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Interaction of Hemoglobin with Red Blood Cell Membranes as Shown by a Fluorescent Chromophore†

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ABSTRACT: Hemoglobin quenching of the fluorescence intensity of 12-(9-anthroyl)stearic acid (AS) embedded in the red blood cell membrane occurs through an energy transfer mechanism and can be used to measure the binding of hemoglobin to the membrane. The binding of hemoglobin to red cell membranes was found to be reversible and electrostatic in nature. Using a theory of energy transfer based on Förster formulation, the quantitative data for the binding were derived. The number of binding sites was found to be $1.4 \pm 0.2 \times 10^6$ molecules per cell and the binding constant was 0.85×10^8 M^{-1}.

Plasma membranes readily available from mammalian erythrocytes have been widely used for studies of membrane structure. For such structural studies it was desirable to utilize membranes (ghosts) free of hemoglobin, and several methods of preparing hemoglobin-free membranes have been devised (Dodge et al., 1963; Hanahan and Ekholm, 1974; Steck and Kant, 1974). Nonetheless, it remains difficult to prepare hemoglobin-free red cell membranes.

Another group of workers has studied the putative binding of hemoglobin to the erythrocyte membrane in an effort to establish the physiological meaning of the interaction (Fischer et al., 1975; Mitchell et al., 1965; Hanahan et al., 1973): their studies suggested that the hemoglobin–membrane interaction is reversible, weak, and of uncertain physiological importance.

There is an apparent paradox in these observations: why is it difficult to remove hemoglobin from the membranes if the binding is weak and reversible? One possible explanation for the discrepancy might be the intercalation of hemoglobin within resealed ghosts rather than the existence of high affinity membrane binding of hemoglobin. Techniques which have previously been developed to study the interaction of hemoglobin with the membrane and which have focused on the separation of the membranes from the supernatant could not distinguish between intercalated or membrane-bound molecules.

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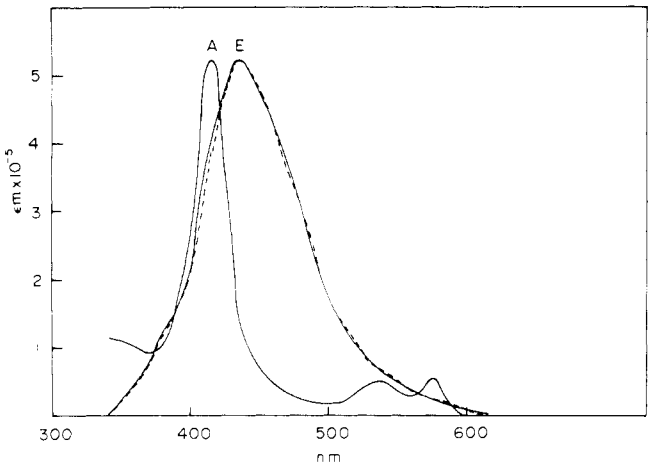

FIGURE 1: (A) The absorption spectra of hemoglobin. The ordinate shows the molar extinction coefficient of the tetramer. (E) The emission spectra of AS in the red cell membrane. (—) The fluorescence intensity before quenching. (- - -) The fluorescence spectra of the quenched sample enlarged by the instrument gain, to the same peak height as the unquenched one.

In the present work we employed a different approach to the study of hemoglobin-membrane interaction using a fluorescent probe, anthroylstearic acid (AS),[1] embedded in the membrane. Such a probe could transfer electronic excitation energy to bound hemoglobin molecules but not to free molecules which might be trapped within resealed ghosts. In addition, measurements of fluorescence intensity are very sensitive and provide an excellent device for measurement of high affinity binding at low hemoglobin concentrations.

We postulated that a constant number of binding sites and high affinity would be correlated with specificity in binding of Hb. The findings reported here show that the red blood cell membrane does bind hemoglobin with a specific high affinity interaction.

Materials and Methods

Horse heart cytochrome *c* was purchased from Sigma Chemical Co. Carboxymethylcellulose was purchased from Whatman Co. AS, anthroylstearic acid, was a product of Molecular Probes, Inc. (fluorescence probes were purchased from Molecular Probes, Inc., Roseville, Minn.). All other chemicals were reagent grade purchased from Fisher, Mallinckrodt, Baker, and Sigma. Red cells from freshly drawn blood were washed three to five times in 0.15 M NaCl buffered with 5 mM potassium phosphate at pH 8.0. Hemolysis was performed at a 1:40 volume ratio of cells to 5 mM phosphate buffer at pH 8.0 (5PB8). Following hemolysis subsequent washes were carried out at a 1:10 volume ratio until the supernatant was free of hemoglobin for four to five washes. The concentration of hemoglobin was detected by absorbance measurements at 415 nm. Following a wash with 10 mM Tris-HCl buffer at pH 8.0 (Brown and Harris, 1970), the ghosts were sedimented by centrifuging the solution for 30 min at 48 000*g* or for 50 min at 25 000*g*. The supernatant and "pellet" were separated following the procedure of Fairbanks et al. (1971). All procedures were carried out at 4 °C.

The optical absorbance of the cell suspension was measured at 415 nm. Cells which contained 0.5% or less of the amount of Hb added in the binding experiments were considered Hb free. Ghosts in 0.15 M solution of NaCl were enumerated in a Model A Coulter Counter with a 100-μm aperture corrected for coincidence.

Labeling of the Ghosts with Anthroylstearic Acid (AS). AS in 1:200 weight ratio to the estimated lipids (Ways and Hanahan, 1964) in the RBC membrane was added to the cell suspension and incubated for 1 h. Although AS is preferentially soluble in the lipid phase some AS micelles were usually found in the water phase. The cells were then washed once more with 5PB8 and sedimented to eliminate any AS in the water phase.

