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Self-association of Band 3, the human erythrocyte anion exchanger, in detergent solution

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Abstract

Dimeric Band 3 purified in n-dodecyl octaethyleneglycol ($C_{12}E_8$) underwent an irreversible, temperature-dependent association, resulting in a complex with a Stokes radius slightly larger than a native tetramer, before forming a higher molecular weight aggregate. Self-association occurred with a half-time of about 1 h at 37°C but did not occur at 0°C after several days. No change in the secondary structure of Band 3, as observed by circular dichroism, occurred during the association process. However, self-association of Band 3 was accompanied by loss of the stilbene disulfonate inhibitor binding site. No association or loss of inhibitor binding occurred with the dimeric membrane domain under similar incubation conditions. The membrane domain dimer was also stable over a wide range of pH (5.5–9.5) and buffer conditions, while Band 3 aggregated below pH 6.5. Inhibitors of anion transport, which stabilize the membrane domain, slowed the association. Band 3, depleted of phospholipids by extensive washing of resin-bound protein with detergent or, incubated with excess detergent, was more prone to aggregation. The membrane domain also showed some aggregation when depleted of lipids. Preparations could be stabilized by adding dimyristoylphosphatidylcholine (DMPC) prior to the 37°C incubation. The effect of inhibitors and DMPC was additive, with a combination of 1 mM 4,4′-dinitrostilbene-2,2′-disulfonate (DNDS) and 1:1 (wt/wt) DMPC:Band 3 stabilizing 90% of the protein to a 24-h incubation at 37°C. The results suggest that self-association of Band 3 dimers is promoted by the cytoplasmic domain but results in alterations to the membrane domain involving the loss of essential phospholipids. Addition of phospholipid or inhibitors to Band 3 results in a stable preparation of the intact protein that may be suitable for crystallization studies.

Keywords: Anion exchanger; Band 3; Crystallization; Detergent; Inhibitor; Oligomer

Abbreviations: BADS, 4-benzamido-4′-aminostilbene-2,2′-disulfonate; $C_{12}E_8$, octaethylene glycol mono-n-dodecyl ether; DiBa, bis-(1,3-dibutylbarbituric acid) pentamethine oxonol; DIDS, 4,4′-di-isothiocyanostilbene-2,2′-disulfonate; DMPC, dimyristoylphosphatidylcholine; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonate; DTT, dithiolthreitol; EDTA, ethylenediaminetetraacetic acid; EM, eosin-5-maleimide; H_2DIDS, 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate; PEG, polyethylene glycol; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate

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1. Introduction

Human Band 3 is a 911 amino acid glycoprotein [1,2] that makes up about 25% by weight of the erythrocyte membrane protein. Band 3 consists of two structural domains, each having distinct functions. The cytosolic amino-terminal domain (M_r = 40 000) possesses binding sites for ankyrin, hemoglobin and glycolytic enzymes (aldolase, phosphofructokinase and glyceraldehyde-3-phosphate dehydrogenase) [3]. The carboxyl-terminal membrane domain of the protein (M_r = 55 000) catalyzes the electroneutral one for one exchange of chloride and bicarbonate [3–6]. Band 3 is a mixture of dimers and tetramers in the membrane and in detergent solutions [7,8]. The protein has been found to aggregate when diluted in detergent solutions and tube stabilized by inhibitors and lipids [9]. Additionally, evidence has suggested that hexamers of Band 3 can form in some detergents [10,11]. It is likely that the base-unit of anion transport in the red blood cell is a dimer of Band 3 [7]. Tetramers appear to provide a site for ankyrin to attach to the membrane [12] and higher oliogmers serve other roles, such as acting as senescence antigens [13,14]. The molecular details of these various Band 3 functions remain elusive due to the lack of atomic resolution structural information.

Few high-resolution structures of membrane proteins have been determined due to the difficulties in obtaining crystals suitable for diffraction studies. Very little is known about the properties of membrane proteins in detergent solutions and the experimental conditions required to maintain protein stability and to promote crystallization. Band 3 serves as a useful model polytopic membrane protein since it is readily purified and a great deal is known about its structure and function [4,6,15–18]. Band 3 [10] or its membrane domain [11,19] can be induced to form 2-dimensional crystalline arrays when reconstituted into phospholipid in the presence of $MgCl_2$. To obtain 2- or 3-dimensional crystals that diffract to high resolution, a stable, homogenous preparation of protein is required. The present study was undertaken to further define the conditions which result in the formation of 2-dimensional crystalline arrays of Band 3 suitable for high resolution analysis. In particular, we examined the stability of Band 3 dimers as a function of time and temperature. The influence of lipids, anion transport inhibitors, pH, ions and protein precipitants was also studied. We have found that the isolated membrane domain is very stable, while the intact protein undergoes self-association with a resulting loss of functionality. A stable preparation of Band 3 could be produced in the presence of inhibitors and phospholipids.

2. Materials and methods

2.1. Materials

Blood was supplied by the Canadian Red Cross Society. BADS was synthesized as described [19]. Materials were purchased from the following suppliers: $C_{12}E_8$, Nikko Chemical Co., Tokyo, Japan; SEC 4000 HPLC columns, Beckman, Canada or Supelco; proteins used as Stokes radii standards, Pharmacia LKB Biotechnology Inc.; DNDS, Aldrich; DIDS, and PEG200 Sigma Chemical Co.; EM, DiBa and H_2DIDS, Molecular Probes; DMPC and cholesterol, Avanti Polar-Lipids, Inc.

