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Structural stability of the erythrocyte anion transporter, band 3, in native membranes and in detergent micelles

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The exothermic thermal denaturation transition of band 3, the anion transporter of the human erythrocyte membranes, has been studied by differential scanning calorimetry, in ghost membranes and in nonionic detergent micelles. In detergent micelles the transmembrane domain of band 3 gave an irreversible denaturation transition (C transition). However, no thermal transition was observed for the N-terminal cytoplasmic domain when band 3 was solubilised in detergent micelles. A reduction in enthalpy (190–300 kcal mol^{-1}) with an accompanying decrease in thermal denaturation temperatures (48–60°C) for the C transition was observed in detergent solubilised band 3 when compared with ghost membranes. Unlike ghost membranes, two thermal transitions for band 3 in detergent micelles were observed for the C transition when in the presence of excess covalent inhibitor, 4,4'-diisothiocyanostilbene-2,2'-disulphonate (DIDS), which derive from the thermal unfolding of a single protein with two different thermal stabilities; DIDS-stabilised (75°C) and DIDS-insensitive (62°C). A reduction in the denaturation temperature for the transmembrane domain of band 3 was observed when compared with intact band 3 although no significant difference was observed in the corresponding enthalpy values. This indicates some cooperativity of the two domains of band 3 in maintaining the transmembrane conformation. The results presented in this study show that detergents of intermediate micelle size (e.g. Triton X-100 and $C_{12}E_8$) are required for optimal thermal stability of band 3.

Introduction

The human erythrocyte band 3 is the major glycosylated integral membrane protein of erythrocytes [1,2]. The 55 kDa transmembrane domain of band 3 protein possesses anion-transporting capabilities [3,4], whereas the 42 kDa cytoplasmic domain of band 3 protein binds several constituents of the cytoplasm [5]. The high abundance of band 3 and its relative ease of preparation makes it an ideal candidate for examining the influence of detergents on the structure and stability of a typical hydrophobic membrane protein.

Detergents can generally be classified into two categories, nondenaturing (e.g. most non-ionic detergents) and denaturing (e.g. SDS) detergents, depending on their ability to stabilise native membrane protein structure after solubilization. In earlier investigations of band 3 in detergent micelles, circular dichroism (CD) and fluorescence measurements have been used as a qualitative measure of the ability of detergents to stabilise secondary structure as found in the native state. Changes in the fluorescence properties of band 3 labelled with the anion transport inhibitor, DIDS, in Triton X-100 showed that the protein unfolds at 56.5°C [6]. Studies of band 3 in octyl glucoside has provided evidence for the formation of high oligomeric state with alterations in the inhibitor binding site [7]. Further studies have shown that the $C_{12}E_8$ solubilized transmembrane domain of band 3 was enriched in α-helical content relative to the same sample in the absence of detergent. Further, it was noted that no change in the CD spectra was detected upon the covalent attachment of DIDS to the membrane associated domain but an enhanced stability to heat denaturation was observed [8]. The polyoxyethylene deter-

Abbreviations: Triton X-100, 1,1,3,4-tetramethylbutylhexanyl polyoxyethylene glycol (with an average of 10 polyoxyethylene chains per molecule); $C_{12}E_8$, polyoxyethylene glycol *n*-dodecyl ether (with an average of eight polyoxyethylene chains per molecule); $C_{12}E_9$, polyoxyethylene glycol *n*-dodecyl ether (with an average of nine polyoxyethylene chains per molecule); $C_{14}E_8$, polyoxyethylene *n*-myristyl ether with an average of eight polyoxyethylene chains per molecule; $C_{16}E_8$, polyoxyethylene *n*-cetyl ether (with an average of eight polyoxyethylene chains per molecule); DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulphonic acid; PMSF, phenylmethylsulphonyl fluoride; SDS, sodium dodecyl sulphate; TPCK, tosylphenylalanine chloromethyl ketone.

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gents are a class of non-ionic surfactants which have been extensively studied and used in crystallisation of membrane proteins [9].

