Molecular Characterization of the Human Erythrocyte Anion Transport Protein in Octyl Glucoside†

Pamela K. Werner* and Reinhart A. F. Reithmeier
Department of Biochemistry, The University of Alberta, Edmonton, Alberta, Canada T6G 2H7
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ABSTRACT: Band 3 protein, the anion transport protein of the human erythrocyte membrane, was purified in the presence of the nonionic detergent octyl glucoside. A molecular characterization was carried out to investigate whether the native structure of the protein was retained in the presence of this detergent. Band 3 bound octyl glucoside below the critical micelle concentration (cmc) of the detergent, approaching saturation above the cmc. At 40 mM octyl glucoside, close to saturating concentrations, 0.64 g of octyl glucoside is bound per gram of band 3 protein, corresponding to 208 molecules of detergent bound per monomer of band 3. Sedimentation velocity and gel filtration studies, performed at 40 mM octyl glucoside, indicated that the band 3–octyl glucoside complex had an average molecular weight of 1.98×10^6, which corresponds to a dodecamer. Sedimentation equilibrium experiments confirmed that band 3 in octyl glucoside exists in a heterogeneous and high oligomeric state. This high oligomeric state did not change dramatically over octyl glucoside concentrations ranging from 6 to 60 mM. The circular dichroism spectrum of band 3 changed only slightly over this range of octyl glucoside concentrations. The α-helical and β-sheet contents of band 3 in 2 mM octyl glucoside were calculated to be 40% and 27%, respectively, indicating that no gross alteration in the secondary structure of the protein had occurred in octyl glucoside. The ability of band 3 to bind 4-benzamido-4′-aminostilbene-2,2′-disulfonate (BADS), a potent inhibitor (K_i = 1 μM) of anion transport, was measured to assess the integrity of the inhibitor binding site of the protein in octyl glucoside. Band 3 in octyl glucoside bound BADS (K_d = 0.8–1.5 μM), but at high octyl glucoside concentrations, an irreversible decrease in the binding affinity (to $K_d \simeq 10$ μM) was observed. The results indicate that while band 3 in octyl glucoside retained a high degree of secondary structure, the inhibitor binding site had been altered during purification in this detergent.

Band 3 protein, the major integral membrane protein of human erythrocytes, is responsible for the exchange of anions such as chloride and bicarbonate across the plasma membrane (Knauf, 1979; Macara & Cantley, 1983). This protein has been purified and characterized in the presence of several detergents (Yu & Steck, 1975a,b; Lukacovic et al., 1981; Nakashima & Makino, 1980). Measurement of the transport activity of purified, detergent-solubilized band 3 is accomplished by incorporating the protein into phospholipid vesicles. There have been many attempts at reconstitution of band 3 (Ross & McConnell, 1978; Wolosin, 1980; Lukacovic et al., 1981; van Hoogevest et al., 1983), but many of these reconstituted preparations have achieved only a fraction of the native transport activity or an activity that has been altered in some other way compared to the native state.

Octyl glucoside has been used successfully for the solubilization, purification, and reconstitution of several intrinsic membrane proteins. This detergent has several properties which make it suitable for reconstitution. It is readily dialyzable due to its high critical micelle concentration (cmc)[1] [20–25 mM (Shinoda et al., 1961)], which allows more efficient removal in reconstitution. This might prevent nonspecific leaks in liposomes due to residual detergent. In addition, it is considered a "mild" detergent that can effectively solubilize membrane proteins without disturbing the native structure of the protein. Because of these properties, octyl glucoside could be expected to be useful as a solubilizing detergent in reconstitution of band 3 and might alleviate some of the problems encountered in previous attempts. The more recent reconstitutions (Köhne et al., 1983; Darmon et al., 1983) have achieved specific transfer rates of up to 80% of those in the native state, as well as activation energies of transport that are close to the native value. This recovery of functionality is more than previous reconstitutions have reported. The reconstitution by Darmon et al. (1983) is of particular interest because it used purified band 3 in octyl glucoside.

Alterations in the function of band 3 protein reconstituted into phospholipid vesicles could be due either to the lack of some specific requirements of the protein, such as the use of inappropriate phospholipids, or to some alteration of the protein during its purification in the presence of detergent. This would be better understood if the intermediate state between the native state in the erythrocyte membrane and the reconstituted state, that of the purified, detergent-solubilized protein, were characterized. The necessity for characterizing both the conformation and function of membrane proteins in this intermediate state has been proposed by Tanford & Reynolds (1976). The intermediate state has been well characterized for band 3 in Triton X-100 (Clarke, 1975; Schubert et al., 1983) and in $C_{12}E_8$ and $C_{12}E_9$ [Nakashima & Makino, 1980 (bovine); Pappert & Schubert, 1983; Lieberman & Reith-

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[1] Abbreviations: BADS, 4-benzamido-4′-aminostilbene-2,2′-disulfonate; cmc, critical micelle concentration; $C_{12}E_8$, *n*-dodecyl octaethylene glycol monoether; $C_{12}E_9$, *n*-dodecyl nonaethylene glycol monoether; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonate; OG, *n*-octyl β-D-glucopyranoside (octyl glucoside); pCMB-Sepharose, [[*p*-(chloromercuri)benzamido]-ethylene]amino-Sepharose 4B.

