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Cytoskeletal restraints of band 3 rotational mobility in human erythrocyte membranes

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The interaction of band 3 with cytoskeletal proteins was investigated in erythrocyte membranes by measuring the rotational mobility of band 3 using the method of transient dichroism. It was found that selective proteolysis of ankyrin, a protein known to link band 3 to the spectrin-actin network, had no significant effect on band 3 rotation. Incubating ghosts to 70 °C, at which temperature ankyrin is expected to be denatured, also had no effect. It thus appears probable that linkage of band 3 to the cytoskeleton via ankyrin does not act as a restraint on band 3 rotational motion. It is suggested that this is a consequence of flexibility in the cytoskeletal structure. In further investigations of the effect of heat treatment, a large enhancement of band 3 rotational mobility was found to result from incubation of intact cells for 1 h at 50 °C. This effect was not observed if ghosts were subjected to the same treatment, nor did it occur if the incubation of cells was performed at 47 °C. These findings, in combination with previous studies of band 3 rotational mobility, indicate that the interactions which restrain band 3 are likely to be more complex than commonly envisaged.

Introduction

The erythrocyte cytoskeleton consists of a network of peripheral proteins which controls cell shape and provides the mechanical strength necessary to prevent rupture of the cell by the high shear forces experienced in blood capillaries. In recent years, the principal structural features of the cytoskeleton have been established mainly by studying the binding properties of isolated components (for reviews, see Refs. 1 and 2). A schematic diagram illustrating the present state of knowledge of the structure of the erythrocyte cytoskeleton is shown in Fig. 1. Essentially, tetrameric units of spectrin are thought to be linked at junctions formed by actin and band 4.1. Two linking proteins, ankyrin (band 2.1) and band 4.1, provide the means by which the spectrin network attaches to the overlying cell membrane. Ankyrin, a 215 kDa polypeptide, possesses binding sites for spectrin and the cytoplasmic domain of band 3, which have been localised to 55 kDa acidic and 82 kDa basic tryptic fragments, respectively [3,4].

Band 4.1 also has a membrane binding site other than spectrin. Early studies have suggested this to be glycophorin A [5], although more recent work has shown this particular association to require the presence of polyphosphoinisotides [6]. A lower-affinity lipid-independent site is available on band 3 [7] and attachment sites on the other glycophorins and phosphatidylserine have also been proposed.

Band 3 is the major integral membrane protein and comprises two domains which can express their function independently. Mild trypsin treatment produces a 43 kDa water-soluble cytoplasmic domain, leaving another domain of 55 kDa spanning the membrane [8]. The membrane-spanning domain facilitates the exchange of Cl^- for HCO_3^- across the erythrocyte membrane [9]. The cytoplasmic domain appears from microscopic and hydrodynamic studies to be highly extended [10] with an axial ratio greater than 10. This region binds rather promiscuously to glycolytic enzymes and haemoglobin [11]. The cytoplasmic domain also binds more tightly both to band 4.2, whose function is unknown, and to ankyrin [1,2].

Rotational diffusion of band 3 may be measured by observing laser flash-induced transient dichroism of the

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; TPCK, tosylphenylalaninechloromethyl ketone; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis.

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triplet probe, eosin-5-maleimide [12]. This probe labels band 3 very selectively when reacted with intact erythrocytes [13]. Phosphorescence depolarisation [14] and fluorescence depletion [15] have also been used to investigate rotational motion of band 3.

Rotational diffusion is sensitive to protein–protein interactions and hence its measurements can provide a valuable method for elucidating such interactions. Previous measurements with band 3 appear to be in reasonable accord with the model shown in Fig. 1. The anisotropy decay calculated from transient dichroism measured at 37°C has at least two components. The faster component is thought to correspond to freely mobile band 3, while the slower component corresponds to a population of band 3 with restricted motion [16]. It has been shown that this restriction is in part removed by release of the bulk of the spectrin-actin network together with ankyrin and band 4.1. Furthermore, removal of the 43 kDa cytoplasmic fragment of band 3 by trypsin treatment produces a similar, though larger, effect [16]. These results were interpreted as indicating restricted motion of a population of band 3 resulting from a linkage via ankyrin or band 4.1 of the cytoplasmic portion of band 3 to the cytoskeleton.

