Molecular Basis of Altered Red Blood Cell Membrane Properties in Southeast Asian Ovalocytosis: Role of the Mutant Band 3 Protein in Band 3 Oligomerization and Retention by the Membrane Skeleton

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Southeast Asian ovalocytosis (SAO) is an asymptomatic trait characterized by rigid, poorly deformable red cells that resist invasion by several strains of malaria parasites. The underlying molecular genetic defect involves simple heterozygous state for a mutant band 3 protein, which contains a deletion of amino acids 400 through 408, linked with a Lys 56-to-Glu substitution (band 3-Memphis polymorphism). To elucidate the contribution of the mutant SAO band 3 protein to increased SAO red blood cell (RBC) rigidity, we examined the participation of the mutant SAO band 3 protein in increased band 3 attachment to the skeleton and band 3 oligomerization. We found first that SAO RBC skeletons retained more band 3 than normal cells and that this increased retention preferentially involved the mutant SAO band 3 protein. Second, SAO RBCs contained a higher percentage of band 3 oligomer-ankyrin complexes than normal cells, and these oligomers were preferentially enriched by the mutant SAO protein. At the ultrastructural level, the increased oligomer formation of SAO RBCs was reflected by stacking of band 3-containing intramembrane particles (IMP) into longitudinal strands. The IMP stacking was not reversed by treating SAO RBCs in alkaline pH (pH 11), which is known to weaken ankyrin-band 3 interactions, or by removing the cytoplasmic domain of band 3 from SAO membranes with trypsin. Finally, we found that band 3 protein in intact SAO RBCs exhibited a markedly decreased rotational mobility, presumably reflecting the increased oligomerization and the membrane skeletal association of the SAO band 3 protein. We propose that the mutant SAO band 3 has an increased propensity to form oligomers, which appear as longitudinal strands of IMP and exhibit increased association with membrane skeleton. This band 3 oligomerization underlies the increase in membrane rigidity by precluding membrane skeletal extension, which is necessary for membrane deformation.

SOUTHEAST ASIAN ovalocytosis (SAO) is a dominantly inherited trait characterized by the presence of oval-shaped red blood cells (RBCs) that are rigid and resistant to invasion by several strains of malaria parasites.[1-8] The underlying molecular defect involves a deletion of nine codons (400 through 408) in the erythroid band 3 protein gene; this defect is tightly linked to the Lys 56-to-Glu substitution (band 3-Memphis polymorphism).[9-12] It is of considerable interest that the primary molecular defect in SAO can be assigned to band 3 protein,[13] the major RBC integral membrane protein, because membrane deformability has been believed to be regulated principally by the properties of the membrane skeleton.[14-16]

Band 3 is the most abundant protein of the RBC membrane, consisting of two domains with distinct structure and function.[17,18] The N-terminal 41-kD cytoplasmic domain of band 3 (cdb3) anchors the membrane skeleton to the membrane via interactions with ankyrin, and it binds hemoglobin and several glycolytic enzymes. The C-terminal 56-kD membrane domain consists of 14 membrane-spanning α helices connected by endoplasmic and ectoplasmic loops. The main function of the membrane domain is to mediate anion exchange across the membrane. Several mutations of the human band 3 have recently been described, including Glu 40-to-Lys and Pro 327-to-Arg substitutions, respectively, leading to spherocytic hemolytic anemia with protein 4.2 deficiency,[19,20] Pro 868-to-Leu substitution reported in association with acanthocytosis and increased anion transport,[21] and a diverse group of band 3 mutations that lead to the phenotype of hereditary spherocytosis and band 3 deficiency.[22-24]

Several biochemical, biophysical, and ultrastructural abnormalities of band 3 in SAO RBCs have been reported, including increased band 3 association with membrane skeleton,[13] increased propensity to form oligomers and to aggregate in the membrane,[25,26] decreased anion transport,[27] and reduced lateral and rotational mobilities.[11,13,26,28,29] However, it has not been established whether or not these abnormalities directly reflect altered properties of the mutant SAO protein, or conversely, whether they represent nonspecific consequences of other alterations of SAO RBCs, such as increased adenosine triphosphate (ATP) consumption resulting in a premature ATP depletion.[30] In normal RBCs, ATP depletion leads to a formation of high-molecular-weight aggregates of membrane proteins[31] and increased retention of membrane skeletal proteins by the RBC membrane.[32] The role of ATP depletion is particularly important in light of the data that the normalization of ATP stores of SAO RBCs leads to a decrease in the resistance of SAO RBCs to malaria parasite invasion.[30]

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To elucidate the contribution of the mutant SAO band 3 protein to the increase in membrane rigidity, we have combined three independent approaches. First, using size exclusion high-performance liquid chromatography (HPLC), we have analyzed the oligomeric forms of band 3 and the enrichment of the skeleton-associated band 3 oligomers by the SAO mutant protein. Second, we have used polarized fluorescence depletion to measure the rotational mobility of band 3 in the membranes of intact RBCs. Third, using freeze-fracture electron microscopy, we have visualized the distribution of intramembrane particles (IMP), which are principally composed of band 3 protein. We find first that the band 3 protein in SAO RBCs, but not in normal RBCs, forms high-molecular-weight oligomers with high band 3-to-ankyrin stoichiometry and that these oligomers are selectively enriched in the mutant SAO band 3 protein. Second, we report that the mutant SAO protein is preferentially retained by the skeleton. Third, we find that the rotational mobility of the band 3 protein in intact SAO red cells is markedly reduced compared with band 3 in normal RBCs and that this reduction is manifested by a decrease in the rapidly rotating fraction of band 3, as well as a marked increase in the rotationally immobile fraction. Finally, on ultrastructural examination, we find that the band 3-containing IMP stack into longitudinal strands and that this stacking is unaffected by membrane expansion by detergents, proteolytic removal of cdb3 from the membrane, or chymotryptic dissection of the third external loop of the transmembrane domain. We conclude that the mutant SAO band 3 protein undergoes linear stacking in the intact membrane, thus restricting lateral and rotational mobilities of band 3 molecules. Because membrane deformation requires extension of the skeletal network as well as lateral movement of band 3 molecules in the plane of the membrane, we propose that the increased oligomerization and membrane skeletal association of the SAO band 3 protein rigidifies the SAO RBC membrane by precluding the skeletal reorganization necessary for RBC deformation.

