

## Red Cell Membranes of Ankyrin-Deficient *nb/nb* Mice Lack Band 3 Tetramers but Contain Normal Membrane Skeletons<sup>†</sup>

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**ABSTRACT:** The role of ankyrin in the formation and stabilization of the spectrin-based skeletal meshwork and of band 3 oligomers was studied by characterizing, in *nb/nb* mouse red cells, the effect of ankyrin deficiency on skeletal ultrastructure, band 3–skeleton associations, and band 3 oligomeric states. Despite severe ankyrin deficiency, *nb/nb* mouse red cell skeletal components formed a relatively uniform two-dimensional hexagonal array of junctional complexes cross-linked by spectrin tetramers. Treatment of *nb/nb* ghosts with the nonionic detergent C<sub>12</sub>E<sub>8</sub> (octaethylene glycol *n*-dodecyl monoether) resulted in nearly complete extraction of band 3. The extracted band 3 was present exclusively as band 3 dimers. Fluorescence photobleaching recovery and polarized fluorescence depletion measurements showed increases in the laterally (33% vs 10%) and rotationally (90% vs 76%) mobile fractions of band 3 in intact *nb/nb* compared to control red cells. The rotational correlation time of the major fraction of band 3 molecules was 10-fold shorter in *nb/nb* compared to control red cells, indicating a significant relaxation of rotational constraints in *nb/nb* cells. These data suggest that, although ankyrin plays a major role in strengthening the attachment of the skeleton to the membrane bilayer, ankyrin is not required for the formation of a stable two-dimensional spectrin-based skeleton. The absence of band 3 tetramers in the membrane of ankyrin-deficient red cells suggests that ankyrin is required for the formation of stable band 3 tetramers.

The ankyrin family of proteins encompasses a group of structurally and functionally related linker/adaptor molecules that serve to attach the membrane skeleton to the overlying plasma membrane bilayer (see reviews 1 and 2). Three genetically distinct ankyrins have been defined: erythroid ankyrin (ANK 1), brain ankyrin (ANK 2), and kidney ankyrin (ANK 3) (2). Erythroid ankyrin (ANK 1) (3–5) mediates the attachment of spectrin to band 3, the principal integral protein of the red cell membrane (1, 2). The spectrin–ankyrin contact region involves the 15th 106-amino acid repeat segment of  $\beta$ -spectrin (6) and residues Thr 1101–Thr 1192 within the spectrin binding domain of ankyrin (7). The ankyrin–band 3 contact is mediated by interactions between several homologous 33 amino acid repeats (repeats 7–24) of ankyrin (8–10) and an incompletely defined ankyrin binding site within the cytoplasmic domain of band 3 (11–13). Two ankyrin–band 3 binding affinities have

been defined: one low-affinity ( $K_d \approx 130$  nM) and the other high-affinity ( $K_d \approx 13$  nM) (14); these two binding affinities may correspond to the two recently identified binding sites for band 3 on ankyrin (10).

Band 3 is a member of a widely distributed family of functionally similar, but genetically distinct, anion exchangers (15). In the native human red cell membrane, about 30%–50% of band 3 is tightly associated with the spectrin-based membrane skeleton through linkages between ankyrin and band 3 tetramers. The remaining band 3 molecules are laterally mobile in the plane of the membrane and can be extracted from the membrane by nonionic detergents such as C<sub>12</sub>E<sub>8</sub> (16, 17). Recent biosynthesis studies indicate that the band 3–ankyrin complex is detected in the pre-Golgi compartment, suggesting that the formation of this complex may facilitate band 3 delivery to the plasma membrane (18).

Several important questions regarding the interactions among spectrin, ankyrin, and band 3 remain to be elucidated. First, it is not established whether ankyrin is required for the recruitment of spectrin to the plasma membrane and for the assembly of spectrin and other proteins into an ordered two-dimensional skeletal network. Second, it is not clear whether the high-affinity ankyrin binding site involves band 3 dimers, band 3 tetramers, or both. Third, it is not known whether the ankyrin interaction with band 3 tetramer involves binding of preformed band 3 tetramer to ankyrin or, conversely, whether ankyrin serves not only to bind band 3 but also to link two preformed band 3 dimers into a stable ankyrin–band 3 tetramer complex.

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The homozygous state of the normoblastosis (*nb/nb*)<sup>1</sup> mutation of the common house mouse *Mus musculus* provides an excellent model to address these questions. The *nb/nb* mutation is characterized by a nearly complete deficiency of erythrocyte ankyrin, to about 10% of the normal value (5, 19–21). Although *nb/nb* erythrocytes are only moderately deficient in spectrin, with a spectrin content of 50%–70% of the normal value (19, 22), these cells are osmotically unstable and exhibit a marked decrease in red cell survival, from the normal value of 48 days to a mere 0.5 day (23). In this communication we report that, first, *nb/nb* spectrin and associated proteins assemble into a highly ordered and mechanically stable hexagonal network which is nearly indistinguishable from the normal skeleton, indicating that ankyrin is not required for membrane skeleton assembly; second, band 3 in *nb/nb* erythrocytes is present exclusively as band 3 dimers, indicating that ankyrin is absolutely required for band 3 tetramer assembly; third, band 3 is only moderately deficient in *nb/nb* erythrocytes (band 3 content, 50% of the normal value), indicating that ankyrin is not absolutely required for delivery of band 3 to the plasma membrane; and fourth, band 3 is nearly completely extracted by nonionic detergent from *nb/nb* erythrocyte membranes, and the lateral and rotational mobilities of band 3 are markedly increased in *nb/nb* membranes, indicating that ankyrin is required for formation of strong attachments between the erythrocyte membrane skeleton and the membrane bilayer. The mechanical instability and markedly reduced survival of *nb/nb* erythrocytes, despite the presence of a fully assembled skeletal lattice, highlights the importance of the ankyrin-mediated skeleton–bilayer linkage in maintaining red cell mechanical stability.

## MATERIALS AND METHODS

**Animals.** Inbred mice of the WBB6F1-+/+ strain were purchased from the Production Colony at the Jackson Laboratory, Bar Harbor, ME. The *nb* mutation was maintained by forced heterozygosity on both the WB/Re (WB) and C57BL/6J (B6) inbred strains (23). Viable *nb/nb* mice were obtained by crossing WB and B6 heterozygotes.

