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Internal Motions of Band 3 of Human Erythrocytes†

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ABSTRACT: Band 3 was labeled with *N*-[[(iodoacetyl)amino]ethyl]-5-naphthylamine-1-sulfonate either exofacially in the intact washed erythrocytes or endofacially by treating inside-out vesicles. Exo labeling resulted in the labeling of several other proteins, besides band 3, which could not be removed from the membrane. Therefore, the exo-labeled band 3 was extracted and purified by chromatography on DEAE-cellulose in Triton X-100. The endo labeling also resulted in the labeling of several other proteins. In this case, washing with NaOH removed all labeled material except band 3 from the vesicles. The lifetime of bound *N*-[(acetylamino)ethyl]-5-naphthylamine-1-sulfonate was heterogeneous, suggesting the positioning of the label in different environments either because different sites were labeled or because of positional freedom of the label at the same point of attachment. The main fraction of emission intensity had a lifetime near 20 ns, as expected for a hydrophobic environment. The rest showed a lifetime of about 3 ns in the exo-labeled band 3 and 9 ns in the endo-labeled band 3. Both lifetimes appeared to be independent of temperature between 5 and 25 °C, suggesting shielding of the probe from the solvent. Quenching phenomena must be responsible for both the 3- and 9-ns lifetimes, not due to residual heme, as proven by the persistence of such quenching in the Triton X-100 extracted protein. The correlation times indicated the presence of a short component, between 2 and 4 ns in the different systems, probably due to the presence of a flexible portion in the structure of the protein. In the endo-labeled system, the anisotropy at long times failed to reach a zero value, consistent with the hypothesis that the rotational motions of the membrane-embedded protein are constrained around a single axis, perpendicular to the plane of the lipid bilayer. Addition of 300 mM NaCl to the samples increased the value of the limiting anisotropy in the endo-labeled system and in the exo-labeled protein decreased the extent of depolarization produced by the motion corresponding to the short correlation time of the system. This suggests an increased rigidity of the system upon addition of NaCl.

Band 3 is the major membrane-spanning glycoprotein of the human erythrocyte with a molecular weight of 95 000 and is the site of the electroneutral anion-exchange reaction. In addition, band 3 acts as a binding site for a number of intracellular proteins such as aldolase (Murthy et al., 1981), glyceraldehyde-3-phosphate dehydrogenase (Yu & Steck, 1975b), phosphofructokinase (Higashi & Richards, 1979), hemoglobin (Shaklay et al., 1977), and band 2.1 of the erythrocyte cytoskeleton (Hargreaves et al., 1980).

The structure and disposition of band 3 in the membrane have been well documented (Drickamer, 1976, 1978; Steck et al., 1976; Appell & Low, 1981; Tsai et al., 1982; Ramjeesingh et al., 1983) although the complete primary structure

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is still unknown. The functional domains of band 3 have been determined (Steck et al., 1975; Appell & Low, 1981; Tsai et al., 1982), and the anion binding site has been localized to a 40-kilodalton (kDa)[1] transmembrane fragment as shown by Ramjeesingh & Rothstein (1982).

Structural studies at the quaternary level have revealed the extent of intermolecular associations of the band 3 protomers that may occur in the membrane. Yu & Steck (1975a) determined that band 3 exists as dimers in the membrane as well as in nondenaturing detergents. Pappert & Schubert (1983) observed a monomer/dimer/tetramer equilibrium in nondenaturing detergents and suggest that this may also exist in the membrane.

The mechanism of anion exchange is not precisely known. From the large array of kinetic data that has been accumulated, two models have emerged to characterize the exchange mechanism. One is the double-displacement mechanism as proposed by Gunn & Frohlich (1980), and the other is the single-displacement mechanism as described by Salhany & Rauenbuehler (1983).

Whatever the precise mechanism, since the anion binding site is alternatively transporting anions from one side of the membrane to the other, the binding site must remain mobile. Therefore, the protein must possess a domain of internal flexibility that is ligand linked in order to have a mobile anion binding site with the ability to "know" which direction to transport the anions.

The notion of internal flexibility in proteins has been documented by theoretical models and experimental data (Karplus & McCammon, 1981; Bennett & Huber, 1984). Computer simulations and spectroscopic techniques have demonstrated correlations between conformational fluctuations and ligand binding in myoglobin (Case & Karplus, 1979). Measurements of decay of fluorescence anisotropy have shown the presence of internal motions in proteins in the nanosecond time region, probably corresponding to groups of atoms defining structural domains in the protein structure (Hanson et al., 1981; Bucci et al., 1979; Oton et al., 1981, 1984; Sassaroli et al., 1982). Recently, it was found that the correlation times detectable in the hemoglobin system are ligand dependent (Sassaroli et al., 1982).

Wahl et al. (1971) were the first to obtain experimental evidence for the existence of internal mobility in membrane proteins, in dansylated membrane fragments prepared from *Electrophorous electricus*. Since then, there have been no subsequent studies concerning the property of segmental motion in membrane proteins with correlation times in the nanosecond range. However, there have been several studies that have investigated rotational and translational diffusion rates of proteins embedded either in natural membranes or in liposomes in the microsecond range. Cherry has been the major contributor to the understanding of the nature of the mobility of band 3 in the erythrocyte membrane (Cherry & Schneider, 1976; Nigg & Cherry, 1979, 1980; Muhlebach & Cherry, 1982). Thus far, there have been no published studies wherein the intramolecular motion of band 3 has been detected.

