Mechanism of band 3 dimer dissociation during incubation of erythrocyte membranes at 37 °C

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The mechanism of dissociation of the stable dimer of band 3 was investigated during the incubation of isolated erythrocyte membranes or resealed ghosts at 37 °C. The kinetics of changes in the structural and functional integrity of the membrane domain of band 3 (MDB3) were measured and correlated with the change in the Stokes radius of band 3. MDB3 integrity was determined as follows: (1) by measuring the fluorescence emission spectrum of 4,4'-di-isothiocyanostilbene-2,2'-disulphonate (DIDS) bound covalently to MDB3; (2) by measuring the number of DIDS covalent binding sites present after incubation of unlabelled resealed ghosts; and (3) by measuring the anion transport $V_{\max}$ by using the same resealed ghosts. Incubation of membranes at 37 °C caused the dissociation of band 3 dimers to monomers but only after a lag period lasting approx. 50 h. The observation of such a lag implies that dissociation involves a sequence of molecular events beginning with some type of initial process. We have discovered that this initial process involves a conformational change in MDB3. There was a shift in the fluorescence spectrum

for DIDS-labelled band 3 and a decrease in the DIDS binding capacity and transport activity of the unlabelled protein. Incubation of membranes at 4 °C inhibited the conformational change in MDB3 and the dissociation of dimers. Furthermore, no conformational change in MDB3 was observed when erythrocytes were incubated at 37 °C. We suggest that MDB3 unfolding is the molecular event responsible for the subsequent dissociation of stable dimers of band 3 to monomers during the incubation of erythrocyte membranes at 37 °C. The monomers so generated are either not functional in anion exchange or they have an attenuated functionality. The absence of a conformational change for band 3 in erythrocytes might imply that haemolysis perturbs the membrane structure and somehow predisposes band 3 to the conformational change that occurs during incubation at 37 °C.

Key words: fluorescence spectroscopy, gel-filtration chromatography, membrane protein folding, membrane transport, stilbene disulphonates.

INTRODUCTION

Band 3 is an obligatory anion exchanger found in erythrocytes and kidney, and is part of a family of anion exchangers that have a ubiquitous distribution in various mammalian tissues [1,2]. A central issue in work on band 3 is which oligomeric form, the monomer or the dimer, constitutes the minimum native structural unit [2]. Solving this problem is essential to gaining an understanding of which oligomeric form constitutes the native functional unit in band 3-mediated anion exchange [3]. The current understanding of the oligomeric structure of band 3, and the factors controlling that structure, has been established almost exclusively by working with band 3 in non-ionic detergent solution (reviewed in [4]). Most groups observe the presence of stable dimers and tetramers when isolated band 3 is analysed in non-ionic detergent solution [5–12], whereas an apparent equilibrium mixture of monomers, dimers and tetramers has been reported in studies in one laboratory [13]. The recent detailed study by Taylor et al. [4] offers evidence that might reconcile some of these differences. They found that when band 3 was isolated under mild conditions to remove the cytoskeleton, stable dimers and higher oligomers were observed. In contrast, when erythrocyte membranes were stripped of cytoskeleton at pH 12, which can lead to a partial unfolding of the membrane domain of band 3 (MDB3) [14], an apparent equilibrium between monomer, dimer and tetramer was detected, suggesting that monomers are generated when MDB3 unfolds.

If maintenance of the native conformation of MDB3 is essential

to the stability of dimeric band 3, one would expect that membrane-bound band 3 should always be minimally dimeric, provided that harsh environmental conditions are not used during sample preparation [4]. However, despite this expectation, a recent paper seems to challenge this point of view: Van Dort et al. [15] report that the incubation of inside-out vesicles (IOVs) in medium of low ionic strength, at 37 °C and pH 8, in the presence of several protease inhibitors, induces monomer formation, albeit over long periods (up to a week). Some indication that the monomers so generated were functional was found in experiments in which the addition of ankyrin to incubated IOVs caused the reaggregation of the monomers to form tetramers [15]. These results imply that an equilibrium between monomer, dimer and tetramer [13] might exist for native band 3 *in situ* at physiological temperature, an equilibrium not seen at lower temperature in non-ionic detergent solutions [4].

After publication of the papers by Van Dort et al. [15] and Taylor et al. [4], we hypothesized that the incubation of IOVs at 37 °C, under the conditions used by Van Dort et al. [15], might lead to a partial unfolding of MDB3, which could destabilize contacts between the subunits and thus explain the appearance of monomers. We designed a series of experiments to test this hypothesis by comparing (1) the kinetics of change in the structural and functional integrity of MDB3 for band 3 incubated at 37 °C with (2) the kinetics of change in the Stokes radius of band 3 derived from the same samples. We used a previously published analytical gel-filtration chromatographic method [10] to determine the Stokes radius of band 3. We also used two well-

Abbreviations used: C₁₂E₈, poly(oxyethylene-8-lauryl ether); C₁₂E₉, poly(oxyethylene-9-lauryl ether); CDB3, cytoplasmic domain of band 3; DIDS, 4,4'-di-isothiocyanostilbene-2,2'-disulphonate; DTT, dithiothreitol; IOV, inside-out vesicle; MDB3, membrane domain of band 3.