There is, however, one observation which so far has not received a satisfactory explanation. The removal of most of spectrin and actin alone from the membrane results in little or no enhancement of band 3 rotational mobility [16,17]. This result is not predicted from the model shown in Fig. 1, since the release of spectrin-actin removes the structure to which band 3 is thought to be attached. It should be emphasised that the viscous resistance to motion of band 3 occurs almost entirely in the lipid bilayer so that the presence of ankyrin per se will not effectively retard rotation.

Recently, a method has been developed which allows the selective degradation of ankyrin in situ by a very mild trypsin treatment of ghosts [18]. The resultant membranes were shown by electron microscopy to be largely detached from their spectrin-actin networks. Ankyrin degradation has been correlated with a lack of response in ghosts to shape-changing agents [18] and to increased susceptibility of cells to fusogens [19].

In the present study we have used the method of selective ankyrin degradation to further investigate the role of the cytoskeleton in restricting band 3 rotational mobility. As an alternative strategy, we have also investigated the effects of various heat treatments of the erythrocyte membrane on band 3 rotational mobility, with a view to correlating the results with protein denaturation.

Materials and Methods

Preparation of eosin-labeled ghosts. The procedure followed that of Nigg and Cherry [13]. Briefly, fresh blood (O^+) was obtained by venipuncture and erythrocytes were isolated with three washes in 5–10 vols of 310 mosM phosphate buffer (pH 7.4). Band 3 was labeled by incubating 1 mg of eosin-5-maleimide (Molecular Probes, Eugene, OR) per 5 ml of packed red blood cells at room temperature for 45 min. Unreacted eosin-5-maleimide was subsequently removed by three washes with 5 mM phosphate-buffered saline (PBS) (pH 7.4). Ghosts were then prepared by lysis and washing with a hypotonic buffer, whose composition varied according to the desired nature of the ghosts.

Selective degradation of ankyrin. Eosin-labeled erythrocytes were hypotonically lysed in 20–30 vols of 10 mM Tris-HCl/1 mM EDTA (pH 7.4) at 4°C and

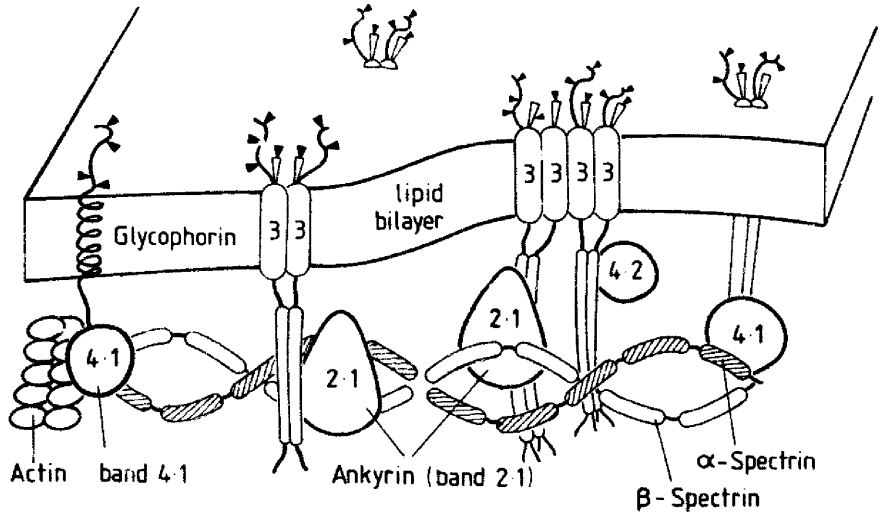

Fig. 1. Schematic diagram of erythrocyte membrane protein architecture.

subsequently washed four times in the same buffer, then twice in 10 mM Tris-HCl buffer (pH 7.4) and finally resuspended to a membrane protein concentration of 2.0–2.5 mg/ml. Selective degradation of ankyrin was based on the method of Jinbu et al. [18]. For best results, TPCK-treated trypsin was added to a concentration of 2.7 μg per mg of ghost protein (1 : 370 w/w) and incubated at 0°C. Incubation times were varied from 0 to 90 s at which point a trypsin inhibitor solution consisting of 0.1 mM lima bean trypsin inhibitor (Sigma type II-L)/0.2 mM PMSF/10 mM Tris-HCl (pH 7.4) was added. The ghosts were then washed four times in 10 mM Tris-HCl (pH 7.4).

