Restriction by Ankyrin of Band 3 Rotational Mobility in Human Erythrocyte Membranes and Reconstituted Lipid Vesicles†

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Received May 8, 1997⊗

ABSTRACT: Rotational diffusion of eosin-5-maleimide-labeled band 3 was measured in erythrocyte membranes at pH 9.4−10.4. Band 3 was found to be more mobile in this pH range than at pH 7.5. Similar results were obtained with spectrin−actin-depleted membranes, where it was further shown that ankyrin is the only detectable protein released from the membrane at pH 10. Further experiments were performed at pH 7.5 to investigate the effects of rebinding purified ankyrin and/or band 4.1 to ghosts stripped of skeletal proteins. Ankyrin was found to reduce band 3 rotational mobility, but band 4.1 had no effect. A fluorescence binding assay revealed that fluorescein isothiocyanate-labeled ankyrin had similar binding parameters to those reported previously using ^{125}I labeling. Finally, the rotational mobility of purified band 3 reconstituted into lipid bilayers was determined before and after ankyrin binding. The results of these reconstitution experiments were globally analyzed, assuming the existence of two populations of band 3 with different correlation times. The faster correlation time is consistent with that expected for either dimers or compact tetramers of band 3. Ankyrin binding reduces the proportion of band 3 contributing to the faster component. This result demonstrates that ankyrin promotes the association of band 3 into more slowly rotating complexes independently of any other components of the erythrocyte membrane. It has been reported that ankyrin contains two binding sites for band 3 [Michaely, P., & Bennett, V. (1995) *J. Biol. Chem. 270*, 22050−22057]. The results of the present study are thus explained by the ability of ankyrin to cross-link band 3 into larger diameter complexes. Cross-linking by ankyrin in part accounts for the slow components in the anisotropy decays of band 3 in the erythrocyte membrane. Other factors which probably influence band 3 aggregation include the membrane "fluidity" and protein concentration.

The anion transport protein band 3 is a major component of the erythrocyte membrane. Its rotational diffusion has been extensively investigated by measuring transient dichroism or phosphorescence anisotropy of the covalently bound triplet probe eosin-5-maleimide (EMA).[1] These experiments reveal the presence in the erythrocyte membrane of different populations of band 3 with different correlation times (Nigg & Cherry, 1979; Cherry, 1992; Matayoshi & Jovin, 1991; Tilley et al., 1990). In particular, it is clear that a significant fraction of band 3 is slowly rotating or immobile over the measurement time of a few milliseconds.

In addition to its anion transport function, band 3 serves as an attachment point for the erythrocyte skeleton (Bennett & Stenbuck, 1979, 1980). The cytoplasmic domain of band 3 binds the linker protein, ankyrin, which also binds to spectrin. It has consistently been shown, however, that removal of most of spectrin and actin from the membrane by low salt extraction has no effect on band 3 rotational mobility (Cherry et al., 1976; Nigg & Cherry, 1980; Tilley et al., 1993). Similarly, band 3 rotational mobility in spectrin-deficient red cells is essentially the same as in normal cells (Corbett et al., 1994). It was suggested that the failure of spectrin−actin to influence band 3 rotation is a consequence of the flexibility of the spectrin molecule (Clague et al., 1990). Flexibility of the cytoplasmic domain of band 3 could also be a factor (McPherson et al., 1992; Wang, 1994)

The cytoplasmic domain of band 3 is clearly involved in interactions which restrict band 3 mobility, since cleavage of this domain by mild proteolysis results in a large increase in rotational mobility (Nigg & Cherry, 1980; Matayoshi & Jovin, 1991). Moreover, an increase in band 3 rotation is also observed upon high salt extraction of ankyrin and band 4.1 subsequent to spectrin−actin removal (Nigg & Cherry, 1980). Band 4.1 is also a linker protein but binds mainly to glycophorin C/D rather than to band 3 (Pinder et al., 1992; Hemming et al., 1994). It thus seems reasonable to suppose that restraints to band 3 rotational mobility result from interactions of ankyrin with the cytoplasmic domain of band 3.

Other experiments, however, have indicated that the situation may be rather more complicated than hitherto supposed. Clague et al. (1989) found that selective proteolytic cleavage of ankyrin failed to enhance band 3 rotational mobility. Subsequent experiments involving the rebinding of ankyrin and band 4.1 suggested that ankyrin is only effective in restricting band 3 mobility if band 4.1 is

† Supported by the Wellcome Trust and the BBSRC.

⊗ Abstract published in *Advance ACS Abstracts,* July 15, 1997.

[1] Abbreviations: EMA, eosin-5-maleimide; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; 5 PB, 5 mM sodium phosphate buffer; 10 PB, 10 mM sodium phosphate buffer; PBS, phosphate-buffered saline; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EGTA, ethylene glycol bis(*β*-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; FITC, fluorescein 5-isothiocyanate.

S0006-2960(97)01074-X CCC: $14.00

also present (Wyatt & Cherry, 1992). On the other hand, an enhanced rotational mobility of band 3 in ankyrin-deficient red cells has been reported (Cho et al., 1994). Here we report the results of further experiments aimed at elucidating the roles of ankyrin and band 4.1 in restricting band 3 rotational mobility.

MATERIALS AND METHODS

Preparation of Ghosts for Rotational Diffusion Measurements. Band 3 was labeled with EMA (Molecular Probes Inc.) in intact cells from which ghosts were prepared as previously described (Nigg & Cherry, 1979). For studies with ghosts depleted of skeletal proteins, band 6 was first removed by washing twice with PBS (pH 7.5). Removal of skeletal proteins was achieved by incubation with 1 mM CAPS, pH 12, on ice for 10 min (Tilley et al., 1993). These membranes are hereafter referred to as "stripped" ghosts. Some measurements were also performed with ghosts depleted of spectrin and actin or with the cytoplasmic domain of band 3 cleaved with trypsin, as described by Nigg and Cherry (1980).