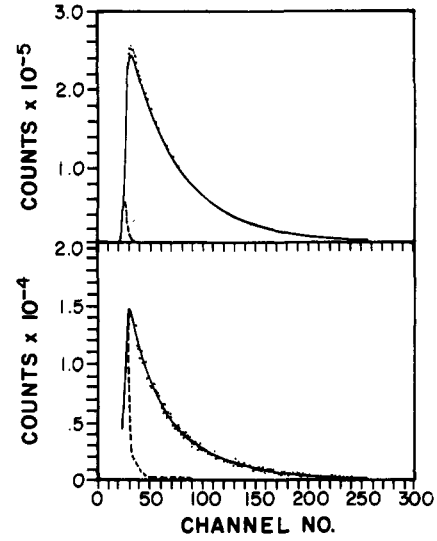

FIGURE 1: Deconvolution of $2V + H$ and $V - H$ transients. The upper gradient is for $2V + H$. The continuous lines are the fitting exponentials reconvolved to the light pulse (dashed lines). The experimental points of $2V + H$ are barely visible under the curve.

We have used time-resolved fluorescence techniques for exploring the existence of segmental motion in band 3 labeled with 1,5-AEDANS. The fluorescence of intrinsic probes was inappropiate because there are too many tryptophans in the protein and none of their positions are known. The lifetime characteristics of 1,5-AEDANS, 10–20 ns depending on the environment (Hudson & Weber, 1973), appeared to be best for exploring correlation times expected to be between 1 and 100 ns.

The data indicate the presence of internal motions in band 3. Also, they suggest a ligand-linked conformational change of the protein which may produce a repositioning of band 3 in the lipid bilayer of the membrane.

Materials and Methods

Fluorescence Measurements. Lifetimes and anisotropy decay of fluorescence were measured by using an Ortec 9200-ns fluorometer, following standard procedures developed in our laboratory (Oton et al., 1981). A 20-nm band-pass filter with a transmission maximum at 360 nm intercepted a nonpolarized exciting beam, and a 470-nm cutoff filter intercepted the fluorescent beam. The zero time of observation was chosen to be the point of maximum intensity of the light pulse. This implied a dead time of the instrument of about 1 ns.

When labeled vesicles were used, the blanks to be subtracted were obtained from suspensions of nonlabeled vesicles whose turbidity was matched to that of the labeled samples by reading the OD at 360 nm, i.e., the excitation wavelength. The absorption of the label was negligible. The total counts for blanks were 5% or less than the counts for the samples.

In many occasions, the decay of anisotropy ended as a plateau with no apparent negative slope. In these cases, the deconvolving algorithms failed to give consistent analyses of the data. Better results were obtained following a procedure suggested by Hanson et al. (1981) in which the sum $(2V + H)$ and difference $(V - H)$ of the decay of emission monitored with vertically (V) and horizontally (H) oriented polarizers were deconvolved separately. From these functions, the deconvolved anisotropy was computed by using

$$A(t) = \frac{(V - H)_t}{(2V + H)_t} \quad (1)$$

[1] Abbreviations: BSA, bovine serum albumin; 1,5-IAEDANS, *N*-[[(iodoacetyl)amino]ethyl]-5-naphthylamine-1-sulfonate; 1,5-AEDANS, *N*-[(acetylamino)ethyl]-5-naphthylamine-1-sulfonate; Tris, tris(hydroxymethyl)aminomethane; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; Triton X-100, (*p-tert*-octylphenyl)nona(oxyethylene); C_{1E9}, dodecylnona(oxyethylene); Mer-AEDANS, 1,5-AEDANS-substituted mecaptoethanol; Cys-AEDANS, 1,5-AEDANS-substituted cysteine; PMSF, phenylmethanesulfonyl fluoride; kDa, kilodalton(s); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid.

Two exponential terms were generally enough for simulating the decays of $2V + H$ and $V - H$. Figure 1 shows a typical result of such analyses. The fit to the data indicated an average 2% deviation between experimental and calculated data.

The deconvolved anisotropy was analyzed by simulations based on multiexponential fits as obtained by the Marquardt algorithm, routinely used in our analyses (Marquardt, 1963).

Polyacrylamide Gel Electrophoresis. SDS–PAGE in tube gels were done according to the method of Fairbanks et al. (1971) using 5% acrylamide and 1% SDS.

Protein Concentration. The concentration of membrane proteins was assayed according to the procedure of Lowry et al. (1951) with BSA as the standard. A correction factor of 1.15 was applied to the concentration of samples of pure band 3, due to the inherent inadequacy of BSA as a standard (Makino & Nakashima, 1981).

Triton X-100 Concentration. The procedure of Garewal (1973) was followed for determining the concentration of Triton X-100 in the various solubilized samples.

Acetylcholinesterase Activity. The procedure developed by Ellman et al. (1961) was followed in order to determine the quality of the sidedness of inside-out vesicle preparations, as modified by Steck et al. (1976).

