Rotational Diffusion of Band 3 in Erythrocyte Membranes. 1. Comparison of Ghosts and Intact Cells

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ABSTRACT: The rotational diffusion of eosin-labeled band 3 in human erythrocyte cells and hemoglobin-free ghosts at 37 °C has been studied in detail by polarized delayed luminescence. The time-resolved anisotropy with both cells and freshly prepared ghosts is similar, decaying with well-resolved rotational correlation times of 0.03, 0.2, and ≥1 ms. Mild proteolytic removal of the water-soluble 41-kDa cytoplasmic domain of band 3 in ghosts results in a drastic increase in the fractional contributions of the two fastest depolarizing components. Our results, taken together with other data in the literature, imply that several classes of band 3 that differ greatly in mobility exist in ghosts and intact cells. The mobility of one class is hindered due to complexation with other membrane or cytoplasmic proteins mediated via the 41-kDa cytoplasmic domain. However, another class of band 3 molecules exists as homo- or heterooligomeric complexes larger than a dimer that are stabilized by hydrophobic interactions involving the intramembranal domain. Finally, the presence of the (previously undetected) 0.03-ms anisotropy component strongly suggests that a significant fraction of band 3 in both ghosts and intact cells is highly mobile and diffuses at the rate expected for a freely rotating dimer in the erythrocyte membrane.

Band 3 (Fairbanks et al., 1971) is the major transmembrane protein of the human erythrocyte membrane and is thought to catalyze the rapid exchange of anions (Cabantchik & Rothstein, 1974a; Passow et al., 1975; Ho & Guidotti, 1975). Much progress has been made in relating the structural features of band 3 to its transport function through the use of covalent and noncovalent inhibitors and in situ proteolytic modification [see, e.g., Cabantchik et al. (1978), Knauf (1979), Passow (1986), and Jennings (1989)]. However, it has become evident that anion transport may be only one of several roles that band 3 serves in the erythrocyte. Band 3 is a glycoprotein with a monomer molecular weight of 95K, and its 41-kDa N-terminal domain resides entirely in the cytoplasm (Steck, 1978). While its membrane-associated segment of MW ~ 55K alone suffices for carrying out anion transport (Grinstein et al., 1978; Lepke & Passow, 1976), the cytoplasmic domain appears to function independently as a binding locus for a diverse number of membrane and cytoplasmic proteins. The former group includes bands 2.1 (also known as ankyrin) (Bennett & Stenbuck, 1979) and 4.1 (Pasternack et al., 1985), both of which may modulate the interaction of band 3 with the cytoskeleton, and band 4.2 (Korsgren & Cohen, 1988), the function of which is unknown. Cytoplasmic proteins which bind to the 41-kDa fragment [for reviews, see Low (1986) and Waseem and Steck (1989)] can be eluted by physiological saline solutions and include glyceraldehyde-3-phosphate dehydrogenase, aldolase, phosphofructokinase, and hemoglobin. Band 3 has also been proposed to mediate urea and water transport, but further direct proof for these roles is required (Solomon et al., 1983; Dix et al., 1985; Benga et al., 1986).

The oligomeric state of self-association of band 3 in the red cell membrane (Jennings, 1984; Schubert, 1988) is at present an incompletely resolved issue. Cross-linking experiments imply that band 3 is either dimeric (Steck, 1972; Kiehm & Ji, 1977; Staros & Kakkad, 1983) or tetrameric (Wang & Richards, 1974; Mikkelsen & Wallach, 1976). Analytical ultracentrifugation of band 3 at dilute concentrations in nonionic detergents also indicates either a dimer (Yu & Steck, 1975; Clarke, 1975; Reitheimer, 1979) or a monomer–dimer–tetramer equilibrium (Nakashima & Makino, 1980; Pappert & Schubert, 1983; Schubert et al., 1983). On the other hand, Cuppoletti et al. (1985) concluded from target size analysis of radiation-induced inactivation of anion transport in intact cells, or degradation of band 3 in stripped ghosts, that band 3 exists as a dimer or possibly in groups of dimers.

One approach to studying the physical state of membrane proteins in native or reconstituted systems is the measurement of their rotational diffusion. This parameter has a strong dependence on size, shape, flexibility, and environmental constraint, and therefore can be used together with independent biochemical information to provide insight into the structural dynamics and association state of the labeled protein. The slow microsecond-to-millisecond rotational diffusion of membrane proteins can be studied at present either by time-resolved triplet-state anisotropy decay, including both absorption and emission techniques (Cherry, 1979; Kinosita & Ikegami, 1980; Kawato & Kinosita, 1981; Jovin et al, 1981; Jovin & Vaz, 1989), or by the time-averaged technique of ST-EPR (saturation-transfer electron paramagnetic resonance) (Thomas, 1978, 1986). Cherry and co-workers, using flash-induced transient dichroism, have carried out an extensive series of studies on band 3 in human erythrocyte ghosts (Cherry et al., 1976; Nigg & Cherry, 1979, 1980; Muehlbach & Cherry, 1982; Clague et al., 1989) and reconstituted in lipid vesicles (Muehlbach & Cherry, 1985). Johnson and Garland (1981) have performed measurements on single ghosts using the technique of fluorescence depletion anisotropy. Time-resolved phosphorescence measurements on band 3 have been previously reported by our laboratory (Austin et al., 1979; Matayoshi et al., 1983), and more recently by Tsuji et al. (1988). Studies on band 3 using ST-EPR were carried out on reconstituted systems by Sakaki et al. (1982) and Bittman et al. (1984), and

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on intact red cells by Beth et al. (1986).

Although the lateral diffusion of band 3 has been studied in both ghosts and intact cells by the method of fluorescence recovery after photobleaching (Sheetz et al., 1980; Golan & Veatch, 1980; Koppel et al., 1981), measurements of its rotational diffusion in intact cells have thus far been at low resolution and limited to our preliminary report (Matayoshi et al., 1983) and the ST-EPR study by Beth et al. (1986). In this paper, we present a comparison of the rotational diffusion of eosin-maleimide-labeled band 3 in ghosts and intact cells. Technical improvements have permitted a much more detailed analysis than has hitherto been possible. In the following paper (Matayoshi et al., 1991), we describe the effect on the rotational diffusion of band 3 occasioned by the binding of cytoplasmic enzymes. These results are of considerable interest in light of the studies with intact cells.

Materials and Methods

Materials. Eosin-5-maleimide was from Molecular Probes (Eugene, OR). Glucose oxidase, catalase, trypsin (DPPC-treated), and soybean trypsin inhibitor were from Sigma.

Labeling of Band 3. All experiments were performed with human blood (O+) drawn within an hour before use. Erythrocytes were washed in phosphate-buffered saline (PBS)[1] and labeled with eosin-5-maleimide essentially as described by Nigg and Cherry (1979). Labeling was also in PBS, and unreacted label was removed by repeated washes of cells until eosin was undetectable in the supernatant. Ghosts were prepared by hemolysis of cells in 20–40 volumes of 10 mM sodium phosphate buffer, pH 7.4, followed by repeated washes in the same buffer. The labeled ghosts were stored in the dark at 0 °C under argon. Protein concentrations were determined after solubilizing membranes in 1% SDS by the method of Lowry et al. (1951). The concentration of eosin in ghosts preparations was determined by the absorbance of solubilized membranes at 527 nm. SDS–polyacrylamide gel electrophoresis was according to Fairbanks et al. (1971).

Sample Treatments. Proteolytic cleavage of band 3 (Steck et al., 1976) was accomplished by incubating ghosts [$\sim$3 mg (mL of membrane protein)$^{-1}$] on ice for 1 h with trypsin (0.1–1.0 μg mL^{-1}, in 10 mM sodium phosphate, pH 7.4). The reaction was terminated by the addition of an excess of soybean trypsin inhibitor. Ghosts were washed 3 times in 10 mM sodium phosphate and used within a few hours. Gel electrophoresis analysis established the effectiveness of these procedures.