2.2. Isolation of Band 3 and the membrane domain

Band 3 was prepared by two different procedures as previously described [20]. All steps were carried out at 0–4°C unless stated otherwise. Briefly, red cells were washed, and ghosts were prepared by osmotic hemolysis with 1 mM PMSF to inhibit proteolysis. The membranes were stripped of peripheral proteins with ice-cold 2 mM EDTA (pH 12) then solubilized with 4 volumes of 1% $C_{12}E_8$. Alternatively, ghost membranes depleted of Band 6 were extracted directly with 1 volume of 1% $C_{12}E_8$ in 5 mM sodium phosphate (pH 8.0). Insoluble material was removed by centrifugation at 100 000 × g for 1 h. The supernatant was applied to a column of aminoethyl-Sepharose 4B, and eluted with a 0 to 0.25 M linear gradient of NaCl in 0.1% $C_{12}E_8$, 5 mM sodium phosphate, pH 8.0. The 55 kDa membrane domain of Band 3 was prepared by trypsin treatment of red cell ghosts, followed by alkali stripping, solubilization in $C_{12}E_8$ and DEAE Sepharose chromatography with elution by a 0 to 0.5 M linear NaCl gradient [20].

Band 3 labeled with H_2DIDS (or DIDS) was prepared by reacting red cells at a 25% hematocrit in 150 mM NaCl, 5 mM sodium phosphate (pH 7.4) with 10 μM H_2DIDS (or DIDS) for 1 h at 37°C. The second isothiocyanate group on H_2DIDS was then cross-linked to Band 3 by incubation of cells in 0.1 M sodium bicarbonate (pH 9.5), for 1 h at 37°C [21]. H_2DIDS-labeled Band 3 or membrane domain was then prepared as above. Band 3 was labeled with EM by reacting red cells at a 10–25% hematocrit with 100–300 μM EM at 37°C for 1–2 h in the dark. This procedure results in greater than 90% inhibition of anion exchange [22]. Treatment with the non-covalent inhibitors DNDS and DiBa was done by dissolving the inhibitors in a suitable buffer then adding them directly to the purified protein solution.

2.3. Reconstitution

Samples (100 μl) of Band 3 or the membrane domain (1 mg protein/ml) in 0.1% $C_{12}E_8$, 100–250 mM NaCl, 5 mM sodium phosphate (pH 8.0) were mixed with an equal weight of DMPC which had been dried under a nitrogen stream. The samples were dialyzed against 50 ml of dialysis buffer at 27°C for various times up to one month as described [11]. The usual dialysis buffer consisted of 100 mM NaCl, 5 mM sodium phosphate (pH 8.0) containing 0.5 mM sodium azide, 0.5 mM dithiothreitol, 10 mM $MgCl_2$ and 10% (w/v) PEG 200. The amount of detergent remaining in the sample was measured using [^{14}C]$C_{12}E_8$.

2.4. Size exclusion high performance liquid chromatography (SE-HPLC)

Chromatography was performed using a 0.75×30 cm or 0.78×30 cm SEC 4000 column [7]. A Spectra-Physics SP8800 HPLC pump was used, at a flow rate of 0.5 ml/min. Typically, 5–20 μl of purified Band 3 (1 mg protein/ml) was injected onto the column. Protein elution was monitored at 215 or 280 nm using a Spectroflow 757 Flowthrough Absorbance Detector (ABI Analytical Co.). The elution buffer contained 0.1% $C_{12}E_8$, 100 mM NaCl in 5 mM sodium phosphate (pH 7.0). The column was calibrated with suitable protein standards which do not bind detergent [23]. Chromatograms were obtained using a Kratos Spectrophotometer and the absorbance changes recorded on a Macintosh computer using MacIntegrator software (Rainin Instruments). The proportion of dimer remaining was determined from the ratio of integrated area under the dimer peak after 37°C incubation to the starting area under the dimer peak prior to 37°C incubation. Preparative separation of dimers and tetramers was accomplished using the same column, applying 0.5 ml samples (1–3 mg protein/ml) and manually collecting the enriched fractions.

2.5. BADS binding assay

The binding of BADS to Band 3 dimers or tetramers was measured by fluorescence enhancement in a Spex Fluorolog fluorimeter [20]. Wavelength settings were 340 nm for excitation and 450 nm for emission. For each determination, 2 ml of filtered 28.5 mM sodium citrate buffer (pH 7.4), was dispensed into a 3 ml fluorescence cell, and 100–250 μl of Band 3 or membrane domain (0.2–1 mg protein/ml) in 0.1% $C_{12}E_8$, 100 mM NaCl, 5 mM sodium phosphate (pH 8.0), was added. In some cases, HPLC fractions (0.1–0.2 mg/ml) were assayed directly. Band 3 samples were titrated with concentrated BADS to a final concentration of 12 μM. Fluorescence was corrected for dilution, self-quenching of the probe, and the background fluorescence of sample and probe.

2.6. Circular dichroism

Band 3 or membrane domain (0.5–1 mg protein/ml) in 0.1% $C_{12}E_8$ solution was diluted 10-fold into distilled water and spectra were recorded at room temperature in a quartz cell with a 0.1-cm path-length, using a Jasco J-720 spectropolarimeter. HPLC fractions containing purified dimers and tetramers (0.1–0.2 mg/ml) were analyzed without dilution. Spectra for buffer alone were subtracted from each spectrum. Protein determinations were performed [24] and corrected to molar concentration. Mean residue ellipticities were calculated using the mean residue weight of Band 3, 112, based on the sequence of human Band 3 [1]. Secondary structures were estimated by best-fit over the whole spectrum, using Jasco software and reference spectra [25].

