Effect of Membrane Potential on Band 3 Conformation in the Human Erythrocyte Membrane Detected by Triplet State Quenching Experiments†

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ABSTRACT: The triplet lifetime and absorption anisotropy decay of eosin-labeled band 3 was measured in resealed erythrocyte ghosts. Membrane potentials were generated by the addition of valinomycin in the presence of a K^+ gradient. Neither negative nor positive membrane potentials had any detectable effect on the rotational diffusion of band 3 nor on the eosin triplet lifetime. The membrane potential did, however, affect quenching of the eosin triplet state by I^- and TEMPO (2,2,6,6-tetramethylpiperidine-*N*-oxyl). Quenching was enhanced by a negative membrane potential (negative inside) and reduced by a positive membrane potential. In addition, it was found that a negative membrane potential enhanced the efficiency of eosin labeling of band 3 in intact erythrocytes. A positive membrane potential had the opposite effect. These results indicate that the eosin binding site on band 3 becomes more accessible to the extracellular aqueous phase in the presence of a negative membrane potential and less accessible in the presence of a positive membrane potential. Quenching by I^- and TEMPO of the triplet state of eosin-labeled band 3 was further investigated as a function of pH. Quenching by TEMPO and its dependence on membrane potential were relatively insensitive to pH. In contrast, the rate of quenching by I^- showed a marked decrease over the range pH 5.5–9.5. Moreover, the effect of a negative membrane potential on I^- quenching also varied with pH. These results are discussed on the supposition that the eosin probe is located in the anion access channel of band 3. It is proposed that the membrane potential influences both the conformation of band 3 and the p*K*s of ionizable groups in the vicinity of the eosin probe.

Electric potential gradients exist across the membranes of essentially all living cells. Typical membrane potentials are in the range of −50 to −100 mV although larger potentials are also observed, for example, −179 mV across the plasma membrane of *Acetabularia acetabulum* and up to −200 mV across inner mitochondrial membranes (O'Shea, 1988). Such membrane potentials correspond to electric field strengths in the order of 10^7 V/m, which is of sufficient magnitude to potentially influence the conformations of membrane constituents. The opening and closing of ion channels in response to changes of membrane potential is fundamental to the mechanism of electrically excitable membranes. In addition, electric fields have been observed to have a variety of functional effects on membranes, as reviewed by Tsong (1990). The phenomena of electrofusion and electroporation have also been the subject of detailed investigation (Zimmermann, 1982).

Although effects of membrane potential on membrane function are well documented, there are relatively few reports of the influence of the electric field on the conformation of membrane constituents, especially membrane proteins. In the case of lipids, effects are anticipated as a consequence of the compressive force which occurs when an electric field is applied across a dielectric (White, 1970). It has in fact been shown (Corda et al., 1982; O'Shea et al., 1984) that membrane potentials increase the fluorescence polarization of the lipid probe 1,6-diphenyl-1,3,5-hexatriene incorporated into phosphatidylcholine bilayers while similar effects have recently been seen in plasma membranes of mouse thymus cells (Lakos et al., 1990). Membrane potentials have been observed to influence the rates of conformational changes in bacteriorhodopsin and other bacterial rhodopsins (Manor et al., 1988) and in (Na,K)-ATPase (Rephaeli et al., 1986; Tsong, 1990). In the case of bacteriorhodopsin, changes in optical absorption (Tsuji & Hess, 1986) and circular dichroism spectra (Brumfeld & Miller, 1988) are induced by an electric field. Jona and Martonosi (1986) have used fluorescence to detect conformational changes of Ca-ATPase arising from changes in membrane potential.

In the present study, we have investigated the effects of membrane potential on the conformation of the erythrocyte anion transporter, band 3. The ability to specifically label this protein with the triplet probe eosin-5-maleimide has a number of advantages for such an investigation. Triplet states are very sensitive to quenching reactions, and hence the accessibility of the probe to aqueous phase quenchers provides a means to monitor conformational changes. Moreover, transient dichroism or phosphorescence anisotropy decay measurements permit rotational diffusion to be monitored (Austin et al., 1979; Cherry et al., 1976a; Moore et al., 1979; Nigg & Cherry, 1979). The rotational diffusion of band 3 in the erythrocyte membrane is partly determined by protein–protein interactions (Nigg & Cherry, 1979, 1980) which could be altered by electric field induced conformational changes. Finally, erythrocyte ghosts are easily resealed and membrane potentials generated by the addition of ionophores in the presence of appropriate ion gradients (Sims et al., 1974). Thus eosin-labeled band 3 in resealed ghosts represents a convenient system for studying electric field effects on a membrane protein.