Heat treatment of ghosts. Eosin-labeled ghosts (4 mg/ml membrane protein) were suspended in an equal volume of 5 mM phosphate buffer (PB) (pH 7.5) containing 1 mM EDTA, and incubated in the dark for 10 min at 50, 60 and 70°C prior to performing transient dichroism measurements. Ghosts were also incubated at 50°C for 1 h as a control for the treatment below.

Heat treatment of intact cells. Intact red blood cells containing eosin-labeled band 3 were incubated for 1 h in the dark at 25, 47 and 50°C at 50% haemocrit in 5 mM phosphate/150 mM NaCl (pH 7.5). Ghosts were prepared from these cells using 5 mM phosphate buffer (pH 7.5) containing 1 mM EDTA for lysis and washing.

Cleavage of band 3 cytoplasmic domain. As shown previously in Refs. 8 and 16, incubation of ghosts with trypsin for longer times than employed for selective degradation of ankyrin results in cleavage of the 43 kDa cytoplasmic domain of band 3. For this treatment, eosin-labeled ghosts (3–4 mg/ml) were combined in a 1 : 1 volume ratio with 5 mM phosphate buffer (pH 7.5) containing 2 μg/ml of trypsin and incubated for 1 h at room temperature. They were then washed three times in the same buffer containing 0.4 mM PMSF and stored at 4°C.

SDS-PAGE. Membrane protein degradation was followed by SDS-PAGE using the methods of Laemmli et al. [20] and Fairbanks et al. [21]. A 10% Laemmli gel provided superior resolution of ankyrin degradation products, but, in this system, ankyrin itself is obscured by band 1. Clear visualisation of ankyrin, migrating just ahead of band 2, was achieved with a 4% Fairbanks gel. Glycoproteins were visualised in 10% Laemmli gels with a periodic acid-Schiff stain [22].

Protein determination. All concentrations of membrane protein cited were determined by the method of Lowry et al. [23], and are quoted in BSA equivalents. For comparative purposes, the amounts of sample added to electrophoresis gels and used in transient dichroism measurements, were normalised to band 3 concentrations derived from the eosin absorbance at 531 nm.

Transient dichroism measurements. The rotation of band 3 was measured in ghosts as previously described in Refs. 13 and 16 by observing the transient dichroism of ground-state depletion signals arising from the excitation of the probe by a linearly polarised light pulse from a Nd-Yag laser (JK Lasers). Excitation was at 532 nm by a flash 15–20 ns in duration, and absorbance changes were recorded at 515 nm. Data were analysed by calculating the absorption anisotropy $r(t)$, given by

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

in which $A_{\parallel}(t)$ and $A_{\perp}(t)$ are the absorbance changes at time, t, after the flash for light polarised parallel and perpendicular, respectively, with respect to the polarization of the exciting flash. All results reported here were obtained by averaging 512 signals with a Datalab DL102A signal averager. The experimental decays were fitted to a double-exponential equation using an iterative non-linear least-squares program.

$$r(t) = r_1 \exp(-t/T_1) + r_2 \exp(-t/T_2) + r_3 \quad (2)$$

r_3 was expressed as a percentage of the initial anisotropy for the purpose of comparing different samples.

All transient dichroism measurements were performed in 66% (w/v) glycerol to remove the possibility of vesicle tumbling over the experimental time-scale. Oxygen was displaced from samples by gently blowing argon over them for 5 min. The samples were then incubated at 37°C for 10 min prior to transient dichroism measurements. Typical sample concentrations were 0.6 mg/ml of membrane protein.