2.7. Analytical techniques

Polyacrylamide gel electrophoresis was performed according to Laemmli [26]. Protein assays were performed using bovine serum albumin as standard [24]. Phospholipids were extracted by the method of Bligh and Dyer [27]. Samples were digested by heating them with sulfuric acid in the presence of hydrogen peroxide. Phospholipid assays were performed by standard methodology [28].

3. Results

3.1. Time and temperature dependence of association

On size exclusion HPLC, purified Band 3, prepared from alkali-stripped membranes, was resolved into a major peak corresponding to a dimer ($R_s = 78$ Å) and a shoulder corresponding to a tetramer ($R_s =$ 100 Å) (Fig. 1A). The membrane domain eluted as a single dimeric peak (Rs = 63 Å) (Fig. 1B) [7]. The stability of the dimeric form of the protein in detergent solution was studied by incubating the purified protein (1–2 mg protein/ml) in 0.1% $C_{12}E_8$, 100 mM NaCl, 5 mM sodium phosphate (pH 8.0) at various temperatures (0–37°C). After incubation at 37°C the amount of Band 3 eluting as a dimer decreased dramatically with the concomitant appearance of a peak with a Stokes radius greater than 100 Å (Fig. 1A, dotted line). This form of the protein appears slightly larger than the native tetramer. This association in $C_{12}E_8$ detergent solution was apparently irreversible, since returning the associated sample to 4°C did not result in dissociation of the complex to dimers.

The association of Band 3 was temperature-dependent. At 4°C there was very little association of Band 3 dimers (Fig. 2). Even after several days storage at 4°C Band 3 remained dimeric, while after a 7-hour incubation at 37°C only about 30% of Band 3 remained dimeric and at room temperature 75% remained dimeric (Fig. 2). The initial association of Band 3 at 37°C proceeded rapidly with a half-time of 1–2 h. Some differences in the rates of association were noted between experiments. For example, between different preparations of Band 3 the proportion of dimer remaining after prolonged incubations could vary by several percent (e.g., 15% dimer remaining after 24 h at 37°C versus 20% in a different preparation). However, triplicate incubation experiments within a single preparation yielded negligible differences in the rates of association, with the largest standard deviation being 1%. SDS gel electrophoresis of the samples showed no proteolysis of the protein

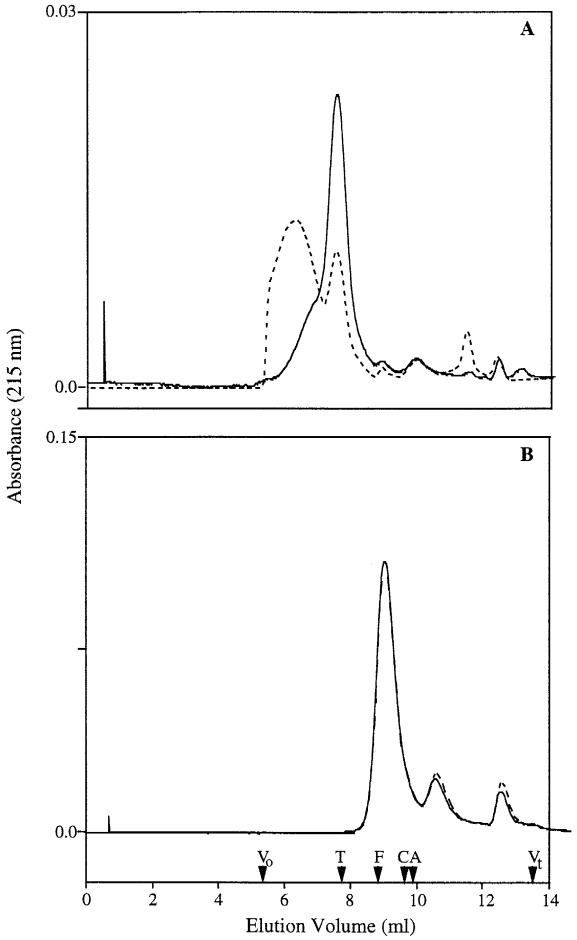

Fig. 1. Size exclusion HPLC chromatogram of intact Band 3 (A) and the 55K membrane domain (B) before (————) and after (---) incubation at 37°C for 1 h. Samples (2.5 μg of Band 3, 10 μg of membrane domain) were injected onto a 30×0.75 cm SEC4000 size exclusion column, run at room temperature in 0.1% $C_{12}E_8$, 0.1 M NaCl, 5 mM sodium phosphate (pH 7.0). Arrows on the bottom scale indicate elution positions for the standard proteins T, thyroglobulin (Rs = 86 Å), F, ferritin (63 Å), C, catalase (52 Å), and A, aldolase (46 Å). The void volume, V_o, was determined from the elution position of Blue Dextran 2000 (average molecular weight 2×10^6) and the total volume, V_t, was determined from the elution position of 2-mercaptoethanol. Absorbance was measured at 215 nm.

