Characterization of the Calorimetric C Transition of the Human Erythrocyte Membrane†

Stephen R. Davio‡ and Philip S. Low*

ABSTRACT: The largest calorimetric endotherm of the human erythrocyte membrane, termed the C transition (68 °C), was shown to derive from the denaturation of the membrane-spanning domain of the anion transport protein, band 3. This identification was based on the following evidence: (i) the fluorescence properties of the highly specific covalent ligand of band 3, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid, abruptly changed during the C transition; (ii) the potent, noncovalent inhibitor of anion transport, dipyridamole, was ejected from erythrocyte membranes during the C transition; (iii) the intrinsic fluorescence of the membrane-spanning domain of band 3 decreased suddenly at the temperature of the C transition; and (iv) the purified 53 000-dalton, membrane-spanning domain of band 3 yielded the C transition upon reconstitution into egg phosphatidylcholine/bovine brain phosphatidylserine vesicles. Although lipid melting was shown not to contribute to the C endotherm, the thermal stability of band 3 was nevertheless observed to be sensitive to its lipid/detergent environment. The stability of the membrane-spanning domain of band 3 was also found to be unaffected by the presence or absence of glycophorin, suggesting that the putative complex between this region of band 3 and glycophorin is either weak or nonexistent.

The advent of highly sensitive differential scanning calorimetry has introduced a new tool for use in characterizing the structure and behavior of biological membranes. If the sensitivity of the calorimeter is high, distinct thermotropic transitions can be resolved for many of the major structural domains of a membrane. Thus, the spinach leaf chloroplast membrane displays six endotherms (Cramer et al., 1981), the bovine milk fat globule membrane yields seven (Appell & Low, 1982a), the purple membrane shows two (Jackson & Sturtevant, 1978), the rat intestinal plasma membrane exhibits at least three (Brasitus et al., 1980), and the human erythrocyte membrane displays five prominent endotherms (Brandts et al., 1977).

Of the major endotherms of the human erythrocyte membrane, only two have been characterized (Figure 1). The A transition (49 °C) has been shown to involve the denaturation of spectrin, a component of the red cell membrane's cytoskeleton (Brandts et al., 1977). This transition is sensitive to adenine nucleotides and other small ligands which perturb the morphology of the cell (Low & Brandts, 1978). The B_2 transition (62 °C at pH 7.4), on the other hand, has been identified with the thermal unfolding of the cytoplasmic domain of band 3 (Lysko et al., 1981; Appell & Low, 1982b). This transition responds dramatically to changes in pH, ADP, ionic strength, and phloretin (Brandts et al., 1978; Low & Brandts, 1978; Appell & Low, 1981, 1982b; Snow et al., 1978). The B_1 transition (56 °C) may involve bands 2.1, 4.1, and 4.2, but the nature of the endotherm is unclear (Lysko et al., 1981). The D transition (78 °C) has not yet been investigated.

The identity of the C transition (68 °C) of the human erythrocyte membrane is of major interest for two reasons. First, most inhibitors of the anion transport pathway of the erythrocyte membrane modify this transition at their inhibitory concentrations (Snow et al., 1978). The noncovalent inhibitors of anion transport have generally been found to lower the temperature of the C transition, while the covalent inhibitors, e.g., 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS),[1] pyridoxal phosphate, etc., significantly stabilize the endotherm. Furthermore, loss of anion transport activity has been directly correlated with the extent of perturbation of the C transition (Snow et al., 1978, 1981; S. R. Davio and P. S. Low, unpublished results). Also, no perturbant has yet been found which modifies the C transition and does not inhibit anion transport. These observations implicate the involvement of transport structures in the C transition. The data also suggest that a careful identification of the membrane components participating in "C" might lead to a clearer definition of the protein and lipid species required for a fully stable and functional anion transporter.

The C transition is also of interest since it displays many properties typical of a lipid melting transition. Thus, C is the only erythrocyte membrane transition that is totally insensitive to lengthy bilateral digestion with papain, trypsin, or chymotrypsin (Brandts et al., 1978). Since no major erythrocyte protein survives this extensive proteolysis, it has been assumed that protein denaturation is not significantly involved in the endotherm. The C transition is also rapidly removed by treatment of the membranes with phospholipase A_2 or phospholipase C (Brandts et al., 1978). This sensitivity to phospholipid hydrolysis strongly implicates the participation of lipid in the endotherm. The C transition is further sensitive to amphiphilic agents at concentrations below those reported to affect protein structure (Krishnan & Brandts, 1979; Snow et al., 1978). Benzyl alcohol, for example, substantially diminishes "C" at concentrations which decrease the enthalpy of lipid melting transitions but which have no effect on protein denaturation processes (Brandts et al., 1978). Finally, Brandts and co-workers (Brandts et al., 1977) were unable to detect any significant change in the circular dichroism of membranes upon scanning through the temperature range of the C tran-

† From the Department of Chemistry, Purdue University, West Lafayette, Indiana 47907. *Received January 29, 1982.* This work was supported in part by Grant GM 24417 from the National Institutes of Health. S.R.D. is a National Institutes of Health predoctoral trainee supported by Grant GM 07211.

‡ Present address: U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD 21701.

[1] Abbreviations: DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; PBS, phosphate-buffered saline (125 mM NaCl and 20 mM sodium phosphate, pH 7.4); NaP_i, sodium phosphate; IOV's, inside-out vesicles obtained from spectrin-depleted erythrocyte membranes; EDTA, ethylenediaminetetraacetic acid; SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid; PAS, periodic acid–Schiff base.

0006-2960/82/0421-3585$01.25/0

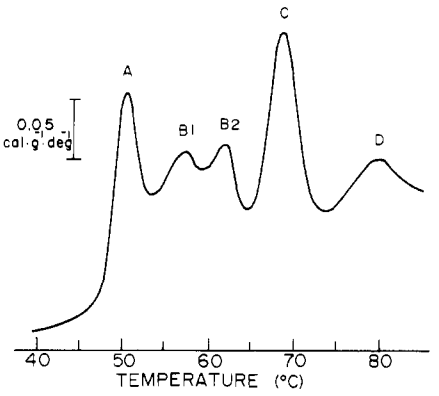

FIGURE 1: Heat capacity as a function of temperature of human erythrocyte membranes suspended in 20 mM sodium phosphate/125 mM NaCl, pH 7.4. The heating rate was 1 °C/min. The sample cell of the calorimeter contained 15 mg of ghost (total membrane dry weight).

sition. This property would not be anticipated if protein structural changes were significantly involved in "C".

The above observations have led to several explanations of the probable composition of the membrane structure which gives rise to the C transition (Snow et al., 1978; Brandts et al., 1978; Davio, 1979; Krishnan & Brandts, 1979). Because of its lipidlike properties, the C endotherm has been proposed to derive at least partially from phospholipid melting. The location of the putative gel phase lipid was thought to be adjacent to band 3, the anion transport protein, because of the dramatic displacement of "C" by covalent ligands of band 3. More recently, as the sensitivity of the C transition to extensive Pronase digestion has become apparent, the above hypothesis has been modified to include the additional participation of band 3 in the transition (Snow et al., 1981).

