in this case would be oriented quite differently within the site. Thus, while the data are strongly supportive of an exchange mechanism, the exact nature of this exchange cannot be deduced from these data. It could be either of the proposed versions, or a different one again in which there are no common hydroxyl binding sites.

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# Rotational Diffusion of the Erythrocyte Integral Membrane Protein Band 3: Effect of Hemichrome Binding†

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ABSTRACT: Human erythrocyte band 3 was covalently labeled within the integral membrane domain by incubating intact erythrocytes with the phosphorescent probe eosinyl-5-maleimide. The rotational diffusion of band 3 in membranes prepared from these labeled cells was measured using the technique of time-resolved phosphorescence anisotropy. Three rotational correlation times ranging from 16 to 3800 μs were observed, suggesting that band 3 exists in different aggregate states within the plane of the membrane. The oxidizing agent phenylhydrazine was used to induce hemichrome formation within intact erythrocytes. The immobilization of band 3 in membranes prepared from these erythrocytes suggests that the binding of hemichromes induces clustering of band 3. The addition of purified hemichromes to erythrocyte ghosts leads to a similar effect. We have also examined the mobility of the cytoplasmic domain of band 3. This region was labeled indirectly using a phosphorescently labeled antibody which binds to an epitope within the cytoplasmic domain. We observed very rapid motion of the cytoplasmic region of band 3, which was only partially restricted upon hemichrome binding. This suggests that the integral and cytoplasmic domains of band 3 may be independently mobile.

Aging of erythrocytes in vivo is accompanied by the gradual oxidation of hemoglobin and the formation of hemichrome-containing Heinz bodies below the membrane surface (Peisach et al., 1975; Jacob & Winterhalter, 1970). The hemichromes have a high affinity for the cytoplasmic domain of band 3, the major integral protein of the red cell membrane. It has been suggested that hemichromes cross-link band 3 molecules into clusters that constitute the so-called "senescence antigen"

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(Schluter & Drenkhahn, 1986). This antigen is recognized by autoantibodies that promote the removal of aged cells from the circulation (Low et al., 1985). Phenylhydrazine can be used to artificially induce rapid oxidation of hemoglobin in vitro, and using immunofluorescence, Waugh et al. (1987) observed morphological clusters of band 3 that coincided with sites of autologous immunoglobulin G (IgG)[1] binding. On the other hand, Lelkes et al. (1988) found no evidence that the positions of Heinz bodies correspond to the clustering of intramembranous particles rich in band 3.

Human erythrocyte band 3 is a 95-kDa glycoprotein that is responsible for bicarbonate–chloride exchange across the plasma membrane. The protein has two distinct domains: a 52-kDa hydrophobic membrane-embedded domain and a structurally independent 43-kDa hydrophilic domain that penetrates approximately 25 nm into the cytoplasm (Low, 1986). The binding of ankyrin to a site within the cytoplasmic domain of band 3 physically links the cytoskeleton to the membrane proper. However, sufficient ankyrin is present to link only about 15% of the band 3 molecules in this way (Bennett & Stenbuck, 1979). The N-terminal region of the cytoplasmic domain is very acidic and contains binding sites for several glycolytic enzymes (Walder et al., 1984; Tsai et al., 1982; Higashi et al., 1979). In addition, this region binds weakly to hemoglobin and very strongly to oxidized hemoglobin (Low, 1986).

There is strong evidence to indicate that band 3 exists in a number of oligomeric forms. Cross-linking studies suggest that band 3 oligomerizes to form at least tight dimers or tetramers (Kiehm & Ji, 1977; Mikkelsen & Wallach, 1976). Interestingly, anion binding may influence the quaternary state of band 3. Salhany and Sloane (1989) have found that binding of the anion 4′,4′-dinitrostilbene-2,2′-disulfonate (DNDS) favored the tetrameric species.

Measurements of rotational diffusion provide evidence for the existence of even higher aggregates of band 3 (Cherry, 1978; Clague et al., 1989; Tilley et al., 1990, 1991; Matayoshi et al., 1991). It has been suggested that band 3 oligomers are of physiological importance in providing a scaffold for the assembly of a multienzyme complex composed of glycolytic enzymes (Kurganov et al., 1985; Low, 1986). However, there is little experimental evidence to support this view (Harris & Winzor, 1989, 1990). Altered band 3 aggregation has been shown to occur in some genetically variant conditions such as ovalocytosis (Tilley et al., 1991) and acanthocytosis (Kay et al., 1988), as well as in pathological conditions of red cells, such as malaria infection (Tilley et al., 1990). The physiological consequences of this aggregation have not been studied in detail.

