Association State of Human Red Blood Cell Band 3 and its Interaction With Ankyrin

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We have studied the association state of band 3, the anion channel and predominant transmembrane protein of the human red blood cell, and the anomalous stoichiometry and dynamics of its interaction with ankyrin, which acts as a link to the spectrin of the membrane skeletal network. Band 3 exists in benign nonionic detergent solutions as a dimer. Tetramer is formed irreversibly in the course of manipulations, particularly in ion-exchange chromatography. The dimer in solution binds ankyrin without self-associating. In ankyrin-free inside-out membrane vesicles and when incorporated into phosphatidylcholine liposomes, only some 10% to 15% of band 3 chains bind ankyrin at saturation. Moreover, in liposomes this was independent of protein/lipid ratio between 1:2 and 1:40. The bound fraction of band 3 remains with the detergent-extracted membrane cytoskeleton, but is released if the ankyrin has been cleaved with chymotrypsin before detergent treatment; thus, the attachment to the membrane cytoskeleton is entirely through ankyrin and not through other constituents such as protein 4.1. The ratio of band 3 to ankyrin in this complex implies that it consists of two chains of band 3 and one chain of ankyrin, at least after detergent extraction. The bound and free populations of band 3 exchange freely in the membrane. In the artificial liposome membrane binding of ankyrin to band 3 dimers cause association of the band 3 into higher aggregates, as seen in freeze-fracture electron microscopy. Successive manipulations of the red blood cell membrane, which are involved in the preparation of ghosts, of inside-out vesicles, and of inside-out vesicles stripped of peripheral proteins are accompanied by progressive aggregation of intramembrane particles, as judged by freeze-fracture electron microscopy. Thus the intramembrane particles are evidently stabilized in the intact cell by the peripheral protein network and the cytosolic milieu. Aggregation may be expected to limit the number of functional ankyrin binding sites. However, although extraneous ankyrin binds to the unoccupied binding site on the spectrin tetramers in intact ghost membranes, little or no ankyrin can bind to the unoccupied band 3 dimers in situ, perhaps by reason of occlusion of binding sites by the membrane skeletal network.

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scribed previously. Band 3 was prepared from white ghosts, obtained by osmotic lysis, as described by Dodge et al. Packaged ghosts were washed once with phosphate-buffered isotonic saline (0.15 mol/L sodium chloride, 5 mmol/L sodium phosphate, pH 8.0) to remove band 6 protein and diluted with the same buffer, containing additionally 0.1 mmol/L EDTA, to the volume occupied by the original packed RBCs. To this was added 1/20 vol of fresh 20% Triton X-100 with mixing and the solution was centrifuged at 540,000g for 5 minutes. The supernatant was made 5% (wt/vol) in sucrose by the addition of solid; the resulting solution (10 to 12 mL), which contained mainly band 3, was applied to a column (2.5 x 40 cm) of Sephaeryl S200-HR, equilibrated with 0.1 mol/L sodium chloride, 20 mmol/L triethanolamine, 0.1 mmol/L EDTA, 0.2 mmol/L dithiothreitol, 3 mmol/L sodium azide, 0.1 mmol/L butylated hydroxyltoluene, and 10 mg/mL Brij 35, pH 7.4. The column was eluted with the same buffer at 35 mL/h; fractions of 10 mL were collected and the protein, monitored by absorption at 280 nm, was eluted before the Triton zone. The fractions containing band 3, as shown by sodium dodecyl sulfate (SDS)-gel electrophoresis, were pooled, applied to a MonoQ anion-exchange column (Pharmacia Biotech Ltd, Uppsala, Sweden) in an fast protein liquid chromatography (FPLC) apparatus and eluted with a buffer containing 20 mmol/L triethanolamine, 0.1 mmol/L EDTA, 0.2 mmol/L dithiothreitol, 0.1 mmol/L butylated hydroxyltoluene, and 10 mg/mL Brij 35, pH 7.4, with a 0.175 to 0.6 mol/L potassium chloride gradient. The band 3 dimer and tetramer eluted at 0.38 and 0.49 mol/L salt, respectively. Samples were screened by SDS-gel electrophoresis and were pure, except for a trace of band 4, although glycoporphin A could be detected by immunoblotting. The protein was concentrated when required by centrifugation in an Amicon Centricon filter (Amicon, Beverly, MA).