We have undertaken a more thorough study of the C transition in order to identify the specific membrane components essential for a normal manifestation of the transition. Using several experimental approaches, we demonstrate that the membrane-spanning domain of band 3 denatures during the C transition. We further show that the purified membrane-spanning fragment of band 3, reconstituted into egg phosphatidylcholine/bovine brain phosphatidylserine vesicles, renders a C endotherm with the same enthalpy as intact band 3 in whole erythrocyte membranes. Since egg phosphatidylcholine and bovine brain phosphatidylserine both have melting temperatures below 0 °C, we conclude that lipid melting does not significantly contribute to the enthalpy of the C endotherm. However, the binding of specific lipids may significantly influence the denaturation temperature of band 3.

Materials and Methods

Materials. Fresh human blood was obtained from the Central Indiana Regional Blood Bank with citrate/dextrose solution added and was used before it became officially outdated. Bovine serum albumin, trypsin, phenylmethanesulfonyl fluoride (PMSF), 2-[[tris(hydroxymethyl)amino]ethanesulfonic acid (Tes), Triton X-100, egg phosphatidylcholine (P-5763), and aminoethylcellulose were obtained from Sigma Chemical Co. Other reagents used were the following: 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) and sodium dodecyl sulfate ($NaDodSO_4$) (Pierce Chemical Co.); bovine brain phosphatidylserine (Avanti Biochemicals, Inc.) and Bio-Beads SM-2 (Bio-Rad).

Preparation of Erythrocyte Membranes at Various Stages of Protein Depletion. Erythrocyte membranes were prepared by hypotonic lysis of washed erythrocytes in 5 mM sodium phosphate (NaP_i), pH 7.4, as described by Dodge et al. (1963). When DIDS-labeled membranes were desired, the covalent labeling was conducted with 10 μM DIDS on washed, whole erythrocytes at 20% hematocrit in phosphate-buffered saline (PBS; 125 mM NaCl/20 mM sodium phosphate, pH 7.4) according to Cabantchik & Rothstein (1974). The labeled cells were washed twice in PBS containing 1% bovine serum albumin and then twice in PBS before membrane preparation.

Trypsin-digested, alkaline-stripped membranes were prepared as follows. Erythrocyte ghosts were first depleted of spectrin and actin and converted to inside-out vesicles (IOV's) by incubation in 20 volumes of 0.5 mM EDTA, pH 8.0, for 30 min at 37 °C (Marchesi et al., 1970). The IOV's were washed in 20 mM sodium phosphate (NaP_i), pH 7.4, and incubated for 30 min on ice in 10 volumes of the same buffer containing 20 μg/mL trypsin. This digestion procedure removes the cytoplasmic domain of band 3 and several peripheral proteins from the membrane, leaving the 53 000 (53K) membrane-spanning domain of band 3 intact. Trypsin was inactivated by incubating the IOV suspension on ice for 20 min with an equal volume of buffer containing 200 μg/mL PMSF. The IOV's were collected by centrifugation, washed once in 20 mM NaP_i, pH 7.4, containing 100 μg/mL PMSF, and washed twice in NaP_i, pH 7.4, without PMSF. The trypsin-digested membranes were then suspended in 7 volumes of ice-cold water adjusted with 1 N NaOH to pH 12. The suspension was immediately centrifuged for 20 min at 48000*g*, and the membrane pellet was washed twice in PBS, pH 7.4. Since the NaOH-stripped membranes are extremely susceptible to proteolytic degradation, they were invariably used in the desired experiments within a few hours of preparation.

For removal of the glycophorins from whole ghosts, the membranes in 20 mM NaP_i, pH 7.4, were suspended in 7 volumes of 0.05% Triton X-100 (v/v) and 20 mM NaP_i, pH 7.4, and stirred on ice for 20 min (Wolosin et al., 1977; Yu et al., 1973). The glycophorin-depleted membranes were retrieved by a 5-min centrifugation at 35000*g*. Two more extractions in 0.05% Triton X-100/20 mM NaP_i, pH 7.4, were conducted to further reduce the amount of glycophorin. The final glycophorin-depleted membrane pellet was washed 2 times in 20 mM NaP_i, pH 7.4, containing 1% bovine serum albumin to remove most of the remaining Triton X-100 (Wolosin et al., 1977) and then finally twice in 20 mM NaP_i, pH 7.4.

Reconstitution. Glycophorin-depleted membranes were cleared of spectrin and actin (Marchesi et al., 1970) and then dissolved in 5 volumes of 1% v/v Triton X-100 in 10 mM Tes, pH 7.4. The Triton X-100 extract, containing ~2.5 mg of protein/mL, was introduced into a flask containing 0.05 g/mL Bio-Beads SM-2, prepared according to Holloway (1973), and the ionic strength was elevated by addition of 0.1 volume of 1.4 M NaCl/10 mM EDTA, pH 7.4 (Wolosin, 1980). The entire suspension was then flushed thoroughly with N_2 and sealed and stirred at 2 °C. After 20 h, the Triton X-100 saturated Bio-Beads were removed by filtration through glass wool, fresh beads were added (0.05 g of beads/mL of suspension), and the suspension was again stirred at 2 °C under N_2. After 8 h, the second batch of beads was replaced with a final batch (0.25 g of beads/mL of suspension) which was stirred for 20 h at 2 °C. The final mixture was filtered through glass wool and spun at 2700*g* for 30 min to remove aggregated proteins and Bio-Bead fragments. The reconstituted vesicles were collected by overnight centrifugation at 140000*g*. The gelatinous membrane pellets were carefully suspended in buffer

(10 mM Tes/140 mM NaCl, pH 8.0) by working the suspended pellet back and forth through a Pasteur pipet. These reconstituted membrane suspensions were stored on ice until used for calorimetry and electrophoresis.

Purification and Reconstitution of the Membrane-Spanning Domain of Band 3. DIDS-labeled, trypsin-digested IOV's were prepared by the procedures described above, except that the proteolysis was conducted for 1 h at pH 8.0 with 10 μg of trypsin/mL. The washed IOV's, suspended in 20 mM NaP_i, pH 8.0, were dissolved in 6 volumes of the same buffer containing 1.5% Triton X-100, 50 μg/mL PMSF, and 25 mM β-mercaptoethanol. The suspension (~35 mL) was stirred on ice for 20 min and centrifuged at 48000*g* for 20 min. The supernatant was applied to a 1.9 × 15 cm aminoethylcellulose column equilibrated in 20 mM NaP_i, 0.5% Triton X-100, and 25 mM β-mercaptoethanol, pH 8.0. The column was washed with two column volumes of the same buffer. The DIDS fluorescence, i.e., the 53K membrane-spanning fragment of band 3, passed directly through the column, whereas essentially all other proteins in the Triton X-100 extract were retarded by the anion exchange resin.

The pooled effluent fractions containing the membrane-spanning fragment were reconstituted by using basically the same procedure as described above, with the following exceptions. Pooled band 3 fractions were swirled in a flask containing a dried mixture of egg phosphatidylcholine and bovine brain phosphatidylserine in a 4:1 weight ratio. The added lipid amounted to about 1 mg/mL band 3 extract. Also, the amounts of Bio-Beads used to remove the Triton X-100 were 0.075, 0.075, and 0.35 g of beads/mL of extract for the first, second, and third batches, respectively.

Calorimetry. Heat capacity measurements were obtained on a Microcal-1 differential scanning calorimeter (Amherst, MA) equipped with matched 1-mL platinum cells. Membrane samples, equilibrated in the desired buffer, were loaded into the sample cell, and an equal volume of the identical buffer system was added to the reference cell of the calorimeter. The heating rate in these experiments was 1 °C/min.

