Conformation and Stability of the Anion Transport Protein of Human Erythrocyte Membranes†

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ABSTRACT: The conformation and stability of purified preparations of band 3, the anion transport protein of human erythrocyte membranes, and its constituent proteolytic subfragments have been studied by circular dichroism. Band 3, purified in the presence of the nonionic detergent *n*-dodecyl octaethylene glycol monoether ($C_{12}E_8$), had an α-helical content of 46%. Denaturation of purified band 3 with guanidine hydrochloride occurred in two phases, one reflecting much more resistance to denaturation than the other. Band 3 can be separated into two domains by limited in situ proteolytic cleavage. The carboxyl-terminal membrane-associated domain (M_r 55 000) purified in $C_{12}E_8$ contained 58% α-helix and was very resistant to denaturation by guanidine hydrochloride. The purified amino-terminal, cytoplasmic domain (M_r 41 000) contained 27% α-helix and was completely converted to a random-coil conformation by 3 M guanidine hydrochloride. The two phases of denaturation observed for intact band 3 corresponded to the two domains of the protein. Irreversible heat denaturation of purified band 3 occurred with half-maximal change in $\theta_{222.5}$ at 48 °C. Covalent attachment of the anion transport inhibitor 4,4′-diisothiocyanostilbene-2,2′-disulfonate to band 3 had little effect on the circular dichroism spectra of band 3 or the membrane-associated domain but resulted in stabilization of band 3 to heat denaturation (half-maximal change in $\theta_{222.5}$ = 61 °C). Circular dichroism studies of membranes that had been digested extensively with proteolytic enzymes and stripped of all extrinsic fragments revealed that the portions of red cell membrane proteins that are embedded in the lipid bilayer contain a very high (86–94%) content of α-helix.

Band 3, the anion transport protein of the human erythrocyte membrane, is the major intrinsic membrane protein of this plasma membrane (Knauf, 1979; Macara & Cantley, 1983). This glycoprotein (M_r 95 000) spans the membrane several times and is composed of two domains (Steck et al., 1976; Appell & Low, 1981, 1982; Davio & Low, 1982). The amino-terminal domain (M_r 41 000) is exposed to the cytoplasm (Steck et al., 1976; Drickamer, 1978) and provides the binding sites for enzymes such as aldolase (Strapazon & Steck, 1976; Murthy et al., 1981) and glyceraldehyde-3-phosphate dehydrogenase (Strapazon & Steck, 1977; Tsai et al., 1982) and the cytoskeleton (Bennett & Stenback, 1979, 1980; Hargreaves et al., 1980; Kaul et al., 1983). The carboxyl-terminal domain (M_r 55 000) is embedded in the lipid bilayer (Steck et al., 1976; Markowitz & Marchesi, 1981; Drickamer, 1976, 1977) and is fully capable of carrying out anion exchange (Grinstein et al., 1978). Band 3 has been reported to contain 43% α-helix (Yu & Steck, 1975) while the cytoplasmic domain had an α-helical content of 37% (Appell & Low, 1981). In this study, we have examined the conformation and stability of band 3 and its two domains by circular dichroism.

Experimental Procedures

Materials. Ultrapure tris(hydroxymethyl)aminomethane (Tris)[1] base and guanidine hydrochloride were purchased from Schwarz/Mann. Specially pure sodium dodecyl sulfate was from the British Drug House; *n*-dodecyl octaethylene glycol monoether ($C_{12}E_8$) was purchased from Nikko Chemical Co., Tokyo. Aminoethyl-Sepharose 4B was synthesized according to Shaltiel & Er-El (1973), and [[*p*-(chloromercuri)benzamido]ethylene]amino-Sepharose 4B was made according to Lukacovic et al. (1981).

Membrane Preparations. Unless stated otherwise, all steps were carried out at 0–4 °C, and membranes were recovered by centrifugation at 15 000 rpm in an SS-34 rotor in a Sorvall RC-5B centrifuge. Erythrocyte ghosts were prepared from outdated blood (kindly provided by the Can. .n Red Cross) by hypotonic lysis in 5 mM sodium phosphate, pH 8.0 (Dodge et al., 1963).

