Binding of Hemoglobin to Red Cell Membranes with Eosin-5-maleimide-Labeled Band 3: Analysis of Centrifugation and Fluorescence Lifetime Data

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ABSTRACT: We have studied the binding of hemoglobin to the red cell membrane by centrifugation and fluorescence methods. The intact red cell was labeled with eosin-5-maleimide (EM), which specifically reacts with lysine 430 of band 3. Even though this residue is not part of the cytoplasmic domain of band 3 (cdb3) associated with hemoglobin binding, fluorescence quenching was observed when hemoglobin bound to inside-out vesicles (IOVs). The use of fluorescence quenching to measure band 3 binding was quantitatively compared with the binding determined by centrifugation, which measures binding to band 3 and non-band 3 sites. For the centrifugation it was necessary to include the non-band 3 association constants determined from chymotrypsin-treated IOVs. The binding of hemoglobin to band 3 was interpreted in terms of the binding of two hemoglobin tetramers to each band 3 dimer. An anticooperative interaction associated with the conformational change produced when hemoglobin binds results in a 2.8-fold decrease in the intrinsic constant of $(1.54 \pm 0.25) \times 10^7\ M^{-1}$ for the binding of the second hemoglobin molecule. From the changes in lifetime produced by binding the first and second hemoglobin molecules, it was possible to show that the conformational change associated with binding the second hemoglobin molecule results in a decrease of the heme−eosin distance from 47.90 to 44.78 Å. Reaction of cyanate with the α-amino group of hemoglobin (HbOCN) is shown to produce a very dramatic decrease in the binding of hemoglobin to both the band 3 and non-band 3 sites. The intrinsic constant for binding the first hemoglobin molecule to band 3 decreases by a factor of 29 to $(5.34 \pm 0.15) \times 10^5\ M^{-1}$. The anticooperative interaction is greater with the intrinsic constant decreasing by a factor of 3.8 for the binding of the second hemoglobin tetramer to band 3. In addition, the nature of the conformational change produced by binding hemoglobin is very different with the second HbOCN increasing the heme−eosin distance to 55.99 Å. The utilization of eosin-5-maleimide-reacted red cell membrane to study hemoglobin binding makes it possible to directly study the binding to band 3. At the same time a sensitive probe of the conformational changes, which occur when hemoglobin binds to band 3, is provided.

The interactions between the red cell membrane and hemoglobin have been known since researchers attempted to obtain white ghosts by hypotonic lysis (*1*). Funk (*2*) and Eisinger et al. (*3*) have shown that hemoglobin binds to the erythrocyte membrane even in vivo. The high-affinity sites for hemoglobin binding to the cytoplasmic surface of the red cell membrane have been localized to the inner segment of band 3 (*4*−*6*), which has been shown to bind a number of red cell proteins. The red cell membrane has other lower affinity sites on the cytoplasmic surface, which have not been as well characterized and are thought to involve glycophorin and phospholipids (*6*, *7*).

Band 3 accounts for approximately 25% of the total membrane protein (*7*) and a major fraction of the hemoglobin binding sites on the red cell membrane (*4*). Band 3 in the red cell membrane exists in equilibrium between a dimer and a tetramer (*8*). The band 3 monomer molecular mass is approximately 93 kDa (*9*) and consists of two major domains, the cytoplasmic and transmembrane domains (*10*). The cytoplasmic domain anchors band 3 to the membrane cytoskeleton linking band 3 to ankyrin, bands 4.1 and 4.2, and providing a site for the binding of cytoplasmic proteins such as hemoglobin, glyceraldehyde-3-phosphate dehydrogenase, aldolase, and phosphofructokinase. The transmembrane domain is responsible for the transport of anions across the membrane (*11*).

The ability to quantitate the interactions of hemoglobin with the membrane can have physiological relevance. It has been shown that hemoglobin interactions with band 3 are influenced by hemoglobin quaternary changes (*12*) and that the binding of hemoglobin affects the conformation of band 3 (*13*, *14*). The hemoglobin-linked band 3 conformational changes are affected by cell age (*15*, *16*) and are coupled to

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the interactions between band 3 tetramers, ankyrin and the membrane cytoskeleton (*17*). The association of several glycolytic enzymes with band 3, in the same region where hemoglobin binds (*17*), inhibits these enzymes and has been implicated in the regulation of glycolysis (*18*). It has also been shown that hemoglobin oxidative processes taking place on the surface of the membrane are not neutralized by the cellular antioxidant systems and can cause oxidative damage (*19*−*22*). Inhibition of the interactions of hemoglobin with the membrane can, therefore, alleviate red cell induced oxidatiove stress.

The binding of hemoglobin to band 3 has been shown to involve insertion of the terminal carboxyl end of band 3 into the internal cavity of hemoglobin (*23*). This binding can be eliminated by cleaving the cytoplasmic end of band 3 with chymotrypsin (*5*, *24*) or by blocking access into the internal cavity of hemoglobin. Reacting the terminal α-amino groups of hemoglobin has been shown to block access to this cavity (*23*). Cyanate reacts with these groups (*25*, *26*) even in intact red cells, and a decrease in the binding of cyanate-reacted hemoglobin with band 3 has been reported (*27*).