Measurement of 1,5-IAEDANS Concentrations. Quantitative determination of 1,5-AEDANS in the labeled samples was done fluorometrically against standards of 1,5-AEDANS reacted with a 10-fold molar excess of mercaptoethanol. To eliminate the turbidity of the samples, 1% SDS was added to both the sample and standard solutions. The excitation and emission wavelengths were at 340 and 490 nm, respectively. The concentration of 1,5-AEDANS in the standards was determined spectrophotometrically by using $\epsilon_{340} = 6.1 \times 10^3$ M^{-1} cm^{-1} (Hudson & Weber, 1973).

Preparation of Membranes. Hemoglobin-free leaky ghosts were prepared from freshly outdated blood obtained from the University of Maryland Hospital Blood Bank. The red cells were washed with isotonic buffer (5 mM sodium phosphate and 150 mM NaCl) at pH 7.5. After thorough removal of the plasma and of the buffy coat, the washed red cells were lysed by addition of 5 mM sodium phosphate at pH 7.5. The ghosts were washed free of hemoglobin either by centrifugation or by the use of the Millipore Pellicon cassette. The resulting ghosts are considered to be hemoglobin-free leaky ghosts (Rosenberry, 1981).

Preparation of Inside-Out Vesicles. Inside-out vesicles were prepared from leaky ghosts following the procedure of Steck & Kant (1974). Their sidedness was determined by the acetylcholinesterase assay as described above. If the assay indicated less than 80% inside-out vesicles, the preparation was discarded.

Removal of Triton X-100. When necessary, removal of the excess of Triton X-100 from the samples was obtained by incubation with Bio-beads SM-2 or XAD-2, following the procedure described by Holloway (1973). The incubations used approximately 600 mg of beads per 5 mL of band 3 solution for a starting concentration of Triton X-100 of 3%. The incubation was conducted at 4 °C in the dark with occasional gentle agitation for about 3–5 h. The incubation was continued until the concentration of Triton X-100 was between 0.05 and 0.1%. For CD measurements, Triton X-100 was completely removed and substituted by $C_{12}E_9$.

Circular Dichroism Measurements. Measurements of the circular dichroism of band 3 in the far-UV region were conducted with a Jasco J-20 spectropolarimeter. If present, Triton X-100 (which strongly absorbs in the UV region) was removed and substituted with $C_{12}E_9$. The measurements were done at 10 °C. The data were analyzed by using

$$[\theta]^{MRW} = 115\theta_{obsd}/10lc$$

where 115 is the mean residue weight as determined by Makino & Nakashima (1981), c is the protein concentration in milligrams per milliliter, θ_{obsd} is the observed ellipticity of the samples in millidegrees, and l is the cuvette path length in centimeters.

Results

Labeling of Intact Red Cells. Washed packed red cells were incubated for 2 h at 37 °C in the dark with 1,5-IAEDANS (5 mg/10 mL of packed red cells). Following the incubation, the red cells were washed extensively with repeated centrifugations in order to remove unreacted 1,5-IAEDANS. Then the labeled ghosts were prepared as described.

Gel electrophoresis indicated that band 3 was not the only labeled protein.

Purification of Exofacially Labeled Band 3. Purified solubilized 1,5-AEDANS-labeled band 3 was obtained according to the method of Yu & Steck (1975a) with slight modifications. The exofacially 1,5-AEDANS-labeled ghosts were prepared as described and subsequently incubated for 30 min at 4 °C with 5 mM sodium phosphate plus 150 mM NaCl at pH 7.5 to remove band 6 which is 3-phosphoglyceraldehyde dehydrogenase. Following the incubation, the membranes were washed with the same buffer twice more. Then the membranes were incubated in 5 volumes of 0.5% Triton X-100 with 50 mM sodium phosphate and 1 mM DTT at pH 7.5 at 4 °C overnight. The extract was centrifuged for 30 min at 30000g to remove the unsolubilized membranes. The supernatant was then loaded onto a Whatman DE-52 column (0.8 cm × 10 cm) that had been preequilibrated with 1% Triton X-100 in 50 mM sodium phosphate and 1 mM DTT at pH 8.0. After the column was loaded, it was eluted with the same buffer until no more fluorescence appeared in the eluate. Then the column was eluted with 1% Triton X-100 plus 88 mM sodium phosphate and 1 mM DTT at pH 8.0 until no more fluorescence was eluted. Finally, 1% Triton X-100 with 170 mM sodium phosphate and 1 mM DTT at pH 8.0 was applied to the column. Until the addition of this last buffer, all of the fluorescent band 3 remained bound to the upper portion of the column. During the elution with this last buffer, most of the fluorescence was removed from the column. The fractions with the most intense fluorescence were chosen by using a long wave UV lamp. These pooled fractions were concentrated by the use of Amicon cones (molecular weight cutoff 30000) and rediluted to reduce the phosphate concentration to about 3 mM. Then the solution was concentrated to a working volume of 5 mL. Figure 2 shows that only labeled band 3 was present in these solutions. In these purified fractions, the molar stoichiometry of labeling was 0.2:1 for 1,5-AEDANS to band 3.