DIDS Treatment of Red Blood Cells. Red blood cells were washed 3 times with 150 mM NaCl and 5 mM sodium phosphate, pH 7.4, at 0 °C. Cells were suspended at a 25% hematocrit in the same buffer. DIDS was added to a final concentration of 100 μM, and the suspension was incubated at 37 °C for 1 h. The cells were washed with the same buffer containing 0.5% bovine serum albumin at 0 °C followed by two washes at 0 °C with the same buffer.

Purification of Band 3 and Its Constituent Proteolytic Fragments. Band 3 was purified as described previously (Lieberman & Reithmeier, 1983). The cytoplasmic domain (M_r 41 000 + subfragments of M_r 23 000 and 20 000) was purified according to Bennett & Stenbuck (1980) as modified by Appell & Low (1981). The membrane-associated domain (M_r 55 000) was purified as follows. Red blood cells (40 mL) or cells pretreated with DIDS were lysed with 5 mM sodium phosphate, pH 8.0 at 0 °C, and ghost membrane was collected by centrifugation at 27000*g* for 20 min. Ghosts were washed 3 times with the same buffer. Ghosts were treated with TPCK-treated trypsin (5 μg/mL) at 0 °C for 1 h. Digestion was stopped by addition of phenylmethanesulfonyl fluoride to a final concentration of 1 mM. After incubation at 0 °C for 15 min, the membranes were washed with 5 mM sodium

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[1] Abbreviations: $C_{12}E_8$, *n*-dodecyl octaethylene glycol monoether; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; Tris, tris(hydroxymethyl)aminomethane; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; SDS, sodium dodecyl sulfate.

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phosphate, pH 8.0. Extrinsic protein fragments were removed from the membrane by extraction with 2 mM EDTA, pH 12. The membranes were washed twice with 5 mM sodium phosphate, pH 8.0, and then solubilized with 5 volumes of 1% $C_{12}E_8$ in the same buffer. The extract was applied to a 5-mL column of DEAE CL-6B. After application of the sample, the column was washed successively with 5, 20, 50, and 100 mM sodium phosphate, pH 8.0, containing 0.1% $C_{12}E_8$. The column effluent was monitored directly at 280 nm with a Pharamcia UV monitor. Fractions (2 mL) were collected and assayed for protein content by sodium dodecyl sulfate gel electrophoresis. The membrane-associated domain (M_r 55000) was eluted with 0.1% $C_{12}E_8$ and 100 mM sodium phosphate, pH 8.0.

Proteolytic Digestion of Membranes. Ghost membranes in 5 mM sodium phosphate, pH 8.0, were digested with trypsin, chymotrypsin, papain, or Pronase at an enzyme concentration of 1 mg/mL at 37 °C for 1 h. Digestion was stopped by addition of phenylmethanesulfonyl fluoride to a final concentration of 1 mM (for trypsin, chymotrypsin, and Pronase) and iodoacetamide to 1 mM (for papain and Pronase) and cooling the samples to 0 °C. Membranes were washed with 5 mM sodium phosphate, pH 8.0, stripped with 2 mM EDTA, pH 12, and washed twice with 5 mM sodium phosphate, pH 8.0. Membranes were solubilized by addition of 5 volumes of 1% $C_{12}E_8$ or 1% sodium dodecyl sulfate in 5 mM sodium phosphate, pH 8.0, at room temperature.

Circular Dichroism. The circular dichroism spectra were recorded on a Jasco Model J-500C spectropolarimeter. A minimum of four spectral scans were accumulated and averaged by a Jasco Model 500N data processor. Unless stated otherwise, spectra were taken at room temperature. Cells with path lengths of 0.01 and 0.05 cm were employed. Ellipticities were calculated by using a mean residue molecular weight of 115. The fraction of α-helix (f_H) was calculated from eq 1