Immediately prior to measurements, samples were deoxygenated by one of three methods. In the first two methods, the sample was placed in the cuvette under a stream of water-saturated argon while being mixed gently either (1) with a Teflon rod on which a small disk at the end is forced up and down through the liquid surface (Austin et al., 1979; Jovin & Vaz, 1989) or (2) with a motor-driven glass rotor placed at the sample surface. In the third method, glucose oxidase (10 μg mL^{-1}), with or without catalase (10 μg mL^{-1}), and 10 mM glucose were included (Horie & Vanderkooi, 1981). All of these methods yielded identical results with ghosts, although the third method was particularly reproducible in terms of the triplet lifetimes. Intact cells, however, proved to be extremely sensitive to any kind of mechanical mixing in which the liquid surface is broken. This point is particularly important because, as discussed below, the mobility of band 3 is acutely sensitive to the consequences of hemolysis. Thus, only the glucose oxidase method was used with cell samples. The problem of cells settling during long measurements was overcome by interrupting the acquisition every few minutes and gently dispersing cells with a thin glass rod with a small disk formed at the end. The latter remained submerged in the cell suspension throughout the measurements. All measurements with ghosts were performed in 10 mM sodium phosphate, pH 7.4, at 37 °C.

Instrumentation. The instrumentation used for making polarized delayed luminescence measurements was constructed in this laboratory and is described in previous publications (Austin et al., 1979; Matayoshi et al., 1983; Jovin & Vaz, 1989). An excimer laser (Model EMG 50, Lambda Physik, Göttingen) is used as the pumping source for a modified dye laser (Model FL 2000, Lambda Physik). The excitation pulses (1–5 mJ) are of $\sim$10-ns duration with a repetition rate of 10–20 Hz. One record (file) consists of $\sim 10^3$ averaged decay events for the two polarized emission components, measured in $\sim$20 cycles of alteration. The excitation wavelengths of 515 and 385 nm are obtained with the laser dyes Coumarin 307 and Polyphenyl 1 (Lambda Physik, Göttingen, FRG), respectively. Eosin phosphorescence (peak near 700 nm) is isolated with Schott (Mainz, FRG) cutoff filters KV550 and RG645 in combination with a Balzers (Balzers, Liechtenstein) 700-nm broad band-pass interference filter, while eosin-delayed fluorescence (peak near 560 nm) is selected by the combined use of the Schott KV550 and a Balzers 550-nm broad band-pass filters. In the latter measurement, spectral isolation from the intense prompt fluorescence is not possible, and gating of the photomultiplier tube only partially alleviates the problem. [An improved gating circuit not used in this work is described in Yoshida et al. (1989).] As a result, photomultiplier recovery is complete only after about 100 μs, in comparison to about 6 μs when measuring eosin phosphorescence. The amplified signal from the photomultiplier tube is digitized by an 8-bit transient recorder (Biomation 8100) with a maximal time resolution of 0.01 μs per channel. Since the input analog signal to the transient recorder is adjusted to a bandwidth at least 5-fold greater than (channel time)$^{-1}$, the latter parameter defines the effective signal bandwidth for each data record.

Analysis of Data. The two polarized emission components, $I_\parallel(t)$ and $I_\perp(t)$, are used to calculate the three time-dependent functions $s(t)$, $d(t)$, and $r(t)$ which are the total emission, difference function, and anisotropy, respectively. For a system of one diffusing component exhibiting a single lifetime, these functions have the form:

$$s(t) = I_\parallel(t) + I_\perp(t) = \sum_k S_{0j} \exp(-t/\tau_j) \quad (1)$$

$$d(t) = I_\parallel(t) - I_\perp(t) \quad (2)$$

$$r(t) \equiv d(t)/s(t) = \sum_i r_{0i} \exp(-t/\phi_i) \quad (3)$$

where k is the number of chemical species with triplet lifetime τ_j and the index i can have values of 1–6 (Jovin et al., 1981; Lipari & Szabo, 1980; Jovin & Vaz, 1989). Anisotropy $r(t)$ data for membrane proteins labeled with triplet dyes are generally analyzed on the basis of a model cylinder or ellipsoid undergoing uniaxial rotation and/or wobbling. The data are fit according to the function:

$$r(t) = \sum_i r_{0i} \exp(-t/\phi_i) + r_\infty \quad (4)$$

where i is limited in practice to two or three (exponential) components. For the case of a single diffusing component, the

[1] Abbreviations: PBS, phosphate-buffered saline (10 mM Na_2HPO_4–NaH_2PO_4, and 150 mM NaCl, pH 7.4); SDS, sodium dodecyl sulfate; DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulfonate; G3PD, glyceraldehyde-3-phosphate dehydrogenase; $r(0)$, initial anisotropy on the microsecond time scale, i.e., long after subnanosecond and nanosecond motions have occurred.

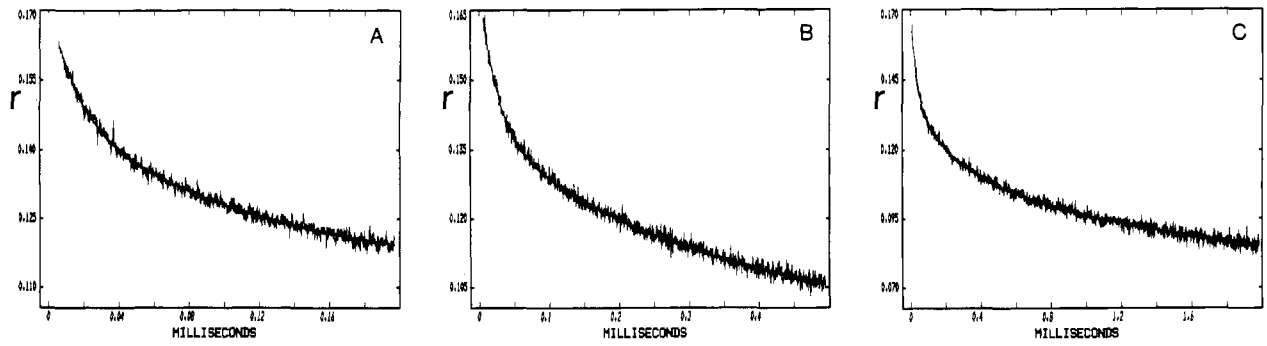

FIGURE 1: Early time behavior of anisotropy decay in ghosts. (A) 0.2 μs per channel; (B) 0.5 μs per channel, (C) 2 μs per channel. The fitted curve overlays the data curve in each case. See Materials and Methods for details of experimental procedures and data analysis.

r_{0i} values are a function of the geometry of the absorption and emission transition moments relative to the rotation axis. The rotational correlation time ϕ_i are related to the rates of depolarization due to rotation and wobbling, and r_∞ is the limiting anisotropy, a measure of the constrained orientational distribution after rotational relaxation. If multiple components with different mobility are present, additional contributions to the amplitudes and decay times in eq 4 must be considered. The different rates of mobility will clearly manifest themselves in the apparent heterogeneity of ϕ_i values, and the relative amounts of each species will be reflected in the amplitudes r_{0i}. Finally, the presence of immobile components will contribute to the limiting anisotropy r_∞.

Strictly speaking, the values of the ϕ_i are determined exclusively by depolarizing motions provided that $s(t)$ is a single exponential. Otherwise, the mathematical form of $r(t)$ is in fact nonexponential, and the apparent ϕ_i values obtained from a multiexponential analysis of $r(t)$ are also a function of the τ_j. In our experience, single-exponential $s(t)$ decays are rarely observed for triplet probes, even in cases where the assumption of chemical homogeneity is reasonable. This is presumably due to the extreme sensitivity to microenvironment and dye conformation of the various photophysical rate constants describing the buildup and decay of the long-lived triplet state. However, if $s(t)$ remains constant, any changes in $r(t)$ observed following a perturbation to the system can be ascribed with certainty to dynamic changes. For example, we can consider a system containing n lifetime components (index j), each of which undergoes the same types of physical motion. This situation is relevant to the case where the dye is attached to a single membrane protein but bound in n (covalent or noncovalent) forms. If a single correlation time ϕ_i suffices to describe the ith depolarizing motion, the observed $r(t)$ is

$$r(t) = [1/s(t)]\sum_{j=1}^{n}\sum_{i=1}^{m} s_{0j} r_{0i} \exp(-t/\phi_{ji}) \qquad (5)$$

where

$$1/\phi_{ji} = 1/\tau_j + 1/\phi_i \qquad \text{and} \qquad s(t) = \sum_{j=1}^{n} s_{0j} \exp(-t/\tau_j)$$

In the case of eosin-labeled band 3, $s(t)$ is heavily dominated (~80%) by a long 2.5-ms component. Thus, $s(t)$ hardly decays during the first few hundred microseconds, and $r(t)$ in eq 5 is approximately exponential at early times. Furthermore, the presence of a small ϕ_{ji} is likely to be due mainly to a small ϕ_i and not "artifactually" to a small τ_j if the relative amplitudes of the latter (i.e., the respective s_{0j}) are low. We have discussed this example at length in order to provide the reader with better insight into the meaning of the $r(t)$ function.