The apparent heat of melting of the C transition (designated ΔH_{app}) was determined by comparing the area under the C transition to the area under an endotherm obtained by delivering a calibrated heat pulse to the reference cell of the calorimeter. In some cases, the area under the C transition was very difficult to determine due to overlapping transitions and due to some uncertainty in the base line. For this reason, and because of the uncertainty in the estimate of band 3 content, the reported values of ΔH_{app} should be considered approximations. Most experiments were conducted at least 3 times, and the observed ΔH_{app}'s were within 20% of the reported values.

Values of ΔH_{app} are given per mole of band 3. The amount of band 3 (intact or proteolyzed) in a given membrane suspension was estimated by determining from gel scans the percentage of the total Coomassie blue staining intensity contributed by band 3. This percentage was then multiplied by the amount of total protein in the sample, as determined by the modified Lowry procedure of Wang & Smith (1975), to give the quantity of band 3. Because glycophorin does not stain by Coomassie blue, it is not accounted for in the gel profile. Thus, quantities of band 3 will be slightly overestimated by this procedure. Assuming that glycophorin comprises ~2% of the total protein in intact membranes (Furthmayr, 1978), our error in determination of band 3 should range between ~2% in intact membranes and ~10% in trypsinized/stripped membranes. Since glycophorin is not present in the purified 53K fragment preparation, the ΔH_{app} obtained on this sample should be more accurate.

Thermotropic Fluorescence Measurements. All fluorescence measurements were obtained on a Perkin-Elmer MPF-44A fluorescence spectrophotometer equipped with two stirred, thermostated quartz cuvettes. A Digitec 5810 temperature probe was inserted into the cuvette for monitoring the temperature, and the heating rate of 1 °C/min was controlled by a Northeast Scientific temperature programmer. The membrane samples were degassed prior to heating to prevent the formation of bubbles.

DIDS fluorescence measurements were obtained by using an excitation wavelength of 370 nm. During heating, the emission spectrum was scanned from 400 to 460 nm, so that both the wavelength of the emission maximum and the fluorescence intensity at 450 nm could be obtained simultaneously.

Dipyridamole polarization was measured with the excitation and emission monochromators set at 400 and 490 nm, respectively. The half-bandwidth was adjusted to 6 nm, and a Corning 3-71 filter was used to eliminate scattered light. The dipyridamole polarization, P, was calculated from the following relationship:

$$P = \frac{V_v - L_v(V_H/L_H)}{V_v + L_v(V_H/L_H)}$$

where V_v, L_v, L_H, and V_H correspond to the fluorescence intensities obtained with excitation and emission polarizers set at 0°, 0°; 0°, 90°; 90°, 90°; and 90°, 0°, respectively.

Intrinsic fluorescence measurements were obtained by exciting at 290 nm and monitoring the emission at 328 nm (the emission maximum).

Analytical Procedures. $NaDodSO_4$–polyacrylamide gel electrophoresis was conducted according to the method of Fairbanks et al. (1971), employing the additional modifications of Steck & Yu (1973). Gel scans were obtained at 550 nm on a Gilford UV–visible spectrophotometer equipped with a gel scanning accessory.

Protein determinations were made by the method of Wang & Smith (1975), with bovine serum albumin as a standard.

Glycophorin concentrations were estimated by using the sialic acid assay of Warren (1959). Additionally, the relative amounts of glycophorin in the different membrane suspensions were determined by running gels on the samples, staining the gels by the PAS procedure (Fairbanks et al., 1971), and integrating the PAS staining intensities.

The amino acid composition of the purified membrane-spanning (53K) fragment of band 3 was obtained on the reconstituted vesicle preparation containing phosphatidylcholine, phosphatidylserine, and band 3. The sample was hydrolyzed for 64 h at 110 °C in 6 N HCl and analyzed on a Durrum D-500 analyzer. Because of the partial release of serine from phosphatidylserine during hydrolysis, the absolute abundances of the component amino acids could not be quantitated by assuming they all derived from the 53K fragment; i.e., some of the serine will be contributed by phosphatidylserine. Thus, the amino acid abundances reported in Table I are not absolute, but instead are relative to an assumed value of 36.6 mol of alanine/mol of band 3 fragment (Markowitz & Marchesi, 1981).

Results

Evidence for Band 3 Denaturation during the C Transition. Because of the sensitivity of the C transition to inhibitors of anion transport, it seemed logical to determine whether denaturation of band 3, the anion transport protein, occurs during

Table I: Amino Acid Composition of the Purified 53K Fragment of Band 3

amino acid	present study[a]	Markowitz & Marchesi (1981)[b]
Asx	29.6	32.0
Thr	25.1	24.0
Ser	42.5	25.9
Glx	40.5	41.5
Pro	27.4	28.0
Gly	41.9	44.0
Ala	36.6	36.6
Val	40.8	43.9
Met	15.7	15.0
Ile	33.4	28.0
Leu	67.1	71.5
Tyr	13.2	13.9
Phe	35.2	35.3
His	6.9	7.6
Lys	17.9	22.0
Arg	21.3	22.6

[a] Relative abundance; see Materials and Methods. [b] Moles per mole of band 3 fragment.

"C". Three experiments were designed to evaluate this possibility. First, erythrocytes were labeled with the highly potent, covalent inhibitor of anion transport, DIDS, and the fluorescence properties of the derived membranes were monitored as a function of temperature. Since DIDS is known to bind almost exclusively to band 3 (Cabantchik & Rothstein, 1974), any major structural perturbation of the transport protein might be expected to cause a change in the fluorescence properties of DIDS. Comparison of the emission spectrum of DIDS-labeled membranes at 25 °C before and after heating to 85 °C demonstrated that an irreversible perturbation of the DIDS environment had resulted from the heat treatment (not shown). Thus, the DIDS emission maximum shifted irreversibly from ~450 to ~420 nm, and the DIDS fluorescence intensity increased ~65%. To determine the temperature at which these changes in DIDS fluorescence occur, we followed both the DIDS emission maximum and the intensity of the DIDS fluorescence at 450 nm (F_{450}) as a function of temperature (Figure 2B). Between 25 and ~70 °C, the emission maximum of DIDS showed a gradual, monotonous decline from ~450 to ~440 nm. However, beginning at 73 °C, the emission maximum dropped abruptly until it resumed its gradual decline at ~80 °C. This precipitous decrease indicates that a cooperative change in the DIDS environment occurs between 73 and 80 °C. Comparison with the calorimetric profile in Figure 2A reveals that this change occurs approximately within the temperature range of the C transition, which is shifted from 68 to 78 °C in these DIDS-labeled membranes. Figure 2B also reveals that the intensity of the DIDS fluorescence undergoes a significant increase during the C endotherm. Significantly, both the DIDS fluorescence transitions and the C endotherm are irreversible.