Fluorescence Measurements. Fluorescence measurements were performed on a spectrofluorometer constructed by one of the authors (Yguerabide, 1973). A 150-W xenon lamp served as the excitation light source. Monochromatic light was obtained using high intensity Bausch and Lomb monochromators. The fluorescence emission was measured at a 90° angle to the incident beam. The bandwidth was 15 nm. The excitation wavelength was 360 nm and the emission intensity was recorded at 480 nm to diminish scattering from excitation light and trivial reabsorbance of the fluorescent light by the high absorbance of Hb at the Soret band (at 415 nm). Fluorescent quenching was measured by adding small aliquots of concentrated Hb solution in the same buffer as the ghosts. Volumes added did not exceed 0.01 of the ghost solutions and the Hb absorbance at the emission wavelength did not exceed 0.01 OD at 1-cm light path length and at 415-nm wavelength.

Titrations of fluorescence were carried out using a 10 × 10 mm cuvette with constant stirring of the cell suspension. Measurements of light absorbance were carried out using a Cary-14 spectrophotometer.

Results

The emission spectrum of AS is shown in Figure 1, together with the absorption of hemoglobin at the Soret band. The addition of Hb to the AS-treated ghosts resulted in a decreased AS fluorescence intensity. The fluorescence emission spectrum is shown in the dashed line of Figure 1. For comparison with the spectrum observed in the absence of Hb, the spectrum of the quenched sample was recorded using a different instrumental gain.

Although the quantum yield has been decreased to 65% of its former value the spectra are unchanged suggesting that trivial reabsorbance did not account for the observed quenching. The addition of Hb A_2 to the ghosts caused the same decrease in fluorescence intensity. In the case of Hb A_2 the decrease in intensity was achieved by the addition of smaller amounts of total hemoglobin than with Hb A.

The overlap of the Hb absorbance and AS fluorescence suggests the Förster mechanism of electronic excitation energy transfer as the mechanism of the quenching in the system described.

R_0 Calculations. The distance between a donor and an acceptor for 50% of the transfer efficiency, R_0, was calculated using Förster's equation (see Appendix), where K^2 = an orientation factor for the dipoles taken to be random. $K = 2/3$; Q_0 = the unquenched quantum yield of AS = 0.4; n = the refractive index of the medium = 1.4; $e(\lambda)$ (= the molar extinction coefficient of Hb at the desired wavelength) was taken to be that of the monomeric heme value (van Assendelt, 1970). J was calculated to be 2.5×10^{-13} cm^3 M^{-1} and R_0 was calculated to be 46 Å. (Based on the molar extinction coefficient for a tetrameric molecule the calculated R_0 becomes 55 Å.)

The Effect of Salt on the Quenching. The dependency of Hb quenching on the ionic strength is shown in Figure 2. The addition of salt reverses the quenching effect seen at lower ionic strength. The reversal of the quenching effect by salt indicates

[1] Abbreviations used are: AS, 12-(9-anthroyl)stearic acid; RBC, red blood cell; Hb, hemoglobin; Cyt *c*, cytochrome *c*; CMC, carboxymethylcellulose; 5PBX, 5 mM phosphate buffer at pH = *X*.

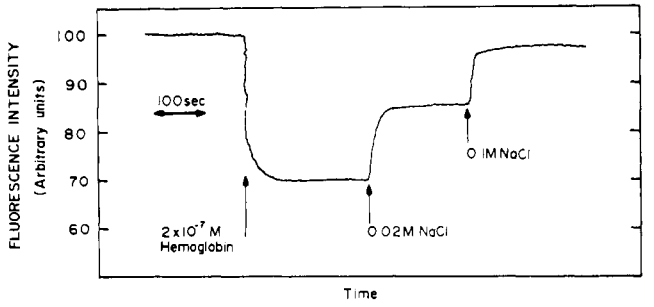

FIGURE 2: The effect of salt on the fluorescence quenching by hemoglobin. NaCl was added after quenching by Hb. The solution was thermostated at 22 °C. Hb concentration was 2×10^{-7} *m*. Cell concentration: 5×10^6 cells/mL. NaCl was added in two steps to the concentrations shown in the figure.

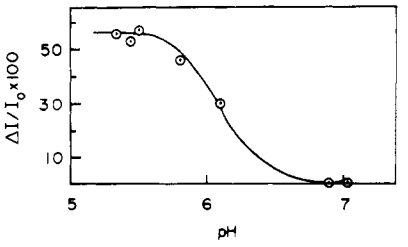

FIGURE 3: The pH dependency of Hb quenching of AS fluorescence in ghosts held at 5PB, temperature = 22 °C. $\Delta I/I_0$ is the relative quenching in each pH. pH values were measured before and after the addition of Hb.

that hemoglobin is bound to the membrane and that the interaction is electrostatic. The results in Figure 2 are also consistent with the postulated quenching by a nontrivial energy transfer.

Effect of pH on Hemoglobin Binding to Membranes. The dependency of the binding on pH is shown in Figure 3. The data were obtained in fluorescence quenching experiments where $\Delta I/I_0$, the fraction of fluorescence quenched, was calculated from the difference in the fluorescence intensity in hypotonic (5×10^{-3} M) and isotonic (0.15 M) solutions at the desired pH values.