As can be inferred from eq 1–3, $r(t)$ records are relatively noisy in comparison to $s(t)$ records because of the manner in which the experimental error in the observables ($\sum I_\parallel$ and $\sum I_\perp$) is propagated. Nonlinear least-squares weighted or unweighted fitting of $s(t)$, $d(t)$, and $r(t)$ to a sum of exponentials was performed by a computer program written by one of us (E.D.M.) which used the Marquardt and modified Gauss–Newton methods. The goodness of fit for any single record is determined by inspection of residuals plots, expanded fit/data overlays, and, if necessary, a plot of the autocorrelation function of the residuals. The residuals plots also reveal whether or not a weighted fit is necessary.

Our data records consist of 1024 digitized points, and data simulation showed that this limits the number of exponentials which can be analyzed with confidence to a maximum of 3 (dependent on the signal/noise ratio, the number of channels represented by 1 decay time, the closeness of the decay times, and the relative amplitudes). Various tests such as the χ^2 statistic are commonly used as an objective test for goodness of fit. These diagnostics require accurate knowledge of the variance of each point in the data. However, under our data acquisition conditions, we have found that the most effective check for testing the validity of an apparent decay time is its quantitative confirmation between at least two records measured at different times per channel. In each record, the exponential component in question is thereby represented by a different number of channels and thus, in the least-squares minimization, effectively is weighted differently relative to other components. The results of exponential analyses given in this paper represent a global analysis of several 1024 channel records taken at different times per channel. In the band 3 experiments, measurements were generally taken at 0.1, 0.2, 0.5, 2, 5, and 10 μs per channel, permitting the unambiguous and quantitative detection of both fast (5–50 μs) and slow (>ca. 5 ms) components. This procedure also overcame the above-mentioned three-component limitation faced for the analysis of data taken at only a single time per channel. Disagreement between records of different resolution is generally taken to imply a merging of two or more highly correlated exponential components. Other tests which we routinely performed to check against fitting artifacts included the analysis of a given record with variable numbers of data channels cut off at the beginning or end.

RESULTS

Anisotropy Decay of Eosin-Labeled Band 3 in Ghosts. Figures 1 and 2 show typical data records of the anisotropy decay [$r(t)$] over a wide range of time windows and signal bandwidth. The results of an exponential analysis of such data can be found in Table I. All high-resolution records (0.1–2 μs per channel) demonstrate conclusively the existence of a fast depolarizing component with correlation time ϕ_1 of 20–30 μs. A second component with nearly an order of magnitude

Table I: Phosphorescence Anisotropy Decay of Eosin-Labeled Band 3 Components

	fresh ghosts		trypsinized band 3 in ghosts		intact cells		intact cells + hemolysate	
component, i	ϕ_i (μs)	r_{0i}	ϕ_i (μs)	r_{0i}	ϕ_i (μs)	r_{0i}	ϕ_i (μs)	r_{0i}
1	26	0.030	30	0.052	28	0.035		
2	203	0.024	150	0.055	334	0.028	495	0.056
3	995	0.033	900	0.020				
4	11000[a]	0.047[a]	3400	0.023	3300	0.053	3100	0.059
	r_∞ = 0.041[a]		r_∞ = 0.028		r_∞ = 0.063		r_∞ = 0.062	

[a] In the case of untreated ghosts, r_{04}, ϕ_4, and r_∞ are poorly determined because $r(t)$ never reaches a well-defined plateau within the 10-ms window. Fixing either r_∞ or ϕ_4 to an assumed value allows the other two parameters to be determined. The $r(0)$ values of different samples are best compared by direct analysis of the the fastest data records (0.1 or 0.2 μs per channel). From such determinations, we find that $r(0)$ at 37 °C for ghosts, trypsinized ghosts, and intact cells lies in the range 0.163–0.169. Variations in ϕ_1 using different ghost preparations did not exceed 20%. Reproducibility between files in a given data set was much better.

Table II: Triplet Decay [$s(t)$] Parameters[a]

	fractional amplitudes			lifetimes (μs)		
	f_1	f_2	f_3	τ_1	τ_2	τ_3
ghosts, control	0.11	0.14	0.75	41	392	2400
ghosts, excitation at 385 nm	0.25	0.13	0.62	40	391	2370
ghosts, trypsinized	0.11	0.10	0.79	39	377	2390
intact cells (no additions)	0.11	0.34	0.55	38	406	2260
+hemolysate	0.34	0.12	0.54	37	277	2400
+hemoglobin, 0.4 mg mL^{-1}	0.30	0.15	0.55	32	276	2240
+hemoglobin, 2.0 mg mL^{-1}	0.36	0.12	0.52	24	298	2290

[a] All data obtained with 515-nm excitation, with the one indicated exception.

longer correlation time ϕ_2 is best revealed by the 2 and 5 μs per channel records. Both ϕ_1 and ϕ_2 have substantial amplitudes, r_{01} = 0.030 and r_{02} = 0.024. With ghost preparations of increasing age, ϕ_1, ϕ_2, and r_{01} appear to be preserved, but r_{02} decreases.

The "slow" records of 5 and 10 μs per channel provide good evidence that in fresh ghosts, components with ϕ_3 = 1 ms and ϕ_4 greater than about 5 ms are present. The amplitude r_{03} = 0.033, while r_{04} is difficult to quantitate because it depends on the values fit or assumed for ϕ_4 and r_∞. The difficulty in obtaining the latter parameters arises because the decay never truly achieves a plateau in the 10-ms window. With increasing age of the ghost preparation, these slow components increase in amplitude relative to the initial anisotropy $r(0)$. While the above analysis has been carried out in terms of discrete components, it is also possible that a distribution of slow components (>1-ms correlation time) is more appropriate in the millisecond diffusion time range; a triplet probe which is longer lived than eosin would be necessary to examine this issue. (Regardless of which form of analysis is used, the conclusions reached below would not be altered.)

It should be emphasized that the extraction of multiple components in noisy anisotropy curves was not the result of fitting to a single record, a feat which is often highly questionable. The problems inherent in multiexponential fitting have been noted by others [see, e.g., Grinvald and Steinberg (1974) and Cherry and Godfrey (1981)]. As discussed under Materials and Methods, our results are based on the analysis of multiple records taken over a wide range of signal resolution, permitting optimal detection and verification of each component. Except for the immobile and very slow (>1 ms) components for fresh ghosts, the components listed in Table I are well separated in time and hence have a low degree of correlation, further facilitating their quantitation.

In contrast to $r(t)$, the $s(t)$ data have an extremely high signal-to-noise ratio. We find that the $s(t)$ data unambiguously require a minimum of three components, with lifetimes of 40, 400, and ~2500 μs and fractional amplitudes of 0.11, 0.34, and 0.55 (Table II). The implications of this result for the interpretation of $r(t)$ data are discussed under Discussion.

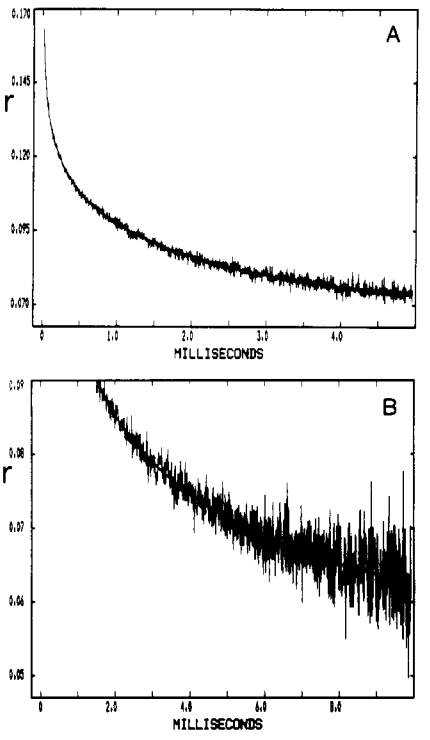

FIGURE 2: Longtime behavior of anisotropy decay in ghosts. (A) 5 μs per channel; (B) 10 μs per channel. Data and fit are overlayed.

Effect of Trypsinization of Band 3. As demonstrated by Steck and co-workers (Steck et al., 1976), band 3 in hemoglobin-free ghosts can be selectively cleaved at its cytoplasmic domain by mild trypsinization. Under the experimental conditions corresponding to the data shown in Figure 3, we find that more than 90–95% of the band 3 has its 41-kDa cytoplasmic polypeptide removed.

