Spin-Label Detection of Sickle Hemoglobin–Membrane Interaction at Physiological pH†

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ABSTRACT: The spin-label electron paramagnetic resonance technique has been used to compare the interactions of normal and sickle hemoglobin molecules with human erythrocyte membranes. The sickle hemoglobin molecules show an enhanced binding to membranes when compared to normal hemoglobin (HbA) molecules. Using a simple equilibrium model for hemoglobin–membrane interactions, we obtain an equilibrium dissociation constant for sickle hemoglobin of about half that of HbA at pH 7.4 in 5 mM phosphate at 20 °C. The interactions are very low affinity in nature and are stronger at lower pH than at pH 7.4 (Fung, 1981a). The difference between normal and sickle hemoglobin persists at both high (pH 7.4) and low (pH 6.7) pH values. The concentrations of hemoglobin at the saturation level are close to physiological concentrations. Removal of spectrin–actin protein molecules from the membranes causes little change in the interactions, indicating that the remaining membrane proteins play the primary role in hemoglobin–membrane interactions. This observation is further supported by data of spectrin–actin-depleted inside-out vesicle samples. The stronger interaction of sickle hemoglobin than normal hemoglobin with membranes is discussed in relation to the formation of irreversibly sickled cells.

The fundamental defect in sickle cell disease is the genetically induced alteration in the hemoglobin (HbS)[1] molecule. It is generally believed that the polymers of deoxy-HbS determine the sickle shape of the blood cells. However, deoxy-HbS polymers in solution dissociate upon oxygenation, and yet some sickle cells retain the sickled shape even after oxygenation. They are irreversibly sickled cells (ISCs). Some sickle cell anemia patients have 5–40% of the circulating erythrocytes as ISCs. Electron microscopy of the ISCs shows no fibers which are characteristic of deoxy sickled cells (Bertles & Dobler, 1969). In vitro, all sickle cells when cycled through many times of deoxygenation and oxygenation become ISCs. Thus, polymerization of HbS is reversible, but sickling of the cells is irreversible. These ISCs suggest alterations in cell membranes. Several abnormal properties in the sickle cell membrane have been detected. For example, decreased amounts of protein Band 4.1 and increased pre-Bands 1 and 2 in ISC membrane suggest cross-linking of the spectrin–actin complex with other proteins (Lessin et al., 1978). Alteration in the distribution of phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine within the bilayer in sickled cells has been observed (Lubin et al., 1981). The calcium pump appears to be impaired (Gopinath & Vincenzi, 1979; Bookchin & Lew, 1980). Phosphorylation is increased in Bands 4.5–4.8 and decreased in Bands 2.–2.1 (Hosey & Tao, 1976; Dzandu & Johnson, 1980). However, the chemical compositions of proteins and lipids within sickle cell membranes have been shown to be similar to those of normal adult erythrocytes (Durocher & Conrad, 1974; Clark et al., 1978; Lubin et al., 1981). Furthermore, hybrid erythrocytes with sickle hemoglobin and membranes of normal cells sickle irreversibly upon prolonged deoxygenation (Clark & Shohet, 1976). It is clear that the sickling event is complex and that the membrane abnormalities appear to be induced by the abnormal hemoglobin, as suggested by many (Lessin et al., 1978; Lux, 1979; Eaton et al., 1979; Smith et al., 1981). However, there is little direct data concerning the possible role of the membrane and the molecular mechanism in sickling and irreversible sickling.

Small amounts of hemoglobin (up to 0.25 g %) bind rather tightly to the membrane in deoxygenated sickle cells but not in deoxygenated normal cells (Fischer et al., 1975; Lessin et al., 1978). Preferential binding of the HbS β chain to stroma has also been reported (Bank et al., 1974; DeSimone et al., 1977). However, the molecular mechanism leading to the binding of hemoglobin to membranes and the hemoglobin-induced membrane defects are uncertain at present. It is not clear how HbS molecules modify membrane properties to cause various clinical symptoms. Recent molecular studies on hemoglobin–membrane interaction show both high- and low-affinity binding sites of hemoglobin on membranes at pH 6.0 but none at higher pH values (Shaklai et al., 1981). We have demonstrated a very low affinity hemoglobin–membrane protein interaction at physiological pH values by spin-label EPR studies (Fung, 1981a). As we noted above, the interaction between intracellular hemoglobin molecules and the proteins on the cytoplasmic surface of the membrane at physiological pH requires techniques with high sensitivity. This interaction is very low affinity in nature, and the hemoglobin concentration at saturation level is close to physiological concentration, which is too high to make use of spectrophotometric methods. However, the spin-label EPR method is sensitive and can be used to monitor this interaction directly, even at high Hb concentrations. We have improved the sensitivity of these methods with statistical pairing procedures (discussed in detail below), to compare the membrane interactions of sickle cell hemoglobin with those of normal hemoglobin molecules at pH 7.4 in 5 mM phosphate buffer. We

