Regulation of Band 3 Mobilities in Erythrocyte Ghost Membranes by Protein Association and Cytoskeletal Meshwork[†]

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ABSTRACT: Rotational diffusion of erythrocyte anion channel protein band 3 was measured in ghost membranes by observing time-resolved phosphorescence anisotropy decays of eosinyl-5-maleimide covalently attached to the protein. Experiments were carried out under conditions similar to those employed by Tsuji and Ohnishi (1986) for translational diffusion measurement of band 3 [(1986) *Biochemistry 25*, 6133–6139] to allow direct comparison of rotational and translational diffusion of band 3. Detailed analysis of diffusive properties of band 3 in ghost membranes was made on the basis of these rotational and translational diffusion data. Rotational diffusion measurements indicated that there are at least three populations of band 3 molecules with high, low, and no rotational mobilities in the time scale of 10^{-4}–10^{-2} s. These populations are in equilibrium, and the fractional ratios are strongly temperature dependent. At 26 °C, 44% of band 3 molecules are mobile (16% have an average rotational correlation time of 0.19 ms, and 28% have an average correlation time of 2.4 ms), and 56% are immobile. These results correlate well with translational diffusion data which indicated 40% mobile and 60% immobile fractions of band 3. The rotational diffusion data together with the translational diffusion data by Tsuji and Ohnishi (1986) and Golan and Veatch [(1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2537–2541] suggest that immobilization of band 3 is largely caused by binding of band 3 oligomers to ankyrin, which abolishes both rotational and translational diffusion of band 3. Dimer/tetramer equilibrium of spectrin, a major constituent protein of the cytoskeletal meshwork, was shifted by varying the ionic strength of the medium or by addition of polyamines. An increase in the dimer fraction of spectrin, which greatly increases the lateral diffusion constant of the translationally mobile component of band 3 without changing the fraction of the translationally mobile component, had little influence either on the mobile/immobile fraction of band 3 in terms of rotational mobility or on the rotational correlation time of the mobile fraction of band 3 in ghost membranes. These results are consistent with a model in which (1) band 3 oligomers, aggregates, and band 3 molecules that are bound to the cytoskeletal network via ankyrin (or via glycophorin and band 4.1) are in temperature-dependent equilibrium in ghost membranes, (2) oligomers and aggregates that are not bound to the cytoskeletal/peripheral protein network are rotationally and translationally mobile, and (3) long-range translational diffusion of mobile oligomers and aggregates is restricted by nonspecific barriers imposed by the cytoskeletal network and the rate of translational diffusion is regulated by the fraction of spectrin dimers (open gate) and tetramers (closed gate).

A number of recent studies have indicated that control of restriction mechanisms of protein mobility in membranes may play key roles in regulation of functional activities of membrane enzymes and receptors. For example, dimerization or formation of larger aggregates of immunoglobulin E–Fc receptors on the surface of rat basophilic leukemic cells initiates the subsequent secondary processes of cellular degranulation and Fc receptor internalization (Metzger, 1978; Menon et al., 1986). Massive aggregation of integral and peripheral membrane proteins takes place at initial stages of desmosome formation during keratinocyte differentiation in culture (Hennings & Holbrook, 1983; Kusumi et al., 1988).

Rotational and translational diffusion of intergral membrane proteins in biological membranes can be restricted by (1) association or aggregation of membrane proteins, (2) specific binding to membrane peripheral/cytoskeletal proteins, and (3) nonspecific barriers made of peripheral/cytoskeletal proteins (cytoskeletal fence model) that nonspecifically limit translational diffusion of membrane proteins by steric hindrance. Recent studies by us and others that indicate involvement of these mechanisms in restriction of membrane protein mobility include the following. Kusumi et al. (1980) and Kusumi and Hyde (1982) found that membrane protein association rather than lipid fluidity is a major cause of changing rotational diffusion of a membrane protein (rhodopsin) in reconstituted membranes above the phase transition temperature. Chang et al. (1981) and Sakaki et al. (1981) found that translational and rotational diffusion of anion channel protein from human erythrocytes (band 3) in reconstituted membranes is decreased by addition of isolated cytoskeletal/peripheral membrane proteins. By using red blood cell ghost membranes, Sheetz et al. (1980), Golan and Veatch (1980), and Nigg and Cherry (1980) indicated that the cytoskeletal network including