Ankyrin was prepared by the method described earlier. Protein concentrations were determined spectrophotometrically using specific absorptivities calculated from the protein sequences, i.e., E280 of 1.0 for band 3 and 0.57 for ankyrin. Membrane cytoskeletons retaining the spectrin-associated ankyrin and its band 3-binding domains. The digestion was stopped with an excess of chymostatin and the ghosts were washed with the same buffer at 35 mL/h; fractions of 10 mL were collected and the resulting solution (2.5 x 40 cm) of Sephaeryl S200-HR, equilibrated with 0.15 mol/L sodium chloride, 5 mmol/L sodium phosphate, and 0.1 mmol/L EDTA, pH 7.6, was treated with 3 μg/mL N-[ethyl-2-14C]maleimide (45 Ci/mmol) for 30 minutes on ice. The residual reagent was quenched with 0.5 mol/L dithiothreitol and unbound radioactivity was eliminated by dialysis against the buffer used for the binding studies, i.e., 0.1 mol/L potassium chloride, 7.5 mmol/L potassium phosphate, 0.2 mmol/L EDTA, 0.5 mmol/L dithiothreitol, 1 mmol/L sodium azide, and 10% (wt/vol) sucrose, pH 7.5. The specific activity of the protein thus labeled was 0.4 to 0.8 μCi/μg. The ankyrin was standardized spectrophotometrically and 1 mg/mL bovine serum albumin was added to the solution.

Inside-out membrane vesicles (right-side out vesicle content <20%, as judged by acetylcholinesterase assay) were prepared by extraction of spectrin and actin at low ionic strength at 36°C, followed by extraction of ankyrin with 1 mol/L potassium iodide. For rebinding assays, vesicles at a total protein concentration adjusted to 0.15 mg/mL in the above binding buffer were incubated with varying concentrations of the labeled ankyrin for 1 to 2 hours at 0°C or 20°C. The vesicles were recovered by pelleting through 15% (wt/vol) sucrose at 48,000g for 30 minutes, and radioactivity in the supernatant and pellet was counted. Vesicles heated at 100°C for 15 minutes served as controls for nonspecific binding. The same procedure was applied to ghosts prepared by lysing washed cells in isotonic phosphate-buffered saline, pH 8.0, with saponin (25 mg/mL) and washing until white with the buffer. This procedure was also applied to detergent-extracted membrane skeletons. Because we were concerned with determination of the number of available binding sites, care was taken to define the plateau of saturation by using the largest possible excesses of ligand protein.

Membrane fusion. White ghosts were resealed by suspension in phosphate-buffered isotonic saline, pH 7.6, and incubation at 37°C. The same procedure was applied to ghosts derived from cells that had been labeled with fluorescein isothiocyanate (0.25 mg/mL) by incubation for 8 hours, with agitation, at a hematocrit of 4% at 4°C in 0.145 mol/L sodium chloride, 10 mmol/L sodium bicarbonate, pH 9.5. We found, in agreement with the earlier work, that the labeling was almost entirely confined to band 3, as shown by the distribution of fluorescence after gel electrophoresis in SDS. Fusion was induced in the ghost suspension with polyethylene glycol, as described by Ahkong and Lucy. To observe migration of fluorescent band 3, labeled cells were mixed with a 10-fold excess of unlabeled cells, allowed to fuse, washed free of fusogen, deposited on polylysine-coated slides, and examined over a period of hours in a fluorescence microscope after the addition of ascorbic acid as an antifading agent. Incorporation of fluorescence into the dark hemisphere of a fused ghost pair was scored as a function of time. To observe fluorescence in the membrane cytoskeletons, the ghosts on the slide were irradiated with isotonic phosphate-buffered saline containing 3.75% Triton X-100. To eliminate unbound band 3, a further extraction with Triton X-100 in buffer supplemented with an additional 0.45 mol/L of potassium chloride was performed.

