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THE INTERACTION OF DEOXYHEMOGLOBIN WITH THE RED CELL MEMBRANE

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Summary:
The interaction of deoxyhemoglobin with the red cell membrane is characterized by comparing the affinity of deoxyhemoglobin for the membrane with that of oxyhemoglobin. The two techniques used, namely light scattering induced changes and quenching of the fluorescence intensity of a membrane embedded probe, demonstrate that deoxyhemoglobin exhibits a much lower affinity for the membrane than that of oxyhemoglobin. The binding constant of $2 \times 10\ M^{-1}$ calculated for deoxyhemoglobin at 5 mM phosphate buffer and pH=6.0 is two orders of magnitude lower than the one calculated for oxyhemoglobin. It is estimated that under physiological conditions the only species capable of interacting with the membrane is the oxyhemoglobin.

From recent studies it is becoming clear that the membrane and cytoplasm of the human erythrocyte are not truly separable (1,2,3). In the last few years we have focused our attention on the possible interaction of hemoglobin, the main component of the red cell cytoplasm, and the red cell membrane (4,5,6). It was possible to demonstrate that HbO_2 binds to the inner surface of the RBC membrane, and when bound, exhibits high affinity binding properties. Since Hb cycles physiologically between its oxygen liganded and unliganded (deoxy) states, it was thought that any physiological significance for the interaction of Hb with the red cell membrane will rely on variations in the affinity of the two states for the membrane. The present study demonstrates the dependency of the Hb affinity for the membrane on its liganded state.

Materials and Methods

Molecular Probes Inc. (Roseville, Minn.) provided 12-9-anthroyl stearic acid (AS). All other chemicals were reagent grade purchased from Sigma, Fisher, Mallinckrodt, British Drug House and Pharmacia Com-

Abbreviations: Hb: hemoglobin; Deoxy-Hb: deoxyhemoglobin; HbO_2:oxyhemoglobin; HbCO: Carbomonoxyhemoglobin; AS: anthroyl stearic acid.

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panies. Hb and unsealed ghosts were prepared as previously described (4). Membrane bound glyceraldehyde-3-phosphate dehydrogenase and aldolase were released by washing the ghosts with 0.1 M NaCl at pH=8.0. The ghosts were labeled with AS as previously described (4). Phosphatidyl serine liposomes were prepared and labeled with AS as described elsewhere (7). Fluorescence and light scattering measurements were carried out on a Hitachi-Perkin-Elmer MPF-2A Spectrofluorimeter. Emission was measured at a 90° angle to the incident beam. For fluorescence measurements the excitation wavelength was 360 nm and the emission intensity was recorded at 480 nm. For light scattering, a wavelength of 650 nm was used for excitation and emission. Cary 118 spectrophotometer was used for light absorption measurements. Kinetics were performed using a stopped flow accessory for the DW-2-UV-VIS Aminco (dual beam) spectrophotometer. Measurements of deoxy-Hb binding data were performed under nitrogen atmosphere and 0.3 mM dithionite. At the end of each titration, absorption spectrum was measured to certify that Hb remained in the deoxy state. Calculation of energy transfer parameters: Overlap integral (J) for AS and the various Hb species in this work were calculated using the absorption bands typical for each. Absorption data were taken from Antonin and Brunori (8). The critical distance R_o, defined as the distance between acceptor and donor at which 50% quenching occurs, was calculated according to Förster's theory (9) using the expression: $R_o^6 = 8.71 \times 10^{23}\ Ke\,\tau o\ n^{-4} KJ$, where Ke = the rate of emission; τo = the lifetime of the probe in the absence of quencher; n = the refractive index of the medium; K = the orientation of the emission and absorption moments of the donor and acceptor, respectively; J = the overlap integral defined above. For a set of one donor and different acceptors, it is reasonable to assume that the first four parameters in the equation do not alter when changing the acceptor and the relation between J and R_o is given therefore by: $J' / J'' = (R_o)^6 / (R_o)^6$.

Results

The affinity of deoxy-Hb for the membrane: Fig. 1 demonstrates the results of a typical experiment where the interaction of deoxy-Hb and HbO_2 for the same ghosts preparation are compared. The results (Fig. 1) show

