey and Reithmeier, 1991). Elution of band 3 above a concentration of 1.3 mg/ml yielded a form of the protein characterized by a mean Stokes radius of 114 Å. This oligomeric state of the protein was also prepared by temperature-induced association of both the dimer (R_S = 76 Å) and the form of the protein characterized by a mean Stokes radius of 100 Å. The 100-Å form, which was previously assigned as the in situ tetramer (Casey and Reithmeier, 1991), was prepared by high pH removal of membrane cytoskeleton before solubilization. The elution peak widths of the 100-Å and 114-Å forms of band 3 were fourfold greater than those observed for dimer, indicating sample heterogeneity of the two faster eluting band 3 preparations. The cause of heterogeneity may be multiple oligomers and/or conformers.

Analytical ultracentrifugation analysis of the hydrodynamic characteristics of the protein was performed in low ionic strength buffer. Sedimentation velocity analysis revealed that both the dimer (R_S = 76 Å) and the form characterized by a Stokes radius of 114 Å were stable during the time course of the experiment (~3 h). The oligomer with a mean Stokes radius of 100 Å participated in a monomer/dimer/tetramer self-association under the conditions of the experiment. Sedimentation equilibrium analysis revealed that the 114-Å form of the protein participated in a dimer/tetramer/higher order self-association that occurred on a time scale slower than the sedimentation velocity analysis.

When the results obtained from this study are considered with the findings of others concerning 1) putative conformational changes associated with band 3 oligomerization (Casey and Reithmeier, 1991; Salhany et al., 1997; Schopfer and Salhany, 1992; Vince et al., 1997); 2) the role of ligands in stabilizing dimeric band 3 in vitro (Maneri and Low, 1989; Schopfer and Salhany, 1992; Vince et al., 1997); and 3) the binding of ankyrin to band 3, which shifts the monomer/dimer/tetramer equilibrium in favor of the tetramer (Huber et al., 1996), a model describing solubilized band 3 hydrodynamic behavior may be proposed:



The brackets indicate a form of the protein that eluted as a single peak by SE-HPLC analysis. This peak is characterized by the Stokes radius given in the subscript. Oligomeric sample heterogeneity and self-association behavior of the single peak were subsequently demonstrated by centrifugation techniques. Thus the $[\text{monomer/dimer/tetramer}]_{100}$ equilibrium, observed by sedimentation velocity analysis, describes self-association of the protein fraction that eluted at a mean Stokes radius of 100 Å. The dimer_{76} refers to the stable dimer, as judged by sedimentation velocity analysis, eluting at a Stokes radius of 76 Å, and the $[\text{dimer/tetramer/higher order associations}]_{114}$ equilibrium, observed by sedimentation equilibrium analysis, describes the self-association of the protein fraction that eluted at a mean Stokes radius of 114 Å. A possible mechanism can be proposed to explain the conversion of dimer_{76} to $[\text{monomer/dimer/tetramer}]_{100}$ in detergent solution. In this mechanism, ligands dissociate from the stable dimer_{76}, causing a change in the conformation of the protein. This conformational change initiates dissociation of dimer_{76} to monomer. The monomers so formed are then able to participate in the $[\text{monomer/dimer/tetramer}]_{100}$ reversible equilibrium. A similar mechanism may be proposed for the conversion of dimer_{76} to $[\text{dimer/tetramer/higher order associations}]_{114}$, except that no evidence of an intermediate monomeric state was observed in the present investigation.

The model presented describes the hydrodynamic behavior of band 3 in detergent solution. It is generally accepted that band 3 exists as a mixture of stable dimers and tetramers in situ. The physiological significance of the observed $[\text{monomer/dimer/tetramer}]_{100}$ band 3 self-association is therefore uncertain, but this form of the protein has been isolated by others using different purification procedures and seems not to arise from an artifact of the purification procedure described in this study (Casey and Reithmeier, 1991; Schopfer and Salhany, 1992). Arguments that may explain why studies fail to reveal evidence for such self-association behavior occurring in situ include the following: 1) ankyrin binds only to tetrameric band 3 and therefore provides a mechanism to drive the monomer/dimer/tetramer self-association toward an exclusively [tetramer-ankyrin] complex (see model); 2) only after removal of ankyrin can the self-associating form of band 3 equilibrate in situ to a mixture of monomers, dimers, and tetramers entirely consistent with the monomer/dimer/tetramer ↔ [tetramer-ankyrin] model presented here (Van Dort et al., 1998); and 3) in situ equilibration of band 3 oligomers occurs very slowly compared to that observed in detergent solution, which may explain why other studies fail to detect the presence of band 3 monomers in situ after removal of ankyrin (Van Dort et al., 1998). This slow equilibration in situ is consistent with modeling studies which predict that protein association within a membrane is greatly enhanced relative to dilute solution, because of the high concentration and preoriented state of self-associating species (Grasberger et al., 1986).