Effect of Ionic Strength on Hemoglobin and Cytochrome c Binding to Membranes. The results shown in Figures 2 and 3 indicate a large contribution of electrostatic interactions in the attachment of hemoglobin to the membranes. Since cytochrome *c* was shown to be nonspecifically bound to RBC membranes (Kant and Steck, 1973), comparisons were made of the binding of cytochrome *c* and hemoglobin to red cell membranes and to carboxymethylcellulose (CMC) as an indifferent cation exchanger. The relative affinities of hemoglobin and cytochrome *c* for CMC reflected the ratio of their electrostatic free energies. The two proteins were mixed with CMC at different ionic strengths and the amount bound was measured. The dependence of the elution on ionic strength is shown in Figure 4B. Much higher ionic strength was required for elution of cytochrome *c* from the cation exchanger than was necessary to elute hemoglobin. The dependency of the interaction of the two proteins with the RBC membrane on ionic strength is shown in Figure 4A. If the affinity or binding constant for each protein to the membrane is expressed as

$$K_{obsd} = K_{ch}K_{\Psi}$$

where K_{Ψ} is the electrostatic contribution and K_{ch} is the remaining chemical affinity, according to Figure 4B:

$$K_{\Psi}^{Cyt} > K_{\Psi}^{Hb}$$

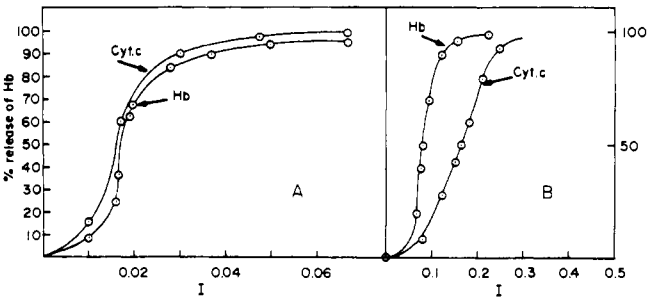

FIGURE 4: Ionic strength dependency of binding of Hb and Cyt *c*. Different ionic strengths were achieved by additions of NaCl. Temperature: 22 °C. (a) Binding of Hb and Cyt *c* to RBC membranes at pH 6.0 as measured by fluorescence quenching. (b) Binding of Hb and Cyt *c* to CMC at pH 6.0 (buffered with 5PB).

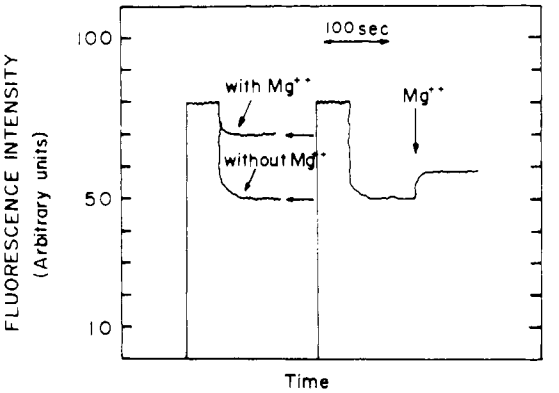

FIGURE 5: The effect of Mg^{2+} on the quenching of AS fluorescence of ghosts at pH 6.0. Cell concentration was 5×10^6 cells per mL. Hb was added in each case to a concentration of 2×10^{-7} M. Temperature = 22 °C, pH 6.0. In each case the reduction in the fluorescence intensity by Hb is seen. On the left, the upper curve shows the effect of Hb on cells pretreated with Mg as follows: The cells were first incubated with 0.1 M NaCl and 1×10^{-3} M $MgCl_2$ at 37 °C for 40 min and then washed three times with 5PB6 containing 1×10^{-3} M $MgCl_2$. The lower curve shows the reduction in fluorescence intensity for untreated cells. On the right, the fluorescence intensity was first reduced by the addition of Hb and, at time indicated by arrow, $MgCl_2$ was added to a concentration of 1×10^{-3} M.

and according to Figure 4A

$$K_{obsd}^{Cyt} = K_{obsd}^{Hb}$$

then

$$K_{ch}^{Hb} > K_{ch}^{Cyt\ c}$$

Thus, hemoglobin exhibits a special affinity for the red blood cell membrane in comparison with cytochrome *c*.

The Effect of Divalent Ions on the Binding. In the set of experiments shown in Figure 5 we used ghosts prepared only with phosphate buffers. Tris buffer has been shown to create permanent leaks in the ghosts while phosphate-treated ghosts can be resealed in the presence of divalent ions (Brown and Harris, 1970). In Figure 5 the addition of 1×10^{-3} Mg^{2+} to the membrane after quenching the fluorescence with Hb is shown. This Mg^{2+} concentration has been found to "reclose" open ghosts (Steck and Kant, 1974). Work with closed ghosts was undertaken in an effort to demonstrate differences in the binding to the inside and outside surfaces of the membrane.

In Figure 5 the difference in fluorescence quenching by hemoglobin of untreated and magnesium treated ghosts is shown. The quenching was less in the case of Mg^{2+}-treated ghosts. Two explanations for this finding would be plausible: part of the ghosts might reclose and could not bind hemoglobin

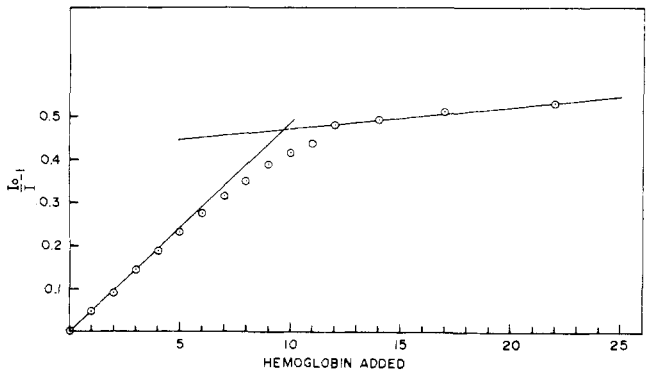

FIGURE 6: The titration of ghosts at pH 6.0, buffered by 5PB. The cell suspension cuvette was thermostated at 22 °C. Concentration of cells was 7.26×10^7 cells/mL. Hb was added from a stock solution stored in the same buffer. Each unit on ordinate scale = 1.05×10^{13} hemoglobin molecules per mL or 10^{11} Hb molecules per cm^2 of one side of the cell surface.