Materials and Methods

Preparation of Eosin-Labeled Resealed Ghosts. Band 3 was selectively labeled by incubating intact erythrocytes from fresh human blood with eosin-5-maleimide (Molecular Probes Inc.) as described by Nigg and Cherry (1979). The cells were then hemolyzed in 20–30 volumes of 4 mM HEPES,[1] pH 7.5.

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A small volume of concentrated salt solution (3 M KCl or 3 M NaCl) was subsequently added to restore isotonicity. For some experiments, KI was also added to a final concentration of 10 mM. After stirring for 5 min, the ghosts were resealed by incubating at 37 °C for 45 min, as described by Richards and Eisner (1982). The resealed ghosts were recovered by centrifugation (17 000 rpm) and washed a further three times in 10 mM HEPES and 150 mM KCl or 150 mM NaCl. All steps except resealing were performed at 0–4 °C. The final molar labeling ratio eosin:band 3 was normally (0.8–1):1.

The efficiency of resealing was checked in separate experiments by incorporating the fluorescent probe calcein inside the ghosts. The addition of $CoCl_2$ enabled the percentage of ghosts impermeable to Co^{2+} ions be determined from the decrease in calcein fluorescence. Co^{2+} forms complexes with calcein and efficiently quenches its fluorescence (Oku et al., 1982).

Generation and Detection of Membrane Potentials. Membrane potentials were generated by suspending the resealed ghosts in media of varying K^+ concentration and adding the ionophore valinomycin. For negative potentials (negative inside), ghosts containing 150 mM KCl were suspended in 150 mM NaCl and 4 mM HEPES, pH 7.5. For positive potentials, ghosts containing 150 mM NaCl were suspended in 150 mM KCl and 4 mM HEPES, pH 7.5. The addition of valinomycin was accomplished by adding 1 μL/mL of ghost suspension of a 1 mM solution of the ionophore in DMSO.

Membrane potentials were detected using the potential-sensitive fluorescent dye 3,3′-dipropylthiadicarbocyanine iodide [diS-C_3-(5)] (Sims et al., 1974). The fluorescent emission of the dye was recorded at 665 nm with a Baird Nova spectrofluorimeter. The excitation wavelength was 630 nm. In a typical experiment, 2 μL of a 0.2 μM solution of the dye in DMSO was added to a fluorimeter cell containing 2 mL of the suspension medium. The fluorescence intensity was continuously recorded while adding first the resealed ghosts and subsequently valinomycin. These measurements were performed on the same sample preparations as used for the transient dichroism and triplet lifetime measurements.

The fluorescence changes were calibrated by entrapping 150 mM K-HEPES inside resealed ghosts and suspending them in buffers containing x mM K-HEPES, y mM Na-HEPES where $x + y = 150$. The potassium diffusion potential created by the addition of valinomycin was calculated from the Nernst equation.

Rotational Diffusion and Triplet Lifetime Measurements. The transient dichroism apparatus used to measure rotational motion was similar to that described in detail elsewhere (Cherry, 1978). Excitation was by a Nd–YAG laser (JK Lasers, Ltd.) using the frequency-doubled emission at 532 nm. The pulse width was about 15 ns and the repetition rate 10 Hz. Transient absorbance changes at time t after the flash arising from ground-state depletion were simultaneously recorded at 515 nm for light polarized parallel [$A_\parallel(t)$] and perpendicular [$A_\perp(t)$] with respect to the polarization of the exciting flash. Up to 512 signals were averaged in a Datalab DL 102A signal averager. Data were analyzed and plotted by calculating the absorption anisotropy, $r(t)$, defined by

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

The anisotropy decay curves were fitted by the double-exponential equation

$$r(t) = r_1 \exp(-t/\phi_1) + r_2 \exp(-t/\phi_2) + r_3 \quad (2)$$

using a nonlinear least-squares analysis. The total absorbance change $A(t)$ is proportional to the concentration of probe molecules in the triplet state and is given by

$$A(t) = A_\parallel(t) + 2A_\perp(t) \quad (3)$$

The triplet lifetimes were determined by fitting $A(t)$ by the equation

$$A(t) = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2) \quad (4)$$

The mean triplet lifetime τ was calculated according to

$$\tau = \frac{A_1\tau_1 + A_2\tau_2}{A_1 + A_2} \quad (5)$$

Data analysis and interpretation are further discussed by Nigg and Cherry (1979) and by Matayoshi and Jovin (1991).

All samples were flushed with argon prior to measurement to obviate quenching of the eosin triplet state by oxygen. The eosin concentration was typically 1–2 μM, and all experiments were performed at 37 °C unless otherwise stated.

