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# Effect of pH on the self-association of erythrocyte band 3 in situ

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**Abstract**

The human erythrocyte anion exchanger (band 3) contains a cytoplasmic domain (cdb3) that exists in a reversible, pH-dependent structural equilibrium among three native conformations. To understand how this conformational equilibrium might influence the association state of band 3, we have incubated stripped erythrocyte membranes in solutions ranging from pH 6.0 to pH 10.5 and have examined the oligomeric state of the protein by size exclusion high performance liquid chromatography. We demonstrate that incubation of membranes in slightly acidic conditions favors dimer formation, whereas extended incubation at higher pHs (pH > 9) leads to irreversible formation of an oligomeric species larger than the tetramer. Since the pH dependence of the conformational state of the cytoplasmic domain exhibits a similar pH profile, we suggest that the conformation of the cytoplasmic domain can modulate the self-association of band 3. Importantly, this modulation would appear to require the structural interactions present within the intact protein, since the isolated membrane-spanning domain does not display any pH dependence of association. The irreversible nature of the alkali-induced aggregation further suggests that a secondary reaction subsequent to band 3 association is required to stabilize the high molecular weight aggregate. Although we were able to eliminate covalent bond formation in this irreversible aggregation process, the exact nature of the secondary reaction remains to be elucidated. 

*Keywords:* Erythrocyte membrane protein aggregation; Band 3 protein conformation; pH dependence of band 3 aggregation

## 1. Introduction

Band 3 ($M_r \sim 101\,000$) is the predominant polypeptide of the human erythrocyte membrane, comprising ~25% of the total membrane protein. Mild proteolytic cleavage of erythrocyte band 3 at the cytoplasmic surface of the membrane yields two major structural domains, a 58-kDa carboxyl-terminal membrane domain that may traverse the bilayer up to 14 times [1,2], and a 43-kDa cytosolic amino-terminal domain [3]. The carboxyl-terminal membrane domain catalyzes the electroneutral exchange of bicarbonate for chloride [4–6] and is the major antigen responsible for immune-mediated removal of senescent and abnormal erythrocytes [7–11]. The cytoplasmic domain (cdb3) binds peripheral proteins to the membrane, including several glycolytic enzymes [12,13], protein 4.1 [14,15], protein 4.2 [16,17], hemo-

Abbreviations: cdb3, cytoplasmic domain of band 3; $C_{12}E_8$, octaethylene glycol monododecyl ether; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; KI-IOVs, 1 M KI-stripped inside-out membrane vesicles; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; SE-HPLC, size exclusion high performance liquid chromatography; tKI-IOVs, trypsin-treated KI-IOVs

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globin [18,19], hemichromes [20], and ankyrin [21], a protein that provides the major link of the spectrin-based membrane skeleton to the bilayer through its interaction with cdb3. The crystal structure of cdb3 has recently been determined at 2.6 Å resolution and reveals that the protein forms a tight symmetric dimer stabilized by extensive interactions within an intermonomeric dimerization domain [22]. In addition, each monomer of cdb3 contains a peripheral protein binding domain that serves as an anchoring site for the aforementioned membrane-associated proteins.

Like many other transmembrane proteins, band 3 is able to self-associate in situ. Most investigators agree that band 3 exists as a mixture of dimers and tetramers in the membrane [23–28]. However, some laboratories report a large predominance of stable dimers [29,30], while others observe a self-associating mixture of monomers, dimers, and tetramers [31–33], or dimers and hexamers [34]. This state of association is not unimportant to erythrocyte integrity, since it can potentially influence several critical membrane functions. For example, band 3 dimers may be the minimum stable structural unit required for anion transport [35], and dimers may contribute disproportionately to the binding and immobilizing of membrane lipid [36], a property thought to be important to maintenance of membrane shape and stability [37]. In contrast, tetrameric band 3 may stabilize less membrane lipid, but simultaneously provide the essential anchor for the spectrin-based membrane skeleton [23,26,27,38], a structure also believed to be critical to membrane stability [39]. In addition, higher oligomeric forms of band 3 can exist and are important for the recognition and removal of senescent cells from circulation [7–11].