$$f_H = \frac{[\theta]_{222} + 2340}{-30300} \quad (1)$$

as described by Chen et al. (1972). The secondary structure of band 3 and its proteolytic fragments was calculated over the wavelength range 190–240 nm according to Chen et al. (1974) using CONTIN, a Fortran IV program developed by the Data Analysis Group, European Molecular Biology Laboratory, Heidelberg. The standard spectra are based on the circular dichroism spectra of 16 proteins of known crystal structure (Chang et al., 1978). Protein concentrations (0.1–1 mg/mL) were determined by the method of Lowry et al. (1951) in the presence of 1% sodium dodecyl sulfate. The absolute protein concentrations of band 3 and the 55000- and 41000-dalton purified fragments were determined by amino acid analysis, using the values for alanine and leucine (Table I). The Lowry protein determinations were multiplied by the following factors to obtain the absolute protein concentrations: band 3, 0.98; M_r 55000 fragment, 0.87; M_r 41000 fragment, 1.07.

RESULTS

In order to determine the circular dichroism spectrum of band 3, this protein must be solubilized from the membrane and purified in the presence of a nondenaturing detergent. The circular dichroism spectrum of the red cell membrane has been extensively studied (Lenard & Singer, 1966; Glaser et al., 1970; Schneider et al., 1970; Glaser & Singer, 1971; Urry et al., 1971; Gordon & Holzwarth, 1971; Holzwarth, 1972). These studies revealed that the circular dichroism spectra of red cell membranes had features of an α-helical structure but had low amplitudes and red shifts of the circular dichroism bands. These effects have been attributed to the particulate nature of membrane samples which causes absorption flattening and differential light scattering (Holzwarth, 1972). The value of the ellipticity at 222 nm of membrane preparations is not, however, significantly affected by these optical artifacts (Glaser & Singer, 1971; Urry et al., 1971) and can be used to estimate the α-helical content of the membrane preparation (Chen et al., 1972, 1974). Yu & Steck (1973), using Triton X-100 as a solubilizing agent, found that solubilizing band 3 did not alter its α-helical content. It was previously shown that band 3 maintains its dimeric structure and inhibitor binding site in $C_{12}E_8$ (Yu & Steck, 1975; Lieberman & Reithmeier, 1983). We conclude that the native structure of band 3 is not greatly affected by solubilization with $C_{12}E_8$.

In order to characterize the secondary structure of band 3 in more detail, the intact protein (M_r 95000), the amino-terminal cytoplasmic domain (M_r 41000), and the carboxyl-terminal membrane domain (M_r 55000) (Figure 1) were purified (Figure 2 and Table I). The cytoplasmic domain is cleaved off the membrane by mild trypsin or chymotrypsin treatments of ghost membranes (Steck et al., 1976). A secondary cleavage point that is difficult to limit produced small amounts of two subfragments (M_r 23000 and 20000) of this domain (Figures 1 and 2). These subfragments remain tightly associated as a complex and copurify with the 41000-dalton fragment. The amino acid analyses of band 3 and its subfragments (Table I) are in good agreement with published analyses (Steck et al., 1978). The circular dichroism spectra of the purified proteins are shown in Figure 3. All spectra were characteristic of an α-helical structure with negative

Table I: Amino Acid Composition (mol %) of Band 3 and Its Constituent Subfragments

amino acid	band 3 (M_r 95000)	membrane domain (M_r 55000)	cytoplasmic domain (M_r 41000)
Asx	7.0	7.6	8.0
Thr	4.7	5.2	4.7
Ser	6.5	7.6	5.6
Glx	12.4	10.8	19.4
Pro	6.1	7.3	8.1
Gly	7.7	7.6	6.4
Ala	7.3	6.5	7.8
Val	7.5	8.3	6.2
Met	2.3	2.5	1.9
Ile	4.3	4.7	2.1
Leu	14.8	13.0	14.6
Tyr	2.2	3.0	1.7
Phe	6.0	5.8	2.9
His	2.8	1.6	3.5
Lys	3.5	4.1	2.0
Arg	4.9	4.4	5.1
no. of determinations	2	5	5

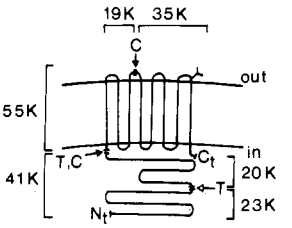

FIGURE 1: Model for the arrangement of the band 3 polypeptide in the erythrocyte membrane. C, chymotrypsin-sensitive sites; T, trypsin-sensitive sites; N_t, amino terminal; C_t, carboxyl terminal; 41K, 41000-dalton cytoplasmic domain; 23K, 23000-dalton fragment; 20K, 20000-dalton fragment; 55K, 55000-dalton membrane-associated domain; 19K, 19000-dalton fragment; 35K, 35000-dalton fragment; Y, carbohydrate attachment site.