Measurements of anisotropy decay and eosin triplet lifetime were performed on the same sample of resealed ghosts in the absence and presence of a membrane potential generated by the addition of valinomycin. The effects of the quenchers I^- and TEMPO on the triplet lifetime were investigated with varying concentrations of the quenchers present in the suspension medium. In the case of I^-, the ionic concentration was maintained at 150 mM by reducing the NaCl or KCl concentration by the appropriate amount. Quenching experiments were analyzed according to the Stern–Volmer equation

$$\tau_0/\tau = 1 + k_q\tau_0[Q] \quad (6)$$

where τ_0 is the triplet lifetime in the absence of quencher, k_q is the quenching rate constant, and [Q] is the quencher concentration. Some experiments were also performed with 10 mM KI trapped inside the resealed ghosts.

Labeling Intact Erythrocytes with Eosin in the Presence of a Membrane Potential. Erythrocytes were washed three times in either 4 mM HEPES and 150 mM NaCl or 4 mM HEPES and 150 mM KCl, depending upon whether a negative or positive potential was to be generated. The erythrocytes were then diluted 1:1 (v/v) in the same buffer, and valinomycin was added (4 μL/mL of cells of a 1 mM solution in DMSO). Immediately after the addition of valinomycin, eosin-5-maleimide was added (1 mg/4 mL of cells). After 35 min, 1 mL of the sample was removed and layered gently upon 400 μL of silicone oil. The cells were spun down on a bench centrifuge, and the excess eosin was removed from the surface. The oil was then carefully pipetted off, and the labeled cells were removed and lysed in 5 mM sodium phosphate (pH 7.4). Ghosts were then prepared by centrifuging the lysed cells at 17 000 rpm.

Protein was determined by the method of Lowry et al. (1951) after solubilizing ghosts in 1% SDS, and the eosin concentration was determined by measuring absorbance at 531 nm. The eosin:band 3 ratio was calculated assuming band 3 accounted for 25% of the total membrane protein and taking the extinction coefficient for bound eosin to be 83 000 M^{-1} cm^{-1} (Cherry et al., 1976b).

Results

Generation of a Membrane Potential. Figure 1 shows fluorescence detection of membrane potentials created in

[1] Abbreviations: diS-C_3-(5), 3,3′-dipropylthiadicarbocyanine iodide; DMSO, dimethyl sulfoxide; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl.

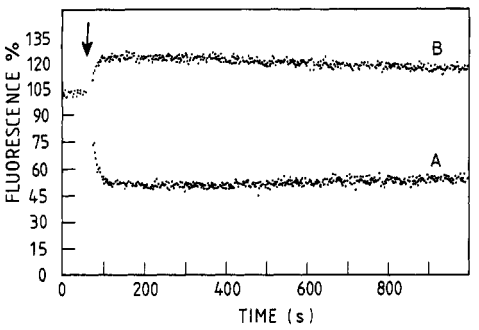

FIGURE 1: Fluorescence detection by DiS-C_3-5 of membrane potential in resealed ghosts. (A) 150 mM NaCl outside and 150 mM KCl inside (negative potential). (B) 150 mM KCl outside and 150 mM NaCl inside (positive potential). The arrow indicates time of addition of valinomycin. The temperature was 20 °C.

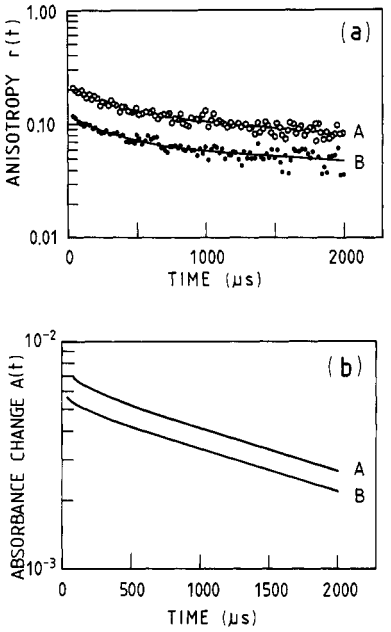

FIGURE 2: (a) Effect of membrane potential on anisotropy decay of eosin-labeled band 3 in resealed ghosts at 37 °C. For clarity, the lower curve has been displaced vertically. (A) No potential. (B) Negative potential. (b) Effect of membrane potential on triplet lifetime of eosin-labeled band 3 in resealed ghosts at 37 °C. (A) No potential. (B) Negative potential.