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meier, 1983] but not in octyl glucoside. Since it has been observed that different results may often be obtained when different detergents are used to solubilize the same protein, a characterization should be done in each particular detergent.

In this study, purified band 3 in octyl glucoside has been characterized. The detergent binding properties of band 3 were determined and the oligomeric structure of band 3 assessed by sedimentation equilibrium, sedimentation velocity, and gel filtration studies. The secondary structure of band 3 was determined by circular dichroism, and the integrity of an inhibitor binding site was assessed by measuring the binding of the stilbenedisulfonate inhibitor BADS to band 3.

Materials and Methods

Materials. $C_{12}E_8$ was purchased from Nikko Chemical Co. (Tokyo), *n*-octyl β-D-glucopyranoside from Calbiochem, and *n*-octyl β-D-[U-^{14}C]glucopyranoside from New England Nuclear (specific activity 314 mCi/mmol). Sepharose 4B was obtained from Pharmacia, aminoethyl-Sepharose was prepared according to Shaltiel & Er-El (1973), and pCMB-Sepharose was synthesized as described by Lukacovic et al. (1981).

Standards used in gel filtration and determination of Stokes radius (in parentheses) (LeMaire et al., 1980) were thyroglobulin (8.6 nm), catalase (5.2 nm), and aldolase (4.6 nm) (Pharmacia); apoferritin (6.1 nm) (Schwarz/Mann); and *Escherichia coli* β-galactosidase (6.9 nm) and myoglobin (1.9 nm) (Sigma Chemical Co.); Mengo virus (14.5 nm) was a gift from Dr. D. G. Scraba, The University of Alberta.

Purification of Band 3. All steps were carried out at 4 °C, unless otherwise stated. Outdated, packed human red blood cells were kindly provided by the Canadian Red Cross. Erythrocyte ghosts were prepared from this blood by hypotonic lysis in 5 mM sodium phosphate, pH 8.0, as described by Dodge et al. (1963), using a Millipore Pellicon cassette system with an HVLP 0.45-μm filter to recover the membranes (Rosenberry et al., 1981). Membranes were extracted with 10 volumes of 2 mM ethylenediaminetetraacetic acid, pH 12. Band 3 was purified by extraction of ghost membranes with 1% $C_{12}E_8$ and chromatography on aminoethyl-Sepharose and pCMB-Sepharose in the presence of detergent as described previously (Lieberman & Reithmeier, 1983). Detergent exchange of octyl glucoside for $C_{12}E_8$ was accomplished while the protein was bound to either aminoethyl- or pCMB-Sepharose. The column with bound protein was equilibrated with at least 3 column volumes of buffer containing 20–40 mM octyl glucoside (cmc = 20–25 mM). Following equilibration in the desired concentration of octyl glucoside, the protein was eluted in 50 mM sodium phosphate, pH 8.0 (0.1% β-mercaptoethanol was added when eluting from pCMB-Sepharose). The amount of $C_{12}E_8$ remaining associated with the eluted protein following this treatment was below the limits of detection of a chemical assay (Garewal, 1973; <0.015 g of $C_{12}E_8$/g of band 3 protein). Unless otherwise stated, all characterizations were performed with fresh, unfrozen preparations of the protein in 50 mM sodium phosphate, pH 8.0, containing 40 mM octyl glucoside.

Detergent Binding. Binding of [^{14}C]octyl glucoside to band 3 was determined at 4 °C under four conditions: equilibrium dialysis, gel filtration, during elution from an affinity resin, and while bound to an affinity resin. Equilibrium dialysis measurements were carried out in 1-mL cells shaken gently by an arm shaker. A solution of band 3 (1 mg/mL) in a given concentration of octyl glucoside was dialyzed against the same concentration of detergent containing the radioactive label. Each side was sampled for counting at up to 12-h intervals. Equilibrium was generally reached within 24 h for detergent concentrations below the cmc but took several days for concentrations above the cmc. Gel filtration measurements (Hummel & Dreyer, 1962) were done by preequilibrating and eluting a column (0.9 × 28 cm) of Sepharose CL4B in radioactive octyl glucoside by using the base line of radioactivity on either side of the protein peak to calculate the specific radioactivity. Samples of each fraction were taken for protein determination and liquid scintillation counting. Protein loading concentrations were 2–3 mg/mL, and the peak concentration of the eluted protein was 0.15–0.2 mg/mL. Measurements during elution from an affinity resin used band 3 complexed with pCMB-Sepharose. The column was equilibrated with radioactive octyl glucoside at a given concentration until a stable base line of radioactivity was reached. The protein was then eluted with the same buffer containing 0.1% β-mercaptoethanol. Fractions were sampled for protein determination and liquid scintillation counting. For measurements using protein complexed to an affinity resin, 1 part protein-saturated pCMB-Sepharose (1:1 slurry in 10 mM octyl glucoside) was added to 4 parts of a solution containing a given concentration of radioactive octyl glucoside. The amount of bound detergent was determined from the difference between the total and free detergent concentrations. The total octyl glucoside concentration was determined by counting aliquots of the resin suspension. The free concentration of octyl glucoside was determined by counting aliquots of the supernatant after pelleting the resin in a microfuge. β-Mercaptoethanol was added to the suspension (final concentration 0.1%) to release the bound band 3, and the protein concentration of the supernatant was determined. The value from this determination was corrected for the original volume to obtain the initial protein concentration.