Endofacial Labeling. After inside-out vesicles were washed clean of the dextran and resuspended in 100 mM sodium phosphate at pH 7.0, they were incubated with 1,5-IAEDANS (10 mg/1 mL of packed vesicles) in a volume of 15 mL for 40 min at room temperature in the dark. Following the incubation, the vesicles were washed extensively with 5 mM sodium phosphate buffer at pH 7.5 to remove the unreacted 1,5-IAEDANS. Finally, the vesicles were incubated with 10 mM NaOH and 0.1 mM EDTA for 30 min and washed 3 times with this solution. In order to maintain the yield of the membranes, it is important to spin at 30000g for at least 20 min. After the final wash, the membranes were resuspended

Table I: Lifetimes of Various Preparations of Band 3 in 0.003 M Phosphate Buffer at pH 7.5[a]

sample	α_1	τ_1 (ns)	α_2	τ_2 (ns)
exofacially labeled ghosts	0.61 ± 0.06	19.6 ± 0.5	0.39 ± 0.20	3.4 ± 0.2
pure exofacial-labeled band 3 at 5 and 25 °C				
0 mM NaCl	0.71 ± 0.07	21.5 ± 1.0	0.29 ± 0.03	3.3 ± 1.0
300 mM NaCl	0.70 ± 0.10	20.3 ± 1.0	0.30 ± 0.06	4.0 ± 2.0
endofacially labeled stripped vesicles				
5 °C, 0 mM NaCl	0.55 ± 0.28	20.6 ± 1.0	0.45 ± 0.08	10.0 ± 2.0
5 °C, 300 mM NaCl	0.56 ± 0.07	21.0 ± 0.5	0.44 ± 0.08	9.9 ± 1.0
25 °C, 0 mM NaCl	0.47 ± 0.06	20.7 ± 0.1	0.53 ± 0.06	10.2 ± 1.0
25 °C, 300 mM NaCl	0.60 ± 0.05	20.2 ± 1.2	0.40 ± 0.05	9.6 ± 2.0

[a] The values of α_i are the normalized preexponential terms corresponding to each τ_i lifetime.

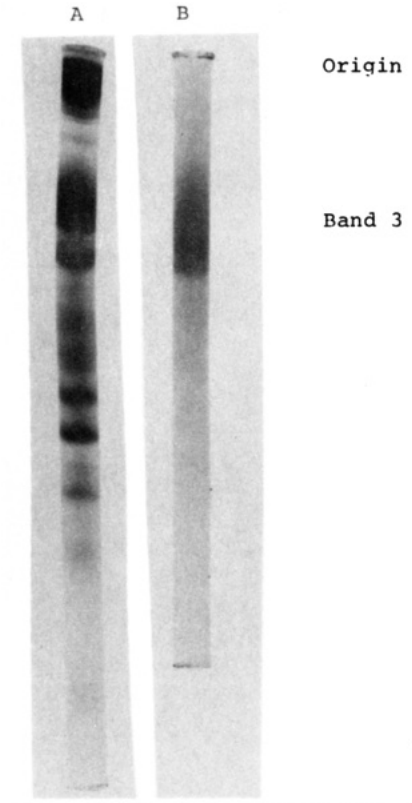

FIGURE 2: Coomassie blue stained SDS–PAGE gels of human erythrocyte membranes. The positions of the various bands are appropriately labeled. The electrophoretic procedure was according to Fairbanks et al. (1971). Gel A is the electrophoretic pattern of hemoglobin-free ghosts. Gel B is the electrophoretic pattern of purified band 3.

in 5 mM sodium phosphate buffer at pH 7.5.

The electrophoresis pattern for these stripped membranes revealed that band 3 protein was the only labeled protein. Coomassie blue staining of the gels indicated that 58% of the total protein content was band 3. On this basis, the stoichiometry of labeling was estimated by measuring the total protein concentration with the Lowry method (Lowry et al., 1951) and correcting for the proportion of band 3. In all preparations, the stoichiometry was near 0.4 mol of 1,5-AEDANS per mole of protein.

Emission Spectra. The emission spectrum of exofacially 1,5-AEDANS-labeled ghosts is shown in Figure 3. The excitation was at 340 nm, and the emission peak was centered at 477 nm. This is blue shifted from Cys-AEDANS or Mer-AEDANS which have an emission maximum centered near 490 nm. The emission spectrum was not affected by solubilization in Triton X-100; however, solubilization by SDS shifted the emission maximum to 490 nm. Very similar spectra were obtained from purified exofacially labeled band 3 in Triton X-100 and from endofacially labeled samples.

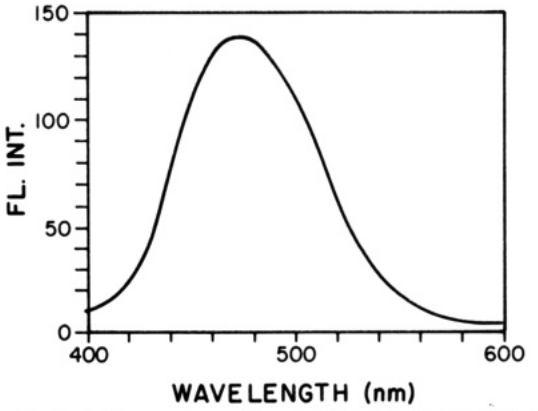

FIGURE 3: Emission spectrum of exofacially 1,5-AEDANS-labeled ghosts in 1.0% Triton X-100. Excitation was at 340 nm.