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established assays to determine the structural integrity of MDB3. One assay involved labelling MDB3 with 4,4'-di-isothiocyanostilbene-2,2'-disulphonate (DIDS) in erythrocytes and then observing the position of the peak fluorescence emission spectrum of DIDS-labelled band 3 as a function of incubation time for the various membrane preparations. This spectral peak is known to shift from an emission wavelength of approx. 450 nm (when excited at 350 nm) to an emission wavelength of about 430 nm when MDB3 is specifically unfolded [16]. The second assay for MDB3 structural integrity was to measure the covalent binding capacity for DIDS of unlabelled band 3 in a given preparation [8]. Finally, MDB3 functional integrity was assayed by monitoring the $V_{\max}$ for dithionite/sulphate exchange in resealed ghosts by using an established and highly band-3-specific spectrophotometric assay [17–19]. In these latter studies, the covalent binding capacity for DIDS, the transport $V_{\max}$ and the Stokes radius of band 3 could all be determined on the same preparations.

MATERIALS AND METHODS

Materials

Recently outdated packed erythrocytes were obtained from the Omaha Chapter of the American Red Cross and were used immediately after receipt. DIDS, PMSF, poly(oxyethylene-8-lauryl ether) ($C_{12}E_8$) and poly(oxyethylene-9-lauryl ether) ($C_{12}E_9$) were obtained from Sigma (St. Louis, MO, U.S.A.). Leupeptin and pepstatin A were obtained from Fluka (Ronkonkoma, NY, U.S.A.). Sepharose CL-4B was obtained from Pharmacia (Piscataway, NJ, U.S.A.). All other reagents were from major suppliers and were of the highest purity available.

Labelling of band 3 with DIDS

Packed erythrocytes were washed four to six times in PBS [5 mM sodium phosphate/150 mM NaCl (pH 8)]. The washed cells were incubated with 25 μ M DIDS in PBS, at 50 % haematocrit, for 45 min at 37 °C. Cells were then washed once in PBS containing 0.5 % BSA, then four more times in PBS.

Preparation of unsealed ghosts used in the incubation studies

The procedures used here were similar to those used by Van Dort et al. [15]. Washed erythrocytes were lysed at a dilution of 1:40 in ice-cold buffer A [5 mM sodium phosphate (pH 8)/0.5 mM EDTA/0.5 mM sodium azide/80 μ g/ml PMSF]. Stock PMSF was prepared in methanol. After dilution, the final concentration of methanol was approx. 96 mM. In certain experiments methanol was absent (see the Results section). In these experiments, enough PMSF/methanol was placed in a flask or a test tube to give the desired final concentration for the volume of IOVs to be added; the methanol was evaporated by flushing with air until the tube or flask was dry. Cells or membranes were then added to this 'PMSF-treated' glassware. After lysis the haemolysate was centrifuged to pellet the ghosts; they were washed at a dilution of 1:40 with buffer A. Ghosts were washed twice more in 20 mM sodium phosphate, pH 8, containing 0.5 mM dithiothreitol (DTT). The ghosts were then brought to 50 % haematocrit in 20 mM sodium phosphate buffer, pH 8, containing 0.5 mM DTT and 20 μ g/ml of leupeptin (made up in water) plus 20 μ g/ml of pepstatin A (made up in methanol or added to tubes in methanol, with the methanol being evaporated subsequently) and 80 μ g/ml PMSF (made up in methanol, but with the methanol removed in certain experiments, as described above). The final pH of the sample was 7.0 and remained constant (± 0.2 pH units) throughout the incubation period. Suspensions of

ghosts were placed either in a 37 °C water bath or in the refrigerator at 4 °C.

Preparations of IOVs used in the incubation studies

The methods used here were similar to those of Van Dort et al. [15]. Washed erythrocytes were lysed in buffer A, the haemolysate was centrifuged and the membranes were washed once more in buffer A. These membranes were then exposed to a low-ionic-strength incubation step by first suspending them (diluted 1:20) in 0.5 mM EDTA/0.5 mM DTT/80 μ g/ml PMSF (pH 8) at 4 °C. The suspension was incubated at 37 °C for 30 min; the membranes were then centrifuged and the supernatant was aspirated. KI-stripped IOVs were prepared by suspending the membranes (diluted 1:100) in 1 M KI/25 mM sodium phosphate/1 mM EDTA/0.5 mM DTT/80 μ g/ml PMSF (pH 7.5) at 4 °C. This suspension was incubated at 37 °C for 30 min, after which the vesicles were centrifuged and washed twice in 20 mM sodium phosphate/0.5 mM DTT (pH 8) (1:50 dilution with each wash). The IOVs were then suspended to 50 % haematocrit with 20 mM sodium phosphate, pH 8, containing 0.5 mM DTT, 20 μ g/ml leupeptin, 20 μ g/ml pepstatin A and 80 μ g/ml PMSF; portions were incubated for extended periods in a 37 °C water bath or were placed in the refrigerator at 4 °C.

Preparation of resealed ghosts used in the incubation studies

Washed erythrocytes (6 ml) were lysed at a dilution of 1:5 in ice-cold 5 mM sodium phosphate, pH 8. The sample was centrifuged and the haemolysate supernatant was collected. Salts and buffer were added to this haemolysate and the mixture was added back to the unsealed ghosts to give the following final concentrations: 90 mM sodium sulphate, 5 mM sodium phosphate, 5 mM Bis-Tris, 5 mM Tris and 1 mM magnesium sulphate, with a final pH of 7.4. Resealing was initiated by incubation at 37 °C for 1 h. The resealed ghosts were then washed three times in the above buffer. The washed resealed ghosts were then placed into a flask pretreated with protease inhibitors in methanol, with the methanol evaporated as described above. The final haematocrit was 50 %, with final protease inhibitor concentrations being the same as described above for the work with unsealed ghosts.