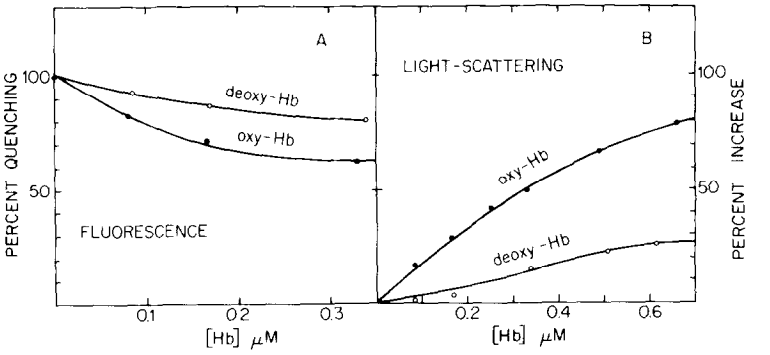

Figure 1. A comparison of the interaction of HbO_2 and deoxy-Hb with the red cell membrane in 5 mM phosphate buffer pH=6.0, temp. 20°C, ghosts concentration 10^{10}/L.
A: Fluorescence intensity measurements of HbO_2 versus deoxy-Hb.
B: Relative light scattering intensity measurements for HbO_2 versus deoxy-Hb.

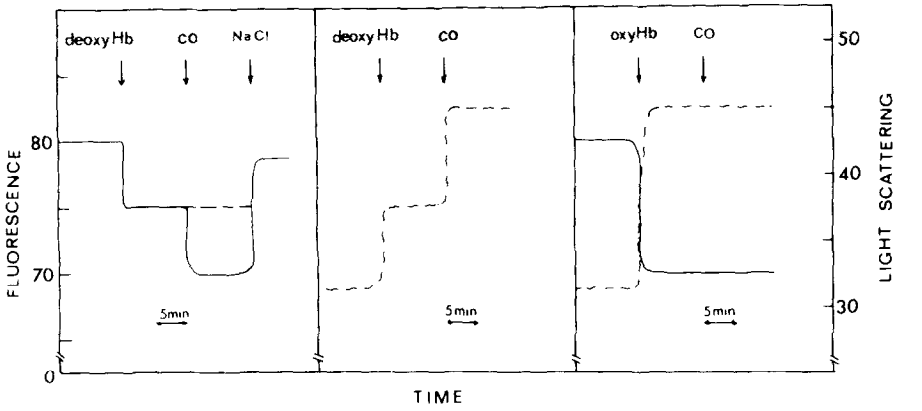

Figure 2. A comparison of relative affinities of Hb species. Ghosts concentration, 10^{10}/L. Phosphatidyl serine vesicles concentration , 0.2 mg/ml; 5 mM phosphate , pH=6.0 for ghosts and 6.7 for liposomes;temp. 20°C. Left side: arrow shows the quenching of AS by the addition of 0.6μM Hb. The change of deoxy to CO is shown by CO arrow.(_)ghosts,(...)liposomes. Middle: light scattering changes in ghosts. 0.6 μM deoxy-Hb added and later altered to HbCO.
Right side: changes induced by HbO_2. Arrow shows the altering of HbO_2 to HbCO.

that although deoxy-Hb is able to quench the fluorescence intensity of the probe, its quenching ability is much lower than that of HbO_2. Light scattering changes up to 0.6 μM are compared for the two Hb in panel B. Here again, light scattering changes caused by deoxy-Hb are much smaller than the ones induced by HbO_2 at the same concentration. Are changes observed on oxygenation a general difference between Hb in liganded and unliganded states? To answer this question and to avoid any changes due to side effects of dithionite or its products on the membrane, the fluorescence and light scattering of deoxy-Hb and HbCO were compared. The left part of Fig. 2 shows the fluorescence quenching of deoxy-Hb added to a sample of ghosts. At that stage, the nitrogen atmosphere in the tonometer was replaced by CO. Under such conditions, the only change is a transformation of the deoxy-Hb into HbCO. The data demonstrate that the transformation of deoxy-Hb into HbCO was followed by further reduction of the fluorescence intensity. Such changes could be the result of differences in spectroscopic parameters of energy transfer. We therefore calculated the relative critical donor-acceptor distances for AS and