From the value for the maximum amount of detergent bound to the protein, an estimate of the fraction of the surface area of a band 3 monomer covered by detergent was calculated, as described by Clarke (1975). A value of 0.354 nm^2 was used for the cross-sectional area of an octyl glucoside molecule, on the basis of the sugar cross-sectional area calculated from the crystal structure of decyl α-glucoside (Dorset & Rosenbusch, 1981).

Analytical Gel Chromatography. Calibration of a Sepharose 4B column (1 cm × 90 cm) with proteins of known Stokes radii (R_s) was carried out at 4 °C in 50 mM sodium phosphate, pH 8.0. Several of the protein standards were run in the same buffer containing either 15 or 40 mM octyl glucoside, and no difference in the elution pattern was noted. Relative elution volumes were determined from continuous monitoring of A_{280} using a Pharmacia UV monitor. Blue dextran and β-mercaptoethanol were used to determine the void and total volumes, respectively. The Stokes radii for all standards except Mengo virus were taken from LeMaire et al. (1983). A Stokes radius of 14.5 nm was calculated from the intrinsic diffusion coefficient given in Scraba et al. (1967).

Analytical Ultracentrifugation. Ultracentrifugation was carried out in a Beckman Model E analytical ultracentrifuge. Sedimentation velocity runs (40 000 rpm, 20 °C) used ultraviolet optics at low protein concentrations and schlieren optics for higher protein concentrations. Sedimentation equilibrium runs (3000 rpm, 20 °C) used ultraviolet optics. Procedures described by Chervenka (1973) were used for the calculation of weight-average molecular weights from sedimentation equilibrium runs, by using a $\bar{v}$ for the protein–detergent complex calculated as described below.

The molecular weight of the protein–detergent complex was calculated from the sedimentation coefficient ($s_{20,w}$), Stokes

radius (R_s), and partial specific volume ($\bar{v}$) by using the equation (Tanford et al., 1974):

$$M_c = \frac{6\pi\eta N_A s^0_{20,w,c} R_{s,c}}{1 - \bar{v}_c\rho} \quad (1)$$

where the subscript c denotes values for the protein–detergent complex, η is solvent viscosity, N_A is Avogadro's number, and ρ is solvent density. The partial specific volume of the protein–detergent complex ($\bar{v}_c$) was calculated (assuming that the partial specific volumes of the protein and detergent components are additive) by using the equation (Tanford et al., 1974):

$$\bar{v}_c = \frac{\bar{v}_P + \delta_D\bar{v}_D}{1 + \delta_D} \quad (2)$$

where $\bar{v}_P$ and $\bar{v}_D$ are the partial specific volumes of the protein and detergent, respectively, and δ_D is the amount of bound detergent (grams per gram of protein). A value for $\bar{v}_P$ of 0.74 cm^3/g, from measurements of acetic acid solubilized band 3 (Dorst & Schubert, 1979), was used. From density measurements of solutions of octyl glucoside at six concentrations above and below the cmc, the $\bar{v}_D$ was calculated to be 0.85 cm^3/g, in close agreement with a published value (Watts et al., 1982). The molecular weight of the protein component of the complex was obtained after correction for the bound detergent by using $M = M_c/(1 + \delta_D)$ (Tanford et al., 1974).

Other Procedures. Sodium dodecyl sulfate gel electrophoresis was performed according to Laemmli (1971). Protein concentrations were determined by the method of Lowry et al. (1951) in the presence of 1% sodium dodecyl sulfate with bovine serum albumin as a standard. Solution densities were measured at 20 ± 0.01 °C on an Anton Paar precision density meter, Model DMA 60 (Anton Paar, Graz, Austria).

Circular dichroism spectra were recorded on a Jasco J-500C spectropolarimeter with a Jasco DP-500N data processor. Spectra were determined from a minimum of two scans at 25 °C by using a 0.05-cm path-length cell. The α-helix and β-sheet contents were estimated from eq 3 by using the ex-

$$[\theta]_\lambda = f_H[\theta]_{H,\lambda} + f_\beta[\theta]_{\beta,\lambda} + f_R[\theta]_{R,\lambda} \quad (3)$$

perimental mean residue ellipticity values, $[\theta]$, at wavelengths of 215, 220, and 225 nm and by using the reference protein values provided by Chen et al. (1972). The f values are the fractions of helix (H), β-form, and random-coil (R) at each wavelength. A mean residue molecular weight of 113 (Yu & Steck, 1975a), which was based on the amino acid composition, was used in the calculation.