This paper examines the effect of hemichromes on the aggregation state of band 3. Use is made of the strong dependence of the rotational diffusion of a membrane protein on the size of the diffusing unit to monitor the association process. Rotational diffusion is measured using the technique of time-resolved phosphorescence anisotropy (TRPA). Two separate domains of band 3 were labeled with phosphorescent probes. When the integral domain of band 3 is labeled with eosinyl-5-maleimide, the binding of hemichromes causes a substantial immobilization of band 3. In contrast, when band 3 is labeled indirectly with an erythrosin-labeled monoclonal Fab fragment specific for the cytoplasmic domain, this region displays substantial flexibility. This flexibility is only partly reduced by the binding of hemichromes. We suggest that a hinge region within the cytoplasmic domain provides a point of articulation that allows substantial flexibility in the linkage of band 3 to the cytoskeleton.

[1] Abbreviations: IgG, immunoglobulin G; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; MAb, monoclonal antibody; TRPA, time-resolved phosphorescence anisotropy; EITC, erythrosinyl 5-isothiocyanate.

## Materials and Methods

*Labeling of Band 3.* Fresh human blood was obtained from the Red Cross Blood Transfusion Service, Melbourne. Band 3 in intact erythrocytes was labeled at its extracellular face with eosinyl-5-maleimide (Molecular Probes, Junction City, OR) as described by Tilley et al. (1990). The specificity of the labeling was determined by examining SDS–PAGE patterns of membrane samples under ultraviolet light.

*Phenylhydrazine Treatment.* One milliliter of 100 mM phenylhydrazine in phosphate-buffered saline, pH 7.4, was added to 19 mL of a suspension of erythrocytes at 10% hematocrit. After incubation at 4 °C for 5 min, the erythrocytes were washed extensively to remove unreacted phenylhydrazine. Heinz body formation was assessed by microscopic observation of methyl violet stained cells (Bates & Winterbourne, 1984). Hemichrome levels in the lysate were monitored by spectral analysis using a Cary 118 spectrophotometer. Ghosts were prepared by hypotonic lysis in 5 mM sodium phosphate, pH 8. The final wash was in 10 mM Tris/10 mM NaCl, pH 7.6, unless otherwise specified. The amount of hemichrome remaining associated with the erythrocyte membranes was quantitated by densitometric scanning of Coomassie Blue stained SDS–polyacrylamide gels (12% acrylamide) of membrane samples (Laemmli, 1970); this analysis assumes that the uptake of Coomassie Blue stain is the same for both band 3 and hemichrome.

*Hemichrome Isolation and Incubation with Erythrocyte Ghosts.* Hemichromes were isolated as described by Waugh et al. (1987). The molar extinction coefficient for hemichromes was taken as $3.9 \times 10^3$ at 630 nm (Winterbourn et al., 1976). Purified hemichromes in 5 mM sodium phosphate, pH 7.4, were mixed with erythrocyte ghosts at a 100-fold molar ratio of hemichrome to band 3. The samples were incubated at room temperature for 5 min and then washed with 10 mM NaCl/10 mM Tris, pH 7.6, to remove unbound hemichromes.

*Preparation and Labeling of MAb and Fab Fragments of the Anti-Band 3 Antibody.* The monoclonal antibody B-9 (IgG class) (Tilley et al., 1990) was affinity-purified using protein A–Sepharose chromatography, and Fab fragments were prepared by incubating the monoclonal antibody (MAb) with activated papain (Oi & Herzenberg, 1979; Tilley et al., 1990). The resulting mixture was passed through a column of protein A–Sepharose to remove Fc fragments and any undigested intact IgG. The Fab fragments were eluted with 0.1 M citrate buffer, pH 4, and concentrated by ultrafiltration. Purity was assessed using SDS–PAGE (12% acrylamide). The final preparation of B-9 Fab was judged to be >95% pure. The MAb or Fab samples (1.0 mg) were dialyzed overnight against bicarbonate buffer, pH 9.5, and incubated with 0.1 mg of erythrosinyl 5-isothiocyanate (Molecular Probes) for 2 h at room temperature. Samples were then dialyzed for 2 days against several changes of 20 mM NaCl/5 mM sodium phosphate, pH 8. Final treatment with SM-2 Bio-beads (Bio-Rad) resulted in a labeled MAb or Fab sample in which <5% of the label was noncovalently associated with the protein. The noncovalently bound probe was determined spectrophotometrically after extraction with chloroform/methanol/water according to the method of Folch et al. (1957).