Because the association state of band 3 exerts an impact on membrane function, several investigators have undertaken to examine factors that might control this subunit association equilibrium. Those factors identified to date include: (i) association with ankyrin [26,27,38], (ii) the binding of denatured hemoglobin [40], (iii) oxidative stress [10,11,41], (iv) the interaction of band 3 with divalent cations and cationic compounds [9,28,42,43], (v) treatment of the membrane with hydrophobic agents [9,44], (vi) DIDS labeling [25,26,45], (vii) temperature [46], (viii) pH [23,33,47], (ix) underglycosylation [48] and (x) deletion of amino acids 400–408 (southeast Asian ovalocytosis) [49]. Since we and others have shown that cdb3 can exist in three native, pH-dependent conformations that interconvert at mid pHs of 7.2 and 9.2 [3,50–53], we have wondered whether this conformational equilibrium might also affect the association state of band 3. Unfortunately, the only data available on the pH dependence of band 3 association have been collected below pH 6.5 and near pH 12, where band 3 is reported to aggregate [23,33,54]. In fact, the only suggestion that the conformational equilibrium in cdb3 might regulate band 3 oligomerization derives from the observation that the pH dependence of this conformational equilibrium follows the pH dependence of ankyrin binding, where the ankyrin interaction converts from high affinity to low affinity at a mid pH of 7.2 and from a low affinity to no affinity at mid pH 9.2 [55]. Since most labs agree that ankyrin preferentially associates with tetrameric band 3 [23,26,27,38], it can be hypothesized that the band 3 tetramer might predominate at low pH.

In the present study, we have evaluated the association state of band 3 in stripped erythrocyte membranes incubated at various pHs between 6.0 and 10.5 using size exclusion high performance liquid chromatography (SE-HPLC). We report that incubation of membranes at pH $< 7.2$ favors dimer formation, whereas extended incubation of band 3 at pHs $> 9$ leads to irreversible formation of an oligomeric species larger than the tetramer. Surprisingly, at no pH do we observe a reversible shift towards band 3 tetramers in the absence of ankyrin addition.

## 2. Materials and methods

### 2.1. Materials

Human blood was collected from healthy donors into acid citrate dextrose upon informed consent and used within 2 days. Materials were purchased from the following suppliers: octaethylene glycol monododecyl ether ($C_{12}E_8$), Nikko Chemical Co., Tokyo, Japan; G-4000$SW_{XL}$ SE-HPLC column, Toso-

Haas, Montgomeryville, PA, USA; protein A, Pierce, Rockford, IL, USA; and Stokes radius calibration standards, Pharmacia, Peapack, NJ, USA. All other reagents were from major suppliers and of the highest purity available.

### 2.2. Preparation of KI-IOVs (1 M KI-stripped inside-out membrane vesicles)

KI-IOVs were prepared from fresh blood as described previously [26]. Briefly, red cells were washed and ghosts were prepared by hypotonic lysis in 5 mM sodium phosphate, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0 in the presence of 40 μg/ml phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml leupeptin, and 2 μg/ml pepstatin A (lysis buffer). The lysate was centrifuged at $23\,400\times g$ for 20 min and the supernatant was removed. The membrane pellets were resuspended and washed three additional times in lysis buffer. The white ghosts were then resuspended in a 100-fold volume excess of buffer containing 0.5 mM EDTA, 0.5 mM dithiothreitol (DTT), 40 μg/ml PMSF, pH 8.0, at 4°C, warmed to 37°C over 30 min, and pelleted at $23\,400\times g$ for 30 min to remove spectrin and actin. The remaining peripheral proteins were removed by incubation for 30 min at 37°C in a 100-fold excess of 1 M KI, 25 mM sodium phosphate, 1 mM EDTA, 0.5 mM DTT, 40 μg/ml PMSF, pH 7.5. The resulting KI-IOVs were pelleted for 30 min at $24\,300\times g$, then washed twice with 5 mM sodium phosphate, 1 mM EDTA, pH 8.0, supplemented with 0.5 mM DTT, and stored at a protein concentration of ~2 mg/ml in the wash buffer at 4°C. Membranes were used within 2 days and protein content was determined by the bicinchoninic acid method (Pierce).

KI-IOVs containing only the membrane-spanning domain of band 3 were prepared by treating ghost membranes (~2 mg/ml) with 5 μg/ml trypsin, as described previously [56]. After a 1 h incubation at 0°C, 1 mM PMSF was added to the membrane suspension and incubation was continued at 0°C for an additional 15 min. The treated membranes were then pelleted by centrifugation at $23\,400\times g$ for 20 min and washed two additional times in lysis buffer. Trypsin-treated KI-IOVs (tKI-IOVs) were prepared from the pelleted ghosts, as described above.