BADS binding to band 3 in solution was determined by fluorescence enhancement titration, as previously described (Rao et al., 1979; Lieberman & Reithmeier, 1983). All measurements were made at 20 °C by using a Perkin-Elmer MPF-44B fluorescence spectrophotometer and were corrected for dilution, background fluorescence, and fluorescence quenching. Protein samples were diluted with 28.5 mM sodium citrate, pH 7.4, for the titration. Excitation of the protein was done at 280 nm, and the fluorescence was measured at 450 nm. Excitation of BADS directly at 340 nm was not used because the background fluorescence was higher and increased dramatically above the detergent's cmc, which made the background correction more difficult. Presumably, this was due to BADS partitioning into micelles.

Results and Discussion

Octyl Glucoside Binding. The preparation of band 3 in octyl glucoside used in these experiments was over 95% pure, as judged by sodium dodecyl sulfate gel electrophoresis (Figure 1). The binding of octyl glucoside to this preparation of band 3 was measured over a range of free detergent concentrations (Figure 2) by using the methods of equilibrium dialysis, gel filtration, and measurements during elution from an affinity resin and while complexed to an affinity resin (described under Materials and Methods). Below the cmc of the detergent, all four methods of measurement gave approximately the same detergent binding curve, clearly indicating that band 3 bound monomers of octyl glucoside. Above the cmc, a maximal amount of octyl glucoside binding to band 3 was approached, although saturation of binding was not achieved at octyl glucoside concentrations as high as 60 mM. At 40 mM octyl glucoside, 0.64 g of detergent is bound per gram of protein, which corresponds to an average of 208 mol of octyl glucoside bound per mole of band 3, well above the aggregation number of octyl glucoside of 52 that has been reported (Roxby & Mills, 1981). The fact that binding occurs below the cmc indicates that monomers of octyl glucoside interact with band 3 and that band 3 does not simply insert into a detergent micelle. The extent of binding indicates a large hydrophobic surface area on the protein, which was to be expected for an intrinsic membrane protein. An estimate of the fraction of the surface

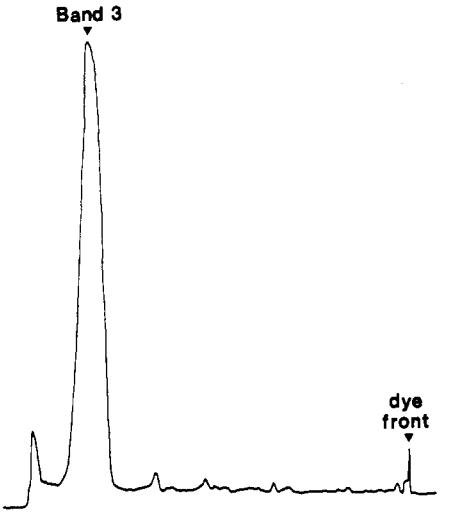

FIGURE 1: Sodium dodecyl sulfate gel electrophoresis of band 3 protein (50 μg) isolated in octyl glucoside. The gel contained 10% polyacrylamide and was stained with Coomassie brilliant blue and scanned at 600 nm.

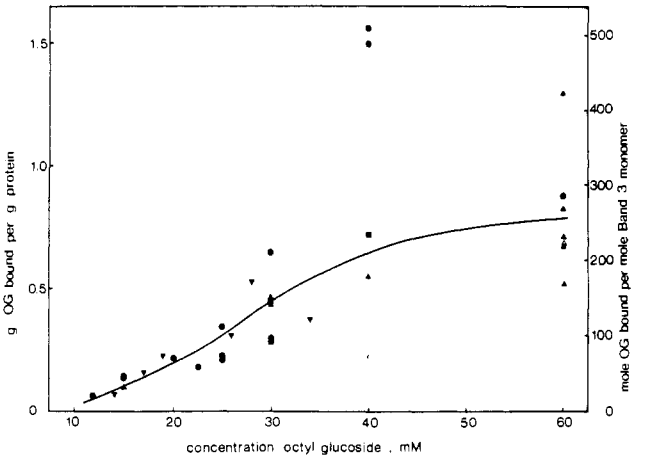

FIGURE 2: Binding of octyl glucoside to band 3 as a function of octyl glucoside concentration. Detergent binding was measured at 4 °C in 50 mM sodium phosphate, pH 8.0, with the stated concentration of octyl glucoside by equilibrium dialysis (●), by gel filtration (■), by measurement during elution from pCMB-Sepharose (▲), or by measurements while bound to pCMB-Sepharose (▼).

area of a band 3 monomer covered by detergent is calculated to be 0.64 for the level of binding in 40 mM octyl glucoside. Our results in octyl glucoside are similar to those of Clarke (1975), who reported that band 3 binds Triton X-100 below the cmc and calculated the fraction of the surface area of the protein interacting with the detergent (at maximal binding) to be 0.50–0.65. The amount of bound detergent must be considered in the hydrodynamic analysis of the band 3–octyl glucoside complex.