MATERIALS AND METHODS

Extractability of band 3 from RBC membranes by the nonionic detergent octaethylene glycol n-dodecyl monoether ($C_{12}E_8$). Blood from individuals with heterozygous SAO was collected in citrate-phosphate-dextrose anticoagulant bags or tubes and shipped on ice overnight to Boston, MA, for evaluation. RBCs were sedimented at 2,000 rpm for 10 minutes and washed with phosphate-buffered saline (PBS).

RBC ghosts (3 mg protein per milliliter), prepared by hypotonic lysis,[33] were mixed with equal volumes of 0.5% $C_{12}E_8$ in isotonic buffer (5 mmol/L NaPi, pH 7.4; 150 mmol/L NaCl) or hypotonic buffer (5 mmol/L NaPi, pH 7.4) at 4°C for 10 minutes. After centrifugation at 150,000*g* for 30 minutes, the supernatants and pellets were dissolved in sodium dodecyl sulfate (SDS) and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) according to Agre et al.[34] Band 3-to-spectrin ratios in isotonic and hypotonic skeletal shells were analyzed by densitometric gel scans. The values were obtained from four experiments.

Retention of the normal and mutant band 3 in SAO membrane skeletons. To assess whether or not band 3 retained by the SAO skeleton is selectively enriched in mutant band 3 protein, we pretreated intact SAO and band 3-Memphis RBCs with chymotrypsin (0.5 mg/mL) in PBS buffer at 37°C for 90 minutes.[35] Membrane prepared from these cells was extracted with $C_{12}E_8$ (0.5%) under isotonic conditions. The extract supernatants and insoluble skeletons were prepared as described above, dissolved in SDS, and analyzed using 7.5% to 15% Laemmli gels.[36] In normal cells, chymotryptic digestion produced an N-terminal 60-kD segment of band 3 resulting from cleavage at Tyr 553.[37] In SAO RBCs containing the linked band 3-Memphis polymorphism, chymotryptic digestion produced both the 60-kD band (the normal allele) and an abnormal 63-kD band.[38] For comparison, samples from a normal individual with morphologically normal RBCs and the band 3-Memphis polymorphism were analyzed in parallel. The relative abundance of mutant to normal band 3 in supernatant and skeleton fractions was assessed by measuring the ratio of 63-kD to total (60-kD + 63-kD) fragments by densitometric gel scans.

Size exclusion HPLC of band 3 extracted from spectrin-depleted membranes by $C_{12}E_8$. RBC ghosts, prepared by hypotonic lysis, were incubated in 0.1 mmol/L NaPi, pH 8.0, buffer at 37°C to remove spectrin.[39] The spectrin-depleted vesicles (2.0 to 2.5 mg/mL protein, final concentration) were dissolved in 0.5% $C_{12}E_8$ in hypotonic buffer (5 mmol/L NaPi, pH 7.4) at 4°C. After centrifugation at 150,000*g* for 30 minutes, the supernatants (45 to 225 μg protein in 50 to 250 μL buffer) were analyzed by size-exclusion HPLC using a TSK-4000 SW_{XL} column (7.8 × 300 mm; Tosohaas, Tokyo, Japan), as described previously.[40] The standard elution buffer contained 0.01% $C_{12}E_8$, 100 mmol/L NaCl, and 5 mmol/L NaPi, pH 7.0. Protein in HPLC fractions was concentrated by centrifugation of 0.5- to 2.0-mL aliquots in a 10,000-molecular weight (mol wt) cutoff ultrafiltration unit (Millipore, Lexington, MA) at 5,000 rpm for 15 to 20 minutes. The concentrated supernatant was removed, dissolved in SDS, and analyzed by SDS-PAGE according to Laemmli.[36]

Enrichment of band 3 oligomers by the SAO band 3 protein. To assess whether or not SAO band 3 oligomers are selectively enriched in mutant band 3 protein, we pretreated intact SAO and normal RBCs with chymotrypsin as described above. Spectrin-depleted membrane vesicles were derived from these cells and extracted with $C_{12}E_8$, and the solubilized supernatants were analyzed by size-exclusion HPLC, as described above. The relative abundance of mutant to normal band 3 in different band 3-containing HPLC peaks was assessed by measuring the ratio of 63-kD to total (60-kD + 63-kD) fragments after SDS-PAGE, as described above.

Freeze-fracture electron microscopy. Washed RBCs from blood collected within 24 hours were fixed in 1.75% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.4, for 1.5 hours at 0°C. The fixed cells were washed five times in 0.05 mol/L sodium cacodylate, pH 7.4, glycerinated (final glycerol concentration, 33%), frozen in freon, and transferred to liquid nitrogen. The samples were fractured in a freeze-etch unit (model BAF 400D; Balzers, Hudson, NH) at −110°C. Samples were rotary-shadowed at a 25° angle with platinum-carbon and at a 90° angle with carbon.[41] Replicas were viewed in a transmission electron microscope (model JEM 100S; JEOL USA, Peabody, MA) at an accelerating voltage of 80 kV.

To test if the IMP stacking persisted at low IMP density, we treated SAO RBCs with prehemolytic concentration of Triton X-100 (0.02%) at 0°C for 10 minutes before these cells were fixed and examined by freeze-fracture electron microscopy. Further, we examined if the IMP stacking in SAO could be reversed by (1) incubation of SAO RBCs in PBS at pH 11.0 (0°C, 30 minutes), which was believed to weaken the ankyrin-band 3 interaction,[42] (2) chymotryptic digestion (0.5 mg/mL, 37°C, 60 minutes) of intact SAO RBCs to cleave band 3 extracellularly at Tyr 553,[37] or (3) trypsin digestion (5 μg/mL, 0°C, 30 minutes) of isolated SAO ghosts (3 mg protein per milliliter) to remove cdb3 from the membrane.[17] Specific band 3 cleavages by the proteolytic enzymes in the above

experiments were verified by SDS-PAGE of the resultant membrane specimen.