Eosin-5-maleimide specifically labels residue 430 of band 3 (*28*). Fluorescence resonance energy transfer (FRET) with heme as an acceptor of the eosin fluorescence will affect the eosin lifetime if it is within 60 Å of the heme (*29*). By this method, we have been able to use the changes in eosin lifetime when hemoglobin binds as a measure of hemoglobin binding to band 3. Since FRET is a sensitive measure of the distance between the donor and acceptor in the range of 20−60 Å, an analysis of the fluorescent changes provides a probe of the band 3 conformational changes, which occur when hemoglobin binds. We have also applied this technique to investigate the effect of reacting hemoglobin with cyanate on the binding of hemoglobin to band 3 and have shown that cyanate inhibits binding of hemoglobin to the membrane.

MATERIALS AND METHODS

Preparation of Eosin-5-maleimide- (EM-) Labeled Red Blood Cells. Fresh whole blood in EDTA anticoagulant, obtained from healthy participants of the Baltimore Longitudinal Study on Aging, was centrifuged, and the plasma and white cells were removed by aspiration. The red blood cells (RBCs) were then washed four times by recentrifuging in phosphate-buffered saline (PBS7.4) (150 mM sodium chloride and 5 mM sodium phosphate at pH 7.4).

Labeling of band 3 in erythrocyte membranes of intact RBCs with eosin-5-maleimide (Molecular Probes, Eugene, OR) was carried out according to the method of Cobb et al. (*28*). EM dissolved in PBS7.4 buffer (0.5 mg/mL) was prepared and used fresh. One milligram of the EM label was added per 5 mL of packed intact RBCs, and the reaction was allowed to proceed in the dark at room temperature for 1 h. The excess EM was removed in four washes of the cells in PBS8 (5 mM sodium phosphate and 150 mM sodium chloride, pH 8.0).

Preparation of Inside-Out Vesicles (IOVs) from Labeled Red Blood Cells. Inside-out vesicles were prepared from the leaky ghosts obtained from labeled erythrocytes according to the method of Steck and Kant (*30*). The cells were hemolyzed by rapidly and thoroughly mixing the packed cells with 5PBS8 (5 mM sodium phosphate, pH 8.0) in a 1:40 volume ratio. The labeled membranous ghosts were pelleted by centrifugation at 14000 rpm for 10 min in an SS-34 Sorvall rotor. After hemolysis, all subsequent steps were performed under reduced light (*28*, *31*), and labeled ghost membranes were washed and recentrifuged six times in order to remove all of the hemoglobin. The spectrum of an aliquot of the supernatant was run from 350 to 450 nm to ensure complete removal of hemoglobin. The EM-labeled leaky ghosts were creamy pink instead of white because of the eosin label.

Each milliliter of the pelleted labeled leaky ghost was diluted to 40 mL in 0.5PBS8 (0.5 mM sodium phosphate, pH 8.0) and incubated for 5 h on ice. The incubated membranes were pelleted at 18000 rpm for 30 min. The membranes were resuspended to the original volume in 0.5PBS8 buffer by low-speed vortexing and passed eight times through a 1 mL syringe fitted with a 27-gauge needle to complete vesiculation. The vesicles were diluted 4-fold in 0.5PBS8 and layered (*30*) on an equal volume of Dextran T-110 (Pharmacia). The mixture was centrifuged at 40000 rpm for 2 h. The top band material, which is the EM-labeled IOVs, was collected and washed four times with repeated centrifugation at 18000 rpm for 15 min. The pellet, which contained unsealed ghost and right-side-out vesicles, was discarded. The total protein concentration was estimated by the procedure of Lowry et al. (*32*), using bovine serum albumin as a standard.

Preparation of Hemoglobin. Hemoglobin was obtained from fresh hemolyzed cells and purified by passing through Sephadex G-100 in PBS7.4. The eluted sample was dialyzed against 5PBS6 (5 mM sodium phosphate, pH 6.0) and its concentration determined from the spectrum acquired in the 640−490 nm region using a Perkin-Elmer Lamda-6 UV−visible spectrophotometer (*33*).

Eosin-Labeled Inside-Out Vesicles Treated with Chymotrypsin. The water-soluble cytoplasmic domain of band 3 was removed from the eosin-labeled IOVs with α-chymotrypsin as previously described (*17*, *28*, *34*). The labeled IOVs were first dialyzed against 0.5PBS7.4 (0.5 mM sodium phosphate, pH 7.4) before the vesicles were mixed 1:1 with 200 μg/mL α-chymotrypsin which was incubated at 37 °C for 45 min. The IOVs were washed three times in 0.5PBS7.4 and then dialyzed against 5PBS6.

Preparation of Cyanate Hemoglobin (HbOCN). For the preparation of hemoglobin with the terminal amino group reacted with cyanate, an aliquot of the washed RBCs was incubated with 300 mM potassium cyanate (KOCN) for 1 h at 4 °C in PBS7.4 (*27*). The cells were washed four times to remove excess KOCN and lysed in 5PBS7.4 (5 mM phosphate buffer, pH 7.4). The hemolysate containing HbOCN was passed through a Sephadex G-100 column and then exhaustively dialyzed against 5PBS6 to lower the ionic strength and the pH.

Hemoglobin Binding to IOVs and IOVC Using the Centrifugation Method. We have used inside-out vesicles for investigating hemoglobin−membrane interactions because the cytoplasmic surface with the hemoglobin binding sites is exposed on the outside surface. In addition, binding to the external surface, which is not normally exposed to hemoglobin, is eliminated. Therefore, binding to IOVs has

been used (*14*) and is thought to reflect binding of hemoglobin to the cytoplasmic surface of the membrane in the cell.