Results

Tryptic cleavage of ankyrin

Mild trypsin treatment of eosin-labeled erythrocyte ghost membranes were performed following the method of Jinbu et al. [18], in order to specifically degrade membrane-bound ankyrin. Repeating the tryptic digestion with the specified trypsin : membrane protein ratio of 1 : 100 (w/w) resulted in nonspecific degradation, including complete removal of band 4.1 within 10 s. A reduced concentration of trypsin (trypsin : membrane protein = 1 : 370, w/w) succeeded in specific degradation of ankyrin within the time-range used (0–90 s). In order to quantitate more exactly the optimum conditions, the activity of the trypsin was assayed. 1 mg of trypsin was found to hydrolyse 233 μmol/min of tosyl arginine methyl ester and 73 μmol/min of benzoyl arginine ester when added to a 10 mM solution of each, using a pH-stat method. Close examination of both the Fairbanks and Laemmli gels (see Fig. 2) revealed a similar degradation pattern to that reported. The majority of ankyrin loss was accomplished within 20 s with the concomitant appearance of a major degradation product corresponding to an M_r of approx. 170000,

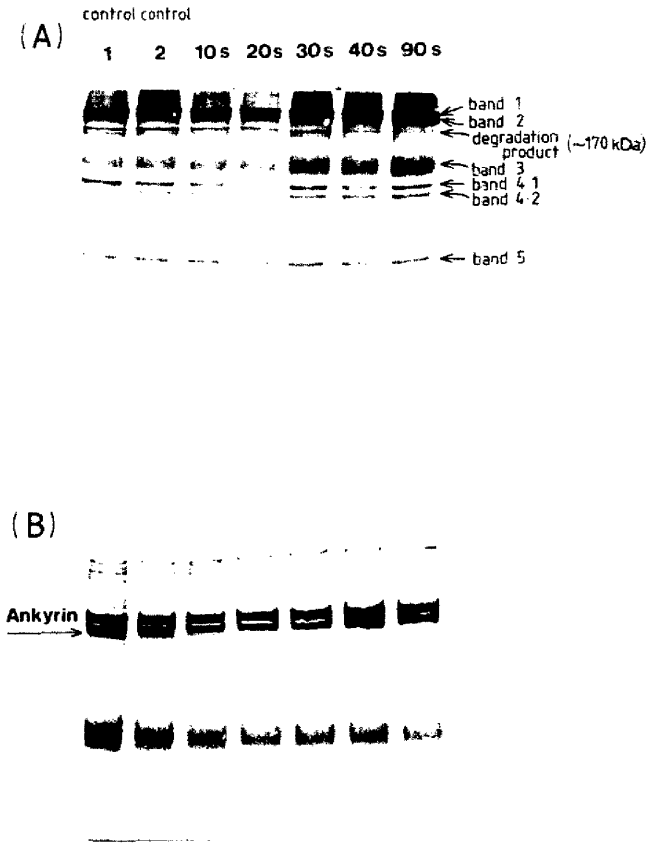

Fig. 2. Selective degradation of ankyrin after exposure of erythrocyte ghosts to trypsin (trypsin : membrane protein = 1:370 (w/w) at 0 °C for the designated times, monitored by two SDS-PAGE systems. (A) 10% Laemmli gel, heavily loaded to ensure clear visualisation of degradation products (B) 4% Fairbanks gel showing ankyrin loss. Control 1 = ghost membrane plus trypsin inhibitor solution; control 2 = ghost membrane plus trypsin inhibitor solution plus trypsin.

which itself undergoes further degradation with time, forming another band within the syndein region (M_r = 150000).

Fig. 3B compares transient dichroism measurements of band 3 rotation in ankyrin-degraded and control ghosts. The anisotropy decays show that there is negligible change in band 3 mobility upon ankyrin degradation to the extent described, in contrast to that produced by ankyrin/band 4.1 removal (Fig. 3A). The more extensively degraded ghosts produced with the higher trypsin level (1 : 100) did show some enhancement of decay, but as band 3 was one of the bands affected, useful information cannot be extracted.