Incubation of erythrocytes at 37 °C

Erythrocytes were labelled with DIDS and washed in PBS as described above. They were then suspended at 50 % haematocrit in 20 mM sodium phosphate/150 mM NaCl (pH 8.0) in a flask pretreated with protease inhibitors in methanol, with the methanol evaporated as described above. The cells were incubated at 37 °C, portions were removed and membranes were prepared and solubilized in detergent as described above for unsealed ghosts.

Analytical gel-filtration chromatography

Gel-filtration chromatography was performed exactly as described previously [10], except that the column buffer consisted of 100 mM NaCl, 5 mM sodium phosphate, pH 8, and 0.1 % $C_{12}E_9$. At each time point during the IOV incubation, 1 ml of a given sample was taken and washed twice in 20 mM sodium phosphate, pH 8. The resulting pellet was then suspended up to 0.5 ml, with final concentrations of 20 mM sodium phosphate and 1 % $C_{12}E_9$. This 0.5 ml sample of solubilized IOVs was then applied to the column. The effluent was passed through a Pharmacia Optical UV-1 monitor, which permitted continuous monitoring at 280 nm; the effluent volume was measured analytically using two Pyrex® 25 ml graduated cylinders (toler-

ance ± 0.3 ml at 20 °C). Selected samples were collected in tubes for the determination of DIDS-band 3 fluorescence. When resealed ghosts were used, IOVs were prepared as described above.

Spectroscopic measurement of the change in fluorescence of the DIDS-band 3 covalent complex

Fluorescence spectroscopy of the DIDS-band 3 covalent adduct was measured as described in several previous reports from this laboratory [8,20,21]. At each time point, 200 μ l of incubated IOVs (or IOVs derived from incubated red cells, or unsealed ghosts) were placed in a 15 ml Corex tube and washed twice in 20 mM sodium phosphate, pH 8, using centrifugation to pellet the IOVs. The pellets were then suspended to 25% haematocrit in 20 mM sodium phosphate, pH 8, containing 1% $C_{12}E_8$; this clarified the IOV suspension. Fluorescence spectra were obtained at 25 °C with a Perkin-Elmer Model 650-40 fluorescence spectrometer. Emission spectra were collected by exciting the sample at 350 nm; excitation spectra were obtained by holding the emission wavelength constant at 460 nm. Excitation and emission slits were set at 2 nm. The excitation and emission path lengths were 10 and 4 mm respectively.

Titration of unlabelled band 3 with DIDS

Titration experiments were performed on matched samples of band 3 derived from the incubation studies with resealed ghosts. Aliquots of the resealed ghosts were removed at various times, placed on ice and lysed with 5 mM sodium phosphate, pH 8, as described above for erythrocytes. The unsealed ghosts generated in this way were washed twice in 20 mM sodium phosphate, pH 8; enough of a 10% (w/v) $C_{12}E_8$ stock solution and of 20 mM sodium phosphate, pH 8, were then added to yield a final 8% haematocrit and a 1% detergent concentration, which solubilized the membranes. Samples at the various time points were matched by measuring the protein concentration by using the bicinchoninic acid method (Pierce, Rockford, IL, U.S.A.) on solubilized membranes. The solubilized membranes were split into several tubes; each was mixed 1:1 (v/v) with various concentrations of DIDS prepared freshly in 20 mM sodium phosphate, pH 8, so as to yield the final DIDS concentration indicated while maintaining a constant concentration of band 3. The samples were left on ice for 45 min, then placed in the 25 °C water bath attached to the spectrofluorimeter for 10 min to reach temperature equilibrium. The fluorescence emission intensity was measured at 460 nm, with excitation at 350 nm.

Dithionite/sulphate exchange with resealed ghosts

The dithionite transport assay was essentially the same as that described in several previous reports [17–19], with the exception that a Hitachi Model 100-60 spectrophotometer with a 1 cm path-length cell was used, requiring the use of a lower haematocrit of resealed ghosts. Resealed ghosts were removed from the 37 °C water bath at various times and converted to metHb resealed ghosts by incubation with 10 mM sodium nitrite in 90 mM sodium sulphate/5 mM sodium phosphate/5 mM Bis-Tris/5 mM Tris/1 mM magnesium sulphate (pH 7.4) for 30 min, followed by washing the metHb resealed ghosts in the same buffer three times (1:100 dilution) to remove the nitrite. The metHb resealed ghosts were then made up to 0.16% haematocrit and placed in a 50 ml flask. The flask was swirled gently; a 2.3 ml aliquot was taken and placed in a capped glass cuvette in the spectrophotometer, which was attached to a circulating-water bath set at 25 °C. Flux measurements were initiated by adding

enough solid dithionite to the cuvette to yield the final concentration desired. The cuvette was capped and inverted four times; kinetic traces were collected with an attached recorder with the spectrophotometer set at 430 nm, which is the Soret peak for deoxyHb. The reactions were all followed to completion; initial rates were measured from the linear portion of the traces after normalization for the total absorbance change of each reaction.

SDS/PAGE

SDS/PAGE analysis of the various preparations was performed with 7.5% (w/v) polyacrylamide gels; the gels were stained with Coomassie Blue, as described previously [22].