Hydrodynamics. It is important to know the oligomeric state of the protein in detergent solution, especially if any investigation of the relationship between oligomeric state and function is to be made, either in detergent solution or in a reconstituted state. Sedimentation velocity runs of band 3 in octyl glucoside produced a single broad boundary. In 40 mM octyl glucoside, where close to the maximum amount of detergent is bound, the sedimentation coefficient decreased linearly with increasing protein concentration over the protein concentration range of 0.3–2.5 mg/mL, giving an intrinsic sedimentation coefficient ($s^0_{20,w}$) of 32 S. This suggests that band 3 isolated in octyl glucoside is in a very high oligomeric state. Sedimentation coefficients reported for band 3 in other nonionic detergents are much lower: in Triton X-100, an $s_{20,w}$ of 6.9 S has been reported and ascribed to a stable noncovalent dimer (Clarke, 1975); in $C_{12}E_9$, two components were resolved with $s_{20,w}$ values of 7.5 and 11.1 S, corresponding to dimer and tetramer (bovine band 3; Nakashima & Makino, 1980). Pappert & Schubert (1983) obtained results similar to those of Nakashima and Makino in $C_{12}E_9$ but observed a single, broad boundary rather than two components, with the $s_{20,w}$ value increasing from 3.8 to 8 S as the protein concentration was increased. They obtained similar results in Ammonyx-LO (Pappert & Schubert, 1983). It is evident that band 3 in octyl glucoside is in an oligomeric state much higher than tetramer, which is the highest oligomeric structure usually observed by sedimentation in other nonionic detergents. Another major difference between the sedimentation behavior of band 3 in octyl glucoside and in $C_{12}E_9$ is that no decrease in $s_{20,w}$ was noted at lower protein concentrations in octyl glucoside, in contrast to observations by Pappert & Schubert (1983). It must be noted that we studied sedimentation only down to a protein concentration of 0.2 mg/mL, while they went down to 0.05 mg/mL. It is possible that dissociation might occur below the lowest protein concentration used in our study. However, it is the sedimentation coefficient at protein concentrations ≥0.2 mg/mL that would be of interest for use in estimates of the molecular weight since the Stokes radius has been determined at these concentrations.

The band 3–octyl glucoside complex, in 40 mM octyl glucoside, eluted from a Sepharose 4B column as a very broad peak of $K_{av} \simeq 0.40$ and sometimes contained a component (up to 10% of the total A_{280}) that eluted at the void volume. From a calibration curve using water-soluble, globular proteins of known Stokes radii, the elution position of the major component corresponded to a Stokes radius of 10.7. This is about 1.5 times the Stokes radius determined for the protein in Triton X-100 (Reithmeier, 1979).

The results from sedimentation velocity and gel filtration measured in 40 mM octyl glucoside were used to calculate a molecular weight of the band 3–octyl glucoside complex of 1.98×10^6. The molecular weight of the complex was corrected for the mass contributed by the bound detergent to obtain a molecular weight of the protein component (described under Materials and Methods), using the amount of octyl glucoside bound at 40 mM (0.64 g/g). This gave a molecular

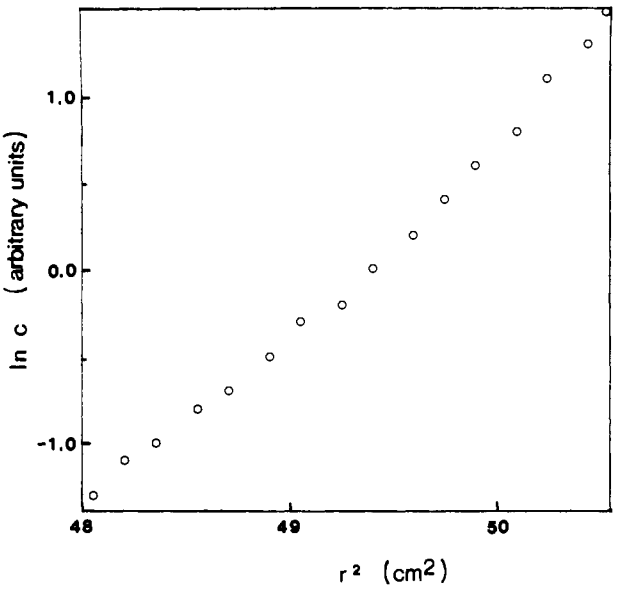

FIGURE 3: Sedimentation equilibrium centrifugation of band 3 in 40 mM octyl glucoside. The initial protein concentration was 1.37 mg/mL, and sedimentation was performed at room temperature with a rotor speed of 3000 rpm in 50 mM sodium phosphate, pH 8.0, and 40 mM octyl glucoside. The ln of the protein concentration (c) is plotted as a function of the square of the distance from the center of rotation (r^2).

weight for the protein component of the band 3–octyl glucoside complex of 1.21×10^6. On the basis of a monomeric molecular weight of 95 000, the calculated molecular weight corresponds to a dodecamer.

The sedimentation coefficient of the band 3–octyl glucoside complex increased with increasing octyl glucoside concentration from 6 to 60 mM (data not shown). However, an analysis similar to that above using $s^0_{20,w}$ and R_s determined in 15 mM octyl glucoside ($s^0_{20,w}$ = 18.2 S, R_s = 9.7 nm, aggregation number $\simeq$ 10) indicated that this could largely be accounted for by the increase in mass that would accompany detergent binding. This suggests that there is no drastic change in oligomeric state over the range of octyl glucoside concentrations used here (6–60 mM). Certainly higher octyl glucoside concentrations did not dissociate band 3 to lower oligomers.