Incorporation of band 3 into liposomes. The procedure of Yu and Branten was followed. Egg phosphatidylcholine (Lipid Products, South Nutfield, UK) at 2 mg/mL was dispersed in 0.1 mol/L sodium chloride, 20 mmol/L triethanolamine, 0.2 mmol/L dithiothreitol, 0.1 mmol/L EDTA, 1 mmol/L sodium azide, 0.1 mmol/L butylated hydroxytoluene, 10 mg/mL Brij 35, 20 mg/mL sodium cholate, pH 7.4, containing 0.17 mg/mL freshly prepared band 3 dimer or tetramer. The solution was dialyzed for 48 hours in the cold against 0.15 mol/L sodium chloride, 5 mmol/L sodium phosphate, 1 mmol/L sodium azide, 8.5% (wt/vol) sucrose, pH 7.5, with six changes of dialysate. The solution was centrifuged for 5.5 hours at 540,000g, at which time the liposomes were recovered as an opalescent zone. The band 3-containing liposomes banded in a different part of the tube from protein-free control liposomes. The liposomes were diluted fourfold in the same buffer without sucrose and were pelleted at 54,000g for 1 hour. The pellet was left at 4°C for up to 7
days to promote fusion of the small liposomes. To determine the orientation of band 3 in the membrane, an aliquot of the preparation was treated with chymotrypsin at 1 μg/mL, which is sufficient to liberate all exposed N-terminal domains. Molecules with the opposite orientation (cytoplasmic domain on the inside of the vesicle) give rise to the stable fragment of molecular weight 58,000 that could be quantitated by densitometry.

Ankyrin binding experiments were also performed on band 3 in larger liposomes produced by sonication; the protein was introduced directly from solution.47

Antibodies. A rabbit polyclonal antibody against the cytoplasmic domain of band 3 was generated by immunising rabbits with the N-terminal fragment prepared from a chymotryptic digest of ghosts.5 Polyclonal antiankyrin was similarly raised in rabbits by immunisation with purified ankyrin.

Column chromatography. Analytical gel filtration chromatography was performed on a Superose 6 (HR10/30) column in a Pharmacia FPLC apparatus (Pharmacia, Uppsala, Sweden). The solvent was 0.1 mol/L sodium chloride, 20 mmol/L triethanolamine, 10 mg/mL BRIJ 35, 0.1 mmol/L EDTA, 0.2 mmol/L dithiothreitol, 0.1 mmol/L butylated hydroxytoluene (BHT), and 0.02% sodium azide, pH 7.4. The eluate was monitored by absorption at 280 nm and the identity of the proteins eluting in each fraction was determined by SDS-gel electrophoresis or by dot-blotting on nitrocellulose membrane and staining with antibodies, using a horseradish peroxidase-conjugated second antibody with the luciferase (ECL) system (Amersham) for detection.

Molecular weight determinations. The band 3 components eluting from the MonoQ column were identified on the basis of their molecular weights. These were determined from the Stokes radii estimated by gel filtration on a Superose 6 column that was calibrated with standard proteins48 and from the corresponding sedimentation coefficients determined in a Beckman model E analytical ultracentrifuge at 60,000 rpm, using schlieren optics. The molecular weight is given by the equation $M = 6N_\alpha R_s(1 - v_\rho)$, (1), where $N_\alpha$ is the solvent viscosity, $s$ is the sedimentation coefficient, $R_s$ is the Stokes radius, $v$ is the partial specific volume, $\rho$ is the solvent density, and $N$ is Avogadro’s number. Allowance was made for the detergent contribution to the buoyancy of the sedimenting particle by masking with $D_2O$, based on extrapolation of sedimentation velocities, measured in $D_2O/H_2O$ mixtures. The buffer was 0.45 mol/L sodium chloride, 20 mmol/L triethanolamine, and 10 mg/mL Brij 35, pH 7.5. The density of the detergent was determined from the corresponding sedimentation velocity by capillary viscometry (Ubbelohde viscometer).

The partial specific volume of the polypeptide was calculated to be 0.750 mL/g; assuming a carbohydrate content of 10%,35 that of the glycoprotein is approximately 0.735 mL/g.