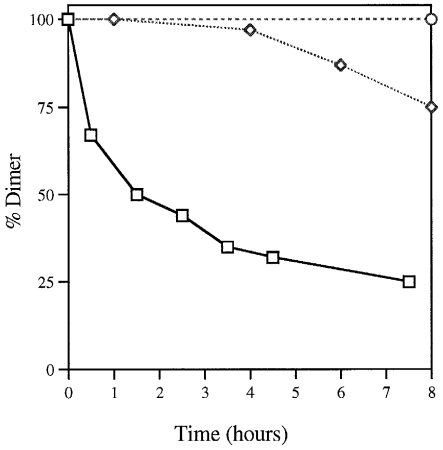

Fig. 2. Temperature and time dependence of association of Band 3 at; 4°C (circles); 22°C (diamonds), and 37°C (squares). Samples were incubated for the indicated length of time, then applied to the size exclusion HPLC column. Results are expressed as the percent of dimer remaining in the sample relative to initial conditions, as determined from the integrated area under the peak in the chromatogram.

occurred during the incubation at 37°C. The gels also showed that the associated samples did not remain aggregated after SDS electrophoresis; although more extensive incubations (e.g., 24 h) generated some aggregated samples which were excluded from SDS gels (data not shown). Incubations at 37°C of Band 3 prepared by direct extraction showed the same temperature-dependent association as did Band 3 prepared by alkali stripping. Band 3 prepared by direct extraction from membranes without alkali stripping is enriched in the dimeric form of the protein since tetrameric Band 3 remains anchored to the cytoskeleton [7]. The membrane domain of Band 3 resisted temperature-induced association at 37°C (Fig. 3). Remarkably, in some preparations even after 7 days at 37°C the purified membrane domain remained solely dimeric with little indication of association. The self-association of Band 3 dimers therefore was greatly enhanced by the presence of the cytosolic domain.

3.2. Secondary structure and inhibitor binding

Circular dichroism studies showed that no change had occurred in the secondary structure of Band 3 or the membrane domain after a 2-h incubation at 37°C (data not shown). The effect of incubation at 37°C on the ability of Band 3 and the membrane domain to bind BADS, an inhibitor of anion exchange, was tested. Associated Band 3 was separated from dimers by preparative size exclusion HPLC. It was found that a substantial proportion of the associated Band 3 had lost its ability to bind the inhibitor with normal affinity (1 μM) while the dimer retained its functionality (Fig. 4A). In contrast, incubation of the membrane domain at 37°C did not result in any loss in BADS binding (Fig. 4B). These results indicate that purified Band 3 is irreversibly altered by incubation at 37°C while the membrane domain is stable.

Stilbene disulfonate inhibitors of anion transport have been noted to stabilize the membrane domain of Band 3 [29,30]. To examine whether various Band 3 inhibitors could stabilize the protein against temperature-induced association, we tested their effects on Band 3 association. The stilbene disulfonates DNDS, DIDS and H_2DIDS all slowed the aggregation of Band 3 at 37°C. Addition of 1 mM DNDS prior to a 24-h incubation at 37°C resulted in 62% of Band 3 remaining dimeric, compared to less than 20% in controls (Fig. 5). Prelabeling the protein with H_2DIDS or DIDS was also effective and stabilized the protein. After a 24-h incubation at 37°C, nearly 80% of H_2DIDS-prelabeled Band 3 remained dimeric. We also examined the effects of inhibitors other than the stilbenes, such as EM and an oxonol dye. EM and the oxonol dye DiBa bind to sites overlapping but separate from that of the stilbene disulfonates [31,32].

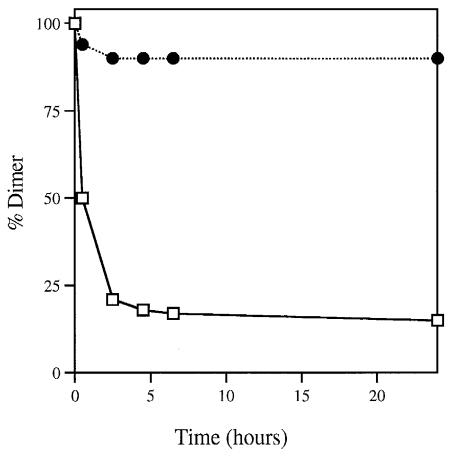

Fig. 3. Time dependence of the association of Band 3 (squares) or the 55K membrane domain (circles) at 37°C. Samples were incubated as detailed in Fig. 2.

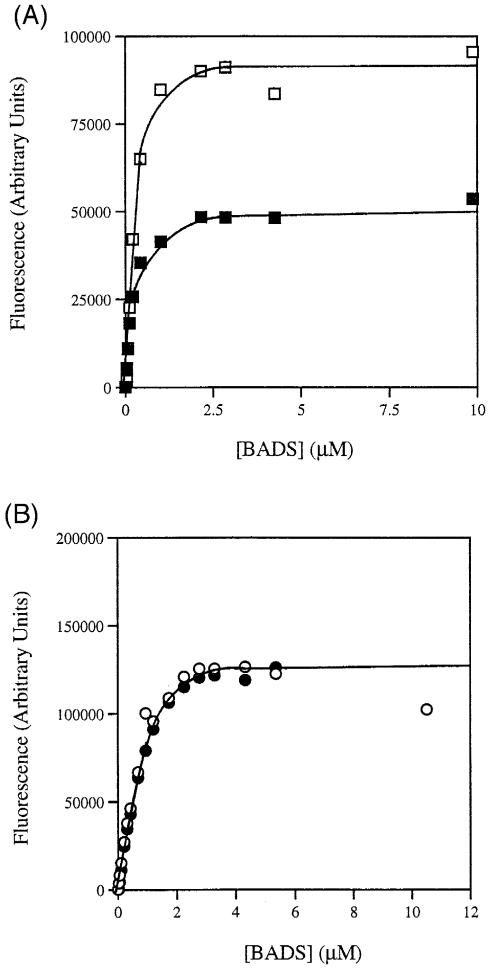

Fig. 4. Binding of the anion exchange inhibitor, BADS, to Band 3 (A) or the membrane domain (B) and the effect of incubation at 37°C for 1.5 h. The Band 3 sample was incubated at 37°C for 1.5 h and then the tetramer (filled squares) and dimer (open) were separated and collected by HPLC. BADS binding was then performed on these isolated fractions. The membrane domain was incubated for 1.5 h at 37°C and BADS binding performed before (open circles) and after (closed) incubation. The excitation wavelength was 340 nm and the emission wavelength was 450 nm. Purified Band 3 or membrane domain in 0.1% $C_{12}E_8$ 0.1 M NaCl, 5 mM sodium phosphate (pH 8.0) was diluted into 2 ml of 28.5 mM sodium citrate (pH 7.0) at 24°C, to a final protein concentration of 50 μg/ml.