Distinct thermotropic transitions have been resolved for many of the major structural domains of the human erythrocyte membrane. The A transition (49°C) and B_1 transition (56°C) have been shown to involve the denaturation of spectrin and bands 2.1, 4.1 and 4.2, respectively [10]. The C transition (68°C) has been identified as the thermal unfolding of the 55 kDa anion transporting domain of band 3 [11]. The B_2 (62°C at pH 7.4) transition has been shown to involve the reversible unfolding of the band 3 N-terminal segment of the cytoplasmic domain [11]. These studies have also shown that the C transition was readily removed by the treatment of membranes with phospholipase A_2, phospholipase C or oleic acid suggesting that lipids at the transport surface of the protein may be important for maintaining protein stability [10]. The C transition of band 3 was also shown to be sensitive to amphiphilic agents, at concentrations below those reported to affect protein structure, and it has been suggested that the anomalous partitioning of amphilic agents into membranes, which could dislodge annular lipids on the band 3 transmembrane surface, may account for this behaviour [6,12].

To date there have been no studies on the thermostability of detergent solubilised band 3. The major difficulty in investigating the denaturation of concentrated protein detergent micelles lies with the phenomenon of post-denaturation aggregation. Apart from the irreversibility of the denaturation process itself, there is an exothermic change in the system due to protein aggregation [13]. At slow heating rates the temperature interval of protein denaturation is long and the processes of denaturation and aggregation become superimposed. In such cases, the exotherms are reduced leading to difficulty in determining enthalpy and the position of the calorimetric baseline. Since the breaking of non-covalent bonds during denaturation (heat absorption) and the subsequent aggregation of molecules (heat release) are separated in time, it might be expected that in the case of rapid heating rates of the solution, the thermal effect of the denaturation process may be monitored before the appearance of the first aggregation phenomenon. In order to avoid the distortions imposed by heat release on aggregation, fast heating rates have been used in this present study (10–20 C°/min).

Here we present an investigation of the heat stabilities of the intact band 3 and its 55 kDa anion transporting membrane domain (TMD) in different acyl chain length polyoxyethylene-type detergent micelles using DSC. Evidence for the irreversibility for band 3 protein thermal unfolding in detergents and *in situ* is presented.

Materials and Methods

Materials

DIDS, TPCK-treated trypsin (type XIII), α-chymotrypsin (type 1-S), $C_{12}E_9$, $C_{10}E_8$, $C_{14}E_8$ and $C_{16}E_8$ were obtained from Sigma Chem. Co., $C_{12}E_8$ was from Nikko Fine Chemicals (Tokyo, Japan), Triton X-100 (reduced) was from Aldrich Chem. Co., and DEAE-Sepharose 6B was from Pharmacia LKB Biotechnology Inc.. All other reagents were analytical grade and obtained from Sigma Chemical Co. or Boehringer Mannheim.

Band 3 purification and preparation for DSC

Band 3 was purified from ghost membranes, prepared from indated human blood by a modified procedure of Schubert et al. 1986 [14]. After the pre-extraction of human erythrocyte membranes with 1% Brij 58, followed by a second extraction in Triton X-100 (0.5% w/v), the supernatant was loaded on to a DE52 column. Detergent exchange was carried out at this stage by washing the column (2.5 × column volume) initially with 10 mM Tris-HCl (pH 8) containing 0.1% w/v of the desired detergent, followed by a second wash in the same buffer containing 130 mM NaCl and finally eluted in the same buffer containing 250 mM NaCl. The protein containing fractions from the column were pooled and diluted immediately with 4 volumes of 10 mM Tris-HCl (pH 8) containing 2 mM dithiothreitol. Samples for DSC were concentrated 10–20-fold to give protein concentrations of approx. 10 mg ml^{-1}. Typically 50 ml of pooled extract was initially concentrated in an ultra-filtration cell with a XM50 membrane and then finally in a Centricon 100 filter (Amicon) Samples were stored at 4°C and used within 24 h of preparation and degassed before obtaining thermograms. Some preparations of protein were incubated with DIDS for 30 min at 30°C prior to calorimetric measurements. When Tris buffer was used the solution pH was calculated assuming a $\Delta pK_a/C°$ of -0.028. Detergent buffers in the absence of protein were also measured to ensure a flat baseline.