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[1] Abbreviations: EPR, electron paramagnetic resonance; ISC, irreversibly sickled cell; HbS, sickle hemoglobin; HbA, normal hemoglobin; W, weakly immobilized component; S, strongly immobilized component; Mal-6, *N*-(1-oxy-2,2,6,6-tetramethyl-4-piperidinyl)maleimide; K_d, equilibrium dissociation constant.

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find that the membrane interaction with sickle cell hemoglobin is consistently stronger than that with normal hemoglobin. Samples at pH 6.7 were also studied and showed interactions stronger than at pH 7.4, as reported previously (Fung, 1981a),[2] with the difference between normal and sickle hemoglobin persisting at lower pH. We have also prepared spectrin–actin-depleted inside-out vesicles and found similar results. However, when spectrin–actin Triton shells were used in place of the membrane samples, no such interactions were observed.

Experimental Procedures

Hemoglobin (Hb) Solution. Normal packed red blood cells were obtained from the Detroit Red Cross Blood Bank. Sickle cells were obtained from the Comprehensive Sickle Cell Center at the Children's Hospital of Michigan. All samples were used within 1 week of receipt, and cellulose acetate electrophoresis was performed on the sickle cell samples to verify that the samples were homozygous.

Normal hemoglobin (HbA) and HbS hemolyzates were prepared in parallel pair from normal and sickle cells, respectively, as described previously (Fung, 1981a). After ammonium sulfate precipitation, the hemoglobin samples were each passed through a desalting column, P-6-DG (from Bio-Rad, CA), which had been equilibrated with 5 mM phosphate buffer at pH 7.4. The pH and conductivity values of the samples were checked after passing through the column to ensure that a thorough equilibration with the buffer had been reached. The hemoglobin solution was CO-gassed and concentrated with ultrafiltration cells (Amicon, MA) to a final concentration of about 10 g %. The HbA and HbS solutions were stored under CO atmosphere and used within 1 week. For each experiment, the Hb solutions were dialyzed overnight in CO-saturated 5 mM phosphate buffer at the appropriate pH (7.4 or 6.7). After dialysis, the Hb samples were centrifuged for 15 min at 17000*g* to remove any particulates and denatured proteins. Absorbance readings at 630 and 569 nm were obtained. Hb concentrations as well as methemoglobin content were determined by using published extinction coefficients (VanAssendelft & Zijlstra, 1975). Hemoglobin solutions with a methemoglobin content larger than 0.5% of the total Hb concentration were not used.

Spin-Labeled White Membrane Ghosts. Membrane ghosts were prepared from washed normal red cells by a high-volume molecular filtration method (Rosenberry et al., 1981). A filtration cell (Pellicon Cassette from Millipore, MA) and 5 mM phosphate buffer at pH 8 were used in this method. Red cells were lysed with 40 volumes of buffer. For 40 mL of packed cells to be processed, about 8 L of buffer was needed to obtain white membrane ghosts. The membrane ghosts prepared with this method were essentially the same as those prepared by centrifugation (Rosenberry et al., 1981).

The ghosts of 4 mg/mL in protein concentration were labeled with *N*-(1-oxy-2,2,6,6-tetramethyl-4-piperidinyl)maleimide (Mal-6). This spin-label (from Syva, CA) was stored in acetonitrile, which was evaporated with a gentle flow of N_2 prior to introduction of ghost membranes. A total of 30–50 μg of Mal-6 was used for each milligram of membrane protein.

After 1 h of incubation with gentle stirring in the dark, the labeled ghosts were washed with 40 volumes of 5 mM phosphate at pH 8.0 to remove excess spin-label. After the third wash, EPR spectra were taken, and washing was terminated when the EPR spectrum of a sample was the same as the one taken from the previous wash. The W/S ratio (see below, EPR Measurements) of the membrane prepared by the molecular filtration method was the same as the ratio of those prepared by the centrifugation method used previously (Fung, 1981a). The labeled proteins are primarily at the cytoplasmic side of the membrane (Fung & Simpson, 1979).

