Band 3 Mobility in Camelid Elliptocytes: Implications for Erythrocyte Shape†

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ABSTRACT: Measurements of time-resolved phosphorescence anisotropy were used to monitor the rotational diffusion of eosin-labeled band 3 in membranes of the elliptocytic erythrocytes of alpacas and camels. The rotational freedom of camelid band 3 was more restricted than for human band 3. Removal of the peripheral membrane proteins from human erythrocyte membranes, by high-pH treatment, increased the band 3 rotational freedom. The same high-pH treatment of alpaca and camel erythrocyte membranes failed to alter the rotational freedom of band 3 in these species and also failed to remove ankyrin. Treatment of human and alpaca erythrocyte membranes with trypsin, which removed the cytoplasmic domain of band 3, caused a marked increase in band 3 rotational freedom in both species. We suggest that ankyrin may modulate the rotational freedom of band 3 in camelid erythrocytes, thereby influences the erythrocyte shape and deformability. The rotational freedom of band 3 in sheep, pig, and rat erythrocyte membranes was also examined and found to be slightly greater than for human band 3. This is consistent with the inability of glyceraldehyde-3-phosphate dehydrogenase to bind to band 3 in the erythrocyte membranes of these species.

Erythrocytes of members of the camelid family are oval disks. These elliptocytic erythrocytes possess decreased deformability (Smith et al., 1979) and increased resistance to hypotonic lysis (Livne & Kuiper, 1973), properties which may promote erythrocyte survival during fluctuations in osmotic pressure experienced during dehydration and rehydration of the desert-adapted camel. In an attempt to determine the molecular basis of the elliptocytic morphology of camelid erythrocytes, we present here a detailed analysis of band 3 rotational mobility in these erythrocytes. We also compare these data with those for band 3 mobility in membranes of other species that have discocytic erythrocytes.

Band 3 is the major erythrocyte integral membrane protein. It is comprised of two structurally independent domains: a 50–55-kDa integral membrane domain and a 41–43-kDa cytoplasmic domain. The integral domain is responsible for Cl^-/HCO_3^- exchange, while the cytoplasmic domain serves an important structural role. The cytoplasmic domain of band 3 is an extended hydrophilic domain penetrating some 25 nm into the cytoplasm (Low, 1986). It contains a binding site for ankyrin through which a proportion of the band 3 population is linked to the underlying cytoskeleton.

The integral membrane domain of band 3 is able to rotate around an axis normal to the bilayer plane at a rate which is determined by the state of band 3 self-association and by interactions of its cytoplasmic tail with peripheral proteins [for a review, see Tilley and Sawyer (1992)]. Thus, measurements of the rotational diffusion of band 3 provide a very sensitive monitor of protein–protein interactions which restrict the rotational freedom of band 3. Transient dichroism and time-resolved phosphorescence anisotropy have been used to measure band 3 mobility in erythrocyte membranes, and suggest a heterogeneous mixture of interconverting aggregates, with the dimer as the minimum aggregate size (Cherry, 1978; Clague & Cherry, 1988; Wyatt & Cherry, 1992; Austin et al., 1979; Matayoshi et al., 1991; McPherson et al., 1992).

Somewhat surprisingly, extraction of spectrin from erythrocyte membranes does not greatly alter band 3 mobility (Cherry et al., 1976; Wyatt & Cherry, 1992). This observation can, however, be rationalized if one considers the substantial flexibility within the cytoplasmic domain of band 3 (McPherson et al., 1992) and in the spectrin molecule itself (Clague et al., 1990).

In this report, we provide evidence that extraction of ankyrin and protein 4.1, along with the cytoskeletal proteins, increases the rotational freedom of band 3. We suggest that ankyrin, in particular, may play an important role in determining the structural organization of band 3 in the erythrocyte membrane. Furthermore, we report that the interaction of band 3 with peripheral proteins appears to be altered in membranes from camelid erythrocytes, which correlates with the altered morphology and decreased membrane deformability of these cells.

MATERIALS AND METHODS

Erythrocyte Sources. Fresh human blood, collected into acid–citrate–dextrose, was obtained from the Red Cross Blood Transfusion Service, Melbourne. All other blood was collected into citrate–phosphate–dextrose in order to avoid osmotic lysis (Smith, 1983). Rat (*Rattus norvegicus*) blood was collected from decapitated rats. Pig blood was collected at a local abattoir. Sheep blood, obtained by venipuncture, was donated by the Department of Agriculture, La Trobe University. Bactrian camel blood was collected by venipuncture at Taronga Zoo, Mosman, Australia. Alpaca blood was collected by venipuncture at the Benleigh Alpaca Stud, Moolap, Australia. All blood was kept at 4 °C during storage and transport, and used within 1 week of collection.