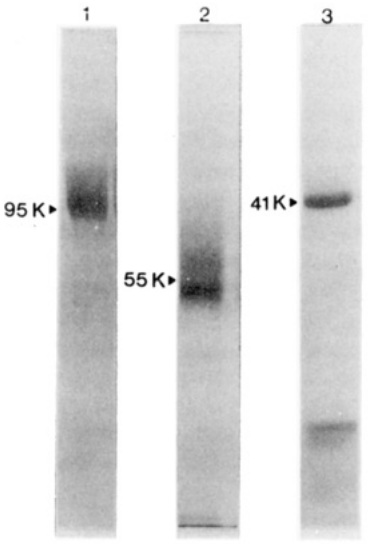

FIGURE 2: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of purified band 3 (lane 1), the membrane-associated domain (lane 2), and the cytoplasmic domain (lane 3).

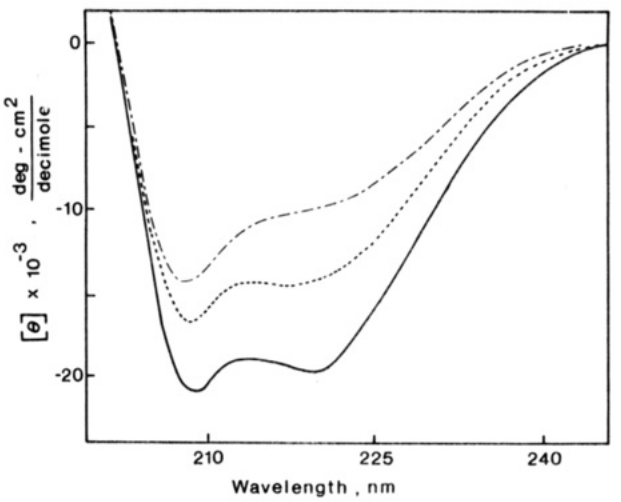

FIGURE 3: Far-ultraviolet circular dichroism spectra of purified band 3 (0.5 mg/mL) in 0.1% $C_{12}E_8$ and 50 mM sodium phosphate, pH 8.0 (---), of the cytoplasmic domain (0.5 mg/mL) in 150 mM NaCl and 20 mM sodium phosphate, pH 8.0 (–·–), and of the membrane-associated domain (0.8 mg/mL) in 0.1% $C_{12}E_8$ and 100 mM sodium phosphate, pH 8.0 (—). All samples contained 0.1% 2-mercaptoethanol.

extremes at 208 and 222 nm. The secondary structure of band 3 and the two domains was calculated according to Chen et al. (1974) over the wavelength range 190–240 nm. The results of these analyses are presented in Table II. Band 3 had a 46% α-helical content with 37% β-structure. The cytoplasmic domain had a lower α-helical content (27%) while the membrane-associated domain was enriched in α-helix (58%) relative to the intact protein. The estimate for the α-helical content of the cytoplasmic domain was lower than that obtained by Appell & Low (1981), who used the ellipticity at 208 nm to calculate a value of 37%. Appell & Low (1981) used a very dilute protein sample (21 μg/mL) and a 1-cm path length to obtain their circular dichroism spectrum while we used more concentrated protein (0.5 mg/mL) and a short path-length cell (0.050 cm). The use of a single wavelength by Appell and Low and the difference in methodology might account for the difference in the estimate of α-helical content. These authors used the Lowry assay to determine the protein concentration, which underestimates the amount of protein in samples of the cytoplasmic domain.