The dimer_{76} was considered stable by both SE-HPLC and sedimentation velocity analysis. The dimer_{76} may be converted to a form with a mean Stokes radius of 100 Å by alkali removal of the cytoskeleton before solubilization and protein purification. The SE-HPLC elution profile of this self-associating species is much broader than that obtained for the nonequilibrating dimer, and describing the faster eluting species as a single oligomer (tetramer) may be inappropriate (Casey and Reithmeier, 1991). Sedimentation velocity analysis under conditions of low ionic strength, where ionic interactions may play a role in band 3 association behavior, revealed that this form of the protein was able to participate in a $[\text{monomer/dimer/tetramer}]_{100}$ self-association (where the subscript refers to the mean Stokes radius measured by SE-HPLC in 100 mM NaCl). However, there was no indication of monomeric band 3 by SE-HPLC analysis performed under conditions of high ionic strength, where charge effects would be screened out (Casey and Reithmeier, 1991). We believe that low ionic strength buffer used during analytical ultracentrifugation analyses more appropriately mimics the in vivo environment experienced by the N-terminal cytoplasmic domain of band 3, a domain that plays a critical role in mediating band 3 self-association (Vince et al., 1997). The choice of low ionic strength buffer is based on the findings that only at low ionic strength can the interaction of cytosolic proteins with band 3 cytoplasmic domain be observed in vitro (Moriyama and Makino, 1987; Strapazon and Steck, 1977; Higashi et al., 1979; Chetrite and Cassoly, 1985; Premachandra, 1986), although the same complexations have been observed in vivo at physiological ionic strength (Ercolani et al., 1992; Low et al., 1987; Harrison et al., 1991; Rogalski et al., 1989). It has been proposed that the high concentration of cytoplasmic hemoglobin will counteract the dissociating effect of physiological ionic strength through an excluded volume mechanism (Liu and Palek, 1984). Furthermore, binding experiments in vitro generally involve concentrations of cell constituents that are orders of magnitude smaller than those found in vivo; when a correction is made for these dilution effects, the in vivo and in vitro results are reconciled (Walder et al., 1984; Kliman and Steck, 1980). The possible role of ionic interactions in mediating band 3 self-association is suggested from the observation that reversible band 3 aggregation within the membrane is induced upon acidification (Casey and Reithmeier, 1991). Presumably the amino acids involved in this charge-mediated association are predominantly located within the N-terminal cytoplasmic domain, because no such pH-dependent association was observed for the transmembrane domain (Vince et al., 1997). Thus it is important to accurately mimic the in vivo environment of the cytoplasmic domain during in vitro oligomeric analysis of the protein, so that band 3 association behavior mediated by N-terminal ionic interactions represents those interactions occurring in vivo. Consideration of such a charge-mediated band 3 self-association may explain differences between the in vitro analyses of the $[\text{monomer/dimer/tetramer}]_{100}$ form of the protein performed at both

high and low ionic strength by SE-HPLC and analytical ultracentrifugation, respectively. Only the low ionic strength analysis revealed self-association behavior.