Prelabeling with EM was effective in slowing Band 3 aggregation; 70% remained dimeric after a 24-h incubation. The oxonol dye DiBa, the most potent inhibitor of Band 3 known [32], was also effective in stabilizing the protein. At a concentration of 10 μM approximately 50% of Band 3 remained dimeric after 24 h, compared to less than 15% in controls. Thus, all of the inhibitors tested provided significant stabilization of Band 3.

3.3. Effect of lipids

To see if preparations of Band 3 could be stabilized by phospholipid, DMPC was added to the samples prior to incubation at 37°C. The addition of DMPC markedly slowed the aggregation of Band 3 at 37°C (Fig. 5). After a 24-h incubation at 37°C in the presence of 1:1 (wt/wt) DMPC:protein about 50% remained dimeric compared with less than 20% in a control Band 3 preparation. The level of protection was dependent upon the amount of lipid added. A ratio of around 1:1 lipid:protein (w/w) provided the best stabilization. Ratios much smaller or larger were not as effective: a ratio of 0.4:1 DMPC:protein resulted in only 35% remaining dimeric after a 24-h incubation at 37°C while at a ratio of 16:1 the association was actually accelerated, with the amount of dimer remaining after incubation being only 10% (data not shown). This latter effect may be due to sequestering of detergent into phospholipid vesicles. The effect of DMPC was additive with that of the inhibitors. A combination of 1 mM DNDS and an equal DMPC to protein ratio (w/w) resulted in excel-

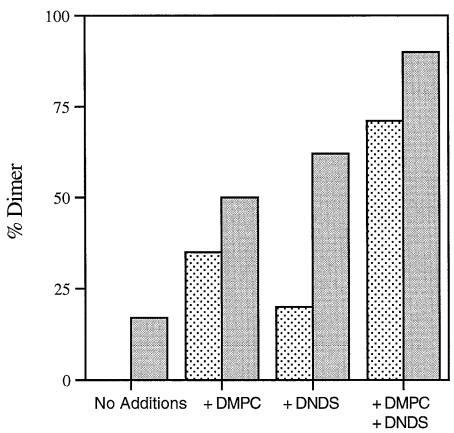

Fig. 5. Stabilization of Band 3 by phospholipids and inhibitors of anion transport. Control (solid bars) and phospholipid-depleted (dotted bars) Band 3 were prepared as described in the text. Incubations were 24 h at 37°C with or without the indicated addition of 1 mM DNDS, a 1:1 weight of DMPC (lipid:protein), or both. The control experiment with phospholipid-depleted Band 3 had no dimer after only 5 h at 37°C.

lent protection of Band 3, with 90% remaining dimeric after a 24-h incubation at 37°C (Fig. 5).

The protective effect of adding DMPC led us to examine whether the rate of Band 3 association was related to the lipid content of the preparation. Band 3 purified by direct extraction of membranes contained 8.9 ± 0.9 phospholipids per monomer, while the same preparation, washed with 30 column volumes while bound to amino-ethyl Sepharose, had only 2.5 ± 0.3 phospholipids per Band 3 monomer. Phospholipid analyses on each of the column fractions showed that the majority of the phospholipids eluted with the column flow-through. The level of phospholipid then rapidly decreased to a stable background until a much smaller peak, coincident with the Band 3 elution peak, was detected. Band 3 purified with extensive column washing was predominantly dimeric, but contained some high molecular weight aggregate, as assayed by HPLC size exclusion chromatography. The lipid-depleted preparation aggregated more rapidly than control preparations (Fig. 5). After 5 h at 37°C, all of the lipid-depleted protein was associated; in contrast, controls retained about 25% dimer. Similar to controls, lipid-depleted Band 3 could be stabilized by addition of DNDS and DMPC, but the levels of recovery were only 20% and 35% respectively compared to 62% and 50% in controls after 24 h at 37°C (Fig. 5).

The protective effect of the inhibitors on the association state of Band 3 led us to examine their effect on the level of associated lipids. A single unit of blood was divided into thirds; one, a control preparation of Band 3, one, labeled with EM and one, labeled with H_2DIDS. The Band 3 used in these experiments was prepared by alkali stripping and the ratio of lipid:protein was in agreement with that determined by direct-extraction. Phospholipid determinations on the Band 3 elution peaks indicated that control Band 3 contained 9.2 ± 1.5 phospholipids per monomer. EM-labeled Band 3 had a ratio of 8.9 ± 0.7, and H_2DIDS-labeled Band 3 contained 11.3 ± 2.7 mol of phospholipid per mol of Band 3. The phospholipid content of the three preparations was very similar. It is likely, therefore, that the stabilizing effect of the inhibitors is not a result of an increased lipid content in the preparation.