Band 3 rotational mobility in intact RBCs. RBC band 3 was fluorescently labeled, as previously described.[43] Briefly, fresh RBCs from blood collected within 24 hours were washed in high potassium PBS (KPBS; 140 mmol/L KCl, 15 mmol/L NaPi, 10 mmol/L glucose, pH 7.4) and mixed with 0.25 mg/mL (final concentration) eosin-5-maleimide for 12 minutes at room temperature. Cells were then washed three times in KPBS with 1% bovine serum albumin (BSA). Under these conditions, greater than 80% of the membrane-associated fluorescence was covalently bound to band 3 in normal and SAO RBCs. The stoichiometry of labeling was approximately 1 eosin molecule per band 3 monomer in both normal and SAO cells, in agreement with the results of Tilley et al[28] and Che et al.[26]

The technique of polarized fluorescence depletion was used to measure the rotational mobility of eosin-labeled band 3 in membranes of intact RBCs, as previously described.[43] Briefly, recovery of fluorescence after a ground state depletion laser pulse depended both on the triplet lifetime(s) of the fluorophore and on the rotational relaxation time(s) of band 3. In addition, the fraction of band 3 molecules that was rotationally immobile on the time scale of the experiment was obtained from the residual anisotropy of the fluorescence intensities excited by parallel and perpendicular probe beams. Using our laser microscopy photometer, two exponential components of anisotropy decay and a residual anisotropy could be resolved, so data were fitted by nonlinear least-squares analysis to the equation $r(t) = r(\infty) + \alpha \cdot e(-t/\tau_1) + \beta \cdot e(-t/\tau_2)$, where r(t) is the anisotropy at time t, $r(\infty)$ is the residual anisotropy, and α and β are the fractions of molecules with rotational correlation times τ_1 and τ_2, respectively. For anisotropy decay curves in which it was apparent that $\tau_1 < 40\ \mu s$, r (0) was set equal to the anisotropy value determined using eosin-labeled RBCs fixed with 1% glutaraldehyde. Band 3 in the membranes of fixed cells exhibited no rotational mobility, and the anisotropy, typically 0.27 to 0.33, was time-invariant. This procedure allowed the reliable determination of α but not τ_1 in such cases.

The preparation of intact RBC samples for polarized fluorescence depletion experiments and the time-resolved photon-counting laser microscope used in such experiments have been described in detail.[43]

RESULTS

Preferential association of mutant band 3 protein with SAO membrane skeleton. Previous studies showed conflicting data on the retention of band 3 in membrane skeletons. In one study using extraction with Triton X-100, no differences were observed in band 3 retention between SAO RBCs and normal RBCs.[10] In another report, increased retention of band 3 by the skeleton was observed and it was attributed to a nonspecific trapping of band 3 by the SAO skeleton.[12] Therefore, we first examined differences between normal RBCs and SAO cells in the retention of band 3 by the skeleton, using a mild nonionic detergent, $C_{12}E_8$.[25,40]

SAO membranes and normal RBC membranes (3 mg protein per milliliter) were extracted with equal volumes of 0.5% $C_{12}E_8$ to determine the proportion of band 3 that was retained by the detergent-insoluble membrane skeleton. Band 3-to-spectrin ratios of the resulting skeletal shells were analyzed by SDS-PAGE followed by dye quantitation after elution from the gels. Increased retention of band 3 in SAO membrane skeletons was detected under both isotonic and hypotonic extraction conditions (Fig 1). After isotonic $C_{12}E_8$ extraction, 59% of band 3 was retained in SAO skeletons, compared with 38% in normal skeletons. No ankyrin were released from the skeleton under these conditions. After extraction under hypotonic conditions, 35% of band 3 was retained in SAO skeletons, compared with 21% in normal skeletons. The band 3 retention in $C_{12}E_8$ shells was not significantly affected by the use of higher extraction volumes (five instead of one) of 0.5% $C_{12}E_8$, or the concentration of $C_{12}E_8$ (0.1% to 1%), or additional washes with 0.5% $C_{12}E_8$ (data not shown).

To explore the possible role of the mutant SAO band 3 in the increased retention of band 3 in SAO membrane skeletons, we investigated whether or not the SAO membrane skeletons were selectively enriched in the mutant SAO band 3 protein. We took advantage of the band 3-Memphis polymorphism[38,44-46] that is tightly linked with the SAO mutation[9-13] and the availability of subjects carrying the band 3-Memphis polymorphism without other associated band 3 abnormalities. After chymotryptic cleavage of band 3 protein at Tyr 553 in intact cells, the band 3-Memphis polymorphism produces a 63-kD proteolytic fragment, rather than the normal 60-kD fragment. The decrease in electrophoretic mobility of the proteolytic fragment of band 3-Memphis is caused by a change in a single amino acid, Lys 56 to Glu 56.[45,46] Figure 2 depicts the analysis of mutant band 3 in SAO membrane skeletons. The percentage of 63-kD fragment in the original SAO ghosts was 40%. In contrast, the amount of the 63-kD fragment was increased in the $C_{12}E_8$-insoluble pellet (52%), whereas it was diminished in the $C_{12}E_8$-soluble supernatant (32%). Thus, the fraction of band 3 associated with the skeleton of SAO RBCs was enriched in mutant band 3 protein. For comparison, we examined RBCs of an asymptomatic carrier of band 3-Memphis and found no enrichment of the skeletal fraction by the 63-kD band 3 fragment. The percentage of 63-kD fragment was 50% in band 3-Memphis ghosts, the $C_{12}E_8$-soluble supernatant, and the insoluble pellet (Fig 2).

Enrichment of band 3 oligomers by the mutant SAO protein. To elucidate the molecular basis of increased retention of the mutant protein by the membrane skeleton, we analyzed the dimeric and oligomeric states of band 3 protein in SAO RBCs and normal RBCs using size-exclusion HPLC. Band 3 oligomeric states were analyzed in the native membrane by extracting spectrin-depleted inside-out vesicles with the nonionic detergent $C_{12}E_8$ and centrifuging at 150,000*g* for 30 minutes. In control cells, the detergent extracted approximately 73% of band 3 from the membrane, leaving the remainder of band 3 protein in the pellet fraction. The amount of ankyrin and protein 4.2 extracted from control cells under such conditions was 83% and 72%, respectively. The amounts of band 3 protein (69%), ankyrin (72%), and protein 4.2 (63%) solubilized from SAO RBCs were all slightly lower than the corresponding amounts extracted from control cells (data not shown).