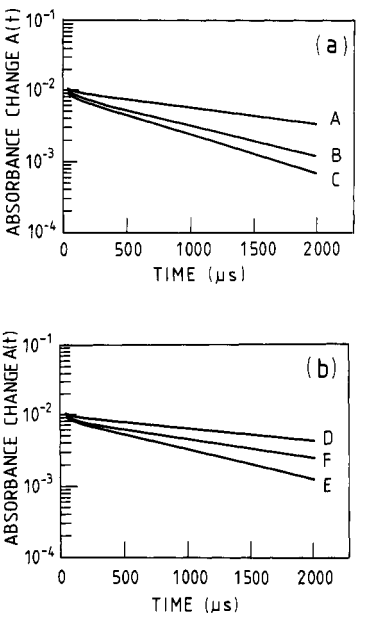

FIGURE 3: Effect of membrane potential on quenching by I^- of the triplet state of eosin-labeled band 3 in resealed ghosts at 37 °C. (a) Negative potential. (b) Positive potential. (A) 150 mM KCl inside and 150 mM NaCl outside. (B) As curve A but with 140 mM NaCl + 10 mM KI outside. (C) As curve B but with addition of valinomycin. (D) 150 mM NaCl inside and 150 mM KCl outside. (E) As curve D but with 140 mM KCl + 10 mM KI outside. (F) As curve E but with addition of valinomycin.

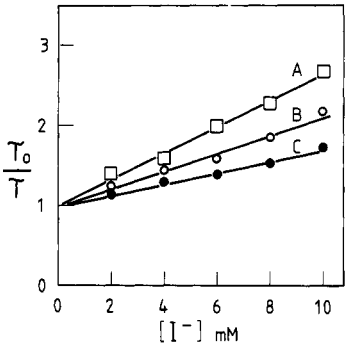

FIGURE 4: Stern–Volmer plots for I^- quenching of the triplet state of eosin-labeled band 3 in resealed ghosts at 37 °C. (A) Negative potential; (B) no potential; (C) positive potential.

resealed ghosts by the addition of valinomycin in the presence of K^+ gradients. The dissipation of the potential was temperature dependent, becoming markedly more rapid above 30 °C. However, under all experimental conditions used for measuring anisotropy decays and triplet lifetimes, the maximum value of membrane potential was essentially maintained for at least 5 min. Calibration of the fluorescence changes indicated that the membrane potentials generated were about ±90 mV. According to the calcein quenching assay, 65–70% of ghosts were tightly resealed such that they were effectively impermeable to Co^{2+}.

Labeling band 3 with eosin-5-maleimide did not affect the magnitude or duration of the membrane potential generated, provided care was taken not to expose the eosin-labeled ghosts for long periods to light. Illumination increased the leakiness of the membranes, probably as a result of eosin-catalyzed photooxidation of membrane constituents (Pooler & Girotti, 1986). Displacing air in the sample with argon eliminated the effect. It was also found that the application of the potential did not have any observable effect on the fluorescence intensity of the eosin probe.

Effect of Membrane Potential on Rotational Diffusion and Triplet Lifetime of Eosin-Labeled Band 3. The effect of a negative membrane potential on the anisotropy decay of eosin-labeled band 3 in resealed ghosts is shown in Figure 2a and on the triplet-state decay in Figure 2b. Data for positive potentials are essentially the same. Analysis of the data showed that the rotational correlations times were indistinguishable from those previously reported (Nigg & Cherry, 1979) irrespective of the presence or absence of a membrane potential. The mean eosin triplet lifetimes were 1270 ± 50 μs in the absence of a membrane potential, 1210 ± 60 μs with a negative membrane potential, and 1230 ± 40 μs with a positive membrane potential present (mean and SD of three measurements).

Effect of Membrane Potential on Quenching of Eosin Triplet State. Figure 3 shows the effect of 10 mM KI on the lifetime of the eosin triplet state in resealed ghosts without a potential present, and with positive or negative potentials generated. The data were fitted to a double-exponential decay as described under Materials and Methods. Mean lifetimes were calculated for various concentrations of quencher and

Table I: Rate Constants for Quenching by I^- and TEMPO of the Eosin Triplet State of Eosin in Solution and Bound to Band 3 in Resealed Ghosts[a]

	I^- $k_q \times 10^{-4}$ (M^{-1} s^{-1})	TEMPO $k_q \times 10^{-6}$ (M^{-1} s^{-1})
eosin in solution	150 (28)	1960 (110)
resealed ghosts		
no potential	4.3 (0.1)	3.3 (0.1)
positive potential	2.2 (0.1)	1.7 (0.2)
negative potential	7.1 (0.1)	5.3 (0.2)

[a] Errors are given in parentheses.

Table II: Eosin:Protein Labeling Ratios for Erythrocytes Labeled for 35 min at Room Temperature[a]

	mole ratio (eosin:protein)
control	0.75:1
positive potential	0.4:1
negative potential	1.2:1

[a] Positive and negative potentials were generated by the addition of valinomycin to cells suspended in 150 mM KCl and 150 mM NaCl, respectively. For the control sample, cells were suspended in 150 mM NaCl without addition of valinomycin.