Table II: Secondary Structure of Band 3 and Its Proteolytic Fragments[a]

sample	M_r	% α-helix	% β-sheet	random coil[b]
band 3	95000	46	37	17
cytoplasmic domain	41000	27	48	25
membrane domain	55000	58	24	18
sum of cytoplasmic + membrane domains[c]		45	34	21
intrinsic fragments[d]				
papain	4000	86	0	14
pepsin	4000	94	0	6

[a] Calculated by a least-squares fit of the circular dichroism spectra from 190 to 240 nm (Chen et al., 1974). [b] Remainder, calculated from $100 - (\alpha + \beta)$. [c] Calculated from $(0.41\alpha_{41K} + 0.55\alpha_{55K})/0.96$; likewise for β-sheet and random coil. [d] Papain or pepsin fragments in 1% $C_{12}E_8$.

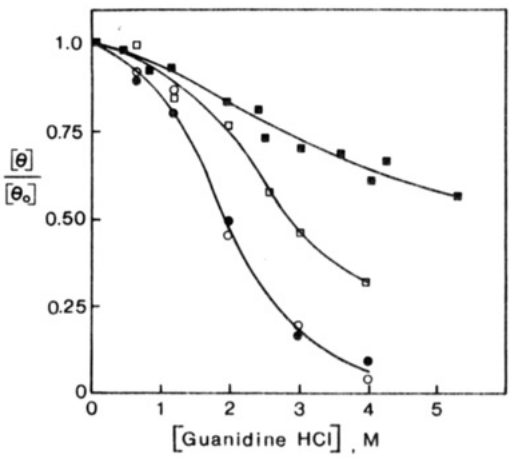

FIGURE 4: Effect of guanidine hydrochloride concentration on the mean residue ellipticity at 222.5 nm of band 3 (□), of the cytoplasmic domain produced by trypsin (○) or chymotrypsin (●) digestion, and of the membrane-associated domain (■). The buffers are identical with those in Figure 4.

The weighted sum of the secondary structure of the membrane and cytoplasmic domain was in excellent agreement with that for the intact band 3 protein (Table II). This indicates that major conformational changes do not occur upon removal of the cytoplasmic domain from band 3. Small conformational changes cannot, however, be ruled out. Indeed, it has been observed that the cytoplasmic domain can no longer be cross-linked with copper(1+) *o*-phenanthroline once it is removed from the membrane (Reithmeier, 1979; Appell & Low, 1981).

The stability of band 3 and its constituent proteolytic fragments to denaturation by pH, guanidine hydrochloride, and temperature was tested. The ellipticity of band 3 at 222 nm was remarkably insensitive to pH over the range 4–10.5. The α-helical content of band 3 was constant between pH 5.0 and 8.0. There was only a 2% decrease in α-helical content at pH 10.5 and a 4% decrease at pH 4.0. Band 3 was, however, sensitive to denaturation by guanidine hydrochloride. Denaturation of band 3 with guanidine hydrochloride was biphasic (Figure 4). A portion of the α-helical content was sensitive to relatively low concentrations of denaturant while a large proportion of the α-helical structure was maintained in concentrations of guanidine hydrochloride as high as 4 M. The purified cytoplasmic domain was readily denatured by low concentrations of guanidine hydrochloride, being almost completely random coil in 4 M guanidine hydrochloride (Figure 4). Identical results were obtained with the fragments isolated by trypsin or chymotrypsin digestion (Figure 4). In sharp contrast, the purified membrane domain was very resistant to denaturation (Figure 4). At 4 M guanidine hy-

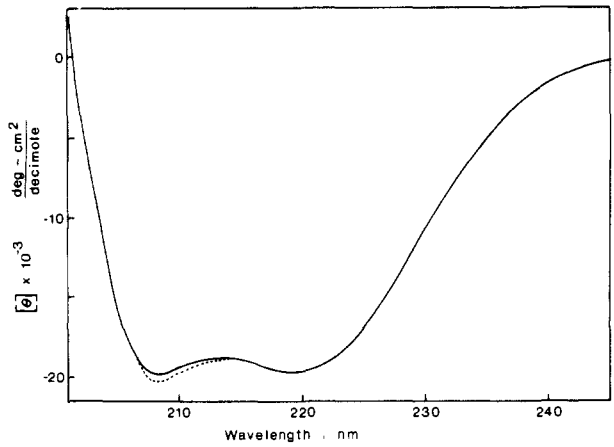

FIGURE 5: Far-ultraviolet circular dichroism spectra of the membrane-associated domain (—) and the membrane-associated domain plus 100 μM DIDS (---) in 0.1% $C_{12}E_8$ and 100 mM sodium phosphate, pH 8.0.