Spectrin–Actin Triton Shells. Mal-6-labeled membrane ghosts, at 4 mg/mL in protein concentration, were suspended in 5 volumes of 1% Triton X-100 in 0.056 M sodium borate buffer at pH 8.0. After incubation for 20 min the suspension was centrifuged for 30 min at 27000*g*. The supernatant was removed and the translucent pellet was dialyzed overnight in phosphate buffer containing 1% Triton. Hemoglobin samples that were used to interact with Triton shells were dialyzed in the same Triton-containing buffer.

Spectrin–Actin-Depleted Inside-Out Vesicles. The methods of Bennett & Branton (1977) were followed to remove the spectrin–actin complex.

Mal-6-labeled membrane ghosts were washed once with 40 volumes of 0.3 mM phosphate buffer at pH 7.6 and incubated in the same buffer for 30 min at 37 °C. After incubation, the sample was centrifuged for 30 min at 30000 rpm (with a Beckman type 40 rotor). The supernatant was collected as the spectrin–actin extract. Concentrations were determined from absorbance readings at 280 nm with ϵ (mg/mL) = 1.0. This supernatant sample generally contained 30–40% of the membrane proteins. The pellet obtained was dialyzed overnight in the same 5 mM phosphate buffer used for the hemoglobin samples. Electrophoresis in 5.6% polyacrylamide gel was performed on this sample as well as on the membrane sample to ensure their identities.

General Procedures. All sample preparations were carried out at 4 °C in a cold room or on ice except where otherwise stated. Protein concentrations were determined by the modified Lowry assay (Peterson, 1977). Samples in Triton X-100 were diluted to 1 mL with Triton buffer rather than deionized water.

EPR Samples. The concentrations of the HbA and HbS samples were brought to 18 mg/mL (usually by adding buffer to concentrated hemoglobin solutions). The membrane samples (ghosts, inside-out vesicles, or spectrin–actin samples) were brought to 2 mg/mL in protein concentration. Various amounts (ranging from 0 to 600 μL) of the hemoglobin solution were introduced to microcentrifuge tubes, and the final volumes of all hemoglobin samples were brought to 600 μL with 5 mM phosphate buffer. Spin-labeled membrane samples (200 μL) (ghosts, vesicles, or spectrin–actin Triton shells) were added to the hemoglobin samples. Samples were incubated on a nutator in the cold room for 30 min, followed by centrifugation. Pellets were introduced into EPR capillary tubes, as described previously (Fung, 1981a). Triplicate samples for both HbA and HbS were prepared at each hemoglobin concentration.

EPR Measurement. Standard EPR procedures used in this laboratory were followed (Fung, 1981a,b). Two EPR signal amplitudes, W and S, of each sample were measured and the W/S ratios were used to obtain the Hb–membrane dissociation constant, K_d, and spectral parameters $\Delta(W/S)_\infty$, for both HbA and HbS, by a nonlinear regression method, as described previously (Fung, 1981a). Briefly, the changes in the W/S ratio observed upon addition of Hb to the membrane were assumed to be the result of Hb binding to the membrane. Then the W/S ratio of membrane in the presence of hemoglobin, $(W/S)_{Hb}$, was the combination of the W/S ratios for

[2] The correct symbols in the legends to Figures 1 and 2 of the paper by Fung (1981a) should be as follows: pH 8.0 (■), 7.4 (▲), and 6.7 (●). The top panels in Figures 1 and 2 show the data of pH 8.0 and bottom panels in Figures 1 and 2 show the data of pH 6.7.

Table I: Hb-Membrane Binding Parameters at pH 7.4 and 6.7 in 5 mM Phosphate Buffer at 20 °C of Typical Runs of the Listed Systems[d]

system	pH	$\Delta(W/S)_\infty$	K_d (10^{-5} M)	N^a	F^b	P^c
membrane-HbA	7.4	0.69 ± 0.08	2.80 ± 0.02	10	258	<0.001
membrane-HbS	7.4	0.96 ± 0.03	1.30 ± 0.09	9	635	<0.001
membrane-HbA	6.7	0.88 ± 0.14	1.14 ± 0.05	13	410	<0.001
membrane-HbS	6.7	1.09 ± 0.04	0.83 ± 0.07	8	989	<0.001
vesicle-HbA	7.4	1.35 ± 0.07	4.10 ± 0.02	12	1380	<0.001
vesicle-HbS	7.4	1.52 ± 0.05	1.70 ± 0.14	17	1677	<0.001
vesicle-HbA	6.7	1.43 ± 0.19	0.20 ± 0.12	12	946	<0.001
vesicle-HbS	6.7	1.81 ± 0.07	0.12 ± 0.09	12	7210	<0.001