*Incubation of EITC–Fab or EITC–MAb with Erythrocyte*

*Membranes.* One milliliter of erythrocyte ghosts (2 mg/mL protein) was incubated with 1.4 mL of EITC–Fab (0.5 mg/mL) or EITC–MAb (0.1 mg/mL) in 20 mM NaCl/5 mM sodium phosphate, pH 8, overnight at 4 °C. Membranes were pelleted through 1 mL of fetal calf serum to remove nonspecifically bound antibody and were then washed twice in 20 mM NaCl/5 mM sodium phosphate, pH 8. EITC–Fab-labeled erythrocyte membranes (0.5 mL of approximately 2 mg/mL protein) were incubated with hemichromes (200 μL of 2 mg/mL protein) for 5 min at room temperature and then washed twice with 20 mM NaCl/5 mM sodium phosphate, pH 8. Goat anti-mouse IgG (50 μL of 2 mg/mL protein) was added to a sample containing 0.25 mL of EITC–MAb-labeled membranes (approximately 2 mg/mL protein) and incubated for 1 h. Freezing of the motion of the EITC–MAb-labeled membranes was achieved by incubating 0.35 mL of EITC–MAb membranes (approximately 2 mg/mL protein) with 170 μL of 1% glutaraldehyde in 20 mM NaCl/5 mM sodium phosphate, pH 8, for 1 h at 20 °C. The reaction was stopped by the addition of glycine to a concentration of 0.2 M. The number of antibody binding sites per band 3 molecule was determined by densitometric scanning of Coomassie Blue stained SDS–PAGE patterns of membrane samples which had been incubated with increasing concentrations of EITC–Fab.

*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 the labeled membranes. The phosphorescence, isolated with a combination of KV550 and RG695 cutoff filters, was measured with a photomultiplier (EMI 9817 QBG) that was gated on 1 μs after the laser pulse to avoid the high-intensity prompt fluorescence. Decays of the parallel [$I_{VV}(t)$] and perpendicular [$I_{VH}(t)$] 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 512 decays of each component. After subtraction of the background signals, the decays were transferred to a PDP 11/23 computer, and the total intensity [$s(t)$] and anisotropy [$r(t)$] decays were generated according to the expressions:

$$s(t) = I_{VV}(t) + 2I_{VH}(t) \quad (1)$$

$$r(t) = [I_{VV}(t) - I_{VH}(t)]/[I_{VV}(t) + 2I_{VH}(t)] \quad (2)$$

The generated curves were fitted to the functions:

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

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

$\alpha_i$ representing the initial intensity of the decay component $i$ having a lifetime $\tau_i$ and $\beta_j$ the partial anisotropy of component $j$ associated with the rotational correlation time $\phi_j$; $r_\infty$ is the limiting anisotropy that reflects, in part, the infinite time distribution function for a hindered rotor. $B$ 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 1–4 exponentials using a Chebychev transformation procedure contained within a data acquisition operating system (DAOS) supplied by Labsoft Associates, Melbourne. Goodness-of-fit was determined by the value of $\chi^2$ or from plots of the weighted residuals.

The phosphorescence anisotropy decays for eosin-labeled band 3 were analyzed according to Tsuji et al. (1988). This analysis interprets the different rotational correlation times for band 3 in terms of populations of different aggregate size. The contribution of each population to the anisotropy decay is derived from the fractional amplitudes:

$$f_j = (\beta_j/r_0)/0.82 \quad (5)$$

$$f_{im} = (r_\infty/r_0 - 0.18)/0.82 \quad (6)$$

where $f_j$ and $f_{im}$ are, respectively, the mobile and immobile populations of band 3, and $r_0$ is the anisotropy at time zero. An intrinsic membrane protein undergoing uniaxial rotation about the membrane normal possesses two correlation times, $\phi$ and $\phi/4$ (Cherry, 1978), where

$$\phi = 4\eta V/kT \quad (7)$$

$\phi$ is the rotational correlation time, $\eta$ is the bilayer viscosity, $V$ is the molecular volume, $k$ is Boltzmann's constant, and $T$ is the absolute temperature. If there is more than one aggregate size of band 3, two correlation times are expected for each species. In practice, the number of populations that can be resolved is limited by the closeness of the correlation times, the curve-fitting procedures employed, and the signal-to-noise ratio in the anisotropy decays.

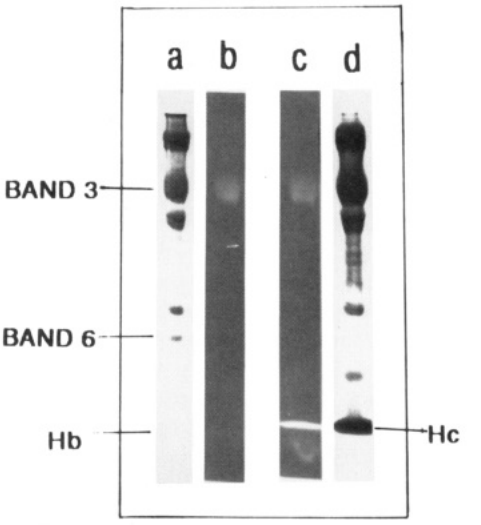


FIGURE 1: SDS–polyacrylamide gels (12% acrylamide) of membranes prepared from control erythrocytes (a, b) and erythrocytes treated with 5 mM phenylhydrazine (c, d). Eosin fluorescence is detected under ultraviolet light. The major eosin-labeled species is band 3 (b, c). Fluorescence from hemichromes (Hc) can also be detected (c). Coomassie Blue staining of the gels (a, d) reveals binding of hemichromes to the membranes prepared from phenylhydrazine-treated erythrocytes (d).