drochloride, where the cytoplasmic domain was denatured, the membrane domain maintained 70% of its $[\theta]_{222.5}$. The two phases of band 3 denaturation are therefore due to the unfolding of the cytoplasmic domain in low concentrations of guanidine hydrochloride and the resistance of the membrane domain to this denaturant. Cleavage of the membrane domain into two tightly associated subfragments (M_r 19000 and 35000) (Figure 1) (Reithmeier, 1979) by prior treatment of cells with chymotrypsin had no effect on the resistance of this domain to denaturation by guanidine hydrochloride.

The effect of covalent attachment of the anion transport inhibitor DIDS to band 3 on the circular dichroism spectra of the intact protein and the membrane domain was determined (Figure 5). Addition of saturating concentrations of DIDS (100 μM) to purified band 3 had no significant effect on the circular dichroism spectrum of the membrane domain (Figure 5). DIDS binding to band 3 therefore did not have a profound effect on the secondary structure of the protein.

A study of the effect of DIDS on the sensitivity of band 3 and its constituent proteolytic fragments to denaturation revealed that the membrane domain was relatively insensitive to denaturation by guanidine hydrochloride in either the presence or the absence of DIDS. DIDS, however, had a significant stabilizing effect on the stability of band 3 to heat denaturation (Figure 6). Heating of purified band 3 above 45 °C resulted in reversible aggregation of the protein and clouding of the sample. This feature did not permit us to follow the heat denaturation of band 3 directly in the spectropolarimeter. Clouding of the DIDS-labeled band 3 occurred at a higher temperature (55 °C). Solutions of DIDS-labeled and unlabeled band 3, however, clarified after cooling to room temperature. Samples were therefore heated at various temperatures for 10 min and cooled to room temperature, and their circular dichroism spectra were taken. The fractional change in ellipticity at 222.5 nm of DIDS-labeled and unlabeled band 3 as a function of denaturation temperature is plotted in Figure 6. There was a 30% decrease in ellipticity at 222 nm upon heating to 100 °C. This was set as 100% maximal change. No difference in the maximum change was observed between the sample labeled with DIDS vs. the unlabeled sample. The half-maximal change for band 3 was 48 °C while the half-maximal change for DIDS-labeled band 3 did not occur until 61 °C. Covalent attachment of DIDS to band 3 therefore resulted in stabilization of band 3 to heat denaturation. Since the DIDS binding site is the membrane domain, the effect of DIDS on the thermal denaturation of the 55000-dalton

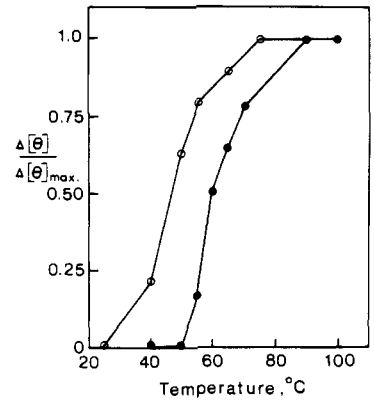

FIGURE 6: Irreversible thermal denaturation of band 3 (○) and DIDS-labeled band 3 in 0.1% $C_{12}E_8$ and 50 mM sodium phosphate, pH 8.0 (●). Samples were incubated at the indicated temperatures for 10 min and then cooled to room temperature. All circular dichroism spectra were taken at room temperature.

fragment was determined as with intact band 3. The DIDS-labeled 55000-dalton fragment was found to be more resistant to heat denaturation (by 8 °C) than the unlabeled fragment. The stabilization of band 3 to thermal denaturation by DIDS binding can therefore be localized to the membrane domain. Stabilization of the membrane domain to heat denaturation by DIDS has also been detected by calorimetry (Snow et al., 1978; Appell & Low, 1982; Davio & Low, 1982a).