Preparation of inside-out vesicles (IOV's)

Spectrin depleted IOV's were prepared by incubating ghosts in 10 volumes of 0.5 mM EDTA (pH 8) at 37°C for 30 min followed by a wash in 10 volumes of 20 mM sodium phosphate (pH 8) [15]. Limited proteolysis was accomplished by stirring the IOV's on ice in TPCK-treated trypsin (10 mg/ml) in 20 mM sodium phosphate, 150 mM NaCl (pH 8) for 1 h. Proteolysis was inhibited by two washes in equal volumes of 1 mM PMSF in 5 mM sodium phosphate at 23000 × *g* and finally in an equal volume of the same buffer without PMSF.

Isolation of the transmembrane domain (TMD) of band 3

The integral domain of band 3 was isolated by solubilising the proteolysed IOV's in 1% w/v of $C_{12}E_8$, 25 mM 2-mercaptoethanol, 5 mM sodium phosphate (pH 8) and stirring on ice for 30 min. After the undissolved membranes were removed by centrifugation ($45\,000 \times g$; 20 min) the supernatant, 2 column volumes, was applied to DEAE-Sepharose 6B column equilibriated in 0.1% w/v of $C_{12}E_8$, 25 mM 2-mercaptoethanol, 5 mM sodium phosphate (pH 8). The extract was recycled for 2 h at a flow rate of 25 ml h^{-1}. The column was then washed with a linear gradient of 5–160 mM sodium phosphate buffer (pH 8) containing 25 mM 2-mercaptoethanol and 0.1% w/v $C_{12}E_8$, the total volume amounting to approximately 12 column volumes.

The protein-containing fractions were collected and pooled ($A_{280} = 0.190$) and concentrated initially in an ultra-filtration cell (XM50 membranes, Amicon) and finally in a centricon 30 (Amicon) to give protein concentrations of approx. 10–20 mg ml^{-1}.

Calorimetry

Calorimetric measurements were obtained on a DSC-7 scanning calorimeter (Perkin Elmer) equipped with an intercooler and a PC for data analysis and storage.

Samples for calorimetry were measured accurately and sealed in stainless steel pans (50 μl). Identical pans containing buffer were placed in the reference pan holder of the calorimeter.

Slow heating rates (10 C°/min or slower) were used for determining temperatures of maximal heat absorption (T_m), but fast heating rates (up to 20 C°/min) were used for enthalpy quantitations. A cooling scan rate of 50 C°/min was used for all measurements. Baselines were obtained by carrying out a second reheat of the samples under identical intrumental conditions.

The apparent enthalpy of irreversible transition (ΔH_{app}) was determined by calculating the area of the thermogram after subtracting the baseline from the initial scan. Slope adjustments on the subtracted curves were made when necessary. Most experiments were conducted at least three times and reproducibility was within 15% in the ΔH_{app} measurements.

Analytical procedures

SDS-polyacrylamide gel electrophoresis was carried out on all purified proteins according to the procedure of Laemmli [16] and staining was performed with Coomassie brilliant blue or 'Stains-all' to detect the presence of any contaminating glycophorin [17]. Erythrocyte membrane proteins on electrophoresis gels were designated according to Fairbanks et al. [1]. Protein determinations were carried out by the modified Lowry assay of Markwell et al. [18] using BSA as standard. Absorbance at 280 nm was also used for measuring protein concentration in detergent solutions ($\epsilon = 2.50 \cdot 10^5$ cm^{-1} M^{-1}, $M_r \approx 97000$ for band 3 and $\epsilon = 2.63 \cdot 10^5$ cm^{-1} M^{-1}, $M_r \approx 55000$ for TMD). Lipids were quantitated by the procedure of Rouser et al. [19].