To further examine the connection between the C transition and the changes in DIDS fluorescence, we have shifted "C" to lower temperatures and have looked to see whether the temperatures of the DIDS fluorescence transitions shift also. Figure 3 reveals that the DIDS-labeled C endotherm is displaced from ~78 to ~65 °C by 21 mM lidocaine. The B_1, B_2, and D transitions are considerably broadened by lidocaine and, hence, are not resolved in this modified scan. Importantly, the thermally induced, irreversible transitions in DIDS fluorescence are also shifted by 21 mM lidocaine into the temperature range of the modified C endotherm. Thus, the precipitous decrease in the DIDS emission maximum is displaced from ~77 to ~61 °C in lidocaine-treated membranes,

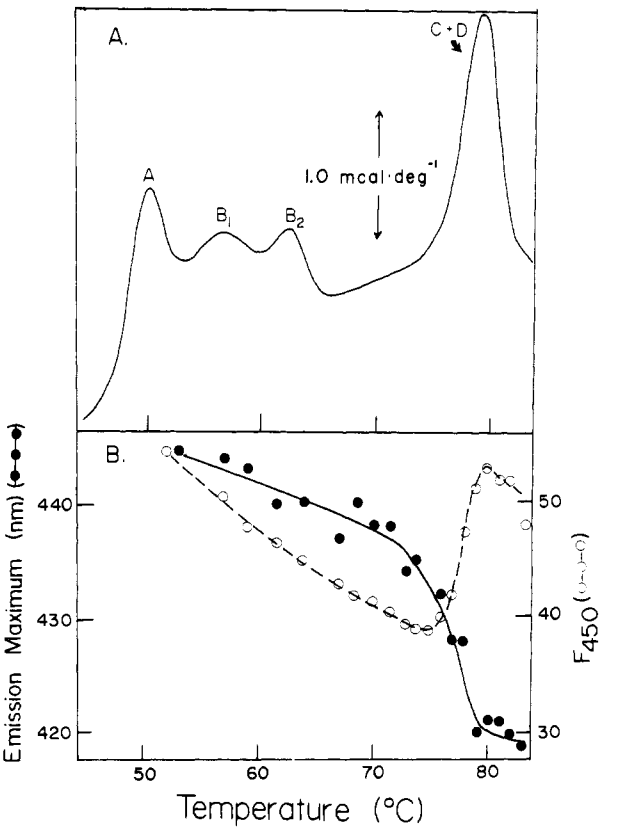

FIGURE 2: Comparison of the temperatures of the calorimetric endotherms of DIDS-modified membranes (A) with the temperatures of the DIDS fluorescence transitions of the same membranes (B). The membranes were suspended in PBS, pH 7.4, and heated at 1 °C/min for both the calorimetric and fluorescence melting experiments. (A) The calorimetric scan of DIDS-labeled membranes was conducted on a 1-mL sample containing 6.1 mg of protein. The C transition is displaced from its normal temperature of 68 °C to 78 °C due to the DIDS labeling (Snow et al., 1978). At 78 °C, the DIDS-modified C transition obscures the underlying, less prominent D transition. (B) The DIDS emission maximum (●) and the DIDS fluorescence at 450 nm (○) were monitored during heating (λ_{ex} 370 nm). The protein concentration was approximately 0.25 mg/mL in the fluorescence cuvette.

and the apparent midpoint temperature of the DIDS fluorescence enhancement transition is shifted to 65 °C. These observations demonstrate that an irreversible structural perturbation of band 3, as detected by the fluorescence properties of DIDS, coshifts with the irreversible C transition in lidocaine-treated membranes.

To be sure that the thermal perturbation of the DIDS fluorescence properties does not derive from the melting of a gel phase lipid surrounding band 3, we have examined the fluorescence transitions of DIDS-labeled band 3 solubilized in 1% Triton X-100. Triton X-100 has been demonstrated to cleanly segregate band 3 from most, if not all, of the surrounding lipids (Yu et al., 1973; Yu & Steck, 1975). Triton X-100 solubilized, DIDS-labeled band 3 displayed fluorescence melting transitions very similar to those of DIDS-labeled band 3 in the presence of 21 mM lidocaine (not shown). The abrupt decrease in the DIDS emission maximum was centered at 56.5 °C, and the midpoint temperature of the fluorescence enhancement transition was 62 °C. Thus, it would appear that the changes in DIDS fluorescence, and by extension the denaturation of band 3, can occur in the absence of a cooperative melting associated lipid phase.

One peculiar feature of the above data which will become more apparent (vide infra) concerns the small temperature

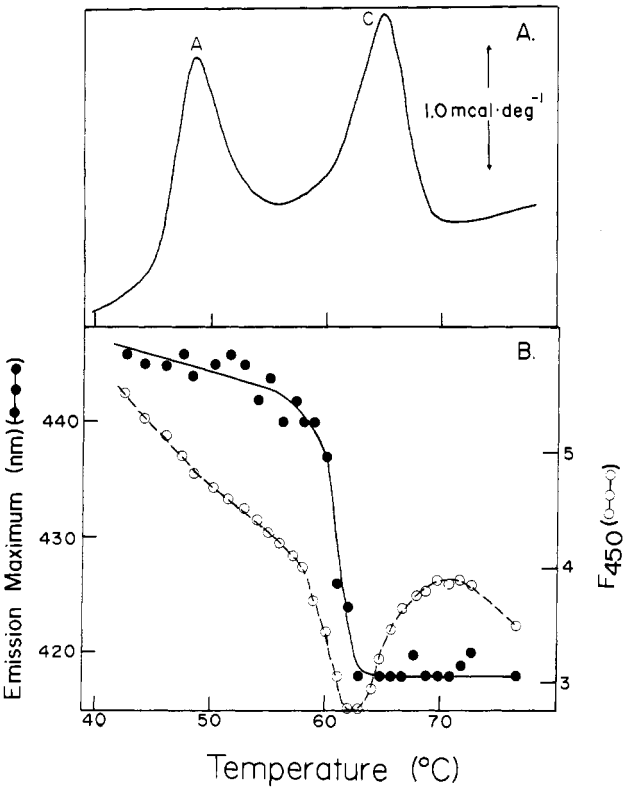

FIGURE 3: Comparison of the shift in temperature of the C transition (A) with the shift in the temperatures of the DIDS fluorescence transitions (B) in DIDS-labeled membranes modified by the presence of 21 mM lidocaine. Conditions are as in Figure 2, except that the protein concentration in the calorimeter cell was 4.03 mg/mL. Lidocaine was introduced by washing the membranes twice in PBS, pH 7.4, containing 21 mM lidocaine.

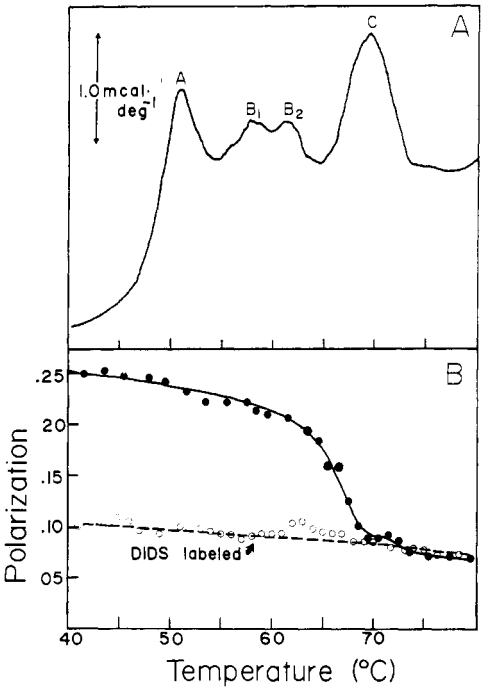

FIGURE 4: Comparison of the temperature of the C transition (A) with the temperature of dipyridamole release (B) from whole erythrocyte membranes. (A) The calorimetric scan was conducted on a 1-mL membrane sample containing 5.0 mg of protein and 1 μM dipyridamole. The C transition is elevated from 68 to 69.5 °C by the dipyridamole. (B) The fluorescence polarization of 1 μM dipyridamole in equilibrium with DIDS-labeled (○) and unlabeled (●) erythrocyte membranes as a function of temperature. The heating rate in both (A) and (B) was 1 °C/min.

difference between the midpoint of the thermotropic DIDS wavelength shift and the midpoint of the C transition. We have consistently observed that the wavelength shift from ~442 to ~418 nm precedes both the midpoint temperature of the C transition and the coincident DIDS fluorescence enhancement by 1–5 °C. The temperature difference becomes more pronounced as "C" is shifted to lower temperatures. This behavior suggests that the denaturation of band 3 may be biphasic. If this is correct, then the emission maximum of DIDS would appear to be more sensitive to the initial phase of the structural rearrangement, while the fluorescence enhancement of DIDS and the concomitant C endotherm may detect the latter phase of the process.