Hemoglobin binding to inside-out vesicles (IOVs) and chymotrypsin-treated inside-out vesicles (IOVC) and cyanate hemoglobin (HbOCN) binding to IOVs were performed. An aliquot of each form of the ghost containing 0.45 mg of membrane protein as determined by Lowry's method (*32*) was incubated at room temperature for 10 min with varying concentrations (0.1−8.0 μM tetramer) of Hb or HbOCN in 5PBS6 with the final volume being 1 mL. The centrifuge tubes containing the samples were spun down at 15000 rpm for 15 min at 5 °C. A spectrum of each supernatant in the Soret region (from 450 to 350 nm) was obtained using a Perkin-Elmer Lamda-6 UV−visible spectrophotometer. The concentration of the free (nonbound) Hb or HbOCN in these samples was obtained by fitting the spectrum with parent spectra of oxyHb and metHb in the same wavelength range. The hemoglobin bound to the membrane was then directly obtained from the difference between the hemoglobin added and the free hemoglobin. The hemoglobin bound was normalized relative to the number of band 3 binding sites, even though the centrifugation method measures binding to band 3 as well as other non-band 3 sites on the cytoplasmic surface of the membrane. The number of band 3 sites on the membrane was determined from the fit for the binding of hemoglobin to IOVs with and without chymotrypsin treatment to remove the band 3 sites (see eqs 7 and 8 below). This analysis gave the best fit for 25.4% of the protein consisting of band 3, which was very close to the 25% reported in the literature (*34*). It was further assumed that one hemoglobin tetramer binds to each 93000 molecular weight band 3 subunit (*27*).

Decrease in the Eosin Mean Lifetime by Hemoglobin To Determine Hemoglobin Binding to Band 3 of the Red Cell Membrane. (A) Lifetime Fluorescence Measurements. The lifetimes of the eosin on eosin-5-maleimide-labeled red cell IOVs suspended in phosphate buffer, pH 6.0, at 0.45 mg/mL protein were measured in a 1 mm path-length cuvette using front face illumination and observation (*35*). This was done to minimize both the effects of scattering from the labeled IOVs and reabsorption from added hemoglobin. The use of IOVs is particularly valuable for fluorescence studies, because the smaller size of these vesicles, than intact ghosts, minimizes scattering artifacts.

Lifetime measurements were performed using the frequency domain (FD) instrumentation previously described (*36*, *37*). Excitation at 488 nm was provided by the vertically polarized output of a CW air-cooled argon ion laser (Omnichrome, 543-AP) that was amplitude modulated by an electrooptical low-frequency modulator (K2.LF, ISS) and input into a frequency domain fluorometer (ISS, Koala). To measure at high frequencies (up to 200 MHz), we used a PTS frequency synthesizer, output into a high-frequency amplifier (ENI) that was used to drive the Pockels cell. Phase angles and modulations were measured relative to scattered light at 488 nm using a 490 ± 5 nm interference filter. Emission was observed using a 550 ± 20 nm interference filter. Measurements were performed using magic angle conditions.

The intensity decays were recovered from the frequency domain data using the multiexponential model in the equation:

$$I(t) = \sum_i \alpha_i \exp(-t/\tau_i) \tag{1}$$

where α_i and τ_i are the preexponential factors and decay times, respectively. The fractional contribution of each decay time to the steady-state intensity was calculated using the equation:

$$f_i = \frac{\alpha_i \tau_i}{\sum_j \alpha_j \tau_j} \tag{2}$$

while the mean lifetime was calculated using the equation:

$$\bar{\tau} = \sum_i f_i \tau_i = \frac{\sum_i \alpha_i \tau_i^2}{\sum_j \alpha_j \tau_j} \tag{3}$$

(B) Decrease in Lifetime due to Hemoglobin Binding. The binding of hemoglobin to band 3 produces a decrease in the eosin lifetime. This decrease of lifetime was corrected for quenching of fluorescence by nonbound hemoglobin in solution. This correction was obtained by measuring the changes in fluorescence lifetime when hemoglobin is added to chymotrypsin (CT) modified IOVs. CT removes the cytoplasmic end of band 3 involved in the binding of hemoglobin to band 3 (*17*, *28*, *34*), and the effect on the fluorescence due to the non-band 3 hemoglobin binding to CT-modified IOVs should be minimal. The small lifetime changes observed with increasing hemoglobin concentration was, therefore, assumed to be due to the quenching of fluorescence by hemoglobin in solution. For this purpose the nonbound hemoglobin concentration determined by centrifugation was used to determine the free hemoglobin concentration which contributes to this quenching process. The additional decrease in lifetime, for IOVs not reacted with CT, in the presence of hemoglobin was attributed to the binding of hemoglobin to band 3.

RESULTS

Lifetime Measurements. Figure 1 shows the typical frequency responses for phase and modulation for eosin-5-maleimide-labeled red cell membrane IOVs. These data were fit to a two-exponential model. The first exponential corresponded to ~95% of the signal and was considered the lifetime for eosin reacted with band 3 in these vesicles. The contribution from the second lifetime was less than 5% and has an insignificant contribution to the overall quenching. Bicknese and co-workers (*38*) had previously observed this minor contribution in a similar study and neglected it. We assume this contribution to be from non-band 3 labeled sites on the membrane far removed from the eosin band 3 site. We have, therefore, also neglected this second minor component.