### 2.3. SE-HPLC of band 3 extracted from KI-IOVs by $C_{12}E_8$

KI-IOVs were incubated at different pHs by diluting a stock solution of membranes into 50 mM sodium phosphate, 50 mM boric acid, 30 mM NaCl, 1 mM EDTA, 0.5 mM DTT, 10 mM 2-mercaptoethanol, 40 μg/ml PMSF, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 0.01% (w/v) sodium azide (buffer A) preadjusted to the desired pH. The final membrane protein concentration was 0.16 mg/ml in all cases. After the desired incubation period at 37°C, the KI-IOVs were pelleted by centrifugation at $24\,300\times g$ for 15 min at 4°C and washed twice with 5 mM sodium phosphate, 1 mM EDTA, pH 8.0 (wash buffer) supplemented with 0.5 mM DTT and 2.5 mM 2-mercaptoethanol. The pelleted membranes were then diluted to ~2 mg/ml with wash buffer, and solubilized by vortexing with an equal volume of 2% $C_{12}E_8$ in wash buffer. After a 15 min incubation on ice, the samples were centrifuged at $43\,000\times g$ for 30 min to remove any residual particles and analyzed by HPLC.

To determine if the alkali-induced changes in band 3 association state were reversible, KI-IOVs were allowed to equilibrate at 37°C in buffer A adjusted to pH 9.5. After 24 h at pH 9.5, the membranes were pelleted by centrifugation at $23\,400\times g$ and washed with buffer A adjusted to either pH 6.5 or 9.5. The washed membranes were then resuspended in buffer A adjusted to either pH 6.5 or 9.5 and placed back at 37°C. At various times, membranes were collected, washed, extracted with $C_{12}E_8$ as described above, and analyzed by HPLC.

SE-HPLC of solubilized band 3 (~20 μl) was performed at room temperature at a flow rate of 0.5 ml/min, as described by Casey and Reithmeier [23], except the column used was a $7.5\times 300$ mm Toso-Haas TSK-4000$SW_{XL}$. The column buffer was 5 mM sodium phosphate, 100 mM NaCl, 0.1% $C_{12}E_8$, pH 7.0 and protein elution was monitored at 215 nm. The column was calibrated with protein standards, and band 3 dimer, tetramer, and oligomer elution peaks were assigned according to the method of Casey and Reithmeier [23]. To allow comparison among the different pH samples, each chromatogram was normalized to one absorbance unit by dividing the ab-

sorbance from each data point by the largest absorbance obtained in the chromatogram.

### 2.4. Immunoblotting of SE-HPLC fractions

To accurately identify the elution positions of the band 3 oligomers in the SE-HPLC analyses, 30 second fractions of column eluate were collected, adjusted to 1% (w/v) sodium dodecyl sulfate (SDS), and dot-blotted (4 μl) onto nitrocellulose membranes. Dot-blots were blocked overnight with 5% milk in TBS-T (20 mM Tris, pH 7.5, 200 mM NaCl, 0.1% polyethylene glycol, 0.05% Tween 20). To develop, the blots were then washed with TBS-T and incubated at room temperature for 3 h in a solution containing rabbit anti-cdb3 diluted 1/2000 in TBS-T with 1% milk. After further washing, the dot-blots were incubated overnight with 0.05 ng/μl $^{125}$I-labeled protein A diluted in TBS-T with 1% milk. Iodination of protein A was conducted as previously described [57]. To quantitate, blots were exposed overnight to a phosphor screen and imaged using a Cyclone Storage Phosphor System (Packard Instrument Company; Downers Grove, IL, USA). The number of digital light units (DLU) per mm$^2$ was determined for each fraction using the OptiQuant software. To allow comparison between the different pH samples, each chromatogram was normalized to 1 DLU/mm$^2$ by dividing the DLU/mm$^2$ from each fraction by the largest DLU/mm$^2$ obtained in the chromatogram.

## 3. Results

### 3.1. Effect of pH on the oligomeric state of band 3

It has previously been demonstrated that the cytoplasmic domain of band 3 (cdb3) exists in a reversible, pH-dependent conformational equilibrium characterized by a ~20% increase in the Stokes radius as pH is elevated from 6.5 to 9.5 [52]. To determine whether this pH-dependent conformational equilibrium might influence the association state of band 3 in situ, we analyzed the oligomerization states of band 3 in KI-IOVs using SE-HPLC. As previously noted [23,26], band 3 extracted from freshly prepared KI-IOVs with the non-ionic detergent $C_{12}E_8$ elutes as a