[a] N is the number of points used in the regression calculations. [b] F test of the regression significance. [c] P is the probability of obtaining F values as large or larger than those given here. [d] Listed uncertainties are the standard errors of estimate as obtained from the regression calculations.

spin-labeled membrane sites without bound hemoglobin, $(W/S)_0$, and those with bound hemoglobin, $(W/S)_b$. Assuming a simple binding model, one obtains $\Delta(W/S)_{Hb} = \Delta(W/S)_\infty (1 + K_d/[Hb])^{-1}$ where $\Delta(W/S)_{Hb} = (W/S)_0 - (W/S)_{Hb}$, $\Delta(W/S)_\infty = (W/S)_0 - (W/S)_b$, and K_d is the equilibrium dissociation constant of the reaction membrane·Hb ⇄ membrane + Hb (Fung, 1981a).

Results

Intact Membrane Ghost Systems. (1) Difference in EPR Spectra between HbA and HbS Samples. Upon addition of hemoglobin molecules, the EPR spectra of the spin-labeled membranes show differences between HbA and HbS. The relative amplitudes of the weakly immobilized component (W) are smaller in the HbS samples than in the HbA samples when equivalent membrane samples were incubated with either HbS or HbA under the same controlled conditions at pH 7.4 ± 0.1 in 5 mM phosphate buffer at 20 ± 0.5 °C. The $\Delta(W/S)$ values (W/S of membrane minus W/S of membrane with hemoglobin) of the spectra were measured as a function of the hemoglobin concentrations, as described previously (Fung, 1981a). Larger $\Delta(W/S)$ values imply that the hemoglobin molecules are interacting with the spin-labeled membrane proteins to a greater extent. Figure 1a shows the initial increase and eventual leveling off of the $\Delta(W/S)$ values upon addition of hemoglobin molecules in a typical run of paired samples of HbA and HbS at pH 7.4. This figure also shows that the values for HbS are consistently higher than those for HbA at each hemoglobin concentration used. Figure 1b shows similar differences for systems at pH 6.7. Hemoglobin has stronger interactions with membranes at lower pH values; thus, the $\Delta(W/S)$ values at pH 6.7 are larger than at pH 7.4 at corresponding Hb concentrations.

Multiple runs with membranes from different blood samples were performed to reduce systematic errors and to ensure that the differences observed between HbA and HbS on membrane protein label mobilities were not unique to membranes of a specific blood sample but were common to many blood samples.

(2) Difference in K_d and $(W/S)_\infty$ Values. Since the W/S ratios are very sensitive to membrane states, and small changes in membrane conditions cause relatively large changes in the W/S ratios (Fung et al., 1982), we have prepared the samples in triplicate at each hemoglobin concentration. The $\Delta(W/S)$ values of triplicate sets of HbA and of HbS were used to obtain the dissociation constants, K_d, and the $\Delta(W/S)_\infty$ parameters of the HbA–membrane and HbS–membrane systems by the nonlinear regression method. The K_d and $\Delta(W/S)_\infty$ values at pH 7.4 and 6.7 of typical runs are shown in Table I. F tests indicate that the regressions are highly significant. Comparing the F, or P, values in Table I with those reported earlier (Fung, 1981a), we find that the triplicate-sample approach is apparently more precise than our previous approach of using only one sample for each hemoglobin concentration in each run (Fung, 1981a).

Table II: Results of Student t Tests of Binding Parameter Differences between HbA and HbS

		$\Delta(W/S)_\infty$		K_d	
system	N^a	t	P	t	P
membrane, pH 7.4	12	4.77	<0.01	2.38	<0.05
membrane, pH 6.7	10	4.74	<0.01	3.00	<0.01
vesicle, pH 7.4	13	3.59	<0.01	5.01	<0.01
vesicle, pH 6.7	3	14.3	<0.01	5.55	<0.05

[a] N is the number of runs of paired experiments of the listed systems as described under Results.