Trypsinization leads to a dramatic effect on $r(t)$ of band 3 (Figure 3A). The results from an analysis of multiple time-resolution records are listed in Table I. Although the fastest component ϕ_1 remains at ~30 μs, its amplitude increases to r_{01} = 0.052. The next fastest component ϕ_2 = 150

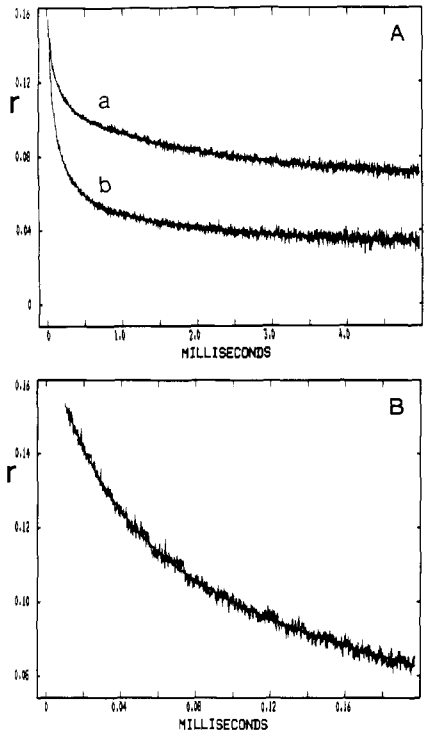

FIGURE 3: Effect on anisotropy decay in ghosts of cleavage of the 41-kDa cytoplasmic domain of band 3. (A) Untreated ghosts (top curve, a); trypsinized ghosts (bottom curve, b); 5 μs per channel. (B) Trypsinized ghosts; 0.2 μs per channel. Data and fit in each case are overlayed.

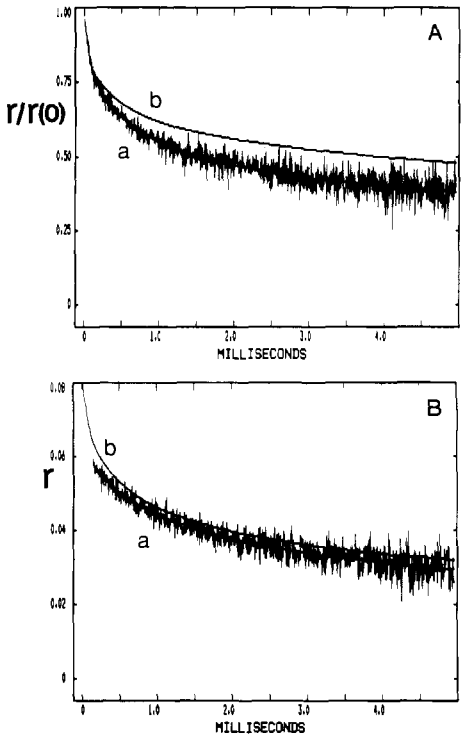

FIGURE 4: Excitation and emission wavelength dependence of $r(t)$. (A) Normalized anisotropy decay, $r(t)/r(0)$, of phosphorescence resulting from excitation at 385 nm (data and fit, a) and excitation at 515 nm (fit only, b). (B) Anisotropy decay (absolute values) for delayed fluoresence (fit and data, a) versus phosphorescence (fit only, b). Excitation in both cases was at 385 nm. See Materials and Methods for technical details of the measurements.

μs is slightly shorter than ϕ_2 of untreated band 3, and its amplitude is approximately doubled. The slower components and r_∞ have greatly reduced amplitudes; they are not attributable to small membrane fragments that should neither arise under the very mild conditions of proteolysis nor be pelleted during sample workup. The trypsinized preparation no longer seems to have the extremely slow (greater than ~5 ms) component which, as discussed above, makes quantitation of ϕ_4, r_{04}, and r_∞ in controls imprecise.

Trypsinization has no perceptible effect on the $s(t)$ decay. We also observe that the total initial anisotropy $r(0)$ is identical within experimental error with that for untreated ghosts. These results are considered further under Discussion.

Excitation and Emission Wavelength Dependence of $r(t)$ and $s(t)$. Some decay measurements were also undertaken using excitation at 385 nm and/or emission at 550 nm (Figure 4A,B). The emission at 550 nm corresponds to the delayed fluoresence of eosin. The following qualitative and semiquantitative conclusions may be drawn from the limited series of measurements.

(a) The phosphoresence initial anistropy $r(0)$ with excitation at 385 nm is about half that obtained with excitation at 515 nm. For a population of emitters with identical absorption properties (but see Discussion), this corresponds to an effective angular difference at 35° between the absorption transition moments at the two wavelengths [see also Corin et al. (1987)].

(b) The initial phase of the $r(t)$ decay with 385-nm excitation contains the same two correlation times found with 515-nm excitation; they differ only in amplitudes, the ratio (r_{01}/r_{02}) being approximately 25% smaller in the case of 385-nm excitation. Qualitatively, no striking differences in the decay kinetics beyond 1 ms are present.

(c) For times beyond ~150 μs, the anisotropy decay of the delayed fluorescence (Figure 4B) is qualitatively similar to that of the phosphorescence. The absolute $r(t)$ values differ by only a few percent, indicating there is only a minor angular displacement between the phosphorescence and fluorescence transition moment directions. This is a useful result because it implies that no significant differences in the form of the $r(t)$ decay for eosin-labeled band 3 are to be expected between triplet emission and absorption techniques, assuming near-parallelism of the 515-nm absorption and 550-nm fluoresence transition moments. This assumption is supported by an observed steady-state prompt fluorescence anisotropy value of ~0.3 at room temperature (data not shown), as well as by previous studies with other eosin derivatives (Corin et al., 1987).

(d) The $s(t)$ decay with 385-nm excitation contains the same three lifetime components obtained from excitation at 515 nm, but the relative amplitudes differ (see Table II). The implications of this observation are discussed in the final section.

Comparison of Present Results with Previous Work on Ghosts. Previously published work on the rotational diffusion of band 3 in ghosts comes from four laboratories (Nigg & Cherry, 1979, 1981; Johnson & Garland, 1981; Austin et al., 1979; Matayoshi et al., 1983; Tsuji et al., 1988). In all of these studies, the 28-μs component was not detected due to insufficient signal bandwidth and/or limited early time resolution following the exciting pulse. In the work cited, analyses were generally based on single records which had been digitized at no less than 10 to 20 μs per channel, with a maximum of 256–512 channels. At such resolution, the fastest components of the $r(t)$ and $s(t)$ decay would be represented by only a few channels of data.

In the experiments presented in this paper, the first valid data point in the fast (0.1 and 0.2 μs per channel) records occurs at 6 μs after the laser pulse. This represents a technical limitation of the photomultiplier gating in our instrument when subjected to the intense prompt fluoresence of eosin, and means