The broad boundary observed in sedimentation velocity and gel filtration suggests that the oligomeric state may be heterogeneous. This was confirmed by sedimentation equilibrium. A curvilinear plot of ln c vs. r^2 was produced (Figure 3), indicating a molecular weight range from 1.8×10^6 to 3.15×10^6 for the band 3–octyl glucoside complex. A range of $(1.16–2.0) \times 10^6$, with an average of 1.6×10^6, was calculated for the protein component. On the basis of a monomeric molecular weight of 95 000, this corresponds to a range from a 12-mer up to a 22-mer with an average of a 16-mer. These results are in reasonable agreement with the molecular weight calculated from combined $s^0_{20,w}$ and R_s, although the sedimentation equilibrium results indicate a somewhat higher oligomeric state. This higher oligomeric state may have been created by the overnight exposure of band 3 to room temperature that occurred during the sedimentation equilibrium experiments. Alternatively, the molecular weight determined by using the Stokes radius could be a low estimate, since the oligomer is in equilibrium with protein species of lower aggregation states.

The results of the hydrodynamic analysis indicate that the oligomeric state of band 3 isolated in octyl glucoside is heterogeneous and very high, having an average aggregation number of n = 12–16. This is much higher than any other

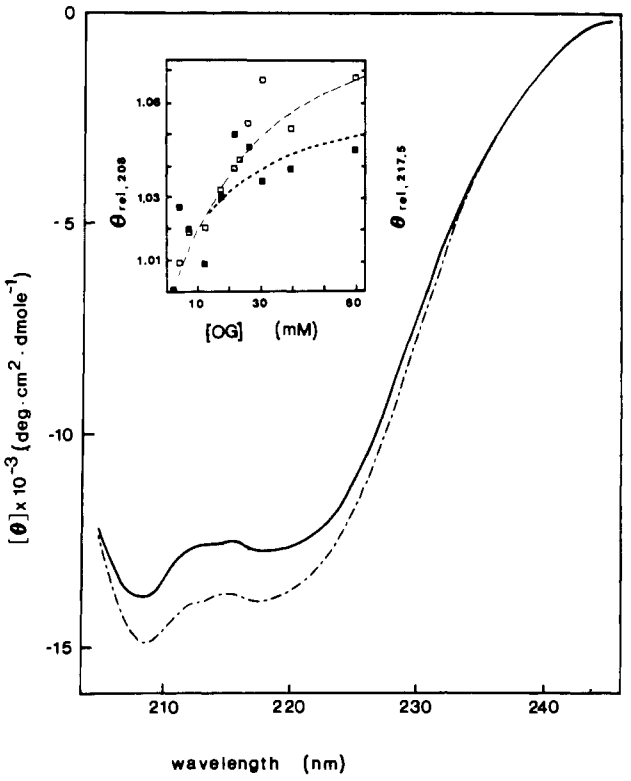

FIGURE 4: Far-ultraviolet circular dichroism spectra of band 3 in octyl glucoside concentrations of 2 (—) and 60 mM (-·-). Spectra were recorded at 25 °C in 50 mM sodium phosphate, pH 8.0, by using a cell of path length 0.05 cm. The results are the average for two samples of protein concentrations 0.562 and 0.525 mg/mL. Inset: effect of octyl glucoside concentration on the relative ellipticity at 208 nm (■) and at 217.5 nm (□).

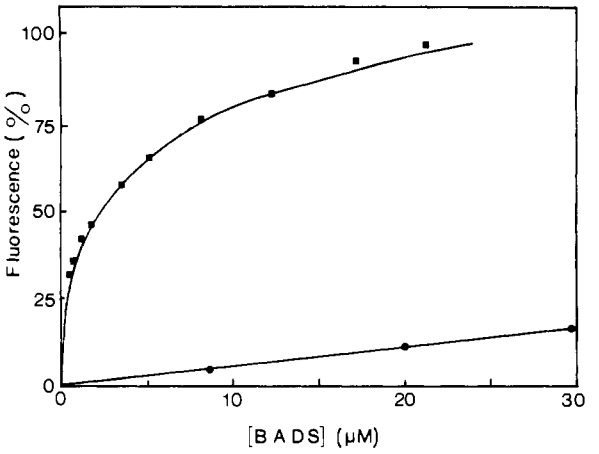

FIGURE 5: Binding of BADS to purified band 3 as determined by fluorescence enhancement titration. Band 3 (■) purified in 20 mM octyl glucoside was diluted with 28.5 mM sodium citrate, pH 7.4, for the assay (final concentration 0.7 mM octyl glucoside). (●) Background fluorescence of BADS in 20 mM octyl glucoside. Excitation was at 280 nm, and emission was measured at 450 nm.

oligomeric state reported for solubilized band 3. Pappert & Schubert (1983) report that their sedimentation equilibrium data of band 3 in $C_{12}E_9$ fit quite well to a model of a monomer/dimer/tetramer self-association equilibrium. Although they point out that their data do not rule out that higher oligomers (hexamers, octamers) may take part in the equilibrium, their sedimentation coefficients at similar protein concentrations are much lower than ours in octyl glucoside. Earlier work on acetic acid solubilized band 3 (Dorst & Schubert, 1979) reported $s_{20,w}$ values of up to 20 S, which they ascribed to band 3 octamers, and indicated that, under appropriate conditions, hexamers and octamers contributed to the observed monomer/dimer/tetramer equilibrium.