## Results and Discussion

*Rotational Mobility of Eosin–Band 3 in Control Membranes.* Figure 1 shows SDS–polyacrylamide gels of membranes prepared from erythrocytes labeled with eosinyl-5-maleimide. Band 3 is the major labeled species, as judged by visual detection of the eosin label under ultraviolet light (Figure 1a,b).

The decays of phosphorescence anisotropy of eosin-labeled band 3 in erythrocyte membranes at 37 °C are shown in Figure 2 (lower curve in each panel). Two different time regimes (0–0.5 and 0–5 ms) were required to resolve both rapid and slow rotational events (Tilley et al., 1990; Matayoshi & Jovin, 1991). The data were analyzed using eq 5 and 6: the results are summarized in Table I. At 37 °C, three mobile populations of band 3 and an apparently immobile population were resolved. The different populations are assumed to represent

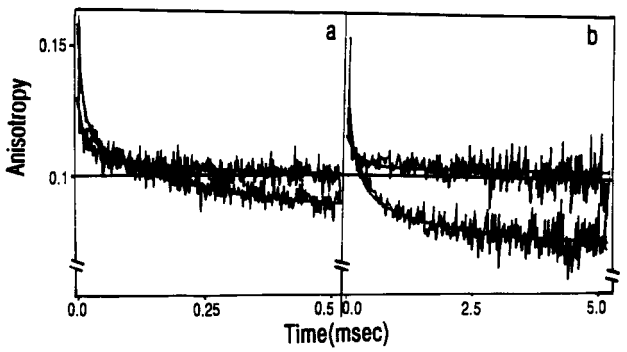


FIGURE 2: Decays of phosphorescence anisotropy of eosinyl-5-maleimide-labeled band 3 in human erythrocyte ghosts at 37 °C. The erythrocyte membrane protein concentration was 0.3 mg/mL. The molar ratio of label to protein was 0.6. Data were collected at two different time resolutions: (a) 0–0.5 ms; (b) 0–5 ms. Data are fitted using eq 4 and analyzed using eq 5 and 6. The bottom curves represent control membranes while the top curves are for membranes prepared from erythrocytes treated with 5 mM phenylhydrazine. Under these conditions, approximately one hemichrome tetramer was bound per band 3 monomer. The best-fit parameters are summarized in Table I.

Table I: Analysis of Phosphorescence Anisotropy Decays of Eosin-Labeled Band 3 in Membranes Prepared from either Control or Phenylhydrazine-Treated Human Erythrocytes[a]

| | control | phenylhydrazine-treated |
|---|---|---|
| $\phi_1$ ($\mu$s) | 16 ± 3 | 14 ± 2 |
| $f_1$ | 0.32 ± 0.01 | 0.20 ± 0.02 |
| $\phi_2$ ($\mu$s) | 280 ± 60 | 170 ± 30 |
| $f_2$ | 0.22 ± 0.01 | 0.10 ± 0.01 |
| $\phi_3$ ($\mu$s) | 2000 ± 600 | |
| $f_3$ | 0.17 ± 0.04 | |
| $f_{immobile}$ | 0.29 ± 0.04 | 0.70 ± 0.01 |

[a] The data were analyzed in terms of three mobile and one immobile population of band 3. Data represent the average ± SD for at least three measurements.

different aggregation states of band 3. The correlation times ($\phi_1$, $\phi_2$, $\phi_3$) and fractional amplitudes ($f_1$, $f_2$, $f_3$) are in agreement with those reported recently by Matayoshi et al. (1991).

*Hemichrome Formation.* Treatment of erythrocytes with 5 mM phenylhydrazine oxidizes the hemoglobin, inducing the formation of intracellular Heinz bodies (Waugh et al., 1987). Spectra of the erythrocyte lysates (Figure 3) showed characteristic features of hemichromes, with a decrease in the 575-nm absorption relative to the 540-nm peak and the appearance of a shoulder at 630 nm (Peisach et al., 1975). SDS–PAGE analysis of the membranes prepared from phenylhydrazine-treated erythrocytes showed the presence of hemichromes (Figure 1d) and also showed that hemichrome binding displaced glyceraldehyde-3-phosphate dehydrogenase (band 6) from the membranes. Densitometric scanning of the Coomassie Blue stained gels indicated that approximately 1 molecule of tetrameric hemichrome was bound per band 3 molecule (Figure 1d). Waugh and Low (1985) have previously shown that up to 2.5 hemichrome tetramers can associate with each cytoplasmic domain of band 3. For the anisotropy measurements, erythrocytes were first labeled with eosin and then subjected to the phenylhydrazine treatment. Fluorescence both from the eosin–band 3 and from the hemichromes was visible when SDS–PAGE gels of membrane samples were examined under ultraviolet light (Figure 1c). There was, however, no measurable phosphorescence from the hemichromes under the conditions of these experiments.