Purification of Ankyrin and Band 4.1. Ankyrin was purified essentially by the method of Gardner and Bennett (1986). Packed red cells devoid of white cells (Pinder et al., 1989) were lysed in 30 volumes of 5 PB, containing 0.2 mM DTT, 1 mM PMSF, pH 7.5, and the ghosts were pelleted by centrifugation at 24000*g* for 10 min. The membranes were then washed 3 times with the same buffer minus PMSF. Solid NaCl was added to 50 mM, and the membranes were then incubated for 10 min at 0 °C.

Subsequently, the membranes were incubated with 10 volumes of 10 PB, containing 100 mM NaCl, 1 mM EDTA, 0.5% v/v Triton X-100, 0.2 mM DTT, and 0.02% w/v PMSF, pH 7.5, for 15 min at 4 °C. The Triton shells were isolated by centrifugation at 25000*g* for 20 min, washed in the same buffer once, and then washed 3 times in 10 PB (i.e., without Triton X-100). Ankyrin was extracted by incubation of the Triton shells in 7.5 PB, 1 M NaCl, 1 mM EDTA, 0.2 mM DTT, and 0.02% w/v PMSF, pH 7.5, for 30 min. The sample was then centrifuged at 40000*g* for 30 min, and the supernatant was dialyzed overnight against 5 L of 7.5 PB containing 10% w/v sucrose, 0.1 mM EDTA, 0.2 mM DTT, 1 mM NaN_3, and 0.02% w/v PMSF, pH 7.5 (buffer A), with one buffer change. Further purification was achieved by applying the dialyzed ankyrin sample to a Whatman DE52 anion exchange column (1.5 × 10 cm, 30 mL/h) equilibrated with buffer A. Bound ankyrin was washed with 40 mL of buffer A before the protein was eluted by applying a stepwise salt gradient consisting of the same buffer with 180 mM NaCl and then 300 mM NaCl. Eluted proteins were identified by SDS−PAGE; ankyrin was eluted at 300 mM NaCl. The ankyrin was then dialyzed against storage buffer (7.5 PB, 0.1 mM EDTA, 0.2mM DTT, 1 mM NaN_3, 20% sucrose, pH 7.5) and stored at −22 °C with freshly added PMSF.

Band 4.1 was prepared essentially according to the method of Ohanian et al. (1984). After removal of the white blood cells, red cells (30 mL packed volume) which had been 3 times washed with PBS containing PMSF 0.05% w/v were suspended in an equal volume of the same buffer and cooled on ice. Lysis was induced by the addition of 60 mL of cold (4 °C) lysis buffer (150 mM NaCl, 24 mM HEPES, 1 mM EGTA, 0.5 mM DTT, 15% v/v Triton X-100, pH 7.0). Portions of 20 mL of this lysate were layered onto cold (4 °C) 30% sucrose (17 mL volume per tube), containing 0.6 M KCl, 24 mM HEPES, 0.5 mM ATP, 0.5 mM DTT, 0.5 mM EGTA, pH 7.4.

Centrifugation at 120000*g* for 45 min produced a transparent pellet, containing spectrin, actin, 4.1, and 4.9, with a trace of band 7. The red supernatant was removed by washing down the walls of the centrifuge tube with distilled water. The pellets were collected and pooled and then washed 3 times in 10 mM Tris-HCl, pH 7.4. The pellet volume was measured, and an equal volume of 2 M Tris-HCl, 0.2 mM DTT, 1 mM EGTA, 0.1% Tween 80, pH 7.0, was added at 25 °C. After 15 min incubation at 37 °C with frequent mixing, the resulting solution was centrifuged at 90000*g* for 30 min to remove undissolved debris, and the supernatants were then kept at 4 °C.

Separation of spectrin and band 4.1 was achieved by gel filtration. The supernatant was passed down a column (120 cm by 2.5 cm) of Sepharose CL-6B, equilibrated with 2 M Tris-HCl, 1 mM EGTA, 0.2 mM DTT, 0.1% Tween 80, pH 7.0, at 12 mL/h. The separation profile was monitored at 280 nm and produced three main protein peaks. The first peak (peak 1) corresponded to spectrin, then a smaller peak (peak 2) contained protein 4.1 and 4.9, with a trace of spectrin, and finally actin was eluted last (peak 3). Further purification of band 4.1 was achieved after dialyzing peak 2 against 7.5 PB, containing 0.2 mM EDTA and 0.1 mM DTT, pH 7.5 (buffer B). Protein 4.9 and other contaminating proteins were then removed by a Whatman DE52 (10 cm × 1.5 cm) anion exchange column equilibrated with buffer B (30 mL/h). A stepwise salt gradient consisting of buffer B with 100, 180 and 300 mM KCl was then applied to release band 4.1 which eluted at 180 mM KCl. The protein was dialyzed against storage buffer and stored at −22 °C.

The protein concentrations of all purified proteins and ghost membranes were determined using the Lowry method and the appropriate correction factors (Lowry et al., 1951). Unless otherwise stated, the protein concentrations are given as BSA equivalents.

SDS−PAGE. The protein contents of the various ghost preparations and protein extractions were analyzed using the Laemmli polyacrylamide gel electrophoresis system (Laemmli, 1970). Gels were stained using Coomassie Blue (0.025% w/v, 40% methanol v/v, 10% acetic acid v/v) and were destained in a solution of 20% v/v methanol, 7% v/v acetic acid. For eosin-labeled samples, all processes were undertaken in the dark to prevent photo-induced covalent cross-linking of the proteins. Eosin fluorescence was photographed under UV illumination with appropriate filters prior to staining with Coomassie Blue.

Rebinding Ankyrin and Band 4.1 to Stripped Ghosts. Rebinding of ankyrin or band 4.1 to stripped ghosts was accomplished essentially as described by Bennett (1983). The purified proteins were dialyzed against 10% sucrose w/v, 90 mM KCl, 7.5 mM PB, 0.2 mM DTT, 0.1 mM EDTA, 0.4 mM PMSF, pH 7.5, and then incubated with stripped ghosts (1.0 mg/mL) in the same buffer at 22 °C for 3 h. Unbound ankyrin or band 4.1 was removed by centrifugation at 22000*g* for 20 min over an equal volume of 20% sucrose, 100 mM KCl, 7.5 mM PB, 0.2 mM EDTA, 0.1 mM DTT, pH 7.4. The sample was collected after 3 washes with 5 PB and resuspended up to the previous volume in buffer containing 66% glycerol in preparation for the transient dichroism measurements. Rebinding was confirmed by SDS−PAGE.

