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Light-Scattering Measurements of Hemoglobin Binding to the Erythrocyte Membrane. Evidence for Transmembrane Effects Related to a Disulfonic Stilbene Binding to Band 3†

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ABSTRACT: Hemoglobin binding to isolated human erythrocyte membranes was studied by using light scattering. The light-scattering change induced upon binding gave saturation curves which were quantitatively comparable to those obtained by centrifugation measurements under stoichiometric conditions. Binding isotherms were constructed as a function of both hemoglobin and membrane concentrations. Two classes of binding sites were identified. A high-affinity class, consisting of $\sim 1 \times 10^6$ hemoglobin tetramer sites per cell, was shown to be coincident with the glyceraldehyde-3-phosphate dehydrogenase binding site on the cytoplasmic portion of band 3, the membrane protein involved in anion transport. A second class of $\sim 4 \times 10^6$ sites/cell had a very much lower affinity and are presumably located elsewhere on the inner surface of the membrane. Following these characterizations, DIDS [4,4′-bis(isothiocyano)-2,2′-stilbenedisulfonate] binding to externally exposed portions of band 3 is shown to alter hemoglobin binding by changing the isotherms from noncooperative to apparent negative cooperative at low membrane concentrations. These results suggest that DIDS binding to band 3, presumably at the anion transport site, results in transmembrane conformational changes which affect the affinity of protein binding sites on the cytoplasmic side of the membrane.

Recent studies have suggested that hemoglobin may bind to band 3 (Shaklai et al., 1977a,b; Salhany & Shaklai, 1979), the transmembrane protein of the erythrocyte involved in anion transport (Cabantchik et al., 1978). Techniques are required which can be used to study hemoglobin binding at concentrations approaching those present within the erythrocyte in order to test the possibility that this interaction may occur under physiological conditions. A relatively unexplored technique which has this potential is light scattering. Since light scattering from particles the size of erythrocytes and their ghosts arises from size and particularly shape changes, binding could be studied if hemoglobin binds to a particular site on the membrane and produces a change in the "shape" of the ghost. Then, the only requirement would be that the resultant hemoglobin-induced light-scattering change be a linear function of the total hemoglobin added under stoichiometric conditions.

After fluorescence measurements were published indicating the conditions for hemoglobin binding (Shaklai et al., 1977a), one of us (J.M.S.) discovered a very large hemoglobin-induced light-scattering effect under those conditions. Myoglobin, on the other hand, caused no light-scattering change under the same conditions. Furthermore, there was no effect of hemoglobin on light scattering at alkaline pH. Following this discovery, Salhany & Shaklai (1979) used the effect preliminarily as an adjunct to measurements of the functional properties of membrane-bound hemoglobin. In the present study, we provide a more detailed characterization of the hemoglobin-induced light-scattering effect. We then use this effect to show that hemoglobin binding to the cytoplasmic surface of the membrane can be influenced by DIDS[1] binding to the site involved in the inhibition of anion transport. This site is located on the outer aspect of band 3 and is thought to be coincident with the anion transport site (Cabantchik et al., 1978).

Materials and Methods

Freshly outdated whole blood or packed cells for this and other projects were obtained from the Omaha chapter of the American Red Cross. Most of the other reagents used have been described in previous publications from this laboratory (Salhany & Swanson, 1978; Salhany et al., 1978; Salhany & Shaklai, 1979). The proteolytic enzymes and G3PD were from Sigma, as was the inhibitor PMSF. DIDS was from Pierce Chemical Co.

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[1] Abbreviations used: G3PD, glyceraldehyde-3-phosphate dehydrogenase; DIDS, 4,4′-bis(isothiocyano)-2,2′-stilbenedisulfonate; PMSF, phenylmethanesulfonyl fluoride; 5P(pH), 5 mM orthophosphate, with the pH given in parentheses; PBS, 5 mM orthophosphate + 150 mM NaCl, pH 8; BSA, bovine serum albumin; $NaDodSO_4$, sodium dodecyl sulfate.

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Preparative Methods. Preparation of unsealed ghosts was as described previously (Salhany et al., 1978). Unsealed ghosts used in this work were routinely stripped of G3PD [band 3 of Fairbanks et al. (1971)] and any residual hemoglobin by washing once in cold 200 mM NaCl plus 5P(8) and then washing twice in 5P(8) alone (Kant & Steck, 1973). Confirmation of band 6 depletion was made by using $NaDodSO_4$ gel electrophoresis according to previously described methods (Fairbanks et al., 1971; Salhany et al., 1978). NaOH-stripped ghosts were prepared as described by Steck et al. (1976). Proteolytic dissections of band 3 were performed as described by Steck et al. (1976) and the products analyzed by using $NaDodSO_4$ gel electrophoresis. Inside-out vesicles were prepared according to the method described by Steck (1974a).

Reaction of erythrocyte membranes with DIDS was performed as follows. Twenty-milliliter samples of PBS-washed (3×) erythrocytes were mixed 1:1 with PBS–DIDS solutions to the final DIDS concentrations indicated. The cells (50% hematocrit final) and DIDS mixtures were allowed to react at 37 °C for 45 min in foil-covered flasks (final pH 6.5). After the reaction period was over, the samples were transferred to centrifuge tubes and spun at 4000 rpm in a CRU 5000 centrifuge at 4 °C. The cells were collected and resuspended in PBS containing 50 μM oxyhemoglobin to react with the free DIDS. The cells were then washed 3 more times in PBS. This procedure was used for those studies where the membranes from DIDS-reacted ghosts were isolated. Isolation procedures and salt stripping were then performed as described above. Certain experiments reported below used membranes from DIDS-reacted cells treated with α-chymotrypsin after DIDS and prior to membrane isolation. DIDS reaction was as just described, and free DIDS was washed away. Chymotrypsin digestion of intact erythrocytes was performed as described by Steck et al. (1976). Enzyme digestion was stopped by using PMSF. Confirmation of digestion was made by analysis of membrane electrophoretic patterns.