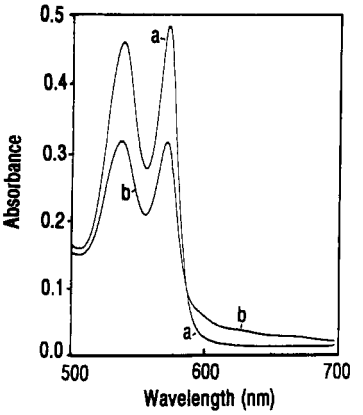


FIGURE 3: Spectra of lysates of control (a) and phenylhydrazine-treated (b) erythrocytes. Intact erythrocytes were incubated with 5 mM phenylhydrazine at pH 7.4, for 5 min, at 4 °C.

Table II: Analysis of Phosphorescence Anisotropy Decays of Eosin-Labeled Band 3 in Human Erythrocyte Membranes with and without Bound Hemichromes[a]

| | control | hemichrome-treated |
|---|---|---|
| $\phi_2$ ($\mu$s) | 19 ± 2 | 12 ± 2 |
| $f_1$ | 0.26 ± 0.01 | 0.27 ± 0.05 |
| $\phi_2$ ($\mu$s) | 250 ± 30 | 120 ± 30 |
| $f_2$ | 0.24 ± 0.01 | 0.17 ± 0.02 |
| $\phi_3$ ($\mu$s) | 3800 ± 1700 | 1600 ± 300 |
| $f_3$ | 0.21 ± 0.05 | 0.14 ± 0.02 |
| $f_{immobile}$ | 0.29 ± 0.05 | 0.42 ± 0.05 |

[a] The data have been analyzed in terms of three mobile and one immobile population. Data represent the average ± SD for at least three measurements.

*Rotational Mobility of Eosin–Band 3 in Membranes Prepared from Phenylhydrazine-Treated Erythrocytes.* Figure 2 (upper curve in each panel) shows the decays of phosphorescence anisotropy of eosin–band 3 in membranes prepared from erythrocytes treated with phenylhydrazine. Analysis of these data (Table I) reveals that the fraction of band 3 which is immobile on the phosphorescence time scale increases from 29% to 70%. Decays could be adequately fitted to a double-exponential function, $\phi_1$ being unaffected and $\phi_2$ marginally shorter (Table I).

The immobilization of band 3 in membranes prepared from phenylhydrazine-treated erythrocytes is consistent with hemichrome-induced cross-linking of band 3 as proposed by Low (1986). It should be noted, however, that the low ionic strength conditions used in the current experiments are not physiological. Waugh et al. (1987) have shown that the interaction between hemichromes and band 3 decreases with increasing ionic strength. However, in intact erythrocytes, the complex between hemichromes and band 3 may well be stabilized by an excluded volume effect due to the very high concentration of hemoglobin [for a discussion, see Harris and Winzor (1990)]. We could not perform our experiments at very high concentrations of hemichromes due to their interference with the phosphorescence emission. The physiological relevance of these results must, therefore, be interpreted with caution. They do, however, provide evidence that under conditions where hemichromes bind tightly to band 3, they can cross-link the band 3 into immobile aggregates.

*Incubation with Hemichromes.* In order to further investigate the immobilization of band 3 mobility by hemichromes, eosin-labeled ghosts were incubated with purified hemichromes. Under the conditions of this experiment, approximately 1 molecule of tetrameric hemichrome was bound per

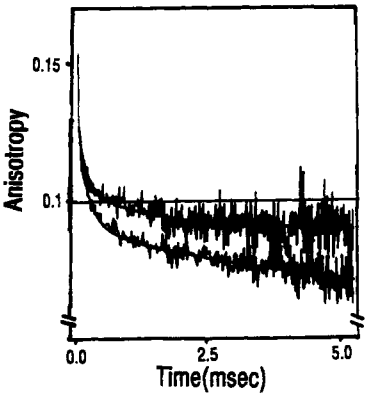


FIGURE 4: Decays of phosphorescence anisotropy of eosin-labeled band 3 in erythrocyte ghosts at 37 °C. The bottom curve represents control membranes while the top curve represents ghosts which have been incubated with purified hemichromes. Under the conditions of this experiment, one hemichrome tetramer was bound per two band 3 molecules. The best-fit parameters are summarized in Table II.

two molecules of band 3. The decay of phosphorescence anisotropy over 0–5 ms at 37 °C is shown in Figure 4. The binding of hemichromes increases the immobile fraction of band 3 from 29% to 42% (Table II). The smaller degree of immobilization which is observed in this case, compared with the phenylhydrazine treatment of intact erythrocytes, probably reflects a lower level of hemichrome binding.