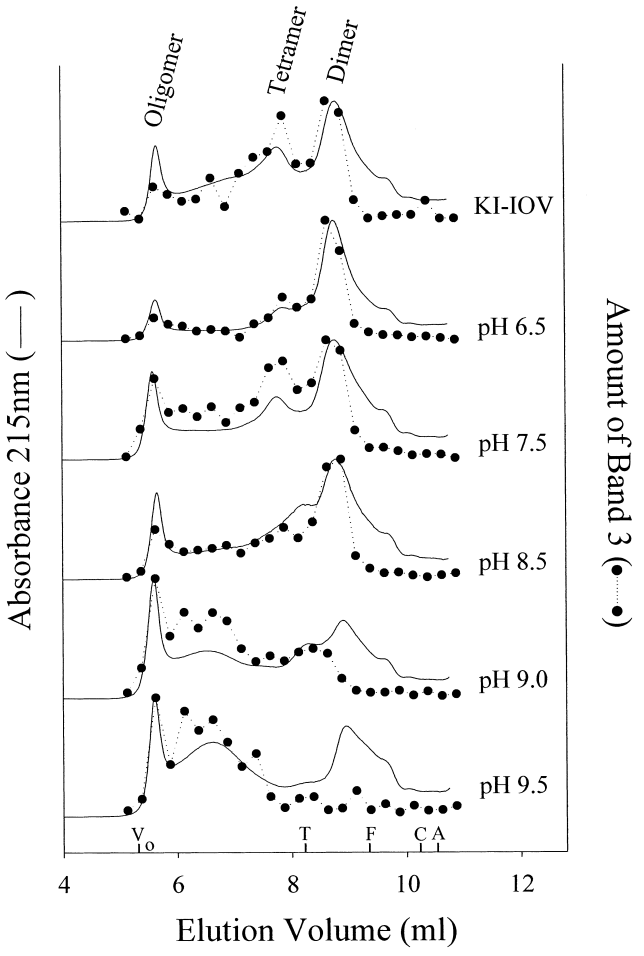


Fig. 1. SE-HPLC analysis of $C_{12}E_8$ detergent extracts of KI-IOVs incubated at different pHs. KI-IOVs were allowed to equilibrate for 24 h at 37°C in 50 mM sodium phosphate, 50 mM boric acid, 30 mM NaCl, 1 mM EDTA, 0.5 mM DTT, 10 mM 2-mercaptoethanol, 40 μg/ml PMSF, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, and 0.01% (w/v) sodium azide at the desired pH. Membranes were then extracted with 2% $C_{12}E_8$ and centrifuged at 43 000×$g$ for 30 min. Supernatants were collected and analyzed by SE-HPLC using a TSK-4000SW$_{XL}$ column. The elution buffer contained 5 mM sodium phosphate, 100 mM NaCl, 0.1% $C_{12}E_8$, pH 7.0, and protein elution was monitored at 215 nm (solid lines). In addition, fractions (0.25 ml) of eluant were collected, and band 3 content was determined by immunoblotting as described in Section 2 (dotted lines). The top chromatogram (labeled KI-IOV) served as a control and represents the elution profile obtained from KI-IOVs that were kept on ice at pH 8.0 during the 24 h incubation. Shown on the $x$ axis are the elution positions of the following protein standards: T, thyroglobulin ($R_S$ = 86 Å); F, ferritin ($R_S$ = 63 Å); C, catalase ($R_S$ = 52 Å); and A, aldolase ($R_S$ = 46 Å). The void volume ($V_0$) was determined from the elution position of blue dextran 2000 (average $M_r = 2\times10^6$).

mixture of dimers, tetramers and higher oligomers (Fig. 1, KI-IOV chromatogram). In further agreement with the characterization of Casey and Reithmeier [23], the band 3 dimer exhibits a Stokes radius of ~76 Å (elution volume = 8.7 ml) and the tetramer a Stokes radius of ~100 Å (elution volume = 7.8 ml). Importantly, the distribution of band 3 oligomeric species observed in Fig. 1 reflects the state of the protein prior to solubilization [23]. Therefore, SE-HPLC of $C_{12}E_8$ detergent extracts is an effective method for evaluating alterations in the oligomeric state of band 3 in situ.

Fig. 1 also includes chromatographic profiles obtained from KI-IOVs incubated for 24 h at 37°C in solutions ranging from pH 6.5 to pH 9.5 (solid lines). Increasing the solution pH over this range results in a decrease in the amount of band 3 dimer and tetramer with a concomitant appearance of several aggregated complexes eluting between 6 and 7 ml. To confirm that these larger oligomers are indeed band 3, we collected the various fractions of eluate and performed quantitative immunoblots for the presence of band 3. As shown in the figure (dotted lines), this analysis confirms that the aggregated complexes indeed contain band 3 and that their emergence at elevated pHs proceeds with a concomitant decrease in the amount of band 3 dimer and tetramer. In addition, the immunoblot of the pH 9.5-treated sample reveals a nearly complete conversion of all band 3 to forms larger than the dimer. Interestingly, the residual peak still present at the dimer position in this pH 9.5 elution profile (solid line) is not band 3. While the proteins present in this fraction have not been fully examined, immunoblot analysis suggests that at least one component must be glycophorin A (data not shown).