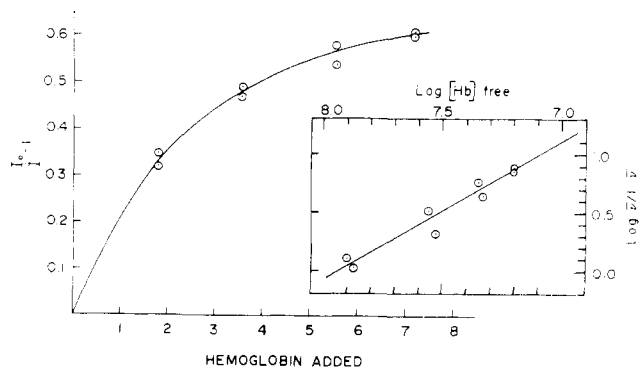

FIGURE 7: The titration of ghosts at pH 6.0, buffered by 5PB. The solution was thermostated at 22 °C. The data are taken from two parallel experiments in which the cell concentrations were 4.4×10^6 cells/mL and 4.6×10^6 cells/mL. Each unit on the ordinate scale = 0.70×10^{13} molecules of Hb per mL. Insert: Hill plot of the binding data derived as explained in the text.

to the inner surface or Mg^{2+} might compete with hemoglobin for a binding site and as a result less Mg^{2+} is bound. If the first explanation holds then the addition of Mg^{2+} after hemoglobin would not effect the fluorescence intensity since Hb has already been incorporated into the cytoplasmic chamber before the membrane reclosed. The findings shown in Figure 5 show that this is not the case. The addition of Mg^{2+} after Hb also resulted in a decrease (albeit smaller) in fluorescence indicating that magnesium competes with Hb for the binding sites.

The Calculation of the Binding Constant and the Number of Sites. The theory of energy transfer as derived by Förster (1959) was applied to the case of a donor embedded in a lipid layer and an acceptor molecule bound on the surface. When the shortest donor–acceptor distance is larger than R_0, the dependence of the fluorescence intensity on the density of the acceptor molecules follows the Stern–Volmer equation (see Appendix):

$$\frac{I}{I_0} = \frac{1}{1 + K_q\sigma} \quad \text{or} \quad \frac{I_0}{I} - 1 = K_q\sigma \qquad (1)$$

where I_0 is the fluorescence intensity before the addition of quencher; I is the fluorescence intensity in the presence of quencher; σ is the density of quencher molecules on the membrane; and K_q is the effective quenching constant.

This equation can be applied to the case of the AS–Hb donor–acceptor pair. AS, a negative chromophore, is more likely to be embedded in the outer layer of the membrane (Sheetz and Singer, 1974). The position of AS in the lipid bilayer was estimated to be 15 Å from the aqueous interface by Waggoner and Stryer (1970). The thickness of the RBC membrane has been shown to be 65–75 Å (Peters, 1973; Weinstein and McNutt, 1970). The heme pocket is located near the surface of the hemoglobin molecule. Consideration of these dimensions indicates a minimal donor–acceptor distance of 50–60 Å for a hemoglobin molecule on the inner surface and of about 20 Å in a case where Hb molecules are attached to the exterior surface. Hemoglobin yields a maximum (Figure 2) of about 30% reduction in fluorescence intensity. Therefore, the 50–60 Å distance is more likely than 20 Å for the donor–acceptor distance. Accordingly, the data are analyzed for the case where $R > R_0$. The theory for such a case predicts $(I/I_0 - 1)$ to be directly proportional to the number of bound hemoglobin molecules as shown in eq 1. Under stoichiometric conditions the added amount of Hb could be considered totally bound and a linear dependency on added Hb amounts should exist. Figure 6 shows the data derived under such conditions. It is apparent that at sufficiently low concentrations of hemoglobin the curve is linear. At higher concentrations of Hb where not all added Hb is bound, a deviation from linearity occurs.

From the slope of the linear region of the curve we get $K_q = 4.9 \times 10^{-13}$ cm^2/molecule. The curve is limited to the range $0 < \sigma < \sigma_{max} n_{max}$ (or σ_{max}) and is limited either by the area of the membrane in a case of nonspecific absorbance or by the number of sites in the case of specific binding.

The constant slow drop in fluorescence intensity could result from trivial reabsorbance of emitted light by higher concentrations of hemoglobin. From the intersection of the straight lines in Figure 6 the maximal number of hemoglobin molecules bound per cell is found to be $n_{max} = 1.4 \times 10^6$. In other experiments the values were in the range $(1.4 \pm 0.2) \times 10^6$ molecules per cell.

In Figure 7 the dependency of fluorescence intensity on the addition of Hb under equilibrium conditions is shown. While stoichiometric conditions are obtained with concentrations of the order of 10^8 cell/mL, equilibrium conditions obtain when the cells are diluted at about 1:20 to the stoichiometric conditions of Figure 6.

Since the quenching ability of bound Hb was known from the data of Figure 6, the intrinsic association constant was calculated as follows. At each point the fraction of bound Hb was taken from the data in Figure 6. From the total amount of added Hb the concentration of free hemoglobin could be calculated. The insert of Figure 7 shows the binding behavior plotted as a Hill plot; the plot is linear, the slope 1.2, and the calculated binding constant is $k = 0.85 \times 10^8$ M^{-1}. Other experiments yielded values in slopes of 1.1 ± 0.1, indicating that the binding is noncooperative.

The data indicate that, while the intensity dropped to 65% of its value in the stoichiometric experiment (Figure 6), it dropped to 62% of its value in the equilibrium experiment. However, a drop equal to or lower than that in Figure 6 would be expected in Figure 7 where saturation is not achieved. Such a finding may indicate the presence of much lower affinity sites which are Hb bound and contribute to the quenching as the free Hb concentration is raised. Studies done at much higher concentration of hemoglobin which might indicate the presence of some weak binding sites are difficult to interpret because reabsorbance of fluorescence by high concentrations of hemoglobin contributes appreciably to the quenching observed.