Changes in Ligand Binding to Band 3 during the C Transition. If band 3 denatures during the C transition, then it might be expected that a ligand which binds to this protein should be released during the C transition. The noncovalent inhibitor of anion transport, dipyridamole, binds specifically and with high affinity to band 3 (K_I ~0.7 μM; Schnell, 1972). Dipyridamole is also a fluorescent molecule. Thus, its binding to band 3 should be conveniently detected as an increase in the fluorescence polarization of the molecule. Consistent with this expectation, titration of an aqueous solution of dipyridamole with erythrocyte membranes results in a gradual rise in the fluorescence polarization of dipyridamole, which extrapolates in a linear double reciprocal plot to a limiting value of 0.5 (not shown). Conversely, release of the bound dipyridamole into the aqueous medium should occur with a decrease in fluorescence polarization, since dipyridamole in buffer has a polarization of 0.

Figure 4 shows the fluorescence polarization of 1 μM dipyridamole in a suspension of erythrocyte membranes as a function of temperature. At low temperatures (25 °C), the polarization is 0.25, reflecting a situation where roughly half of the dipyridamole molecules are membrane bound and roughly half are free. However, as the suspension is heated, the polarization of dipyridamole decreases suddenly within the temperature range of the C transition, demonstrating an abrupt increase in the motional freedom of the inhibitor at these temperatures. To establish that dipyridamole is being released from band 3, and not from another site on the membrane, we have examined the temperature dependence of dipyridamole binding to membranes in which all band 3 sites have been blocked with DIDS (lower curve, Figure 4). Significantly, the fluorescence polarization remains low at all temperatures between 40 and 80 °C, indicating that DIDS interferes with dipyridamole binding to its high-affinity site. Since DIDS is bound almost exclusively to band 3 (Cabantchik & Rothstein, 1974), dipyridamole must be released from this protein during the C transition. Loss of ligand binding ability is an expected consequence of protein denaturation.

Changes in the Intrinsic Fluorescence of Band 3 during the C Transition. The third experiment to evaluate the participation of band 3 in the C transition was to monitor the intrinsic fluorescence of the membrane-spanning (53K) fragment of band 3 as a function of temperature. An abrupt change in the intrinsic fluorescence of the fragment during the C transition would indicate a perturbation of the environment of the fragment's tryptophan residues. Figure 5 shows the gels of membranes that have been spectrin depleted, trypsin digested (gel B), and then stripped of peripheral proteins and proteolytic fragments by a single wash in a cold NaOH solution of pH 12 (gel C). The predominant polypeptide remaining in the membrane after this procedure is a very broad-looking 53 000-dalton protein which has been identified as the mem-

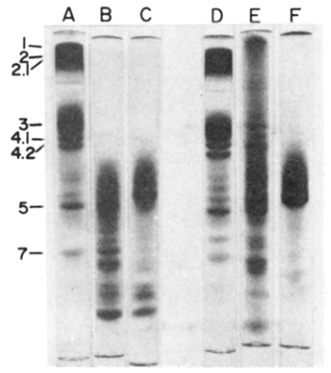

FIGURE 5: Sodium dodecyl sulfate–polyacrylamide disc gel electrophoresis of membranes at various stages in the isolation of the membrane-spanning (53K) domain of band 3. The gels are stained with Coomassie blue. (Gels A–C) Whole membranes (gel A) were depleted of spectrin and actin, digested with 20 μg/mL trypsin for 30 min on ice (gel B), and then stripped with a cold NaOH solution of pH 12 (gel C), as described under Materials and Methods. (Gels D–F) Whole membranes (gel D) were depleted of spectrin and actin, digested with 10 μg/mL trypsin for 1 h on ice (gel E), and then dissolved in 1.5% Triton X-100. After the 53K band 3 fragment passed through an aminoethylcellulose column equilibrated at pH 8, the fragment was reconstituted into egg phosphatidylcholine/bovine brain phosphatidylserine vesicles (gel F), as described under Materials and Methods.

brane-spanning fragment of band 3 (Steck et al., 1976; Markowitz & Marchesi, 1981).[2] The 53K segment of band 3 comprises ~70% of the total Coomassie blue staining material in these trypsin-digested/stripped membranes. Several smaller peptides ranging in size from M_r 10 000 to M_r 20 000 make up the remainder of the proteins on the gel. Glycophorin fragments should also be present, but except for the very minor component, glycophorin C, these peptides contain no tryptophan (Furthmayr, 1978) and, hence, should not fluoresce (Teale, 1960).

The calorimetric profiles of unlabeled and DIDS-labeled membranes that have been treated as described above are shown in Figure 6A, and the temperature dependences of the intrinsic fluorescence of the same membrane preparations are displayed in Figure 6B. That the surviving calorimetric transition is, in fact, C is evident from three observations: (i) calorimetric scans at each stage of the above negative purification procedure retain an unmodified C transition; (ii) the temperatures of the DIDS-modified (79 °C) and unmodified (69 °C) transitions are within 1 °C of the temperatures of the C transitions in whole membranes; and (iii) the surviving transition retains the peculiar sensitivity to DIDS which is unique to the C endotherm of whole membranes. For the unlabeled and DIDS-labeled preparations, respectively, the intrinsic fluorescence decreased irreversibly 17% and 21% during the C transition (Figure 6B). Furthermore, when the C transition was removed by treatment with 0.17 M acetic acid, the intrinsic fluorescence transition also disappeared (not shown). More detailed experiments reported elsewhere have demonstrated a general correlation between the enthalpy of the C transition and the magnitude of the intrinsic fluorescence decrease at that temperature (Davio, 1981). Since the membrane-spanning fragment of band 3 is the predominant polypeptide in these preparations, it is highly probable that the observed irreversible transition derives from the fragment's

[2] The breadth of the 53K gel band has been attributed either to heterogeneity in the carbohydrate moieties attached to band 3 (Yu & Steck, 1975; Findlay, 1974) or to heterogeneity in the binding of Na-DodSO$_4$, since the same peptide map is obtained from protein in the leading and trailing edge of the band (Markowitz & Marchesi, 1981).