Electron microscopy. Fused liposomes, containing band 3 dimer and tetramer with and without added ankyrin, and the membranes of intact RBCs, of ghosts, and of vesicles depleted of peripheral proteins by successive extractions of the host membrane at low and high ionic strengths were examined by freeze-fracture electron microscopy. The samples were prepared as a thin film between Balzer specimen holders and frozen with a BioRad liquid nitrogen jet freezer (BioRad, Richmond, CA). They were fractured at −150°C in a Polaron E7500 freeze-fracture apparatus (Fisons Scientific Equipment, Loughborough, UK) and immediately replicated with platinum carbon at a 45° shadowing angle, followed by application of a carbon film. Replicas were recovered by floating on distilled water, cleaning with sodium hypochlorite, and picking up on G400 high-transmission hexagonal copper grids. The replicas were examined in a Philips EM301G transmission electron microscope (Philips, Eindhoven, Holland) at 100 kV accelerating voltage. The apparent projected diameters of the particles were measured orthogonally to the shadowing direction.

RESULTS

Association state of band 3. freshly prepared band 3, in the presence of Brij 35, eluted from the gel filtration column as a single symmetrical zone (Fig 1), with an elution volume corresponding to a Stokes radius of 7.9 nm. After chromatography on a MonoQ ion-exchange column, variable proportions of a second component were generated. This eluted before the parent zone on gel filtration, with an estimated Stokes radius of 10.3 nm. There was little or no rapid interconversion of the two components on gel filtration, because the bulk of both eluted at the same volume when rechromatographed after dilution of up to 10-fold.

Both components were examined by sedimentation veloc-
ity in the analytical ultracentrifuge. Correcting for bound detergent, as indicated, sedimentation coefficients, $s_{20,w}$, of 4.4S and 7.1S were obtained for the two components. From equation 1, the corresponding molecular weights emerged as 230,000 and 470,000 (calculated values, approximately 220,000 and 440,000). The two components are thus identified as dimer and tetramer, and the freshly extracted protein consisted of pure dimer. No monomer was present, as was also shown by preparing band 3 from cells after cross-linking with the oxidant, copper-$\alpha$-phenanthroline. Gel electrophoresis in SDS showed that cross-linking was complete, and the cross-linked and untreated proteins eluted at identical volumes from the gel filtration column, except for a trace of highly aggregated material in the former. We were unable to generate any measurable proportion of species higher than the dimer by successive cross-linking from the outside and the inside of the cell with bis(succinimidyl) suberate and copper-$\alpha$-phenanthroline, respectively, or by the procedure described by Salhany et al.

![Fig 2. Elution from gel filtration column (Superose 6, HR10/30) of band 3-ankyrin complexes. (A) Complex prepared by dissociation from isotonic membrane skeletons. The elution volumes of free band 3 dimer (D) and tetramer (T) are indicated. (B) Elution of complexes formed by 1:1 mol:mol mixtures of ankyrin with band 3 dimer (---) and with band 3 tetramer (----); the elution of free ankyrin at the same concentration as in both complexes is also shown (-----). The elution volumes of free band 3 dimer and tetramer are as indicated. Fractions were analyzed for the presence of both proteins by immunoblot to show that the displacement of the elution positions reflected formation of complexes.](image)

The dimer and tetramer showed little or no concentration-dependent interconversion in solution. Therefore, we take the tetramer to be an artefact of the preparation in which an ion-exchange step is involved. However, both components were found to bind ankyrin. Figure 2B shows that the elution volumes were displaced towards the void volume after incubation with ankyrin. The apparent change in Stokes radius was relatively small, suggesting that the asymmetric or flexible structure of the band 3 is rendered more compact in its complex with ankyrin. Judging by the disappearance of the free ankyrin from the elution profile upon incubation
with the band 3 dimer, it appeared that the band 3 dimer was largely or entirely capable of binding ankyrin. The tetramer, on the other hand, appeared to bind about 50% less ankyrin on a weight basis and was therefore only partly functional. Neither ankyrin nor its complexes with band 3 formed stable sedimenting boundaries in the analytical ultracentrifuge; a likely explanation is that the ankyrin bound sufficient nonionic detergent to generate a hydrodynamic particle sedimenting too slowly to observe. We therefore have no definitive measure of the compositions of the complexes, but, because the dimer and tetramer yielded products of different size on binding ankyrin, the dimer evidently did not associate, nor the tetramer dissociate, in forming ankyrin complexes.