Discussion

The present studies (Figures 1, 2, and 3) show that the

quenching of fluorescence intensity occurs by a distance dependent energy-transfer mechanism and not by reabsorbance of emitted light which is independent of distance. Therefore, quenching of AS fluorescence is a result of hemoglobin binding to the RBC membrane.

The binding constant determined by these experiments indicates a high affinity interaction between Hb and the red cell membrane. The interaction reported here is electrostatic in nature and is reversible. The comparison between Hb and cytochrome *c* shows that, although the interaction is basically electrostatic in both cases, a specific form of interaction is involved in the binding phenomena in the case of hemoglobin.

There is an important difference between the interaction of Hb with the red cell membrane and the interaction of Hb with models of artificial membranes composed of the different lipids of the red cell membranes. although the binding of Hb to the lipid membrane is an electrostatic interaction (as shown by the inability of the Hb to bind at high ionic strengths), it is an irreversible interaction (Papahadjopoulos and Kimelberg, 1973). In the case of the red blood cell the reversibility shows that hemoglobin is not incorporated into the lipid layer as it had been suggested by the earlier use of lipid model systems.

Additionally, the binding between Hb and the RBC membrane appears to be noncooperative. The area of the red blood cell membrane has been shown to be 147 μm^2 (Gul and Smith, 1974). The maximum number of molecules determined to be bound in this study is 1.4×10^6 per cell. For the binding to take place on only one surface, hemoglobin molecules would be an average of 100 Å apart. The hemoglobin radius has been determined as 30 Å (Muirhead et al., 1967). Hemoglobin molecules could indeed be spread apart with no interaction on the membrane surface.

Fischer and co-workers (1975) have reported the binding capacity of erythrocyte ghosts for hemoglobin to be 55 μg per mg of membrane protein. Using the values of Dodge et al. (1963), for the protein content of erythrocyte ghosts, the calculated adsorption capacity turns out to be 0.36×10^6 hemoglobin molecules per erythrocyte membrane. They also reported different binding capacities for different hemoglobins. As mentioned earlier, in our experiments the same quenching effect was achieved with Hb A_2 as with Hb A, although a smaller amount of A_2 was needed for the effect. Such a finding indicates that the number of sites is the same, while the affinity is different and depends on the positivity of the protein which is added. In studies by Fischer et al. (1975) on Hb A_2 the number of bound Hb A_2 molecules per ghost is 0.73×10^6 where their curve shows that the values are closer to saturation. This value is closer to the number found in the present work.

Mitchell et al. also reported quantitative binding capacity of red cell membranes for hemoglobin (Mitchell et al., 1965). In their studies a value of 2.4 mg bound per 1 mL of packed cells was reported. Packed ghosts usually contain $5\text{-}7 \times 10^9$ ghosts per mL. Taking 6×10^9 ghosts per mL, the binding capacity would be 3.5×10^6 Hb molecules bound per ghost cell. This value exceeds the findings in the present work. It is difficult to compare the quantitative data from different laboratories using different techniques. However, the data by Fischer et al. appear to represent conditions below saturation, while the work by Mitchell et al. suggests the existence of another low affinity class of sites for hemoglobin binding.

The data of Figure 6 are in agreement with the assumption that the Hb molecules are interacting with the inner surface of the membrane. $\overline{R_M}$, the effective minimal distance between the donor and the acceptor (i.e., the closest distance to which the acceptor can approach the donor on the membrane as shown in Figure 8), can be calculated from the slope of Figure 6 and the following expression derived in the Appendix (see eq 39A) for such a slope:

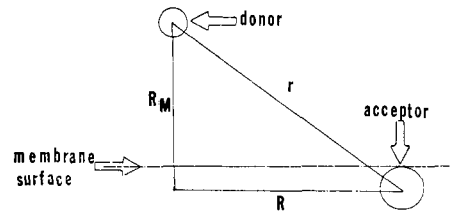

FIGURE 8: Coordinate system showing the distance r between donor and acceptor, the position R of an acceptor molecule with respect to a point immediately below the donor molecule, and R_M the closest distance to which the acceptor can approach the donor. The value of R_M is determined by how much the acceptor can penetrate the membrane.

$$K_q = \frac{\pi R_0}{2} \frac{1}{\overline{R_M^4}} \tag{2}$$

Using the value $K_q = 4.9 \times 10^{-3}$ cm^2/molecule and $R_0 = 46$ Å, we get 42 Å for the calculated average minimal donor-acceptor distance. It should be noted that the actual minimal distances R_{M_j} between the heme groups on an Hb molecule are related to the $\overline{R}$ by the expression (see eq 35A)

$$\left(\frac{1}{\overline{R_M}}\right)^4 = \sum_{j=1}^{4} \left(\frac{1}{R_{M_j}}\right)^4 \tag{3}$$

Calculation of the R_{M_j} from this equation requires an assumption as to how the Hb molecule is oriented on the surface of the membrane. If, for example, two of the heme groups on an Hb molecule are very close to the membrane surface, the other two would be about 60 Å away from the surface. In this case the two distant heme groups would not contribute much to eq 3 because of their large R_{M_j} and, assuming that R_{M_j} is the same for the other two we get from eq 3

$$R_{M_j} = \sqrt[4]{2}\,\overline{R_M}$$

From this equation we calculate the value of 50 Å for the actual minimal distance between the two closest heme groups and AS. Consideration of other possible orientations of Hb leads to the conclusion that the minimal distance between acceptor and donor is around 40–60 Å. This range is in good agreement with the assumptions concerning the location of AS in the outer lipid layer and the location of hemoglobin on the interior surface of the membrane.

The high affinity interaction together with the suggested location of the hemoglobin interaction make it tempting to speculate about the physiological importance for the binding of hemoglobin to the RBC membrane. More evidence about the location of the hemoglobin-membrane interaction may indicate the role of the interaction in red cell function.