Data analysis

Gel-filtration profiles were quantified by hand from the continuous recordings of the absorbance of the column effluent. A single Gaussian function was fitted to the data by using Sigma Plot 4.0 (Jandel Scientific, San Rafael, CA, U.S.A.), to determine quantitatively the exact position of the effluent peak. The peak positions of the fluorescence excitation and emission spectra were determined by an inspection of recorded scans. A single exponential function was fitted to data that consisted of the wavelength of the fluorescence emission peak against sample incubation time. The DIDS titration data were analysed by plotting the observed fluorescence against DIDS concentration. The linear background fluorescence from free DIDS was subtracted; the data were normalized for the total change in fluorescence and for any slight difference in protein concentration detected by the bicinchoninic acid protein determinations. The intercept of the linear vertical and the horizontal lines in the plot gave a value for the concentration of DIDS binding sites present in the sample (see [20] for further details). Initial rates from the transport data were determined and plotted against dithionite concentration, then fitted to a hyperbolic function to determine $V_{\max}$.

RESULTS

Change in the apparent Stokes radius of DIDS-labelled and unlabelled band 3 derived from IOVs or resealed ghosts incubated at 37 °C

We used a gel-filtration chromatographic method [10] to study the change in size of band 3 derived from IOVs incubated at 37 °C for various durations. Figure 1 shows the results from a typical chromatographic profile for solubilized DIDS-labelled IOVs before incubation at 37 °C. The solid points are the absorbance of the effluent measured at 280 nm, whereas the open circles are the fluorescence intensities of selected samples measured at the excitation wavelength characteristic of the DIDS-band 3 covalent complex [8,20,21]. A single Gaussian function gave a good fit to the absorbance data (solid line in Figure 1). Although glycophorin A was present in the column effluent, it does not bind DIDS under the specific labelling conditions used here. This was demonstrated by subjecting solubilized DIDS-labelled membranes to aminoethyl-Sepharose 4B chromatography, which separates glycophorin A from band 3 [7] (results not shown). To be certain that any shifts in the position of the peak determined by absorbance measurements corresponded to a change in the Stokes radius of band 3, DIDS fluorescence measurements were performed on the chromatographic profiles from IOVs incubated at 37 °C for various durations. The position of the peak determined on the basis of the absorbance measurements, or by DIDS fluorescence, was always the same.

Figure 1 Gel-filtration chromatography of solubilized DIDS-labelled IOVs

Band 3 was labelled stoichiometrically with DIDS in intact red cells; IOVs were then prepared as described in the text. The sample was solubilized in 1% $C_{12}E_9$ and chromatographed on Sepharose CL-4B equilibrated with 0.1% $C_{12}E_9$ /100 mM NaCl/5 mM sodium phosphate (pH 8.0). The effluent was monitored continuously at 280 nm (●); selected tubes were collected and scanned for DIDS fluorescence ($\lambda_{ex} = 350$ nm) (○). V_0 and V_t indicate the column void and total volumes respectively, determined as described previously [10]. The peak position was determined by fitting a single Gaussian function to the absorbance data (solid line). The elution volumes of standard proteins with known Stokes radii are indicated at the top: thyroglobulin (TG, 8.9 nm), apoferritin (AF, 6.9 nm) and myoglobin (Mb, 1.9 nm).

Figure 2 Effect of incubation of IOVs at 37 °C on the gel-filtration chromatographic profile of band 3

DIDS-labelled IOVs were incubated at 37 °C for 13 days as described in the text. Initial and incubated samples were solubilized as described in the legend to Figure 1. The points show A_{280} ; the solid lines in each profile are drawn on the basis of a fit to a single Gaussian function, with a peak centre indicated by the vertical lines. The peak positions are as follows: initial, 35.9 ml; incubation for 13 days, 39.5 ml.

IOVs were incubated in the presence of the protease inhibitors PMSF, pepstatin A and leupeptin, essentially as described by Van Dort et al. [15]. Figure 2 shows the change in the peak elution volume of DIDS-labelled band 3 after incubation of IOVs at 37 °C for 13 days. There was an increase in the peak

Figure 3 Plot of the distribution coefficient $(-\log K_{av})^{1/2}$ against Stokes radius

Protein standards (○) were used to establish the line in the figure, on the basis of a linear least-squares fit. The values of the distribution coefficient were calculated for native band 3 and for band 3 incubated for 13 days at 37 °C (■). K_{av} was calculated from eqn. (1). Calculated values of the distribution coefficient were placed on the line determined by the standards, to estimate the Stokes radii of the various band 3 samples. The standard proteins and their Stokes radii are as follows: thyroglobulin (TG), 8.9 nm; apoferritin (AF), 6.9 nm; aldolase (AL), 4.8 nm; BSA (SA), 3.6 nm; catalase (CL), 4.5 nm; carbonic anhydrase (CA), 2.0 nm; myoglobin (Mb), 1.9 nm; cytochrome *c* (CC), 1.64 nm. The Stokes radii of the experimental proteins were determined as: native band 3, 8.96 nm; band 3 incubated for 13 days at 37 °C, 6.98 nm.

elution volume as a function of incubation time. We performed SDS/PAGE analyses on these samples and found that the protease inhibitors prevented cleavage of the band 3 polypeptide chain, in agreement with the results of Van Dort et al. [15] (results not shown).