In some experiments, both ankyrin and band 4.1 were rebound sequentially to the same stripped ghosts. Following the above procedures, ankyrin was rebound first, the membranes were then washed 3 times in 5 PB, and finally band 4.1 was rebound. Alternatively, band 4.1 was rebound prior to ankyrin. Control experiments were performed using spectrin−actin-depleted ghosts or trypsin-treated ghosts.

Fluorescence Assay for Ankyrin Rebinding. After dialysis against 0.1 M $NaHCO_3$, 0.2 mM DTT, 1 mM EDTA, pH 9.0, ankyrin (1.3 mg/mL) was incubated with a 15-fold molar excess of FITC at 22 °C in the dark for 3 h. Unreacted FITC was removed by passing the reaction mixture through a Sephadex G-25 gel filtration column (Pharmacia PD-10 unit, 1.5 × 5 cm) equilibrated with the same buffer at room temperature. Labeled ankyrin was then dialyzed against 20% w/v sucrose, 7.5 PB, 1 mM EDTA, 0.2 mM DTT, 1 mM NaN_3, pH 7.5, and stored at −22 °C in the dark. The fluorescein:ankyrin molar ratio was calculated from the fluorescein absorbance measured at 492 nm using an extinction coefficient of 6.8×10^4 cm^{-1} mol^{-1}.

Fluorescein-labeled ankyrin (FITC-ankyrin) was rebound to stripped ghosts as described above and the fluorescence intensity of the sample measured using a Baird Nova Spectrofluorimeter. The sample was excited at 495 nm and fluorescence emission measured at 518 nm. The concentration of rebound FITC ankyrin was determined from a standard curve constructed by measuring the fluorescence of FITC-ankyrin over a range of known concentrations.

High-pH Measurements. Control ghosts for transient dichroism were prepared in 5 PB, pH 7.5. For high-pH studies, the pH of the sample was adjusted with NaOH at room temperature and measured as soon as practical thereafter. This resulted in exposure of the sample to high pH for 5−10 min while the sample was equilibriated at 37 °C and deoxygenated. Some samples were incubated for at least 30 min at high pH and then returned to normal pH by addition of HCl. Experiments were also performed with samples washed 3 times at pH 10 and resuspended in 5 PB, pH 7.5, containing 66% glycerol.

Purification and Reconstitution of Band 3. EMA-labeled band 3 was extracted from erythrocyte ghosts in Triton X-100, purified, and reconstituted essentially as described by Mühlebach and Cherry (1985). For reconstitution, band 3 and egg phosphatidylcholine solubilized in Triton X-100 were mixed in a ratio of 7:1 (lipid:protein, w/w), and the detergent was removed by dialysis over 4 days. The reconstituted vesicles were recovered by centrifugation, and ankyrin was bound using the same conditions as for stripped ghosts. After ankyrin binding, the vesicles were suspended in 5 PB, pH 7.5, containing 75% glycerol for transient dichroism measurements.

Rotational Diffusion Measurements. The transient dichroism apparatus used to measure rotational motion was similar to that described in detail elsewhere (Cherry, 1978). Excitation was by a Nd-YAG laser (JK Lasers, Ltd.) using the frequency-doubled emission at 532 nm. The pulse width was about 15 ns and the repetition rate 10 Hz. Transient absorbance changes at time t after the flash arising from ground-state depletion were simultaneously recorded at 515 nm for light polarized parallel [$A_{\parallel}(t)$] and perpendicular [$A_{\perp}(t)$] with respect to the polarization of the exciting flash. Up to 512 signals were averaged in a Datalab DL 102A signal averager. All samples were flushed with argon prior to measurement to obviate quenching of the eosin triplet state by oxygen. The eosin concentration was typically 1−2 μM. Data were analyzed and plotted by calculating the absorption anisotropy, $r(t)$, defined by

$$r(t) = \frac{A_{\parallel}(t) - A_{\perp}(t)}{A_{\parallel}(t) + 2A_{\perp}(t)} \quad (1)$$

The anisotropy decay curves for band 3 in erythrocyte membranes were measured at 37 °C and fitted by the double exponential equation:

$$r(t) = r_1 \exp(-t/\phi_1) + r_2 \exp(-t/\phi_2) + r_3 \quad (2)$$

by a nonlinear least-squares analysis.

The anisotropy decay curves for reconstituted band 3 were measured at 28 °C and fitted by a global analysis essentially as described by Morrison et al. (1986). For uniaxial rotation of a single rotating species, $r(t)$ is given by (Cherry, 1979)

$$r(t) = r_0\{1.2 \sin^2\theta \cos^2\theta \exp(-t/\phi) + 0.3 \sin^4\theta \exp(-4t/\phi) + 0.1(3\cos^2\theta - 1)^2\} \quad (3)$$

θ is the angle between the transition dipole moment of the probe and the membrane normal, and r_0 is the experimental anisotropy at $t = 0$. As shown previously (Morrison et al., 1986), the anisotropy decays for reconstituted band 3 are best fitted by a model consisting of two rotating species. The present data obtained in the presence and absence of ankyrin were thus fitted simultaneously to a two-component model with global parameters ϕ_A, ϕ_B (the correlation times of the two species) and θ. The proportions of the two components, c_A, c_B, were independent parameters. A further refinement was that a term was included to account for any residual vesicle tumbling with the vesicle radius r_v as an additional global parameter, so that

$$r(t) = [c_A r_A(t) + c_B r_B(t)] \exp[(-3kT/4\pi\eta_a r_v 3)t] \quad (4)$$

where η_a is the viscosity of the aqueous phase and r_A, r_B are given by eq 3. The latter term only marginally affects the fits as vesicle tumbling is very slow in 75% glycerol (the correlation time at 28 °C is about 2.5 ms for 100 nm diameter vesicles).