**Preparation and Stability of Red Cell Membrane Skeletons (Triton Shells).** *nb/nb* mice were anesthetized by injection with phenobarbital sodium. 100 USP units of heparin sodium was injected into the tail vein of anesthetized mice. Red cells were then collected by cardiac puncture. Red cell ghosts were prepared by hypotonic lysis and two washes with 5 mM Na<sub>3</sub>PO<sub>4</sub>, pH 8.0, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (24). Triton shells were prepared by mixing a 50% suspension of ghosts with 11 vol of Triton X-100 solution (3% Triton X-100 in 5 mM Na<sub>3</sub>PO<sub>4</sub>, pH 7.4) at 0–4 °C. Triton shells in the mixture were visualized directly without staining by using a phase contrast light microscope attached to a Newvicon camera (MTI Inc., Michigan City, IN) and a video monitor. The stability of the Triton shells was measured in a concentric cylinder-rod shearing apparatus at a defined shear stress of 36 dynes/cm<sup>2</sup> (25). The percentage of intact Triton shells was plotted against the time of shearing.

**Negative Staining Electron Microscopy.** Triton-treated red cell ghosts were layered on a discontinuous gradient of 10%

and 60% sucrose and centrifuged in a swinging bucket rotor (26). The intact membrane skeletons were collected from the 10%/60% sucrose interface, diluted with 0.1 mM Na<sub>3</sub>PO<sub>4</sub>, pH 7.0, applied to a carbon-coated grid, fixed with 2.5% glutaraldehyde, negatively stained with 1% uranylacetate, and examined in a Jeol JEM-100S electron microscope, as described (26).

**Spectrin Extraction from Red Cell Ghosts and Non-Denaturing Gel Electrophoresis.** Spectrin was extracted from red cell ghosts by incubation in low ionic strength buffer at 0 °C, as described (24). Spectrin species in the extract were separated by electrophoresis in 0.3% agarose–2.5% acrylamide gels, as described (24).

**Band 3 Extraction from Red Cell Ghosts using the Nonionic Detergent Octaethylene Glycol *n*-Dodecyl Monoether (C<sub>12</sub>E<sub>8</sub>).** Red cell ghosts (3 mg of protein/mL) were mixed with an equal volume of 0.5% C<sub>12</sub>E<sub>8</sub> in hypotonic buffer (5 mM Na<sub>3</sub>PO<sub>4</sub>, pH 7.4) at 4 °C for 10 min. After centrifugation at 150000g for 30 min, the supernatant and pellet were each dissolved in SDS and analyzed by SDS–polyacrylamide gel electrophoresis (27). Band 3 retained by the C<sub>12</sub>E<sub>8</sub> skeleton was quantified by densitometric gel scanning and expressed as the band 3 to spectrin ratio.

**Size Exclusion High-Performance Liquid Chromatography (HPLC) of C<sub>12</sub>E<sub>8</sub>-Extracted Band 3.** To analyze the oligomeric species of band 3 in normal and *nb/nb* mouse red cell membranes, spectrin was extracted from the membranes by incubation in low ionic strength buffer (0.1 mM Na<sub>3</sub>PO<sub>4</sub>, pH 8.0) at 37 °C for 15 min. Spectrin-depleted inside-out vesicles (2.0–2.5 mg of protein/mL) were then dissolved in 0.5% C<sub>12</sub>E<sub>8</sub> in hypotonic buffer (5 mM Na<sub>3</sub>PO<sub>4</sub>, pH 7.4) at 4 °C. After centrifugation at 150000g for 30 min, the supernatant (45–225 μg of protein in 50–250 μL of buffer) was analyzed by size-exclusion HPLC using a TSK-4000 SW<sub>XL</sub> column (7.8 × 300 mm, Tosohaas, Tokyo, Japan), as described (16, 17). The standard elution buffer contained 0.01% C<sub>12</sub>E<sub>8</sub>, 100 mM NaCl, 5 mM Na<sub>3</sub>PO<sub>4</sub>, pH 7.0.

**Lateral and Rotational Mobilities of Band 3 in Intact Red Cells.** Band 3 lateral and rotational mobilities were measured in intact normal and *nb/nb* mouse red cells, as described (17, 28). Band 3 was specifically labeled by incubating intact red cells with eosin-5-maleimide (Molecular Probes, Eugene, OR). Fluorescence gel scanning confirmed that >90% of the eosin fluorescence comigrated with band 3 on SDS–polyacrylamide gel electrophoresis. The fluorescence photobleaching recovery technique was used to measure the lateral mobility of eosin-labeled band 3 in membranes of intact red cells (28). The polarized fluorescence depletion technique was used to measure the rotational mobility of eosin-labeled band 3 in membranes of intact red cells (17). Band 3 lateral and rotational mobilities were measured at 37 °C.

**Freeze-Fracture Electron Microscopy.** Intramembrane particles were examined in intact red cells, as described (17). Washed RBCs were fixed in 1.75% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 1.5 h at 0 °C. The fixed cells were washed five times in 0.05 M sodium cacodylate, pH 7.4, glycerinated (final glycerol concentration, 22%), frozen in freon, and transferred to liquid nitrogen. Samples were then fractured in a freeze-etch unit (model BAF 400D; Balzers, Hudson, NH) at –110 °C and rotary-shadowed at a 25° angle with platinum-carbon and at a 90° angle with carbon. Replicas were viewed in a transmission

<sup>1</sup> Abbreviations: *nb*, normoblastosis; C<sub>12</sub>E<sub>8</sub>, octaethylene glycol *n*-dodecyl monoether; EDTA, [ethylene dinitrilo]tetraacetic acid; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; RBC, red blood cell; IMP, intramembrane particle.

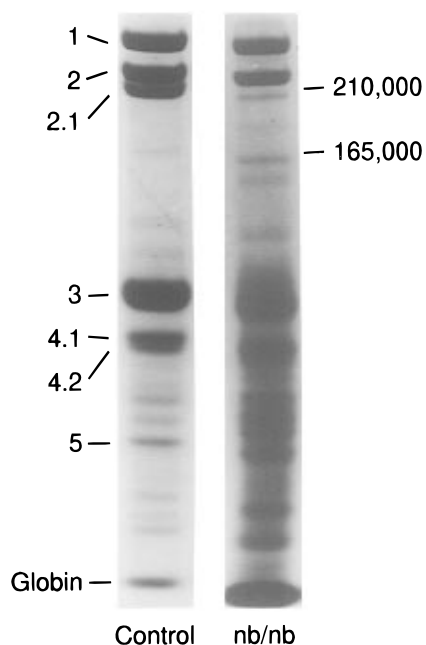


FIGURE 1: Electrophoresis of solubilized normal and *nb/nb* mouse red cell membranes. Red cell membranes from normal and *nb/nb* mice were prepared by hypotonic lysis of isolated red cells (24). 20  $\mu$ g of total membrane protein was solubilized in SDS and electrophoresed in a 3.5%–17% exponential gradient Fairbanks gel (27). Spectrin to band 3 and ankyrin (210 and 165 kDa) to band 3 ratios were obtained from densitometric tracing of the gels. *nb/nb* mouse red cell membranes showed a marked deficiency of ankyrin (<10% of normal).

electron microscope (model JEM 100S; Jeol USA, Peabody, MA) at an accelerating voltage of 80 kV.