Band 3 Labeling. Band 3 was labeled at the extracellular face with eosinyl-5-maleimide (Molecular Probes, Eugene, OR) as described by Tilley et al. (1990). A labeling ratio of approximately 0.8 eosin per band 3 molecule was observed in all species examined. The specificity of labeling was deter-

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mined by examining sodium dodecyl sulfate (SDS)[1]–polyacrylamide gels of membrane samples under ultraviolet light. Ghosts were prepared by hypotonic lysis in 5 mM sodium phosphate, pH 8.0. The final wash was in 10 mM sodium phosphate, pH 7.4, unless otherwise specified.

Time-Resolved Phosphorescence Spectroscopy. Rotational diffusion of phosphorescently-labeled band 3 in erythrocyte ghosts was measured using the instrument described by Tilley et al. (1988). Briefly, a nitrogen-pulsed dye laser (8–10-ns pulse width, 515 nm) provided vertically polarized light that illuminated a 7 × 7 mm cuvette containing a suspension of labeled membranes. The phosphorescence, isolated with a combination of KV550 and RG695 cutoff filters, was measured with an EMI 9817 QBG photomultiplier that was gated on 1 μs after the laser pulse to avoid the intense spike of prompt fluorescence. Decays of the parallel, $I_V(t)$, and perpendicular, $I_H(t)$, components of the polarized transient phosphorescence were collected serially by 90° rotation of the emission polaroid after every 64 decays. The analogue signal from the photomultiplier was digitized and delivered to a 1024-channel Nicolet 1170 signal averager that accumulated 256 decays of each component. After subtraction of the background signals, decays were transferred to a PDP-11/23 or IBM-compatible computer, and the total intensity, $s(t)$, and anisotropy, $r(t)$, decays were generated according to the equations:

$$s(t) = I_V(t) + 2I_H(t) \quad (1)$$

$$r(t) = [I_V(t) - I_H(t)]/s(t) \quad (2)$$

The generated curves were fitted to the functions:

$$s(t) = \sum \alpha_i \exp(-t/\tau_i) + B \quad (3)$$

$$r(t) = \sum \beta_j \exp(-t/\phi_j) + r_\infty \quad (4)$$

α_i representing the partial intensity of the decay component i having a lifetime τ_i and β_j the partial anisotropy of component j associated with the correlation time ϕ_j. r_∞ is the observed limiting anisotropy. For a hindered rotor, r_∞ reflects, in part, the limits on redistribution of the chromophore at times much longer than the phosphorescence lifetime. r_∞ and β_j are also partly determined by the angles that the excitation and emission dipoles make with the axis of rotation (Jovin et al., 1981). B is a fitting parameter that accounts for any offset in the total intensity decay and was close to zero in the experiments described below. Data were fitted to 1 to 3 exponentials using a Chebychev transformation procedure contained within a data acquisition operating system supplied by Labsoft Associates, Melbourne, Australia. Goodness-of-fit was determined by the value of χ^2 or from plots of the weighted residuals.

In all experiments, the total intensity decays, $s(t)$, over the 0–5-ms time window could be adequately fitted using three exponentials (McPherson et al., 1992). The lifetimes did not differ significantly between different experiments, nor between different species, indicating that the local environment of eosin conjugated to band 3 was similar in all cases.

High-pH Treatment of Erythrocyte Ghosts. Erythrocyte ghosts were washed once in 1 mM CAPS[1] buffer, pH 12 at 4 °C. The ghosts were then suspended in 10 volumes of the same CAPS buffer and incubated on ice for 5 min. The stripped inside-out membrane vesicles were washed 2 times in 10 mM sodium phosphate, pH 7.4, and suspended in 60% glycerol in 10 mM sodium phosphate, pH 7.4.

[1] Abbreviations: CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone.

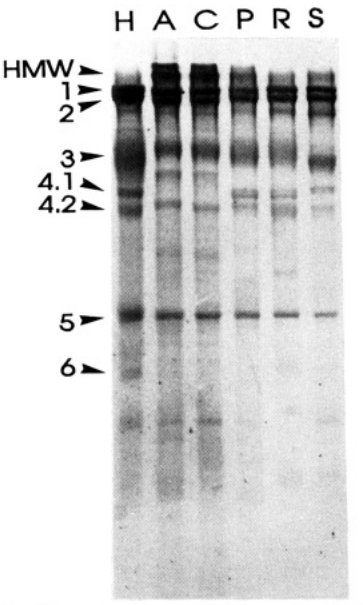

FIGURE 1: SDS–polyacrylamide gel (10% acrylamide) of membranes prepared from human (H), alpaca (A), camel (C), pig (P), rat (R), and sheep (S) erythrocytes. The labeling of the major bands is according to Steck (1974).