It is generally agreed that band 3 exists as a mixture of dimers and tetramers in the erythrocyte membrane under physiological conditions (Jennings, 1984). Cross-linking studies in intact cells produced dimers but no higher oligomers (Staros & Kakkad, 1983). Freeze-fractured membranes show particles of tetrameric size (Weinstein et al., 1980), and the resonance energy transfer data on the distance between stilbenedisulfonate sites suggest that dimers and tetramers of band 3 exist in the membrane (Macara & Cantley, 1981). Our results suggest that band 3 in octyl glucoside is in a much higher oligomeric state than in the erythrocyte membrane. This indicates that octyl glucoside affects the oligomeric state of proteins in different ways. It has been found to dissociate some proteins, for example, pilin arrays (Watts et al., 1982) and cytochrome P-450–NADPH–cytochrome P-450 reductase complexes (Dean & Gray, 1982). The high oligomeric state of band 3 in octyl glucoside might prevent the use of this detergent for reconstitution of single functional units of band 3. However, it is possible that reassociation of the protein with phospholipids could allow dissociation, since phospholipid has been found to dissociate oligomers of the sarcoplasmic reticulum Ca^{2+}-ATPase (Andersen et al., 1983).

Circular Dichroism. The far-ultraviolet circular dichroism spectrum of band 3 in octyl glucoside, shown in Figure 4, is typical of a protein containing a high amount of α-helix, as indicated by the double trough between 200 and 250 nm. Calculation of the amount of secondary structure (eq 3, see Materials and Methods) indicated that the protein has an α-helical content of 40% and a β-sheet content of 27% in 2 mM octyl glucoside. With increasing concentrations of detergent, the shape of the spectrum remained the same, and there was a slight increase in negative ellipticity. In 60 mM octyl glucoside, the molar ellipticities at the minima of 208 and 217.5 nm were approximately 5% and 7% more negative than in 2 mM octyl glucoside, indicating 45% α-helix and 26% β-sheet. An α-helical content of 40–45% and a β-sheet content of 26% are similar to those reported for band 3 purified in other detergents: 46% α-helix and 37% β-sheet in $C_{12}E_8$ (Oikawa et al., 1985) and 43% α-helix in Ammonyx-LO (Yu & Steck, 1975a). It has been claimed that the native structure of the protein was retained in this latter detergent (Yu & Steck, 1975a). Our results therefore suggest that there are no gross alterations in the secondary structure of band 3 when it is purified in octyl glucoside. The slight increase in negative ellipticity observed at higher concentrations could indicate an actual increase in the amount of secondary structure.

BADS Binding. The anion transport activity of band 3 can be measured only when the protein is inserted in a sealed membrane. However, the functional integrity of the soluble purified protein in octyl glucoside was determined by the ability of band 3 to bind BADS, a potent inhibitor (K_i = 1 μM) of anion transport. This provides an estimate of the integrity of the stilbenedisulfonate binding site that can be compared to the value for the native protein. The measurement of BADS binding in solution is based on a fluorescence enhancement titration in which excitation of the protein at 280 nm results in energy transfer to BADS bound in the hydrophobic inhibitor binding site of band 3.

Band 3 purified in octyl glucoside bound BADS, as shown by the saturable fluorescence enhancement upon addition of BADS (Figure 5). The affinity of BADS binding obtained from such a titration varied with the concentration of octyl glucoside present in the assay. Figure 6 shows the effect of

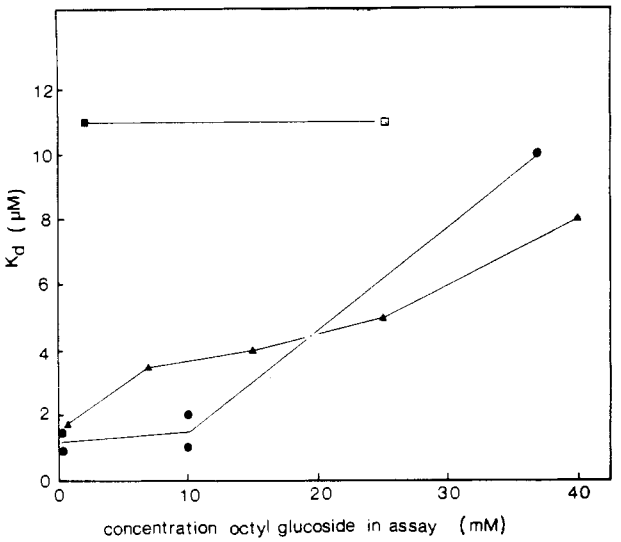

FIGURE 6: Effect on the concentration of octyl glucoside on the affinity of BADS binding to band 3. The K_d of BADS binding was determined by using fluorescence enhancement titration at the stated assay concentration of octyl glucoside for band 3 prepared in 2 (●), 15 (▲), and 40 mM (■) octyl glucoside. The protein prepared in 40 mM octyl glucoside was exposed to 250 mM octyl glucoside for 6 h at 4 °C prior to assay at 25 mM octyl glucoside (□).