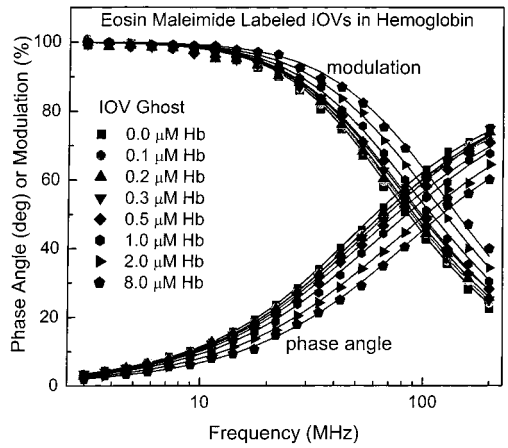

FIGURE 1: Phase and modulation frequency responses for eosin-5-maleimide-labeled red cell membrane IOVs at different hemoglobin concentrations in 5 mM phosphate-buffered saline, pH 6.0.

Table 1: Mean Lifetimes Obtained from a Global Fit of the Frequency Response for a Series of Hb and HbOCN Concentrations Added to Eosin-Labeled IOVs in 5 mM PBS Buffer, pH 6.0

$[Hb]_{tot}$ (μM)	IOV + Hb	IOV + HbOCN	$[Hb]_{tot}$ (μM)	IOV + Hb	IOV + HbOCN
0	3.08	3.08	0.5	2.777	2.98
0.1	2.963		1	2.601	2.95
0.2	2.972		2	2.259	2.78
0.3	2.914		4	2.004	2.68
0.4	2.816		8	1.926	2.51

Also shown in Figure 1 is the change in the frequency response as hemoglobin is added to the IOVs. The observed shift in the frequency response to higher frequencies as a function of increasing hemoglobin concentration indicates a decrease in the lifetime of the eosin-5-maleimide label.

The mean lifetimes calculated by a global fit of the frequency response for a whole series of hemoglobin concentrations are given in Table 1.

Analysis of Binding Data. The binding of hemoglobin to the cytoplasmic surface of the membrane was assumed to consist of the binding to non-band 3 sites (eq 4) and the binding of two hemoglobin tetramers to each band 3 dimer (eqs 5 and 6).

$$\text{membrane} + \text{hemoglobin} \rightleftharpoons \text{membrane}-\text{hemoglobin} \quad (4)$$

$$(\text{band 3})_2 + \text{hemoglobin} \rightleftharpoons (\text{band 3})_2-\text{hemoglobin} \quad (5)$$

$$(\text{band 3})_2-\text{hemoglobin} + \text{hemoglobin} \rightleftharpoons (\text{band 3})_2-(\text{hemoglobin})_2 \quad (6)$$

In the analysis of the binding to non-band 3 sites (eq 4), the number of non-band 3 sites (P_1) is expressed relative to the number of band 3 sites with a single equilibrium constant (K_1) used to average the association constants for all non-band 3 sites. The binding of the two hemoglobin tetramers to band 3 (eqs 5 and 6) has separate equilibrium constants (K_2 and K_3) representing the binding of the first tetramer of hemoglobin and the second tetramer of hemoglobin, respectively. Changes in these equilibrium constants make it possible to include cooperative and/or anticooperative interactions, which reflect the conformational changes on band 3 that occur as hemoglobin binds (*13*, *24*, *39*).

(A) Centrifugation. The binding of hemoglobin to the non-band 3 sites on chymotrypsin-treated IOVs was fit by the equation:

$$\text{bound Hb/band 3 sites} = (P_1)[\text{Hb}]_{\text{free}}(K_1)/[1 + [\text{Hb}]_{\text{free}}(K_1)] \quad (7)$$

derived from the reaction given in eq 4 with P_1 = the number of non-band 3 binding sites (relative to band 3 binding sites) and the equilibrium constant K_1 = the average association constant for the binding of hemoglobin to sites on the cytoplasmic surface of the red cell membrane which do not involve band 3.

The data for centrifugation binding of hemoglobin to the non-CT-treated membranes were fit by including in addition to the non-band 3 sites (with the parameters obtained from the CT data) binding of two hemoglobin tetramers to a band 3 dimer (*39*) as indicated by the reactions given in eqs 5 and 6. Different binding constants for the first and second hemoglobin molecule were assumed with the total number of hemoglobin binding sites equal to the number of band 3 sites (eq 8):

$$\text{bound Hb/band 3 sites} = (P_1)[\text{Hb}]_{\text{free}}(K_1)/[1 + [\text{Hb}]_{\text{free}}(K_1)] + \{[\text{Hb}]_{\text{free}}(K_2) + 2[\text{Hb}]^2_{\text{free}}(K_2K_3)\}/\{2[1 + [\text{Hb}]_{\text{free}}(K_2) + [\text{Hb}]^2_{\text{free}}(K_2K_3)]\} \quad (8)$$

with K_2 = the equilibrium constant for the binding of the first hemoglobin tetramer to the band 3 dimer and K_3 = the equilibrium constant for the binding of the second hemoglobin tetramer to the band 3 dimer. In fitting the data to eq 8 values of K_2 and K_3 as well as the number of band 3 sites were obtained. The number of band 3 sites was found to correspond to 25.4% of the membrane protein.

(B) Fluorescence. After correction of the mean lifetime for quenching due to free hemoglobin (see above), the normalized binding to band 3 was calculated by comparing the change in lifetime at any hemoglobin concentration with the change in lifetime when the band 3 sites are saturated:

$$\text{normalized binding of Hb to band 3} = [(\text{lifetime})_0 - (\text{lifetime})_i]/[(\text{lifetime})_0 - (\text{lifetime})_\infty] \quad (9)$$

The fluorescent binding data were fit by first setting the parameters for the non-band 3 sites, which do not change the eosin lifetime, equal to zero and adding an additional parameter which determines the change in lifetime when the band 3 sites are saturated, $[(\text{lifetime})_0 - (\text{lifetime})_4] = P_2$.