Trypsin Treatment of Erythrocyte Ghosts. Erythrocyte ghosts were suspended in 1 volume of 10 mM sodium phosphate, pH 7.4, and TPCK-treated trypsin (Sigma type XIII) was added to a final concentration of 50 μg/mL. The suspension was incubated for 5 min on ice. Proteolysis was stopped by the addition of excess soybean trypsin inhibitor (Sigma) followed by incubation on ice for 5 min. The trypsin-treated membranes were washed 2 times in 10 mM sodium phosphate, pH 7.4, and suspended in 60% glycerol in 10 mM sodium phosphate, pH 7.4.

Western Blot Analysis of Membrane Samples. Proteins were electrophoretically transferred from SDS–polyacrylamide gels to Western blots, and immunoreactive zones were detected using a polyclonal anti-ankyrin antibody which was kindly supplied by Dr. D. Dhermy and Dr. M.-C. Lecomte of INSERM U 160, Hôpital Beaujon, Paris, France.

Microscopic Examination. Whole erythrocytes and erythrocyte membranes were viewed using a Zeiss research microscope with phase-contrast optics. Eosin-labeled membranes were viewed by fluorescence microscopy.

RESULTS

Gel Electrophoresis of Membranes from Different Species. SDS–polyacrylamide gels of membrane preparations from human, pig, rat, sheep, alpaca, and camel erythrocytes show that band 3 is a diffuse Coomassie blue-staining band in all species (Figure 1). As has been noted previously (Ralston, 1975), band 3 in the membranes of alpaca and camel erythrocytes has a slightly higher apparent molecular weight and is less diffuse on SDS-gel electrophoresis than is human erythrocyte band 3. Band 4.1 also has a higher apparent molecular weight in camelid erythrocytes. In overloaded gels, some very high molecular weight material which ran above spectrin was observed in camelid erythrocyte membranes. Band 6 (glyceraldehyde-3-phosphate dehydrogenase) is present at a significant level only in human erythrocyte membranes. Examination of gels of eosin-labeled membranes under ultraviolet light indicated that specific labeling of band 3 with eosinyl-5-maleimide was achieved in all the species examined (data not shown).

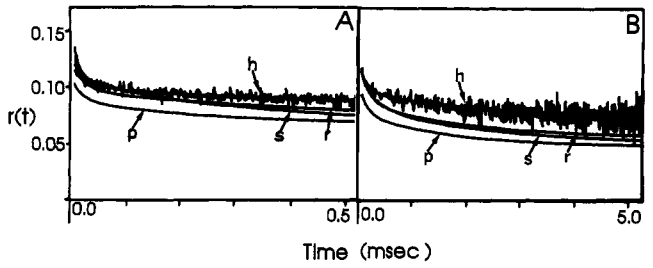

FIGURE 2: Decays of phosphorescence anisotropy [$r(t)$] of eosin–band 3 in human (h), pig (p), rat (r), and sheep (s) erythrocyte membranes at 37 °C. The anisotropy data for human eosin–band 3 are shown along with the fitted curves generated using eq 4. For clarity, only the fitted curves are shown for pig, rat, and sheep erythrocyte membranes. Data were collected at two different time regimes: (A) 0–0.5 ms; (B) 0–5 ms.

Rotational Mobility of Eosin-Band 3 in Human Erythrocyte Ghosts. Decays of phosphorescence anisotropy of eosin-labeled band 3 in human erythrocyte membranes are shown in Figure 2 (top curves). Two time regimes were required in order to resolve both rapid and slow rotational events. Panel B shows the decay of anisotropy over a 5-ms time period after excitation of the sample by a flash of light from the pulsed laser. Panel A gives a higher resolution of the early time period, 0–0.5 ms. Each of these anisotropy decays has been fitted to two exponentials. Goodness-of-fit of the individual decay curves was assessed from the values of χ^2 or by visual inspection of plots of weighted residuals. Three decay events are resolved within the noise of the phosphorescence decay data. The event with a correlation time of about 250 μs can be resolved using either time base. The data from a series of measurements were fitted using eq 4, and the correlation times and the fractional amplitudes for each component of the anisotropy decay are summarized in Table I. The three correlation times which are resolved reflect the existence of discrete interconverting aggregate states of band 3 [for discussion, see Matayoshi and Jovin (1991) and Tilley and Sawyer, (1992)]. The proportion of band 3 molecules involved in the different aggregate states is reflected in the fractional amplitudes associated with each decay component. Fractional amplitudes (f_j) were calculated using the equation: $f_j = \beta_j/r_0$, where r_0 is the initial anisotropy. It should be stressed, however, that the values which are obtained for both the correlation times and the fractional amplitudes depend on the constraints which are imposed on the fitting procedure [see discussions by Mühlebach and Cherry (1982) and Morrison et al. (1986)]. The most meaningful indicator of the relative mobility of band 3 in the different species is r_∞/r_0.