## RESULTS

**Ultrastructure and Stability of *nb/nb* Red Cell Membrane Skeletons.** Consistent with previous reports (5, 19–21), SDS polyacrylamide gel electrophoresis of *nb/nb* mouse red cell membranes revealed a marked deficiency of ankyrin (<10% of the normal value) (Figure 1). On the basis of the spectrin to band 3 ratio measured in these gels, the spectrin content of *nb/nb* red cell membranes was decreased to about 50% of normal. To determine whether the spectrin associated with the membranes of ankyrin-deficient *nb/nb* red cells was assembled into a two-dimensional network, Triton-insoluble skeletal shells were prepared from *nb/nb* red cell membranes and examined by phase contrast (Figure 2) and negative staining electron microscopy (Figure 3). By phase contrast light microscopy, Triton shells prepared from spherocytic *nb/nb* red cell ghosts in hypotonic buffer appeared as small spherical structures resembling *nb/nb* ghosts in size and shape (Figure 2).

A representative high-resolution electron microscopic image of a spread membrane skeleton from a normal mouse red cell is shown in Figure 3, top panel. The micrograph featured junctional complexes, presumably containing short F-actin filaments and other proteins such as band 4.1 and band 4.9, cross-linked by spectrin tetramer molecules to form a two-dimensional network similar to that observed in human red cell membrane skeletons (26). Globular structures, presumably representing ankyrin or ankyrin/band 3 complexes, were attached near the midregion of the extended spectrin tetramer molecules. In the spread *nb/nb* mouse membrane skeleton (Figure 3, bottom panel), the number of these ankyrin-containing globular structures was markedly

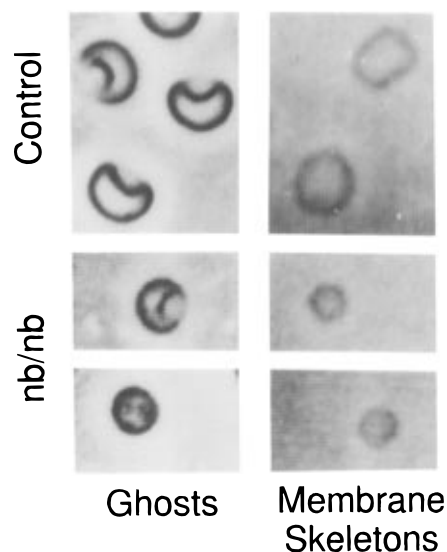


FIGURE 2: Morphology of red cell ghosts and membrane skeletons from normal and *nb/nb* mouse red cells. Membrane skeletons were prepared by incubating red cell ghosts at 0 °C for 10 min with 11 vol of Triton X-100 (3% Triton X-100 in 5 mM Na<sub>3</sub>PO<sub>4</sub>, pH 7.4). Aliquots (10  $\mu$ L) of ghost or skeleton suspensions were applied to glass slides without staining, covered with coverslips, and examined under a phase contrast light microscope using a contrast enhancement Newvicon camera connected directly to a video monitor. Although both ghosts and membrane skeletons from *nb/nb* red cells were smaller and more spherical than those from control red cells, structurally intact Triton shells (but not shell fragments) were obtained from *nb/nb* ghosts, and *nb/nb* skeletons resembled *nb/nb* ghosts in size and shape.

decreased from the normal value of 0.74 per spectrin tetramer ( $n = 78$ ) to 0.09 per spectrin tetramer ( $n = 145$ ). This decrease was consistent with the severe ankyrin deficiency in *nb/nb* mouse red cells. Other than the paucity of ankyrin-containing structures, however, the spread *nb/nb* membrane skeletons appeared to be normal, i.e., there was an orderly hexagonal lattice containing normal size junctional complexes cross-linked by extended spectrin tetramer molecules (Figure 3, bottom panel).

The mechanical stability of the *nb/nb* red cell membrane skeleton was tested by subjecting Triton-insoluble skeletal shells to shearing in a concentric cylinder-rod shearing apparatus at a defined shear stress and examining the time course of skeletal fragmentation. As shown in Figure 4, Triton shells from *nb/nb* mouse red cells had a mechanical stability similar to that of Triton shells from normal mouse red cells. This observation indicated that skeletal components in the *nb/nb* mouse red cell membrane possessed normal horizontal interactions (29) that were capable of producing a mechanically stable skeleton. As illustrated by the unstable membrane skeletons found in hereditary pyropoikilocytosis, a set of hemolytic anemias associated with spectrin mutations and defects in spectrin dimer–dimer interactions (24, 29), the formation of stable spectrin tetramers represents the major horizontal interaction required for a mechanically stable skeleton. Biochemical analysis of spectrin species extracted from *nb/nb* red cell membranes at 0 °C in low ionic strength buffer showed that the spectrin was mainly in tetrameric form, similar to that found in control mouse membranes (Figure 5).

**Band 3 Retention in *nb/nb* Red Cell Membrane Skeletons.** Ankyrin provides the major high-affinity attachment site for band 3 on the red cell membrane skeleton. It was therefore hypothesized that band 3 would be less associated with the