We used methanol in making stock solutions of PMSF and pepstatin A because we observed that the use of propan-2-ol, the more traditional medium for carrying these inhibitors, led to a significant degradation of band 3. This effect of propan-2-ol might be related to the findings of Forman et al. [23] that *n*-alkanols alter band 3 function in a manner related to alcohol chain length. This presumably reflects the ability of *n*-alkanols to partition into the membrane. We chose to use methanol because of its relatively low tendency to partition into the lipid bilayer [23]. Alternatively, we eliminated organic solvents altogether by placing measured amounts of the methanolic solutions of the protease inhibitors in the incubation vessel, then evaporating the methanol away before adding the IOVs. The results were the same as those in the presence of methanol.

By using the peak elution volume (V_e) and the values of the void (V_0) and total (V_t) volumes (Figure 1), we calculated the partition coefficient K_{av} : (1) for the native band 3 dimer before incubation at 37 °C, (2) for band 3 derived from IOVs incubated for 13 days at 37 °C, and (3) for each protein standard. We used the relationship [24]:

$$K_{av} = (V_e - V_0)/(V_t - V_0) \quad (1)$$

The Stokes radii for native and incubated band 3 were determined by using a plot of the distribution coefficients $(-\log K_{av})^{1/2}$ against the Stokes radii of standard proteins (Figure 3). Stokes radii for band 3 were: native band 3, 8.96 nm; band 3 incubated for 13 days at 37 °C, 6.98 nm. Incubation for 13 days at 37 °C decreased the apparent Stokes radius by approx. 1.98 nm.

Figure 4 Plot of the peak elution volume from gel-filtration chromatograms against the incubation duration at 37 °C

The peak positions were taken from chromatographic profiles similar to those shown in Figures 1 and 2. The symbols and the numbers (n) of independent time courses studied are as follows: ●, DIDS-labelled band 3 from IOVs ($n = 5$); ○, unlabelled band 3 from IOVs ($n = 1$); □, DIDS-labelled band 3 from resealed ghosts ($n = 1$). The solid curve passing through the points was drawn on the basis of results of fitting eqns. (2) and (3) of the text to the data for DIDS-labelled IOVs (●): $E = E_0 + \Delta E[f(t, k_{1-3})]$, with $f(t, k_{1-3})$ defined as in eqn. (3). The following constants were determined by the fit: $E_0 = 35.84$ ml, $\Delta E = 3.01$ ml; $k_1 = 0.016$ h⁻¹; $k_2 = 0.016$ h⁻¹; $k_3 = 0.017$ h⁻¹. The P value for the fit was less than 0.0001.

Scheme 1 Schematic model for unfolding of MDB3 and dissociation of the band 3 dimer

The symbols represent the conformation of MDB3: circles, native MDB3; squares, unfolded MDB3. See the text for further details.

We next measured the change in peak elution volume as a function of incubation time for DIDS-labelled band 3 derived from IOVs incubated at 37 °C. The results from five independent time-course studies are shown in Figure 4 (●). It is apparent that the increase in peak elution volume (i.e. the decrease in Stokes radius) occurs only after a substantial lag time has elapsed. The observation of such a lag period in any kinetic process is usually indicative of some type of sequential mechanism, where one or more initial steps must occur before the observed process. We proposed that MDB3's unfolding within each subunit of the native band 3 dimer must occur before dimer dissociation. Scheme 1 illustrates a tentative model depicting a consecutive, irreversible three-stage reaction mechanism with four molecular species, and three kinetic steps: (1) MDB3 unfolding to form a partly unfolded dimer (associated kinetic constant k_1), (2) MDB3 unfolding within the second subunit to form the fully unfolded dimer (associated kinetic constant k_2) and (3) dissociation of that dimer to yield two unfolded monomers (associated kinetic constant k_3).

In analysing data on the change in peak elution volume with time, we assume that only two species are present after the initiation of incubation at 37 °C, each with a different Stokes radius: the dimer (with either folded or unfolded MDB3) and the dissociated monomer. We assume that all of the band 3 starts as native dimer. Because our analytical gel-filtration method [10] does not have the resolution to separate dimers from monomers

Figure 5 Fluorescence excitation and emission spectra of free DIDS and DIDS-band 3 derived from initial IOVs and IOVs incubated at 37 °C

Free DIDS was dissolved in water; spectra were measured immediately to avoid hydrolysis [21]. DIDS-labelled IOVs were prepared as described in the text. A portion of the sample was solubilized in 1% C₁₂E₉ in 20 mM sodium phosphate, pH 8. The remaining IOVs were incubated for 288 h at 37 °C in the presence of protease inhibitors as described in the text after which they were harvested and washed in 20 mM sodium phosphate, pH 8.0, and solubilized in 1% C₁₂E₉ in 20 mM sodium phosphate buffer, pH 8.0. For all emission spectra the excitation wavelength was held constant at 350 nm, whereas for all excitation spectra the emission wavelength was held constant at 460 nm. These standard settings account for the fact that the peak excitation amplitude is different from the peak emission amplitude for each sample. The excitation and emission peaks were, respectively: free DIDS, 350/415 nm; DIDS-IOVs, initial, 370/452 nm; DIDS-IOVs, 288 h at 37 °C, 360/430 nm. The peak at 395 nm in the emission spectrum of free DIDS is due to the Raman scattering of water. The spectra were collected in a water-jacketed cuvette held at 25 °C.