In fitting the fluorescence data, the K's for binding hemoglobin to band 3 obtained from the centrifugation are used and the only adjustable parameter is P_2:

$$\text{bound Hb to band 3/band 3 sites} = [(\text{lifetime})_0 - (\text{lifetime})_i]/P_2 = \{[\text{Hb}]_{\text{free}}(K_2) + 2[\text{Hb}]^2_{\text{free}}(K_2K_3)\}/\{2[1 + [\text{Hb}]_{\text{free}}(K_2) + [\text{Hb}]^2_{\text{free}}(K_2K_3)]\} \quad (10)$$

Hemoglobin binding to the band 3 dimer has been shown to produce a conformational change in band 3 (*13*, *40*, *41*). Changes in K_2 and K_3 indicative of cooperative or anticooperative interactions during the binding of hemoglobin to band 3 are consistent with such a conformational change. These conformational changes would be expected to alter

Table 2: Constants for the Binding of Hemoglobin to Band 3 IOVs

	Hb binding	HbOCN binding
equilibrium constant for non-band 3 sites (M^{-1})	$(2.49 \pm 0.19) \times 10^6$	0
no. of non-band 3 binding sites/band 3 binding sites	0.69 ± 0.02	
intrinsic constant for binding the first Hb tetramer (M^{-1})	$(1.54 \pm 0.25) \times 10^7$	$(5.34 \pm 0.15) \times 10^5$
intrinsic constant for binding the second Hb tetramer (M^{-1})	$(5.48 \pm 0.22) \times 10^6$	$(1.40 \pm 0.20) \times 10^5$

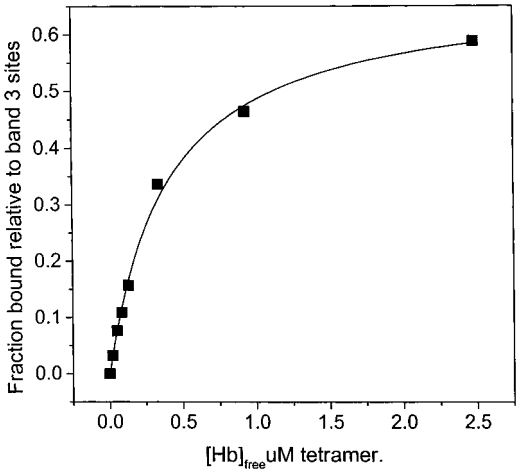

FIGURE 2: Binding of hemoglobin to chymotrypsin-treated IOVs in 5 mM phosphate-buffered saline, pH 6.0.

the distance between the hemoglobin binding site and the eosin probe, thereby influencing the change in lifetime produced by binding hemoglobin. To include this factor in our analysis of the fluorescence binding data, the change in lifetime after binding the first hemoglobin tetramer to the band 3 dimer was permitted to be different than the change in lifetime observed when hemoglobin is bound to both sites. This effect is included in the binding equation by adding a parameter, P_3, which modifies the first term in the numerator of eq 10:

$$\text{bound Hb to band 3/band 3 sites} = [(\text{lifetime})_0 - (\text{lifetime})_i]/P_2 = \{(P_3)[\text{Hb}]_{\text{free}}(K_2) + 2[\text{Hb}]^2_{\text{free}}(K_2K_3)\}/\{2[1 + [\text{Hb}]_{\text{free}}(K_2) + [\text{Hb}]^2_{\text{free}}(K_2K_3)]\} \quad (11)$$

Binding of Hemoglobin to IOVs Reacted with Chymotrypsin. Chymotrypsin cleaves the cytoplasmic end of band 3, which includes the binding site for hemoglobin on band 3. As indicated by the centrifugation results (Figure 2), hemoglobin still binds to the chymotrypsin-reacted IOVs. Equation 7 was used to fit the data in Figure 2. The parameters obtained from this fit are given in Table 2 and assumed to represent the binding of hemoglobin to non-band 3 sites on the cytoplasmic surface of the membrane (*6*).

Binding of Hemoglobin to Red Cell IOVs. The same preparation of IOVs was used for centrifugation and fluorescence lifetime studies. The concentration of nonbound hemoglobin (free hemoglobin) determined by centrifugation was used for both sets of data. Figure 3 shows the binding data obtained from both the centrifugation data and the fluorescent lifetime data. The leveling off of the fluorescent data, in a concentration range where the centrifugation data indicate increased binding, supports the contention that the fluorescence only observes the higher affinity band 3 sites, while the centrifugation also detects a group of lower affinity sites not located on band 3 (see Figure 2).