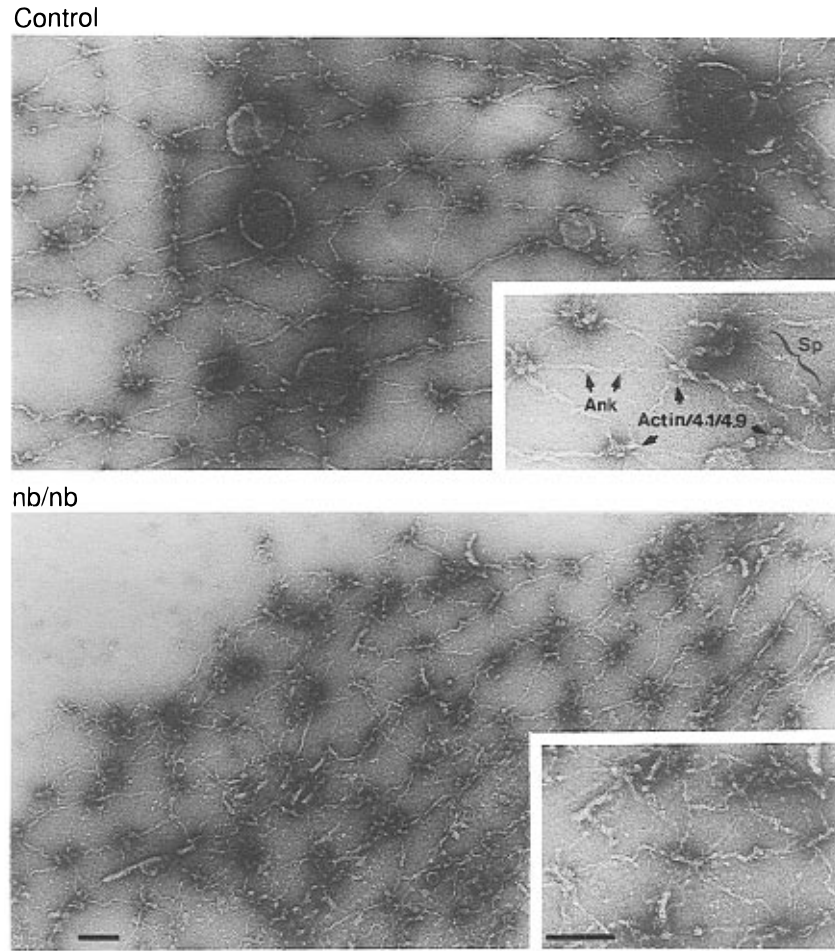


FIGURE 3: Representative electron micrographs of uniformly spread membrane skeletons from normal and *nb/nb* mouse red cells. The normal skeleton showed a primarily hexagonal lattice of junctional complexes, presumably containing short F-actin filaments, band 4.1, and band 4.9, which were cross-linked by spectrin tetramers. Globular structures, presumably representing ankyrin or ankyrin/band 3 complexes, were attached to spectrin filaments at the predicted ankyrin binding site, i.e., 80 nm from the distal end of spectrin. The ankyrin-deficient *nb/nb* skeletal network appeared to have normal skeletal elements, including junctional complexes and spectrin filaments arranged in a hexagonal lattice, except that very few ankyrin structures were detected.

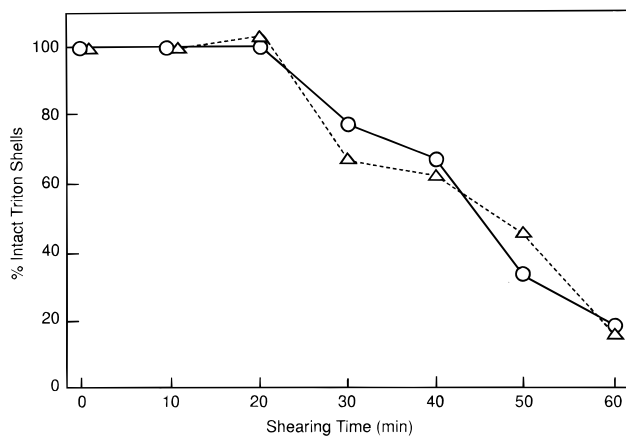


FIGURE 4: Mechanical stability of normal and *nb/nb* mouse red cell membrane skeletons. Triton shells prepared from normal and *nb/nb* red cell ghosts were introduced into a concentric cylinder-rod shearing apparatus (25) and sheared at 36 dyn/cm<sup>2</sup> at room temperature. Fragmentation of the Triton shells was visualized as described in the legend to Figure 2. The stability of the skeletons was expressed as the percentage of the original number of intact skeletons remaining in suspension as a function of shearing time. (○) Control sample; (△) *nb/nb* sample. *nb/nb* red cell membrane skeletons had mechanical stability similar to that of normal skeletons.

skeleton in ankyrin-deficient *nb/nb* red cells than in normal red cells. To study this association quantitatively, the

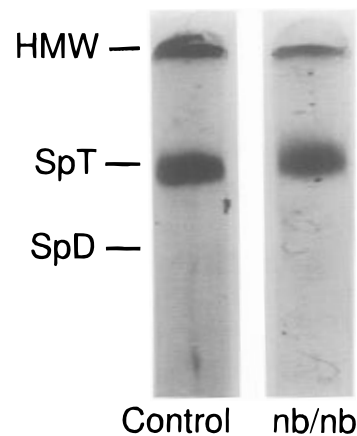


FIGURE 5: Non-denaturing agarose-polyacrylamide gel electrophoresis of spectrin extracts (0 °C, overnight incubation) from normal and *nb/nb* mouse red cell membranes. SpT, spectrin tetramers; SpD, spectrin dimers; HMW, high molecular weight complexes. Tetramer was the predominant molecular species of spectrin in both normal and *nb/nb* membranes.

retention of band 3 in C<sub>12</sub>E<sub>8</sub>-insoluble *nb/nb* red cell membrane skeletons was examined. After C<sub>12</sub>E<sub>8</sub> extraction of ghosts, band 3 retention in the *nb/nb* skeleton was much less than that in the normal skeleton. Based on the band 3 to spectrin ratio, 8% of band 3 was retained by the *nb/nb* skeleton while 28% of band 3 was retained by the normal skeleton (Figure 6). (Conversely, 92% of band 3 was