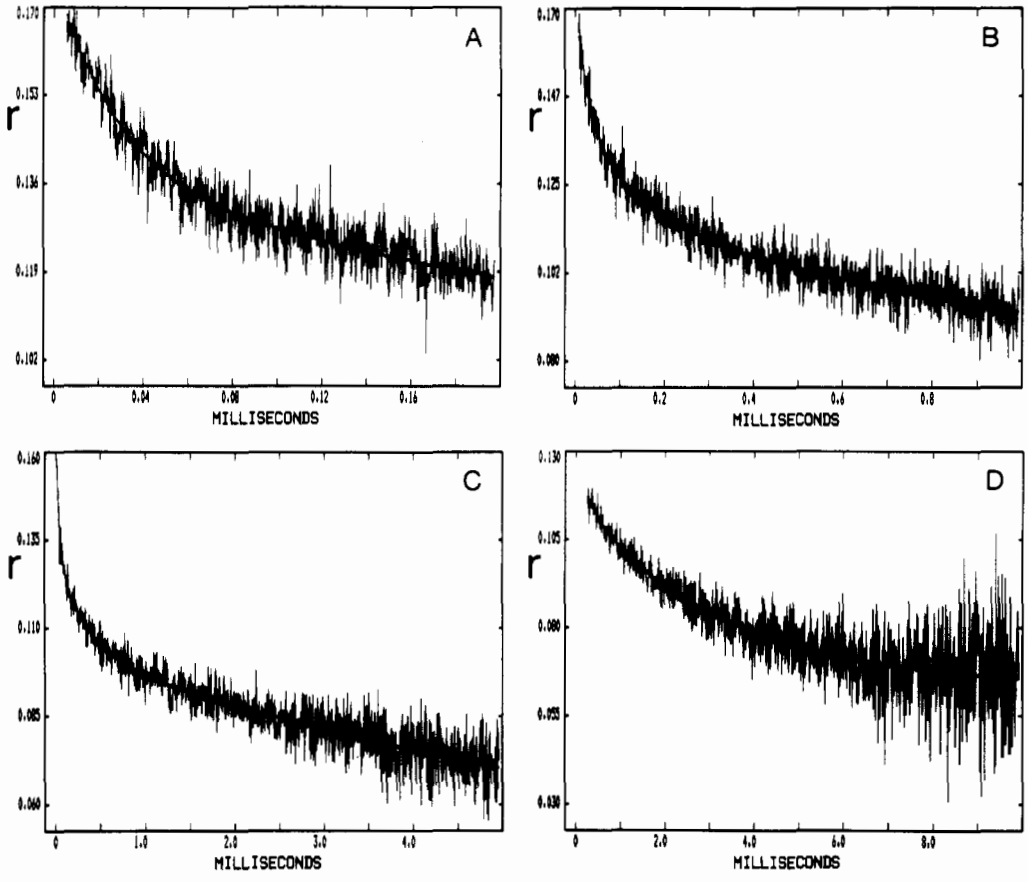

FIGURE 5: Anisotropy decay in intact erythrocytes. (A) 0.2 μs per channel; (B) 1 μs per channel; (C) 5 μs per channel; (D) 10 μs per channel. Data and fit in each case are overlayed.

that in the presence of the 28-μs component, we would be unable to detect a component faster than about 6 μs in the $r(t)$ decay.

The dramatic effect on band 3 rotational diffusion brought about by tryptic removal of its 41-kDa fragment was first reported by Nigg and Cherry (1980), using the method of triplet-state absorption; our observations are in qualitative agreement.

Anisotropy Decay of Band 3 in Intact Cells. The high sensitivity and spectral distribution of triplet emission techniques make feasible the acquisition of $r(t)$ data from band 3 in intact erythrocytes. Hemoglobin absorption and cell scattering limit the maximum sample concentrations severely, which results in much lower signal to noise ratios in comparison with ghost measurements. However, it is certainly possible to recover well-separated correlation times. Typical decay records are shown in Figure 5.

The analyzed results in Table I show that the fastest component of 28 μs is identical with that observed for hemoglobin-free ghosts. The second component with correlation time 334 μs differs apparently from the ghost system. Although the $r(t)$ kinetics for cells definitely show some small differences from ghosts in the ~100–500-μs region, one must be cautious in this instance about overinterpreting such differences quantitatively. Because of the high noise level, one cannot, for example, exclude that the analyzed component of 334 μs is in reality the average of two highly correlated components of ~200 and 400 μs. [Obviously, one cannot rule out similar possibilities with the ghost data. However, quantitative comparisons of the apparent fitted parameters for any two sets of noisy $r(t)$ data require at least that the signal to noise ratios be similar.] Regardless of the true explanation for the apparent 334-μs component, we cannot rule out the possibility that a small amount of hemolysis has taken place, despite our attempts to minimize it. As is discussed below, small amounts of hemolysis evidently cause immobilization of band 3.

As in the case of ghosts, a substantial amplitude fraction of the $r(t)$ decay from intact cells is due to components with correlation times greater than 1 ms. The high level of noise does not allow the existence of more than one slow component to be established. Qualitatively, one concludes that the millisecond depolarizing components show no striking differences between ghosts and cells. On the other hand, the $s(t)$ data from intact cells have very low noise, and can be compared quantitatively with the corresponding ghost results. In Table II, it can be seen that while the lifetime components are identical in the two systems, their relative amplitudes differ. We return to this result under Discussion.

Effect of External Hemoglobin Binding. In a previous paper, we reported that band 3 was relatively immobilized in intact erythrocytes (Matayoshi et al., 1983). We have since then determined that extremely small amounts of hemolysis (caused by continuous stirring of cells during the long signal averaging period) can lead to this phenomenon. In Figure 6A,B, we demonstrate that simple addition of hemolysate to the external medium causes total loss of the fast 28-μs rotational diffusion component.

In Figure 6A, the $r(t)$ decay (fit curve) for control samples is overlaid on the same plot, to facilitate the comparison. (The data shown in this figure correspond to an end result less extreme than for the case of real cell hemolysis.) The analyzed results in Table I indicate the loss of the fast component, although this conclusion is already clear by visual inspection of the data curve shown in Figure 6B, which was taken at a signal bandwidth 25 times greater than the record of Figure 6A. The value of $r(0)$ also appears slightly increased.

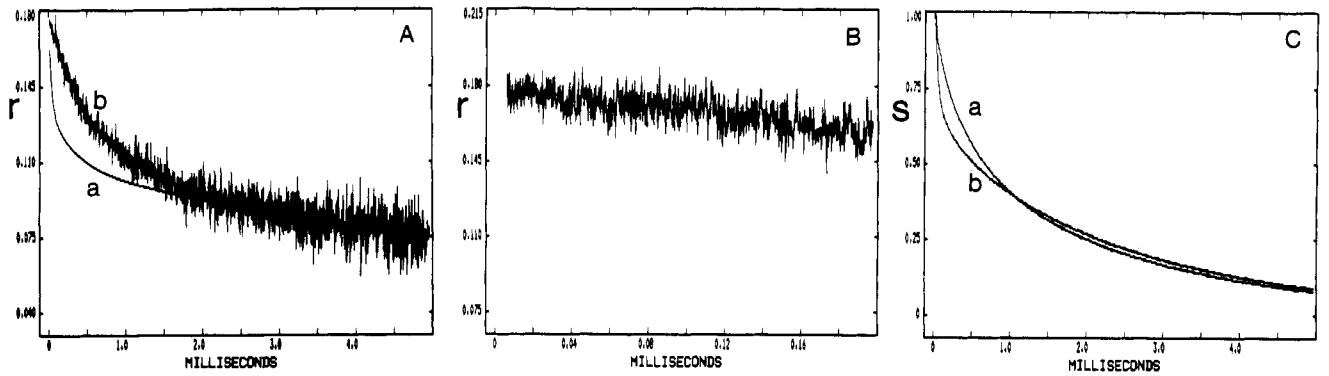

FIGURE 6: Immobilization of band 3 in intact cells by hemoglobin. (A) Effect on $r(t)$ of hemolysate addition to external medium. Untreated intact cells (bottom curve, a, fit only); intact cells in the presence of crude hemolysate, equivalent to 0.2 mg mL^{-1} hemoglobin (top curve, b, data and fit); 5 μs per channel. (B) Hemolysate-treated cells; 0.2 μs per channel (data only). (C) $s(t)$ for hemolysate-treated cells (curve b, data only); $s(t)$ for control (untreated) intact cells (curve a, data only).

The $s(t)$ decay curves corresponding to the samples in Figure 6A are shown in Figure 6C. A marked effect on $s(t)$ is also evident, and the fitting analysis (Table II) indicates that the main effect has been a shift away from the 2.3-ms component and an increase in fractional amplitude contributed by the 37-μs component. A moderate reduction of the ~400-μs component is observed, while the other two lifetimes remain constant.

In order to ascertain whether the binding of hemoglobin was in fact responsible, rather than some other component of the hemolysate, we also measured samples of intact cells to which purified (recrystallized) human hemoglobin was added. The results, given in Table II, clearly indicate an analogous effect. A significant effect on the decay time was evident at the lower hemoglobin concentration shown. The higher concentration corresponds to the limit dictated by the absorbance at the excitation wavelength. We note that the effect of purified hemoglobin (a commercial preparation) is much weaker at the same concentrations, compared with the crude hemolysate (obtained from the same cells). This is presumably due in part to the different state of hemoglobin in the two cases, for example, the fractional concentration of methemoglobin.

Under Materials and Methods, we discussed possible artifacts in $r(t)$ due to heterogeneity in $s(t)$. One must therefore question whether the observed change in $r(t)$ induced by hemoglobin is a trivial consequence of the change in $s(t)$, rather than a true change in band 3 mobility. However, an increase in the 37-μs lifetime component would be likely, if anything, to increase the relative amount of a fast ($\phi < 37$ μs) anisotropy component, whereas precisely the opposite effect was observed. Reduction of the long-lifetime component per se is also unlikely to produce an apparent loss of the 28-μs anisotropy component because we have noted (results not shown) that artificially quenching the long-lifetime component in ghosts with oxygen does not lead to such a loss.