Acknowledgment

We wish to thank Evangelina Yguerabide for her assistance in this work.

Appendix I: The Theory of Excitation Energy Transfer between a Donor and Randomly Distributed Acceptors Bound to a Membrane

1. *Rate Expression.* We consider the case where a donor fluorescent probe is internally positioned at a known distance from one surface of a membrane and N quencher molecules are randomly attached to that surface. The density of quencher molecules is $\sigma = N/A$ where A is the area of the membrane. Each quencher may have more than one acceptor per molecule.

For example, hemoglobin has four acceptor heme groups per molecule. We consider the general case of n acceptor groups per quencher molecule.

Let $p_D(t)$ be the probability that a donor molecule excited at $t = 0$ is still excited at time t, i.e., $p_D(t) = n(t)/n(0)$ where $n(0)$ and $n(t)$ are the number of excited molecules at time zero and time t. The rate expression for $p_D(t)$ in the presence of quencher molecules can be written (assuming that the density of excited molecules is very low compared with the density of quencher molecules, which is usually the case) as:

$$\frac{dp_D(t)}{dt} = -\{k_e + k_i + \sum_{i=1}^{N}\sum_{j=1}^{n}(k_{ij}(r)p_A(t)\} \quad (1A)$$

where k_e and k_i are the specific rates of emission and internal quenching respectively and $k_{ij}(r)$ is the specific rate for excitation transfer from the donor to the jth acceptor positioned at a distance r from the quencher. The summation over i accounts for the N quencher molecules interacting with the donor molecule and the summation over j accounts for the n acceptor groups per quencher molecule.

According to Förster, the specific rate for dipole-dipole excitation energy transfer from a donor to an acceptor group separated by a distance r (in Å) and having orientations described by the angles Ω is given by the expression

$$k(\Omega,r) = \frac{1}{\tau_0}\left(\frac{R_0}{r}\right)^6 s^{-1} \quad (2A)$$

where τ_0 is the lifetime in the absence of quencher, i.e.

$$\frac{1}{\tau_0} = k_e + k_i \quad (3A)$$

and R_0 (Å) is defined by the expression

$$R_0 = (8.71 \times 10^{23} J K^2 k_e \tau_0 n^{-4})^{1/6} \quad (4A)$$

where n is the refractive index of the medium between the donor and acceptor. The overlap integral J is in turn defined by the expression

$$J = \frac{\int F_D(\lambda)\epsilon_A(\lambda)\lambda^4 d\lambda}{\int F(\lambda)d\lambda} (\text{photon·cm}^6/(\text{m mol})) \quad (5A)$$

where $F_D(\lambda)$ refers to the fluorescence spectrum of the donor (plotted as photons vs. wavelength in cm), $\epsilon_A(\lambda)$ is the molar decadic coefficient in L mol^{-1} cm^{-1}, and λ is the wavelength in cm. K is an orientation factor dependent on the relative orientation of the emission and absorption moments of the donor and acceptor, respectively. It is defined by the expression

$$K = \cos\theta_{DA} - 3\cos\theta_D\cos\theta_A \quad (6A)$$

where θ_{DA} is the angle between the donor emission and acceptor absorption moments, θ_D is the angle between the donor emission and a line l joining the donor and acceptor groups and θ_A is the angle between the acceptor and the line l. The symbol Ω in eq 2A refers to the fact that at any distance r, the rate of excitation transfer is dependent on the orientation of the donor and acceptor molecules. For simplicity, we have assumed in eq 1A that K can be represented by an average value $\overline{K^2}$ which is independent of r, i.e., $\overline{K^2}$ is the same for all acceptor groups regardless of their positions. In this case, $k(\Omega,r)$ is not dependent on orientation and we can simply write $k(r)$ in its place as shown in eq 1A. The assumption of an average $\overline{K^2}$ independent of position is approximated in many real systems. Thus, for the case discussed in the main text of this paper, where AS is the donor and heme is the acceptor, polarized fluorescence measurements indicate that the anthracene moiety of AS has a large distribution of orientations within the bilayer and moves rapidly over these orientations (Yguerabide and Stryer, 1971; Sherwood and Yguerabide, unpublished results). This motion together with the D_{4h} symmetry of Fe-porphyrin introduces some averaging over $\overline{K^2}$ tending to make its value independent of the position of the probe (Yguerabide, 1973).

2. *Solution of Rate Expression.* Integrating eq 1A for the condition where the system is excited by a very short pulse of light gives

$$p_D(t) = e^{-t/\tau_0}e^{-\sum_{i=1}^{N}\sum_{j=1}^{n}k_{ij}(r)t} \quad (7A)$$

which can be rewritten as

$$p_D(t) = e^{-t/\tau_0}\prod_{j=1}^{n}\prod_{i=1}^{N}e^{-k_{ij}(r)t} \quad (8A)$$

In a system consisting of an ensemble of membranes, such as in a membrane dispersion, each quencher molecule, and consequently, each acceptor group, has a random distribution of distances with respect to the donor. Thus, for any one acceptor group, e.g., the jth acceptor on the ith quencher, we can replace $e^{-k_{ij}(r)t}$ by the average value $\overline{e^{-k_{ij}(r)t}}$ obtained by averaging $e^{-k_{ij}(r)t}$ over a random distribution of distances with respect to the donor. Since the average is independent of i, we can rewrite eq 8A in the simpler form

$$p_D = e^{-t/\tau_0}\prod_{j=1}^{n}\overline{(e^{-k_{ij}(r)t})}^N \quad (9A)$$

In order to evaluate the average in this expression, it is necessary to define a coordinate system for carrying out the integration. A convenient system is shown in Figure 1A. For a random distribution of positions on a surface, the probability dP for finding a quencher molecule within the element of area $dA = 2\pi R dR$ on the surface of the membrane is given by the expression