The membrane domain of Band 3 could also be stripped of associated lipids by extensive column washing. An experiment similar to above on the membrane domain showed that the ratio of phospholipid to protein was 16.6 (2.7 for control preparations and 8.3 ± 1.3 for an extensively washed ± 30 column volumes) preparation. These numbers are consistent with a reported lipid depletion of the membrane domain [33]. In this study the mol ratio of lipids associated with the membrane domain ranged from 12–18 after washing with 2–8 column volumes. More extensive washing (30–100 column volumes) caused this number to decrease to around 8 [33]. Finally, this number too, could be reduced after washing with 100 column volumes to 5 phospholipids per membrane domain monomer. The higher number of lipids associated with control preparations of the membrane domain relative to intact Band 3 may be one of the reasons for the greater stability of this domain. The enhancement of association observed for lipid-depleted Band 3 was also found with the lipid-depleted membrane domain (data not shown). The degree of association was, however, considerably less that in either control or lipid-depleted preparations of intact Band 3. For example, after an 18-h incubation at 37°C, approximately 50% of the lipid-depleted membrane domain remained dimeric. In contrast, a similar incubation with control Band 3 resulted in only 20% remaining dimeric and in the case of lipid-depleted Band 3, all of the sample was aggregated after only 5 h at 37°C. Furthermore, the effect on the membrane domain of lipid-depletion could also be slowed by the addition of DMPC.

In another attempt to strip the protein of associated phospholipids, a 10-fold excess (1% (wt/vol)) of $C_{12}E_8$ was added to the Band 3 solution containing 0.1% free detergent prior to incubation. This treatment resulted in extremely rapid association and after 1 h at 37°C no dimer remained. Thus high concentrations of detergent promote Band 3 aggregation. This suggests that membrane proteins require an optimal concentration of detergent to maintain a native structure and that the use of high concentrations of detergent may be detrimental.

3.4. Reconstitution conditions

2-Dimensional crystalline arrays of the membrane domain of Band 3 formed in the presence of $MgCl_2$, PEG 200, and at pH 8 [11]. In order to rationalize the

requirements for these components in crystallization trials of intact Band 3 and the membrane domain, we investigated their effect on Band 3 structure. Aggregation of Band 3 in solution at 37°C could be promoted by the addition of $MgCl_2$. After incubation of a Band 3 sample at 37°C for 3 h, 49% of the control sample remained dimeric while 31% was dimeric in the presence of 10 mM $MgCl_2$ and only 9% in 100 mM $MgCl_2$ (data not shown). The effect of $MgCl_2$ was irreversible since the protein remained associated whether the HPLC column buffer contained $MgCl_2$ or not. $MgCl_2$ had little effect on DIDS-labeled Band 3 and did not promote aggregation of Band 3 at 0°C. 10% PEG had little effect on the aggregation of Band 3 at 0°C. The aggregation state of Band 3 was very sensitive to pH (Fig. 6). Band 3 dimers incubated at room temperature for 1 h remained dispersed at pH-values of 6.5 and above while the protein aggregated at lower pH values. In contrast the membrane domain was not sensitive to aggregation over the pH range 5.5–9.5 (Fig. 6).

Band 3 or the membrane domain could be reconstituted into lipid vesicles by detergent dialysis. The removal of $C_{12}E_8$ by dialysis was slow (Fig. 7) due to the low cmc of the detergent (0.056 mM). A significant amount of detergent was retained by lipid,

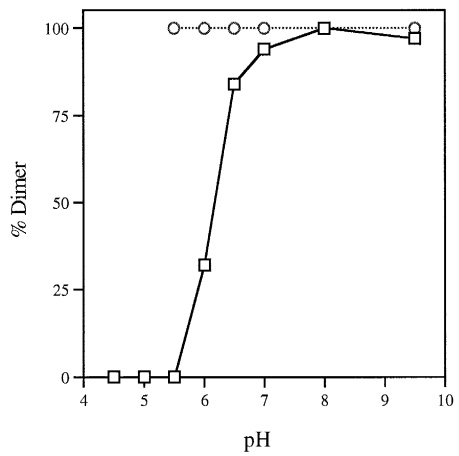

Fig. 6. Effect of pH on the percentage of Band 3 dimer (squares) or the 55K membrane domain (circles). Samples were diluted 1:1 with 130 mM sodium phosphate at the indicated pH, incubated at 24°C for 1 h, then chromatographed on HPLC. Results are expressed as the proportion of dimer remaining in the sample relative to control conditions (pH 8.0) as determined by integration of the area under the chromatogram.

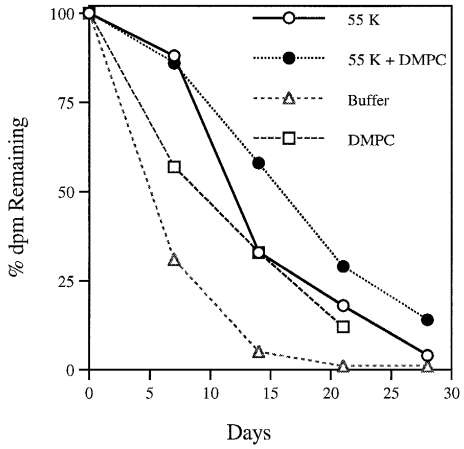

Fig. 7. Time course of detergent removal during reconstitution. Samples (200 μl) of purified membrane domain (1 mg protein/ml), DMPC (1 mg/ml), membrane domain plus DMPC and detergent alone were dialyzed against 50 ml of reconstitution buffer for 1 month. All samples initially containing 0.1% free [^{14}C]$C_{12}E_8$. Duplicate aliquots (5 μl) were taken for counting at timed intervals. The initial total detergent concentration for samples with the membrane domain was 3.7 mM, while for buffer and DMPC alone containing samples this number was 1.85 mM.

protein or a combination of both (Fig. 7). The level of total detergent in the presence of 1 mg/ml DMPC and 1 mg/ml of the membrane domain of Band 3 was reduced from 3.7 mM to 0.5 mM after 4 weeks dialysis. Re-solubilization of the reconstituted membrane domain with $C_{12}E_8$ and analysis by size exclusion HPLC revealed that the protein sample was not irreversibly aggregated and that the membrane domain could be dispersed in detergent (data not shown). In contrast, the reconstituted intact protein was highly aggregated after a week of dialysis under identical conditions. After 1 week at 27°C most Band 3 was also aggregated in detergent solution. In contrast, preliminary experiments with H_2DIDS-labeled Band 3 contained some dimer ($>30\%$) after a week of reconstitution at 27°C. This suggests that inhibitor-labeled protein may be one means of forming crystals of the intact protein.