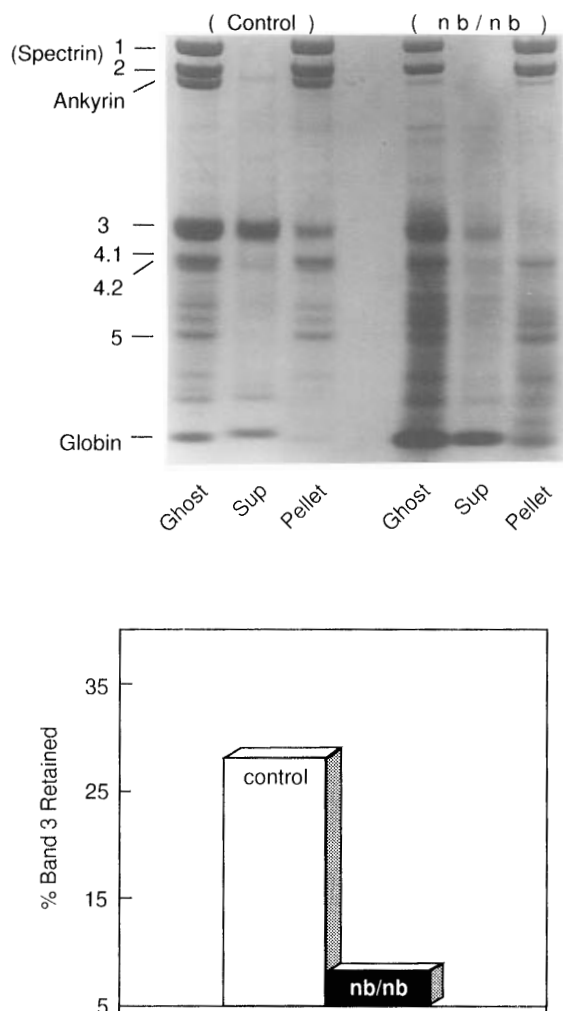


FIGURE 6: Band 3 retention in normal and *nb/nb* mouse red cell membrane skeletons. Red cell ghosts were incubated with 0.5%  $C_{12}E_8$  in hypotonic buffer (5 mM  $Na_3PO_4$ , pH 7.4) at 4 °C for 10 min. After centrifugation, the supernatants (Sup) and pellets were analyzed by SDS-polyacrylamide gradient gel electrophoresis. The nonionic detergent  $C_{12}E_8$  extracted 72% and 92% of band 3 from normal and *nb/nb* mouse red cell membranes, respectively.

extracted from the *nb/nb* membrane, while 72% of band 3 was extracted from the normal membrane, by the nonionic detergent.) Thus, a much smaller fraction of band 3 was tightly associated with the membrane skeleton in *nb/nb* red cells than in normal red cells. A control sample from a mouse with a high reticulocyte count (65%) induced by Friend virus infection did not exhibit the decrease in band 3 retention seen in *nb/nb* red cell membrane skeletons (data not shown).

**Oligomeric State of Band 3 Extracted from *nb/nb* Red Cell Membranes.** Ankyrin has been postulated to play a role in the oligomerization of band 3. The *nb/nb* mouse red cell was used as a model system to investigate whether band 3 is capable of forming tetramers in the absence of ankyrin. The oligomeric state of band 3 in  $C_{12}E_8$  extracts derived from *nb/nb* and normal mouse red cell membranes was analyzed by size-exclusion HPLC. To analyze band 3 species in control membranes, the spectrin-actin network was removed from the membrane by low salt extraction, and the resulting spectrin-depleted inside-out vesicles were dissolved in  $C_{12}E_8$  and analyzed by size-exclusion HPLC. The elution profile of band 3 species in control membranes contained both band 3 dimers and ankyrin/band 3 tetramer complexes (Figure 7, top panel), similar to the profile reported previously for band

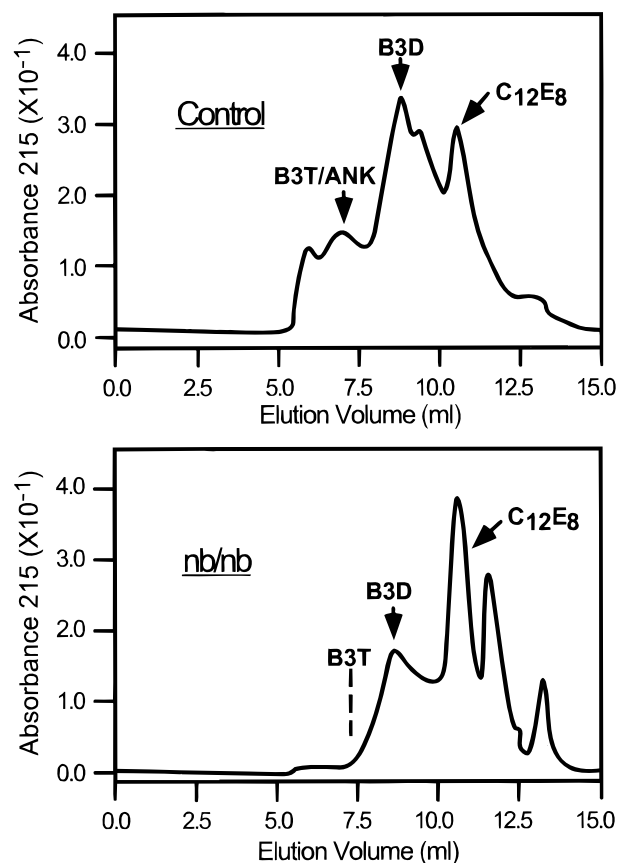


FIGURE 7: Size-exclusion HPLC elution profiles of band 3 from normal and *nb/nb* mouse red cell membranes. Normal red cell ghosts were incubated in low salt buffer to remove spectrin. The spectrin depleted vesicles were dissolved in 0.5%  $C_{12}E_8$ , 100 mM NaCl, 5 mM  $Na_3PO_4$ , pH 7.0. The band 3 oligomer peak (B3T/ANK) and band 3 dimer peak (B3D) eluted at 6.0–7.9 and 9.0 mL, respectively. Band 3 protein was extracted directly from *nb/nb* red cell ghosts using  $C_{12}E_8$ , and band 3 species were analyzed by size exclusion HPLC. The extracted band 3 from *nb/nb* membranes was exclusively in dimeric form.

3 species in human red cells (17). Since more than 90% of band 3 was readily extracted from the membrane by  $C_{12}E_8$ , band 3 species were analyzed in the  $C_{12}E_8$  membrane extract without prior removal of membrane spectrin. The elution profile of band 3 species in *nb/nb* membranes showed that the extracted band 3 was present exclusively as band 3 dimers (Figure 7, bottom panel). No band 3 tetramers were detected at the predicted position. These data indicate that the 92% of band 3 which was extracted by  $C_{12}E_8$  from *nb/nb* membranes was in dimeric form. This fraction was much greater than the band 3 dimer content in the normal mouse membrane, which was estimated to be 60%–70% of total band 3. A high reticulocyte count control sample did not exhibit the greater band 3 dimer content seen in the *nb/nb* mouse sample (data not shown).