Lifetimes of Exofacially Labeled Ghosts. The lifetimes of exofacial 1,5-AEDANS-labeled ghosts were generally best fitted with two decay components. As shown in Table I, a long lifetime component near 19 ns dominated the fluorescence intensity ($\alpha\tau$), being 85% of the total. A shorter component near 3 ns contributed less than 15% of the total emission intensity.

The lifetimes were not sensitive to temperature between 5 and 25 °C or to addition of 300 mM NaCl. Solubilization with 1% SDS gave a single lifetime of 13 ns. In contrast, solubilization with Triton X-100 produced no change in the lifetime characteristics.

Lifetimes of Exofacially Labeled, Purified Band 3. The lifetimes of a solution of purified exofacially labeled band 3 in 0.2–0.4% Triton X-100 also showed the presence of multiple lifetimes, as shown in Table I.

Lifetimes of Endofacially Labeled Vesicles. Also in this case, at least two lifetimes were detectable in the samples. The longer component was near 20 ns as in all other systems. No lifetimes were detectable below 9 ns. Also in this case, solubilization with Triton X-100 did not affect the lifetimes (Table I).

Correlation Times of Purified Band 3. The deconvoluted anisotropies were best simulated by two correlation times, one below 10 ns, probably corresponding to some internal librational mode of the protein, and the other near and above 300 ns, probably corresponding to the rotational diffusion of the entire protein (see Table II and Figure 4).

The correlation times were independent of temperature, and sensitive to NaCl, which prolonged the longer correlation time, suggesting the formation of higher polymers in the system.

Correlation Times of Endofacially Labeled Vesicles. In this case, the deconvolved anisotropies showed the presence of short correlation times, followed by a plateau of nonzero anisotropy as expected for rotations confined to a single axis. As shown in Table II and Figures 5–7, the rotational behavior

Table II: Correlation Times of Labeled Band 3 in 0.003 M Phosphate Buffer at pH 7.5[a]

sample	temp (°C)	[NaCl] (mM)	A_1	ϕ_1 (ns)	A_2	ϕ_2 (ns)	A_0	A_∞
exo	5, 25	0	0.014 ± 0.002	3.6 ± 1.0	0.037 ± 0.003	290 ± 50	0.051 ± 0.002	
	5, 25	300	0.015 ± 0.002	8.9 ± 3.0	0.051 ± 0.008	1010 ± 500	0.067 ± 0.006	
endo	5	0	0.012 ± 0.001	8.2 ± 4.0			0.045 ± 0.001	0.033 ± 0.001
	5	300	0.011 ± 0.003	12.7 ± 4.0			0.056 ± 0.002	0.045 ± 0.003
	25	0	0.016 ± 0.001	11.1 ± 4.0			0.041 ± 0.001	0.025 ± 0.001
	25	300	0.012 ± 0.003	4.7 ± 0.5			0.039 ± 0.002	0.027 ± 0.002

[a] Exo = exofacially labeled purified band 3 in 0.4% Triton X-100. Endo = endofacially labeled inside-out vesicles. The values of A_i are the preexponential terms corresponding to each ϕ_i. $A_0 = A_1 + A_2 + A_\infty$; the standard errors listed for A_0 are the weighted averages of the errors of the added terms.

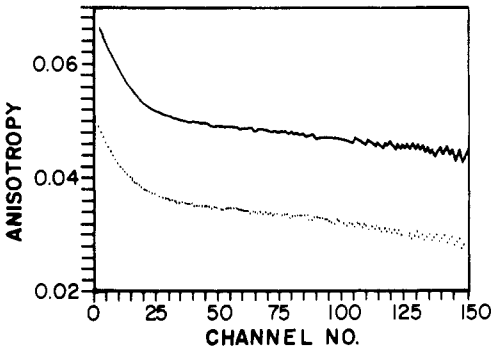

FIGURE 4: Salt dependence of deconvolved anisotropy decay of pure exofacially 1,5-AEDANS-labeled band 3 at 25 °C in 0.2% Triton X-100. The channel time is 0.4 ns/channel. Protein concentration, 1 mg/mL. (···) 0 mM NaCl; (—) 300 nM NaCl.

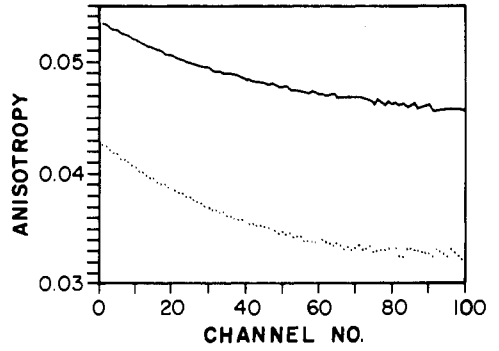

FIGURE 5: Temperature dependence of the anisotropy decays of endofacially labeled vesicles washed with 10 mM NaOH and 0.1 mM EDTA prior to measurement. The vesicles were suspended in 3 mM phosphate buffer at pH 7.5. The time scale is 0.4 ns/channel. Total protein concentration near 1 mg/mL. (—) 5 °C; (···) 25 °C.

was dependent on both temperature and ionic strength.

The similarity of the data obtained with vesicles and with solubilized material suggests that the secondary scattering produced by the turbidity of the vesicles had only a small effect on the polarization of the emitted light. Also, it should be stressed that inspection of the blanks failed to show a dependence of the secondary scattering on the ionic strength of the solvents.