completely, the observed peak elution volume is the weighted sum of the absorbances of the two species present in the sample; the peak should shift to a larger volume as dimers are converted to monomers during incubation at 37 °C [10]. The empirical equation describing such a shift in peak elution volume as a function of time is:

$$E = E_0 + \Delta E[f(t, k_{1-3})] \quad (2)$$

where E is the observed peak elution volume, E_0 is the peak elution volume at time $t = 0$ and ΔE is the change in peak elution volume that occurs when dimers convert to monomers. The function $f(t, k_{1-3})$ is the integrated rate equation for the appearance of the last product in a three-stage consecutive irreversible reaction (Scheme 1) [25] and has the form:

$$f(t, k_{1-3}) = 1 - [k_2 k_3 / (k_2 - k_1) (k_3 - k_1)] e^{-k_1 t} - [k_1 k_3 / (k_1 - k_2) (k_3 - k_2)] e^{-k_2 t} - [k_1 k_2 / (k_1 - k_3) (k_2 - k_3)] e^{-k_3 t} \quad (3)$$

Figure 6 Plot of the peak wavelength of the DIDS fluorescence emission spectrum ($\lambda_{\text{excite}} = 350$ nm) against incubation time for band 3 derived from DIDS-labelled IOVs (●), ghosts (■) or erythrocytes (RBCs) (◆), incubated at 4 or 37 °C

A function representing a single-exponential decay process ($\lambda = \Delta\lambda e^{-kt} + \lambda_i$) was fitted to the IOV and ghost 37 °C data measuring the kinetics of the fluorescence spectral shift. The term $\Delta\lambda$ is the difference between the initial and final emission wavelengths, λ_i is the wavelength at the end of the exponential transition, and k is the apparent rate constant. The apparent rate constants, k , are: IOVs at 37 °C, 0.016 h^{-1} ; ghosts at 37 °C and low ionic strength (low μ), 0.008 h^{-1} ; ghosts plus 150 mM NaCl, 0.03 h^{-1} .

The curve in Figure 4 is the result of the fit of eqns. (2) and (3) to the DIDS-labelled IOV data (●), with values for the constants given in the legend to Figure 4.

We also studied the change in Stokes radius for unlabelled band 3 derived from IOVs incubated at 37 °C (Figure 4, ○) and for band 3 derived from DIDS-labelled resealed ghosts incubated at 37 °C (Figure 4, □). The overall behaviour of both types of preparation was essentially the same as that of DIDS-labelled IOVs. Finally, we found that incubation of the various membrane preparations at 4 °C inhibited the change in Stokes radius of band 3 (results not shown).

Fluorescence spectral shift in DIDS-labelled MDB3 occurs during the lag period for the change in Stokes radius

To test the hypothesis that MDB3 unfolding represents the initial step in the dissociation of the band 3 dimer, we looked for a characteristic blue shift in the fluorescence spectrum of the DIDS-band 3 covalent complex that occurs when MDB3 unfolds [16]. When DIDS makes a covalent complex with band 3 at 'lysine A', there is a shift towards the red in both the fluorescence excitation and emission peaks [20]; this is illustrated in Figure 5. Free DIDS in aqueous solution has an excitation peak at approx. 350 nm and an emission peak at 415 nm. The peak at 395 nm in the emission spectrum of free DIDS is due to the Raman scattering of water. The DIDS-band 3 covalent complex [DIDS-IOVs (initial)] has an excitation peak at 370 nm and an emission peak at 452 nm. When DIDS-labelled IOVs were incubated for 288 h at 37 °C, the characteristic blue shift in the fluorescence spectrum was observed (Figure 5). The excitation peak was then located at 360 nm, a 10 nm blue shift from the initial condition, whereas the emission peak was shifted to 430 nm, 22 nm towards the blue.

Figure 7 Fluorescence titration of covalent binding of DIDS to band 3

(A) DIDS titration of band 3 derived from resealed ghosts. Unsealed ghosts were prepared from the resealed ghosts and solubilized in C_{12}E_8 as described in the text. Samples were divided and various amounts of DIDS were added from a stock solution. The reaction proceeded on ice for 45 min in 20 mM sodium phosphate, pH 8. The samples were then placed in the fluorimeter at 25 °C for 10 min. Fluorescence emission intensity was measured at 460 nm ($\lambda_{\text{ex}} = 350$ nm). The background fluorescence from DIDS was subtracted on the basis of the observed linear extrapolation after saturation. (B) Normalized change in fluorescence for the DIDS reaction with band 3 from resealed ghosts incubated for various durations at 37 °C: 1, initial; 2, incubated for 5 days; 3, incubated for 8 days. The change in fluorescence for each titration was normalized by using the total change in fluorescence for that particular titration, with corrections for slight differences in the total protein determined independently for each sample (see the text for details).