Complex formation between dimeric band 3 and ankyrin could also be detected by gel electrophoresis of the undenatured proteins. Immunoblotting with antiankyrin and antiband 3 showed the formation of a new zone, corresponding to a complex, on the addition of the band 3 to ankyrin, with slower anodic mobility.

**Stoichiometry of the band 3-ankyrin interaction in situ.** Binding of labeled ankyrin to membrane vesicles was in all cases taken as close to saturation as ankyrin concentrations permitted to allow the saturation level to be defined, independently, so far as possible, of the association constant. However, profiles were also analyzed by computer fitting. Binding was relatively rapid; in our hands, there was no evidence of a very slow binding phase over about 2 hours. The profiles were consistent with a single binding process and a titer of approximately $0.8 \times 10^5$ binding sites per cell. This finding confirms that saturation occurs at grossly substoichiometric ratios of ankyrin:band 3 subunits. To determine whether the restriction on ankyrin binding is related to the presence in the membrane of higher association states than the dimer, binding experiments were also performed on band 3 in liposomes (which evinces the same low binding capacity...
binding was checked by demonstrating competition with the 43K cytoplasmic fragment of band 3. The amount of ankyrin bound could be reduced by 75% by an 18-fold excess of free cytoplasmic fragment. Regulation of ankyrin rebinding by a slow equilibrium between binding-competent and binding-incompetent states of band 3 was also excluded by the absence of further ankyrin binding, after 24 hours of incubation of ankyrin-saturated membrane vesicles.

**Dependence on ankyrin of band 3 association with the membrane skeleton.** As predicted from the band 3:ankyrin ratio in the cell, some 20% of the total membrane band 3 should be recovered with isolated membrane cytoskeletons if the attached band 3 is a dimer. The band 3-ankyrin complex can be released from its site on the spectrin by high salt concentrations. To establish that the band 3 remaining with the membrane cytoskeleton during extraction with nonionic detergent in isotonic salt is all bound through ankyrin, we treated unsealed ghosts with low concentrations of chymotrypsin. Jinbu et al showed that trypsin at very low concentrations cleaves the ankyrin between its band 3-binding and spectrin-binding domains, while causing little damage to other proteins. We have found that this specificity of proteolysis is improved by the use of chymotrypsin. Gel electrophoresis in the presence of SDS in the buffer system of Fairbanks et al, in which the ankyrin bands are resolved from spectrin, showed disappearance of intact ankyrin without significant degradation of the other components after such treatment (data not shown). When membrane cytoskeletons were prepared from the resulting ghosts, more than 95% of the band 3 was released into the detergent-containing supernatant, compared with 80% from untreated membranes. We conclude that the band 3 is indeed essentially all attached to the membrane cytoskeleton through ankyrin and not by interactions with any other proteins (such as 4.1, 54, 55).

**Characterization of membrane skeleton-associated band 3.** That the band 3-ankyrin-membrane cytoskeleton complex, as isolated by extraction with nonionic detergent, is a true stoichiometric entity is shown by the constancy of its composition upon successive re-equilibrations with the isotonic detergent medium. After three further extractions, the band 3-spectrin molar ratio remained the same as in the initial preparation, ie, 0.2 to 0.25 in all of five separate experiments. This value is in agreement with earlier data.

By immunoassay (ELISA) the ratio of band 3:ankyrin in intact cells was found to be 8.2:1, which is in reasonable agreement with values calculated from earlier published data; by reference to the concentrations in intact membranes, the ankyrin was shown to have been recovered in its entirety in the membrane skeletons, and the molar ratio of band 3:ankyrin was found to be 1.8:1. It thus appears that the quaternary structure of the bound and free populations of band 3 is the same or becomes the same after separation from the membrane. In addition, we examined the band 3-ankyrin complex released from membrane skeletons by increasing the salt concentration. This eluted from the gel filtration column between the positions for the band 3 dimer and tetramer (Fig 2A). To determine whether the complex contained one subunit of band 3 or two, the band 3 was cross-linked in situ before preparation of the membrane skeleton. This was performed in three ways, ie, (1) with 3,3'-