Effect of Sodium Acetate. To test the relevance of changes in ionic strength and of different ions on the correlation times of labeled band 3, measurements were conducted in the presence of 300 mM sodium acetate. This salt failed to produce detectable modifications of the correlation times and anisotropies of the samples.

Circular Dichroism. As shown by Figure 8 the CD spectra of purified, exofacially labeled band 3 in $C_{12}E_9$ were sensitive to solvent conditions.

Discussion

Sidedness of Labeling. Labeling of either intact erythrocytes or inside-out vesicles produced different patterns of labeled proteins in the ghosts. Spectrin was labeled only in the inside-out vesicles, and bands 4 and 5 were labeled only in the intact erythrocytes. Drastic differences were also detectable

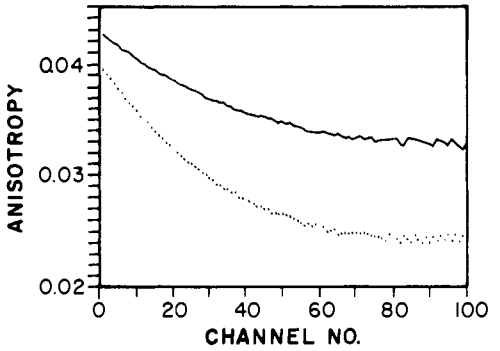

FIGURE 6: Salt dependence of anisotropy decays at 5 °C of endofacially labeled vesicles in 0.003 M phosphate buffer at pH 7.5. The time scale is 0.4 ns/channel. Total protein concentration near 1 mg/mL. (···) 0 mM NaCl; (—) 300 mM NaCl.

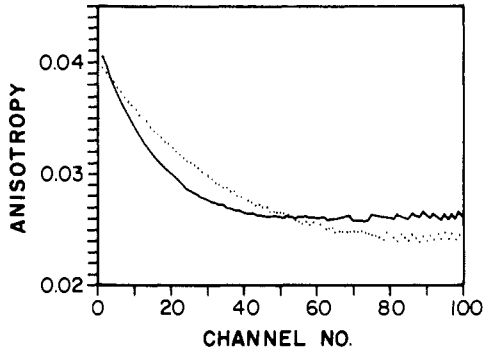

FIGURE 7: Salt dependence of anisotropy decays at 25 °C of endofacially labeled vesicles in 0.003 M phosphate buffer at pH 7.5. The time scale is 0.4 ns/channel. Total protein concentration near 1 mg/mL. (—) 300 mM NaCl; (···) 0 mM NaCl.

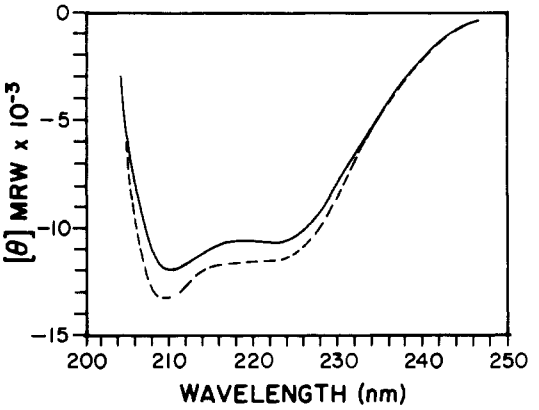

FIGURE 8: Far-UV CD spectra of pure exofacially 1,5-AEDANS-labeled band 3 at 10 °C in 0.003 M phosphate at pH 7.5 and 0.5% $C_{12}E_9$. Protein concentration 0.1 mg/mL. Cuvette path lengths were 1 mm. (—) No NaCl; (---) 300 mM NaCl.

after digestion with trypsin or chymotrypsin of the labeled samples (Steck et al., 1976). The digestion produced the appearance of a membrane-embedded 65-kDa fragment only in the exofacially labeled ghosts and released fluorescence in the soluble peptides only in the endofacially labeled vesicles.

Finally, and most importantly, the external labeling of band 3 produced the appearance of a 3-ns lifetime component, which was totally undetectable in the labeled inside-out vesicles.

These findings indicate that in the two cases the labels were positioned in different regions of band 3 and suggest that 1,5-IAEDANS was not able to cross the erythrocyte membrane.

Exofacial Labeling of Band 3. Exofacial labeling of red cells by 1,5-IAEDANS gave electrophoretic patterns indicating that several proteins were labeled. Band 3 had 80% of the emission intensity. Washing the membranes with 10 mM NaOH and 0.1 mM EDTA did not remove any of the labeled proteins, thus indicating that 1,5-IAEDANS was modifying only membrane-bound proteins. The gels showed that a number of proteins with molecular weights less than that of band 3 were labeled. Addition of PMSF during the preparation of the exofacial-labeled membranes did not alter the labeling pattern, indicating that PMSF-sensitive proteolysis was not a factor.

The position of the label was investigated by digesting the labeled intact cells with trypsin or chymotrypsin. This produces the fragmentation of band 3 into two membrane-bound fragments of 35 and 65 kDa (Steck et al., 1976). The labeling pattern in SDS–PAGE electrophoresis indicated that the label was confined to the 65-kDa peptide, which contains the binding site for anions in band 3 (data not shown).