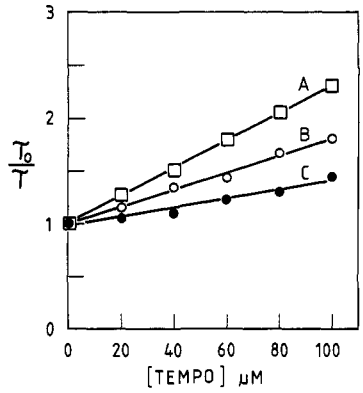

FIGURE 5: Stern–Volmer plots for TEMPO quenching of the triplet state of eosin-labeled band 3 in resealed ghosts at 37 °C. (A) Negative potential; (B) no potential; (C) positive potential.

used to construct the Stern–Volmer plots shown in Figure 4. A Stern–Volmer plot was also obtained for quenching by I^- of eosin maleimide free in solution (4 mM HEPES, 150 mM KCl). The k_q values obtained from the Stern–Volmer plots are given in Table I.

Similar experiments were performed with the spin label quencher TEMPO. Stern–Volmer plots are shown in Figure 5, and values of k_q are given in Table I. From Figures 4 and 5 and Table I, it is clear that for both quenchers a positive potential decreases the rate of quenching while a negative potentials increases the rate. The addition of valinomycin in the absence of a K^+ gradient had no effect on quenching rates.

Effect of Membrane Potential on Eosin Labeling of Band 3. Table II shows the results of experiments in which intact erythrocytes were labeled with eosin maleimide in the presence or absence of K^+ diffusion potentials. The labeling conditions in the absence of a K^+ potential resulted in an eosin:band 3 stoichiometry of <1, which permitted both increases and decreases in labeling ratio to be detected. The decay of the membrane potential after addition of valinomycin is much slower in intact cells than in resealed ghosts so that the potential was maintained for the duration of the labeling.

Table II shows that cells labeled under a negative potential had an increased eosin:band 3 ratio when compared to control cells. Cells labeled when the potential was positive had a decreased eosin:band 3 ratio. SDS gels revealed that essentially all of the label in each case was associated with band 3. There is very probably only one eosin binding site per band 3 monomer. The slightly higher stoichiometry measured in the presence of a negative membrane potential may reflect uncertainties in the parameters used to calculate the eosin:band 3 ratio.

Table III: Eosin Triplet-State Lifetimes for Resealed Ghosts with I^- Inside or Outside[a]

	lifetime (μs)		
	10 mM KI outside	10 mM KI inside	No KI
no potential	840 (36)	1300 (47)	1270 (48)
negative potential	690 (68)	1140 (59)	1210 (59)

[a] For I^- inside, ghosts containing 140 mM KCl + 10 mM KI were suspended in 150 mM NaCl. For I^- outside, ghosts containing 150 mM KCl were suspended in 140 mM NaCl + 10 mM KI. Values are mean and standard deviation from three data sets.

Table IV: Effect of pH on the Rate Constants for Quenching by I^- and TEMPO of the Triplet State of Eosin-Labeled Band 3 in Resealed Ghosts in the Absence and Presence of a Negative Membrane Potential[a]

	no potential $k_q \times 10^{-4}$ (M^{-1} s^{-1})	negative potential $k_q \times 10^{-4}$ (M^{-1} s^{-1})	R
TEMPO			
pH 5.5	300 (20)	530 (30)	1.8 (0.2)
pH 7.5	330 (40)	590 (70)	1.8 (0.3)
pH 9.5	150 (50)	290 (60)	1.9 (0.7)
iodide			
pH 5.5	11.7 (3.1)	33.7 (2.8)	2.9 (0.8)
pH 7.5	5.5 (0.4)	8.0 (0.4)	1.5 (0.1)
pH 9.5	4.6 (0.2)	3.2 (0.3)	0.7 (0.1)

[a] Errors are given in parentheses. R is the ratio of rate constants in the presence and absence of a negative membrane potential.

Quenching Effects of I^- Inside Resealed Ghosts. Table III presents the results of eosin triplet lifetime measurements in which 10 mM KI was entrapped inside resealed ghosts prior to the generation of a membrane potential. Mean lifetimes are given and compared with values obtained from experiments with the same concentration of KI outside the ghosts. No significant quenching by internal I^- was detected in the absence of a membrane potential. In the presence of a negative membrane potential, a small change was recorded which was, however, within the limits of experimental uncertainty.

I^- did not significantly leak out of the resealed ghosts. This was checked by centrifuging the resealed ghosts after the triplet lifetime measurement had been performed and analyzing the supernatant for I^- on a Dionex ion exchanger. The sensitivity of this machine for I^- was 1 ppm ($\equiv 5 \times 10^{-5}$M). No I^- was detected on the ion exchanger. The ratio of the entrapped volume to the total volume was 1:20, so that total leakage of 10 mM I^- would have produced a concentration of 5×10^{-4}M.