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**Fig 4. Histograms showing apparent dimension, orthogonal to the shadowing direction, of intramembrane particles in liposomes with incorporated band 3 dimer (A), tetramer (B), and dimer, followed by binding of ankyrin (C).**

as in membrane vesicles. Band 3 was incorporated into liposomes at protein:lipid weight ratios of 1:2 and 1:40 (compare the band 3:lipid weight ratio of approximately 1:2.5 in the intact RBC membrane), and ankyrin binding profiles were obtained as before; no significant difference in binding stoichiometry could be discerned (saturation at band 3:ankyrin ratio of 11:1 in both cases). This result excludes the participation of any rapid association-dissociation equilibrium in the binding process. The specificity of the measured...
depleted of spectrin and actin by extraction at low ionic strength (C), and similar vesicles also depleted of ankyrin by extraction at high ionic strength (D). Bar = 100 nm.

**Fig 5.** Freeze-fracture electron micrographs of membranes of intact RBCs (A), ghosts prepared by hypotonic lysis (B), membrane vesicles depleted of spectrin and actin by extraction at low ionic strength (C), and similar vesicles also depleted of ankyrin by extraction at high ionic strength (D). Bar = 100 nm.

dithiobis(sulphosuccinimidyl propionate), which introduces a reducible extracellular cross-link between the constituent chains of the dimer; (2) with copper-o-phenanthroline, to generate a cross-link between the cytoplasmic domains; and (3) with the first of these reagents, applied to the intact cells, followed by the second on ghosts derived from them. The band 3 recovered with membrane skeletons, prepared by extraction with isotonic Triton solution in the absence of reducing agent, was estimated in all cases by SDS-gel electrophoresis (under reducing conditions); cross-linking was found not to have changed the amount remaining with the membrane skeleton and it follows that the ankyrin-band 3 complex attached to the membrane skeleton (Fig 2A) contained two subunits of band 3.

Exchange of free and ankyrin-bound band 3 in the membrane. Bennett and Whitfield et al were unable by peptide mapping to show a significant difference in the covalent character of ankyrin-bound and free band 3. To show that there is no other intrinsic difference between the two populations in the cell, we have sought evidence for exchange between them. Resealed ghosts, labeled on band 3 with a fluorescein fluorophore, were fused with a 10-fold excess of unlabeled ghosts. This ensured that fluorescent ghosts in a fused pair were overwhelmingly (80%) associated with an unlabeled partner. As observed by Fowler and Branton, there was progressive penetration of the fluorescent label into the dark hemisphere, reflecting the diffusion of band 3 in the plane of the membrane. These fused cells were treated with isotonic buffer, containing Triton X-100 to extract the fraction of band 3, that was not bound to ankyrin and leave the membrane skeletons, with the attached fraction of band 3, on the slide. As has been noted, the membrane skeletal network shrinks on extraction of the lipid. This is apparent in Fig 3, but it can be seen that the fused ghosts gave rise to paired membrane cytoskeletons. Moreover, where these were derived from fused pairs of ghosts, one of which had been rendered fluorescent, they also showed uniform fluorescence. When the slide was further irrigated with a high-salt medium, also containing Triton X-100, only vestigial fluorescence remained (consistent with very weak labeling of membrane skeletal proteins, detectable on the SDS gels, and resulting probably from penetration of traces of the reagent through the membrane). Thus the ankyrin-bound band 3, which remained with the membrane cytoskeletons after extraction in isotonic conditions but was dissociated from them in the hypertonic medium, had similar fluorescence in both partners of the fused pairs and had consequently entered the unlabeled membrane cytoskeleton by exchange with free band 3 within the dead time of the experiment (approximately 1 hour for fusion and subsequent manipulations).