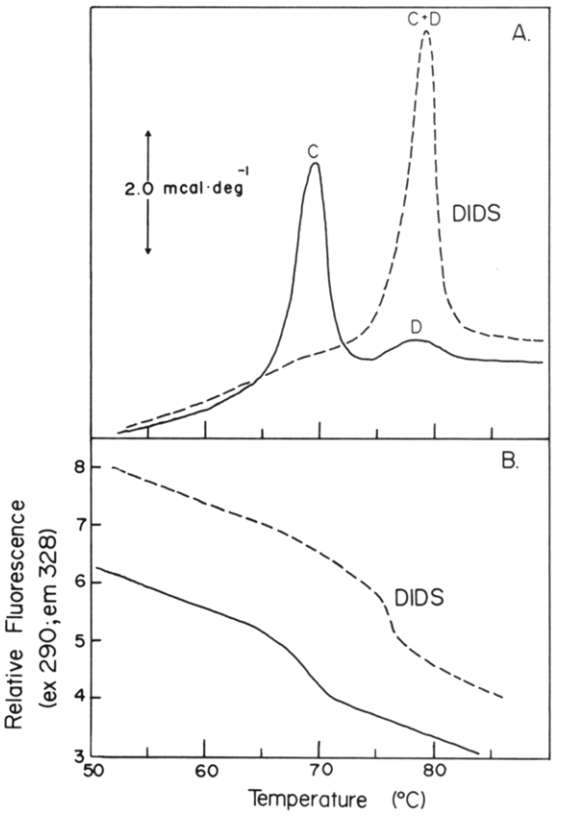

FIGURE 6: Comparison of the C transitions in DIDS-labeled (---) and unlabeled (—) trypsin-digested/NaOH-stripped membranes (A) with the intrinsic fluorescence transition (B) of the same membrane preparations. (A) The calorimetric scans each represent the melting of 5 mg of protein. The approximate enthalpies of the transitions are 190 and 240 kcal/mol of band 3 for the control and DIDS-labeled preparations, respectively. The enhanced enthalpy of the latter is probably due to the contribution of the underlying D transition, which is centered at 78 °C in both DIDS-labeled and control membranes. (B) The intrinsic fluorescence was monitored with excitation and emission monochromators set at 290 and 328 nm, respectively.

denaturation. In summary, the above experiments demonstrate that the membrane-spanning fragment of band 3 denatures during the C transition.

Investigation of the Possible Participation of Membrane Components Other than Band 3 in the C Transition. The data presented in Figure 6 also demonstrate that peripheral proteins are not involved in the C transition. Gel C of Figure 5 shows that the protein-depleted membranes lack all of the peripheral proteins present in unmodified membranes. Since the temperatures of the DIDS-modified and unmodified C transitions in these preparations are similar to the corresponding values in whole membranes, it would appear that the stability of the "C domain" is not significantly dependent on peripheral proteins. However, it should be mentioned that the apparent enthalpy of the C endotherm is reduced from ~380 kcal/mol of band 3 in intact membranes to ~190 kcal/mol of band 3 in the protein-depleted membranes. This decrease in enthalpy is probably due to limited NaOH denaturation of band 3 and not to loss of an essential peripheral protein, since the NaOH stripping procedure also reduces the enthalpy of the C transition in membranes previously depleted of all peripheral proteins by milder procedures (Davio, 1981). Furthermore, if the decrease in enthalpy were due to the loss of a stabilizing peripheral protein, then the temperature of the C transition ($T_c = \Delta H_c/\Delta S_c$) would also be expected to change, and this does not occur.

Two experiments were conducted in order to evaluate the possible involvement of integral proteins other than band 3

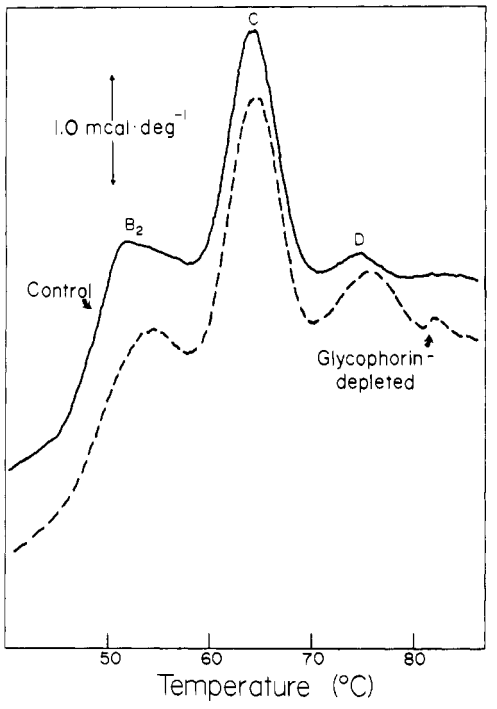

FIGURE 7: Calorimetry of glycophorin-depleted and control reconstituted membranes. The glycophorin-depleted membranes (---) contained 10.8 mg of protein, while the control sample (—) contained 10.5 mg of protein (see Materials and Methods for preparation).

in the C transition. First, the glycophorins of the erythrocyte membrane were removed by three consecutive extractions in 0.05% Triton X-100, as described by Wolosin et al. (1977). The membranes were then depleted of spectrin and actin, dissolved in 1% (v/v) Triton X-100, and reconstituted into their own lipid. Polyacrylamide disc gel electrophoretic analysis of the preparation demonstrated that glycophorin A was reduced to $\sim^1/_3$ of its original content relative to the concentration of band 3. Figure 7 compares the calorimetric scan of these glycophorin-depleted, reconstituted membranes with the scan of control, reconstituted membranes. The control membranes were treated in the same manner except the glycophorin extraction step was omitted. The identical solubilization and reconstitution steps were necessary to ensure that both samples had similar lipid and residual Triton X-100 contents. The similarity in the two scans attests to the lack of involvement of glycophorin in the C transition. Despite a significant difference in glycophorin content, the midpoint temperature of the endotherm in both cases is 64.5 °C. The enthalpies of the transitions of the glycophorin-depleted and control membranes are 310 and 360 kcal/mol of band 3, respectively, and these values are similar within the accuracy of the measurements.

Finally, to conclusively demonstrate that denaturation of the membrane-spanning domain of band 3 alone can account for the C transition, it was necessary to purify the 53K fragment in native form and to compare its calorimetric transition with the C endotherm of intact membranes. The purification of the membrane-spanning domain was conducted on DIDS-labeled membranes, since (i) the location of band 3 in column fractions could be easily detected by monitoring the fractions for DIDS fluorescence and (ii) DIDS-labeled band 3 was found to be more resistant to unwanted proteolysis. The 53K fragment of band 3 was purified by passing the Triton X-100 extract of spectrin-depleted, trypsin-digested membranes (Figure 5, gel E) through an aminoethylcellulose column. The membrane-spanning fragment was not retarded by the column,

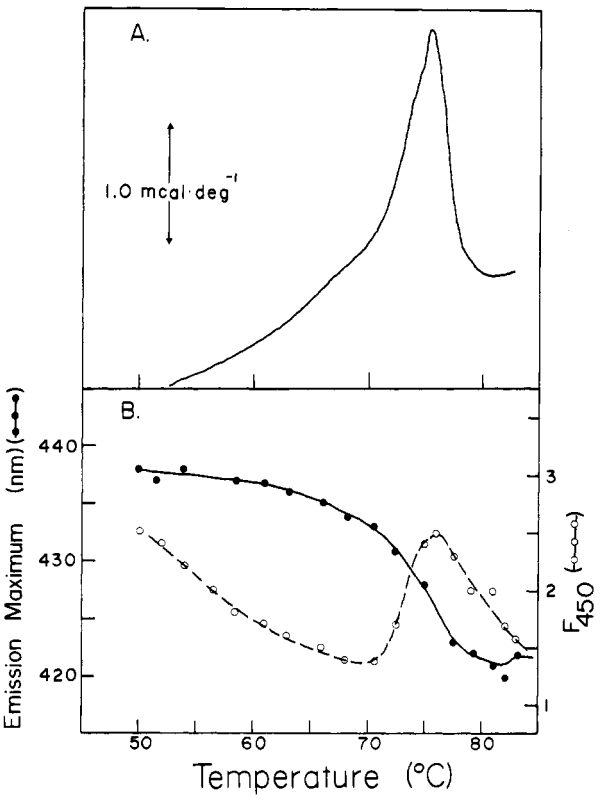

FIGURE 8: Calorimetric (A) and DIDS fluorescence transitions (B) of the purified DIDS-labeled, 53 000-dalton membrane-spanning domain of band 3. The calorimetric scan represents the melting of 2.7 mg of protein. All other conditions are as in Figure 2.

whereas nearly all other proteins and proteolytic fragments were retained on the anion exchange resin. The membrane-spanning fragment was greater than 90% pure as judged by polyacrylamide disc gel electrophoresis (Figure 5, gel F). No PAS-staining material was present in the column eluate. The band 3 containing fractions were reconstituted immediately into egg phosphatidylcholine/bovine brain phosphatidylserine vesicles as described under Materials and Methods.