Effect of pH. Quenching of the triplet-state of eosin-labeled band 3 by I^- and TEMPO was measured at pHs of 5.5, 7.5, and 9.5 in resealed ghosts. Quenching rate constants in the presence and absence of a negative membrane potential were calculated from the slopes of Stern–Volmer plots and are presented in Table IV. The Stern–Volmer plots were linear in all cases. Quenching of eosin free in solution was found to be independent of pH over the range investigated.

Discussion

Generation of Membrane Potentials. It is well established that fluorescence of the dye diS-C_3-(5) responds to changes in membrane potential (Sims et al., 1974; Waggoner, 1979). The results in Figure 1 clearly show that ghosts have been resealed and that membrane potentials are generated upon the

addition of valinomycin in the presence of a K^+ gradient. According to the calcein quenching assay, up to 35% of resealed ghosts may be permeable to small ions. Since the calibrations of the dye fluorescence changes were performed under identical conditions, the estimate of a membrane potential of ±90 mV should apply to the tightly sealed ghosts. If no potential is generated across the remaining ghost membranes, any observed effect of membrane potential will be somewhat underestimated. The membrane potential eventually dissipates due to the finite permeability of the membrane to other ions. Under all experimental conditions used in this study, the potential was effectively constant over 5 min after the addition of valinomycin, which was ample time for the collection of spectroscopic data.

Effect of Membrane Potential on Band 3 Rotational Mobility and Eosin Triplet Lifetime. Rotational diffusion of membrane proteins is very sensitive to protein aggregation. Band 3 in erythrocyte membranes as well as in reconstituted systems appears to be rather susceptible to self-association as a function of temperature, pH, or cholesterol content of the membrane (Cherry et al., 1976a; Dempsey et al., 1986; Mühlebach & Cherry, 1985; Nigg & Cherry, 1979). It is thus quite conceivable that if an electric field induced a conformational change of band 3, then this would cause protein aggregation which would be readily detectable in a rotational diffusion experiment. No measurable change in band 3 rotational mobility was, however, observed. Alternatively, a conformational change might cause a change in the triplet lifetime of the eosin probe. Again, no effect due to the presence of a membrane potential was detected.

These results are at variance with preliminary data which we previously reported (Cherry, 1986). The discrepancy is due to the inadvertant admittance of oxygen to the cuvette when valinomycin was injected in these earlier experiments. If oxygen is rigorously excluded, the results shown in Figure 2 are obtained. The controls for these earlier experiments appeared satisfactory because the small amount of oxygen involved had no effect in the absence of a membrane potential. Thus, fortuitously, we were led to the conclusion that the accessibility of the eosin probe to quenchers was changed by a membrane potential. To study this effect in detail, we switched to using I^- and TEMPO as quenchers as their concentration is more easily controlled than that of oxygen.

Effect of Membrane Potential on Band 3 Conformation. Before discussing the present results further, it is useful to consider the likely location of the eosin probe on band 3. Jennings (1989) has recently summarized the evidence that band 3 contains an outward-facing access channel leading to the anion translocation site. Part of this evidence rests on experiments with anion transport inhibitors, which include eosin maleimide and stilbene disulfonates. Resonance energy transfer and fluorescence quenching experiments indicate that the binding site(s) for these inhibitors lie(s) at a significant distance from the exofacial surface of band 3 (Macara et al., 1983; Rao et al., 1978). The present studies show that eosin maleimide bound to band 3 is strongly protected from the triplet quenchers I^- and TEMPO. Quenching rate constants are reduced about 40 times for I^- and about 600 times for TEMPO when eosin is bound to band 3. These findings are also consistent with the eosin binding site being in a cleft in the protein. The simplest supposition is that this cleft corresponds to the access channel for anions. It does not necessarily follow that the binding site for eosin maleimide corresponds to the anion translocation site, and indeed NMR evidence suggests that some inhibitors may block the channel without competing for the translocation site (Falke & Chan, 1986). Recent studies by Cobb and Beth (1990) have established that Lys 430 is labeled by eosin maleimide although this in itself does not establish the location of the probe in the channel.

The present studies reveal a number of effects of membrane potential on eosin-labeled band 3. Quenching of the eosin triplet state by both I^- and TEMPO is enhanced by a negative potential and diminished by a positive potential (Table I and Figures 3–5). Membrane potential also influences the efficiency of labeling band 3 with eosin malemide (Table II). These results are most simply explained by supposing that the membrane potential influences the conformation of band 3 in the region of the access channel. In the case of the uncharged quencher TEMPO, it is very likely that its access to the eosin probe is mainly determined by the size and flexibility of the channel. Access is increased by a negative potential and decreased by a positive potential.