Association state of free and ankyrin-bound band 3. There remains the possibility that the association state of band 3 in the membrane (if not in solution) changes when ankyrin is bound. We tried to resolve this question by means of freeze-fracture electron microscopy. Yu and Branton showed that band 3 was dimeric after incorporation into liposomes by counting the number of intramembrane particles corresponding to a given concentration of protein. However, the precision of this method is inadequate to detect the reduction in the number of particles corresponding to the small proportion of the band 3 that binds ankyrin at saturation. We therefore attempted to measure the size distribution of the particles before and after binding of ankyrin. We assume only that the dimensions of the tetramers in the membrane are greater than those of the dimer in at least one direction. Because the shadow obscures the boundary of the particle, length measurements are meaningful only along the axis orthogonal to the direction of shadowing. Figure 4 shows histograms of the apparent extent of the particles along the axis of measurement. The average apparent projected lengths, <r>, of the dimer and tetramer particles were 7.9 and 11.8 nm, respectively. If these can be approximated as rods or ellipses, then the corresponding true lengths, r, can be obtained by averaging over all orientations with
The uptake of the labeled ankyrin was relatively rapid, reaching a plateau in less than 1 hour; at much longer times, the binding is accompanied by the increase of diffusional mobility of the intramembrane particles in RBCs and membrane vesicles can be discerned in the electron micrographs shown by Shotton et al.29 There was no increase in the number of particles seen in the E-faces. It thus appears that extraction of the membrane skeleton destabilizes the band 3 dimers in the membrane and causes aggregation. These aggregates may be incapable of binding ankyrin and thus explain the low saturation titre.

Binding of excess ankyrin by band 3 in situ. The above observations raise the question of whether the free band 3 dimers in the intact membrane are capable of binding ankyrin. Tsuji and Ohnishi30 have reported that a certain quantity of additional ankyrin can bind to unsealed ghosts and that binding is accompanied by the increase of diffusional constraints on band 3. We have observed similar binding. The uptake of the labeled ankyrin was relatively rapid, reaching a plateau in less than 1 hour; at much longer times, the radioactivity incorporated could be seen to increase, presumably as a result of exchange against the endogenous fraction. This was consistent with a slow, but measurable off-rate of the bound radioactive ankyrin (data not shown). The profile shown in Fig 6 was determined after 2 hours of incubation and corresponds to binding of 1.9 × 10^5 molecules of ankyrin per RBC. Of this, however, 1.0 × 10^5 can be accounted for by the unoccupied site on each spectrin tetramer, and about 60% of it was recovered in membrane skeletons prepared by extraction with isotonic detergent (which also contained an unchanged proportion of band 3). The symmetry of the heterogeneous head-to-head association between the α- and β-chains of the spectrin dimers making up a tetramer dictates that the normally unoccupied ankyrin binding sites point away from the membrane. Ankyrin bound at these sites cannot therefore interact with membrane-associated band 3. Thus, allowing for incomplete recovery of membrane skeletons after preparative manipulations, we conclude that more than half of the extraneous ankyrin taken up by ghosts was bound to the unoccupied spectrin site and the remainder probably to free band 3. Because there are 5 × 10^5 band 3 dimers with no bound ankyrin in the cell, saturation of both types of binding site for ankyrin would correspond to the uptake of approximately 6 × 10^5 molecules of ankyrin per cell. Thus, the bulk of the free band 3 in the ghost cannot bind ankyrin.

We measured binding of ankyrin to membrane skeletons, prepared by extraction with isotonic salt and retaining therefore their original complement of endogenous (unlabeled) ankyrin. Curve fitting gave the ratio of binding sites to spectrin tetramers as 1.6:1, i.e., approximately 1.6 × 10^5 molecules per cell equivalent. This value may be distorted by exchange of endogenous against labeled ankyrin, which would be expected to occur more readily than in ghosts, in which the ankyrin forms part of a complex attached both to the membrane skeletal network and the membrane.

Membrane skeletons extracted at high ionic strength, which contained no endogenous ankyrin, bound 2 molecules of labeled ankyrin per spectrin tetramer, as expected, i.e., approximately 2 × 10^5 molecules per cell equivalent (dissociation constant, approximately 0.2 μM/L compared with 0.1 μM/L, determined by Tyler et al31).
in both Triton X-100 and Brij 35 for periods of at least several days. We cannot account for the conflict with the sedimentation equilibrium data of Pappen and Schubert. If there were a rapid association equilibrium detectable in free solution, the entropic advantage derived from confining the associating species in the plane of the membrane, together with the excluded-volume effect and the very high concentration of the band 3, would be expected to ensure that the highest state of association prevailed in situ (see Grasberger et al.63 who have calculated a factor of about 10^6 for the increase in the effective dimerization association constant for molecules thus constrained). The most direct available measurement of association state in the membrane comes from radiation inactivation analysis and suggests that band 3 is dimeric. In liposomes, as Yu and BrantonZ9 have shown, band 3 is dimeric, regardless of concentration over a wide range.