The phenylhydrazine treatment did not appear to have a direct effect on the lifetime of the eosin probe. In control membranes, the decay of phosphorescence intensity of eosin–band 3 was fitted to three exponentials having the following lifetimes: $\tau_1 = 30 \pm 3$ μs, $\tau_2 = 340 \pm 20$ μs, $\tau_3 = 2200 \pm 50$ μs. These lifetimes were not altered in membranes prepared from phenylhydrazine-treated erythrocytes. The similarity in the time constants for the decay of the total intensity also indicates that the changes in anisotropy cannot be attributed to fortuitous changes in lifetime. Membranes prepared from phenylhydrazine-treated erythrocytes that were not labeled with eosin gave no phosphorescence signal.

*Structural Implications.* A single protein species undergoing uniaxial rotation around an axis normal to the membrane surface generates two correlation times, $\phi$ and $\phi/4$ (Cherry, 1978). The three apparent correlation times that we observe for eosin–band 3 rotation differ from each other by a factor greater than 4, suggesting that they represent three different populations of band 3 rather than the $\phi$ and $\phi/4$ values pertaining to a single population (Clague et al., 1989). Band 3 has 10–12 membrane-spanning segments. Assuming these segments to be helical, the radius of the dimer would be about 40–48 Å, giving rise to a correlation time (eq 5) of 23–33 μs at 37 °C (Saffman & Delbruck, 1975; Matayoshi & Jovin, 1991). Thus, the short correlation time (16 μs) may be attributed to the global rotation of band 3 dimers which are unconstrained by interactions with peripheral proteins. We emphasize, however, that our measurements of rotational mobility cannot distinguish between global rotation of band 3 and internal flexibility within the integral membrane domain. Motion of a labeled segment within the membrane-embedded domain of band 3 might contribute to the depolarization of the phosphorescence signal [for discussion, see Tilley et al. (1990, 1991) and Matayoshi and Jovin (1991)].

The longer correlation times (approximately 250–280 and 2000–3000 μs, respectively) are significantly larger than those predicted theoretically for the rotation of a dimer or tetramer of band 3 and may be attributed to slowly rotating oligomers of band 3 that may also be stabilized by cross-linking interactions with peripheral proteins at the cytoplasmic surface.

Binding of hemichromes to the erythrocyte membrane, whether by prior treatment of intact cells with phenylhydrazine or the binding of purified hemichromes to erythrocyte ghosts, results in an increase in $r_\infty$ and a significant decrease in the fractional amplitudes of the anisotropy decays associated with the longer correlation times: $f_1$ is relatively unaffected while $f_2$ and $f_3$ are decreased. The increase in $r_\infty$ therefore reflects the immobilization of oligomeric forms of band 3 which previously possessed correlation times $\phi_2$ and $\phi_3$. The results therefore suggest that a degree of polyvalency of band 3 may be required for effective hemichrome binding. However, the constancy of $f_1$ also lends support to the suggestion that the short correlation time may represent segmental motion within band 3 (possibly associated with the ion channel function), which remains unaffected by hemichrome binding.

The immobilization of band 3 is consistent with cross-linking of the cytoplasmic domains of band 3 molecules by hemichromes, as proposed by Low et al. (1985) and Schluter and Drenckhahn (1986). The results are in contrast to those of Lelkes et al. (1988), who found no evidence, on the basis of freeze–fracture electron microscopy studies, of aggregation of intramembranous particles in phenylhydrazine-treated erythrocytes. However, we suggest that the time-resolved phosphorescence anisotropy technique may provide a more sensitive measure of the aggregation state of band 3 than the observation of intramembranous particles by freeze–fracture electron microscopy.

*Mobility of the Cytoplasmic Domain of Band 3.* The cytoplasmic and integral membrane domains of band 3 are connected by an amino acid sequence that is susceptible to proteolytic attack (Low, 1986). The two domains are thought to be structurally and functionally independent, and thus the removal of the cytoplasmic domain does not destroy the anion transport property of the transmembrane domain (Rice & Steck, 1977). In addition, Low (1986) has proposed that the cytoplasmic domain consists of two subdomains connected by a flexible hinge region at residues 176–191. Flexibility at this point permits the adoption of three different native conformations depending on the pH. Fluorescence resonance energy transfer measurements from a cluster of tryptophan residues on the N-terminal side of the hinge region to a labeled cysteine cluster on the C-terminal side of the hinge region support the notion that this region is highly flexible (Low et al., 1984). In contrast to the flexibility of the cytoplasmic domain, the motion of the relatively rigid integral domain, with its series of closely packed transmembrane segments, is more likely to reflect the global rotation of the molecule as a whole. This is not to discard the possibility that a degree of internal motion within the transmembrane domain is required for the protein to perform its anion transport function.