Results

A single protein band corresponding to band 3 in erythrocyte membranes was observed in all detergent micelle preparations when analysed by electrophoresis and thus the protein preparations were judged to be > 95% pure. Additionally, no contaminating glycophorin bands were observed in any of the band 3 detergent purifications as judged from the 'Stains-all' gel. The residual endogenous phospholipids in band 3 preparations were undetectable (less than 1 mol of phospholipid per mol of band 3) although this does not preclude the presence of residual unphosphorylated erythrocyte lipids [20]. Similar results were obtained with TMD preparations.

Ghost membranes

Several endothermic transitions were observed by calorimetry for ghost membranes (Fig. 1). When ghost

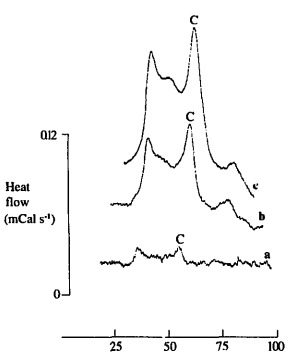

Fig. 1. Thermograms showing the thermal denaturation of the C transition in erythrocyte ghost membranes suspended in 310 mosmolar phosphate (pH 7.4), as a function of heating rate. Heating rates are 1 C°/min (a), 5 C°/min (b) and 20 C°/min (c). Protein content for each sample was 1 mg. Traces have been displaced vertically for clarity.

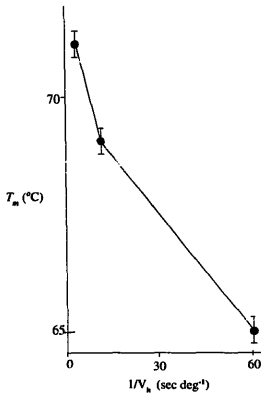

Fig. 2. The variation of temperature (T_m) of the C transition of erythrocyte ghost membranes as a function of the heating rate ($1/V_h$) of the membranes (1 mg) suspended in 310 mosmolar phosphate (pH 7.4). T_m was determined three times at each heating rate.

membranes were reheated in the calorimeter after the first heating scan, no endotherms were observed under any of the experimental conditions investigated. The thermal unfolding of all proteins appeared to follow irreversible denaturation under the conditions of the DSC determinations. Some samples containing dithiothreitol were also scanned which eliminated any possible oxidation of -SH groups during heating; no differences arose from this modification and the DSC scans never showed reversible transitions on reheating.

As shown in Fig. 1a, the T_m values for the various exothermic transitions of erythrocytes are dependent on calorimetric scanning rate; increased scanning rate resulted in increased T_m values for the C transition from 60°C to 71°C for ghost membranes in phosphate buffer, as illustrated in Fig. 2. Other workers have reported a similar range of T_m values for the C transition of erythrocyte ghosts [10] but the dependence of T_m on the heating rate has not been reported. Similar intrumental conditions for T_m measurements were used in all subsequent experiments in order that direct comparisons of thermal stabilities could be made between band 3 in ghosts and band 3 and TMD in various detergents. The scanning rate chosen for subsequent work was therefore 20 deg min^{-1}.

Band 3 detergent micelles

When detergent buffers were heated in the absence of protein, no thermal transition was observed suggesting that there were no contributions from detergent phase separation under the experimental conditions used in the present study.

The DSC thermograms for band 3-detergent micelles were generally monophasic and showed only one well defined irreversible endothermic transition (the C transition) under the experimental conditions used. However, additional endothermic transitions were observed in samples pre-incubated with DIDS. Of the two transitions, the lower transition occurred at T_m values which were very close to those observed in the absence of DIDS, whilst the higher transition occurred at T_m values which were at a temperature 5–15 degrees higher relative to the lower unchanged value for the C transition (Fig. 3). Other workers have also reported similar findings with band 3 detergent micelles [8].

Similar T_m values (50–52°C) were observed for the C transition band 3 in $C_{10}E_8$, $C_{14}E_8$ and $C_{16}E_8$ detergent micelles (Fig. 4). A somewhat higher T_m value was observed relative to the other detergents studied, namely $C_{12}E_9$, $C_{12}E_8$ and Triton X-100, for which a single irreversible endothermic transition was seen with T_m of approximately 61°C for these particular detergents in the absence of DIDS.