**Lateral and Rotational Mobilities of Band 3 in Intact *nb/nb* Red Cells.** Measurements of membrane protein lateral and rotational mobilities provide independent assessments of the degree of protein association with the membrane skeleton and the state of protein self-association. The fluorescence photobleaching recovery and polarized fluorescence depletion techniques were used to measure the lateral and rotational mobilities, respectively, of eosin maleimide labeled band 3 in membranes of intact normal and *nb/nb* mouse red cells. In normal mouse red cells, 10%  $\pm$  6% of band 3 molecules were laterally mobile (mean  $\pm$

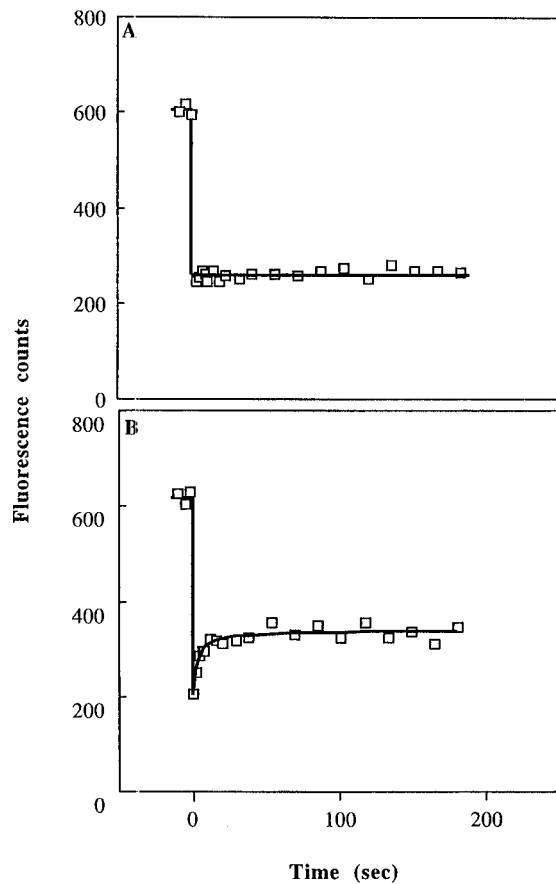


FIGURE 8: Typical fluorescence photobleaching recovery curves depicting the lateral mobility of eosin labeled band 3 in intact red cells from normal mice (A) and *nb/nb* mice with ankyrin deficiency (B). Data points represent fluorescence counts at experimental times before and after the photobleaching pulse, which occurred at time = 0. Ankyrin deficient red cells showed increased band 3 lateral mobility relative to normal red cells, indicating increased freedom of band 3 lateral movement. (A) Fractional mobility = 3%, diffusion coefficient cannot be measured reliably. (B) fractional mobility = 34%, diffusion coefficient =  $1.3 \times 10^{-10}$  cm<sup>2</sup>/s.

SD,  $n = 34$  cells). The diffusion coefficient of the laterally mobile band 3 molecules could not be estimated reliably for this degree of lateral immobilization. In *nb/nb* mouse red cells, the band 3 fractional mobility was  $33\% \pm 9\%$  and the lateral diffusion coefficient was  $(1.9 \pm 1.0) \times 10^{-10}$  cm<sup>2</sup>/s ( $n = 25$  cells) (Figure 8). The laterally mobile fraction of band 3 in *nb/nb* red cells was significantly greater than that in normal red cells ( $p < 0.0001$ , Student two-tailed  $t$  test). The band 3 lateral diffusion coefficient in *nb/nb* mouse red cells was an order of magnitude greater than that in normal human red cells but was comparable to that in spectrin deficient (30) or spectrin/ankyrin-deficient (Cho et al., unpublished) human red cells.

Band 3 rotational mobility in *nb/nb* mouse red cells also differed significantly from that in normal mouse red cells. Polarized fluorescence depletion data in normal mouse red cells were adequately fitted by a single exponential, indicating the presence of one major rotationally mobile population of molecules (see Figure 9A). In normal mouse red cells,  $76\% \pm 14\%$  of band 3 molecules were rotationally mobile (mean  $\pm$  SD,  $n = 3$  samples). The rotationally mobile band 3 molecules had an average rotational correlation time of  $1.0 \pm 0.3$  ms. In *nb/nb* mouse red cells, polarized fluorescence depletion data showed an initial rapidly decaying anisotropy component and a prolonged slowly decaying anisotropy component. Although the  $\chi^2$  value for a two-