For verification that the isolated polypeptide is in fact the 53K membrane-spanning domain of band 3, an amino acid analysis on the reconstituted membranes was conducted (Table I). For all amino acids except serine, there is excellent agreement between the composition of our purified fragment and that of the 53K fragment purified by Markowitz & Marchesi (1981). The disagreement between our level of serine and theirs comes from the fact that our analysis was conducted on the 53K fragment reconstituted into membranes containing 25 mol % phosphatidylserine. Some of this phospholipid hydrolyzes to form free serine during the hydrolysis of the polypeptide. The 53K fragment of Markowitz and Marchesi was purified under denaturing conditions, i.e., in $NaDodSO_4$, and was verified to be a fragment of band 3 by peptide mapping. These researchers have also determined that the peptide backbone is homogeneous throughout the width of the broad 53K band. Given the agreement in amino acid composition between our peptide and theirs, we have assumed that our fragment preparation is also homogeneous.

The calorimetric profile of the reconstituted membranes containing the purified 53K fragment of band 3 is shown in Figure 8A. The C transition in this profile is centered at 75 °C and has a ΔH_{app} of 360 kcal/mol of 53K fragment. This value of ΔH_{app} is very close to the value obtained for the C transition in intact membranes ($\sim$380 kcal/mol of band 3). Since the exogenous lipids in the reconstituted vesicles are both

highly fluid at 75 °C, the ΔH_{app} must derive solely from the denaturation of the integral fragment of band 3. Thus, lipid melting does not appear to be essential for a normal C transition. The melting temperature of "C" in the reconstituted membranes is ~3 °C lower than the DIDS-labeled C transition in intact membranes. We believe that this may be a result of residual Triton X-100 bound to band 3, since reconstitution of unpurified, Triton X-100 extracts containing essentially all membrane lipids and intrinsic proteins also yields vesicles with a C transition melting ~4–5 °C lower than normal (not shown). Alternatively, the depressed transition temperature could be due to (i) destabilization of band 3 by digestion products of proteins or phospholipids formed during solubilization and reconstitution or (ii) displacement of tightly bound, stabilizing lipid molecules from band 3 by the detergent.

The DIDS fluorescence transitions upon heating the reconstituted fragment preparation (Figure 8B) further verify that the observed endotherm in the calorimetric scan is "C". This predictable behavior of DIDS fluorescence is consistent with the idea that the structural changes which occur during the C transition of reconstituted membranes are the same as those which occur during "C" in intact membranes.

Discussion

The above data lead us to conclude that the C transition is a denaturation transition of the membrane-spanning domain of band 3. Any measurable contribution of lipid melting to the irreversible C transition can be dismissed, since the transition is observed both in detergent extracts of erythrocyte membranes and also in vesicles reconstituted from the 53K fragment of band 3 plus low melting lipids. It is possible, however, that only a subdomain of the 53K fragment is required for the C endotherm, since the endotherm has also been observed in extensively proteolyzed membranes containing the smaller (17K) transmembrane fragment of band 3 as the predominant polypeptide (Brandts et al., 1978).

The identification of the C transition now permits the use of sensitive differential scanning calorimetry to assay the structural integrity of the membrane-spanning domain of the anion transport protein in situ. Thus, without resorting to band 3 purification, we can assess the influence of perturbants and inhibitors on the transporter's structure by examining their effect on the temperature, cooperativity, and enthalpy of the C transition. In line with this principle, previous reports that benzyl alcohol, aliphatic alcohols, phenothiazines, tertiary amine local anesthetics, phenol, octylamine, phenylbutazone, dipyridamole, DIDS, SITS, pyridoxal phosphate, and 2,4-dinitrofluorobenzene modify the C transition (Snow et al., 1978; Krishnan & Brandts, 1979) can now be interpreted in terms of their structural perturbations of band 3. The ability of each member of this diverse collection of compounds to inhibit anion transport in erythrocytes seems more reasonable in view of their common capacity to perturb the transport protein's structure.

In the absence of a sensitive microcalorimeter, the other properties of the C transition can be employed to evaluate the structural state of band 3. For example, the wavelength of maximum DIDS fluorescence was used by ourselves to estimate the fraction of native band 3 molecules obtained by several potential purification procedures. Separation of band 3 on a concanavalin A affinity column was not employed, since a measurable fraction of the DIDS emission of the isolated product was centered at 420 nm. Purification by aminoethylcellulose chromatography, however, was eventually selected since all of the DIDS emission of the derived 53K fragment was centered at 450 nm. Similarly, the polarization of dipyridamole fluorescence can be used to evaluate the ligand binding ability of band 3 preparations, so long as the transport protein has not been previously labeled with DIDS.

While lipid melting does not appear to contribute to the enthalpy of the C transition, it should not be assumed that the fluid lipid environment does not significantly influence the stability of band 3. On the contrary, in the absence of significant associated lipid, i.e., in 1% Triton X-100, the DIDS fluorescence transitions occur ~17 °C below normal. If 20 mM oleic acid is added to erythrocyte membranes or if the membranes are digested with phospholipase A_2 or C, the C transition completely disappears (Davio, 1981; Brandts et al., 1978). Furthermore, if the isolated, DIDS-labeled 53K fragment of band 3 is reconstituted into egg phosphatidylcholine alone, the resulting vesicles yield an endotherm centered at ~71 °C, i.e., ~4 °C lower than in the added presence of 25 mol % bovine brain phosphatidylserine (Appell & Low, 1982b). It is, therefore, anticipated that the stability and perhaps even the catalytic properties of band 3 may be sensitive to the lipid at the transport protein's surface.

The comparison of the properties of the C transition before and after removal of glycophorin from the membrane bears upon another question of current interest. Several electron microscope studies suggest that band 3 and glycophorin are localized in the same intramembrane particles observed on the freeze–fracture faces of erythrocyte membranes (Pinto da Silva & Nicolson, 1974; Tillack et al., 1972). Other data, however, indicate that the participation of glycophorin in these intramembrane structures may be either minimal or nonexistent (Edwards et al., 1979; Gahmberg et al., 1978; Gerritsen et al., 1978; Lutz et al., 1979). Cherry and co-workers (Nigg et al., 1980) have recently demonstrated that divalent antibodies specific for glycophorin A reduce the rotational motion of a fluorescent probe located largely on band 3, implicating substantial interaction between the two transmembrane proteins. However, no association of band 3 and glycophorin can be demonstrated in detergent extracts of erythrocyte membranes (Yu & Steck, 1975). We have observed that the temperature, enthalpy, and cooperativity of band 3 denaturation, i.e., of the C transition, were unaffected by removal of glycophorin from the membranes. This indicates that glycophorin does not significantly stabilize the membrane-spanning domain of band 3. Thus, either the putative glycophorin/band 3 complex is too weak to be detected by sensitive calorimetry or its stability depends on interactions with the cytoplasmic pole of band 3.