In both normal and SAO RBCs, HPLC separation resolved the $C_{12}E_8$ extract into two major protein peaks and one detergent peak (Fig 3). The slower, more abundant peak was identical in mobility to that of band 3 dimers, whereas the faster peak represented complexes containing ankyrin and band 3 oligomers. Often, a small but variable amount of

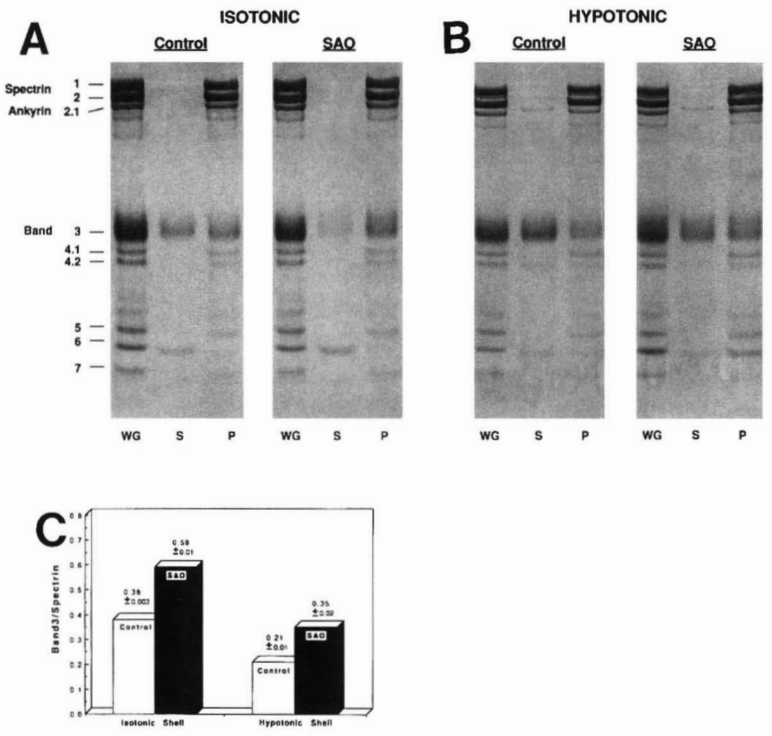

Fig 1. Extractability of band 3 from SAO membranes by $C_{12}E_8$. (A) $C_{12}E_8$ extraction of normal and SAO ghosts was performed under isotonic conditions. (B) $C_{12}E_8$ extraction of ghosts was performed under hypotonic conditions. (C) Band 3:spectrin ratio from $C_{12}E_8$-insoluble normal and SAO shells. Note that the band 3:spectrin ratio was markedly increased in SAO skeletons compared with normal skeletons, indicating substantial band 3 retention.

void volume peak (Vo) also appeared in HPLC profiles. The identification of the band 3 dimer peak on size-exclusion HPLC agreed with that determined previously by using sedimentation velocity analysis and partial specific volume determination.[40] Importantly, differences between normal RBCs and SAO RBCs were found in the relative distribution of the band 3 dimer and ankyrin/band 3 oligomer peaks. The amount of the ankyrin/band 3 oligomer peak (including the overlapping Vo peak) was considerably greater in SAO RBCs (62%) than in normal cells (42%). There was a corresponding decrease in the dimer fraction of band 3 protein in SAO cells (38%) compared with normal cells (58%).

In both normal and SAO RBCs, the band 3 dimer fraction contained not only band 3 but also protein 4.2 and a small amount of protein 4.1, as shown in SDS-polyacrylamide gels (Fig 3). Further, glycophorin A was detected in a fraction that eluted slightly slower than the band 3 dimer fraction (data not shown). Notably, no ankyrin was detected in the band 3 dimer fraction in either normal or SAO RBCs. In both SAO and normal RBCs, the band 3 oligomer fraction contained not only band 3 but also protein 4.2 and ankyrin. However, in contrast to the band 3 dimer fraction, the band 3 oligomer fraction was devoid of glycophorin and protein 4.1. Importantly, the band 3-to-ankyrin stoichiometry in the oligomer fraction of SAO RBCs differed from normal cells. Densitometric scans of gels corresponding to the oligomer fraction showed that SAO RBCs contained 6.0 copies of band 3 for each copy of ankyrin, whereas normal cells contained about 4.4 copies of band 3 for each copy of ankyrin. The molar ratio of protein 4.2 to band 3 was 1:6 in both dimer and oligomer fractions from both SAO and normal RBCs.

We next examined whether or not the oligomer fraction is selectively enriched in the mutant band 3 protein by monitoring the percentage of 63-kD fragment of band 3 in the band 3 dimer and oligomer fractions derived from chymotrypsin-treated SAO RBCs and band 3-Memphis RBCs. The relative amount of 63-kD fragment in SAO ghosts was 40%; this value was markedly increased in the band 3 oligomer fractions (50% to 55%; HPLC fractions 1 through 3, Fig 4) and markedly depleted in the dimer fractions (25% to 35%; HPLC fractions 5 through 7, Fig 4). The enrichment of mutant band 3 in the oligomer fractions was specific for SAO RBCs: in RBCs carrying only the band 3-Memphis variant, the percentage of the 63-kD fragment was nearly identical in band 3 dimer and oligomer fractions (Fig 4).

In complementary studies, band 3 species obtained by HPLC separation of normal and SAO samples without prior chymotrypsin digestion (Fig 3) were subjected to limited tryptic digestion in $C_{12}E_8$ solution. This treatment generates 22-kD and 25-kD fragments that represent the first 180 amino acid peptides from normal and SAO band 3, respectively. The percentage of the abnormal 25-kD fragment derived from the mutant protein was likewise found to be increased in the oligomer fraction (40%) compared with the dimer fraction (25%). This finding supports the conclusion that the oligomer fraction is preferentially enriched by the mutant band 3 protein. Furthermore, because there was a similar degree of enrichment by the mutant protein of oligomeric band 3 fraction and the band 3 fraction associated with the skeleton, the data suggest that band 3 oligomers, enriched in the mutant SAO protein, are preferentially associated with membrane skeleton.

Irreversibility of IMP stacking in SAO RBC membranes.