The time course of the blue shift in the DIDS-band 3 fluorescence emission peak during the incubation of IOVs at 37 °C was exponential (Figure 6, ●). The apparent rate constant for this change was 0.016 h^{-1} . Lowering the temperature to 4 °C inhibited the spectral shift (Figure 6) and, as mentioned, also inhibited the decrease in the Stokes radius of band 3. When DIDS-labelled unsealed ghosts were studied at low ionic strength (Figure 6, ■; low μ ghosts, 37 °C), we observed a 50% slower rate (0.008 h^{-1}) for the blue shift in the fluorescence emission peak; however, the extent of the spectral shift was the same. The rate of the spectral shift for band 3 in unsealed ghosts was accelerated approx. 3–4-fold after the addition of 150 mM NaCl ($k = 0.03 \text{ h}^{-1}$; Figure 6, ■; ghosts, 37 °C + 150 mM NaCl) under otherwise identical experimental conditions. In contrast, the incubation of DIDS-labelled erythrocytes at 37 °C (see the Materials and methods section) showed no significant change in DIDS fluorescence over the first 50 h of incubation (Figure 6, ◆; RBCs, 37 °C). It proved to be difficult to prevent the haemolysis of erythrocytes after 50 h, which accounts for the abbreviated time course shown for the erythrocyte data in Figure 6.

Figure 8 Dithionite/sulphate exchange for resealed ghosts incubated for various durations at 37 °C

Transport was assayed as described in the text at 25 °C. The observed initial rate (k) was plotted against dithionite concentration in double-reciprocal form. The k^{-1} axis intercept is proportional to $V_{\max}$ when various samples from the same ghost preparation are compared (see [17] for details).

Kinetics of the change in DIDS binding capacity and transport $V_{\max}$ for unlabelled band 3 in resealed ghosts incubated at 37 °C

We studied the decrease in the capacity of band 3 derived from resealed ghosts incubated at 37 °C to bind DIDS covalently. Figure 7(A) shows a typical titration in which the intercept between the stoichiometric binding line and the saturation line was used to determine the amount of DIDS bound covalently to the band 3 subunit population. Figure 7(B) shows the change in the covalent binding capacity of DIDS for various protein-matched band 3 samples as a function of incubation time for resealed ghosts at 37 °C. This incubation decreased the DIDS binding capacity of the band 3 population (Figure 7B).

To determine the effect of incubation at 37 °C on the number of functional transport sites, we measured transport $V_{\max}$ for the dithionite–sulphate exchange by using resealed ghosts incubated at 37 °C. Figure 8 shows that transport $V_{\max}$ was decreased systematically with increasing incubation time at 37 °C, for ghosts assayed at the same haematocrit and derived from the same incubated stock suspension.

The quantitative results for the decrease in transport $V_{\max}$ and for the decrease in the DIDS binding capacity as a function of incubation duration at 37 °C are shown in Figures 9(A) and 9(B) respectively. These data come from independent DIDS binding and transport experiments performed on two different blood samples. Both measures of MDB3 integrity decreased by approx. 50 % at 150 h. Using this as the half-time, we calculated an apparent rate constant of approx. 0.005 h^{-1} . This is smaller than the rate constants observed for the DIDS spectral shift (0.008 to 0.03 h^{-1} ; Figure 6), indicating that the loss of MDB3 integrity is somewhat slower for unlabelled band 3 than it is for the DIDS-labelled protein. Cross-correlation of the change in DIDS binding capacity with the corresponding change in transport $V_{\max}$ for matched samples of resealed ghosts followed the 1 : 1 correlation line (Figure 9C). This indicated that there is a proportional loss in each of these two independently determined measures of MDB3 integrity as a function of incubation duration at 37 °C.

Figure 9 Change in the percentage of DIDS reactive band 3 and in the transport $V_{\max}$ for resealed ghosts measured as a function of incubation time at 37 °C

Data similar to those shown in Figure 8 (transport) and Figure 7 (DIDS binding), from two sets of independent experiments, are plotted in (A) and (B) respectively. The initial DIDS binding capacity and transport $V_{\max}$ served as the 100% point. Both transport activity and DIDS binding capacity were lost at essentially the same rate ($t_{1/2} = 150 \text{ h}$, or $k = 0.005 \text{ h}^{-1}$). (C) A cross-correlation plot of DIDS binding capacity against transport $V_{\max}$ with the data from (A) and (B). There is a 1 : 1 correspondence.

DISCUSSION

The gel-filtration studies presented here have confirmed the findings of Van Dort et al. [15] that the incubation of erythrocyte membranes at 37 °C leads to a decrease in the apparent Stokes radius of DIDS-labelled and unlabelled band 3. We observed an approx. 2.0 nm decrease in the apparent Stokes radius of band 3 after incubation for 13 days at 37 °C (Figures 2 and 3). A change of this magnitude is expected when band 3 dissociates from dimers to monomers. For example, it is known that treatment with 2,3-dimethylmaleic anhydride causes the dissociation of

band 3 dimers to monomers [7] and that such treatment decreases the apparent Stokes radius of band 3 by approx. 1.9 nm [26], in agreement with the magnitude of the Stokes radius shift that we observed during the incubation of membranes at 37 °C (Figure 3).