increasing concentrations of octyl glucoside on the affinity of BADS binding for preparations purified in three different concentrations of octyl glucoside. Preparations that were purified in 2 mM octyl glucoside, and exposed to a maximum concentration of 20 mM octyl glucoside during detergent exchange, bound BADS with high affinity (0.8–1.5 μM). This compares quite well to the affinity of BADS for band 3 in membranes ($K_d \simeq 1$ μM) and the inhibition constant of BADS inhibition of phosphate uptake in cells ($K_i \simeq 1$ μM) (Lieberman & Reithmeier, 1983). It is also consistent with reported results in which direct solubilization of ghosts with octyl glucoside (0.1% or ~3.5 mM) had no effect on the affinity of BADS binding (Lieberman & Reithmeier, 1983). Increasing the octyl glucoside concentration gradually increased the K_d of BADS binding to 8–10 μM at an octyl glucoside concentration of approximately 40 mM (Figure 6, preparations in 2 and 15 mM). Above this detergent concentration, it became technically difficult to do the measurements, due to the high background fluorescence of BADS.

The increase in K_d observed in higher octyl glucoside concentrations did not appear to be reversible. In a preparation purified in an octyl glucoside concentration above the cmc (40 mM) and then diluted to a concentration below the cmc for the binding assay, the K_d remained at ~11 μM, rather than decreasing to the K_d measured at the same assay concentration for preparations in 2 or 15 mM octyl glucoside. Exposure to an even higher concentration of octyl glucoside (to 250 mM) for 6 h, conditions which might approximate those used in reconstitution from octyl glucoside, did not have any further effect on the affinity of BADS binding. It should be noted that the maximum fluorescence enhancement was the same for the measurements done at assay concentrations of octyl glucoside of 25 mM or lower, indicating that the number of BADS binding sites did not change as the affinity changed. However, exact numbers of binding sites per protein cannot be determined by using this method of measurement. The decrease in affinity may be due to some alteration in the inhibitor binding site of the protein or to interaction of octyl glucoside with the inhibitor binding site. The former seems more likely, since the altered affinity is not reversible upon dilution into low concentrations of detergent. There have been previous reports of alterations in stilbenedisulfonate binding and inhibitory potency in reconstituted band 3. It was found that the concentrations of DIDS or DNDS required to inhibit transport in band 3 proteoliposomes (100 μM DIDS or 500 μM DNDS) were an order of magnitude higher than those required in cells (Cabantchik et al., 1980; Wolosin, 1980; van Hoogevest, 1983). Köhne et al. (1981) found that their reconstituted preparation had a lowered and more heterogeneous affinity for DNDS, with an I_{50} value estimated to be about 6-fold higher than in cells.

Although band 3 purified in octyl glucoside retains a significant amount of α-helix and β-structure, the affinity of BADS for the stilbenedisulfonate binding site is reduced 10-fold. In fact, a 5% increase in negative ellipticity at 208 and 217.5 nm was observed at high concentrations of detergent, indicative of an increase in α-helical content. This change in secondary structure is consistent with the idea that a structural alteration led to the observed decrease in affinity for BADS. It must be noted that addition of 1% sodium dodecyl sulfate to purified band 3 has been reported to have had little effect on the circular dichroism spectrum (Oikawa et al., 1985), yet it destroyed the stilbenedisulfonate binding site (Lieberman & Reithmeier, 1983). In these cases, the measurement of inhibitor binding affinity provided more information about the alterations than did the estimate of α-helical content from the circular dichroism spectrum. Although circular dichroism and estimation of secondary structure are an important part of the characterization of a detergent-solubilized membrane protein, the net circular dichroism spectrum may not be sensitive to small structural changes that could result in functional alteration.

Thus, an assay of protein function is necessary to evaluate whether purification in the presence of a particular detergent results in alteration of the native properties. This point has been made by Tanford & Reynolds (1976), who stressed the importance of carrying out a structural and functional characterization of the intermediate state for a purified, detergent-solubilized membrane protein that is to be reconstituted. Such a characterization would make it possible to determine whether alterations in structure or function that might be observed in the reconstituted state had occurred before or after this intermediate state. It would then be possible to decide which aspect of the purification and reconstitution needed improvement.

Reconstitution studies which use band 3 in octyl glucoside (Darmon et al., 1983) must take into account the oligomeric state and the alteration in inhibitor binding we detect in the octyl glucoside solubilized protein. A potential use of the highly aggregated band 3 in octyl glucoside would be as a starting material for formation of two-dimensional arrays that would be suitable for electron diffraction.

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