The conformation of the portion of band 3 that interacts with the lipid bilayer was determined by digesting membranes extensively with various proteases. In this way, only those portions of the red cell membrane proteins buried in the membrane will be protected from digestion. Extraction with alkali removed all extrinsic protein fragments, leaving only intrinsic fragments. Treatment of membranes with trypsin or chymotrypsin degraded band 3 to fragments with molecular weights of ~15000 while papain or pepsin reduced band 3 to smaller fragments. The molecular weights of the papain fragments could not be accurately determined by sodium dodecyl sulfate gel electrophoresis since their mobility depended on the gel system employed. However, the papain fragments comigrated with the pepsin fragments (M_r 4000) previously characterized by Ramjeesingh et al. (1984). The circular dichroism spectrum of the membrane fraction containing these fragments was predominated by light scattering (Figure 7). Solubilization of the membrane with $C_{12}E_8$ or sodium dodecyl sulfate revealed an α-helical spectrum. On the basis of the ellipticity at 222 nm, this preparation had an α-helical content of 67% in the membrane state, 64% in 1% $C_{12}E_8$, and 57% in the 1% SDS. Calculation of the secondary structure of the fragments in $C_{12}E_8$ over the range 190–240 nm gave values of 86% α-helix, 0% β-sheet, and 14% random coil for the papain-treated membranes and 94% α-helix, 0% β-sheet, and 6% random coil for the pepsin-treated membranes (Table II). A similar enrichment in the α-helical content of the membrane-associated portion of the Ca^{2+}-ATPase of sarcoplasmic reticulum has been reported (Toogood et al., 1983). The parts of band 3 buried in the bilayer have a very high α-helical content, reminiscent of bacteriorhodopsin (Long et al., 1977; Jap et al., 1983; Mao & Wallace, 1984).

Discussion

The band 3 polypeptide (M_r 95000) of human erythrocyte membrane is composed of two domains (Macara & Cantley, 1983). The amino-terminal domain (M_r 41000) is responsible for the binding of the cytoskeleton and glycolytic enzymes to the red cell membrane. We have found that this domain has

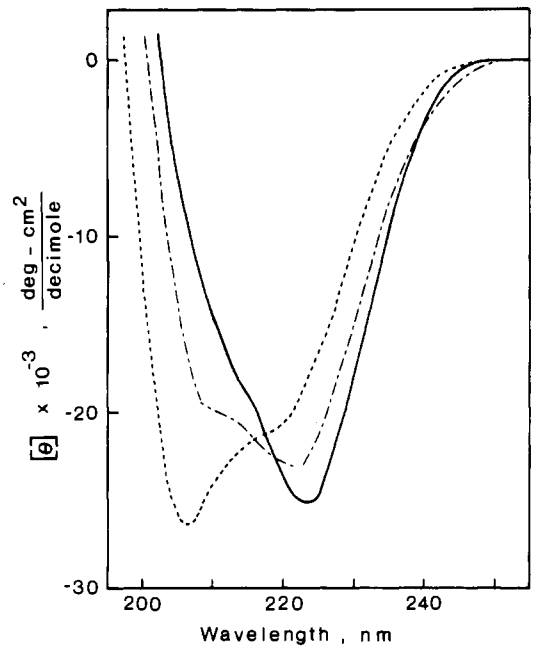

FIGURE 7: Circular dichroism spectra of intrinsic membrane fragments prepared by extensive papain digestion: (—) membrane in 5 mM sodium phosphate, pH 8.0; (–·–) membrane solubilized in 1% $C_{12}E_8$ and 5 mM sodium phosphate, pH 8.0; (- - -) membrane solubilized in 1% sodium dodecyl sulfate and 5 mM sodium phosphate, pH 8.0. The protein concentration was 0.44 mg/mL.

a moderate α-helical content and is denatured by guanidine hydrochloride in a manner similar to water-soluble proteins. The carboxyl-terminal domain (M_r 55 000) is embedded in the membrane and is capable of carrying out anion transport (Grinstein et al., 1978). This domain is dimeric and binds the total amount of nonionic detergent that is bound by intact band 3 (Reithmeier, 1978). Interestingly, the glucose and nucleoside transporters of the red cell membrane have a molecular weight similar to that of the membrane domain and are also oligomeric (Woo et al., 1983; Carter-Su et al., 1982).