While extraction with Triton did not modify the lifetime of band 3, solubilization of the protein in SDS produced a red shift of the emission spectrum and a homogeneous lifetime of 13.0 ns, consistent with exposure of the label to an aqueous environment, and with a protein-embedded probe in the native protein.

The label 1,5-IAEDANS is an alkylating agent quasi-specific for cysteinyl residues. It can also react with unprotonated amino groups and very slowly with histidine residues. Under the experimental conditions used in this investigation, it may have reacted with some of the low-pK lysyl residues shown by Passow et al. (1980) to be present in band 3. The α-amino group is acetylated in this protein, and its alkylation is very improbable. Also, Ramjeesingh et al. (1980) have shown that in the external face of band 3 there is a sulfhydryl group near the anion binding site, which is not reactive with *N*-ethylmaleimide. The strong negative charge of the sulfonic group present in 1,5-IAEDANS may direct this reagent to the anion binding site of band 3, producing an affinity labeling of the sulfhydryl group present there.

In the same fashion, the presence of especially reactive methionines near the binding site can produce labelings of these residues. Keller et al. (1982) have shown the existence of such reactive groups in calmodulin.

It should be stressed that there are no obvious groups which appear to be ready to react with the probe on the external side of band 3. This implies that the reactive site had unusual chemical characteristics, proposing a specificity of labeling. Very probably, only one site was coupled with 1,5-AEDANS.

The lifetimes of both the exofacially labeled ghosts and the purified band 3 solutions showed the presence of at least two components. Also, in both cases, the lifetimes appeared practically independent of temperature between 5 and 25 °C. This can be taken as an indication of shielding of the probe from the aqueous environment of the buffer (Kirby & Steiner, 1970). This is very consistent with the length of the longer lifetime component, which suggests a hydrophobic environment surrounding the probe. The shorter component can be explained only on the basis of quenching phenomena produced by collisions or charge-transfer reactions with nearby amino acid residues. Quenching by residual hemes in the various preparations seems to be ruled out by the presence of a very similar strong quenching in both the exofacially labeled ghosts and the band 3 extracted from them. For the same reason, the presence of oxygen radicals as quenchers appears to be improbable.

If the hypothesis of a single labeling site is true, the lifetime heterogeneity can be explained by the freedom of the probe to oscillate between different positions in times longer than the longest lifetime component, so as to spend a distinguishable amount of time in each of them.

Endofacial Labeling of Band 3. The cytoplasmic portion of band 3 carries five sulfhydryl groups, and no attempt was made to identify the position of the label. The lifetime was heterogeneous, and the stoichiometry of labeling was less than 1:1. However, in this case, the expected similar affinity of the various sulfhydryl groups for the label may have produced different substitutions in different molecules. Also, in these samples, the lifetimes were independent of temperature, suggesting shielding of the probe from the aqueous environment. This implies that also in this case the shorter lifetime components were due to quenching phenomena.

Correlation Times of Band 3. The anisotropy of the immobilized probe was measured in 100% glycerol at −2 °C. It was found to be 0.11. This low value was probably due to the broad-band selection of wavelengths in the excitation beam and to the nonselectivity of the cutoff filter in emission (Hudson & Weber, 1973). The initial anisotropy detectable in our samples was in all cases much lower, indicating a fast motion of the probe, with correlation times less than 1 ns. In all cases, the anisotropy decay showed the presence of a rotator with short correlation times, between 3 and 12 ns, much shorter than what is expected from a protein with molecular weight in excess of 100K.

The low value of the anisotropy initially detectable in our samples (A_0) suggests that the depolarization produced by the rotation of the probe per se was fully expressed during the "dead time" of the instrument. Also, the correlation times of a few nanoseconds detected in the samples were too long to be produced by a freely rotating probe. We cannot exclude the possiblity that these resulted from thermally dependent hindered rotation of the probe on the surface of the molecule. However, this appears to be improbable in view of the extensive depolarization already produced by the fast, nonmeasurable motion mentioned above. Therefore, the short correlation times probably reflected the presence of internal flexibility in band 3 detectable in both the exo- and endofacial sides of the molecule. These motions would be formally equivalent to those of spherical rotators of sizes between 30 and 100 amino acids in water.

Table II shows that the correlation times were sensitive to the presence of 300 mM NaCl, suggesting an involvement of that part of the molecule which participates in the binding of chloride. This hypothesis is supported by the observation that sodium acetate failed to modify the correlation times, consistent with the much slower, or absent, transport of acetate ions by band 3 (Gunn et al., 1975).

The mechanism by which the regulation occurs is not clear. At 5 °C, the presence of Cl^- ions retarded the movement for

endofacially labeled inside-out vesicles while at 25 °C it made it faster. It is possible that the higher fluidity of the lipid bilayer at this temperature had an effect on the motion of the protein.

In the exofacially labeled, Triton X-100 extracted band 3, the longer detectable correlation time was consistent with a dimeric state of the molecule, in the absence of Cl^- ions (Table II). These correlation times increased in the presence of 300 mM NaCl, well above the limits of the experimental errors, suggesting the formation of higher polymers. These observations are consistent with the findings of Dorst & Schubert (1979).

It is interesting to note that the correlation times reported in Table II were very little affected by temperature. This supports the hypothesis that the short correlation times reflect internal motions which are sensitive to NaCl but not to the bulk viscosity of the solvent. For the longer correlation time detectable in the exofacially labeled purified band 3, which reflects the rotation of the entire molecule, the effect of a decreasing viscosity of the solvent with temperature was masked by the noise of the measurements, due to the low values of the terminal slopes of the transients.