$$dP = \frac{2\pi R dR}{A} \quad (10A)$$

However, since $k_{ij}(r)$ depends directly on r instead of R, it is more convenient to have dP expressed in terms of r. This can be achieved by making use of the relation

$$r^2 = R^2 + R_M^2 \quad (11A)$$

which yields

$$r dr = R dR \quad (12A)$$

and allows one to write

$$dP = \frac{2\pi r dr}{A} \quad (13A)$$

The average of eq 9A can then be explicitly written as

$$\overline{e^{-k_{ij}(r)t}} = \int_{R_{M_j}}^{r_1} e^{-k_{ij}(r)t}\frac{2\pi r dr}{A} \quad (14A)$$

where R_{M_j} is the closest distance to which the jth acceptor group can approach the donor and r_1 is the radius of the area A containing the N quencher molecules. Introducing eq 14A into 9A gives

$$p_D(t) = e^{-t/\tau_0}\prod_{j=1}^{n}\left(\int_{R_{M_j}}^{r_1} e^{-k_{ij}(r)t}\frac{2\pi r dr}{A}\right)^N \quad (15A)$$

Equation 15A can be written in a still more convenient form by using the following transformations. Let

$$x = \frac{N}{A}\int_{R_{M_j}}^{r_1}(1 - e^{-k_{ij}(r)t})2\pi r dr \quad (16A)$$

$$= N - \frac{N}{A}\int_{R_{M_j}}^{r_1} e^{-k_{ij}(r)t}2\pi r dr \quad (17A)$$

Also let

$$y = -\frac{x}{N} \tag{18A}$$

$$y = -\frac{1}{A}\int_{R_{M_j}}^{r_1}(1 - e^{-k_{ij}(r)t})2\pi r\mathrm{d}r \tag{19A}$$

Then we have from eq 17A

$$1 - \frac{x}{N} = \frac{1}{A}\int_{R_{M_j}}^{r_1} e^{-k_{ij}(r)t}2\pi r\mathrm{d}r \tag{20A}$$

and from eq 18A

$$1 - \frac{x}{N} = 1 + y \tag{21A}$$

Finally, combining eq 18A, 20A, and 21A, we can write

$$\left(\int_{R_{M_j}}^{r_1} e^{-k_{ij}(r)t}\frac{2\pi r\mathrm{d}r}{A}\right)^N = (1 + y)^{-x/y} \tag{22A}$$

We now consider the behavior of y as the radius r_1 of A becomes much greater than R_0. For most donor–acceptor pairs, R_0 usually has a value which is not much greater than 80 Å. On the other hand, the planar radius of most intact biological membranes is usually greater than 1 μm. Therefore, in practice, r_1 can be assumed as much greater than R_0. Now, according to eq 19A, y has an absolute value of 1 if $Q = (1 - e^{k_{ij}(r)t})$ is 1 for all values of r within A. This function indeed has the value of 1 for $r \ll R_0$, but is very small for $r \gg R_0$. Of course, the quantity Q depends not only on r but also on t and for large values of t, Q increases toward 1 at any value of r. However, from a practical standpoint, only values of t, perhaps, less than $5\tau_0$ are important. For these values of t, Q will be much less than 1 for $r \gg R_0$. Consideration of eq 19A then shows that the value of y decreases as r_1 increases and can be considered as practically zero for $r_1 \gg R_0$. Now, from the definition of the base e we have

$$\lim_{y\to 0}(1 + y)^{1/y} = e \tag{23A}$$

Thus, introducing eq 23A into eq 22A gives

$$\lim_{\substack{A\to\infty \\ r_1\to\infty}}\left(\int_{R_{M_j}}^{r_1} e^{-k_{ij}(r)t}\frac{2\pi r\mathrm{d}r}{A}\right)^N = e^{-\sigma\int_{R_{M_j}}^{\infty}(1-e^{-k_{ij}(r)t})2\pi r\mathrm{d}r} \tag{24A}$$

In the right-hand side of eq 24A, we have written infinity for the upper limit of the integral, although membranes usually have a finite size. This is justified because for $r_1 \gg R_0$ the integral taken between r_{min} and the finite value of r_1 does not differ significantly from that taken between r_{min} and infinity. Moreover, selection of infinity as the upper limit facilitates the evaluation of integrals and the resultant expressions are conveniently independent of the actual value of r_1. In addition, it should be recalled that N is the number of quencher molecules within the area A so that, as $A \to \infty$, N must also tend to infinity in such a way that the ratio $\sigma = N/A$ is the density of quencher molecules on the membrane. Finally, introducing eq 24A into eq 15A gives the relatively simple expression

$$p_D(t) = e^{-t/\tau_0}\prod_{j=1}^{n} e^{-\sigma L_j(t)} \tag{25A}$$

where

$$L_j(t) = \int_{R_{M_j}}^{\infty}(1 - e^{-k_{ij}(r)t})2\pi r\mathrm{d}r \tag{26A}$$

In order to get an explicit analytical expression for $p_D(t)$ it is necessary to integrate eq 26A using eq 2A for $k_j(r)$. This integral, however, cannot be evaluated analytically in general, but it assumes simple forms in special cases. One simple case encountered in practice is for the condition where the closest distance R_{M_j} which can be attained between donor and acceptor is greater than R_0. For this condition, we can use the approximation

$$e^{-k_{ij}(r)t} \simeq 1 - k_{ij}(r)t \tag{27A}$$

for all values of r available to the acceptor. The approximation holds to within 6% for values of $k_{ij}(r)t < 0.3$. The approximation is at its worst for $r = R_{M_j}$. The smallest value of R_{M_j} for which the approximation is valid to within 6% can be calculated by noting that values of t greater than about $7\tau_0$ are practically insignificant. The condition on R_{M_j} necessary for eq 27A to hold is then