In addition to the formation of novel aggregated species following incubation at higher pH; there were three further alterations in the chromatographic properties of band 3 as a function of pH. First, incubation of KI-IOVs in slightly acidic conditions (pH 6.5) consistently yielded less high molecular weight oligomer and less tetramer than control (KI-IOV) and pH 7.5-treated membranes. This observation suggests that the hydrodynamically more compact form of cdb3 occurring at pH 6.5 may somehow favor dimer formation, in contrast to our initial hypothesis. Second, the sharply resolved dimer and tetramer peaks seen in the elution profile at pH 7.5 gradually convert to a species of intermediate mobility (eluting between the dimer and tetramer) as the pH is increased to 9.0. We assume this implies either the formation of a trimer, the rapid interconversion of dimers and tetramers, or the partial unfolding of the dimer, leading to a conformation characterized by a larger hydrodynamic volume. Further studies will obviously be necessary to evaluate these possibilities more rigorously. Finally, there was a slight increase in the amount of oligomeric band 3 species eluting at the void volume as the pH was increased from 6.5 to 9.5. That is, a component even larger than the above oligomer eluting between 6 and 7 ml also increases at high pH. The nature of this aggregate has not been investigated.

### 3.2. *Quantitation of the pH-dependent change in band 3 subunit association*

To quantitate the observed shift in band 3 association state as a function of pH, the percent dimer in

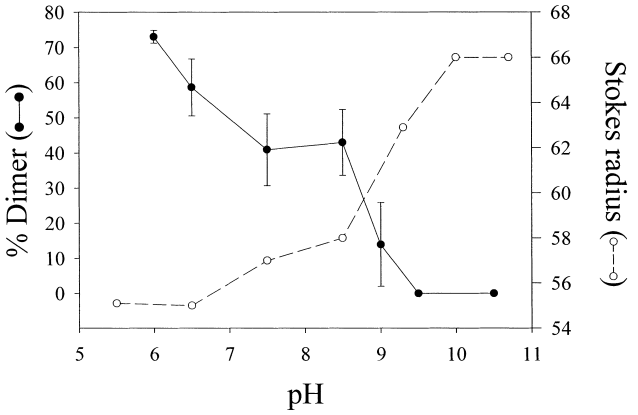


Fig. 2. Dependence of band 3 dimer content on incubation pH. KI-IOV samples were treated and analyzed as detailed in Fig. 1. Results are expressed as the percent dimer relative to the total band 3 content in each sample (solid line), as determined from the integrated areas under the peaks in each chromatogram. The dimer area was integrated from 8.23 ml to 9.52 ml and the total band 3 area was integrated from 5.00 ml to 9.52 ml. The non-band 3 contribution to the dimer peak, obtained from the pH 9.5 analysis, was subtracted in all cases. Data collected at pHs 6.5, 7.5, 8.5, 9.0, and 9.5 represent the mean ± 1 S.D. of five experiments on separate KI-IOV preparations. The data collected at pH 6.0 and 10.5 represent the average of three chromatographs conducted on a single KI-IOV preparation. The pH-dependent change in cdb3's Stokes radius (dashed line) was re-plotted from the data in [52].

each chromatogram (Fig. 1, solid lines) was calculated from the corrected areas under the dimer peak (integrated from 8.23 ml to 9.52 ml) relative to the total band 3 area (integrated from 5.00 ml to 9.52 ml) in each chromatogram. For correction of the band 3 dimer content, the contribution of the non-band 3 species eluting at pH 9.5 was subtracted from each chromatogram, based on the dot-blots shown in Fig. 1. As seen in Fig. 2, the observed pH dependence of dimer content is triphasic, with the dimer favored at low pH and larger oligomers favored at high pH. Although insufficient data have been collected to determine the p$K_a$ of the two inflection points, the shape of the curve still resembles the previously published titration curve of the conformational change in cdb3, as indicated by the change in Stokes radius [52]. This correlation suggests that the conformation of cdb3 may influence the association state of band 3 in situ. More specifically, the compact form of cdb3 that occurs under slightly acidic conditions may favor dimer formation, whereas the elongated state of cdb3, induced by alkaline treatment, may allow formation of oligomeric species larger than the tetramer. No treatment identified to date, except the addition of ankyrin [23,26,27,38], appears to favor tetramer formation.