Rotational Mobility of Eosin-Band 3 in Sheep, Pig, and Rat Erythrocyte Membranes. Decays of phosphorescence anisotropy of eosin–band 3 in rat, pig, and sheep erythrocyte membranes are also shown in Figure 2. Data from a series of measurements were analyzed using eq 4, and the decay parameters are presented in Table I; three rotational correlation times are observed in all species examined. The initial anisotropy values for the different species were within 12% of the r_0 value for human eosin–band 3 except in the case of the pig erythrocyte membranes [r_0(pig) = 0.12 ± 0.01; r_0(human) = 0.16 ± 0.02]. Differences in the initial anisotropy may indicate different degrees of motion of the eosin–band 3 on the submicrosecond time scale. Such motions might result from local probe reorientation or from segmental flexibility of the labeled domain [see Matayoshi and Jovin (1991) for a discussion]. The differences in the initial anisotropy could suggest that the eosin labeling site is slightly different in the different species. However, analysis of phosphorescence intensity decays reveals similar lifetimes in all species, indicating that the local environment of eosin conjugated to band 3 was similar in all cases. The intensity decays were fitted to three exponentials using eq 3. The phosphorescence lifetimes (τ_i) for eosin–band 3 in pig erythrocyte membranes were found to be 14 ± 1, 330 ± 40, and 2220 ± 20 μs. The corresponding fractional amplitudes (α_i/I_0) were 0.38 ± 0.01, 0.03 ± 0.01, and 0.59 ± 0.02. These values are very similar to the lifetimes (16 ± 2, 370 ± 30, and 2620 ± 30 μs) and fractional amplitudes (0.37 ± 0.01, 0.05 ± 0.01, and 0.58 ± 0.01) of eosin–band 3 in human erythrocyte membranes.

The values of the time constants for the decay of anisotropy on the phosphorescence time scale do not vary significantly between species, consistent with the interpretation that similar types of band 3 aggregates exist in all species. Analysis of the fractional amplitudes associated with the three correlation times suggests that motion of band 3 on the phosphorescence time scale is somewhat less restricted in the nonhuman species. The values of r_∞/r_0 for eosin–band 3 observed for pig, rat, and sheep erythrocyte membranes are slightly lower than for the human membranes, indicating a small increase in rotational mobility. This increase is consistent with the absence of glyceraldehyde-3-phosphate dehydrogenase (band 6) in the membrane samples of these species (Figure 1). The binding of glyceraldehyde-3-phosphate dehydrogenase to band 3 has previously been shown to decrease band 3 mobility (Matayoshi et al., 1991).

Rotational Mobility of Eosin–Band 3 in Camel and Alpaca Erythrocyte Membranes. The camel and the alpaca belong to the family Camelidae. The camelid family has evolved an anucleate elliptocytic erythrocyte (Smith et al., 1979), with unusual properties, including a high ratio of protein to lipid, resistance to deformation, and a high resistance to osmotic lysis (Khodadad & Weinstein, 1983). The phosphorescence lifetimes (τ_i) of eosin–band 3 in membranes prepared from camel membranes were 13 ± 2, 320 ± 70, and 2670 ± 20 μs. The corresponding fractional amplitudes (α_i/I_0) were 0.38 ± 0.01, 0.03 ± 0.01, and 0.59 ± 0.01. These values are very similar to the lifetimes and fractional amplitudes of eosin–band 3 in human erythrocyte membranes.

Phosphorescence anisotropy decays were collected using two time bases (0–0.5 and 0–5 ms). The data for the 0–5-ms time period for eosin–band 3 in membranes prepared from alpaca erythrocytes are shown in Figure 3 (top curve). Fitted data for a series of measurements on camel and alpaca membranes are summarized in Table I. Only minor differences were found in the values of the three correlation times for eosin–band 3 in membranes prepared from the different species. However, the fractional amplitudes associated with each anisotropy decay component were lower in camelid erythrocytes than in other species, leading to higher values of r_∞/r_0 for camel and alpaca band 3. Thus, a higher proportion of band 3 molecules are rotationally restricted in the erythrocyte ghosts prepared from the blood of these animals.