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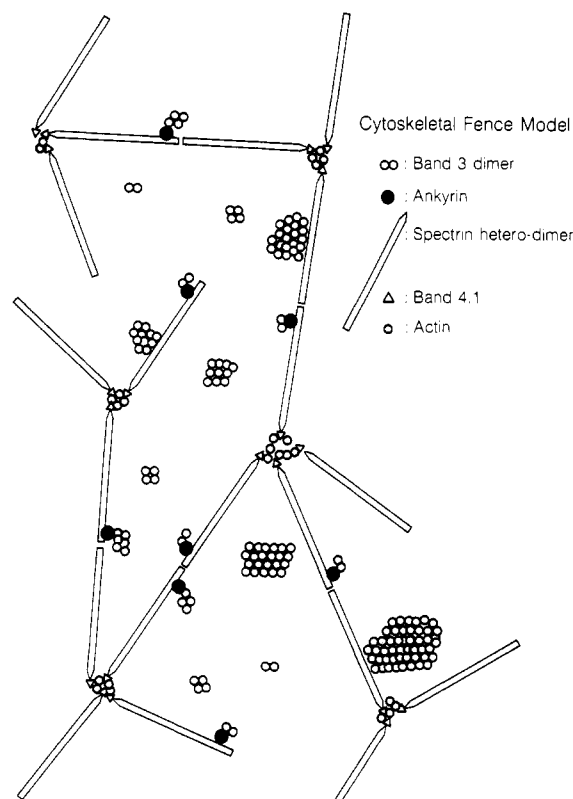
FIGURE 1: Schematic model for restriction of band 3 diffusion in erythrocyte ghost membranes (cytoskeletal fence model).

spectrin polymers located on the cytoplasmic surface of the erythrocyte membrane gives restriction to both rotational and translational diffusion of band 3.

More recently, Tsuji and Ohnishi (1986) have further studied the restriction mechanisms for band 3 lateral diffusion by the cytoskeletal network in terms of the state of polymerization of spectrin and the involvement of ankyrin using the fluorescence photobleaching recovery (FPR)[1] technique. On the basis of their experimental results, they presented a model that incorporates mechanisms for the restriction of translational diffusion of band 3 by the erythrocyte cytoskeletal network (Figure 1). Key features of their model (spectrin dimer/tetramer equilibrium gate model—SPEQ gate model)[2] are the following. (1) Band 3 proteins that are bound to ankyrin are anchored onto the cytoskeletal network via ankyrin and show no translational mobility. (2) Band 3 proteins that are not associated with ankyrin cannot bind to the cytoskeletal network. However, these molecules are trapped in the meshwork formed with spectrin, band 4.1, and actin in the cytoplasmic domain; i.e., band 3 proteins can diffuse freely inside the mesh, but the diffusion beyond the mesh is restricted by the cytoskeletal network. (3) The major diffusion barrier is spectrin tetramers, and the gate opens as tetramers dissociate to dimers. Thus, band 3 diffusion beyond the mesh takes place only through spectrin dimer gates, and the translational diffusion constant as observed by the FPR method, in which large areas compared with the mesh size are observed, is strongly

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[1] Abbreviations: FPR, fluorescence photobleaching recovery; SPEQ gate model, spectrin dimer/tetramer equilibrium gate model.

[2] "The cytoskeletal fence" indicates that lateral diffusion/transport of membrane proteins is restricted by nonspecific barriers formed with a cytoskeletal/peripheral protein network underneath the cellular plasma membranes. The SPEQ gate model provides a more specific and concrete model for the restriction mechanisms of protein motion in the erythrocyte membranes.

dependent on the polymerization state of spectrin. (4) There is equilibrium between spectrin tetramers and dimers. Liu and Palek (1980) and Tsuji and Ohnishi (1986) found that this equilibrium is dependent on concentrations of inorganic salt and some polyamines. When these concentrations were varied, the translational diffusion constant of the mobile fraction changed by a factor of more than 10 without changing the amount of the mobile fraction. Effects of polyamines and ionic concentrations on ghost membranes have also been investigated by Farmer et al. (1985) and Ballas et al. (1983).

One of the major thrusts for the present study is to give a critical examination of the SPEQ gate model by studying the rotational diffusion of band 3 in erythrocyte ghost membranes with various dimer/tetramer ratios of spectrin. Dimer/tetramer equilibrium was altered by varying ionic strength or concentrations of several polyamines as was done by Tsuji and Ohnishi (1986). According to the SPEQ gate model, it is expected that a shift of the dimer/tetramer equilibrium of spectrin, which strongly affects the lateral diffusion of band 3 as measured by the FPR method, should not influence rotational diffusion of band 3 since these molecules are either mobile inside each mesh or immobilized by binding to the cytoskeletal network via ankyrin.