### 3.3. Kinetics of the pH-dependent change in band 3 subunit association

The SE-HPLC profiles in Fig. 1 show that conversion of band 3 dimers to higher oligomers at pH 9.5 is complete by 24 h. The kinetics of this change in band 3 association at pH 9.5 and pH 6.5 are shown in Fig. 3. Freshly prepared KI-IOVs (at pH 8.0) containing approximately 45% dimer (Fig. 3, $t=0$ h) were diluted into solutions of pH 9.5 or pH 6.5 and incubated at 37°C. At the indicated time points, an aliquot of each suspension was processed and analyzed by HPLC, as described in Section 2. As shown in the figure, incubation of KI-IOVs at pH 9.5 led to complete disappearance of the dimer and its conversion to higher oligomers with a half time of ~4 h. Also shown is a rapid ~18% increase in the amount of dimer at the expense of tetramer within the first hour of the incubation at pH 6.5. During the remaining 23 h, there was only an additional ~7% increase in the amount of dimer. These data support

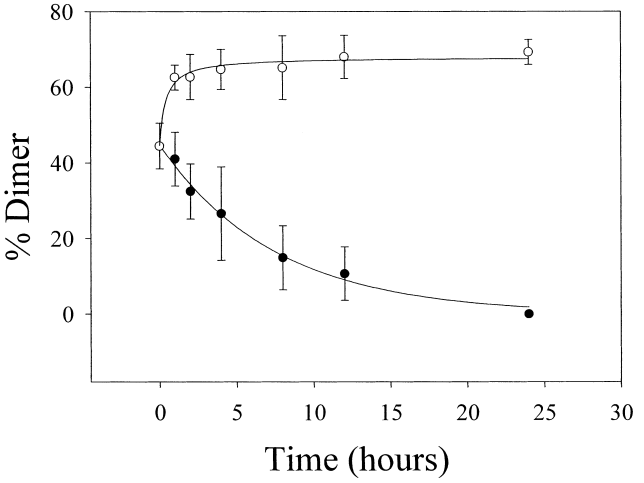


Fig. 3. Time dependence of the change in band 3 association state during incubation of KI-IOVs at pH 6.5 and 9.5. KI-IOVs were equilibrated at 37°C in 50 mM sodium phosphate, 50 mM boric acid, 30 mM NaCl, 1 mM EDTA, 0.5 mM DTT, 10 mM 2-mercaptoethanol, 40 μg/ml PMSF, 0.2 μg/ml leupeptin, 0.2 μg/ml pepstatin A, 0.01% sodium azide adjusted to either pH 6.5 (○) or 9.5 (●). At the indicated time points, the membranes were extracted and analyzed by SE-HPLC, as detailed in Fig. 1. The percent dimer in each chromatogram was determined from the integrated area under the peaks, as described in Fig. 2. Each point represents the mean ± 1 S.D. of three experiments on separate KI-IOV preparations.

the hypothesis that the more compact form of cdb3 occurring at low pH favors the band 3 dimer, whereas the elongated form of cdb3 at pH 9.5 allows band 3 aggregation.

### 3.4. Effect of pH on association of the membrane-spanning domain of band 3

As mentioned above, erythrocyte band 3 contains two major structural domains, a 58-kDa carboxyl-terminal membrane domain and a 43-kDa amino-terminal cytosolic domain. To determine how each domain individually contributes to the pH-dependent changes in band 3 association state, we removed cdb3 from the KI-IOVs with trypsin and evaluated the effect of pH on the association state of the membrane-spanning domain. In agreement with previous reports [23,58], the membrane-spanning domain extracted from the trypsinized KI-IOVs migrated almost exclusively as a dimer in $C_{12}E_8$ detergent solution (Fig. 4, tKI-IOV chromatogram). Further, prior incubation of the trypsinized KI-IOVs for 24 h at