The observed decrease in band 3 mobility in camel membranes could conceivably derive from the increased ratio of integral membrane protein to lipid in these membranes (Khodadad & Weinstein, 1983), which may promote self-association of band 3 within the plane of the bilayer (Mühlebach & Cherry, 1985). Alternatively, it may reflect an alteration in the interaction of band 3 with one of the peripheral membrane proteins. In order to distinguish between these possibilities, human and camelid erythrocyte membranes were stripped of peripheral membrane proteins by high-pH

Table I: Analysis of Phosphorescence Anisotropy Decays of Eosin–Band 3 in Membranes from Human, Rat, Pig, Sheep, Alpaca, and Camel Erythrocytes[a]

	human	pig	rat	sheep	alpaca	camel
ϕ_1 (μs)	17 ± 5	17 ± 2	13 ± 6	23 ± 15	18 ± 9	23 ± 9
f_1	0.16 ± 0.02	0.15 ± 0.03	0.20 ± 0.02	0.17 ± 0.02	0.12 ± 0.06	0.07 ± 0.03
ϕ_2 (μs)	210 ± 90	250 ± 60	280 ± 200	240 ± 80	310 ± 160	380 ± 130
f_2	0.17 ± 0.02	0.21 ± 0.02	0.20 ± 0.02	0.23 ± 0.02	0.11 ± 0.04	0.14 ± 0.03
ϕ_3 (μs)	2050 ± 870	2180 ± 900	1340 ± 260	1900 ± 540	2260 ± 1100	2180 ± 720
f_3	0.20 ± 0.05	0.24 ± 0.05	0.20 ± 0.02	0.25 ± 0.02	0.15 ± 0.06	0.19 ± 0.02
r_∞/r_0	0.46 ± 0.05	0.41 ± 0.07	0.40 ± 0.03	0.35 ± 0.04	0.62 ± 0.10	0.60 ± 0.08

[a] Fractional amplitudes were calculated using the equation $f_j = \beta_j/r_0$, where β_j is the partial anisotropy and r_0 is the initial anisotropy. Data represent the average ± SD for at least three measurements.

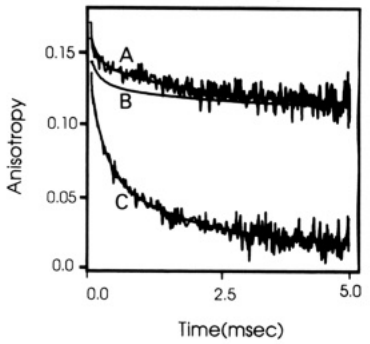

FIGURE 3: Decays of phosphorescence anisotropy over the 0–5-ms time window of eosin–band 3 in alpaca erythrocyte membranes. (A) Control membranes; (B) pH 12-treated membranes (only the fitted curve generated using eq 4 is shown); (C) trypsinized membranes.

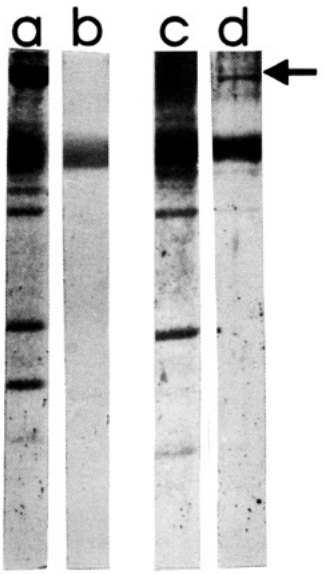

FIGURE 4: SDS–PAGE gel (10% acrylamide) of control human erythrocyte membranes (lane a), pH 12-treated human erythrocyte membranes (lane b), control alpaca erythrocyte membranes (lane c), and pH 12-treated alpaca erythrocyte membranes (lane d). A protein that is consistent with the molecular weight of ankyrin is arrowed (lane d).

treatment using a protocol adapted from that of Steck (1974).

Rotational Mobility of Eosin–Band 3 in Human and Camelid Erythrocyte Membranes Treated with High pH. Treatment of human erythrocyte membranes with 1 mM CAPS buffer, pH 12, on ice for 5 min resulted in extensive vesiculation of the membranes as judged by light microscopic examination. The treatment allowed efficient extraction of the peripheral membrane proteins (Figure 4, lane b). The decay of phosphorescence anisotropy over the 0–5-ms time window of eosin–band 3 in pH 12-treated erythrocyte membranes is shown in Figure 5 (curve B). Analysis of data

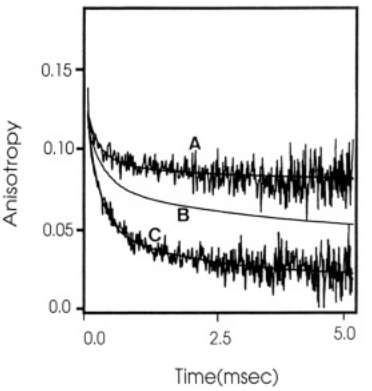

FIGURE 5: Decays of phosphorescence anisotropy over the 0–5-ms time window of eosin–band 3 in human erythrocyte membranes at 37 °C. (A) Control membranes; (B) pH 12-treated membranes (only the fitted curve generated using eq 4 is shown); (C) trypsinized membranes.

for a series of measurements (Table II) suggests that the mobility of eosin–band 3 is significantly increased by the removal of the peripheral proteins (r_∞/r_0 is decreased from 0.46 ± 0.05 in controls to 0.34 ± 0.06 in high-pH-treated membranes).