There was no direct correlation between the detergent type and ΔH_{app} values for the band 3 detergent micelles under the experimental conditions used (Table I). The ΔH_{app} values for the endothermic transition of band 3 in detergents were always less (a decrease of $\approx$ 120 kcal mol^{-1}) than those reported for native membranes, which gave a value of 360 kcal

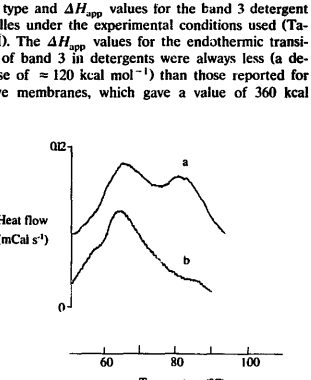

Fig. 3. The heating thermogram (20 C°/min) of band 3 (0.6 mg protein) in 0.1% $C_{12}E_8$ detergent micelles in the presence (a) and absence (b) of 2 mM H_2DIDS in 50 mM NaCl, 10 mM Tris-HCl (pH 7.4). Baseline subtractions were performed as described in text.

TABLE I

The effect of DIDS on the enthalpy of denaturation (ΔH_{app}) of various band 3 preparations in detergent micelles and in various erythrocyte ghost membrane preparations

Preparation	ΔH_{app} without DIDS (kcal mol^{-1})	Fractional increase in ΔH_{app} with DIDS [a]
Band 3 in $C_{10}E_8$	228	n.d.
Band 3 in $C_{12}E_8$	216	0.08
Band 3 in $C_{14}E_8$	250	n.d.
Band 3 in $C_{16}E_8$	248	n.d.
TMD in $C_{12}E_8$	212	0.17
Band 3 in $C_{12}E_9$	241	0.21
Band 3 in TX-100	295	0.05
Proteolysed IOV's	191	0.14
Ghost membranes	360	0.05

[a] The fractional increase in ΔH_{app} of the same preparations in the presence of DIDS was measured by calculating the ratio of the difference ΔH_{app} (+DIDS)–ΔH_{app} (no DIDS) relative to the value for ΔH_{app} (no DIDS). n.d., values not determined.

mol^{-1}. Since two endothermic transitions were observed in the presence of DIDS (see Fig. 3), the ΔH_{app} was calculated by measuring the area under each peak, which essentially represents the unfolding of a single protein with different enthalpy values for each of the two populations of band 3 which unfold.

Enthalpy measurements from thermograms of band 3-detergent micelles have not been previously reported. The band 3-Triton X-100 detergent micelles gave large values of ΔH_{app} (296 kcal mol^{-1}) which were very close to the value of 360 kcal mol^{-1} obtained for native membranes (Table I). In the presence of DIDS, the values of ΔH_{app} were increased by approx. 5% for the same sample. The increase in the length of oxyethylene chain of the detergent by one unit ($C_{12}E_9$ relative to $C_{12}E_8$) resulted in a 12% increase in ΔH_{app} value (Table I).

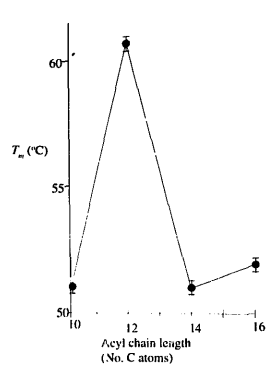

Fig. 4. The effect of varying acyl chain lengths of polyoxyethylene type detergents (C_nE_8, n = 10–16) on the C transition of purified band 3 (0.60 mg of protein). The T_m values for Triton X-100 and $C_{12}E_9$ lie on the point where n = 12.