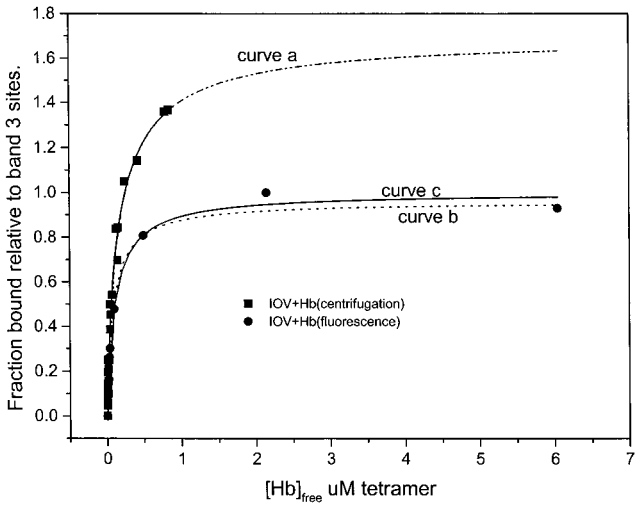

FIGURE 3: Binding of hemoglobin to red cell membrane IOVs: (■) centrifugation data; (●) fluorescence lifetime data. Curves: a, fit to eq 8; b, fit to eq 10; c, fit to eq 11 (see text).

Table 3: Effect of Hemoglobin on Fluorescence Lifetime of Eosin-Labeled IOVs

	Hb	HbOCN
eosin lifetime	3.08	3.08
lifetime with one Hb/dimer	2.34 (2.14)[a]	2.39 (2.57)[a]
lifetime with two Hb/dimer	2.09 (2.14)[a]	2.74 (2.57)[a]

[a] Number obtained by setting $P_3 = 1$.

The centrifugation data were fit by eq 8 (Figure 3, curve a) by setting the non-band 3 sites equal to those obtained for the CT-modified membranes. The equilibrium constants obtained for the binding of hemoglobin to band 3 are given in Table 2. The two constants obtained for the binding of the first hemoglobin tetramer and the second hemoglobin tetramer to band 3 are indicative of anticooperative binding where the binding of the first hemoglobin tetramer decreases the intrinsic constant for binding the second hemoglobin tetramer (see below).

The fluorescence data were fit using the same equilibrium constants for the binding to band 3 obtained from the centrifugation data. Curve b shows the fit obtained with eq 10, where the only adjustable parameter is the decrease in fluorescence lifetime when the band 3 sites are saturated with hemoglobin. Since the curve has leveled off, the decrease in the lifetime from an I_0 of 3.08 to an I_∞ of 2.09 is close to the lifetime obtained at the higher hemoglobin concentrations (Table 1).

The use of eq 11 to fit the data is shown in Figure 3, curve c. This equation provides an improved fit where the squares of the deviations decrease from 0.00231 to 0.00124. When this equation is used, the lifetime for binding the first hemoglobin tetramer to the band 3 dimer and the lifetime for saturating both hemoglobin sites are adjusted (Table 3). This adjustment is consistent with the evidence for interaction

Table 4: Eosin−Heme Distance (Å) Calculated from Fluorescence Quenching

	Hb	HbOCN
one Hb tetramer/band 3 dimer	47.90 (45.35)[a]	48.64 (51.77)[a]
two Hb tetramers/band 3 dimer	44.78 (45.35)[a]	55.99 (51.77)[a]

[a] Number obtained by setting $P_3 = 1$.

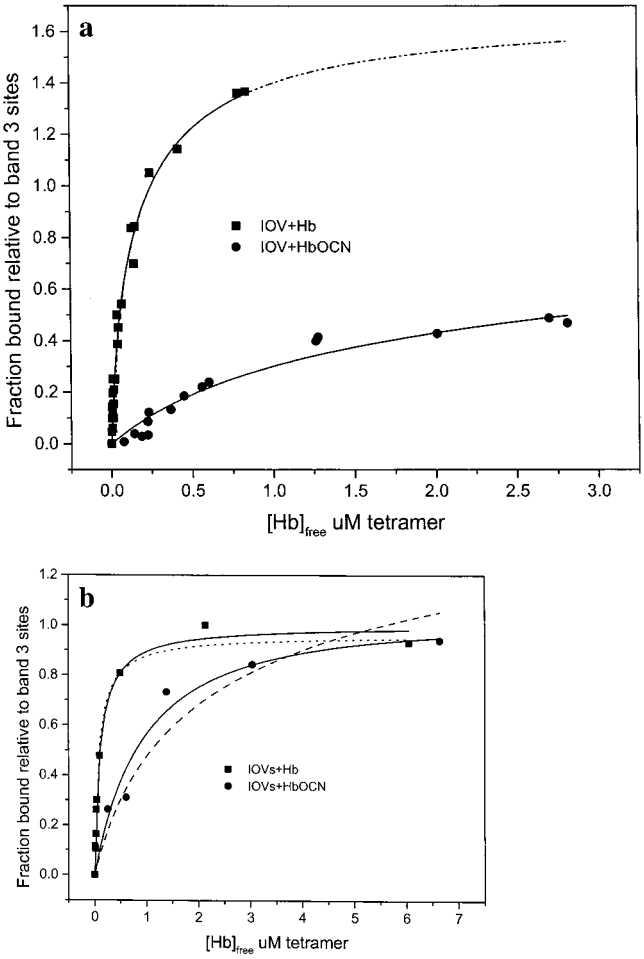

FIGURE 4: Comparison of binding of nonmodified Hb (■) and HbOCN (●) to eosin-labeled IOVs by (a) the centrifugation method and (b) the fluorescence lifetime method; Curves: (—) fit to eq 11 for both Hb and HbOCN; (•••) fit of Hb data to eq 10; (- - -) fit of HbOCN data to eq 10.

between sites which alter the values of K_2 and K_3. The value of 0.75 obtained for P_3 means that the binding to the first site has less of an effect on the lifetime (Table 3). This difference is interpreted in terms of a conformational change after the second hemoglobin is bound that brings the hemoglobin molecule closer to the eosin (Table 4).