Treatment of alpaca erythrocyte membranes with 1 mM CAPS buffer, pH 12, did not cause vesiculation of the membranes; the high-pH-treated alpaca membranes retained their elliptocytic morphology. Analysis of the extracted membranes by SDS–PAGE showed that most of the peripheral membrane proteins had been removed except for a protein with a molecular weight equivalent to that of ankyrin (Figure 4, lane d). The identity of the ankyrin band was confirmed by Western blot analysis (not shown). Similar results were found for camel membranes. Measurements of rotational diffusion (Figure 3, Table II) revealed that, in contrast to the human samples, treatment of alpaca and camel membranes with high pH did not increase band 3 mobility (r_∞/r_0 is 0.62 ± 0.10 in alpaca controls and 0.67 ± 0.06 in high-pH-treated membranes).

Rotational Mobility of Eosin–Band 3 in Trypsin-Treated Human Erythrocytes. Trypsin treatment of erythrocyte membranes cleaves the cytoplasmic domain of band 3 from the integral membrane portion (Reithmeier, 1979). Under the incubation conditions used in our experiments, the 52-kDa membrane-embedded fragment of eosin–band 3 remained intact (Figure 6); however, all the peripheral proteins appeared to be degraded to some extent. Rotation of the eosin-labeled integral domain of band 3 in trypsinized membranes should, therefore, be free from any rotational constraints imposed by binding of the cytoplasmic domain to peripheral membrane proteins.

The phosphorescence anisotropy decays over the 0–5-ms time window of eosin–band 3 in trypsin-treated human

Table II: Analysis of Phosphorescence Anisotropy Decays of Eosin–Band 3 in Erythrocyte Membranes of Different Species Treated with either 1 mM CAPS, pH 12, or Trypsin[a]

	human, pH 12	human, trypsinized	alapca, pH 12	alpaca, trypsinized	camel, pH 12
ϕ_1 (μs)	19 ± 4	27 ± 3	16 ± 7	30 ± 6	10 ± 2
f_1	0.18 ± 0.04	0.11 ± 0.01	0.12 ± 0.04	0.13 ± 0.02	0.16 ± 0.04
ϕ_2 (μs)	230 ± 100	200 ± 90	440 ± 260	320 ± 130	390 ± 190
f_2	0.25 ± 0.04	0.41 ± 0.07	0.10 ± 0.03	0.41 ± 0.07	0.08 ± 0.02
ϕ_3 (μs)	2000 ± 1400	1820 ± 300	2300 ± 600	1500 ± 300	2500 ± 1500
f_3	0.23 ± 0.05	0.31 ± 0.03	0.11 ± 0.04	0.36 ± 0.02	0.16 ± 0.07
r_∞/r_0	0.34 ± 0.06	0.17 ± 0.04	0.67 ± 0.06	0.10 ± 0.05	0.60 ± 0.08

[a] Data represent the average ± SD for at least three measurements.

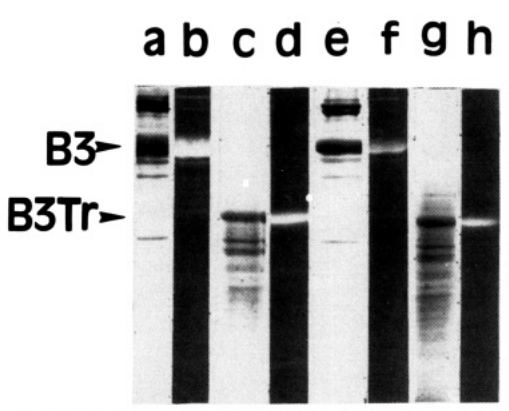

FIGURE 6: SDS–PAGE gels (10% acrylamide) of human and alpaca eosin-labeled erythrocyte membranes viewed with Coomassie blue staining (lanes a, c, e, and g) and under ultraviolet light (lanes b, d, f, and h): control human (lanes a and b), trypsinized human (lanes c and d), control alpaca (lanes e and f), and trypsinized alpaca (lanes g and h) erythrocyte membranes. Intact band 3 (B3) is seen in control erythrocyte membranes, and the 52-kDa membrane domain of band 3 (B3Tr) is seen in trypsinized erythrocyte membranes.

membranes are shown in Figure 5 (bottom curve): a value of 0.17 ± 0.04 was obtained for r_∞/r_0 (Table II). This value is in reasonable agreement with results obtained by other workers (Nigg & Cherry, 1980; Matayoshi & Jovin, 1991). The value of r_∞/r_0 for eosin–band 3 in these membranes reflects the intrinsic constraints on rotational diffusion of band 3 imposed by the two-dimensional nature of the lipid bilayer and by self-association of the integral domain within this bilayer. The value of r_∞ is also determined, in part, by the angle that the excitation and emission dipoles make with each other and with the axis of rotation [see Jovin et al. (1981) for a discussion].