TMD in $C_{12}E_8$ micelles

The TMD $C_{12}E_8$ micelles showed a single reversible endothermic transition with T_m values lowered by 20 C° relative to the C transition in native membranes (the T_m value obtained for the TMD in $C_{12}E_8$ micelles was 48°C). The denaturation temperature obtained for detergent solubilised TMD was reduced when compared with detergent solubilised intact band 3. However, the ΔH_{app} values for TMD $C_{12}E_8$ micelles (213 kcal mol^{-1}) compared well with values obtained for intact band 3 (216 kcal mol^{-1}) in the same detergents. The fractional increase in ΔH_{app} value for the TMD $C_{12}E_8$ micelles in the presence of DIDS was approximately 2-fold greater relative to the value of the intact band 3 $C_{12}E_8$ micelles under the same conditions (Table I).

Discussion

Band 3 in native membranes

The irreversibilty of the thermal transitions observed with erythrocyte ghost membranes confirm the denaturation of the membrane proteins and are shown in Fig. 1. These thermograms are obtained from the subtraction of a reheat scan from the first heating scan. Previous studies have identified the reversible unfolding of the cytoplasmic domain (B_2 transition) in native membranes and in erythrocyte ghost membranes and in solution [11]. However, in the present study it was not possible to identify the reversible folding transition of the cytoplasmic domain in membranes because all thermograms were obtained after subtraction of a reheat scan from the initial scan.

At slower heating rates, the exothermic transition due to protein aggregation is subtracted to a greater extent because both aggregation and denaturation occur at the same time resulting in decreased observed ΔH_{app} values. From Figs. 1a and 1b it can be seen that the different heating rates used to obtain thermograms cause a change in the protein denaturation transition temperature, with a simultaneous change in the shapes of the thermograms. Two causes are likely to be responsible for these effects; namely isothermal denaturation of the protein at low-heating rates and overheating of the protein at high heating rates, both resulting in similar changes in the T_m (T_m decreased with decreasing V_h, Fig. 2) and in the shape of the

curve. Therefore, the temperature and enthalpy of protein denaturation is distorted. It is not surprising that membrane proteins in bilayers show thermal denaturation characteristics (T_m and ΔH_{app}) remarkably similar to those determined for concentrated solutions of globular proteins, since similar secondary structures (e.g. α-helices) exist in both types of proteins. Previous workers have suggested that the irreversibility of these denaturations in membrane proteins was due to geometrical constraints imposed by the planar bilayer and not because of aggregation or other secondary events due to protein structural changes [13]. The results presented here do not support this view as the results for band 3 in this study were obtained in detergent micelles and not in bilayers.

Band 3 in detergent micelles

Although no systematic thermal denaturation studies are available for other membrane proteins in various detergents for direct comparison with the current results, earlier studies have reported large changes in the secondary and quaternary structures for the 38 kDa DIDS binding fragment of proteolytically cleaved band 3 in $C_{12}E_9$ upon heating through the C transition [21]. A large change in the emission fluorescence at 62°C in DIDS-labelled band 3 in Triton X-100 was also identified as the thermally induced denaturation of the C transition of the band 3 [6]. The DSC thermograms of band 3 in detergent micelles (Fig. 3) show that the anion transporting domain denatures irreversibly. The reversible band 3 N-terminus transitions, which were relatively much smaller and occur within the region of the main C transition, cannot be identified under the experimental conditions of this study.

The lower T_m of band 3 in detergent micelles compared with the native membrane (T_m decreased by 6 C°) suggests that the native membrane retards the band 3 denaturation process. The band 3 detergent micelles gave broader denaturation transitions compared with native membranes (peak widths at half-heights of 15 C° for the detergent micelles compared with 8 C° in native membranes) indicating a reduction in the cooperativity of the denaturation process (reduced band 3 domain organisation in detergent micelles). Similarly, previous authors have also found marked thermal destabilisation of the Ca^{2+}-ATPase in deoxycholate solubilised preparations when compared with the native sarcoplasmic reticulum membranes [30].