Removal of the cytoskeleton by incubation at pH 12 before solubilization converts $dimer_{76}$ to band 3 described by a mean Stokes radius of 100 Å. As discussed above, charge effects may play a role in the conversion of $dimer_{76}$ to $[monomer/dimer/tetramer]_{100}$. The well-established pH dependence of the cytoplasmic domain conformation may also induce such an oligomeric conversion (Low et al., 1984; Low, 1986). In terms of the proposed model, different conformations of the cytoplasmic domain would define $dimer_{76}$, $[monomer/dimer/tetramer]_{100}$, and $[dimer/tetramer/higher\ order\ associations]_{114}$. The conformer defining $dimer_{76}$ does not self-associate, whereas the two conformers defining $[monomer/dimer/tetramer]_{100}$ and $[dimer/tetramer/higher\ order\ associations]_{114}$ promote self-association of the protein in vitro. Evidence for the existence of at least two distinct forms of the cytoplasmic domain in situ comes from the ability of tetrameric band 3 alone to bind hemoglobin and cytosolic proteins, whereas dimeric band 3 was unable to bind such enzymes (Mulzer et al., 1990; Thevenin and Low, 1990; von Ruckmann et al., 1997; Schuck and Schubert, 1991). Kinetic analysis of ankyrin binding to the cytoplasmic domain of band 3 has revealed a two-phase binding (Thevenin and Low, 1990). The initial fast phase involved predominantly the binding of ankyrin to low affinity sites. The apparent pK_a values describing this reversible pH dependence (7.2 ± 0.1 and 9.2 ± 0.1) defined states of band 3 with high, moderate, and no capacity to bind ankyrin (in order of increasing pH). Because these pK_a values also characterize three distinct conformation states of the isolated cytoplasmic domain, it was hypothesized that the reversible structural equilibrium in the band 3 cytoplasmic domain could influence ankyrin binding (Low et al., 1984; Low, 1986). As preferential binding of ankyrin to band 3 tetramer is observed, it seems reasonable that the conformation of the cytoplasmic domain of band 3 tetramers is in the high-affinity state. Conversely, the N-terminal conformer associated with $dimer_{76}$ has a lower affinity for ankyrin binding. Circular dichroism studies on the isolated oligomeric forms of band 3, however, indicate that no large changes in protein secondary structure accompany association of the protein (Casey and Reithmeier, 1991; Werner and Reithmeier, 1985). An alternative mechanism describing cytoplasmic domain-mediated band 3 associations may be proposed. Instead of a large change in conformation, perhaps the different oligomeric forms are characterized by a change in spatial orientation of the cytoplasmic domain, relative to the transmembrane domain (Low et al., 1984).

In view of this observed pH-induced conversion of $dimer_{76}$ to the form characterized by $R_S = 100$ Å by treatment of membranes at pH 12, the seemingly contradictory data of Casey and Reithmeier (1991) require some comment. Casey and Reithmeier were able to copurify both the $dimer_{76}$ and the higher oligomer, after removal of the cytoskeleton by incubation at pH 12, by solubilization in octaethylene glycol mono-*n*-dodecyl ether ($C_{12}E_8$) and subsequent anion exchange chromatography. Several factors may explain the observed differences between the two studies. First, Reithmeier (personal communication) has observed that Hg^{2+} (originating from synthesis of the resin) is present in samples of band 3 purified by sulfhydryl chromatography, the method of band 3 preparation used in this study, and this divalent cation may promote protein associations. It has been demonstrated that Mg^{2+} was necessary for the formation of 2-D arrays of band 3, thereby implying that divalent cations promote protein-protein interactions (Dolder et al., 1993; Wang et al., 1994). Furthermore, binding of Zn^{2+} to the cytoplasmic domain of band 3 has been shown to induce conformational changes in both the cytoplasmic and the transmembrane portions of the protein (Tu and Xu, 1994; Tu and Yang, 1995). Similarly, Ca^{2+} also binds to the cytoplasmic domain of band 3 and causes changes in the conformation of this domain (Thevenin et al., 1994; Salhany and Cordes, 1991). Thus divalent cation-induced conformational changes in the cytoplasmic domain may promote $dimer_{76}$ association. Second, the number of lipids copurifying with band 3 has been shown to be critical to the oligomeric state of the protein. Band 3 copurifies with five to seven tightly bound long-chain phospholipids, and removal of these phospholipids by prolonged washing of the matrix-bound protein promotes aggregation (Maneri and Low, 1989). Differences between the two studies may be due to differences in the column washing procedures employed. Finally, the choice of detergent may be important in stabilizing a particular oligomeric form of the protein. Calorimetric analysis of band 3 in both TX-100 and $C_{12}E_8$ indicated a similar stabilization of the protein in the two detergents (Sami et al., 1992). However, the stability of different oligomeric forms of the protein in the different detergents was not examined. It is therefore possible that dimeric band 3 is more stable in $C_{12}E_8$, whereas TX-100 may promote formation of $tetramer_{100}$.