RESULTS

Effects of High pH on Band 3 Rotational Diffusion in Ghosts. The effect of high pH on the anisotropy decay curves of EMA-labeled band 3 in ghosts is shown in Figure 1. The treatment produces a substantial increase in the rotational mobility of band 3. Similar results were obtained from experiments performed over the range pH 9.4−10.4. The parameters obtained from fitting the anisotropy decays to eq 2 are presented in Table 1. The data are noisier at high pH due to a reduction in triplet lifetime and an apparent loss of triplet quantum yield. This leads to quite large errors, particularly at pH 10.4. The only parameter which shows a significant change from the control value is the slower correlation time, ϕ_2, which decreases approximately 2 times at high pH. Within experimental error, there is rather little change in ϕ_2 between pH 9.4 and pH 10.4.

To investigate the reversibility of the effect, ghosts were returned to normal pH after incubation at high pH. Figure 1, curve C, shows that the initial part of the anisotropy decay is very similar to that of the control but the latter part of the

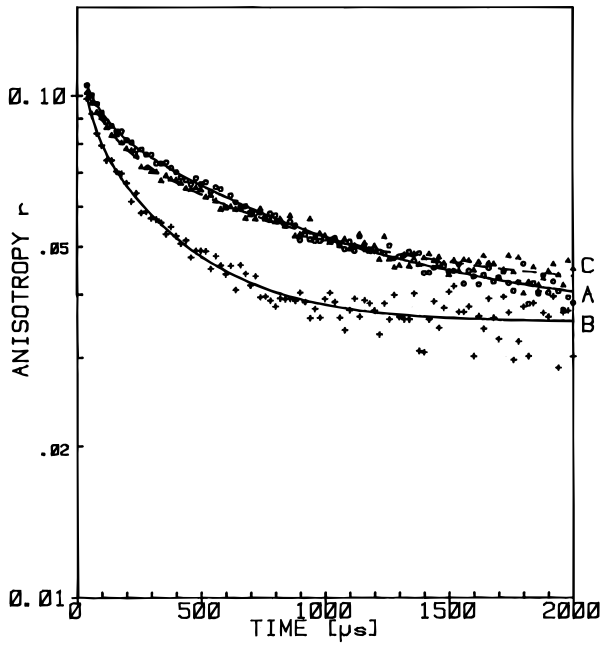

FIGURE 1: Effect of high pH on band 3 rotational mobility in ghosts. (A) ○ = pH 7.5 control; (B) + = ghosts measured at pH 9.9; (C) △ = ghosts incubated at pH 9.9, and then measured at pH 7.5 (dashed line). All measurements were performed in 5 PB at 37 °C.

decay is somewhat flatter than that of the control. Thus, the effect of high pH is largely reversible although a small amount of band 3 aggregation appears to be induced by the treatment. If ghosts are washed before returning to normal pH, the effect of high pH is completely irreversible.

Disruption of the erythrocyte cytoskeleton can cause fragmentation of the ghosts leading to an enhancement of the anisotropy decay by vesicle tumbling. Experiments performed in 66% glycerol, however, gave qualitatively similar results to those performed in the absence of glycerol. These experiments were also performed with incubation times at high pH of up to 1 h, but no further increase in rotational mobility was observed. In addition, visual examination by fluorescence microscopy of the eosin-labeled ghosts used for transient dichroism revealed little fragmentation at pH 9.4. Somewhat higher fragmentation was observed to occur at pH 10.4, but the fragmented vesicles only accounted for a minor fraction of the membrane present.

The effect of high pH is similar in ghosts depleted of spectrin and actin (data not shown). To determine whether any protein is released by the high-pH treatment, the supernatant from spectrin−actin-depleted ghosts which had been incubated and centrifuged at pH 10.0 was subjected to SDS−PAGE. The supernatant was found to contain a single protein (Figure 2A) which, by comparison with gels of total ghost protein, was indentified as ankyrin.

Effects of Rebinding Ankyrin and/or Band 4.1 on the Rotational Diffusion of Band 3 in Stripped Ghosts. The effects of rebinding ankyrin and band 4.1 to stripped ghosts on the anisotropy decay of EMA-labeled band 3 are shown in Figures 3 and 4. The addition of ankyrin caused a significant reduction in mobility of band 3 in stripped ghosts (Figure 3, curve C). In contrast, no effect on the anisotropy decay was observed upon rebinding band 4.1 up to a concentration of 3.0 mg/mL (Figure 4, curve C).

Experiments were also performed to test the effect on band 3 rotation of sequential rebinding of ankyrin and band 4.1. Figure 3, curve D, shows that after rebinding ankyrin, no further effect is detected upon subsequent rebinding of band 4.1. Similarly, when ankyrin is added subsequent to rebinding of band 4.1, its effect on band 3 rotation is essentially the same as in the absence of band 4.1 (Figure 4, curve D). The insets in Figure s 3 and 4 show the values of r_3 obtained by fitting the anisotropy decays by eq 2.

Control experiments were performed using ghosts treated with trypsin to remove the cytoplasmic domain of band 3 and also with membranes depleted of spectin−actin but not ankyrin and band 4.1. In both cases, ankyrin addition had no effect on band 3 rotation (data not shown).

The successful rebinding of ankyrin and band 4.1 was checked by SDS−PAGE in each experiment. Figure 2 shows examples of the results obtained.

Fluorescence Assay of Ankyrin Binding. A more quantitative assay of ankyrin binding to stripped ghosts was performed with FITC-ankyrin. Figure 5 shows the fluorescence determination of bound FITC-ankyrin as a function of the amount of FITC-ankyrin added. Several methods were used to correct for non-specific binding. A rather similar level of nonspecific binding was obtained with trypsin-treated membranes and with spectrin−actin-depleted membranes (in which the endogenous ankyrin is still present). A somewhat lower level of nonspecific binding was obtained in the presence of a 10-fold excess of unlabeled ankyrin. In view of this difference, two separate calculations were made of specific binding and used to construct the Scatchard plots shown in Figure 6. From the slopes of these plots, the dissociation constant for FITC-ankyrin binding to stripped ghosts was determined to be in the range 55−65 nM. The *x* axis intercepts give saturation binding in the range 140−200 μg of ankyrin/mg of stripped ghost protein. Assuming stripped ghosts contain approximately 50% of the protein of normal ghosts, this converts to about 10^5 ankyrin binding sites/cell.