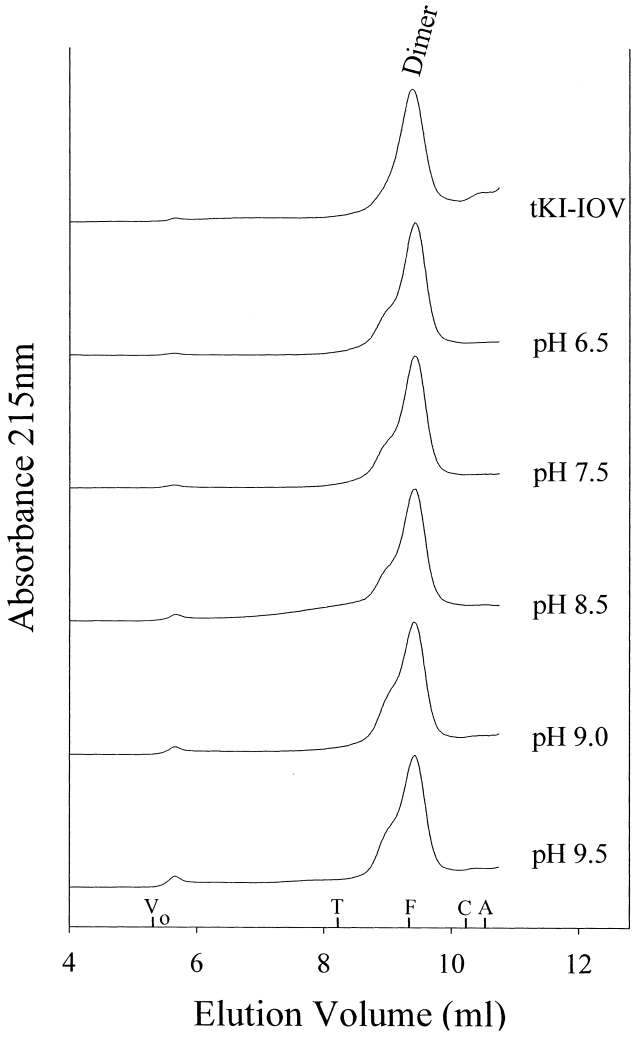


Fig. 4. SE-HPLC analysis of the membrane-spanning domain of band 3 following incubation of tKI-IOVs at different pHs. Red blood cell ghost membranes were treated with trypsin to remove the cdb3, and KI-IOVs were prepared. The tKI-IOVs were equilibrated at the indicated pHs for 24 h at 37°C and analyzed by SE-HPLC as detailed in Fig. 1. A typical result from two independent experiments is shown.

37°C in solutions ranging from pH 6.5 to pH 9.5 yielded no change in oligomeric species (Fig. 4). There was a slight increase in the amount of band 3 eluting at the void volume as the pH was increased from 6.5 to 9.5. However, since the quantity of this aggregated protein at pH 9.5 was always less than 2% of the total protein analyzed, we conclude that the alkali-induced aggregation of band 3 is not driven by a tendency of the membrane-spanning domain to associate at high pH.

### 3.5. Reversibility of the alkali-induced association of band 3

To evaluate whether the band 3 oligomerization seen in situ at high pH reflects a possible regulatory equilibrium within the band 3 population, we explored the natural reversibility of this interaction. For this purpose, alkali-induced oligomers were generated by incubating KI-IOVs for 24 h at 37°C in pH 9.5 buffer and then re-incubating the KI-IOVs at either pH 6.5 or 9.5 prior to solubilization in $C_{12}E_8$ detergent. We found that resuspension of the pH 9.5-treated KI-IOVs into a pH 6.5 solution resulted in no dissociation of the alkali-induced band 3 oligomers over a 24 h incubation period (data not shown). Since reducing agents were present during all steps, the irreversible association of band 3 in KI-IOVs at pH 9.5 is probably not due to disulfide bond formation. In addition, SDS–polyacrylamide gel electrophoresis demonstrated that greater than 90% of the band 3 in the pH 9.5-treated KI-IOVs migrated as a monomer (data not shown), suggesting that the association was not stabilized by covalent bond formation. Still, the aggregation was sufficiently avid to prevent dissociation back to dimers within the 24 h re-equilibration period at pH 6.5.

## 4. Discussion

In previous research, we have noted that the pH dependence of ankyrin binding to KI-IOVs corresponds closely to the pH dependence of the conformational equilibrium in cdb3, with both titrations displaying three structural states characterized by transitions centered at pHs 7.2 and 9.2 [3,50–53,55]. Since ankyrin strongly prefers to bind the low pH conformation of cdb3 [55] and the tetrameric form of intact band 3 [23,26,27,38], we hypothesized that the low pH conformation might, in fact, promote tetramer formation in situ. We further speculated that titration of cdb3 from high pH through the structural transitions at pHs 9.2 and 7.2 might convert band 3 from a monomer to dimer to tetramer, respectively. However, in contrast to these expectations, the conformational equilibrium in cdb3 was found to correlate only with the stability of the band 3 dimer (Fig. 2). In fact, the default oligomer

formed when the dimer destabilized was not the anticipated tetramer, but rather an irreversible aggregate. These data suggest that the conformational equilibrium in cdb3 does not function physiologically to regulate band 3 subunit equilibrium, but rather may enable cdb3's participation in some other structure-dependent process. Candidate processes that might depend on cdb3's conformational flexibility include the formation or optimization of membrane–skeleton interactions, especially since the affinity of ankyrin for KI-IOVs correlates directly with the conformational state of cdb3 [55]. Alternatively, since the conformational equilibrium also occurs in kidney cdb3 [59], which has no affinity for ankyrin or any other known erythrocyte protein [59,60], the structural changes may function more to regulate the membrane-spanning domain of band 3. Further studies will obviously be necessary to resolve why cdb3 changes conformation so easily.