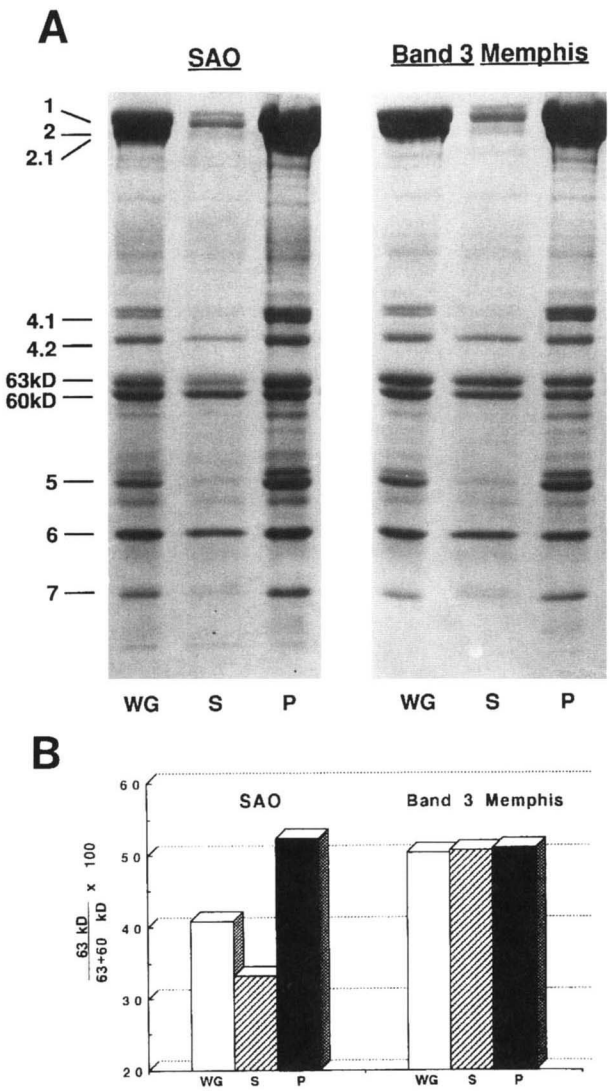

Fig 2. Analysis of mutant band 3 in SAO membrane skeletons. Intact SAO and band 3-Memphis RBCs were treated with chymotrypsin to cleave the band 3 quantitatively into the 35-kD [amino acids (AA) 554 to 911] fragment and the complementary 63-kD or 60-kD fragments (AA 1 to 553). The 63-kD and 60-kD fragments were derived from the mutant and normal alleles of band 3, respectively. White ghosts (WG) prepared from these cells were extracted with $C_{12}E_8$ (0.5%) under isotonic conditions. (A) Extract supernatants (S) and insoluble skeletons (P) were dissolved in SDS and analyzed using Laemmli gels. (B) The ratio of 63-kD to total (60-kD + 63-kD) fragments in the supernatant and skeleton fractions was analyzed by densitometric gel scans. Note that the percentage of 63-kD fragment was 50% in band 3-Memphis ghosts, the $C_{12}E_8$-soluble supernatant, and the insoluble pellet. In contrast, the percentage of 63-kD fragment was 40% in SAO ghosts, markedly increased in the $C_{12}E_8$-insoluble pellet (52%), and depleted in the $C_{12}E_8$-soluble supernatant (32%).

Band 3 protein is the principal constituent of IMP. Recently, a linear stacking of IMP has been demonstrated in SAO RBC membrane[26] using a unidirection shadowing technique. However, because of the very high density of IMP in the membrane and the inherent limitations of unidirectional shadowing, it is difficult to exclude artifactual IMP aggregation. Therefore, we examined the distribution of IMP using rotary shadowing, which provides a better visualization of particles in a close proximity to one another.

Using rotary shadowing, we found that the densities of IMP in P- and E-fracture faces of SAO red cells were 3,640 $\pm$ 120/μm^2 and 430 $\pm$ 20/μm^2, respectively, which were similar to IPM densities in normal cells of 3,680 $\pm$ 100/μm^2 and 470 $\pm$ 30/μm^2, respectively. The density was of the same magnitude as the number of band 3 dimers in the normal RBC membrane, namely 600,000 band 3 dimers per cell.[47] However, the distribution of the IMP in SAO red cells was not uniform, with many particles stacked together to form linear strands that resembled strings of beads (Fig 5B). Analysis of IMP in 12 0.1-μm^2 fields of fractured P face showed that 17% to 19% of band 3 dimers were stacked into such structures. The structures were 50 to 250 nm in length (average, 94 nm) and contained 4 to 21 IMP each (average, eight IMP). IMP organized into stacked structures 150 to 200 nm in length were unique to SAO RBCs: we did not find any such structures in spherocytic RBCs from patients with hereditary spherocytosis (HS), including HS asso-

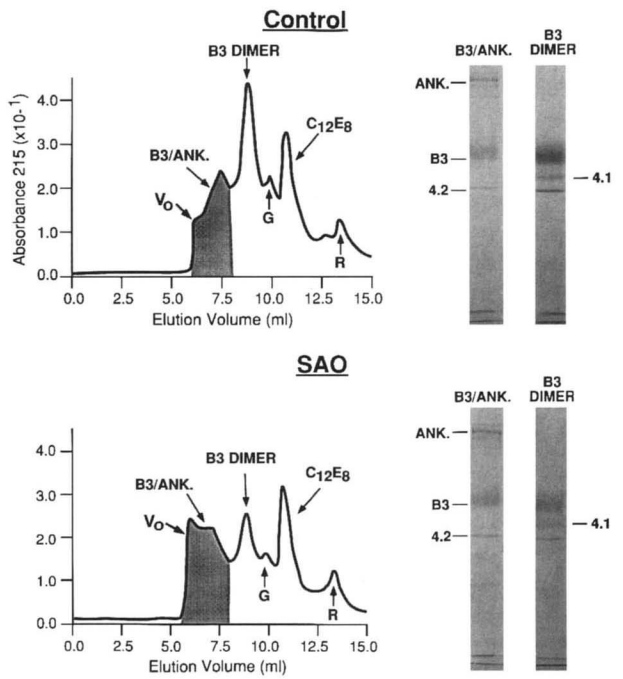

Fig 3. Size-exclusion HPLC of $C_{12}E_8$ extracts from spectrin-depleted membrane vesicles. Normal and SAO RBC ghosts were incubated in low salt buffer to remove spectrin. The spectrin-depleted vesicles were dissolved in 0.5% $C_{12}E_8$ in hypotonic buffer at 4°C. Samples were centrifuged at 150,000*g* for 30 minutes. Supernatants were collected and analyzed by size-exclusion HPLC using a TSK-4000 SW column. The elution buffer contained 0.01% $C_{12}E_8$, 100 mmol/L NaCl, and 5 mmol/L NaPi, pH 7.0. Note the marked increase in the amount of band 3 oligomer peak (B3/ANK) eluting at 6.0 to 7.9 mL in the $C_{12}E_8$ extract from SAO vesicles as compared with the amount eluting from normal vesicles. Note also the marked decrease in the band 3 dimer peak (B3 DIMER) eluting at 9.0 mL from SAO as compared with normal vesicles. The protein composition of each peak was analyzed using a 10% Laemmli gel. V_o, void volume peak; G, glycophorin A; R, residual globin.