4. Discussion

The results presented in this paper show that Band 3 undergoes a temperature-dependent association in the presence of $C_{12}E_8$. This change in structure is greatly enhanced by the cytosolic domain and is

likely due to the loss of essential phospholipids. Certain lipids are known to stabilize the protein; saturated lipids are more effective than unsaturated ones and the longer the chain length the more stabilization is provided [34]. Phosphatidylcholine and cholesterol stabilize the membrane domain best while negatively-charged lipids destabilize Band 3 [9,34]. In DMPC, the lipid used for reconstitution, the membrane domain denatures at 47°C [34]. At least three populations of lipids exist in reconstituted Band 3 preparations; those bound very tightly, those in a restricted annulus around the protein and those whose fluidity is affected by long range interactions of the protein [33,35,36]. The most important lipids required to maintain Band 3 dimeric structure are likely those which are the most tightly associated (i.e., those lipids which co-purify with the protein). The purified membrane domain of Band 3 can be washed extensively such that only 5–7 tightly-bound phospholipids per monomer co-purify with the protein [33]. These are mainly long-chain saturated fatty acids such as stearate [33]. Our standard preparations of the membrane domain possessed greater than double the number reported above, likely because the protein is typically washed with only 2 column volumes while resin-bound. The ratio of phospholipid to protein for intact Band 3 was half the ratio found for the membrane domain. Loss of these phospholipids likely results in denaturation of the protein, and exposure of hydrophobic patches that result in protein aggregation. The protective effect of DMPC suggests that the association of Band 3 dimers is due to exposure of hydrophobic sites caused by removal of essential phospholipids. Alternatively, the removal of phospholipids may result in protein denaturation and subsequent aggregation if the lipids are required to maintain protein structure. The importance of lipids in maintaining membrane protein structure has also been noted in other membrane proteins. The glucose transporter (GLUT1) also associates when stripped of lipids by harsh detergent treatment [37]. Similarly, the plant light-harvesting complex II (LHC II) when purified in a detergent which stripped lipids away was difficult to crystallize [38]. Finally, the high resolution crystal structure of cytochrome-*c* oxidase revealed the presence of 8 tightly complexed phospholipids [39].

Inhibitors of anion transport like H_2DIDS are known to stabilize the protein against denaturation [29,30]. The stilbenedisulfonates bind to a site facing the outside of the cell and lock the protein into the outward-facing conformation [5]. Self-association of Band 3 was decreased in the presence of stilbenedisulfonates. Other classes of inhibitors such as EM and DiBa were also effective. Like H_2DIDS, EM locks Band 3 into one conformation by binding to an exofacial site. The stabilizing effect of inhibitors may be directly on the protein structure. By combining inhibitors and phospholipid, the protection seen was improved additively, suggesting separate types of stabilizing interactions. These inhibitor-bound, phospholipid-stabilized, preparations of Band 3 are the subject of current crystallization trials.

The association of Band 3 suggests that the intact protein in detergent solution is unstable at 37°C. Calorimetric studies have shown that C_{12}-detergents provide the greatest degree of stability for Band 3 in solution [40]. It was also noted [40] that Band 3 in detergent solution was less stable than in the native membrane. In order to retain the native structure of Band 3 in detergent solutions, the protein should be stored at low temperature (0–4°C) and crystallization trials should be carried out at these temperatures. At 37°C there is a rapid ($t_{1/2}$ = 1 h) conversion of dimer to a complex slightly larger than native tetramers. The production of a complex with a discrete size suggests that the initial association of dimers is a specific process. It is possible that this complex resembles the structure of Band 3 tetramers found in situ. The temperature-dependent association of isolated Band 3 has also been observed in other detergents [10]. Self-association of Band 3 at 37°C also occurs in DMPC vesicles, but this process is a reversible one [36]. In contrast to intact Band 3, the isolated membrane domain is a very stable dimer, even at 37°C. Calorimetry studies on ghost membranes have shown that there is no loss in the thermal stability of Band 3 after proteolytic treatment to generate the membrane domain [41]. It has been reported that the membrane domain is less stable in detergent solution [40]. We find that the isolated membrane domain is more stable than the intact protein in detergent solution. The self-association likely results from a conformational change in Band 3 which requires the cytosolic domain. It is possible that Band 3 dimers are brought together by interac-

tions between their cytosolic domains. It is known, for example, that native Band 3 tetramers require the cytosolic domains [7]. The interaction of the cytosolic domains may affect the membrane domain, perhaps de-stabilizing it, and resulting in its irreversible denaturation. This indicates that the two domains of Band 3 can influence each other [40,42].

Higher temperatures would promote the more rapid exchange of lipids between Band 3 and detergent micelles and enhance the interaction of hydrophobic protein domains ultimately involved in aggregation. As temperature increases, the hydration of the $C_{12}E_8$ oxyethylene moiety is decreased. This corresponds to a decrease in the head-group volume which results in lessening the head-groups' repulsions [43,44]. Thus, elevated temperatures may allow $C_{12}E_8$ micelles to fuse together, further enhancing the irreversible association of the membrane protein domains within a larger, single detergent micelle. The association and loss of Band 3 function induced by elevated temperature has also been observed upon dilution of the protein [9]. The aggregation induced by dilution was also slowed by phospholipids, inhibitors and chloride. Removal of the cytosolic domain also prevented the association upon dilution and slowed the conformational change. The remarkable similarity of the association induced by dilution and incubation at 37°C suggests that the two processes induce the same changes in Band 3.