Table III: Average Values of Binding Parameter Ratios of HbA and HbS

system	N^a	$\Delta\%^b$	$K_d(A)/K_d(S)$
membrane, pH 7.4	12	25 ± 25	2.2 ± 1.7
membrane, pH 6.7	10	17 ± 8	1.4 ± 0.1
vesicle, pH 7.4	13	26 ± 21	2.8 ± 1.4
vesicle, pH 6.7	3	19 ± 7	1.5 ± 0.2

[a] N is the same as that in Table II. [b] $\Delta\%$ is defined as $100[\Delta(W/S)_\infty(S) - \Delta(W/S)_\infty(A)]/\Delta(W/S)_\infty(A)$.

Twelve sets of the $K_d(A)$, $K_d(S)$, $\Delta(W/S)_\infty$ (A), and $\Delta(W/S)_\infty$ (S) values at pH 7.4 were also analyzed collectively with the Student's t test; the analysis shows that the differences between $K_d(A)$ and $K_d(S)$ and between $\Delta(W/S)_\infty(A)$ and $\Delta(W/S)_\infty(S)$ are both highly significantly (Table II).

Among this set of K_d values some $K_d(A)$ [or $K_d(S)$] values differ among themselves between runs, probably due to slight variation in membranes from different blood samples, but the value of $K_d(A)$ is generally higher than that of $K_d(S)$ in the same run. Thus, $K_d(A)/K_d(S)$ ratios were calculated for each run; the average value of the ratios is 2.2 ± 1.7 with $N = 12$ for system at pH 7.4 (Table III). This indicates that the HbS affinity for membranes at pH 7.4 in 5 mM phosphate at 20 °C is about twice that of HbA. Similarly, the $\Delta(W/S)_\infty(S)$ is higher than $\Delta(W/S)_\infty(A)$ (Table III).

When we lower the pH of the samples, the K_d values decrease and the $\Delta(W/S)_\infty$ values increase, indicating a stronger interaction (Table I), in good agreement with our previous results (Fung, 1981a). The differences between HbA and HbS systems persist at pH 6.7 (Tables II and III).

We have used HbA and HbS which are purified by a Bio-Rex 70 (Bio-Rad, CA) column (Schroeder & Huisman, 1980) and obtained similar results.

Spectrin–Actin Systems. Since the cytoplasmic side of the membrane consists largely of spectrin–actin molecules (Steck & Kant, 1974), it is interesting to see whether the Hb molecules interact with the spectrin–actin complex and/or with the

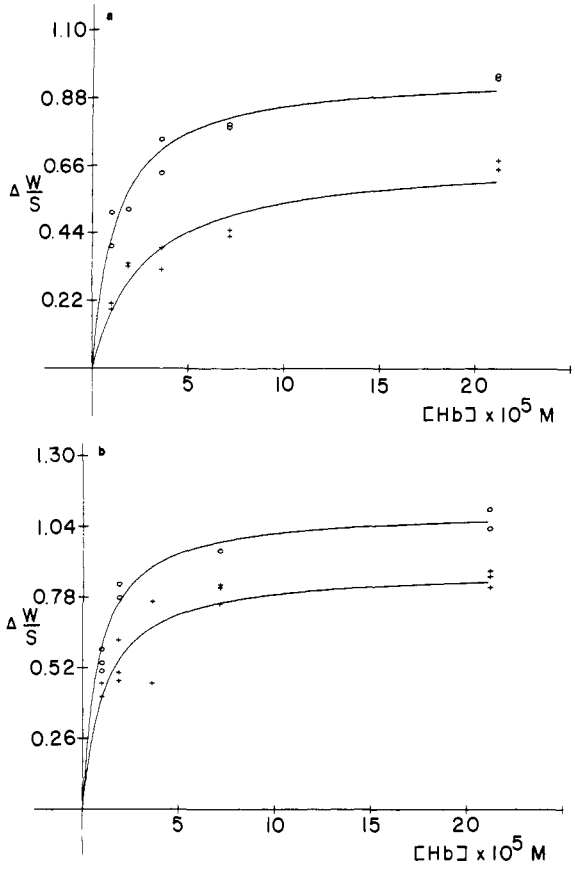

FIGURE 1: Change in $\Delta(W/S)$ of labeled membranes as a function of hemoglobin concentration in a typical run of paired samples of HbA and HbS interacted with spin-labeled membranes. Values of triplicate samples for both HbA (+) and HbS (O) at each hemoglobin concentrations show differences between HbA and HbS. Some of the $\Delta(W/S)$ values of the triplicate sample sets have very similar values and appear as a single point on the plot. The smooth curves are obtained by a nonlinear regression method using the equations discussed in the text. (a) System at pH 7.4 in 5 mM phosphate at 20 °C. (b) System at pH 6.7 in 5 mM phosphate at 20 °C.