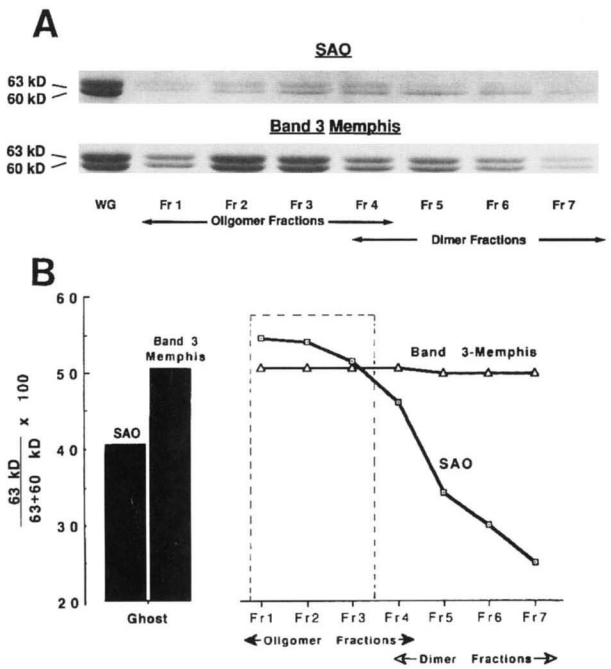

Fig 4. Distribution of mutant and normal band 3 in dimer and oligomer fractions of band 3 from SAO and band 3-Memphis RBCs. Intact SAO and band 3-Memphis RBCs were treated with chymotrypsin to cleave band 3 extracellularly. Spectrin-depleted membrane vesicles derived from these chymotrypsin-pretreated cells were extracted with $C_{12}E_8$ and analyzed by HPLC, as described in the legend to Fig 3. (A) SDS-polyacrylamide gels of HPLC fractions corresponding to oligomers and dimers of band 3. (B) The ratio of 63-kD to total (60-kD + 63-kD) fragments in SAO and band 3-Memphis ghosts and in the respective HPLC fractions was analyzed by densitometric gel scans. Note that the relative amount of 63-kD fragment was 50% in both band 3-Memphis ghosts and in oligomer and dimer species of band 3 fractionated by HPLC. In contrast, the relative amount of 63-kD fragment was 40% in SAO ghosts, but was markedly enriched in the band 3 oligomer fractions [fractions (Fr) 1 to 3] and markedly depleted in the dimer fractions (Fr 5 to 7).

ciated with band 3 deficiency, in elliptocytic RBCs containing mutant spectrins, or in normal cells (data not shown). The abnormal stacking of IMP in SAO membranes became even more evident in cells pretreated with low concentrations (0.02%) of Triton X-100 before freeze-fracture (Fig 5C). At such low concentrations, Triton X-100 reduced somewhat the IMP density in P-fracture face from 3,640 ± 120/μm^2 to 3,340 ± 145 μm^2 without a detectable change of the size of individual IMP. It is likely that Triton, at prehemolytic concentrations, expands the membrane without disturbing the linear stacking of IMP present in the SAO membrane. However, this modest expansion of RBC surface area did not produce an obvious increase in cell dimensions or cell shape (data not shown).

We next attempted to disaggregate the linear strands of IMP by subjecting SAO RBCs to treatment that disrupts band 3-ankyrin interaction or produces proteolytic cleavage of band 3 leading to a release of cdb3 from the plasma membrane (Fig 5D through F). These conditions included (1) treatment of SAO RBCs at elevated pH (eg, pH 11), which weakens the ankyrin-to-band 3 interaction[42] (Fig 5D), (2) pretreatment of SAO RBCs with α-chymotrypsin, which cleaves band 3 at Tyr 553 in the third exoplasmic loop[37] (Fig 5E), and (3) treatment of SAO RBC ghosts with trypsin to remove cytoplasmic domain of band 3 from the membrane by cleavage at residue Lys 360[17] (Fig 5F). None of these conditions leads to a disassembly of the stacked IMP, implying that the stacking of IMP in SAO membranes does not require the covalent bond between Tyr 553 and Asn 554, the tight interaction between ankyrin and band 3, or the presence of cytoplasmic domain of band 3. The definitive site(s) involved in the stacking of SAO band 3 remains to be established.

Rotational immobilization of band 3 in intact SAO RBC membrane. Measurements of membrane protein rotational mobility provide an independent assessment of the state of protein self-association and degree of protein association with the membrane skeleton. We used the polarized fluorescence depletion technique to measure the rotational mobility of eosin maleimide-labeled band 3 in membranes of intact normal and SAO RBCs. Unlike other systems that require a relatively large phosphorescence signal from many optically transparent cells to measure rotational mobility (in the case of RBCs, this requirement necessitates the use of white ghosts rather than intact cells), the technique used here took advantage of the sensitivity of fluorescence detection to perform measurements on several hundred intact RBCs in one to three layers on a microscope slide. The instrument design[43] avoided the problem of inner filter effects, as excitation and emission light paths were not filtered by the hemoglobin in intact cells. In addition, the system used here allowed performance of band 3 lateral and rotational mobility measurements on matched samples of intact normal and SAO RBCs. Lateral mobility was measured using the fluorescence photobleaching recovery technique, which used the same optical and electronic apparatus as that used for rotational mobility measurements.

In agreement with previous reports,[43,48] rapidly rotating (25%), slowly rotating (52%), and rotationally immobile (23%) forms of band 3 were observed in the membranes of intact normal RBCs. In contrast, intact SAO cells manifested a very large (85%) rotationally immobile band 3 fraction and a small (15%) rapidly rotating band 3 fraction. The slowly rotating band 3 fraction was absent in SAO cells (Table 1). These data are similar to reports of band 3 rotational mobility in ghosts prepared from SAO RBCs,[27,29] suggesting that the physical state of band 3 molecules is not markedly perturbed by the preparation of ghosts from intact SAO cells. As previously reported,[13] the lateral mobility of band 3 in SAO RBCs was also markedly reduced as compared with that in normal cells. The profound rotational and lateral immobilization of band 3 in SAO cells is consistent with the increased band 3 oligomerization and increased band 3 binding to the membrane skeleton that were demonstrated using ultrastructural and biochemical techniques. In intact RBCs carrying only the band 3-Memphis variant, the rotational and lateral mobilities of band 3 were not significantly different from those in control RBCs (data not shown). These data further suggest that the nine-amino acid deletion