We should also point out that the efficacy of the crude hemolysate addition is much less than that produced by mild (stirring-induced) hemolysis of the cells. That is, for a given level of "immobilization", the external hemoglobin concentration due to hemolysis is manyfold lower than the concentration required by direct hemolysate addition. Presumably, the form of the binding of hemoglobin to band 3 which is necessary for "immobilization" is somehow facilitated at the time of hemolysis.

Discussion

A major aim of this work was to compare the rotational diffusion of band 3 in human erythrocytes and hemoglobin-free ghosts. In doing so, however, we found it necessary to reexamine the anisotropy decay of the triplet state of eosin-labeled band 3 in greater detail and resolution than was done previously. In light of this information, we further wanted to study how known perturbations to band 3 affect its diffusion. The effect of removing the 41-kDa water-soluble cytoplasmic fragment by trypsinization has been described in this paper, while our studies on the binding of the two cytoplasmic enzymes to band 3 are presented in the paper which follows (Matayoshi et al., 1991).

The general method of time-resolved anisotropy decay of the triplet state is an attractive approach to the study of rotational diffusion in membranes. However, as with all versatile techniques, considerable care is required before conclusions or reasonable speculations are made. In the following discussion, we consider in detail the interpretation of the decay data.

Multicomponent Nature of the Total Intensity Decay, $s(t)$. We have found that in all ghost or cell preparations of eosin-maleimide-labeled band 3, the total intensity decay is comprised of three widely separated components. Thus, an obviously important question to ask is whether the signal measured arises from a chemically homogeneous population and, if not, whether small-impurity species can explain the effects we observe in the anisotropy decay. Although we detect only one fluorescent eosin band on SDS gels of red cell membrane proteins, this test alone does not suffice to exclude some heterogeneity of labeling: the integrated fractional intensities for ghosts, corresponding to the 40-, 400-, and ~2500-μs components, are 0.002, 0.03, and 0.97, respectively. The two smaller intensities would be undetectable on gels. However, there are other observations which should be considered.

The eosin moiety by itself is a potent inhibitor of anion transport in red cells (Nigg et al., 1979). Therefore, eosin-maleimide is a covalent affinity label for band 3, much in the same manner as DIDS and its noncovalent analogues (Cabantchik et al., 1978). Specificity for band 3, even in the case of DIDS, is probably not better than about 95% (Cabantchik & Rothstein, 1974b). Although we would hardly expect better specificity in the case of eosin-maleimide, the amplitudes of the components of the anisotropy decay (to be discussed shortly) are not small and unlikely to arise from species which constitute less than about 10% of the total labeling. Furthermore, the perturbations specific to band 3 in this and the following paper (Matayoshi et al., 1991) serve as an internal consistency check on this premise. Another possibility, however, is that eosin reacts with more than one lysine residue in the transport pocket. It has been shown, for example, that DIDS can react with at least two lysine residues of band 3 (Jennings & Passow, 1979; Jennings, 1982).

Eosin-maleimide does not penetrate the intact cell under the labeling conditions employed, thereby eliminating the vast majority of potentially reactive groups. [We failed to detect eosin in extracted water-soluble ghost proteins, a finding consistent with this notion; see also Macara et al. (1983).] The only cell surface membrane proteins present in substantial numbers are band 3 and glycophorin. Glycophorin contains no sulfhydryl groups, and at pH 7.4, labeling of amino groups is minimal. The labeling to what is probably an amino group on band 3 is due to the special circumstances of the transport site and its high noncovalent affinity for the eosin moiety. This supposition is also supported by the result (data not shown) that prereacting of intact cells with *N*-ethylmaleimide does not block eosin-maleimide labeling of band 3, as well as recent studies identifying the reactive locus as Lys-430 in the 17-kDa transmembrane fragment (Cobb et al., 1990).

We should point out, however, that chemical homogeneity of covalently labeled sites is not incompatible with heterogeneity of the total intensity decay. It is not unreasonable to suppose that there are several conformational modes (noncovalent) available to the covalently attached dye. Eosin has two negative charges which could in principle give rise to several modes of binding in the anion transport pocket of band 3. From this point of view, it is particularly interesting that $s(t)$ values from ghosts and intact cells (515-nm excitation) and ghosts (385-nm excitation) all exhibit the same lifetime components but with slightly different amplitudes. In fact, there are only two simple explanations for the difference obtained with ghosts at the two excitation wavelengths: (1) heterogeneity of emitting species, in which the components have slightly different absorption properties; (2) excitation at 385 nm facilitating occupation of higher triplet states. Should (1) be the case, the differences in amplitudes in the $s(t)$ decay between ghosts and intact cells might reflect the distribution of eosin binding modes at the transport site. The binding of external hemoglobin would also cause a shift in this distribution. Should (2) be true, the observed shift in amplitudes between ghosts and intact cells excited at 515 nm might reflect a subtle difference in the environment sensed by the probe, leading to an alteration of its photophysical properties.

Multicomponent $r(t)$ Decay of Band 3. The interpretation of the multicomponent $r(t)$ is difficult in principle and practice. In theory, a single diffusional species by itself can give rise to as many as five exponential decay times. Even the simple model of pure cylindrical rotation in a membrane can lead to two exponentials. In general, the apparent presence or absence of exponentials depends on the geometry of the rotation axes relative to the absorption and emission transition moments, since this information is contained within the preexponential amplitudes (Jovin et al., 1981; Jovin & Vaz, 1989).

One rationale for performing $r(t)$ decay at different excitation wavelengths is to check for possible anisotropic diffusion effects. This procedure provides a simple means for changing the optical geometry, thereby biasing certain depolarizing modes upon excitation at the different wavelengths. The results of the measurements on ghosts using 385-nm excitation ruled out the existence of significant geometric effects, at least with respect to the fastest component in the $r(t)$ decay. However, as pointed out in the preceding section, comparisons between decay data obtained from excitation at 385 versus 515 nm might be complicated by absorption due to heterogeneous species which differ in absorption at the two wavelengths. Thus, the results in Figure 4A can only serve as a qualitative "negative" test, in that no extreme differences were observed.

Another potential complicating factor in the $r(t)$ kinetics is the existence of multiple immobile components differing both in lifetime and in $r(0)$ value. In this case, nonexponential $r(t)$ kinetics (rising or falling in time) are observable, from which it is inferred (falsely) that depolarizing motions are present, when none in fact exist. The $r(t)$ kinetics in this situation are dictated by the decay of the excited state, i.e., $s(t)$, for each immobile species. As we have pointed out elsewhere (Matayoshi et al., 1983), pure cases of this effect can be inferred by analyzing the difference decay function $d(t)$. It is clear, however, that a mixture of kinetics resulting from immobile as well as truly mobile species can be difficult to sort out. The liklihood of variable $r(0)$ values on the microsecond time scale is high, and we have observed many examples in our laboratory. In contrast, the paucity of similar examples in nanosecond fluorescence anisotropy decay measurements is not surprising, since comparatively little motion can occur on the subnanosecond time scale.

Although immobile components are probably present in the case of band 3, the above effect is unlikely to play a dominating role. Very substantial changes in $r(t)$ occur following various treatments of ghosts [this work and Matayoshi et al. (1991)], despite the constancy of the $s(t)$ decay. Alternatively, it could be argued that the primary result of these treatments is to alter the amplitudes (r_{0i}) for the ith immobile species, while $s(t)$ remains constant. However, we tend to discount this possibility, since the total initial anisotropy $r(0)$ remains constant, and any radical changes in one or more r_{0i} would have to occur in a compensating manner, that is, so as to maintain $r(0)$. In addition, any perturbations large enough to change r_{0i} significantly are likely also to affect the $s(t)$.

It was suggested in the preceding section that the three $s(t)$ components might represent different covalent or noncovalent states of eosin at the transport site of band 3. If an equilibrium between the noncovalent states exists, one might ask whether conversion between these states underlies some of the depolarization components. We feel this explanation is also unlikely, because interconversion on the microsecond to millisecond time scale would be revealed by a coupling between the diffusion processes and the kinetics of the excited state. As just pointed out, $s(t)$ and $r(t)$ appear to be entirely uncorrelated.