Rotational Mobility of Eosin–Band 3 in Trypsin-Treated Camelid Erythrocyte Membranes. Trypinization of alpaca membranes caused extensive vesiculation as judged by light microscopy, suggesting substantial destabilization of the membrane structure. Phosphorescence anisotropy decays of eosin–band 3 in trypsin-treated alpaca erythrocyte membranes are shown in Figure 3 (bottom curve). The data analysis for a series of measurements involving two time regimes is presented in Table II. The value of r_∞/r_0 (0.10 ± 0.05) is consistent with removal of restrictions on band 3 mobility. The value of r_∞/r_0 for trypsinized alpaca membranes is slightly lower than that observed for eosin–band 3 in trypsinized human erythrocyte membranes (Table II). This suggests that the level of self-association of the integral membrane domain of band 3 into oligomers is slightly less extensive in alpaca erythrocyte membranes.

DISCUSSION

Rotational Mobility of Eosin–Band 3 in Erythrocyte Membranes. Measurements of rotational diffusion of eosin–band 3 in erythrocyte membranes of different animals reveal three rotational correlation times in all cases. From previous studies, it has been suggested that the longer correlation times (about 250 and 2000 μs) derive from tetramers and higher oligomers of band 3 which result from cross-linking of band 3 dimers by peripheral proteins [see Tilley and Sawyer (1992) for a review]. The short correlation time (20–30 μs) may represent the global rotation of band 3 dimers, but may also involve a contribution from the motion of the labeled segment within the membrane-embedded domain of band 3 (McPherson et al., 1992; Tilley & Sawyer, 1992). For the six species examined, the correlation times were similar, suggesting that similar types of aggregates exist in all species. By contrast, the fractional amplitudes of the decay components varied substantially. This is consistent with the idea that band 3 exists in rapidly and slowly rotating forms, the proportions varying from one species to another.

Interpretation of the phosphorescence data relies on the assumption that eosin is covalently attached to band 3 in a similar manner in all species. The validity of this assumption is supported by the finding that the phosphorescence lifetimes of eosin–band 3 were very similar in all species examined; however, it is possible that the labeling site varies slightly from one species to another. The site of binding of eosinyl-5-maleimide is thought to lie within the highly conserved anion channel of band 3 (Cobb & Beth, 1990). Sequence variations between species occur mainly within the cytoplasmic domain (Moriyama & Makino, 1987; Lieberman & Reithmeier, 1988).

The erythrocytes of humans, rats, sheep, and pigs all have a discocytic morphology in the resting state. Of these species, the mobility of band 3 appears to be slightly higher in membranes prepared from the nonhuman erythrocytes. The observed differences in mobility may reflect differences in the binding of glyceraldehyde-3-phosphate dehydrogenase, which has previously been shown to decrease band 3 mobility (Matayoshi et al., 1991). Differential binding of glycolytic enzymes in different species has been reported previously (Ballas et al., 1985; Moriyama & Makino, 1987) and may result from an altered N-terminal region of the band 3 cytoplasmic domain or from the presence of interfering cytoplasmic components (Jay, 1983; Ballas et al., 1985). The data suggest that a discocytic morphology is associated with a relatively high proportion of rapidly diffusing band 3 molecules.

Rotational Freedom of Band 3 in Camelid Erythrocytes. The rotational freedom of band 3 in membranes of camelid erythrocytes is substantially reduced compared with all other species examined. Even upon exposure of the camelid erythrocyte ghosts to basic conditions, which removed most of the cytoskeletal proteins, the rotational freedom of band 3 remained highly restricted. These data lead to the suggestion that the organizational integrity of the camelid erythrocyte membrane is not dependent on the presence of the cytoskeleton.

Support for this suggestion comes from the observation that the high-pH-treated camelid ghosts retained their elliptical shape. Similar observations have been reported previously (Ralston, 1975; Eitan et al., 1976; Khodadad & Weinstein, 1983). By contrast, human erythrocyte ghosts fragment into small inside-out vesicles upon removal of spectrin (Lew et al., 1988). A notable difference in the SDS–polyacrylamide gel profiles of pH 12-treated human and camelid erythrocyte membranes is that ankyrin is apparently not removed by high-pH treatment of camelid ghosts. It is therefore possible that the restriction of camelid band 3 mobility derives, at least in part, from interaction of its cytoplasmic domain with ankyrin. However, it is also possible that alterations in intermolecular interactions between band 3 molecules make the camelid erythrocyte membranes more resistant to the brief high-pH treatment.