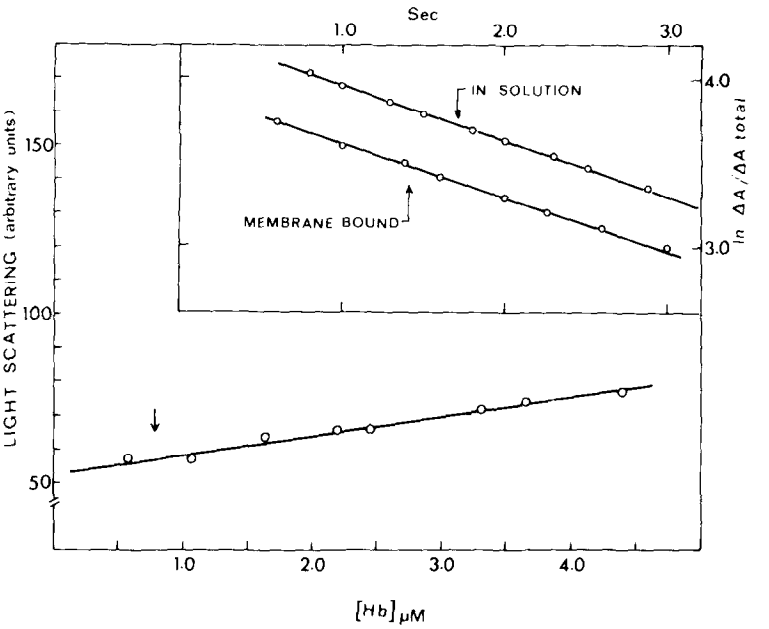

Figure 3. Light scattering changes induced by addition of deoxy-Hb to 5×10^{11} ghosts/L in 0.3 mM dithionite.
Insert: kinetic of CO binding of membrane-bound versus solution deoxy-Hb. 2 cm pass length. Hb concentration 0.75 μM before mixing and CO concentration 6 μM before mixing. The rate constant calculated for both membrane bound and solution Hb is $K = 1.1 \times 10^5 M^{-1} sec^{-1}$.

deoxy-Hb, HbO_2 and HbCO. The results showed the following values: $R_o^{deoxy\text{-}Hb} / R_o^{HbO_2} = 1.03$ and $R_o^{deoxy\text{-}Hb} / R_o^{HbCO} = 1.01$. These data predict the same quenching of fluorescence intensity per bound molecule of the various Hb species. It has been shown previously (7) that Hb binds to phosphatidyl-serine liposomes, an interaction expected to be the same for all Hb species. In this case no reduction in fluorescence intensity was observed by passing CO into the nitrogen atmosphere. (Fig. 2, left). The light scattering induced changes of the same kind of experiment (Fig. 2, middle panel), also demonstrate an elevation of the amount of Hb bound to the biomembrane after transferring the unliganded deoxy-Hb into HbCO. The right section of Fig. 2 demonstrates that changing O_2 into CO changed neither fluorescence nor light scattering signals. In Fig. 3, light scattering changes versus deoxy-Hb concentration at ghost concentration of 5×10^{11} per liter are shown. Under these conditions, the signal of

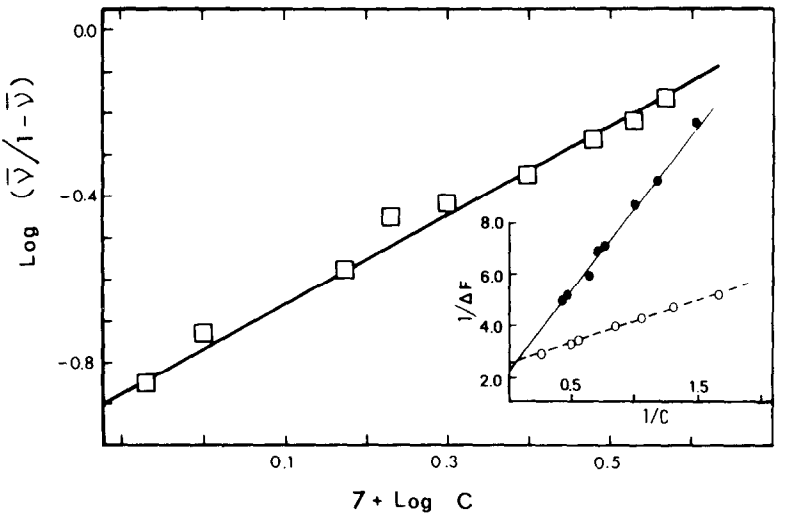

Figure 4. Hill plot for binding of deoxy-Hb to red cell ghosts. Ghosts concentration : 10^{10} cells/L; 5 mM phosphate buffer, pH=6.0;temp. 20^0C; n=1.1 and K= 2 x 10^6M $^{-1}$.(see text for calculations)
Insert: double reciprocal plots of fluorescence intensity versus Hb concentration. ΔF= percent of intensity quenched. C=free Hb concentration (tetramer). 8.0 x10^9 ghosts/L (...)HbO_2; (_) deoxy-Hb.

light scattering is linear with the amount of deoxy-Hb, showing that all Hb is bound. The arrow in Fig. 3 points at the Hb concentration in the kinetic experiment shown in the insert of that figure. We compared kinetics of CO binding to membrane bound deoxy-Hb and deoxy-Hb in solution. The results demonstrate definitely that they both have the same kinetic binding constant typical of the tetramer in the T state.