Band 3 is thought to exist minimally as a dimer in the membrane (see the introduction), and we propose that the fastest correlation time $\phi_1 = 28$ μs corresponds to the mobility (rotational and/or wobbling diffusion) of a dimer of band 3. For the case where diffusion is modeled as pure rotation of a cylindrical protein around the membrane normal, Saffmann and Delbruck (1975) derived the expression:

$$D_r = kT/4\pi\eta a^2 h \qquad (6)$$

where η is the membrane viscosity, h the cylinder height, and a the cylinder radius. It is instructive to estimate the expected D_r for band 3 within the framework of this simple model. The width of the erythrocyte lipid bilayer can be taken from X-ray data as 48 Å (McCaughhan & Krimm, 1980). An unambiguous estimation of the dimensions of band 3 (equivalent cylinder) is difficult, but a minimum dimer radius of about 40–48 Å (corresponding to four helices) would be necessary to account for the 10–12 membrane-spanning regions per monomer proposed in the models of Jay and Cantley (1986) and Kopito and Lodish (1985). The "microviscosity" of ghost membranes at 37 °C, as sensed by small fluorescent probes which form intramolecular excimers, is ~100 cP (Zachariasse et al., 1982). [The use of viscosities derived from small-probe diffusion for purposes of estimating free protein diffusion in

membranes may not be so unreasonable, in view of studies on the translational diffusion of membrane proteins (e.g., Vaz et al., 1982).] Assuming the above values, the minimum rotational correlation time ($1/D_r$) of a band 3 dimer at 37 °C can be estimated to be 23–33 μs, a range that encompasses the fastest correlation time that we observe experimentally. We conclude that a substantial fraction of band 3 rotates freely as a dimer, unhindered by protein–protein interactions. This interpretation necessarily depends upon the validity of the assumptions regarding molecular shape and membrane microviscosity. If they are quantitatively in error, we must consider that the data could also accommodate a tetrameric structure, for which the rotational correlation time would be roughly twice that of the dimer. The previously reported rotational correlation time for the fast component of ~0.2 ms (Nigg & Cherry, 1980, 1979; Tsuji et al., 1988) tended to argue against a predominance of any small molecular species.

We wish to emphasize that the $r(t)$ data do not provide a unique physical mechanism for the depolarization kinetics. There is a present no evidence which favors either rotational or wobbling diffusion of the whole or part of the protein. Pure cylindrical rotation, at a rate described by eq 6, gives rise to two correlation times of relative magnitude 1:4. However, the apparent presence or absence of one or the other exponential is not an adquate means for judging the validity of the pure rotation model, since as pointed out above, the amplitudes (r_{0j} and r_∞) can become undetectably small under certain geometric conditions of the emission and absorption transition moments. The depolarization due to pure wobbling can be approximated be a single exponential plus a finite r_∞ value (Kinosita et al., 1977). The wobbling rate D_w is expected to follow an expression of the form

$$D_w = kT/6n_cV_ef \quad (7)$$

where n_c is the "cone viscosity" and V_e and f are the effective volume and shape factor, respectively (Kinosita et al., 1981). The wobbling rate of band 3 oligomers would thus have a dependence on oligomer size which is similar to D_r.

If ϕ_1 represents the rotational diffusion of an uncomplexed band 3 dimer, what meaning can be attributed to the slower correlation times? Can ϕ_1 and ϕ_2 (or any other pair), for example, reflect different exponentials arising from the diffusive motion of any one band 3 species (eq 3)? We would maintain that this is not the case, inasmuch as there appear to be no fixed amplitude relationships among any of the components. However, on the assumption that band 3 interacts with other membrane proteins primarily through its external 41-kDa cytoplasmic fragment, our measurements on trypsinized band 3 suggest that different sized aggregates of band 3 do coexist. This conclusion follows because the shortest correlation time (ϕ_1) persists after trypsinization, and the next two longer components (150 and 900 μs) are also rather similar to their values in untreated ghosts. Thus, ϕ_1–ϕ_3 might correspond to fundamental aggregate sizes of band 3, and the change in amplitudes following trypsinization would accordingly reflect a change in aggregate distribution and/or the removal of (diffusion-hindering) interactions between aggregates and other membrane proteins. While it is tempting to use ϕ_2–ϕ_4 and their respective amplitudes to estimate the size and relative fraction of band 3 oligomers larger than the dimer, such calculations are not reliable. One must bear in mind both the theoretical and experimental limitations in an exponential analysis of the anisotropy decay of an inhomogeneous system (see discussion under Materials and Methods).

An important question bearing on the above comparison is whether removal of the 41-kDa fragment perturbs band 3–lipid and/or band 3–band 3 interactions, and hence to what extent is it valid to compare the complex anisotropy decays of cleaved and uncleaved band 3. The constancy of $r(0)$ and $s(t)$ that we observe after trypsinization argues strongly against even a small conformational change in the transmembrane portion of band 3. A variety of other studies also support the notion that the transmembrane and cytoplasmic domains of band 3 are essentially independent. Using calorimetry, Appell and Low (1982) found that the thermal stability of the integral region of band 3 was unaffected by cleavage of the 41-kDa fragment, implying minimal interaction between these two domains. Grinstein et al. (1978) and Lepke and Passow (1986) demonstrated that the absence of the cytoplasmic fragment is of little consequence to the capacity of band 3 for carrying out sulfate transport. It therefore seems unlikely that any significant structural change in the 55-kDa fragment accompanies tryptic modification. On the other hand, Salhany et al. (1980) reported that DIDS binding of band 3 caused changes in the characteristics of hemoglobin binding to the cytoplasmic domain. Our results do not necessarily conflict with the latter result, since in our studies we cannot absolutely rule out an eosin-maleimide-induced stabilization of the transmembrane region.

We have observed that proteolytic removal of the 41-kDA cytoplasmic domain of band 3 roughly doubles the amplitudes of the putative band 3 dimer (ϕ_1) and next largest band 3 oligomer (ϕ_2) (Table I). Furthermore, this procedure (also under more vigorous conditions or at higher concentrations of trypsin than the experiment under Figure 3) drastically reduces but never completely eliminates the slow millisecond components (ϕ_3, ϕ_4). We conclude from these results that the mobility of some band 3 oligomers is hindered because of interactions with other membrane or cytoplasmic proteins mediated by the 41-kDa domain and that removal of the latter enables "free" rotational diffusion. The persistence after trypsinization of a subpopulation of band 3 displaying extremely hindered mobility (ϕ_3, ϕ_4) implies that another group of band 3 molecules exists natively as large homo- or heterooligomers that are stabilized by hydrophobic interactions with the membrane-bound domain of band 3. While our measurements of the anisotropy decay kinetics reflect the resultant mobility of band 3 only and cannot distinguish between homo- vs heterooligomers, it should be noted that at present, evidence in the literature is lacking for the existence of heterooligomers of band 3 which are stabilized by transmembrane interactions. Glycophorin, the second most abundant integral membrane protein, would appear to be the most likely candidate for such complexes. Indeed, Nigg et al. (1980) found that the rotational diffusion of band 3 was strongly hindered following the cross-linking of glycophorin with anti-glycophorin antibodies, and the authors concluded that band 3–glycophorin complexes preexist in the membrane. However, an equally valid explanation is that band 3 has been dragged in and trapped between cross-linked glycophorin molecules. In another study, Davio and Low (1982) reported that the thermal stability of band 3 in ghosts was unaffected by extraction of glycophorin, suggesting that band 3–glycophorin interactions are either weak or nonexistent. The observed lack of association of band 3 with glycophorin in detergent solutions (Yu & Steck, 1975) is also consistent with the latter conclusion.

The value of r_∞ for pure cylindrical rotation is related to $r(0)$ by a multiplicative constant, whose value depends on the absorption and emission transition moment orientations. On the assumption of cylindrical rotation and an accurate

knowledge of $r(0)$, it is therefore possible to fit data records by fixing the value of r_∞, which Nigg and Cherry (1979) chose to do for much of their transient dichroism data. However, the analysis of trypsinized ghosts (Table I) indicates that this cannot be justified here, since while $r(0)$ remains constant, r_∞ is drastically reduced. The apparent magnitude of r_∞ is determined not only by the degree of hindrance to isotropic rotation experienced by the mobile species but also by the fraction of relatively immobile species as well. We would suggest in this regard that the increase in r_∞ observed with increasing age of ghost preparations is due to a slow aggregation of band 3. Other changes in the r_{0i} and ϕ_i with age presumably reflect this process as well. (Note, however, that $\phi_1 \sim 25$ μs is constant.)