The rotational diffusion of band 3 molecules that are not bound to ankyrin may be determined by the level of band 3 self-association, association with glycophorin, and/or effective viscosity of the lipid environment.

Rotational diffusion of band 3 in erythrocyte ghost membranes was studied by time-resolved phosphorescence anisotropy decay measurements of eosin-labeled band 3 (Austin et al., 1979; Garland & Moore, 1979; Moore et al., 1979; Kawasaki et al., 1988). Our results of intact ghost membranes will be compared with published data acquired by flash-induced time-dependent dichroic decay measurements (Cherry et al., 1976; Nigg & Cherry, 1979, 1980; Nigg et al., 1980; Mühlebach & Cherry, 1985).

MATERIALS AND METHODS

Labeling of Band 3 and Preparation of Ghost Membranes. Human red blood cells were obtained either from the Southeastern Wisconsin Blood Bank (freshly outdated, type B, Rh−) or from one of the authors (K.K., type B, Rh+, used fresh). The erythrocytes were washed 4 times in 5 mM Na_2HPO_4/NaH_2PO_4 and 150 mM NaCl, pH 8.0. Labeling of band 3 in erythrocyte membranes with eosinyl-5-maleimide was carried out according to the method of Nigg et al. (1979) and Nigg and Cherry (1979) with slight modification. Eosinyl-5-maleimide (1 mg; Molecular Probes, Eugene, OR) was dissolved in 2 mL of 5 mM Na_2HPO_4/NaH_2PO_4 and 150 mM NaCl, pH 7.8, and mixed with 5 mL of washed packed erythrocytes. The mixture was incubated for 30 min at room temperature ($\sim$26 °C) in the dark. Unreacted probe was removed by repeating centrifugation and resuspension of the erythrocytes 3 times. The labeled erythrocytes were osmotically lysed by addition of 50 volumes of ice-cold 5 mM Na_2HPO_4/NaH_2PO_4, pH 8.0. The ghost membranes were washed with the same buffer by repeating centrifugation (45000g, 30 min) and resuspension 4 times. The pattern of sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the eosin-labeled ghost membranes showed a strong fluorescence band almost exclusively at the position of band 3. No fluorescence was detected when lipids were extracted by the method of Bligh and Dyer (1959). The number of bound eosins was estimated to be 0.8–1.0 eosinyl-5-maleimide/band 3 by measuring the optical density of labeled ghost membranes at 530 nm and assuming the molar extinction coefficient of

eosin to be 104000 (Matsuura et al., 1988) and 1.2×10^6 band 3 copies present per cell (Fairbanks et al., 1971).

Eosin-labeled ghost membranes were resuspended with 5 mM Na_2HPO_4/NaH_2PO_4 and 10 mM NaCl, pH 7.8, at a total protein concentration of 350 $\mu g/mL$. For studies on the effects of NaCl concentration or polyamines, an eosin-labeled ghost membrane pellet was resuspended with solutions of 10–150 mM NaCl buffered with 5 mM Na_2HPO_4/NaH_2PO_4 at pH 7.8 or 0–0.5 mM spermine, spermidine, or putrescine (Nakarai Chemical Co., Inc., Kyoto, Japan) buffered with 10 mM Tris-HCl and 10 mM NaCl at pH 7.6, and the suspension was incubated for 20 min at 37 °C. Under these conditions, no loss of spectrin and actin from the ghost membranes was detected when ghost membranes and the supernatant after centrifugation of the membranes were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Tsuji & Ohnishi, 1986). The ghost membranes were further washed by centrifugation 3 times with the selected solution and finally resuspended in 1.5 mL of the solution to a total protein concentration of 350 $\mu g/mL$. The concentration of eosin of this sample was about 1 μM.

Time-Resolved Phosphorescence Anisotropy Decay Measurements. An eosin-labeled ghost membrane suspension was placed in a test tube (4-mm i.d.) or a 5 mm $\times$ 5 mm quartz cuvette with a long neck. These sample cells were sealed with a rubber septum, and molecular oxygen in the sample cell was removed by blowing a stream of argon (99.999% pure, Spectra Gas) onto the membrane suspension through a long needle (with a second needle for releasing the pressure inside the cell) for 20 min at room temperature.