We further investigated the role of the peripheral membrane proteins in controlling camelid band 3 mobility. The membrane-embedded domain of band 3 was freed from all external constraints by proteolytic removal of the cytoplasmic domain of band 3 and proteolytic degradation of the peripheral proteins. Under these conditions, the rotational freedom of the camelid band 3 was greatly increased. Thus, it appears likely that cross-linking of band 3 through its cytoplasmic domain, rather than inter-band 3 interactions within the plane of the membrane, is responsible for the restrictions on its mobility. This cross-linking could occur directly through self-association of the cytoplasmic domain of band 3, as well as indirectly, via ankyrin. A role for both ankyrin and protein 4.1 in controlling band 3 mobility has previously been suggested (Wyatt & Cherry, 1992). These workers found that the rotational mobility of band 3 was decreased when ankyrin and protein 4.1 were added back to human erythrocyte membranes which had been stripped of peripheral membrane proteins.

The normal human erythrocyte is a biconcave disk with an average disk width of 2.5 μm and a diameter of about 8 μm. Members of the family Camelidae are unique among mammals in that their erythrocytes are characteristically flat ellipsoids. The camelid erythrocyte shape is surprisingly immutable, resisting morphological changes upon ATP depletion or treatment with stomatogenic agents (Omorphos et al., 1989). Similarly, microtubule-inhibiting agents have no effect, nor does calcium, nor even heat treatment at 60 °C for 10 min (Ralston, 1975). For the camel, which can survive long periods without water, these properties may allow erythrocytes to survive changes in osmotic pressure during dehydration and rapid rehydration (Cohen, 1978). The ability of human erythrocytes to adopt a variety of morphologies in the circulation is considered to offer an advantage in the microcirculation, where the diameter of the capillaries may be less than that of the red cell (Cohen, 1978). However, at least for the guanaco and the llama, the survival time of camelid erythrocytes in the circulation is not impaired (Vacha, 1979; Kaneko & Cornelius, 1962; Berlin & Berk, 1975).

Alterations in one or more of the major membrane proteins presumably underlie the unusual properties of the camelid erythrocytes. In agreement with a previous report (Ralston, 1975), we find that camelid band 3 has a slightly higher molecular weight, as does protein 4.1. Omorphos et al. (1989) reported an absence of ankyrin in camelid erythrocyte membranes. This is not supported by our results, nor by those of Khodadad and Weinstein (1983). Western blot analysis of llama erythrocyte membranes using a polyclonal anti-ankyrin antibody has also confirmed the presence of ankyrin in this species (Dr. D. Dhermy and Dr. M.-C. Lecomte, INSERM U 160, France, personal communication).

Whatever the underlying molecular alteration, protein–protein interactions within the camelid erythrocyte membrane appear to be markedly enhanced. Ralston (1975) reported that extraction of spectrin under conditions of low ionic strength was much less efficient than for human erythrocyte membranes, suggesting that spectrin must be more tightly bound to the erythrocyte membrane. Furthermore, the nonionic detergent Triton X-100 failed to extract the major integral membrane proteins (Ralston, 1975; Omorphos et al., 1989), suggesting tighter interactions between the integral membrane proteins and the peripheral proteins. Our observation that ankyrin is not efficiently extracted during brief exposure to high pH also suggests altered protein–protein interactions in camelid erythrocyte membranes. One possible explanation of this finding is a tighter interaction of ankyrin with band 3.

We propose that the decreased rotational mobility of band 3 in camelid erythrocyte membranes is directly responsible for the decreased deformability of camelid erythrocytes. Increased cross-linking of band 3 molecules through their cytoplasmic tails will decrease both the rotational and the lateral mobility of band 3. This, in turn, might be expected to restrict the lateral expansion of the spectrin molecules which are anchored to the band 3 molecules. As "spring-like" extensions of spectrin molecules are thought to provide the molecular basis for membrane deformability (Elgsaeter et al., 1986), a highly cross-linked membrane would be expected to resist deformation (Tilley et al., 1993).

In conclusion, we suggest that both the degree of cross-linking of band 3 and the strength of the spectrin/ankyrin/band 3 interaction are important factors in determining red cell morphology and deformability. In camelid erythrocyte membranes, the degree of cross-linking of band 3 appears to be increased due to interactions involving peripheral membrane proteins. These increased intermolecular interactions within camelid erythrocyte membranes may be responsible for the decreased membrane deformability.

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