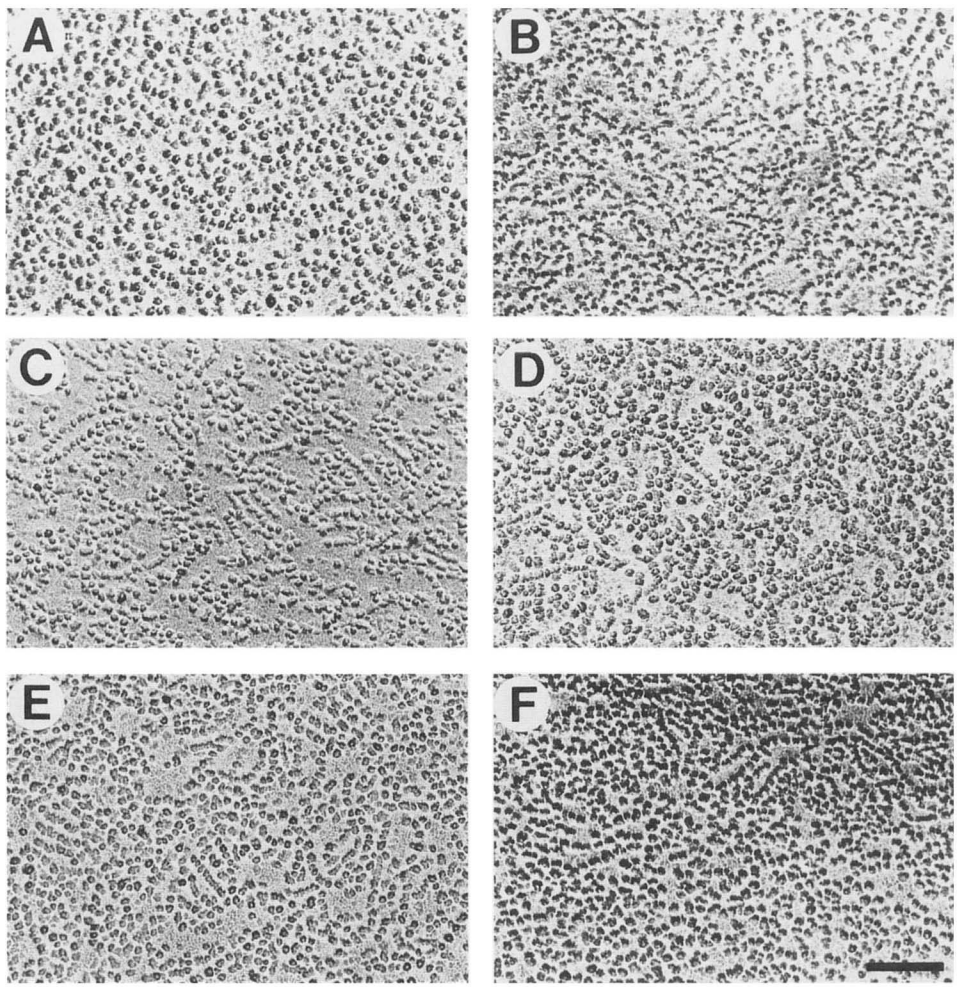

Fig 5. Freeze-fracture electron micrographs of normal (A) and SAO (B through F) RBCs after rotary replication: (A) untreated normal RBC, (B) untreated SAO RBCs, (C) SAO RBC pretreated with 0.02% Triton X-100, (D) SAO RBC pretreated in alkaline buffer at pH 11, (E) SAO RBC pretreated with α-chymotrypsin, and (F) SAO RBC ghosts pretreated with trypsin to remove cdb3 from the membrane. Note the stacking of IMP into long structures on the P-face of untreated SAO, but not normal RBCs. The stacking of IMP in SAO RBCs was not reversed by dispersion with 0.02% Triton, incubation at pH 11 to disrupt ankyrin-band 3 interaction, chymotrypsin cleavage of Tyr 553-Asn 554 covalent bond of band 3 at the extracellular surface, or trypsin cleavage to remove the cdb3 from the membrane. The data suggest that the cdb3 is not involved in the formation of stacking of IMP in SAO RBCs. Bar = 100 nm.

at residues 400 through 408 of normal band 3, rather than the linked Lys 56-to-Glu substitution, represent the molecular defect underlying the rotational and lateral immobilization of band 3 in intact SAO RBCs.

DISCUSSION

This study presents complementary biochemical, ultrastructural, and biophysical evidence that band 3 in SAO RBCs has an increased propensity to assemble into large oligomers that are selectively enriched in the mutant SAO band 3 protein. Furthermore, the amount of band 3 associated with the membrane skeleton is increased in SAO RBCs compared with normal RBCs, and this increase preferentially involves the mutant SAO band 3 protein.

Table 1. Rotational Mobility of Band 3 in Intact Normal and SAO RBCs

	Control (n = 2)	SAO (n = 2)
% Band 3 rotating rapidly (α)	25	15
Rotational correlation time for rapidly rotating band 3 (τ_1)	60 μs	$<$40 μs
% Band 3 rotating slowly (β)	52	*
Rotational correlation time for slowly rotating band 3 (τ_2)	1.0 ms	•
% Immobile component [r(∞)]	23	85

Data were identical for the two SAO samples and within $\pm$5% for the two control samples.

* Slowly rotating component of band 3 was not observed in SAO samples.

Our ultrastructural studies using rotary shadowing electron microscopy confirm recent observations of linear stacking of IMP.[26] Importantly, we further show the linear stacking is preserved in preparations of SAO membranes in which the IMP have been artificially dispersed by incorporating low concentrations of Triton X-100 into the membrane and, thus, increasing membrane surface area (Fig 5C). Furthermore, our data show that the removal of cdb3 by proteolytic cleavage, the cleavage of band 3 at the third exoplasmic loop, or the treatment of intact RBCs by pH 11, which weakens the ankyrin-band 3 contact, did not affect IMP stacking (Fig 5D through F), indicating that the oligomer formation was not related to conformational changes involving cdb3, or cdb3-ankyrin interaction.

Increased oligomerization of band 3 in SAO RBCs was also detected by Sarabia et al.[25] In the latter study the amount of band 3 oligomers was substantially smaller than that reported here, presumably reflecting differences in preparation