The apparatus used for measurement of time-resolved phosphorescence anisotropy decay will be published in detail elsewhere. Briefly, eosin probes attached to band 3 were excited at 518 nm with pulsed laser light (10-ns pulse width) which was vertically or horizontally polarized with a Glan–Thompson polarizer (Photochemical Research Associates, London, Ontario, Canada). Time-dependent phosphorescence emission was detected at a right angle (L-format) with short-cut filters (Fuji Film, Tokyo) and a Glan–Thompson polarizer for vertically polarized light. The laser system (Lambda Physik Model 101 excimer laser and Model 2000 E dye laser) was operated at a 40-Hz repetition rate, and the phosphorescence signal was accumulated for 20 000–50 000 excitation pulses (10–20 min). Phosphorescence light was detected by a single photon counting method using a Hamamatsu R952 photomultiplier tube (Hamamatsu Photonics, K. K., Hamamatsu, Japan) with a type 1716 constant-fraction timing discriminator (Photochemical Research Associates) and a LeCroy 3500 multichannel analyzer with a type 352 multichannel scaling unit. The anisotropy decay, $r(t)$, was calculated as

$$r(t) = \frac{I_{vv}(t) - I_{hv}(t)}{I_{vv}(t) + 2I_{hv}(t)} \quad (1)$$

where $I_{vv}(t)$ [or $I_{hv}(t)$] is the corrected phosphorescence intensity at time t when the excitation polarizer was set at the vertical (or horizontal) orientation and the emission polarizer was set at the vertical orientation. The $r(t)$ curve was fit to two exponential decays and a constant (Nigg & Cherry, 1979)

$$r(t) = r_1 \exp(-t/\tau_1) + r_2 \exp(-t/\tau_2) + r_3 \quad (2)$$

by using an iterative nonlinear least-squares analysis program on a PDP-11/23 computer (see Results). Initial anisotropy, r_0, an anisotropy values at time 0, is defined as

$$r_0 = r(0) = r_1 + r_2 + r_3 \quad (3)$$

Table I: Lifetime (in Microseconds) of Eosinyl-5-maleimide Conjugated to Band 3 in Ghost Membranes

temp (°C)	t_1 (int %)[a]	t_2 (int %)	t_3 (int %)
32	82 (0.5)	625 (8.1)	2607 (91.4)
37	92 (0.7)	647 (9.2)	2525 (90.2)
42	105 (0.9)	557 (7.5)	2361 (91.6)

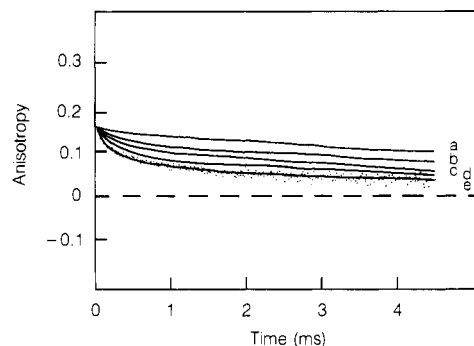[a] Fraction of integrated intensity of each decay component as a percentage.

FIGURE 2: Time-dependent phosphorescence anisotropy decay curve of eosinyl-5-maleimide covalently attached to band 3 in erythrocyte ghost membranes at (a) 10, (b) 20, (c) 30, (d) 37, and (e) 42 °C. Dots are observed values for (e), and lines indicate the fitted curves by a computer.

RESULTS

Analysis of Rotational Diffusion. Triplet lifetimes of eosinyl-5-maleimide bound to band 3 in ghost membranes were obtained by analyzing the total phosphorescence decay curve, $S(t)$, defined as

$$S(t) = I_{vv}(t) + 2I_{hv}(t) \quad (4)$$

$S(t)$ exhibited triple-exponential decays (Table I). These multiple lifetimes may be due to the existence of different binding sites. However, we do not think it is likely because simple solutions of eosinyl-5-maleimide ($\sim$0.01 μM) in an aqueous buffer or in ethanol also showed similar triple-exponential decays. The pattern of sodium dodecyl sulfate–polyacrylamide gel electrophoresis was essentially the same as that of Nigg and Cherry (1979; see Figure 1), which indicated that 75–85% of the eosin fluorescence runs together with the characteristic zone of band 3. Using selective extraction of proteins and lipids, they found that about 85% of the label was associated with band 3. They also found that the anion transport is stoichiometrically inhibited by binding of eosinyl-5-maleimide and is completely inhibited after binding of $\sim$1 eosin/band 3 (Nigg & Cherry, 1979; see Figure 2), which is suggestive of specific labeling. In the present work, 0.8–1 eosin per band 3 was attached. The lifetime data in Table I are in agreement with those by Nigg and Cherry (1979) in that the long-lived component is the major species (over 90% of the total intensity).