Effect of Binding Ankyrin to Band 3 in Reconstituted Vesicles. Anisotropy decays for EMA-labeled band 3 in reconstituted vesicles in the presence and absence of ankyrin are shown in Figure 7. Ankyrin clearly slows the rotational diffusion of band 3. The anisotropy decays were analyzed by global analysis using a two-component model as described under Materials and Methods. The parameters obtained are listed in Table 2.

DISCUSSION

High-pH Experiments. It was previously shown that high-salt removal of ankyrin and band 4.1 from spectrin−actin-depleted ghosts results in enhanced rotational mobility of band 3 (Nigg & Cherry, 1980). This observation in itself does not distinguish the roles of ankyrin and band 4.1 in restricting band 3 mobility.

The binding of ankyrin to band 3 is known to be pH-dependent (Thevenin & Low, 1990). This arises from pH-dependent conformational changes in the cytoplasmic domain of band 3 which have pK_a values of 7.2 and 9.2 (Low et al., 1984). By about pH 9.4, ankyrin is fully dissociated from band 3 in erythrocyte ghosts while other skeletal interactions remain intact. At higher pH, further dissociation of skeletal proteins occurs, leading to loss of membrane stability (Low et al., 1991).

The pH-dependent binding of ankyrin thus provides a method of investigating the effect on band 3 rotational mobility of selectively breaking the band 3−ankyrin linkage.

Table 1: Fitted Parameters of Double Exponential and Constant (eq 2) Fit to Transient Dichroism Data for Ghosts after High-pH Treatment.[a]

pH	r_1	ϕ_1 (μs)	r_2	ϕ_2 (μs)	r_3
control	0.027 ± 0.003	79 ± 17	0.056 ± 0.001	830 ± 60	0.036 ± 0.001
9.4	0.034 ± 0.009	93 ± 49	0.047 ± 0.009	550 ± 130	0.035 ± 0.002
9.8	0.032 ± 0.021	51 ± 47	0.051 ± 0.008	423 ± 79	0.035 ± 0.001
10.4	0.027 ± 0.050	93 ± 340	0.042 ± 0.060	350 ± 350	0.041 ± 0.003
9.9	0.039 ± 0.038	43 ± 51	0.055 ± 0.011	340 ± 70	0.035 ± 0.001
7.5[b]	0.029 ± 0.004	96 ± 28	0.044 ± 0.003	750 ± 110	0.041 ± 0.002

[a] The error values are one standard deviation given by the least-squares algorithm. The control was measured at pH 7.5 prior to exposing ghosts to high pH. [b] Sample incubated at pH 9.9; returned to pH 7.5 for measurement.

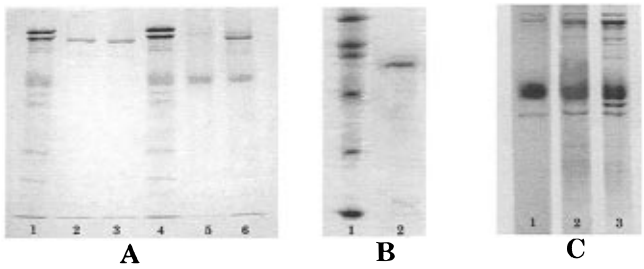

FIGURE 2: Results of SDS−PAGE. (A) Ghosts (lane 1), purified ankyrin (lane 2), supernatant from spectrin−actin-depleted ghosts washed at pH 10 (lane 3), ghosts (lane 4), stripped ghosts (lane 5), stripped ghosts with ankyrin rebound (lane 6). (B) Standards (lane 1: myosin, 205 kDa; galactosidase, 116 kDa; phosphorylase, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa); purified band 4.1 (lane 2). (C) Stripped ghosts (lane 1), stripped ghosts with ankyrin rebound (lane 2), stripped ghosts with ankyrin and band 4.1 rebound (lane 3).

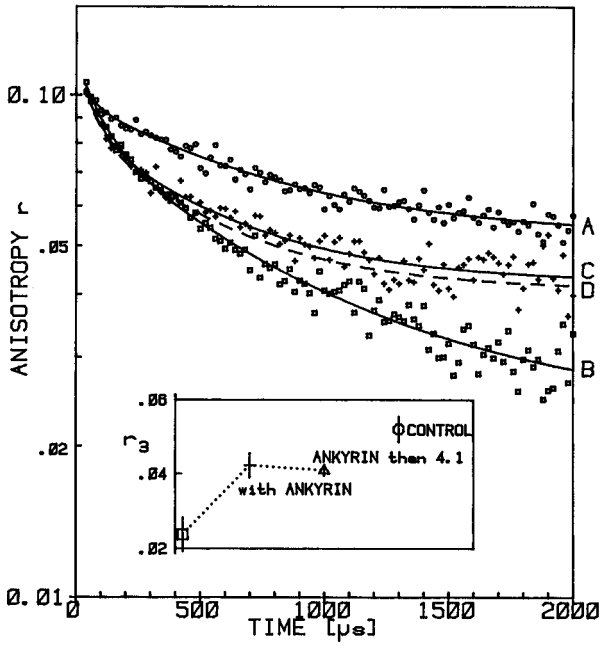

FIGURE 3: Effect of sequential rebinding of ankyrin and band 4.1 on anisotropy decay curves for eosin-labeled band 3 in erythrocyte membranes. (A) ○ = control ghosts; (B) □ = stripped ghosts; (C) + = stripped ghosts (1 mg/mL) incubated with ankyrin (2 mg/mL). (D) was the same as (C) but subsequently incubated with band 4.1 (3 mg/mL) (dashed line, data points omitted for clarity). All measurements were performed in 5 PB containing 66% glycerol at 37 °C. The inset shows the values of r_3, the time-independent anisotropy in eq 2, obtained under the different experimental conditions.