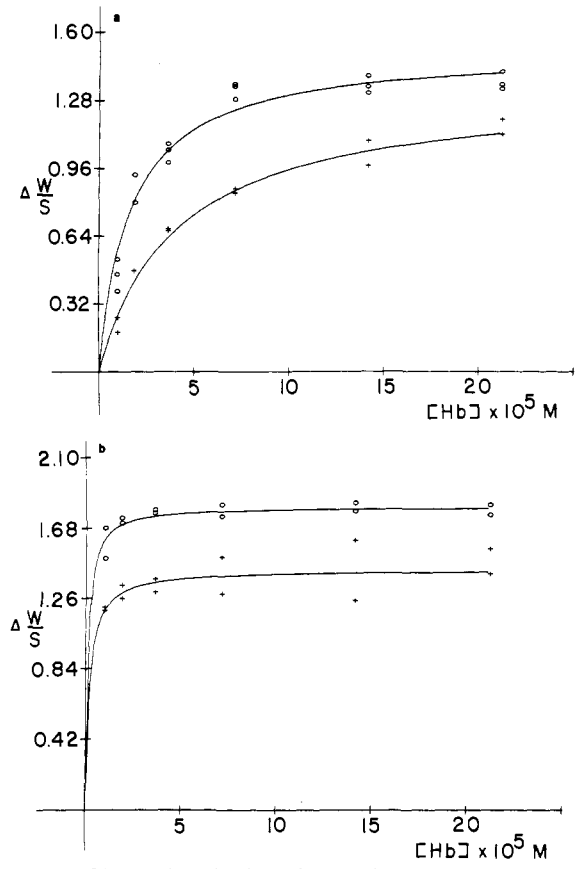

FIGURE 2: Change in $\Delta(W/S)$ of labeled spectrin–actin-depleted inside-out vesicles as a function of hemoglobin concentration in a typical run of paired samples of HbA and HbS interacted with spin-labeled vesicles. Symbol notations are as in Figure 1. (a) System at pH 7.4 in 5 mM phosphate at 20 °C. (b) System at pH 6.7 in 5 mM phosphate at 20 °C.

remaining portion of the membrane surface. However, we find that spectrin–actin samples extracted with Triton X-100 solution from the spin-labeled membranes show little reduction in the W/S values when either HbA or HbS molecules were added (data not shown).

Spectrin–Actin-Depleted Inside-Out Vesicles. The inside-out vesicles show similar decrease in the W/S values as in the intact membranes when Hb molecules were added at pH 7.4 (Figure 2a). The results from these samples (Tables I–III) and those of spectrin–actin samples show that the interaction of hemoglobin with membrane in the intact form is largely due to the interaction with non-spectrin–actin proteins on the cytoplasmic surface of the membrane. Similar results were observed for systems at pH 6.7 (Figure 2b; Tables I–III).

Discussion

Recently, with the spin-label molecules attached mainly to the proteins on the cytoplasmic side of the erythrocyte membrane surface, we have shown that the EPR spectral parameter, the W/S ratio or the $\Delta(W/S)$ value, is a very sensitive parameter for studying very low affinity hemoglobin–membrane interactions at physiological pH (Fung, 1981a). Since our labels are on the protein molecules, the hemoglobin–membrane interaction is actually a hemoglobin–membrane protein interaction. Others have shown that carboxyhemoglobin does not interact with phospholipid molecules (Bossi et al., 1975). In the present study, we show that a simple triplicate-sample approach further improves the W/S ratio sensitivity, allowing us to study interactions between different systems in a quantitative manner with highly significant confidence levels.

We have studied the difference between HbS and HbA systems in order to gain some insights toward understanding the complex problem of ISC. We have chosen the ghost membranes in 5 mM phosphate at pH 7.4 for this study since they represent a well-characterized membrane system and closely resemble the intact erythrocyte in their responsiveness (Steck & Kant, 1974). Once the interaction of Hb with this system is characterized, more complicated systems, such as membranes in higher salt concentration and effects of intracellular materials, can be studied in a systematic manner.