Heat treatments

SDS-PAGE (stained with Coomassie blue) of ghosts prepared from heat-treated (50 °C) intact cells revealed no degradation of any major protein band. However, several new bands were present, presumably due to cytoplasmic proteins rendered 'sticky' by the heat treatment; increased levels of bands 7 and 8 were also evident. The transient dichroism data of these ghosts shows a marked increase in the mobility of band 3 (Fig. 4A). however, no such increase was observed with ghosts prepared from cells incubated at 47 °C. There was no obvious difference between gels of the 47 °C and 50 °C samples. The effect of the 50 °C treatment on the anisotropy decay appears to be similar to that produced by trypsin removal of the cytoplasmic domain of band 3 in a control ghost sample (Fig. 4B). The r_3 value of the decay was the same for both cases, 26% compared with 38% for control ghosts, although the heat-treated sample exhibits a slightly faster initial rate of decay. Trypsin removal of the cytoplasmic domain of band 3 in

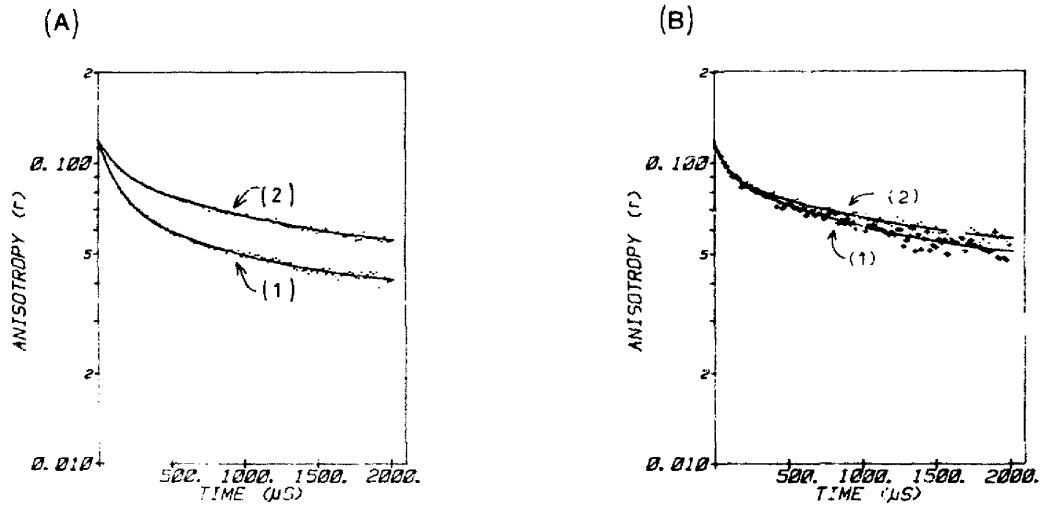

Fig. 3. Anisotropy decay curves for eosin-labeled band 3 in erythrocyte ghosts showing (A) enhanced decay for stripped ghosts (curve 1) (depleted of spectrin, actin, band 4.1 and ankyrin) relative to control ghosts (curve 2); and (B) negligible change in decay curves for ankyrin-degraded ghosts (+) (curve 1) (30 s incubation with trypsin, see Fig. 2) relative to control ghosts (·) (curve 2). Stripped ghosts were prepared and gave anisotropy decays as previously described in Ref. 16. All measurements were made at 37 °C on ghosts suspended in 5PB, 66% (w/v) glycerol (pH 7.5).