Figure 1 and Table 1 show that incubation of ghosts at pH 9.4 and above results in enhanced rotational mobility of band 3. The effect is rapid since no further change is observed if exposure to high pH is prolonged beyond the time of a few minutes required for sample preparation. The same effects are observed in spectrin−actin-depleted membranes where

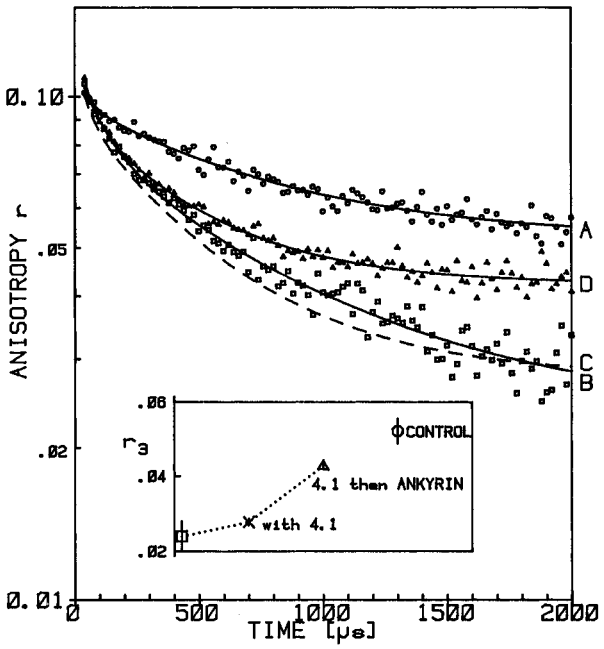

FIGURE 4: Effect of sequential rebinding of band 4.1 and ankyrin on anisotropy decay curves for eosin-labeled band 3 in erythrocyte membranes. (A) ○ = control ghosts; (B) □ = stripped ghosts; (C) stripped ghosts (1 mg/mL) incubated with band 4.1 (3 mg/mL) (dashed line, data points omitted for clarity). (D) △ = as (C) but subsequently incubated with ankyrin (2 mg/ml). All measurements were performed in 5 PB containing 66% glycerol at 37 °C. The inset shows the values of r_3, the time-independent anisotropy in eq 2, obtained under the different experimental conditions

it is shown that ankyrin is the only protein detected in the supernatant of membranes subjected to the high-pH treatment (Figure 2A).

The increased rotational mobility is not the consequence of tumbling of small vesicles following membrane fragmentation as judged by microscopy and by experiments in a high viscosity medium (see Results). Additionally, Figure 1 shows that the effect of high pH was largely reversed upon returning membranes to pH 7.5, while membrane fragmentation is irreversible. In fact, band 3 was somewhat less mobile in this sample than in the control, suggesting that some irreversible aggregation of band 3 occurred during the high-pH treatment. Pinder et al. (1995) previously suggested on the basis of freeze−fracture electron microscopy that removal of skeletal proteins promotes band 3 aggregation.

The effects of high pH on band 3 rotation are in excellent harmony with the binding studies of Thevenin and Low (1990). They showed that binding of ankyrin to stripped ghosts is abolished above pH 9.3, the transition between binding and nonbinding states of ankyrin is reversible, and the dissociation of ankyrin after a pH jump is rapid. There is thus little doubt that the enhanced rotational mobility of band 3 which we observe at high pH is a consequence of

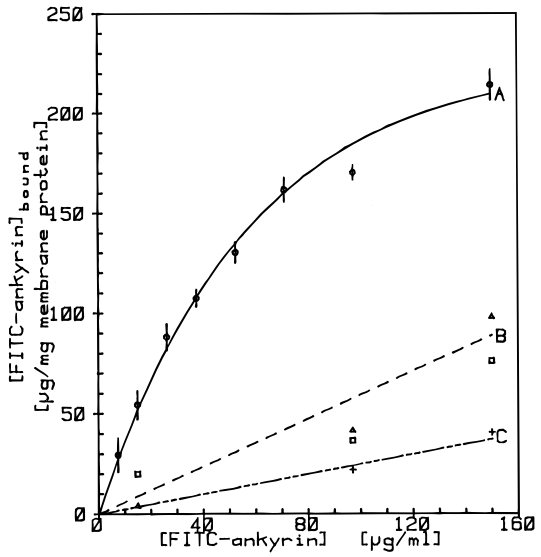

FIGURE 5: Fluorescence assay of ankyrin rebinding. (A) Rebinding of FITC-ankyrin to stripped ghosts; (B) rebinding of FITC-ankyrin to spectrin−actin-depeted ghosts (△) or to trypsin-treated ghosts (□); (C) rebinding of FITC-ankyrin to stripped ghosts in the presence of a 10-fold excess of unlabeled ankyrin (+).

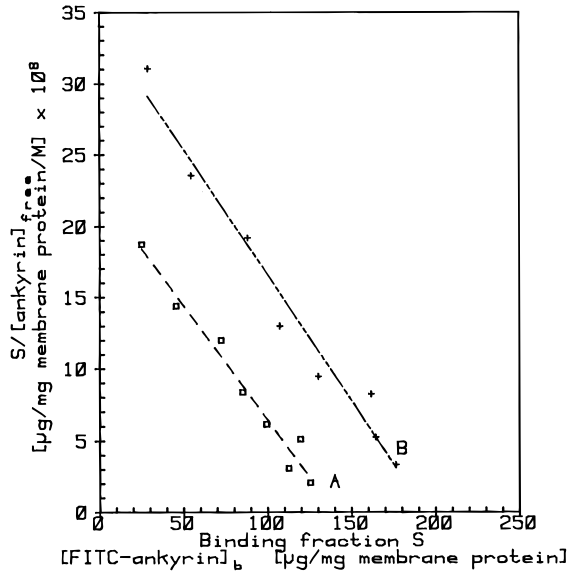

FIGURE 6: Scatchard plots constructed from the data in Figure 5. (A) Data corrected for nonspecific binding using curve B in Figure 5; (B) data corrected for nonspecific binding using curve C in Figure 5.

dissociation of ankyrin from band 3. Simultaneous release of band 4.1 is clearly not required.