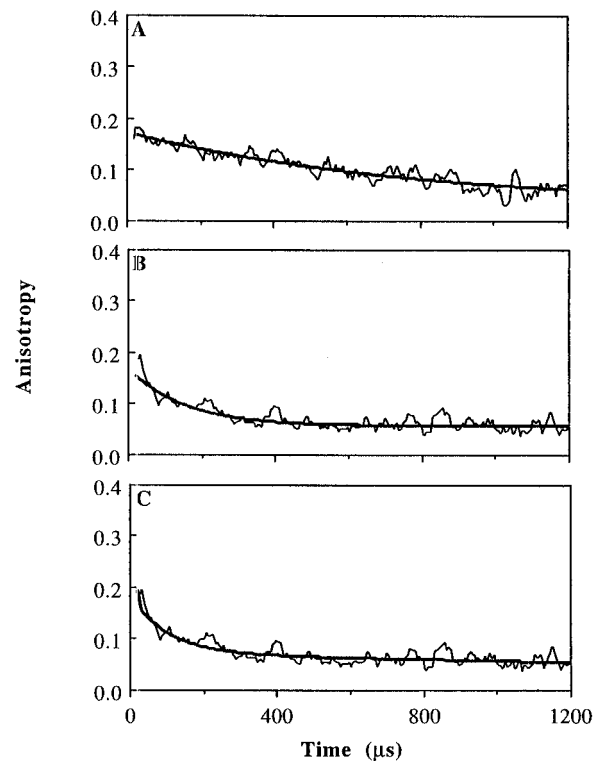


FIGURE 9: Typical polarized fluorescence depletion curves showing the rotational mobility of eosin labeled band 3 in intact red cells from normal mice (A) and *nb/nb* mice with ankyrin deficiency (B, C). For each sample approximately 500 red cells were illuminated by the laser beam, so the data represented the average rotational mobility of approximately  $5 \times 10^8$  band 3 molecules per sample. Rotational mobility parameters were determined by nonlinear least-squares analysis. Data in panels A and B were fitted to a single exponential; data in panel C were fitted to the sum of two exponentials.  $\alpha$  and  $\beta$  are fractions of band 3 molecules rotating with correlation times  $\tau_1$  and  $\tau_2$ , respectively, and  $r(\infty)$  is the fraction of rotationally immobile band 3 molecules. Although data from normal red cells (A) were adequately fitted by a single exponential, the single-exponential fit of data from ankyrin-deficient red cells (B) did not adequately fit the initial rapidly decaying anisotropy component (compare B and C). Relative to normal red cells, ankyrin-deficient red cells showed an increase in the rotationally mobile fraction of band 3 molecules and the appearance of a rapidly rotating fraction of band 3 molecules. Both of these changes indicated increased freedom of band 3 rotational movement. (A)  $\alpha = 83\%$ ,  $\tau_1 = 0.80$  ms,  $\beta = 0\%$ ,  $r(\infty) = 17\%$ ,  $\chi^2 = 0.42$ . (B)  $\alpha = 68\%$ ,  $\tau_1 = 0.15$  ms,  $\beta = 0\%$ ,  $r(\infty) = 32\%$ ,  $\chi^2 = 0.30$ . (C)  $\alpha = 63\%$ ,  $\tau_1 = 97$   $\mu$ s,  $\beta = 31\%$ ,  $\tau_2 = 3.1$  ms,  $r(\infty) = 6\%$ ,  $\chi^2 = 0.29$ .

exponential fit to the anisotropy decay data was only marginally smaller than that for a single exponential fit, the initial rapidly decaying anisotropy component was fitted much better by the two-exponential fit (compare Figures 9B, C). (In contrast, a second, rapidly rotating component could not be found by fitting the anisotropy decay data from the normal mouse red cell samples to the sum of two exponentials.) Using the two-exponential fit,  $90\% \pm 6\%$  of band 3 molecules were rotationally mobile in *nb/nb* mouse red cells:  $58\% \pm 7\%$  rotated rapidly with a rotational correlation time of  $97 \pm 1$   $\mu$ s, and  $32\% \pm 1\%$  rotated slowly with a rotational correlation time of  $4.2 \pm 1.6$  ms ( $n = 2$  samples) (Figure 9). Signal-to-noise considerations prevented the collection of meaningful data at times longer than 1.2 ms after the photoselection pulse, so the rotational correlation time of the slowly rotating component must be considered as an approximate value only. Compared to normal red cells, *nb/nb* red cells appeared to have a greater rotationally mobile fraction of band 3 molecules ( $p < 0.1$ ). The major

rotationally mobile fraction of band 3 molecules rotated faster in *nb/nb* compared to normal red cells ( $p < 0.05$ ). While normal mouse red cells had no significant fraction of rapidly rotating band 3 molecules (i.e., molecules that rotated with a rotational correlation time in the 100  $\mu$ s range), more than 50% of band 3 molecules in *nb/nb* mouse red cells rotated rapidly.

**Intramembrane Particles (IMP) in *nb/nb* Red Cell Membranes.** Band 3 protein is the principal constituent of IMP. Rotary shadowing was used to examine the size and densities of IMP in P (protoplasmic)- and E (extracellular)-fracture faces of *nb/nb* red cells. The size of IMP in the P-fracture face of *nb/nb* mouse red cells was not significantly different from that in normal mouse red cells (data not shown). The IMP density in the P-fracture face of *nb/nb* mouse red cells was heterogeneous, varying from cell to cell over the range 2062–3620 IMP/ $\mu$ m<sup>2</sup>. In contrast, the IMP density in normal mouse red cells was  $3446 \pm 102$  IMP/ $\mu$ m<sup>2</sup> ( $n = 12$ ), without much variation from cell to cell. It was hypothesized that the heterogeneity of IMP density observed in *nb/nb* red cells was due to the elevated number of reticulocytes in the blood of *nb/nb* mice (reticulocyte count, 50%–70%). In a reticulocyte-enriched control obtained from a Friend virus infected mouse (reticulocyte count, 70%–90%), a wide range of IMP densities, varying from cell to cell over the range 2612–3480 IMP/ $\mu$ m<sup>2</sup>, was also detected.

## DISCUSSION

In this report, we demonstrate that severe ankyrin deficiency does not affect the ability of spectrin and other membrane skeletal components to assemble into a highly ordered hexagonal network in *nb/nb* mouse red cells. Because this network is associated with the inner surface of the *nb/nb* red cell membrane, spectrin molecules, in the absence of ankyrin, must bind to the membrane through secondary connections. Potential spectrin binding sites include protein 4.1, protein 4.2, actin, and other membrane proteins and lipids (2, 30–32). It is striking that spectrin, actin, protein 4.1, and other skeletal components are capable of forming a mechanically stable membrane skeleton in *nb/nb* red cells. Mechanical stability requires intact “horizontal” interactions, including spectrin dimer–dimer associations and spectrin-actin-protein 4.1 linkages (29). The present model suggests that, in the normal red cell membrane, spectrin–ankyrin–band 3 associations provide the key “vertical” interactions, that is, interactions perpendicular to the plane of the membrane that are important for coupling of the membrane skeleton to the lipid bilayer and therefore for stability of the lipid bilayer membrane (29). Defects in vertical interactions often produce unstable lipid bilayers which are prone to be released as skeleton-free lipid vesicles (31). This lipid loss, in turn, results in loss of membrane surface area and spherocytosis (29). Results from the present study of ankyrin-deficient *nb/nb* mouse red cells are consistent with this hypothesis. Without adequate amounts of ankyrin in the membrane, band 3 is not retained in normal amounts by the *nb/nb* red cell skeleton (Figure 6). Without adequate amounts of band 3, the cells lose substantial surface area and became spherocytic.

It is generally believed that band 3 is present as dimers and tetramers in normal red cell membranes, and that band 3 tetramers represent the high-affinity ankyrin binding sites (14, 16, 17). However, it is not clear how these band 3

tetramers are formed and maintained. A recent study has shown that, during erythroid development, ankyrin forms a complex with band 3 in the endoplasmic reticulum or the Golgi compartment and is subsequently delivered to the plasma membrane as an ankyrin–band 3 complex (18). This finding raises the possibility that, in the presence of ankyrin, band 3 tetramers are formed in the endoplasmic reticulum. Alternatively, band 3 tetramers could be formed regardless of the presence or absence of ankyrin. Our observation that band 3 is present exclusively as band 3 dimers in ankyrin-deficient *nb/nb* red cells suggests strongly that ankyrin is absolutely required for band 3 tetramer assembly.