The isolation of $dimer_{76}$ exclusively was shown to be dependent on the concentration of purified protein. Above a protein concentration of 1.3 mg/ml, the form of band 3 characterized by a mean Stokes radius of 114 Å copurified with $dimer_{76}$. This corresponds to the binding of one detergent micelle per band 3 monomer. Casey and Reithmeier (1993) showed that, although a variety of detergents may be used to isolate dimeric band 3, the detergent concentration in all cases must be above the critical micelle concentration to maintain dispersed dimers. Furthermore, for rTX-100, the minimum detergent concentration required for stabilization of the band 3 dimer corresponded to the binding of one detergent micelle/band 3 monomer. To fulfill this binding requirement at a rTX-100 concentration of 0.1% (w/v), the concentration used in the present study, the protein concentration must not exceed 1.2 mg/ml. The same form of band 3 ($R_S = 114$ Å by SE-HPLC analysis) may also be prepared by temperature-induced aggregation, by an increase in the detergent concentration, or by dilution, suggesting that dissociation of copurifying ligands is concomitant with forma-

tion of this oligomer (Schopfer and Salhany, 1992; Vince et al., 1997). The production of a common form of band 3, independent of the method of formation, indicates that the initial association of the protein is a specific process. Whether ligand dissociation is followed by dissociation of dimers to transient monomers or by association of dimers themselves remains to be established.

The addition of ligands to dimeric band 3 has been demonstrated to stabilize $dimer_{76}$ in detergent solution (Schopfer and Salhany, 1992; Vince et al., 1997). Of physiological relevance is the stabilization observed by the addition of chloride (but not sulfate) and of lipids. Chloride induces conformational changes in the transmembrane domain of band 3 distinct from those associated with sulfate binding (Ruffing et al., 1996). Thus the transmembrane domain conformation may also contribute to $dimer_{76}$ stability. Conformational changes in the transmembrane domain of band 3 upon conversion of $dimer_{76}$ to the other forms characterized by mean Stokes radii of 100 and 114 Å was suggested by the different binding affinities for BADS. Alternatively, it has been observed that other stilbene disulfonates are capable of dissociating higher oligomers of band 3 to form dimer exclusively (Van Dort et al., 1998; Schuck et al., 1995). Therefore, the apparent reduced affinity of BADS for the different aggregates of band 3 compared to dimer may instead reflect the ability of BADS to dissociate these different oligomeric forms of the protein to dimer. It should be noted, however, that not all stilbene disulfonates are able to stabilize band 3 dimers, and the affect of BADS binding on the association state of band 3 has not been characterized (Schuck et al., 1995). The mechanism of aggregation induced by loss of phospholipids may be driven by the need to minimize interactions between hydrophilic solvent and the hydrophobic transmembrane helices (Mouritsen and Bloom, 1984; Cornea and Thomas, 1994). Alternatively, essential phospholipids may play a structural role in stabilization of $dimer_{76}$ (Vince et al., 1997). Binding of ligands to the transmembrane domain may also affect the conformation/spatial orientation of the cytoplasmic domain through an allosteric mechanism. The binding of stilbene disulfonates to the transmembrane domain has been shown to affect the conformation of the band 3 cytoplasmic domain (Batenjany et al., 1993), and stilbene disulfonates are also able to stabilize $dimer_{76}$ in vitro (Schopfer and Salhany, 1992; Vince et al., 1997). Similarly, binding of hemoglobin to the cytoplasmic domain of band 3 affected sulfhydryl reactivity at a site over 100 Å from the hemoglobin-binding site (Salhany and Cassoly, 1989). It was suggested that such long-range conformational changes would imply that the cytoplasmic domain is poised to function as a cytosolic arm or lever to modulate the global structure of the porter.

In conclusion, the choice of purification protocol allowed isolation of either stable dimeric band 3 or a form of the protein (R_S = 100 Å) that was able to participate in self-association. The mechanism of conversion of the stable dimer to the self-associating form is unknown but may involve an initial dissociation of ligands that stabilize the dimer, followed by a change in the conformation/spatial orientation of the cytoplasmic domain. Both of these forms of the protein aggregated to a species characterized by R_S = 114 Å that participated in dimer/tetramer/higher order self-association.

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