Our results indicate that in detergent solution the dimer binds ankyrin without further self-association, which is consistent with observations on the (dimeric) N-terminal cytoplasmic fragment of band 3,32,64,65 Mulzer et al.66 have given evidence for ankyrin binding only to band 3 tetramers, in rapid equilibrium with dimer and monomer. Such a scheme seems to be excluded, at least under our experimental conditions.

In the model membrane of phosphatidylcholine vesicles, on the other hand, ankyrin does appear to induce association of the band 3 dimers to which it binds. We must suppose that such association limits the ankyrin binding capacity. It is unlikely that this represents a stoichiometric, reversible process, for then the negative free energy of association between an ankyrin-band 3 dimer complex and additional free band 3 dimers would have to be vastly in excess of that for the ankyrin-band 3 dimer association. It is hard to envisage a plausible mechanism for such a relationship.

Exchange between free band 3, isolated as the unbound fraction in the membrane, and band 3 bound to extracted membrane cytoskeletons has been shown.62 We have shown here that such exchange also occurs freely in the intact membrane. Because of the time taken for fusion of the ghosts in our experiments, we cannot determine the time scale of the exchange reaction, other than to set an upper limit on it of about 1 hour at 37°C. This finding nevertheless excludes any possibility that the bound and unbound populations of band 3 in the cell differ intrinsically in their capacity to bind ankyrin. This conclusion was also tentatively drawn by Bennett15 and Whitfield et al.57 on grounds of the indistinguishable peptide maps of the free and ankyrin-bound fractions. It is also possible to exclude regulation of ankyrin binding by band 4.2 protein, which associates tightly with band 3, on the grounds that it is not required for in vitro band 3-ankyrin interaction and its elimination from inside-out membrane vesicles does not affect ankyrin rebinding.33

Our results leave the problem of the anomalous ankyrin rebinding capacity of the ankyrin-depleted membrane still incompletely resolved. On the basis of the arguments enumerated above, one may eliminate explanations of the stoichiometry based on (1) structural differences between the free and ankyrin-bound band 3 fractions; (2) exclusive binding by a form of high association state, either in rapid association equilibrium or not; and (3) whether or not the band 3 bears attached 4.2. The possibility seems not to have been considered that the similar ankyrin contents of the native membrane and saturated membrane-derived inside-out vesicles might have different explanations. The concentration of ankyrin on the native membrane is evidently determined by the supply of the protein, already coupled to band 3;64 this, together with the additional (free) band 3, arrives in the membrane after assembly of the spectrin network.69 Moreover, if, as the rather low affinity of ankyrin for band 3 (see above) suggests, stably attached ankyrin must be secured by ternary interactions with band 3 and spectrin, its concentration must be limited by that of spectrin tetramers. By contrast, a nonstoichiometric aggregation of band 3, when the system is no longer stabilized by the membrane skeletal complex (Fig 5), may limit the functional fraction of ankyrin binding sites. A highly nonrandom distribution of band 3 in the ghost membrane, although on a much larger scale of distance, has been observed.70

We cannot explain why the large proportion of free band 3 in the native membrane will apparently bind no ankyrin: obstruction of access by the membrane skeleton remains a possibility. It may be noted at the same time that other, perhaps related, constraints on the behavior of band 3 remain unexplained; thus, some 35% of the band 3 is rotationally immobile on a time scale of seconds, presumably by reason of unidentified interactions with other molecules.71-73 Clague et al.74 have given reasons why this is not to be identified with the ankyrin-bound population. The participation of as yet unidentified membrane proteins remains a possibility.

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REFERENCES

8. Jöns T, Drendkhahn D: Identification of the binding interface involved in linkage of cytoskeletal protein 4.1 to the erythrocyte ion exchanger. EMBO J 11:2863, 1992

12. Nigg EA, Gahnberg CG, Cherry RJ: Rotational diffusion of band 3 proteins in membranes from En(a−) and neuraminidase-treated normal human erythrocytes. Biochim Biophys Acta 600:536, 1980


55. Gascard P, Pawelczyk T, Lowenstein JM, Cohen CM: The...


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