The present EPR data show that the HbS molecules not only interact with membranes at physiological pH but also affect the W/S ratio of membranes to a larger extent than do HbA molecules. The decrease in W/S ratio [or the increase in $\Delta(W/S)$ values] is due to the presence of hemoglobin molecules which interact with membrane components to cause small changes in protein label mobilities. As reported earlier, this immobilization of the spin-labels that are alkylated to membrane proteins is hemoglobin specific; other proteins such as BSA do not produce the same effect (Fung, 1981a). Since most of the spin-labels are on the cytoplasmic surface of the erythrocyte membrane and the inside-out vesicle samples show no decrease in W/S when hemoglobin is added, we concluded earlier that the hemoglobin molecules interact with the membrane proteins on the cytoplasmic surface of erythrocyte (Fung, 1981a). The present studies of membranes with HbS mole-

cules further indicate that HbS molecules have larger effects than HbA molecules. Because of the substitution of the hydrophobic valine residue for glutamic acid, HbS is more positively charged than HbA. We have previously suggested that the very low affinity interactions are probably, at least in part, short-range attractive electrostatic interactions (Fung, 1981a). Thus, the slight charge difference between HbA and HbS with their p*I*'s of 6.87 and 7.09, respectively (Stryer, 1981), may cause this difference in their affinity with the highly negatively charged cytoplasmic surface of the membranes. Furthermore, even though the gross structures of the relaxed form of HbA and HbS are very similar (Perutz et al., 1951), we have shown, by NMR studies of the aromatic regions of the oxy- or carboxy-HbA and -HbS molecules, that some of the surface histidine residues may have different local conformations in HbA and HbS (Fung et al., 1975). Elbaum et al. (1975) have found that there are differences in the surface properties between HbA and HbS. Thus, the $\beta 6$ mutation has caused not only the charge and hydrophobicity differences but also conformational changes beyond the $\beta 6$ region, even in the oxy state. These changes in oxy-HbS, even though sufficiently minor that they were not detected by X-ray studies, causes the molecule to show additional affinity with the cytoplasmic surface of the membranes in 5 mM phosphate buffer.

It has been shown that the spectrin–actin network extracted from ISCs with Triton X-100 containing buffer remains in the sickled shape (Lux et al., 1976). We have interacted both HbA and HbS with Triton-extracted spectrin–actin samples and found that hemoglobin molecules do not restrict the motion of these membrane protein spin-labels. However, the spectrin–actin-depleted inside-out vesicles, upon addition of (carbon monoxy)-Hb, show increases in $\Delta(W/S)$ values similar to those in the intact membranes. These results show that the hemoglobin–membrane interaction in 5 mM phosphate buffer is mainly with the non-spectrin–actin proteins that are on the cytoplasmic surface of the membrane and is not with the spectrin–actin network.

Recently, using a fluorescence probe embedded in membranes, Shaklai et al. (1981) have shown that HbS molecules show stronger binding in membranes than do HbA molecules in 5 mM phosphate at pH 6.0. Earlier they have used similar techniques on an HbA system to show that about 3% of the intracellular hemoglobin associates with the membrane at pH 6 and that this binding involves both high affinity (with a K_d of about 10^{-8} M) and low affinity (with a K_d of about 10^{-7} M) sites on membranes (Salhany & Shaklai, 1979). However, when the pH of the system was raised above pH 7, HbA completely dissociated from the membrane and a fraction of HbS was released (Shaklai et al., 1981).

The present study does not allow us to judge the significance of the HbS–membrane interaction in the formation of ISCs directly. However, at the present time, very little is known about the mechanism of ISC formation. An understanding of this HbS–membrane interaction and the effects of HbS on membrane properties in ISC studies is needed. Our studies indicate that the concentration of Hb molecules involved in the restriction of motions of the spin-labels that are attached to the membrane protein is relatively high. It is close to the intracellular hemoglobin concentration level. We suggest that this integration may eventually cause some of the intracellular hemoglobin molecules to associate tightly with erythrocyte membrane. The initial step of the very low affinity interaction may simply be to bring hemoglobin molecules closer to the membrane surface and shift the equilibrium of membrane·Hb $\rightleftarrows$ membrane + Hb toward membrane·Hb, and subsequently a fraction of the hemoglobin molecules show higher affinity with the membrane. When the very low affinity interaction becomes stronger (as in the case of HbS and at lower pH medium), it may further lead to a tight association of a small amount of Hb with membranes. This may explain what occurs for the HbS–membrane system in acid pH medium (Fischer et al., 1975). In vivo, where the Hb concentration is high to give high activity coefficient, this interaction may become more significant. Recently elegant ^{13}C NMR experiments show HbS molecules form polymers in cells at high oxygen saturation values (Noguchi & Schechter, 1981), and this unexpectedly low solubility of HbS in sickle erythrocytes can be largely accounted for by the theory of protein nonideality (Minton, 1977). As discussed previously, in our data analysis, we have not taken the thermodynamic nonideality of the hemoglobin into consideration (Fung, 1981a). Detailed thermodynamic approach may change the half-saturation concentration values. It is also important to study these interactions under physiological ionic strength condition.