When the phosphorescence decay is not monoexponential, analysis of $r(t)$ curves becomes complicated. In our band 3 system, the phosphorescence decay is expressed as

$$S(t) = S_1 \exp(-t/t_1) + S_2 \exp(-t/t_2) + S_3 \exp(-t/t_3) \quad (5)$$

We can safely neglect the component of the fastest decay (t_1) because of its very small contribution to the phosphorescence signal. The anisotropic decay with two triplet lifetimes can be expressed as

$$r(t) = \frac{S_2 \exp(-t/t_2)r_2(t) + S_3 \exp(-t/t_3)r_3(t)}{S_2 \exp(-t/t_2) + S_3 \exp(-t/t_3)} \quad (6)$$

where $r_i(t)$ is the anisotropy decay associated with the com-

ponent of the lifetime of t_i ($i = 1, 2,$ and 3)(Rigler & Ehrenberg, 1973). This equation indicates that the anisotropy decay is dependent on the lifetimes. In our analysis, we neglected the second component ($t_2 \sim 600$ μs) because its contribution is quite small (less than 10%, see Table I). This is probably the best approach we can take at the present moment because using eq 6 to analyze $r(t)$ is unfeasible due to the problem of the signal-to-noise ratio of the anisotropy decay curves. The same approach was taken by Nigg and Cherry (1979) for their analysis of flash-induced time-dependent dichroic decays.

Phosphorescence anisotropy decay curves measured at various temperatures between 9 and 44 °C are shown in Figure 2. This figure indicates a strong temperature dependence of rotational diffusion of band 3 in ghost membranes. These anisotropy decay curves can be analyzed for multiple rotating species of band 3 with various rotational diffusion constants according to Mühlebach and Cherry (1985) and Nigg and Cherry (1979). The decay curves were fit to two exponentials and a constant as expressed in eq 2.

r_3 is important because the immobilized fraction can be estimated by using the equations

$$r_3/r_0 = (1 - f_{im})(r_\infty/r_0) + f_{im} \qquad (7)$$

$$r_\infty/r_0 = (1/4)(3 \cos^2 \theta_N - 1)^2 \qquad (8)$$

where f_{im} is the fraction of immobilized band 3 in the present experimental time scales (5–10 ms) and θ_N is the angle between the emission dipole moment of the eosin probe and the membrane normal (Kawato et al., 1982; Müller et al., 1984; Mühlebach & Cherry, 1985). It should be noted that eq 8 is correct even when the sample contains multiple rotating species of band 3. r_∞/r_0 was estimated to be 0.18 by measuring low-salt-treated ghost membranes that had lost most of the cytoskeletal proteins (Kawasaki et al., 1987). On the basis of this estimation, f_{im} can be evaluated as

$$f_{im} = \frac{r_3/r_0 - 0.18}{0.82} \qquad (9)$$

According to Nigg and Cherry (1979), it is likely that the r_1 and r_2 components reflect the existence of at least two populations of band 3 proteins with different mobilities. Thus, we analyze the decay curves on the basis of a simplified model of three populations of band 3 in terms of rotational mobilities: r_1, fast; r_2, slow; and r_3–r_∞, immobilized or very slow. The fractions for r_1 and r_2 components are given as

$$f_1 = (r_1/r_0)/0.82 \qquad (10)$$

$$f_2 = (r_2/r_0)/0.82 \qquad (11)$$

Figure 3 shows the temperature dependence of anisotropy decay parameters, $r_1, r_2, r_3, \tau_1,$ and τ_2. The difference of τ_1 and τ_2 is a factor between 7 and 20 as seen in Figure 3. It is probable that there are two predominant association states of band 3 fractions which are not anchored by the cytoskeletal network in the ghost membrane. Another possibility is that band 3 dimers that show a rotational correlation time of τ_1 are in dynamic equilibrium with immobilized band 3 (either by anchorage to the cytoskeletal network or by massive aggregation) and that τ_2 appears as a result of rapid exchange of band 3 molecules between free and immobilized fractions in the observation time scale of 5–10 ms.