The portions of band 3 (and other intrinsic red cell membrane proteins) embedded in the membrane have a high content of α-helix and are very resistant to denaturation by guanidine hydrochloride. A similar resistance to denaturation has been observed for the membrane-associated regions of intrinsic membrane proteins such as cytochrome b_5 (Tajima et al., 1976), glycophorin (Schulte & Marchesi, 1979), and the Ca^{2+}-ATPase (Rizzolo & Tanford, 1978). The resistance of membrane-embedded portions of intrinsic membrane proteins to denaturation by guanidine hydrochloride may be a common feature of intrinsic membrane proteins. This resistance may be due to the hydrophobic nature of the lipid-associated sequences (Rizzolo & Tanford, 1978). Those portions of membrane proteins exposed to solvent like the cytoplasmic domain of band 3 are readily denatured by guanidine hydrochloride. Thus, guanidine hydrochloride denaturation can be used to distinguish lipid-associated portions of membrane proteins from those portions exposed to water.

An α-helical conformation for proteins embedded in a hydrophobic environment has been predicted by energetic considerations (Green & Flanagan, 1976; Guidotti, 1977; Dunker & Jones, 1978). Indeed, the membrane-spanning portions of glycophorin (Schulte & Marchesi, 1979) and bacteriorhodopsin (Long et al., 1977) are highly α-helical as determined by circular dichroism. The portions of band 3 embedded in the membrane may be considered as α-helical rods that span the membrane. Small segments of the intrinsic fragments have been sequenced (Mawby & Findlay, 1982; Brock et al., 1983). When arranged in an α-helical conformation, the polar residues fall on one side of an α-helix. We propose that these polar residues face the interior of a bundle of helical rods that span the bilayer. Transport of anions would occur through this polar pore. Similar models for membrane transport have been proposed on the basis of experimental and theoretical analyses of the conformation and packing of the transmembrane segments of membrane proteins (Wallach & Zahler, 1966; Inouye, 1974; Dunker & Zaleske, 1977; Dunker & Marin, 1978).

DIDS and other stilbenedisulfonates that inhibit anion transport bind to an externally facing site in the membrane domain of band 3 (Knauf, 1979). This binding has little effect on the α-helical content of band 3 but does result in stabilization of the protein to heat denaturation. Irreversible heat-induced structural transitions in the red cell membrane have been extensively studied by calorimetry (Jackson et al., 1973; Brandts et al., 1977, 1978; Snow et al., 1978; Davio & Low, 1982a,b; Appell & Low, 1981, 1982) and thermal gel analysis (Lysko et al., 1981). These studies have shown that band 3 undergoes two separate transitions corresponding to the irreversible denaturation of the cytoplasmic and membrane domains. The denaturation temperature of the cytoplasmic domain is sensitive to ionic strength and pH (Brandts et al., 1978; Appell & Low, 1981, 1982). The transition takes place at 57 °C at pH 8.0, the pH used in this study. The membrane domain unfolds at 68 °C but is stabilized by 10 °C by DIDS (Snow et al., 1978; Appell & Low, 1982; Davio & Low, 1982a). We were unable to clearly distinguish two phases of *heat* denaturation of band 3 by circular dichroism. The unfolding of DIDS-labeled band 3 by heat as determined by circular dichroism was clearly stabilized by 10 °C (Figure 7). The denaturation of band 3 in detergent solutions occurred at lower temperatures than in the native membrane (Davio & Low, 1982a). DIDS-labeled band 3 in Triton X-100 unfolded at 56.5 °C as determined by changes in the fluorescence properties of DIDS (Davio & Low, 1982a). We found that the half-maximal change in ellipticity at 222 nm of DIDS-labeled band 3 in $C_{12}E_8$ occurred at 61 °C, in good agreement with the fluorescence studies. This stabilization of band 3 supports the notion that stilbenedisulfonates bind to a cleft in band 3. Indeed, fluorescence quenching studies have shown that these inhibitors become exposed to the interior of the cell after initially binding to the outside of the cell (Macara et al., 1983). These inhibitors may perhaps be thought of as "partially transported" anions.

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