In summary, high levels of HbS molecules (close to physiological concentration) exhibit an interaction, which we call a very low affinity type, with membrane proteins that are mainly on the cytoplasmic side of erythrocytes and are mostly non-spectrin–actin proteins at pH 7.4. The interaction is slightly stronger at lower pH values. K_d and $\Delta(W/S)_\infty$ values are obtained for HbS–membrane systems by using a simple binding model and nonlinear regression method. These values show that the very low affinity interaction in HbS is stronger than that of HbA. We suggest that the very low affinity interaction of HbS may eventually promote strong association of Hb molecules with the membrane surface.

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Mercuric Reductase from R-Plasmid NR1: Characterization and Mechanistic Study†

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ABSTRACT: Mercuric reductase, a flavoprotein which catalyzes the NADPH-dependent reduction of mercuric ion to metallic mercury, has been purified from *Escherichia coli* containing the cloned mercury resistance genes from plasmid NR1. The purification, which involves Blue Dextran affinity chromatography and ion exchange, gives a 1000-fold purification with an overall yield of 30%. The purified enzyme has a molecular weight of 110 000 and is composed of two identical subunits of 56 000 each. The enzyme was found to be incapable of reducing any of the other metal ions tested although Cd, Ag, Cu, and Au were shown to be potent inhibitors. Anaerobic titration of the enzyme with NADPH indicated that the enzyme-bound FAD could not be fully reduced to $FADH_2$ unless arsenite was included in the reaction mixture. In the absence of arsenite, NADPH formed a charge-transfer complex with partially reduced enzyme with an absorbance maximum around 540 nm. The similarity of these spectra with glutathione reductase suggested the presence of oxidation–reduction active cysteine residues at the active site. This was verified by the appearance of two additional thiols in NADPH, reduced enzyme. In the presence of Hg^{2+}, the purified enzyme requires ethylenediaminetetraacetic acid (EDTA) or thiol reagents for activity. With EDTA, there is an initial, rapid reaction velocity which slowly ($t_{1/2}$ = 0.14 s) decreases to no detectable rate, indicating the slow formation of an irreversibly inhibited complex. The completely inhibited enzyme can be slowly reactivated by the addition of 2-mercaptoethanol. In the presence of thiol reagents there was marked biphasic kinetics characterized by an initial rapid reaction velocity which slowly reaches a much slower steady-state rate. This was shown to be a hysteretic phenomenon induced by sulfhydryl compounds. These results are discussed in terms of the proposed mechanism.

The enzyme mercuric reductase (reduced NADP:mercuric ion oxidoreductase), which catalyzes the NADPH-dependent reduction of mercuric ion to elemental mercury, occupies a key position in the mercury detoxification system found in many bacteria (Summers & Silver, 1978). Organisms which possess the required plasmid-encoded genes for mercuric reductase are able to reduce mercuric ion to elemental mercury which is volatile. The volatile mercury evaporates from the growth medium. Studies aimed at understanding the properties of mercuric reductase will give insight into the molecular mechanism of this rather unique biological detoxification system. This information will be useful from the standpoint of mercury toxicology and the overall mercury cycle in our environment. Mercuric reductase has previously been isolated from *Pseudomonas* K62 (Furukawa & and Tonomura, 1972; Tezuka & Tonomura, 1976, from two plasmid-bearing *Escherichia coli* (Izaki et al., 1974; Schottel, 1978), and most recently from a strain of *Pseudomonas aeruginosa* carrying the plasmid pVS1 (Fox & Walsh, 1982). From these studies the enzyme has been found to contain FAD, utilize NADPH as an electron-donating cofactor, and require thiol reagents for activity. Fox & Walsh (1982) have also shown that mercuric reductase appears to have a mechanism similar to lipoamide dehydrogenase and glutathione reductase. These flavoproteins catalyze the transfer of electrons between pyridine nucleotides and disulfide substrates through the oxidation–

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