As is shown in Figure 3, r_1 decreases while r_3 and τ_2 increase with lowering of the temperature. τ_1 is rather constant over 9–44 °C. Fractional ratios of these components at various temperatures are summarized in Table II. At 37 °C, Table II indicates that about 80% of band 3 molecules ($f_1 + f_2$) are
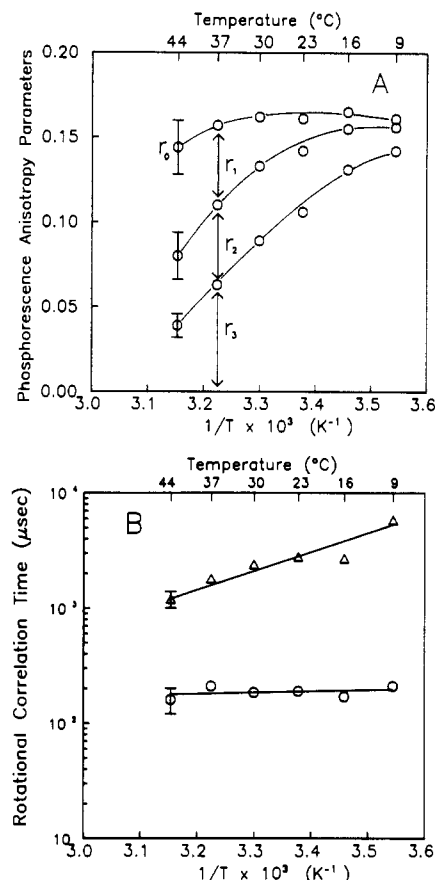

FIGURE 3: Temperature dependence of phosphorescence anisotropy decay parameters. (A) r_0, r_1, r_2, and r_3. (B) τ_1 (O) and τ_2 ($\triangle$).

Table II: Analysis of Time-Resolved Phosphorescence Anisotropy Decays of Eosinyl-5-maleimide Bound to Band 3 in Ghost Membranes

temp (°C)	f_1	f_2	f_{im}	r_0	τ_1 (μs)	τ_2 (μs)
23	0.13	0.27	0.60	0.160	190	2750
26	0.15	0.29	0.56	0.161	185	2550
30	0.19	0.39	0.42	0.162	180	2400
37	0.30	0.52	0.18	0.157	150	1850
44	0.49	0.38	0.13	0.144	170	1200
SD	±0.07	±0.06	±0.06	±0.01	±50	±300

undergoing rotational diffusion in the submilli- and millisecond time scale. More comprehensive analysis of the rotational diffusion of band 3 in ghost membranes will be published elsewhere (Kawasaki et al., 1988).

Effects of NaCl Concentration on Rotational Diffusion of Band 3. Spectrin is a major constituent protein of the cytoskeletal/peripheral protein meshwork in erythrocyte membranes. Spectrin exists in dimeric and tetrameric forms in equilibrium which can be shifted by varying the NaCl concentration (Liu & Palek, 1980; Tsuji & Ohnishi, 1986) or by adding some polyamines (Tsuji & Ohnishi, 1986). A decrease of tetramers and an increase of dimers were observed with a decrease of NaCl concentration from its physiological value (150 mM NaCl) through 10 mM NaCl. The molar ratios of dimers/tetramers are 1/1 and 2/1 at 150 and 10 mM NaCl, respectively (Tsuji & Ohnishi, 1986). Tsuji and Ohnishi (1986) studied the effects of NaCl concentration on the *lateral diffusion* of band 3 in ghost membranes using the FPR method. They found that the diffusion constant of the mobile fraction increased by a factor of 10 (from 5.0×10^{-12} cm^2/s to 5.3×10^{-11} cm^2/s) while the ratio of mobile and immobile components remained the same (40/60) as the NaCl concentration was lowered from 150 to 10 mM. Effects of NaCl

concentration on the *rotational diffusion* of band 3 in ghost membranes have been investigated at 37 °C in this work. The results indicated that neither the rotational correlation time nor the relative population of rapidly diffusing, slowly diffusing, and immobile fractions was affected by changes in NaCl concentrations.

Effects of Some Polyamines on Rotational Diffusion of Band 3. Some polyamines such as spermine, spermidine, and putrescine affect equilibrium between dimers and tetramers of spectrin at 10 mM NaCl(Tsuji & Ohnishi, 1986). These polyamines shift the equilibrium toward tetramers and reduce the formation of dimers at 10 mM NaCl. This effect is larger in the order of spermine > spermidine > putrescine.

Tsuji and Ohnishi (1986) found that the lateral diffusion constant of the mobile fraction of band 3 as measured by FPR decreased by a factor of 5 and more than 10 in the presence of 0.05 and 0.2 mM spermine, respectively. The relative population of mobile and immobile components was not affected. Effects of the presence of spermidine were smaller, and almost no effect was observed by addition of putrescine.