Mobility of Band 3 in Intact Cells. The experiments in which either a crude hemolysate or purified hemoglobin was added to a suspension of intact cells demonstrate that binding of hemoglobin to the outside membrane surface can occur. The binding is presumably either to band 3 or in its immediate vicinity, since both $s(t)$ and $r(t)$ are affected. It is noteworthy that hemolysis seems to greatly facilitate the requisite binding. The detection of hemoglobin binding at the outer surface is in apparent disagreement with previous reports in the literature using other techniques (Shaklai et al., 1977; Fung, 1981) for reasons which remain unclear. Detection of binding in our experiments is unambiguous, since both $s(t)$ and $r(t)$ decays are strongly affected. We have not pursued any quantitation of the binding, as it has no obvious physiological importance.

The general features of the $r(t)$ decay for ghosts and intact cells are similar, implying that the procedure of preparing ghosts leaves protein–protein and protein–lipid interactions involving band 3 largely intact and that gross alteration of membrane structure has not occurred. The measurements on intact cells and ghosts were performed at different ionic strengths. However, the anisotropy decay of band 3 in ghosts depleted of G3PD (Matayoshi et al., 1991) was insensitive to the salt concentration in the range 0.01 M sodium phosphate–0.15 M NaCl (data not shown). Furthermore, the results imply that the large 41-kDa cytoplasmic fragment of band 3 does not sense an abnormally large viscosity either at the membrane surface or in the cytoplasm of the intact cell. The 41-kDa cytoplasmic fragment has been proposed to exist as an elongated noncovalent dimer complex of 25-Å diameter, which extends 250 Å out from the membrane surface (Low, 1986). Of interest in this regard is the interaction between band 3 and hemoglobin (Shaklai et al., 1977; Salhany & Shaklai, 1979; Salhany et al., 1980; Fung, 1981; Sayare & Fikiet, 1981; Eisinger et al., 1982; Casoly, 1983; Murthy et al., 1984; Walder et al., 1984; Chetrite & Cassoly, 1985; Low, 1986). At physiological pH, the association constant was estimated to be only of the order $\sim 10^3$–10^4 M^{-1} (Fung, 1981; Chetrite & Cassoly, 1985; Walder et al., 1984; Cassoly, 1983), but at a concentration of 5 mM tetramer within the cell, substantial band 3–hemoglobin interaction is to be expected. Chetrite and Casoly (1985) found that at physiological pH and ionic strength, only deoxyhemoglobin binds to the band 3 cytoplasmic fragment and that 2,3-diphosphoglycerate dissociated this complex. On the basis of these data, they estimated that in vivo about half of the band 3 per cell is complexed with hemoglobin. Our results (Table I) indicate that if band 3 and hemoglobin are associated in the intact cell to such an extent, then the rotational diffusion of band 3 is apparently unaffected by hemoglobin binding at the cytoplasmic surface. This is in contrast to the immobilization of band 3 brought about by the binding of G3PD and aldolase (Matayoshi et al., 1983, 1991). If ϕ_1 corresponds as proposed to a population of freely diffusing band 3 dimers, its presence in intact cells, ghosts, and trypsinized ghosts implies that, for this group, viscous interactions within the bilayer are dominant over the retarding presence of any large exterior mass.

Conclusions. Our results demonstrate the existence in both ghosts and intact cells of several classes of band 3 that differ greatly in mobility. A substantial number of band 3 molecules are immobilized partially or totally because of complexation with other membrane or cytoplasmic proteins that is mediated by its 41-kDa cytoplasmic domain. However, a significant fraction of band 3 also exists as homo- or heterooligomeric complexes larger than a band 3 dimer that are stabilized by hydrophobic interactions with its membrane-bound domain. Finally, there exists a highly mobile class of band 3 in both ghosts and cells exhibiting a correlation time which is consistent with that expected for free rotational diffusion of a band 3 dimer in the erythrocyte membrane. The mobility of this group appears to be determined purely by viscous interactions within the lipid bilayer and is independent of the presence or absence of the extrinsic 41-kDa cytoplasmic fragment.

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Rotational Diffusion of Band 3 in Erythrocyte Membranes. 2. Binding of Cytoplasmic Enzymes

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ABSTRACT: Time-resolved phosphorescence anisotropy has been used to study the rotational diffusion of eosin-labeled human erythrocyte band 3 in the presence of an enzyme bound at its cytoplasmic pole. With increasing amounts of G3PD (glyceraldehyde-3-phosphate dehydrogenase) added to ghosts, the infinite time anisotropy (r_∞) increases, and at saturating concentrations, very little decay of the anisotropy $r(t)$ occurs at all. These phenomena are reversed by elution of the enzyme with 150 mM NaCl. Prior proteolytic removal of the N-terminal 41-kDa cytoplasmic fragment of band 3 also disenables the G3PD effect. When ghosts are stripped of their residually bound G3PD, a small reduction in the fraction of immobile band 3 is observed. Finally, titration of band 3 sites with aldolase shows effects on the $r(t)$ qualitatively similar to those observed with G3PD. On the basis of our interpretation of the heterogeneous anisotropy decay of eosin-labeled band 3 [Matayoshi, E. D., & Jovin, T. M. (1991) *Biochemistry* (preceding paper in this issue)], we conclude that the binding of G3PD and aldolase results in the immobilization of band 3 oligomers.

In the preceding paper (Matayoshi & Jovin, 1991), we compared in detail the rotational diffusion of eosin-maleimide-labeled band 3 in human erythrocyte ghosts and intact cells. A question of particular interest which arises from these studies is whether the rotational mobility of band 3 in the intact cell is affected by the binding of cytoplasmic proteins. The N-terminal 41-kDa region of band 3, which is thought to protrude as an elongated structure into the cytoplasmic space (Low, 1986), contains binding sites for the three glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase (G3PD)[1] (Yu & Steck, 1975; Tsai et al., 1982), aldolase (Strapazon & Steck, 1976, 1977; Murthy et al., 1981), and phosphofructokinase (Higashi et al., 1979; Jenkins et al., 1985), as well as hemoglobin (Shaklai et al., 1977; Salhany et al., 1980; Sayare & Fikiet, 1981; Casoly, 1983; Murthy et al., 1984; Walder et al., 1984; Chetrite & Casoly, 1985; Low, 1986; Premachandra, 1986). In addition, the catalytic activities of G3PD, aldolase, and phosphofructokinase are altered upon complexation with band 3 amino-terminal fragments. The interaction of these cytoplasmic proteins with band 3 is significant in vitro only under conditions of low ionic strength, and it might therefore be questioned whether such binding has any relevance to the erythrocyte (Maretzki et al., 1989; Masters, 1989). Indeed, in two studies, it was concluded that G3PD in the intact cell is not membrane-bound (Brindle et al., 1982; Rich et al., 1985). On the other hand, support for the existence of membrane-bound glycolytic enymes in the intact cell was obtained in other studies (Kliman & Steck, 1980; Solti et al., 1981; Fossel & Solomon, 1981; Jenkins et al., 1984). Steck and co-workers estimate that perhaps half to two-thirds of the total cellular content of G3PD, aldolase, and phosphofructokinase are bound to band 3 in the intact cell (Kliman & Steck, 1980; Jenkins et al., 1984). Such occupancy would require 15-20% of the 1 million band 3 monomers per cell. If one further assumes that about half of the band 3 is complexed with hemoglobin (Chetrite & Casoly, 1985), there would be sufficient band 3 to bind both hemoglobin and the glycolytic enzymes, as well as other membrane proteins such as bands 2.1, 4.1, and 4.2. Although the question as to whether cytoplasmic proteins are bound to band 3 in vivo must be considered still unresolved, it is evident that such interactions could be advantageous for coordinating cell flexibility and metabolism with transport functions, as has been noted by many authors [e.g., see Salhany and Gaines (1981), Gillies (1982), and Low (1986)].

In this paper, we report that the binding of G3PD and aldolase to band 3 in ghosts inhibits its rotational diffusion. A preliminary account of this work has appeared previously (Matayoshi et al., 1983).

Materials and Methods

Materials. Rabbit muscle G3PD and rabbit muscle aldolase were obtained from Sigma. All other materials were as described in Matayoshi and Jovin (1991).

Sample Treatments. Residually bound G3PD was removed from ghosts by incubation of ghosts for 20 min at room temperature either in 10 mM sodium phosphate/0.4 M NaCl, pH

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[1] Abbreviations: G3PD, glyceraldehyde-3-phosphate dehydrogenase; DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulfonate.

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