Finally, the earlier observations suggesting that "C" may be a lipid melting transition can be partially explained. First, Brandts et al. (1977) observed no major change in the circular dichroism at 222 nm of whole erythrocyte membranes upon heating through the C transition. We have recently examined the circular dichroism of the isolated 53K fragment of band 3 in both detergent and phospholipid vesicles and have observed a 30% decrease in the negative ellipticity at 222 nm upon heating through the C transition (preliminary results). While this change in ellipticity is not small, it may have been very difficult to resolve in whole membranes, where the membrane-spanning (53K) domain of band 3 represents only ~15% of the total protein.

Brandts et al. (1978) also demonstrated that the C transition is resistant to membrane proteolysis by papain, trypsin, and chymotrypsin. Even though these enzymes clearly cleave band 3, the membrane-spanning region of the protein is sufficiently protected by the bilayer to remain largely intact (Jennings & Passow, 1979; Steck et al., 1976). Since the C transition

derives from the unfolding of the membrane-spanning domain(s) of band 3, the endotherm should also survive this treatment.

The disappearance of the C transition after phospholipase digestion is more difficult to explain. Apparently, the stability of band 3 is very sensitive either to the loss of certain digested lipids or to the appearance of their digestion products in the membrane. It was mentioned earlier (Davio, 1981) that oleic acid, a product of phospholipase A_2 digestion, eliminates the C transition from whole membranes.

Lastly, the unanticipated sensitivity of band 3 to amphiphilic agents, e.g., benzyl alcohol, at concentrations previously thought to be too low to affect protein stability, may derive in part from the high partition coefficients of the amphiphilic agents in the membrane. For example, band 3 may be bathed in 100 mM benzyl alcohol when only 25 mM benzyl alcohol is added to the medium (Seeman, 1972). However, band 3 still appears to be unusually sensitive to amphiphiles, since 100 mM benzyl alcohol completely eliminates the C transition, yet it reduces the melting temperature of ribonuclease by only 3 °C (Schrier et al., 1965). It should be interesting to learn if the structures of other transmembrane channels are equally sensitive to amphiphiles and anesthetics.

References

Appell, K. C., & Low, P. S. (1981) *J. Biol. Chem. 256*, 11104–11111.

Appell, K. C., & Low, P. S. (1982a) *Biochim. Biophys. Acta* (in press).

Appell, K. C., & Low, P. S. (1982b) *Biochemistry 21*, 2151–2157.

Brandts, J. F., Erickson, L., Lysko, K., Schwartz, A. T., & Taverna, R. D. (1977) *Biochemistry 16*, 3450–3454.

Brandts, J. F., Taverna, R. D., Sadasivan, E., & Lysko, K. A. (1978) *Biochim. Biophys. Acta 512*, 566–578.

Brasitus, T. A., Tall, A. R., & Schachter, D. (1980) *Biochemistry 19*, 1256–1261.

Cabantchik, Z. I., & Rothstein, A. (1974) *J. Membr. Biol. 15*, 207–226.

Cramer, W. A., Whitmarsh, J., & Low, P. S. (1981) *Biochemistry 20*, 157–162.

Davio, S. R. (1979) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 38*, 341.

Davio, S. R. (1981) Ph.D. Dissertation, Purdue University, West Lafayette, IN.

Dodge, J. T., Mitchell, C., & Hanahan, D. J. (1963) *Arch. Biochem. Biophys. 100*, 119–130.

Edwards, H. H., Mueller, T. J., & Morrison, M. (1979) *Nature (London) 203*, 1343–1346.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606–2617.

Findlay, J. B. C. (1974) *J. Biol. Chem. 249*, 4398–4403.

Furthmayr, H. (1978) *J. Supramol. Struct. 9*, 79–95.

Gahmberg, C. G., Tauren, G., Virtanen, I., & Wartiovaara, J. (1978) *J. Supramol. Struct. 8*, 337–347.

Gerritsen, W. J., Verkley, A. J., Zwaal, R. F. A., & Van Deenan, L. L. M. (1978) *Eur. J. Biochem. 85*, 255–261.

Holloway, P. W. (1973) *Anal. Biochem. 53*, 301–308.

Jackson, M. B., & Sturtevant, J. M. (1978) *Biochemistry 17*, 911–915.

Jennings, M. L., & Passow, H. (1979) *Biochim. Biophys. Acta 554*, 498–519.

Krishnan, K. S., & Brandts, J. F. (1979) *Mol. Pharmacol. 16*, 181–188.

Low, P. S., & Brandts, J. F. (1978) *Arch. Biochem. Biophys. 190*, 640–646.

Lutz, H. U., von Däniken, A., Semenza, G., & Bächi, T. (1979) *Biochim. Biophys. Acta 552*, 262–280.

Lysko, K. A., Brandts, J. F., Carlson, R., Taverna, R., & Snow, J. (1981) *Biochemistry 20*, 5570–5576.

Marchesi, S. L., Steers, E., Marchesi, V. T., & Tillack, T. W. (1970) *Biochemistry 9*, 50–57.

Markowitz, S., & Marchesi, V. T. (1981) *J. Biol. Chem. 256*, 6463–6468.

Nigg, E. A., Bron, C., Girardet, M., & Cherry, R. J. (1980) *Biochemistry 19*, 1887–1893.

Pinto da Silva, P., & Nicolson, G. L. (1974) *Biochim. Biophys. Acta 363*, 311–319.

Schnell, K. F. (1972) *Biochim. Biophys. Acta 282*, 265–276.

Schrier, E. E., Ingwall, R. T., & Scheraga, H. A. (1965) *J. Phys. Chem. 69*, 298–303.

Seeman, P. (1972) *Pharmacol. Rev. 24*, 583–655.

Snow, J. W., Brandts, J. F., & Low, P. S. (1978) *Biochim. Biophys. Acta 512*, 579–591.

Snow, J. W., Vincentelli, J., & Brandts, J. F. (1981) *Biochim. Biophys. Acta 642*, 418–428.

Steck, T. L., & Yu, J. (1973) *J. Supramol. Struct. 1*, 220–232.

Steck, T. L., Ramos, B., & Strapazon, E. (1976) *Biochemistry 15*, 1154–1161.

Teale, F. W. J. (1960) *Biochem. J. 76*, 381–388.

Tillack, T. W., Scott, R. E., & Marchesi, V. T. (1972) *J. Exp. Med. 135*, 1209–1221.

Wang, C., & Smith, R. L. (1975) *Anal. Biochem. 63*, 414–417.

Warren, L. (1959) *J. Biol. Chem. 234*, 1971–1975.

Wolosin, J. M. (1980) *Biochem. J. 189*, 35–44.

Wolosin, J. M., Ginsburg, M., & Cabantchik, Z. I. (1977) *J. Biol. Chem. 252*, 2419–2427.

Yu, J., & Steck, T. L. (1975) *J. Biol. Chem. 250*, 9170–9175.

Yu, J., Fischman, D. A., & Steck, T. L. (1973) *J. Supramol. Struct. 1*, 233–248.