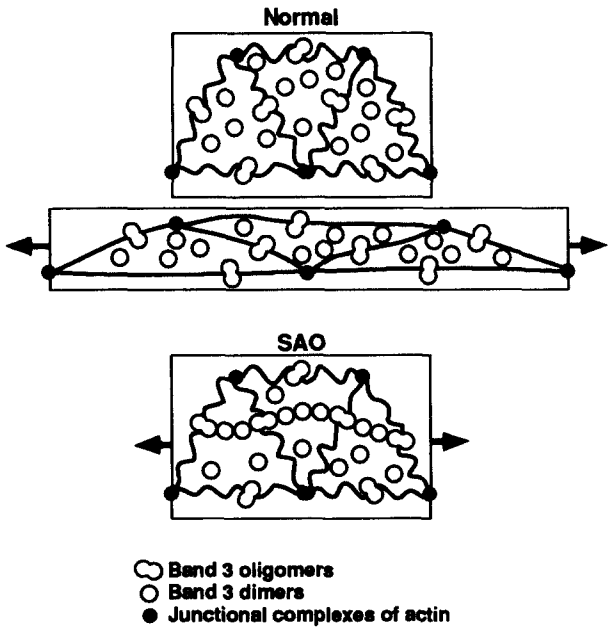

Fig 6. Model of increased membrane rigidity in SAO RBCs induced by band 3 self-association and increased association of band 3 with the membrane skeleton. In normal RBCs, spectrin molecules behave like elastic springs, capable of undergoing unidirectional extension. During the extension process, spectrin either transiently detaches from ankyrin/band 3 oligomers in the membrane or drags the ankyrin/band 3 complexes along. In SAO RBCs, the altered self-association of band 3 into longitudinal structures, which are tightly linked to the skeleton, precludes extension of the underlying skeleton and deformation of the membrane.

of the extract. Specifically, we have found that the results of oligomer assays are critically dependent on the speed of centrifugation, because at high centrifugal forces the large oligomers cosediment with the insoluble pellet. The studies of Sarabia et al[25] and Moriyama et al[12] and the present work also show increased retention of band 3 in the SAO membrane skeleton. Moriyama et al[12] attributed this finding to nonspecific trapping of band 3 in the membrane skeleton. However, our findings of altered rotational mobility and the preferential enrichment of the SAO band 3 oligomers by the mutant band 3 protein suggest that the band 3 retention is a direct consequence of enhanced SAO band 3 oligomerization and membrane skeletal association. Our data differ from those of Schofield et al[10] who did not detect an increased association of SAO band 3 with the skeleton, presumably because these investigators used Triton X-100 extraction under hypotonic conditions, which may have disrupted the altered band 3-band 3 and band 3-ankyrin associations in SAO RBCs. It should be mentioned that at isotonic salt concentration, the retention of ankyrin and band 3 by the skeleton is considerably greater than both at hypotonic conditions (Fig 1) and at high salt concentration.[49,50] The band 3-to-spectrin stoichiometry detected in the isotonic skeleton was not altered by changing the extraction volume or the number of washes (data not shown).

Our data demonstrating a preferential retention of the SAO band 3 protein by the spectrin skeleton also differ from results of Moriyama et al.[12] These investigators did find an increased band 3 retention by the skeleton, but they did not detect a preferential retention of the mutant band 3 in SAO spectrin shells. The reason for this discrepancy is not clear. It is possible that in the study by Moriyama et al[12] the amount of band 3 retained in Triton shells was relatively low, thereby diminishing the accuracy of the subsequent quantitative measurement of mutant SAO band 3 in these shells. The validity of our data demonstrating the enrichment of mutant band 3 in spectrin shells from SAO membranes is further strengthened by our finding of the complementary reduction of mutant band 3 in the $C_{12}E_8$-soluble supernatant (Fig 2).

Finally, our study is consistent with previous reports of markedly decreased band 3 rotational mobility in ghosts prepared from SAO RBCs.[26,28,29] Rotational immobilization could be caused by increased band 3 self-association (aggregation) in the plane of the membrane or by increased direct binding interactions between the cytoplasmic domain of band 3 and membrane skeletal proteins. Several lines of evidence point to the relative importance of the former over the latter mechanism in regulating band 3 rotation in SAO RBCs. First, there is conclusive ultrastructural and biochemical evidence for increased band 3 oligomerization in SAO RBCs. Second, theoretical studies predict that the rotational mobility of particles in membranes is especially sensitive to particle size.[51] Linear oligomers, eight IMP in length (on average), are expected to have rotational diffusion coefficients approximately 100-fold less than those of an individual IMP, and thus to appear rotationally immobile on the time scale of the experiments reported here. Third, Che et al[26] found that removal of spectrin and actin from SAO ghost membranes has a minimal effect on band 3 rotational mobility and that further removal of ankyrin and protein 4.1 does not increase band 3 rotational mobility to the level manifested by band 3 in similarly treated membranes from normal cells. These data suggest that the rotational and lateral immobilization of band 3 in SAO RBCs is caused by the mutant SAO band 3 oligomerization, which is mediated by conformational changes in the transmembrane domain resulting from the deletions of amino acid residues 400 through 408. On the other hand, it is unlikely that increased binding interactions between the cytoplasmic domain of band 3 and membrane skeletal proteins, especially ankyrin, contribute to band 3 immobilization, as evidenced by our data that IMP stacking was unaffected by removal of cdb3 by proteolytic cleavage or by weakening of the ankyrin-band 3 contact (Fig 5D and F).

It is not known whether the linear band 3 aggregates in SAO membranes are composed exclusively of mutant band 3 or whether they contain both normal and mutant band 3. About 25% of the mutant band 3 was detected in the dimer fraction (Fig 4), which presumably represents band 3 molecules that are not bound to the membrane skeleton. In contrast, both the skeleton-associated and the oligomeric band 3 fractions were enriched in the mutant protein. This finding implies that SAO band 3 is more prone than normal band 3 to form oligomers. Further, as the presence of 40% mutant band 3 molecules in SAO membranes leads to an increase in the rotationally immobile component of band 3 from 23%

to 85% (Table 1), it is likely that mutant band 3 is capable of forming at least some hetero-oligomers. However, we cannot exclude the possibility that a small amount of mutant band 3 does form homodimers or homo-oligomers in SAO.

On the basis of these studies, we propose the following mechanism by which the SAO band 3 protein mutation leads to a marked increase in membrane rigidity (Fig 6). The previously held hypothesis suggested that membrane deformability is principally regulated by the skeleton and, in particular, by the propensity of spectrin to undergo unidirectional extension without rupture. In this model, spectrin molecules act as elastic springs.[15,16,52] While acknowledging this important property of spectrin, we propose that membrane deformability also requires that band 3 oligomers, which represent the principal attachment points of the skeleton to the membrane, are laterally and rotationally mobile. Aggregation and stacking of such connecting points into longitudinal strands, such as occurs in the case of the SAO band 3 mutation, precludes both lateral mobility of the band 3 protein and, consequently, the lateral extension of the underlying skeleton. Membrane skeletal immobilization, in turn, produces a marked decrease in membrane deformability. This model can explain most, if not all, abnormalities found in SAO RBCs, including the high-molecular-weight protein aggregation, the lateral and rotational immobilization of band 3, and the increased membrane rigidity.

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