The lateral mobility of band 3 in normal mouse red cells is markedly restricted compared to that in normal human red cells. Approximately 40% of band 3 molecules in normal human red cells are laterally immobilized by high-affinity (i.e., long-lived) binding interactions between ankyrin and band 3 tetramers. Because the ankyrin to band 3 ratio is similar in mouse and human red cells, this high-affinity binding mechanism can account for only ~40% of the 90% of band 3 molecules that are laterally immobilized in normal mouse red cells. In certain pathologic human red cells, the fraction of laterally immobilized band 3 molecules approaches 100% due to band 3 self-aggregation mediated by oxidative cross-linking or other, as yet unidentified, mechanisms (28, 33, 34). Because the major band 3 species in normal mouse red cells are dimers and tetramers, however, self-aggregation cannot account for the decreased fractional mobility of band 3 in normal mouse red cells. Unless mouse red cells manifest a second, non-ankyrin-related high-affinity binding site for band 3 on the membrane skeleton (a possibility which is unlikely), the most likely molecular mechanism for increased lateral immobilization of band 3 in mouse red cells is an increase in steric hindrance interactions between the cytoplasmic domain of band 3 and the spectrin-based membrane skeleton. This conclusion is strengthened by the findings that, in both normal and *nb/nb* mouse red cells, the difference between the rotationally mobile and the laterally mobile fractions of band 3 molecules is ~60%. If the non-ankyrin-linked, laterally immobile band 3 species were immobilized by high-affinity binding interactions rather than by steric hindrance interactions, then an increased degree of rotational immobilization would also be expected.

The fractional mobility of band 3 in ankyrin-deficient *nb/nb* mouse red cells is 30%–35%. The increased fractional mobility of band 3 in *nb/nb* compared to normal mouse red cells is consistent with the increased extractability of band 3 by nonionic detergent in *nb/nb* cells, and with the conclusion that band 3 is more weakly associated with the membrane skeleton in *nb/nb* cells than in normal cells. Furthermore, the increase in band 3 fractional mobility in ankyrin-deficient cells compared to normal cells suggests that high-affinity binding to ankyrin serves to immobilize 20%–25% of band 3 molecules in the normal mouse red cell membrane. The observation that 65%–70% of band 3 molecules remain laterally immobile in ankyrin-deficient *nb/nb* membranes indicates that molecular mechanisms other than high-affinity binding to ankyrin must mediate the lateral immobilization of ~50% of band 3 molecules in normal mouse red cells. As noted above, steric hindrance interactions are likely to represent the major such mechanism.

The rotational mobility of band 3 in normal mouse red cells is also restricted compared to that in normal human

red cells. In normal human red cells, ~15% of band 3 molecules are rotationally immobile, ~60% of band 3 molecules rotate slowly (rotational correlation time, 1–3 ms), and ~25% of band 3 molecules rotate rapidly (rotational correlation time, 50–200  $\mu$ s). It is thought that the rotationally immobile fraction represents band 3 molecules bound with high affinity to ankyrin; the slowly rotating fraction consists of band 3 molecules transiently bound (i.e., bound with low affinity) to ankyrin, protein 4.2, and possibly other membrane skeletal proteins; and the rapidly rotating band 3 fraction represents band 3 dimers and tetramers rotating free of constraints other than the viscosity of the lipid bilayer (17, 30, 32, 33, 35–40). Despite the observation that 60%–70% of band 3 molecules in normal mouse red cells are present in dimer form, no rapidly rotating band 3 molecules are seen. These data indicate that there are no rotationally “free” band 3 dimers in normal mouse red cells but that all band 3 molecules are bound for a significant fraction of the time to high- and low-affinity binding sites on the membrane skeleton. The rotationally immobile band 3 molecules in normal mouse red cells, like those in normal human red cells, are likely immobilized through high-affinity binding interactions between band 3 and ankyrin. Indeed, there is an excellent correlation between the fraction of rotationally immobile band 3 molecules and the fraction of band 3 that remains associated with the membrane skeleton following nonionic detergent extraction in normal mouse red cells.

Band 3 rotational mobility is significantly increased in ankyrin-deficient *nb/nb* mouse red cells compared to that in normal mouse red cells. In *nb/nb* mouse red cells, the ~60% of band 3 molecules that rotate rapidly presumably represent band 3 dimers that are free of rotational constraints other than the viscosity of the lipid bilayer. Because this rapidly rotating band 3 population is not observed in normal mouse red cells, these data suggest that, in normal mouse cells (i.e., in cells that are not ankyrin deficient), ~60% of band 3 dimers are rotationally slowed by low-affinity binding interactions with ankyrin. As in normal mouse red cells, in *nb/nb* mouse red cells there is an excellent correlation between the rotationally immobile band 3 fraction and the  $C_{12}E_8$ -inextractable band 3 fraction. These data suggest that high-affinity binding interactions between band 3 and a membrane skeletal component(s) serve to immobilize the rotationally immobile band 3 molecules.

Taken together, the rotational mobility data reported here are consistent with a model in which, in normal mouse red cells (i) 20%–25% of the band 3 molecules are rotationally immobile due to high-affinity binding interactions with ankyrin; (ii) all of the rotationally mobile band 3 molecules rotate slowly due to low-affinity binding interactions between band 3 and other membrane proteins; and (iii) low-affinity binding interactions between band 3 and ankyrin account for rotational slowing of ~60% of all band 3 molecules, or ~80% of the rotationally mobile band 3 molecules. Other membrane proteins that could participate in low-affinity interactions with band 3 include protein 4.2, protein 4.1, and glycophorin A. In *nb/nb* mouse red cells, the rotational mobility data suggest that (i) ~10% of band 3 molecules are rotationally immobile, probably due to high-affinity binding interactions between band 3 and the residual ankyrin; (ii) ~60% of band 3 molecules are free of rotational constraints other than the viscosity of the lipid bilayer; and (iii) ~30% of band 3 molecules rotate slowly due to low-affinity binding interactions with protein 4.2, protein 4.1,

glycophorin A, and possibly other integral membrane or membrane skeletal proteins.

It has recently been observed that band 3 rotational mobility is sensitive to the ankyrin content of the intact human red cell membrane. In red cells from patients with hereditary spherocytosis, ankyrin deficiency affects primarily the slowly rotating population of band 3 molecules. Over the range of normalized ankyrin/band 3 ratios from 0.8 to 1.2, there is a direct linear correlation between the ankyrin/band 3 ratio and the average band 3 rotational correlation time. These data suggest that low-affinity binding interactions between band 3 and ankyrin account for rotational slowing of a significant fraction of band 3 molecules in human red cells (Cho et al., unpublished). Thus, in both human and mouse red cells, interactions between band 3 and ankyrin represent the major molecular mechanism that slows the rotation of band 3 molecules.

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