The present results show that the thermostability of band 3 in detergent micelles was significantly decreased (as indicated from the lower values of T_m, see Fig. 4) in the presence of detergent compared with native membranes, but that there was no significant correlation between the length of the detergent acyl chain and the maximal thermal denaturation temperature (T_m). C_{12}-detergents and Triton X-100 were found to give the highest values for T_m indicating that band 3 is more thermally stable in these detergents. These results were surprising since detergents have been envisaged as being associated with membrane proteins in a similar manner to lipids; that is less constrained than in the native membrane but with the acyl chains associated with the protein hydrophobic domain and the head groups occupying sites on either side of the protein hydrophilic surface [22]. Increasing or decreasing the acyl chain length would then be expected to inhibit the ability of the detergent headgroups to occupy these sites. Additional evidence suggests that a large difference in the putative size of the protein hydrophobic core (4.2 nm for band 3 [23]) and the hydrophobic moiety of the detergent (1.16 nm for $C_{12}E_8$ [24]) are not optimally matched but are probably better for C_{12}-detergents and Triton X-100. An alternative model of detergent membrane protein interaction which could account for these observations shows that the optimal matching between the protein and detergent molecules can only be obtained if the detergent molecules form a prolate or monolayer ring around the protein hydrophobic core; the detergent acyl chains would be orientated perpendicularly to the hydrophobic core unlike the lipids in an extended bilayer [25]. Although Triton X-100 is structurally different to the polyoxyethylene type detergents it is still classed as an intermediate size detergent (micelle aggregation size similar to the C_{12} poloxyethylene type detergents) which probably accounts for its similar effect on band 3 stability as the C_{12} detergents. Evidence from previous studies on the Ca^{2+}-ATPase also shows that detergents of intermediate size (C_{12} and Triton X-100) are optimal for retention of activity of the detergent solubilised membrane proteins [31].

Under the conditions of high protein concentrations used in the present investigations we propose that the protein will be oligomerised. However, we have not observed any significant changes in the T_m values of highly oligomerised band 3 samples. In subsequent transport studies, identical band 3 detergent solubilised preparations, that were reconstituted into phospholipid vesicles, were found to be fully active as shown by > 90% DIDS inhibited sulphate transport.

Two band 3 populations were observed on DIDS binding: DIDS sensitive as shown by the increased thermostability of the C transition and DIDS insensitive. A possible reason for the existence of these two band 3 populations could be that the existence of different oligomers of band 3 in detergent micelles have different binding affinities for DIDS. It is possible that higher oligomers of band 3 exist in a conformation that restricts the access of DIDS to the binding site. We are at present investigating DIDS binding to different oligomers of band 3 using analytical centrifugation and non-denaturing gel electrophoreisis. The C transi-

tion of detergent solubilised band 3, in the absence of DIDS, does not appear to be significantly effected by the oligomeric state of the protein. Band 3 is believed to exist in a monomer-dimer-tetetramer equilibrium in Triton X-100 [14] but only one transition was observed in the non-DIDS containing samples by DSC (Fig. 3b). Thus, band 3 appears to be behaving like a globular protein, where oligomeric state has been found not to significantly affect denaturation temperatures [13].

The finding that similar values of enthalpy were observed for the C transition of intact band 3 and the TMD in $C_{12}E_8$ micelles suggests that there is little significant change in the secondary structure of the transmembrane region upon the removal of the N-terminal region of the protein. However, the difference in the T_m values obtained for the two preparations (the TMD in $C_{12}E_8$ micelles gave a T_m value reduced by approximately 12 C° when compared with intact band 3 in the same detergent) does reflect a cooperation between the two domains of band 3 in the maintenance of the TMD conformation. Given that little difference is observed between the enthalpy values for intact band 3 and the TMD, it would appear that this cooperativity involves interactions at the tertiary rather than at the secondary structural level.

The failure to observe any correlation between the T_m values with ΔH_{app} in Table I suggests that detergents may alter the intrahydrophobic contacts, but do not suppress H-bonding capabilities within the transmembrane region of band 3 (Triton X-100 and $C_{12}E_8$ give similar T_m values but different enthalpies, see Fig. 2 and Table I). Similar thermodynamic results were obtained with lysozyme in different alkyl chain length alcohol-water mixtures [29].

The studies reported here give an indication of the relative stability of different detergent membrane proteins. Such information would be important in isolation, purification and reconstitution methods for membrane proteins and may thus help in defining optimal condition for crystallisation of membrane proteins.

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