Quantitative binding parameters for deoxy-Hb to the red cell membrane: Light scattering changes require higher Hb concentrations than fluorescence measurements. Some agglutination was observed at Hb concentration larger than 5 μM. Since this interferes with light scattering changes, it was decided to use fluorescence intensity measurements wherever quantitative parameters were calculated. The double reciprocal plots of fluorescence intensity versus Hb concentration are shown in the insert of Fig. 4. The fraction of fluorescence quenched at infinity (intercepts) is practically the same for HbO_2 and deoxy-Hb (40% and 43% respectively). As their energy transfer parameters were also found to be the same, it is

concluded that the number of heme sites for oxy and deoxy-Hb on the membrane is the same, 1.6 x 10^6 sites per cell for each tetramer (4).

Calculation of the binding constants of the deoxy state: The theory of excitation energy transfer between a donor and randomly distributed acceptors bound to a membrane as summarized previously (4) showed that the relative flourescence intensity is related to the density of bound molecules by the Stern-Volmer analogue equation: $I_o/I-1 = K_q\sigma$. I_o and I are fluorescence intensities of the probe before and after quenching, respectively, σ represents the density of quenchers per cm^2 of membrane area and K_q is the effective quenching constant. The ratio of the constants of HbO_2 and deoxy-Hb will be: $K_q^{Hb} / K_q^{HbO_2} = (R_o^{Hb}/R_o^{HbO_2})^6 = 1.16$. From the number here calculated and $K_q^{HbO_2}$ of 4.9 x 10^{-13} cm^2/molecule previously calculated (4), the effective quenching constant for deoxy-Hb becomes: 5.7 x 10^{-13} cm^2/molecule. Using K_q^{Hb}, the correlation of the density of bound deoxy-Hb and the fluorescence quenching, I_o/I, was obtained. From experimental dependency (Fig. 1) of I_o/I on Hb_{total}, the correlation of σ and Hb_{total} was calculated. Free deoxy-Hb concentration was obtained using the following expression: $Hb_{free}=Hb_{total} K\sigma$, where σ is the density of bound hemoglobin and K the molarity of available sites considering the ghosts concentration in the experiment. The membrane area per ghost was taken as 147 μ^2 (10). Y, the fractional saturation of Hb sites on the membrane is given by $Y = \sigma/\sigma_\infty$. The dependency of Y on free Hb concentration is demonstrated as a Hill plot in Fig. 4. The slope of n=1.1 is consistent with a noncooperative binding. The binding constant calculated is $2x10^6 M^{-1}$.

Discussion

The findings of the present study unequivocally show that although deoxy-Hb is capable of interacting with the red cell membrane, its affinity is much lower than that of HbO_2. Why should Hb in the liganded state bind with higher affinity to the membrane? As pointed out in pre-

vious studies (4,5) although positive charge is a prerequisite for the binding, overall molecular charge is not the dominating factor. It has been made clear by several investigators (11,12) that quaternary structure of deoxy-Hb is constrained by salt bridges which are absent from the quaternary structure of the HbO_2 derivative. It seems that the ligand conformational changes occurring in the molecule affect to a significant extent the environment of those side chains which are involved in the interaction with the membrane. Potential chains are the positively charged amino groups of the 40α and 127α lysines, but participation of hydrophobic interactions should be considered. The physiological significance of the liganded high affinity Hb at the membrane surface is an intriguing question. From pH and ionic strength dependency of the apparent affinity, we estimated that the calculated binding constant measured at low pH and ionic strength will decrease in five orders of magnitude under physiological conditions. Therefore K_{app} of deoxy-Hb calculated in this work as 2×10^6 M^{-1} will become 20 M^{-1} under physiological conditions. Such a binding constant will render the bound deoxy-Hb negligible even at high physiological Hb concentration of 5 mM. On the other hand an apparent binding constant of $10^8 M^{-1}$ calculated for HbO_2 reduced by five orders will still be effective enough to saturate membrane sites physiologically. We therefore conclude that under physiological conditions deoxy-Hb probably fails to interact with the membrane and HbO_2 may be the only species capable of binding. It should be noted that membrane bound Hb is 1% of its total amount in the cell and therefore the role of membrane bound Hb in ligand binding should be considered in kinetic rather than steady state aspects. In previous work (6) we observed that in an oxygen "off" CO "on" kinetic experiment, a large fraction of membrane bound hemoglobin exists as a high affinity form. These data were interpreted as binding of Hb in dimer state only to the membrane. The findings of this work show that deoxy-Hb when

membrane bound is in a tetrameric form. Therefore, a unitary binding of the dimer should be ruled out. An alternative explanation of the "ligand exchange" kinetic data would be the failure of the membrane bound Hb molecule to undergo a fast R → T transition. This means that the membrane bound Hb stays in a high affinity form while exchanging oxygen molecules. If this situation holds physiologically, one can predict the ability of membrane bound Hb to accelerate oxygen diffusion by serving as "trap gates" (13) for oxygen molecules diffusing to the tissues when oxygen tension inside the red cell drops.

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