Binding of Cyanate-Modified Hemoglobin (HbOCN) to Red Cell IOVs. Figure 4 shows that reacting hemoglobin with cyanate reduces the binding of hemoglobin to IOVs. This decrease in binding is observed both from the centrifugation data and from the fluorescence data. To fit both the centrifugation and fluorescent data with the same equilibrium constants, it was necessary to neglect binding to the non-band 3 sites. This observation is consistent with a decrease in the affinity of hemoglobin to all membrane sites when the hemoglobin is modified by cyanate. Since the non-band 3 sites already have a much lower affinity than the band 3 sites, this results in negligible binding of HbOCN to non-band 3 sites.

Figure 4a shows the fit obtained for the binding of HbOCN determined by centrifugation. The equilibrium constants obtained from eq 10 are given in Table 2. Figure 4b shows the fit to the binding determined by fluorescence. Shown in this figure are the curves obtained by using both eq 10 (dotted or dashed line) and eq 11 (solid line). As observed for the binding of nonmodified hemoglobin, eq 11 produces an improvement of the fit (the squares of the deviations decrease from 0.00112 to 0.00056). The analysis of the curves indicates that, in addition to the decrease in affinity for band 3 when hemoglobin is modified with cyanate, the binding of HbOCN produces a smaller change in fluorescence lifetime (Table 3) with the lifetime decreasing from 3.08 to only 2.74 (using eq 11) or 2.57 (using eq 10). However, in this case the binding of the second hemoglobin tetramer to the band 3 dimer decreases the change in fluorescence lifetime instead of increasing the change in fluorescence lifetime (Table 3).

The comparison of the fluorescent data (Table 3) for the binding of hemoglobin and cyanate-modified hemoglobin to the band 3 dimer indicates that in both cases the initial binding of hemoglobin produces a very similar change in lifetime. It is only after binding the second hemoglobin molecule that there are appreciable differences in the lifetime, with Hb increasing the change in lifetime and HbOCN decreasing the change in lifetime.

DISCUSSION

Binding of Hemoglobin to the Red Cell Membrane. Hemoglobin has been shown to bind with a relatively high affinity (*3*−*6*, *42*−*44*) to the cytoplasmic surface of band 3 and with a much lower affinity to a number of other sites on the membrane. Band 3 binding and non-band 3 binding have generally been separated by using chymotrypsin to cleave the cytoplasmic end of band 3, which is involved in the binding of hemoglobin. By labeling band 3 with eosin and following the quenching of the eosin fluorescence by hemoglobin (Figure 1), it was possible to directly observe the binding of hemoglobin to band 3. As expected, this binding was eliminated by reacting the membranes with chymotrypsin.

Refined binding of hemoglobin to band 3 was obtained by combining the centrifugation (before and after chymotrypsin treatment) with the fluorescence data (Figure 3). The same equilibrium constants for binding to band 3 were used in analyzing the centrifugation data and the fluorescent data. This refinement made it possible to consider the binding of two hemoglobin tetramers to the band 3 dimer. From the constants obtained by fitting eq 8, it is possible to determine the intrinsic constants for binding to the two sites. The intrinsic constant for binding to the first site (K_{i2}) is $K_2/2$ or $(1.54 \pm 0.25) \times 10^7$ M^{-1}, and the intrinsic constant for binding the second hemoglobin to the band 3 dimer (K_{i3}) is K_2K_3/K_{i2} or $(5.48 \pm 0.22) \times 10^6$ M^{-1} (Table 2). This analysis indicates that the interaction between sites produces a slight decrease in affinity (the binding is anticooperative). These equilibrium constants are in the range of the values reported in the literature (*4*, *39*), which range from 6.67×10^6 to 8.5×10^7 M^{-1}.

It has been shown that hemoglobin binding does affect the band 3 conformation (*13*), which would be expected to alter the hemoglobin−band 3 binding constants. However, this study is the first study where differences between these constants have been delineated.

Effect of Cyanate on the Binding of Hemoglobin to the Red Cell Membrane. Cyanate reacts with the terminal α-amino groups at the amino end of the hemoglobin molecule (*25*, *26*). Reaction with these groups are expected to prevent the binding of band 3 into the dyad axis of hemoglobin (*23*). This decrease in binding of hemoglobin to band 3 has been previously reported (*27*). However, our data provide the first detailed analysis of the effect of the reaction of cyanate with hemoglobin on the binding of hemoglobin to red cell membranes (Figure 4). Cyanate is shown to decrease binding not only to band 3 but also to the non-band 3 sites. The observed inhibition of binding to the non-band 3 sites may involve changes in electrostatic interactions involving hemoglobin. The decrease in the affinity for these non-band 3 sites has made it impossible to determine the apparent binding constant to these sites. It should be noted that a decrease of the affinity for these sites by the same magnitude as found for the band 3 sites (Table 3) would result in negligible binding to these sites. A comparison of the two hemoglobin sites for hemoglobin bound to band 3 indicates that the binding of HbOCN is even more anticooperative than the binding of hemoglobin with the intrinsic constants decreasing from $(5.34 \pm 0.15) \times 10^5$ to $(1.40 \pm 0.20) \times 10^5$ M^{-1}.