$$7\left(\frac{R_0}{R_{M_j}}\right)^6 < 0.3 \tag{28A}$$

which yields

$$R_{M_j} > 1.68R_0 \tag{29A}$$

Thus, for the case where eq 27A applies, after substituting this expression into eq 26A, we obtain

$$L_j(t) = \frac{1}{\tau_0}\int_{R_{M_j}}^{\infty}\left(\frac{R_0}{r}\right)^6 2\pi r\mathrm{d}r \tag{30A}$$

$$= \frac{\pi R_0^2}{2\tau_0}\left(\frac{R_0}{R_{M_j}}\right)^4 \tag{31A}$$

Finally, introducing eq 31A into 25A yields

$$p_D(t) = e^{-t/\tau_0}\prod_{j=1}^{n} e^{-\sigma\pi R_0^2(R_0/R_{M_j})^4/2\tau_0} \tag{32A}$$

$$= e^{-(1/\tau_0 + k_q\sigma)t} \tag{33A}$$

where k_q is the effective quenching rate constant defined by the expression

$$k_q = \frac{\pi R_0^2}{2\tau_0}\left(\frac{R_0}{\bar{R}_M}\right)^4 \tag{34A}$$

where the $\bar{R}_M$, the effective minimal distance, is given by the expression

$$\left(\frac{1}{\bar{R}_M}\right)^4 = \sum_{j=1}^{n}\left(\frac{1}{R_{M_j}}\right)^4 \tag{35A}$$

Note that if, for example, R is expressed in cm, then the rate constant k_q has units of cm^2/(molecule s).

3. *Steady-State Fluorescence Intensity Expression.* The fluorescence intensity obtained from the system described above under conditions of excitation with a steady beam of light is given by the expression

$$I = a\int_0^{\infty} p_D(t)\mathrm{d}t \tag{36A}$$

where a is the constant of proportionality. Introducing eq 33A into 36A and integrating gives

$$I = \frac{a}{\frac{1}{\tau_0} + K_q\sigma} \tag{37A}$$

from which we can write

$$\frac{I_0}{I} = 1 + K_q\sigma \tag{38A}$$

where I_0 is the intensity in the absence of quencher (i.e., for $\sigma = 0$) and where K_q is an effective quenching constant defined as

$$K_q = \tau_0 k_q = \frac{\pi R_0^6}{2} \frac{1}{\overline{R_M^4}} \quad (39A)$$

K_q has units of reciprocal density, e.g., cm^2/molecule.

4. *Discussion of Most Useful Expressions.* Of the equations derived above, the most useful in the analysis of experimental data are as follows. For a membrane dispersion which is excited by a very short (instantaneous) pulse of light, the decay of fluorescence intensity $I(t)$ from the initial value $I(0)$ is, from eq 33A, given by the expression

$$I(t) = I_0 e^{-t/\tau_0} \quad (40A)$$

where the lifetime is given by

$$\frac{1}{\tau} = \frac{1}{\tau_0} + k_q \sigma \quad (41A)$$

The effective quenching rate is given by

$$k_q = \frac{\pi R_0^2}{2\tau_0} \left(\frac{R_0}{\overline{R}_M}\right)^4 \quad (42A)$$

with

$$\frac{1}{\overline{R}_M} = \sum_{j=1}^{n} \left(\frac{1}{R_{M_j}}\right)^4 \quad (43A)$$

where n is the number of acceptor groups per quencher molecules. The steady-state intensity in turn is given by

$$\frac{I_0}{I} = 1 + K_q \sigma \quad (44A)$$

where the effective quenching constant K_q, from eq 39A and 40A, is

$$K_q = \frac{\pi R_0^2}{2} \left(\frac{R_0}{\overline{R}_M}\right)^4 \quad (45A)$$

As described above, these equations apply for the case where the closest distance R_{M_j} to which any acceptor group can approach the donor is greater than about $1.7R_0$. Although the equations were developed under the additional assumption that the acceptor groups are all on one side of the bilayer only and that all groups have the same distance R_s from the surface, the results can easily be generalized to cases where these additional assumptions do not apply. Thus, for example, if quencher molecules are present on both surfaces of the membrane, but the donor is only on one side, the above equations apply except that

$$\sigma = \sigma_I + \sigma_O \quad (46A)$$

where σ_I and σ_O are the quencher densities on the inner and outer surfaces and eq 42A becomes

$$\left(\frac{1}{\overline{R}_{M_j}}\right)^4 = f_I \sum_{j=1}^{n} \left(\frac{1}{R_{M_j}}\right)_I^4 + f_O \sum_{j=1}^{n} \left(\frac{1}{R_{M_j}}\right)_O^4 \quad (47A)$$

where f_I and f_O are the fractions of quencher molecules in the inner and outer surfaces, respectively.

Equation 40A indicates that the decay of fluorescence intensity for the case here considered is a simple exponential function with a single lifetime τ that is related to σ as shown in eq 41A. The specific rate quenching constant k_q can be evaluated experimentally by measuring τ for various values of σ. k_q is then given by the slope of a plot of $1/\tau$ vs. σ. $\overline{R}_M$ can then be calculated with eq 42A. It should be noted that eq 40A and 41A have the same form as the well-known equation for collision quenching by the so-called Stern–Volmer mechanism. The steady-state expression, eq 44A, also has the same form as the steady-state Stern–Volmer equation for collisional quenching. The value of the effective quenching constant K_q can be determined experimentally from the slope of a plot of I_0/I vs. σ. $\overline{R}_M$ can then be evaluated with eq 45A. It should be noted that K_q for the excitation energy transfer case is not dependent on τ_0, see eq 45A, in contrast to the Stern–Volmer quenching constant. This difference is due to the fact that the specific rate for energy excitation transfer quenching depends on τ_0 which results in cancellation of this lifetime in the expression defining K_q, see eq 34A and 39A. On the other hand, the specific rate for collisional quenching by the Stern–Volmer mechanism depends on translational diffusion parameters only and not on τ_0.

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