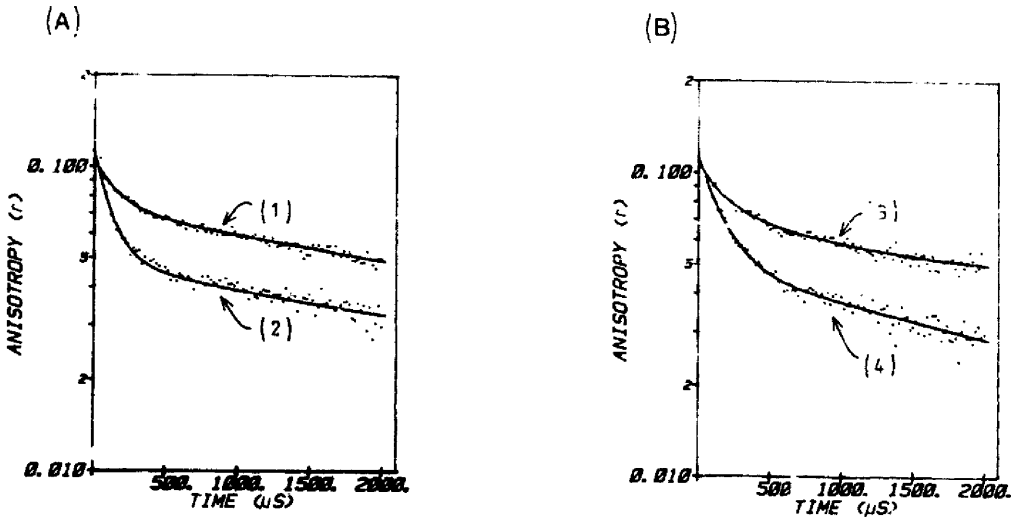

Fig. 4. Anisotropy decay curves for eosin-labeled band 3 in (A) ghosts prepared from intact cells pre-incubated at (1) 47 °C, (2) 50 °C; (B) control ghosts (3) and ghosts from which the cytoplasmic domain of band 3 has been cleaved by trypsin (4). Trypsin-treated ghosts gave anisotropy decays as previously described in Ref. 16. All measurements were made at 35 °C on ghosts suspended in 5PB, 66% (w/v) glycerol (pH 7.5)

ghosts derived from heat-treated cells (50 °C) produced no extra enhancement of band 3 mobility.

Normal ghosts incubated at 50, 60 and 70 °C showed no enhancement of band 3 mobility. There were no significant changes in gels of these heat-treated ghosts compared with the control.

Gels stained by the periodic acid-Schiff procedure were examined for evidence of glycophorin degradation. No significant changes were observed for the major bands corresponding to glycophorin A as a result of any of the heat treatments.

Discussion

The previous findings [16] that high salt removal of ankyrin and band 4.1 or the tryptic removal of the 43 kDa band 3 cytoplasmic domain increase the rotational mobility of a population of band 3 molecules were thought to most probably reflect removal of the means of band 3 attachment to an immobile cytoskeletal network. The failure, reported here, of selective ankyrin degradation by trypsin to influence band 3 rotation (Fig. 3) is not in accord with this interpretation. It might be argued that even though ankyrin has been cleaved, it is well within the capacity of the high-molecular-weight daughter product to contain both the band 3 and spectrin binding sites, and thus the ability to link the two proteins. It could also be suggested that the degraded ankyrin remains intact on the membrane, as no physical separation of the fragments need necessarily occur. However, this would directly contradict the electron microscopic evidence of Jinbu et al. [18], which clearly shows a detachment of spectrin from the membrane as a result of tryptic digestion to an extent that falls within the range shown in Fig. 2. For this latter observation to be met and to comply with the tryptic digestion patterns already established for ankyrin in solution [3], it is necessary to conclude that it is the 55 kDa spectrin binding domain, which is at least in part cleaved from the ankyrin. It thus appears very improbable that linkage of band 3 to spectrin survives the ankyrin cleavage and hence the present transient dichroism measurements indicate that ankyrin-mediated attachment to spectrin is not a factor in restricting the rotational mobility of band 3. This conclusion is further supported by experiments in which cleavage of ankyrin by a Ca^{2+}-activated endogenous proteinase also failed to enhance band 3 mobility (Clague, Harrison and Cherry, unpublished data).

Since the linkage of band 3 to spectrin via ankyrin is well-established by binding studies, the failure of this attachment to restrict band 3 rotation is somewhat unexpected. A plausible explanation can, however, be based on the flexibility of the cytoskeleton. This flexibility most probably resides principally in spectrin, both ESR measurements [24] and recent transient dichroism (Clague, Harrison and Cherry, unpublished data) studies indicating that this protein undergoes microsecond motion even when attached to the membrane. This motion is much faster than the millisecond rotation of the slow component of band 3. It is thus quite likely that the flexible cytoskeleton fails to provide the necessary restriction to angular displacements of band 3 over $\pm\pi/2$ which is required to account for the slow component. This proposal also readily explains the previously puzzling observation [16,17] that low ionic strength extraction of spectrin and actin alone has little or no effect on band 3 rotational mobility.