Since eosin is negatively charged, it could perturb the forces experienced by band 3 in an electric field. Moreover, there is some evidence that anion transport inhibitors themselves cause a conformational change (Macara et al., 1983). To investigate whether the membrane potential can affect the conformation of unmodified band 3, we examined how the potential affects the labeling efficiency by eosin maleimide. We found that band 3 is more efficiently labeled in the presence of a negative membrane potential and less efficiently labeled in the presence of a positive potential, compared with control cells (Table II). This is compatible with the binding site becoming more or less accessible to the eosin probe and is entirely consistent with the results of the quenching experiments described above.

A conformational change could conceivably be mediated by the lipid bilayer. Membrane potentials compress the lipid bilayer and produce changes in "fluidity" which are detected by fluorescent lipid probes (Corda et al., 1982; O'Shea et al., 1984). The conformation of band 3 could be sensitive to these changes in lipid packing. However, the effects of membrane potential on lipids are rather small. The change in "fluidity" has been estimated to be equivalent to a 1–2 °C change in temperature (O'Shea et al., 1984). Such a small change would have an insignificant effect on quenching rate constants. Moreover, the effects of membrane potential on lipids are always observed to by symmetrical with respect to the polarity of the potential. In contrast, Table I shows that quenching of the eosin triplet state is asymmetric. A negative membrane potential increases the quenching rate constant whereas a positive potential decreases it. It thus appears that the membrane potential acts directly on band 3.

Quenching from the Cytoplasmic Side. In the quenching experiments so far discussed, the quencher was present on the outside of resealed ghosts. To assess whether the eosin probe is accessible from the cytoplasmic side, I^- was sealed inside ghosts. In the absence of a membrane potential, no significant quenching by internal I^- was detected (Table III). The eosin probe thus appears to be only accessible to quencher from the outside. This is the reverse of the finding by Macara et al. (1983), who found that eosin fluorescence is quenched by Cs^+ only from the inside. Possibly the positively charged Cs^+ ion is excluded from the exofacial access channel. However, it is unclear why Cs^+ but not I^- should quench from the inside. It may be relevant that Cs^+ appears to quench by a static rather than a collisional mechanism.

In the presence of a negative membrane potential, internal I^- caused a small change in the eosin triplet lifetime which was in the same direction as that observed with the quencher

outside. As the change is close to the limit of experimental error, we are uncertain whether or not there is a genuine effect. If there is, it is not due to I^- leaking out of the ghosts. Analysis showed that, at the end of the quenching experiment, the concentration of I^- in the external medium was $<5 \times 10^{-5}$ M. From Figure 4 it is clear that this upper limit is too small to have any effect on the eosin triplet lifetime.

Effects of pH. In order to try to gain further insight into the mechanism of the membrane potential effect, experiments were performed at different pH. In the case of the uncharged quencher TEMPO, the effect of the membrane potential was effectively constant over the pH range 5.5–9.5. As shown in Table IV, the ratio (R) of quenching rate constants in the presence and absence of a negative membrane potential is slightly less than 2 over this range. We interpret this lack of pH dependence as indicating that the membrane potential produces a conformational change by acting either on charged groups with pKs outside this range or on helix dipoles.

The pH dependence of I^- quenching is more complex. In the absence of a membrane potential, there is a strong enhancement of quenching at acidic pH (Table IV) which is not present for TEMPO. This could be explained by the presence of a titratable group or groups in the vicinity of the eosin probe. Increased positive charge (or decreased negative charge) at acidic pH could increase the local concentration of I^- and hence increase its rate of quenching. However, additional mechanisms are needed to explain the effects of membrane potential. In contrast to quenching by TEMPO, the parameter R increases with decreasing pH. A number of mechanisms can be envisaged which would effect quenching by charged but not by uncharged quenchers. These would most likely involve an effect of membrane potential on the pKs of one or more titratable group in the vicinity of the eosin probe. Consequent changes in their charge would then affect the local concentration of I^-. It seems unlikely that the two acidic groups on eosin itself are involved since their pKs are below pH 3.5 in aqueous solution (Grzywacz & Smagowski, 1965), although these pKs could conceivably be changed by the protein environment.

Thus our interpretation of the data in Table IV is that the membrane potential has two separate effects. First, it induces a change in band 3 conformation which is responsible for the change in quenching of the eosin triplet state by TEMPO. Additionally, it changes the pKs of groups near and possibly on the eosin probe which further affect the rate constant for I^- quenching.