Kinetic studies of monomer formation for DIDS-labelled band 3 derived from IOVs revealed a lag period of approx. 50 h (Figure 4). The occurrence and the length of this lag is consistent with the finding of Van Dort et al. [15] that there was no change in the fraction of dimer present for DIDS-labelled IOVs at 37 °C until after the first approx. 50 h of incubation. The observation of such a lag implies that some type of sequential process is involved, with some type of molecular event or sequence of events occurring before the dissociation of band 3 dimers. We propose that MDB3 unfolding is the molecular event that takes place during the lag and that this unfolding is a necessary first step in the dissociation of the band 3 dimers during the incubation of erythrocyte membranes at 37 °C (Scheme 1). The evidence supporting this proposal falls into two categories. The first category involves the evidence indicating that MDB3 unfolds. The observed spectral shift in DIDS-MDB3 is a characteristic of MDB3 unfolding [16]; the magnitude of the shift that we observed (452 to 430 nm) is the same as that seen when SDS is added to DIDS-labelled band 3 (J. M. Salhany, unpublished work). This latter comparison suggests that all of the subunits unfold during incubation at 37 °C. In support of this statement is the fact that there was no evidence for the development of two spectral peaks during incubation, as might be expected if, for example, only half of the subunits changed structure. The second category of evidence involves a comparison of the rate constants assigned to the lag period for dimer dissociation (k_1 and k_2 from the fit in Figure 4), with the rate constants for the shift in the fluorescence of DIDS-labelled MDB3 during incubation at 37 °C (Figure 6). Fitting the data for the change in Stokes radius in Figure 4, for DIDS-labelled IOVs, to eqns. (2) and (3) yielded values for k_1 and k_2 of 0.016 h⁻¹. Within the context of the model in Scheme 1, the overall rate constant for the unfolding of MDB3 is $[1/(1/k_1 + 1/k_2)]$ [25], which is in turn equal to $\frac{1}{2}k_1$, or 0.008 h⁻¹ (Because $k_1 = k_2 = 0.016$ h⁻¹). This value of 0.008 h⁻¹ is clearly within the range of rate constants seen for the spectral shift due to DIDS in Figure 6 (0.008 to 0.03 h⁻¹).

The loss of MDB3 integrity occurs at a slightly slower rate for unlabelled band 3 (Figures 9A and 9B) than that for DIDS-labelled band 3 (Figure 6). These results contrast with the observation of Van Dort et al. [15], who found a more rapid appearance of monomers for unlabelled band 3. One way in which to rationalize the data is to expand the model in Scheme 1 to allow the unlabelled protein to dissociate after the unfolding of the first MDB3 subunit. Another possibility is to suggest that there exists a 5–10-fold larger ratio of k_3 to k_1 and k_2 for unlabelled compared with DIDS-labelled band 3. Such an increased ratio would have the effect of greatly decreasing or eliminating the lag, which arises from the fact that the kinetic constants in Scheme 1 are nearly equal for DIDS-labelled band 3 (Figure 4). The resulting first-order time course for unlabelled band 3, with the larger ratio of k_3 to k_1 and k_2 , would now have monomers appearing sooner.

Our results generally support the conclusions of Taylor et al. [4], suggesting that band 3 subunit conformation is an important factor in determining which quaternary state of band 3 is observed in a given sample. Our results can also accommodate the finding of Van Dort et al. [15] that the addition of ankyrin to IOVs that had been incubated at 37 °C caused the association of monomers to tetramers. This is illustrated in Figure 10. The basic requirement for the binding of ankyrin to the band 3 tetramer is

Figure 10 Schematic diagram of a possible mechanism for the association of partly unfolded band 3 with ankyrin

(A) Depiction of two conformations of a band 3 monomer: native band 3 with native conformation for both MDB3 (within the bilayer) and CDB3 (protruding from the bilayer), and partly unfolded band 3 with an unfolded membrane domain but a native cytoplasmic domain. (B) Reversible association of four partly unfolded band 3 monomers to form two partly unfolded dimers, and one partly unfolded tetramer with ankyrin attached.

that the cytoplasmic domain of band 3 (CDB3) should have a native conformation [27]. Our model puts no constraint on the structure of CDB3, which can retain its native conformation even when MDB3 unfolds [27] (Figure 10A). If we assume that such partly unfolded band 3 exists in a monomer–dimer–tetramer equilibrium in the lipid bilayer, then the addition of ankyrin, which binds preferentially to the band 3 tetramer [13], should shift the distribution of species from partly unfolded monomers to partly unfolded tetramers (Figure 10B).

Our results and those of Taylor et al. [4] support the view that the native structural unit of band 3 is the stable dimer. However, it is not clear whether or not the monomers that form after incubation at 37 °C are functional, although their functionality is significantly attenuated (Figures 8 and 9A). It was not technically possible to follow the kinetics shown in Figures 9A and 9B to completion. We therefore do not know whether the transport activity goes to zero at the end of the process, or whether some finite value is reached. This is an important point because even poorly active monomers would imply that the subunits within the native dimer constitute the basic functional unit. If this were so, formation of the native stable dimer could serve to increase the turnover number of the subunits, possibly through homotropic, allosteric subunit interactions [19].

At present we do not know why the incubation of IOVs or unsealed ghosts at 37 °C leads to the unfolding of MDB3. It seems revealing that the incubation of intact erythrocytes at 37 °C did not lead to an unfolding of MDB3 over the first 50 h of incubation (Figure 6). We conclude tentatively that the haemolysis of erythrocytes somehow predisposes membranes to the slow temperature-dependent unfolding of MDB3. The haemolysis of erythrocytes is known to produce conformational changes in band 3. Haemolysis produces changes in pyridoxal 5'-phosphate binding to MDB3 [28], in MDB3 thiol reactivity [29] and in the rotational mobility of band 3 [30]. In addition, Stadler and Schnell [31] have presented results demonstrating that the partial substrate inhibition effect, which is a characteristic of the anion exchange mechanism (reviewed in [3]), is attenuated in resealed ghosts in comparison with erythrocytes. It will be

important in future work to identify the cellular factors that serve to stabilize band 3 structure *in situ* at 37 °C.

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