The ability to inhibit hemoglobin binding to the membrane can have important physiological implications. It has been shown that the reactive oxygen species generated during autoxidation are not efficiently neutralized by the cellular antioxidant enzymes when hemoglobin is bound to the membrane (*19*−*21*). The potential relevance of this factor is indicated by studies which indicate increased membrane−hemoglobin interactions during aging (*22*). The functional relevance of red cell induced oxidative stress is indicated by the correlation between red cell lipid peroxidation and impaired hemorheological properties (*45*). The finding that cyanate inhibits the binding of hemoglobin to the membrane makes cyanate hemoglobin a good candidate to investigate ways to limit red cell induced oxidative stress and to attenuate damage to the red cell membrane caused by the binding of native hemoglobin to band 3.

Use of Fluorescence Lifetime To Determine the Distance between the Heme and the Eosin Moiety. Eosin-5-maleimide reacts with lysine-430 of band 3 (*28*), which is far removed from the cytoplasmic end of band 3 where hemoglobin binds. This residue is not even located in the relatively large cytoplasmic domain of band 3 (*17*). The residue which binds EM is part of the transmembrane domain and is accessible from both sides of the membrane (*46*, *47*). The lifetime of the eosin fluorescence is, nevertheless, affected by binding hemoglobin. It is this change in lifetime which makes it possible to use fluorescence to measure the binding of hemoglobin (see above).

Changes in lifetime can be attributed to changes in the polarity in the region of the eosin moiety and/or fluorescence resonance energy transfer (FRET), which is dependent on the distance between the donor (eosin) and acceptor (the heme). The absence of a shift in the eosin emission spectra indicates that polarity changes such as those generated by changes in solvent accessibility are negligible when hemoglobin binds. The effect on the lifetime is, therefore, primarily attributed to energy resonance transfer, which depends on the distance between the donor and acceptor. This method is particularly sensitive to distances in the region of 20−60 Å (*29*). We have calculated a Forster distance (R_0) from the fluorescent properties of the eosin and the overlap between the eosin emission and hemoglobin absorption using the equation (*29*):

$$R_0 = 0.211[\kappa^2 n^{-4} Q_D J(\lambda)]^{1/6} \qquad (12)$$

where κ^2 is a parameter defining orientation of the transition dipoles of the donor and acceptor usually assumed to equal $^2/_3$, n is the refractive index which is assumed to be 1.4 for biomolecules in aqueous solution, Q_D is the quantum yield of the donor in the absence of the acceptor, and $J(\lambda)$ is the overlap integral between the donor emission and acceptor absorption. The transfer efficiency is then related to the distance between the donor and acceptor (r) and the Forster distance (R_0) by the equation (*29*):

$$E = R^6_0/(R^6_0 + r^6) = 1 - \tau_{DA}/\tau_D \qquad (13)$$

where τ_D is the lifetime in the absence of the acceptor (without hemoglobin) and τ_{DA} is the lifetime with the acceptor (hemoglobin) bound. The eosin−heme distance calculated from this analysis using eq 10, which neglects the conformational change taking place when hemoglobin binds to band 3, indicates a distance of 45.35 Å for normal hemoglobin and a distance of 51.77 Å when hemoglobin reacts with cyanate (HbOCN). These distances are consistent with the location of eosin far removed from the hemoglobin binding site. The greater distance for the HbOCN is consistent with the expectation that cyanate blocks access of the cytoplasmic end of band 3 from entering the cavity between the hemoglobin chains. Not only does this perturbation of band 3 binding by cyanate weaken the band 3 binding but the more superficial binding of hemoglobin results in a larger eosin−heme distance.

Band 3 Conformational Changes Associated with the Binding of Hemoglobin. The binding data indicate a small but significant decrease in the intrinsic constant for binding the second hemoglobin to band 3 (a 2.8-fold decrease with normal hemoglobin and a 3.8-fold decrease for cyanate-reacted hemoglobin). These results are attributed to the conformational change on band 3 when hemoglobin binds (*13*).

This same change in conformation would be expected to affect the quenching of eosin by hemoglobin. Equation 11, which includes different values for the change in lifetime when the first hemoglobin is bound and when the second hemoglobin is bound, provides this information. This analysis (Tables 3 and 4) suggests that the initial binding of unmodified hemoglobin to band 3 is <1 Å closer to eosin than is HbOCN. However, the conformational change induced when the second hemoglobin tetramer binds is completely different. Normal hemoglobin, which interacts more strongly with band 3, results in the heme−eosin distance decreasing by more than 3 Å. On the other hand, HbOCN produces a conformational change which results in

an increase in the eosin−heme distance by more than 7 Å. This observation is a clear indication of a change in the hemoglobin-induced band 3 conformational change when access to the dyad axis of hemoglobin is blocked.

CONCLUSION

The simultaneous fitting of data from fluorescence and centrifugation to study the binding of hemoglobin provides new insights into the binding which could not be obtained with only one of these methods. The centrifugation thus provides data which quantitatively measure the extent of hemoglobin binding. However, its analysis is complicated by the inclusion of non-band 3 binding sites. The fluorescence, on the other hand, only measures the binding to band 3.

The conformational change thought to take place when hemoglobin binds to band 3 results in a change in equilibrium constants for the second Hb bound to the band 3 dimer, as indicated by the centrifugation data. Simultaneous fitting of the fluorescence and centrifugation data for the binding of hemoglobin to band 3 provides additional insights into this conformational change. Since fluorescence quenching is sensitive to the distance between the hemoglobin and the eosin moiety, it is able to delineate the conformational change which takes place when hemoglobin binds to band 3 and actually provide information regarding the change in the eosin−hemoglobin distance associated with this conformational change.

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