The continued inability to identify any solution condition that can promote band 3 tetramer formation in situ was not expected, especially since band 3 isolated from freshly prepared KI-IOVs exists as a mixture of dimers and tetramers [23–28]. Because band 3 tetramers are not formed in the absence of ankyrin [38], one is compelled to conclude that ankyrin may be the sole driving force for band 3 tetramerization in vivo. Still, the fact that such ankyrin-induced tetramers remain stable for many hours at 4°C following ankyrin removal [23–25,27,28], and since they survive for at least 24 h at 37°C (Fig. 1), one must surmise that a weak natural affinity exists to stabilize the dimer–dimer associations once they are induced. Thus, non-interacting band 3 dimers should have a diffusion coefficient of approximately $10^{-9}$ cm$^2$/s in cytoskeleton-depleted membranes [61,62], suggesting that any unassociated dimers would diffuse apart at nearly 1 micron/s (calculated from the relationship $x^2 = 2Dt$; where $D$ is $10^{-9}$ cm$^2$/s and $t$ is 1 s) if unrestrained by dimer–dimer interactions. The dimer–dimer contacts revealed in the crystal structure of cdb3 [22] could provide the hypothesized stabilizing interactions required to prolong tetramer lifespan in situ.

Although the conformational equilibrium in cdb3 may not serve to regulate band 3 tetramer content, the correlation between cdb3 conformation and band 3 aggregation in situ (Fig. 2) still suggests that aggregation depends in some way on the structure of cdb3. Among several possible mechanisms, the most obvious would have been that cdb3 itself might aggregate in a pH-dependent manner. However, no stable oligomers of purified cdb3 have ever been observed upon extended incubation at high pH ([52] and data not shown). A second conceivable mechanism was that the membrane-spanning domain of band 3 might spontaneously aggregate in the absence of cdb3, and that this aggregation would have only been allowed when cdb3 was titrated to a permissive (i.e. high pH) conformation. However, once again, the membrane-spanning domain displayed no disposition to aggregate at any pH where intact band 3 was seen to associate (Fig. 4). It would, therefore, seem likely that band 3 aggregation might require an intact protein, perhaps in a membrane environment. In this scenario, the conformational change in cdb3 might be envisioned to force exposure of previously cryptic aggregation sites in the membrane-spanning domain, leading to irreversible clustering of the polypeptide. Interestingly, Salhany et al. recently reported that conformational changes within the membrane-spanning domain of band 3 induced the dissociation of band 3 dimers at 37°C [46]. However, whether pH-dependent structural changes within cdb3 induce such conformational changes within the membrane-spanning domain has not been determined.

Although our results largely confirm those of Vince et al. [54] wherever overlap occurs, one apparent discrepancy warrants further explanation. These authors observed no band 3 aggregation upon incubation of purified band 3 in solutions of elevated pH, while we obviously noted extensive aggregation in our model system. However, two significant differences in the methodologies must be considered. First, Vince et al. incubated their band 3 preparations for only 1 h before HPLC analysis. As indicated in Fig. 3, we also observed no conversion of band 3 dimer to oligomer at this short incubation time. Second, the high pH incubations of Vince et al. were conducted on detergent-solubilized band 3, not the membrane-associated polypeptide. Since modeling studies predict that protein association within a membrane should be enhanced at least $10^6$-fold relative to the solubilized counterparts [63], the failure to observe band 3 aggregation in their study could also be

caused by the lower protein concentration and/or absence of rotational and translational restrictions in the solubilized system.

In summary, regulatory changes in the band 3 subunit association equilibrium do not appear to be controlled by the conformational equilibrium in cdb3, even though this equilibrium may directly regulate ankyrin affinity. It now seems more likely that ankyrin is solely responsible for band 3 tetramer formation and that prolonged tetramer survival following ankyrin removal is simply a consequence of weak dimer–dimer interactions that are insufficient to maintain the tetrameric state indefinitely.

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