If linkage of band 3 to spectrin via ankyrin is not responsible for the slow component of band 3 rotation,

what then is the mechanism? In general terms, it very probably involves crosslinking of a number of band 3 molecules to form a slowly rotating aggregate. At this point, it may avoid confusion if one factor which has been discussed elsewhere is put aside for the purpose of the present discussion. Evidence from studies with reconstituted band 3 indicates that band 3 undergoes a temperature-dependent self-association independent of the presence of other proteins [25,26]. Whilst such an association may occur to some extent in the erythrocyte membrane at 37°C, we wish here to consider only additional restraints involving interaction with other proteins.

Any mechanism which involves binding of proteins to the cytoplasmic domain of band 3 requires the linkage with the membrane-bound domain to be rigid. Perhaps the best evidence that this is the case comes from the observation that addition of glyceraldehyde-3-phosphate dehydrogenase, which binds to the cytoplasmic domain, strongly decreases band 3 rotational mobility [27,28]. This presumably occurs because the tetrameric enzyme is capable of crosslinking band 3. It should be noted that the amount of enzyme normally bound to ghost preparations does not have a detectable effect on band 3 mobility.

The number of band 3 molecules required to form an aggregate with millisecond rotation is not very large, an aggregate of diameter equivalent to four or five dimers or tetramers would be sufficient. Such structures would probably not be readily identifiable in freeze-fracture electron micrographs of erythrocyte membranes because the high density of intramembranous particles, corresponding to integral membrane proteins, means that many particles are of necessity in close proximity. In fact, it has been shown that the particle distribution in such micrographs is non-random [29].

Ankyrin and band 4.1 are both candidates for aggregating band 3, in view of the increase in rotational mobility observed upon their removal (subsequent to spectrin and actin). A role for ankyrin is not necessarily ruled out by the selective degradation experiments reported here, since comparison with previous data indicates that it is the spectrin, rather than the band 3 binding site which is destroyed. The implication is that most of the ankyrin mass remains bound to band 3 and thus might conceivably promote band 3 aggregation. Korsgren and Cohen [30] have very recently reported evidence that band 4.2 binds to ankyrin and band 4.1 in addition to band 3. This multiple-binding property of 4.2 provides a crosslinking mechanism which could well explain the rotational mobility data.

Band 4.1, on the other hand, is known to have both a high-affinity binding site for glycophorin and a lower-affinity site for band 3 [5,7]. Given that both glycophorin and band 3 are probably at least dimeric in the membrane [1,31], the formation of band 3-glycophorin aggregates mediated by band 4.1 is an alternative possibility. Experiments involving crosslinking with antibodies directed against glycophorin have in fact previously indicated the existence of glycophorin-band 3 complexes [32].

Experiments with heat-treated membranes were performed to provide an alternative method of studying the band 3–ankyrin interaction. Incubation of ankyrin at temperatures above 65°C has been shown to destroy the reassociation of purified ankyrin with ankyrin-stripped ghost membranes due to denaturation [33,34]. It would thus seem probable that the 70°C heat treatment of ghosts perturbs the ankyrin interaction with band 3 on the membrane, although this could not be checked by looking for coextraction with spectrin/actin in low salt media, as this process was inhibited by the treatment. No change in band 3 rotation was detected as a result of the 70°C incubation. Whilst this finding must be interpreted with caution, it does further support the view that interaction with ankyrin is not responsible for the slow component of band 3 rotation.