Effects of polyamines on the rotational diffusion of band 3 have been investigated in the present study. At lower concentrations, spermine (0.01 and 0.05 mM) and spermidine (0.1 and 0.2 mM) gave no influences on $r(t)$ curves. At these concentrations, effects of polyamines on the lateral diffusion of band 3 were considerably larger (Tsuji & Ohnishi, 1986). At higher concentrations of spermine (0.2 and 0.5 mM) and spermidine (0.5 mM), an increase of τ_2 by a factor of about 2 was observed. These effects are much smaller than those on lateral diffusion (a factor of 10). Addition of spermine and spermidine induces little changes in the relative population of rapidly diffusing, slowly diffusing, and immobile fractions. Taken together, effects of spermine or spermidine on the rotational diffusion of band 3 are small. Putrescine gave no detectable changes in the $r(t)$ curve.

DISCUSSION

We have measured the rotational diffusion of band 3 in ghost membranes under conditions similar to those employed for lateral diffusion measurements using FPR by Tsuji and Ohnishi (1986). Measurements of both rotational and translational diffusion of band 3 could reveal various molecular processes such as the following: (1) contact interaction of band 3 molecules with the cytoskeletal network (via ankyrin) which would stop FPR translational diffusion of band 3 and greatly decrease rotational diffusion (while oscillative rotational diffusion of band 3 in a limited angle may still be detectable); (2) the role of the cytoskeletal protein meshwork in restricting translational diffusion over the meshwork (Tsuji & Ohnishi, 1986) without influencing the rotational diffusion of band 3 inside the mesh; (3) association or aggregation of band 3 (or band 3 and other membrane proteins such as glycophorin) which would reduce rotational diffusion (D_R; rotational diffusion constant) much more than translational diffusion (D_T; translational diffusion constant), as shown by Saffman and Delbrück (1975):

$$D_R = \frac{kT}{4\pi\mu h a^2} \qquad (12)$$

$$D_T = \frac{kT}{4\pi\mu h}\left(\log\frac{\mu h}{\mu' a} - \gamma\right) \qquad (13)$$

where protein diffusion is described by using a model of Brownian motion of a cylindrical particle (radius = a, height = h) in a continuous sheetlike medium of viscosity μ and
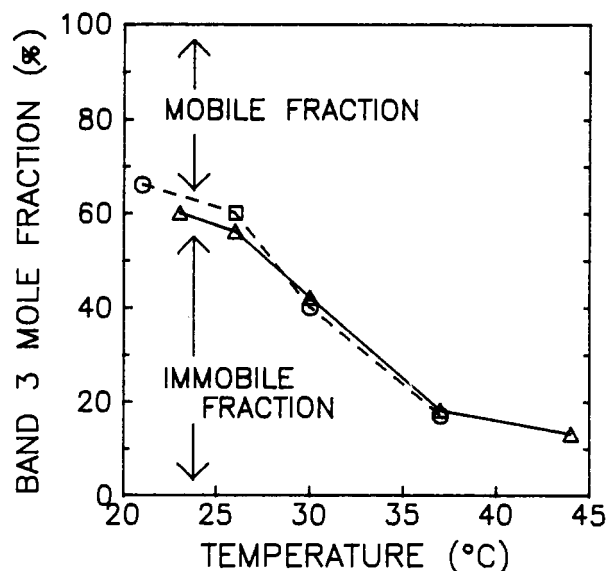

FIGURE 4: Immobile fraction (f_{im} in percent) and mobile fractions ($f_1 + f_2$) for rotational (△) and translational (○, □) diffusion are plotted as a function of temperature. (○) Taken from Golan and Veatch (1980). (□) Taken from Tsuji and Ohnishi (1986).

thickness h surrounded by aqueous phases of viscosity μ'. T is absolute temperature, and k and γ are the Boltzmann constant and Euler constant (=0.5772), respectively.

Rotational diffusion measurements in the present work indicated the presence of at least two mobile components whose effective rotational correlation times are 0.19 and 2.4 ms at 26 °C. Assuming that 0.19 and 2.4 ms are the characterstic times ($1/D_R$) of rotational diffusion of f_1 and f_2 components, respectively, with no restriction by the cytoskeletal network, translational diffusion constants are calculated to be on the order of between 10^{-7} and 10^{-9} cm^2/s by using eq 12 and 13 ($30 < a < 300$ Å). The translational diffusion constant of band 3 molecules in ghost membranes obtained by Tsuji and Ohnishi (1986) is 5.3×10^{-11} cm^2/s at 26 °C for the mobile component (40% of total band 3). This value is substantially smaller than those expected from rotational diffusion constants, and this difference is indicative of the presence of control mechanisms that substantially reduce the long-range translational diffusion rate of the mobile population of band 3 without affecting rotational diffusion, i.e., a cytoskeletal protein meshwork that imposes a nonspecific barrier effect on translational diffusion (process 2 above), which we call a cytoskeletal fence model. In fact, trypsin-cleaved band 3, which has lost a 40 000-dalton cytoplasmic portion of band 3 for nonspecific interaction (collision) with the cytoskeletal/peripheral protein meshwork, showed a D_T of 4.1×10^{-10} cm^2/s at 25 °C, comparable to the D_T expected from the rotational diffusion constant of the mobile population of band 3 molecules (Tsuji & Ohnishi, 1986).