The availability of a monoclonal antibody (MAb) specific for the cytoplasmic domain (Tilley et al., 1990) presented an opportunity of indirectly labeling this region with a phosphorescent probe. The precise site of binding of this MAb is not known. However, the site may be near tyrosine-8, the major phosphorylated residue within the cytoplasmic domain, since Western blot analysis shows that all phosphorylated fragments of the cytoplasmic domain of band 3 react with the monoclonal antibody (Tilley et al., 1990). The eosin-labeled Fab fragment of the anti-band 3 MAb was used as a probe, rather than the intact antibody, to ensure monovalent binding and to exclude consideration of the flexibility of the hinge region within the antibody itself [see Pecht et al. (1991) for a discussion].

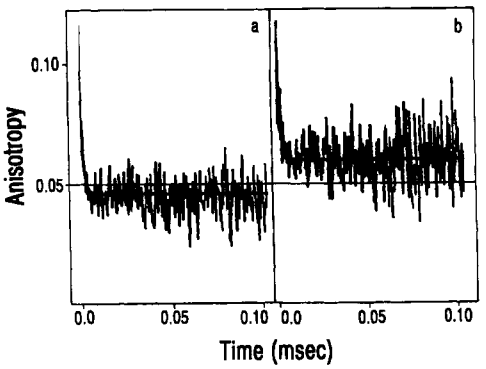


FIGURE 5: Decays of phosphorescence anisotropy of erythrosin-labeled Fab fragment bound to the cytoplasmic domain of band 3 in erythrocyte ghosts at 20 °C. The erythrocyte membrane protein concentration was 0.3 mg/mL. The ratio of EITC–Fab to band 3 molecules was 0.8:1. Data were collected over the time period 0–0.1 ms. Panel a represents control membranes, while panel b represents membranes which have been incubated with purified hemichromes. The best-fit parameters are given in Table III.

Table III: Analysis of Phosphorescence Anisotropy Decays of Erythrosin-Labeled Fab Bound to Band 3 in Human Erythrocyte Membranes with and without Bound Hemichromes[a]

| | $\phi$ ($\mu$s) | $r_\infty$ |
|---|---|---|
| control | 2.6 ± 0.6 | 0.035 ± 0.007 |
| hemichrome-treated | 2.6 ± 0.2 | 0.059 ± 0.004 |

[a] The data have been fitted to a single-exponential decay. Data represent the average ± SD for at least three measurements.

Scatchard analysis of the binding of EITC–Fab to membranes indicated the existence of one high-affinity binding site per band 3 molecule, as well as evidence for low-affinity sites (data not shown). Under the incubation conditions employed for the anisotropy measurements, 0.8 EITC–Fab fragment was bound per band 3 molecule.

The region of the band 3 cytoplasmic domain to which EITC–Fab binds appears to undergo very rapid motion on the sub-microsecond time-scale. At 20 °C, we have resolved a very short correlation time of approximately 3 $\mu$s (Figure 5, Table III). It should be emphasized, however, that this correlation time is at the limit of the time resolution of our instrument. Note that upon incubation with hemichromes, $r_\infty$ increases from 0.035 to 0.059, indicating some restriction of the rotational freedom of the cytoplasmic domain. There is, however, no measurable change in the correlation time.

The limiting anisotropy is partly determined by the angles that the absorption and emission dipoles make with the axis of rotation of the chromophore and, in part, by the distribution function for the population of chromophores at times long relative to the correlation time [see Jovin et al. (1981) for a review]. Thus, the increase in $r_\infty$ described above could reflect an increase in the size of an immobile population or, alternatively, a change in the position of the chromophore relative to the membrane normal and the axis of rotation. In either case, the short correlation time and the low value of the limiting anisotropy indicate that the cytoplasmic domain of band 3 has substantially more intrinsic flexibility than the transmembrane domain.

Observation of rapid motion of the cytoplasmic domain in these experiments with EITC–Fab is in reasonable agreement with the observations of Cassoly (1982), who used spin-labeled hemoglobin to indirectly label the cytoplasmic domain. Measurements of saturation-transfer EPR provided a correlation time of 8 $\mu$s for the hemoglobin–band 3 complex.