Our primary aim in these studies was to demonstrate the applicability of triplet state spectroscopy to the study of protein conformational changes induced by a membrane potential. Since the eosin probe inhibits anion transport (Nigg & Cherry, 1979), we cannot investigate whether most of our findings apply to band 3 in a functional state. (The observation that the labeling efficiency of band 3 by eosin maleimide is dependent on membrane potential is, however, an intrinsic property of the protein.) Although membrane potential is believed not to influence the anion translocation event (Gunn & Fröhlich, 1979; Milanick & Gunn, 1984; Wieth et al., 1980), Jennings and co-workers (Jennings, 1989; Jennings et al., 1990) have recently detected an effect of membrane potential on the $k_{1/2}$ for extracellular SO_4^{2-} influx in human erythrocytes. They propose that the membrane potential is in part experienced in the access channel of band 3, thus affecting the concentration of SO_4^{2-} ions at the base of the access channel. Subject to the reservation of possible perturbation by the eosin probe, the present experiments suggest that the potential could also influence band 3 conformation and possibly the pKs of ionizable groups in the anion access channel.

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ATP-Sensitive K^+ Channels in Insulinoma Cells Are Activated by Nonesterified Fatty Acids†

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ABSTRACT: Both $^{86}Rb^+$ efflux experiments and electrophysiological studies have shown that arachidonic acid and other nonesterified fatty acids activate ATP-sensitive K^+ channels in insulinoma cells (HIT-T15). Activation was observed with arachidonic, oleic, linoleic, and docosahexaenoic acid but not with myristic, stearic, and elaidic acids. Fatty acid activation of ATP-sensitive K^+ channels was blocked by antidiabetic sulfonylureas such as glibenclamide. The activating effect of arachidonic acid was unaltered by indomethacin and by nordihydroguaiaretic acid, indicating that it is not due to metabolites of arachidonic acid via cyclooxygenase or lipoxygenase pathways. Moreover, the nonmetabolizable analogue of arachidonic acid, eicosatetraynoic acid, was an equally potent activator. Activation of ATP-sensitive K^+ channels by fatty acids was potentiated by diacylglycerol and was inhibited by calphostin C, an inhibitor of protein kinase C. These findings indicate that fatty acid activation of ATP-sensitive K^+ channels is most likely due to the participation of arachidonic acid (and other fatty acid)-activated protein kinase C isoenzymes. Activation of ATP-sensitive K^+ channels by nonesterified fatty acids is not involved in the control of insulin secretion since arachidonic acid stimulates insulin secretion from insulinoma cells instead of inhibiting it.

ATP-sensitive K^+ (K_{ATP})[1] channels play a key role in insulin secretion from pancreatic β-cells (Ashcroft, 1988). Exposure of β-cells to glucose induces closure of K_{ATP} channels via a variation of the intracellular ATP/ADP ratio and thereby a depolarization which leads to activation of Ca^{2+} channels, elevation of intracellular Ca^{2+}, and insulin secretion (Dunne & Petersen, 1991). K_{ATP} channels are specifically blocked by sulfonylureas such as glibenclamide which are drugs capable of restoring insulin secretion in patients affected by non-insulin-dependent diabetes (Schmid-Antomarchi et al., 1987). They are activated by hormones such as somatostatin and galanin which inhibit insulin secretion (De Weille et al., 1988, 1989; Dunne et al., 1989).

Arachidonic acid (AA) and its metabolites are known to be modulators of K^+ channels in several cell types. For example, 5-lipoxygenase-derived metabolites of AA (leukotriene B_4 and C_4) modulate the activity of muscarinic cardiac K^+ channels (Kim & Clapham, 1989; Kim et al., 1989; Kurachi et al., 1989), while AA derivatives from the 12-lipoxygenase pathway (12-HPETE) act directly on K^+ channels of the S-type in nervous cells from *Aplysia* (Belardetti et al., 1987, 1989; Piomelli et al., 1987). A class of K^+ channels from smooth muscle cells is activated directly by nonesterified fatty acids (NEFAs) (Ordaway et al., 1989).

Experimental Procedures

Materials. Indomethacin, NDGA, 1,2-dioleoylglycerol (DOG), 1-oleoyl-2-acetylglycerol (OAG), oligomycin, penicillin, polyornithine, streptomycin, trypsin, and all NEFAs with the exception of ETYA (Fluka, Mulhouse, France) were from Sigma (L'Isle d'Abeau, France). Calphostin C was from Kamiya Biomedical Co. (Thousand Oaks, CA). Ham's F12k medium was from Gibco (Paisley, U.K.). Glibenclamide and tolbutamide were from Hoechst Laboratories (Paris, France);

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[1] Abbreviations: AA, arachidonic acid; DOG, 1,2-dioleoylglycerol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; ETYA, eicosatetraynoic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; K_{ATP} channels, ATP-sensitive K^+ channels; NEFAs, nonesterified fatty acids; NDGA, nordihydroguaiaretic acid; OAG, 1-oleoyl-2-acetylglycerol.

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