The ratio of detergent:protein is a crucial factor in Band 3 stabilization. Past work has shown that Band 3 can be purified in many different detergents; however, at detergent concentrations below the cmc the protein aggregates irreversibly [8]. This is likely the result of too few detergent molecules being present to coat the exposed hydrophobic domains of the protein. Interestingly, in this study we have found that the opposite extreme, high concentrations of detergent, also promote aggregation. This phenomenon is probably the result of excess detergent micelles stripping the protein of essential lipids. This point must be considered when purifying membrane proteins. In each case it is necessary to optimize the detergent concentration used for solubilization and purification. It may be possible initially to use excess detergent for solubilization since a high content of lipids is present at this stage. The assumption that excess detergent will have no detrimental effect on the protein during the purification may not be correct. For example, stripping of essential, tightly bound lipids can result and the protein may become aggregated and denatured.

The effect of pH on Band 3 aggregation may be useful for crystallizing the protein. By capitalizing on the tendency of Band 3 to aggregate at acidic pH it may be possible to promote specific self-association by carefully approaching the aggregation boundary. In contrast to the intact protein, the membrane domain was stable over a wide range of pH. This also may be useful in crystallization as it indicates that a wide variety of pH conditions may be screened without fear of protein aggregation. Another means of promoting self-association of the intact protein may be to manipulate the $MgCl_2$ concentration.

The ratios of detergent, protein and lipid are important for successful reconstitution and crystallization. The purified membrane domain binds 1 mg of detergent/mg protein, corresponding to a concentration of detergent bound to the membrane domain of 1.85 mM at 1 mg protein/ml. The protein maintains this level of detergent binding over a wide range of free detergent levels, above 0.2 mM free detergent [8]. The amount of free detergent at the beginning of the reconstitution experiments was 1.85 mM (0.1%), well above the cmc for $C_{12}E_8$ of 0.056 mM. Thus, the total detergent (free plus bound) initially in the solution was at a concentration of 3.7 mM. For the experiments described here, the purified protein at 1 mg/ml (18.2 μM) was reconstituted with an equal weight of DMPC (1.47 mM). Under these conditions there are approximately 80 DMPC molecules per membrane domain. This value is close to the upper limit of 70 lipids excluded from each Band 3 monomer by temperature-induced aggregation as determined by NMR [36]. The initial ratio of free detergent to phospholipid was 1.2:1 (1.85 mM:1.47 mM). This value is intermediate between detergent-saturated liposomes, which occur at a mole ratio of 0.66 detergent to phospholipid, and mixed micelles, which occur at a mole ratio of 2.2 [45]. During the intermediate stages of this transition populations of both micellar and lamellar structures coexist, with the proportion varying depending on the mole ratio [44,45]. Interestingly, this ratio is very close to that found to be ideal in a reconstitution system of Band 3 using phosphatidylcholine and Triton X-100 [46].

The formation of the 2D arrays is believed to occur by a two-stage process [47,48]. In the first stage the protein-detergent and lipid-detergent micelles coalesce resulting in lipid bilayer formation and simultaneous protein insertion. This stage in our system probably begins immediately and is complete when the mol ratio of detergent to phospholipid drops to 0.66. The second stage is dominated by lipid–protein and protein–protein interactions and results in the formation of 2D-lattices within the bilayer. This process occurs as detergent is removed from the saturated liposomes by dialysis and components such as $MgCl_2$ act to promote protein:protein interactions within the bilayer, leading to the formation of the 2D-arrays. At the end of the dialysis the total detergent concentration of the sample decreased from the starting value of 3.7 mM to 0.5 mM. This value, which is still above the detergent's cmc, is insufficient to provide the 1.85 mM of detergent needed to saturate the membrane domain of Band 3 at 1 mg/ml. However, much of the remaining detergent could be associated with the protein. In reconstitutions with radiolabeled detergent the amount of $C_{12}E_8$ remaining after dialysis was greater when the protein was present than in the presence of buffer alone. A tight interaction between Band 3 and Triton X-100 has been shown in other reconstitutions [46].

Intact Band 3 in 1% octyl-POE forms crystals in good yield when reconstituted with DMPC at a lipid to protein ratio of 0.75–1 (w/w) in 25–50 mM NaCl, 20 mM $MgCl_2$ in 10 mM Hepes (pH 7.0) at temperatures up to 37°C [10]. Band 3 in short alkyl chain detergents (C_6–C_{10}), however, formed oligomers at room temperature that were not dissociated by SDS. Few oligomers were observed using C_{12} detergents at room temperature [10]. Band 3 held at 4°C in octyl-POE did not form these oligomers but oligomers up to the size of hexamers were induced by incubation at room temperature. The functionality of these oligomers was not tested but the fact that they cannot be dissociated by SDS indicates that they may be denatured. Indeed previous results [49] have shown that Band 3 in short chain detergents such as octyl glucoside has a decreased affinity for stilbene disulfonates and is in a highly aggregated state.

In conclusion, the membrane domain of Band 3 is a very stable entity that is not sensitive to temperature-dependent association. Band 3 associates in a temperature-dependent manner due to the interaction between cytosolic domains and the loss of stabilizing phospholipids. Band 3 can be stabilized with inhibitors or by supplementing detergent solutions with phospholipids. These types of stabilized Band 3 preparations are the subject of current crystallization trials.

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