The presence of the lipid bilayer in the measurements performed on inside-out vesicles produced a nonzero anisotropy at long times ($A_\infty \neq 0$). This may be interpreted as restriction of the rotation of the protein around only one axis, perpendicular to the plane of the membrane in which it was embedded. Eventually, a complete depolarization must be produced by the rotation of the vesicles; however, this rotation (probably in the millisecond range) was too slow to be detectable in our measurements.

The total depolarization produced by isotropic rotators may be expressed as the product of the contributions of all rotational modes (Dale & Eisinger, 1975):

$$A = \prod_i d_i \tag{2}$$

where A is the detected anisotropy and

$$d_i = \tfrac{3}{2} \cos^2 \theta_i - \tfrac{1}{2} \tag{3}$$

Here, θ_i is the average value of the angular change in orientation of the emission dipole produced by the ith rotational mode. In eq 2, the initial term arises from the shift in orientation of the emission dipole with respect to the absorption dipole and is given by

$$d_0 = 0.4(\tfrac{3}{2} \cos^2 \theta_0 - \tfrac{1}{2}) \tag{4}$$

If the fluorophore is completely immobilized, as in a rigid medium at low temperature, then d_0 is the only term contributing to A in eq 2.

When the fluorophore is a fluorescent label attached to a protein with organized structure, a frequently encountered case is that for which the anisotropy as a function of time may be represented by

$$A(t) = A_1 e^{-t/\phi_1} + A_2 e^{-t/\phi_2} \tag{5}$$

Here, ϕ_1 is a short correlation time corresponding to a rapid localized motion of the fluorophore, which is restricted in range so as to be confined to the surface of a cone of semiangle θ_1'; ϕ_2 is the correlation time reflecting the overall motion of the protein. A_1 and A_2 are the amplitudes corresponding to the two rotational modes. For this case, we have (Kinosita et al., 1977; Dale & Eisinger, 1975)

$$A_2/A_0 = \tfrac{1}{4}(3 \cos^2 \theta_1' - 1)^2 \tag{6}$$

where A_0 ($=A_1 + A_2$) is the limiting anisotropy at zero time. If a third rotational mode of the fluorophore exists which is too rapid to detect, then A_0 will differ from the anisotropy of a completely immobilized fluorophore by the factor $\tfrac{3}{2} \cos^2 \theta_1 - \tfrac{1}{2}$ where θ_1 is the change in orientation of the emission dipole produced by the very rapid rotation.

Table III: Values of the Semiapex Angle of the Cone of Rotation of Labeled Band 3 Protein As Computed from Equations 6 and 8

sample	temp (°C)	[NaCl] (mM)	θ_1' (deg)	θ_1^* (deg)
exo	5, 25	0	18.4	
	5, 25	300	16.7	
endo	5	0		18.0
	5	300		15.2
	25	0		22.5
	25	300		19.5

A second case of interest is that for which the labeled protein is embedded in a lipid bilayer which constrains its rotation to a single axis perpendicular to the plane of the bilayer. In this case, eq 5 has the form, if localized rotation of the label is neglected (Heyn et al., 1977; Rigler & Ehrenberg, 1976)

$$A(t) = A_1 e^{-t/\phi_1} + A_2 \tag{7}$$

and

$$A_2/A_0 = \tfrac{1}{4}(3 \cos^2 \theta_1^* - 1)^2 \tag{8}$$

Here, θ_1^* is the angle between the emission dipole and the axis about which rotation is confined (assuming that the absorption and emission dipoles are coincident). Also, in this case, $A_0 = A_1 + A_2$ is the detectable anisotropy at zero time. If localized motion of the label is present, θ_1^* will correspond to an average.

For the Triton X-100 solubilized band 3 protein, eq 6 may be used to compute an apparent value of θ_1', which provides a measure of the angular range accessible to the localized rotational mode (Table III). At both 5 and 25 °C, the presence of 300 mM NaCl results in a decrease in the magnitude of θ_1'. This is consistent with a restriction of the motion of the label produced by Cl^- ion. This in turn implies the existence of a specific domain in band 3 protein whose motions are sensitive to Cl^- ions, possibly corresponding to the binding site on the protein.

In the case of the endofacially labeled band 3 protein present in the inside-out vesicles, eq 8 was used to compute θ_1^*. The apparent angle formed by the emission oscillator with the axis of rotation is likewise sensitive to the presence of NaCl. However, the computed values are probably influenced by any localized rotation involving the probe.

It should be stressed that as shown in Table III, all of the average angles defined by the motions associated with the shorter correlation times consistently decreased in the presence of NaCl. The inability of sodium acetate to reproduce those data suggests that those motions originate from domains involved in the functional activity of the molecule.

On a speculative ground, it is interesting to note the large sizes of the rotational cones listed in Table III. It is very consistent with the function of the molecule which has to "move" internally in order to transport ions from one end to the other of itself.

Circular Dichroism. Consistent with the data in regard to correlation time, the optical activity of the solubilized system appeared to be sensitive to the presence of NaCl. The ellipticity at 222 nm suggested the presence of about 40% α-helix in the protein.

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