The addition of divalent EITC–MAb to erythrocyte ghosts caused little change in the values of the correlation time of

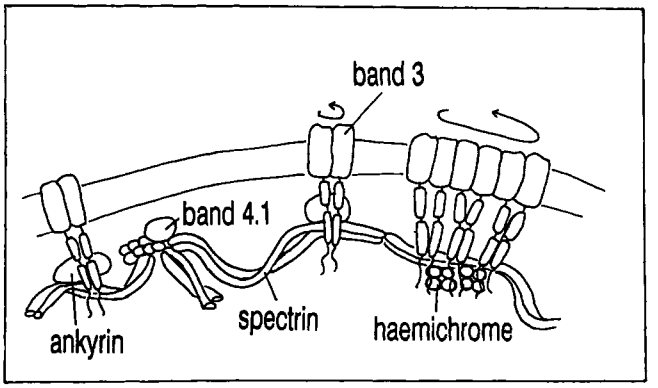


FIGURE 6: Diagram showing cross-linking of band 3 molecules by spectrin and hemichromes. Hemichrome binding to band 3 is assumed to result in the formation of aggregates, with an associated increase in the radius of the diffusing unit and, consequently, a decrease in the mobility of the integral membrane domain of band 3. Spectrin, on the other hand, is an extended molecule with substantial internal flexibility, and its linkage, via ankyrin, to the flexible cytoplasmic domain of band 3 does not affect the rotational mobility of the integral domain. It should be noted that eosin–band 3 molecules have to rotate through an average angular displacement of only 180° for the anisotropy of the emission to decay to its limiting value.

$r_\infty$ ($\phi$ = 1.4 ± 0.3 $\mu$s, $r_\infty$ = 0.037 ± 0.002) compared to the situation with EITC–Fab. The addition of a second antibody (goat anti-mouse IgG) to this system increased $r_\infty$ to 0.048 ± 0.002. Thus, it appears that cross-linking the cytoplasmic domain of band 3 by antibodies is associated with only partial immobilization of its rotational motion. Freezing of the motion of the cytoplasmic domain was achieved by treatment of the EITC–MAb-labeled membranes with the cross-linking agent glutaraldehyde. Under these conditions, a limiting anisotropy of 0.084 ± 0.004 was obtained.

Our data suggest that the cytoplasmic domain of band 3 has a high degree of internal flexibility. Some rotational freedom of the cytoplasmic domain is retained even upon cross-linking by hemichromes or by the divalent monoclonal antibody. This contrasts with the ability of these ligands to cause substantial immobilization of the integral membrane domain of band 3 [see Figure 4 and Tilley et al. (1990)]. The integral membrane domain is restricted to uniaxial motion around an axis normal to the lipid bilayer, and any cross-linking element which causes oligomerization of the integral domain will decrease its mobility (see Figure 6). The cytoplasmic domain is not constrained by the viscous lipid bilayer and is probably able to undergo some torsional and segmental motion even in the presence of the hemichromes.

This demonstration of internal flexibility within the cytoplasmic domain of band 3 may explain why the removal of spectrin from erythrocyte ghosts is not associated with an increase in the mobility of band 3. Cherry (1978) observed that removal of most of the spectrin by low ionic strength extraction caused little, if any, increase in the mobility of band 3 labeled at its extracellular eosinyl-5-maleimide site (Cherry et al., 1976; Cherry, 1978). This result has been confirmed by Matayoshi and Jovin (1991) and in our own laboratory. At least 10–15% of the band 3 molecules are linked to spectrin through ankyrin (Bennett & Stenbuck, 1979), and it might be expected that this linkage would immobilize a part of the band 3 population. These results are, however, explicable in light of the current demonstration of internal flexibility in the cytoplasmic domain of band 3. The spectrin molecule itself is an additional source of cytoskeletal flexibility. Learmonth et al. (1989) found that spectrin retains a large degree of rotational freedom when reassociated with the erythrocyte

membrane. Similar observations have been made by Clague et al. (1990) using the technique of transient dichroism.

In conclusion, band 3 exists as a polydisperse population of aggregates within the plane of the membrane, each population contributing to the anisotropy decay. Indirect labeling of the cytoplasmic domain of band 3 with a specific eosin–MAb fragment shows that this domain is intrinsically flexible probably due to motion about a hinge region near residues 176–191. The long-range cross-links that occur through ankyrin and spectrin do not substantially inhibit the mobility of the transmembrane domain of band 3 due to the intrinsic flexibility of its cytoplasmic domain and of spectrin itself. On the other hand, cross-linking via hemichromes (or glycolytic enzymes such as aldolase or glyceraldehyde-3-phosphate dehydrogenase; Matayoshi et al., 1991) results in a significant increase in the radius of the membrane-embedded diffusing unit and thus causes substantial immobilization of band 3. These differences are depicted in Figure 6. We surmise that the degree of immobilization of the integral domain of band 3 induced by different ligands depends on the intrinsic flexibility of the linkage element.

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