Rebinding Experiments. Although the above experiments demonstrate that release of ankyrin alone is sufficient to increase band 3 mobility, they do not resolve the question of whether or not the immobilizing effect of ankyrin requires the presence of band 4.1. To further investigate this issue, we studied the effect of rebinding ankyrin and/or band 4.1 on band 3 rotational mobility in stripped ghosts. In agreement with an earlier report (Wyatt & Cherry, 1992), we observed no detectable change in band 3 mobility upon binding band 4.1 (Figure 4). It is known that band 4.1 can bind to band 3 although it has a considerably higher affinity for glycophorin C/D. Hemming et al. (1994) have shown that about 20% of band 4.1 is bound to band 3 in the human erythrocyte membrane. The present results suggest, however, that any binding of band 4.1 to band 3 in the erythrocyte

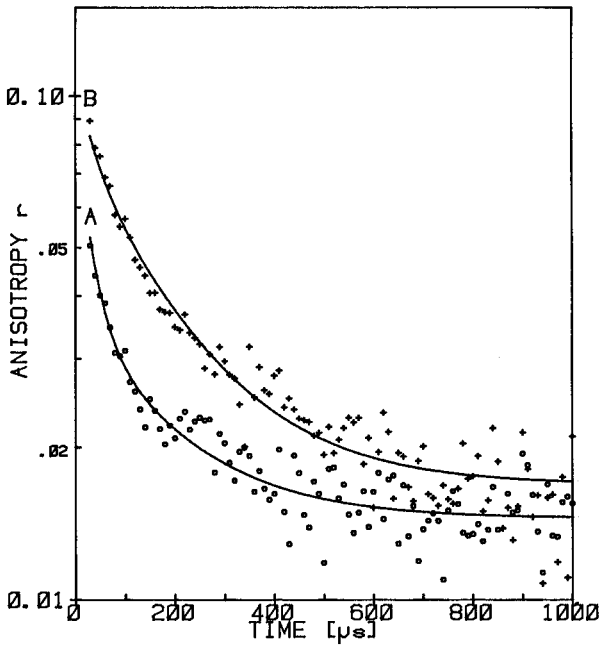

FIGURE 7: Effect of ankyrin binding on anisotropy decay curves of eosin-labeled band 3 in reconstituted vesicles. (A) ○ = reconstituted band 3; (B) + = reconstituted band 3 after incubation with ankyrin. The solid lines are the best fit by global analysis as described in the text. All measurements were performed in 5 PB containing 75% glycerol at 28 °C.

Table 2: Fitted Parameters of Global Analysis of Transient Dichroism Data for Reconstituted Band 3 in Lipid Vesicles, before and after Ankyrin Addition[a]

sample	θ (deg)	ϕ_A (μs)	C_A (%)	ϕ_B (μs)	C_B (%)
control[b]	42 ± 3	21 ± 10	79 ± 49	191 ± 31	21 ± 7
+ankyrin[b]			49 ± 49		51 ± 10
control[c]	40 ± 3	23 ± 10	79 ± 40	164 ± 30	21 ± 7
+ankyrin[c]			42 ± 42		58 ± 14

[a] The data in Figure 7 are fitted by two rotating species (eq 4), with global values for θ and ϕ. The error values are one standard deviation given by the least-squares algorithm. [b] Omitting vesicle tumbling. [c] Including vesicle tumbling ($r_v = 53 \pm 10$ nm).

membrane is unlikely to influence band 3 rotational mobility significantly.

In contrast to band 4.1, ankyrin does reduce the rotational mobility of band 3 in stripped ghosts (Figure 3). The effect appears to be independent of whether or not band 4.1 is also bound (Figures 3 and 4). These results are different from those previously reported (Wyatt & Cherry, 1992), where no effect was observed with ankyrin alone. We can only suppose that the discrepancy arises from variation in the activities of the different ankyrin preparations. In the present experiments, the binding activity of ankyrin was checked using a fluorescence assay (Figures 5 and 6). Using identical rebinding conditions to those for preparation of samples for transient dichroism, we obtain a K_d of ~60 nM and a binding capacity of ~190 μg/mg of membrane protein. These values fall within the range of those previously determined (Bennett & Stenbuck, 1980; Hargreaves et al., 1980; Thevenin & Low, 1990). The ability to label ankyrin with a fluorescent probe without significantly perturbing its binding activity could find further application in studies of ankyrin.

Overall, the rebinding and high-pH experiments reported here strongly indicate that ankyrin binding reduces the rotational mobility of band 3 in erythrocyte membranes. The

involvement of other proteins is not ruled out, but no evidence was obtained to support a role for band 4.1.

Reconstitution Experiments. In order to further examine the band 3−ankyrin interaction, we investigated the effect of ankyrin on the rotational mobility of band 3 in reconstituted lipid vesicles. Qualitatively, these experiments consistently show that ankyrin reduces band 3 rotational mobility in this system. They thus provide conclusive evidence that ankyrin alone can produce this effect without the involvement of other proteins.

A complication in quantitatively evaluating these results, however, is that different reconstitutions exhibit a variable amount of a slowly rotating component. The data in Figure 7 correspond to a reconstitution in which the slower rotating component was relatively small. This data set was analyzed by global analysis in which it was assumed that two populations of band 3 are present with rotational correlation times ϕ_A and ϕ_B. These correlation times are global parameters while their relative proportions are independent variables.

The results of the global analysis are presented in Table 2. The value of θ of 42 ± 3° is in excellent agreement with our previous analysis of band 3 rotation in reconstituted vesicles (Morrison et al., 1986) as is the correlation time of 21 ± 10 μs obtained for the faster rotating species. The inclusion of a term for vesicle tumbling in the analysis has very little effect on these parameters (lower part of Table 2). The faster correlation time may be analyzed in relation to the low-resolution structure of the membrane domain of the band 3 dimer determined by electron microscopy of 2-dimensional crystals (Wang et al., 1994). The structure contains a basal domain, 4 nm thick, which is thought to span the lipid bilayer. The dimensions of this domain in the plane of the membrane are approximately 11 × 4 nm, and the cross-sectional area A is about 30 nm^2. If the domain is regarded as a cylinder with elliptical cross section, then its correlation time for rotation about the membrane normal is given by (Dornmair et al., 1985; Saffman & Delbrück, 1975)

$$\phi = \frac{4\eta A h}{\nu k T} \tag{5}$$

where h is the height of the cylinder, η is the membrane viscosity, and ν is a shape factor given by

$$\nu = \frac{2(a_1/a_2)}{1 + (a_1/a_2)^2} \tag{6}$$

where a_1 and a_2 are the major and minor axes of the ellipse, respectively. Taking $a_1 = 5.5$ nm, $a_2 = 2$ nm, $h = 4$ nm, and $A = 30$ nm^2 for the basal domain of the band 3 dimer, then at 28 °C

$$\phi\ (\mu s) = 18\eta \tag{7}$$

where η is in poise. Thus the correlation time of 21 ± 10 μs is consistent with a dimer rotating in a membrane of viscosity 1.2 ± 0.6 P.