The results of rotational and FPR lateral diffusion measurements[3] of band 3 obtained in this work and by Tsuji and Ohnishi (1986) and Golan and Veatch (1980) are compared in Figure 4. The fraction of the rotationally immobile component matches well with the fraction of the immobile component in the FPR experiment at temperatures between 23

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[3] Lateral diffusion data based on FPR measurements are sensitive to both thermal Brownian diffusion and cytoskeletal meshwork restriction. In order to discuss Brownian diffusion at the molecular level separately from the long-range macroscopic diffusion at the optical microscopic level as observed by the FPR method, we call the former molecular lateral diffusion and the latter FPR lateral diffusion.

and 37 °C. These data imply that the translationally immobile component is also rotationally immobile, indicating that the immobile band 3 population is due to binding to the cytoskeletal meshwork and/or formation of massive clusters. Since D_T is rather insensitive to clustering, the match in numbers between translationally and rotationally immobile fractions suggests that immobilization of band 3 is caused by binding to the cytoskeletal proteins.

Band 3 molecules are bound to the cytoskeletal meshwork via ankyrin (Nigg & Cherry, 1980) or via glycophorin and band 4.1 (Pinto da Silva & Nicolson, 1974; Tyler et al., 1980; Anderson & Lovrien, 1984). While the molar ratio of ankyrin to band 3 monomer is in the range of 10–15% (Bennett & Stenbuck, 1979), many band 3 molecules could indirectly bind to an ankyrin molecule by self-association of band 3. Ankyrin can bind 20–30% band 3 as dimers, 40–60% as tetramers, and 60–90% as hexamers. In addition, since larger band 3 oligomers possess more binding sites for ankyrin, the overall affinity of larger oligomers must be larger than that of monomers and dimers. If the dissociation constant of band 3 dimer for ankyrin is K, that of hexamer may be $K/3$. Furthermore, if the band 3 hexamer can bind to two ankyrin molecules at the same time, the dissociation constant is smaller than K^2. Thus, larger oligomers and aggregates tend to bind to ankyrin, and an increase in the extent of self-association of band 3 may lead to more binding to ankyrin. This avidity effect due to the multiple binding sites for ankyrin induced by self-association of band 3, coupled with a good agreement of immobile fractions in rotational and translational diffusion measurements, suggests that immobilization of band 3 is largely caused by binding of band 3 oligomers and aggregates to ankyrin which, in turn, is bound to the cytoskeletal network of spectrin. Large temperature dependence of the immobilized fraction of band 3 may be due to dependence of both the band 3/band 3 interaction and the band 3/ankyrin interaction on temperature.

Tsuji and Ohnishi (1986) presented a SPEQ gate model in which opening of the gate in the network is regulated by a dimer (open)/tetramer (closed) equilibrium of spectrin on the basis of their observation of the relationships between the dimer/tetramer equilibrium of spectrin and FPR translational diffusion of band 3. In the present paper, we have examined if the shift of spectrin dimer/tetramer equilibrium changes the rotational diffusion of band 3. If the SPEQ gate model well describes restriction mechanisms by the cytoskeletal/peripheral protein networks on band 3 diffusion in ghost membranes, the rotational diffusion rate of the mobile component and the fraction of rotationally mobile components should be little influenced by changes in the spectrin dimer/tetramer ratio. When we shifted the spectrin dimer/tetramer equilibrium by varying the NaCl concentration, rotational diffusion of band 3 was not affected. When the shift of equilibrium was made by incubation with some polyamines, only a slight influence was observed at higher concentrations of polyamines, and the extent of the effect on rotational diffusion was much less than that on FPR translational diffusion. Thus, we have shown that the SPEQ gate model can explain diffusive behaviors of band 3 in ghost membranes. We have also demonstrated that much information on membrane protein interaction is gained by observing both rotational and translational diffusion of membrane proteins.

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