In view of the lack of effect of effect of heating ghosts to 70°C for 10 min or 50°C for 1 h, it was a considerable surprise to observe a large increase in band 3 rotational mobility in ghosts derived from intact cells pretreated at 50°C (Fig. 4). The onset of this effect is very sharp, since it was not seen in cells incubated at 47°C. De Bruijne and Van Steveninck [35] have shown that heating erythrocytes to 48.4°C antagonises the effects of anaesthetics in decreasing red cell deformability, and that ghosts prepared from these cells suffered a loss of low ionic-strength extractability of spectrin, band 7 and band 8. Furthermore, spectrin could be extracted quite normally from ordinary ghosts treated at the same temperature. It is known that membrane-bound spectrin undergoes a partial denaturation over the same temperature range [36] (47–50°C) which has been shown to dissociate spectrin into monomers and to destroy the ability of β-spectrin (which binds to ankyrin) to associate with spectrin-depleted ghosts [37]. However, the presence of cytoplasmic components is not required and hence this alone cannot provide a simple explanation to either set of results. Degradation of major erythrocyte proteins, another possible explanation, could not be detected in any of the heat-treated samples. So for now the cause must remain buried within the complexity of the intact cell, but elucidation of the factors responsible may well shed new light on the erythrocyte cytoskeleton–membrane interactions.

After fitting the data to Eqn. 2, the immobile fraction of the decay (r_3) for both ghosts from heat-treated cells and for ghosts in which the cytoplasmic domain of band 3 was cleaved, was found to be 26%. The latter value is in good agreement with previous studies [16,38]. It has also been shown [38] that cleavage of the cytoplasmic domain of band 3 following prior incubation of

TABLE I

Effects of different treatments on band 3 rotational mobility in erythrocyte membranes

All measurements were performed on ghosts at 37°C, except (j) where the result was obtained at 20°C in vesicles with lipid:protein ratio of 7.5 (w/w). Enhancement of band 3 mobility is observed as a reduction in the contribution of slowly rotating species to the anisotropy decay. The value of r_3 obtained from fitting the anisotropy decays to Eqn. 2, and expressed as a percentage of the initial anisotropy, provides a comparative measure of the effects of different treatments.

	r_3(%)
A. Treatments which have no effect on band 3 rotational mobility	
(a) Removal of spectrin and actin by low salt [16]	
(b) Selective proteolytic degradation of ankyrin	
(c) Heat treatment of ghosts up to 70°C	
B. Treatments which enhance band 3 rotational mobility	
(d) Removal of spectrin, actin, ankyrin and band 4.1 [16]	31
(e) Removal of cytoplasmic domain of band 3 by *trypsin* [16]	26
(f) Heat treatment of intact cells at 50°C	26
(g) Combination of (f) and (e)	26
(h) Heat treatment of intact cells for 24 h at 37°C plus (e) [38]	12
C. Comparative values of r_3(%)	
(i) Control ghosts [13]	38
(j) Band 3 reconstituted into egg PC vesicles [25]	12

intact cells for 25 h at 37°C reduces this value to 12% for ghosts measured at 45°C. A similar value of r_3 is found for band 3 reconstituted into lipid vesicles [25].

The effects of various treatments on the rotational mobility of band 3 encompassed in the present work and that of previous studies are collected together in Table I. Taken together, the various observations strongly suggest that a multiplicity of important restraints are imposed on band 3 rotational mobility. However, the correlation in anisotropy decays following heat treatment of cells and cleavage of the cytoplasmic domain of band 3 in ghosts shown in Fig. 4, plus the lack of further mobilisation after band 3 cleavage of the heat-treated samples, indicate that, in these instances, at least, the same restraint is being removed.

In summary, then, the present study leads to the conclusion that attachment of band 3 to the cytoskeleton via ankyrin does not restrict band 3 rotational mobility, presumably because of rapid molecular motion within the cytoskeletal structure. The slow component of band 3 rotational diffusion is likely to be due to small aggregates. Whilst band 4.1 appears to be a possible candidate for promoting such aggregates, the effects resulting from heat treatment of cells and other experiments listed in Table I indicate that the associations of band 3 are very probably more complex than have previously been appreciated. The associations of less-studied proteins, such as bands 4.2, 4.9, 7 and 8, may well prove to be relevant to a full explanation of the experimental results.

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