In calculating the correlation time of a band 3 tetramer, there are two possibilities: the dimers may be end to end (T_e) with cross section 22 × 4 nm or side by side (T_s) with cross section 11 × 8 nm. In either case, the cross-sectional area is twice that of the dimer, i.e., 60 nm^2. Inserting these values into eqs 4 and 5 gives

$$\phi\ (\mu s) = 66\eta \text{ for } T_e \tag{8}$$

$$\phi\ (\mu s) = 25\eta \text{ for } T_s \tag{9}$$

In these cases, the measured correlation times would require membrane viscosities of 0.3 ± 0.15 P and 0.8 ± 0.4 P for T_e and T_s, respectively. The value for T_e seems unreasonably small; it is at the lower end of membrane microviscosities determined by lipid probes (Rehorek et al., 1985) and considerably lower than a previous value determined from the rotational diffusion of bacteriorhodopsin (Cherry & Godfrey, 1981; Dornmair et al., 1985). Thus, T_e is unlikely to account for the faster component but the measurements do not distinguish between dimers and T_s. This would only be possible if the membrane viscosity appropriate to protein rotation were to be quite accurately known. It can, however, be argued that the faster rotating component, is more likely to be the dimer since there is previous evidence from electron microscopy that band 3 is dimeric when reconstituted into liposomes (Yu & Branton, 1976). In addition, band 3 is dimeric in crystals grown from concentrated solutions of band 3 in detergent micelles (Wang et al., 1994). Pinder et al. (1995) argue that tetramers observed in nonionic detergents are a consequence of manipulations after extraction from the membrane. For simplicity, we will assume in the following discussion that the faster rotating species is in fact the band 3 dimer.

In the presence of ankyrin, there is a clear increase in the proportion of the slower rotating species at the expense of the faster rotating species (the large standard deviations for the proportion of the faster rotating component arise because variations in this parameter can be compensated in the fit by changes in r_o). Thus, ankyrin promotes the association of dimers into a more slowly rotating species. In harmony with this result, Pinder et al. (1995) observed an increase in the diameters of intramembranous particles when ankyrin was added to band 3 reconstituted into liposomes. Their data, as well as previous results by Hargreaves et al. (1980), indicate that ankyrin binding to reconstituted band 3 is substoichiometric, thus accounting for the fact that the mobility of only a fraction of band 3 is affected by ankyrin binding.

The slower component cannot be interpreted in any detail although the correlation time is clearly too long to correspond to T_e or T_s. The variability of this component in different reconstitutions suggests that the slower rotating species may be irreversible aggregates induced by the purification and reconstitution procedure. There may well be multiple components present, the more so after ankyrin binding. Analysis of the anisotropy decays by more complex models with additional components is, however, not justified by the data.

McPherson et al. (1993) have suggested that ankyrin cross-links the cytoplasmic domain of band 3 on the basis of studies with camelid erythrocyte membranes. They found that ankyrin is resistant to extraction in these membranes in which the rotational mobility of band 3 is also greatly reduced. Cross-linking by ankyrin is also the simplest explanation of ankyrin-induced association of band 3 observed in the present study. The 89 kDa band 3 binding

domain of ankyrin consists of 4 subdomains, each containing 6 repeats of a 33 amino acid sequence (Bennett, 1992). Michaely and Bennett (1995) have reported the identification of two distinct binding sites for band 3, one in subdomain two and the other requiring the participation of both the third and fourth subdomains. This finding provides a rationale for the ability of ankyrin to slow the rotational diffusion of band 3 by a cross-linking mechanism.

Clague et al. (1989) previously found that selective mild proteolysis of ankyrin in erythrocyte membranes did not enhance band 3 rotational mobility. The result is in fact in good agreement with the present findings, since the 89 kDa band 3-binding domain of ankyrin would have remained bound to band 3 in these experiments.

Association of Band 3 in the Erythrocyte Membrane. The state of association of band 3 in the erythrocyte membrane has been the subject of much debate. Matayoshi and Jovin (1993) observed a fast component of phosphorescence anisotropy decay of EMA-labeled band 3 which they assigned to freely rotating dimers. Tilley et al. (1993) also observed a fast component but suggested it might in part correspond to the internal flexibility of band 3. Saturation transfer electron paramagnetic resonance measurements have recently determined a correlation time consistent with band 3 dimers (Husted & Beth, 1995). All rotational diffusion measurements of band 3 in normal erythrocyte membranes performed with optical probes, however, indicate the presence of species which are rotating too slowly to be either dimers or tetramers. Even in stripped ghosts and trypsin-treated ghosts, slow components still persist. Pinder et al. (1995) have suggested that dissociating band 3 from the membrane skeleton in itself promotes band 3 aggregation. It was also previously found that in trypsin-treated ghosts, both temperature and cholesterol content influence the proportion of slowly rotating components (Mühlebach & Cherry, 1982). In reconstituted membranes, slowly rotating species appear as the protein concentration in the membrane is increased (Mühlebach & Cherry, 1985). It thus seems likely that there is specific or nonspecific aggregation of band 3 in the erythrocyte membrane which is influenced by membrane "fluidity" and protein concentration.

Thus, although the results of this study indicate that ankyrin can slow the rotation of band 3, probably by a cross-linking mechanism, this is not the sole origin of the slowly rotating components in the erythrocyte membrane. Michaely and Bennett (1995) in fact suggest that ankyrin cross-linking may be limited to forming tetramers from dimers. This is not sufficient in itself to explain the slowly rotating components. If, however, aggregates of band 3 exist independently of ankyrin, then the effect of ankyrin cross-linking on band 3 rotation might be enhanced, since a single ankyrin molecule could influence the mobility of a much larger number of band 3 molecules.

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BI971074Z