In those studies where dithionite–sulfate heteroexchange into DIDS-reacted erythrocytes was studied, a slightly different treatment was performed prior to reaction with DIDS. The difference consisted of conversion of intracellular HbO_2 to methemoglobin by suspending PBS-washed erythrocytes (10% hematocrit) in 20 mM sodium nitrite in PBS and allowing reaction for 30 min at room temperature. The cells were then washed 3 times in 90 mM sodium sulfate plus 5P(8). Following this treatment, the cells were reacted with DIDS as described above except that the medium was now sulfate–phosphate instead of chloride–phosphate.

G3PD (from yeast) was obtained as the lyophilized protein and was solubilized, extensively dialyzed [or desalted on a gel filtration column (G25)], and volume reduced with vacuum dialysis as previously described (Gaines et al., 1977). G3PD preparations were at 4 °C.

Total protein analyses were performed as described (Lowry et al., 1951) by using a BSA standard.

Analytical Procedures and Data Analysis. The 90° light-scattering measurements were performed by using the 90° light-scattering cell of the Gibson-Durrum stopped-flow apparatus with monochromatic incident light of 700 nm. At this wavelength, there is no significant absorption contribution from hemoglobin over the concentration ranges studied here. Stopped-flow light-scattering kinetic measurements were made with the same instrument interfaced to a computerized data acquisition system previously described (Demma & Salhany, 1977). The basic experiment involves measurement of the amount of 90° scattered light in terms of photomultiplier voltage for a given ghost suspension mixed first with buffer and then with various concentrations of oxyhemoglobin.

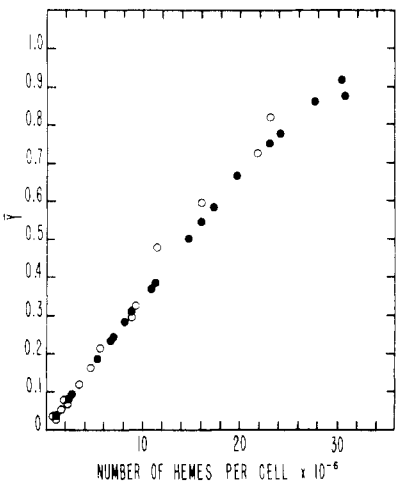

FIGURE 1: Comparison of light scattering with centrifugation for hemoglobin binding to erythrocyte membranes. The fractional saturation Y (i.e., the fractional voltage change or fraction of hemoglobin bound) is plotted vs. the total number of hemes added per cell in millions. The open circles (○) are from light-scattering measurements, and the closed circles (●) are the centrifugation results. The concentration of ghosts before centrifugation or after mixing in the light-scattering experiments was ~1.3 mg/mL. The temperature was 25 °C, and the buffer was 5P(6). Calculation of the total number of hemes added per cell was made by using the total concentration of heme added, Avogadro di Quaregna's number, and a constant from the work of Lepke et al. (1976) relating the number of ghosts present to the ghost protein concentration (i.e., 5.13×10^{-10} mg/ghost).

Centrifugation measurements were made with a Sorvall RC2B at 25 °C by using either SS34 or GSA rotors and spinning rates of 10000 rpm for 15 min.

Dithionite–sulfate heteroexchange experiments were performed by using the on-line split-beam stopped-flow method described by Salhany & Swanson (1978).

Curve-fitting procedures used in this work employed a program capable of weighted linear or nonlinear fits of up to 20 adjustable parameters (Dye & Nicely, 1971). The program gives an estimate of the variance–covariance matrix with multiple correlation coefficients and estimates of the standard error for each parameter derived from this matrix.

Results

Comparison of Hemoglobin Binding As Determined by Light Scattering and by Centrifugation. If light scattering can measure the fraction of hemoglobin bound to ghosts, then one should expect the light-scattering saturation curve to be coincident with that determined by centrifugation when both are measured under the same conditions. Centrifugation and light scattering were compared under stoichiometric conditions (i.e., [sites] per unit of sample volume > [heme]). Under these conditions, the centrifugation process (i.e., shifts in the equilibrium) should be without major consequence to the comparison. The results of this comparison are shown in Figure 1. There was good agreement between the two methods, thus establishing that the fractional light-scattering change measures the fraction of membrane-bound hemoglobin.

Light-Scattering Kinetics and Evidence for the Reversibility of Binding. Having demonstrated that light scattering measures the fraction of hemoglobin bound to membranes, it was next necessary to establish that the hemoglobin-induced light-scattering effect is reversible. In order to accomplish this, we elected to investigate the kinetics of hemoglobin release from the membrane with pH jump (Shaklai et al., 1977a).

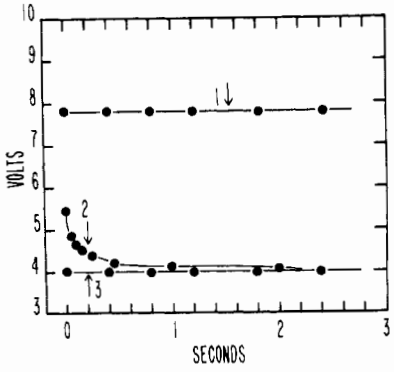

FIGURE 2: pH reversibility of hemoglobin binding as determined by light-scattering kinetic measurements. The results are plotted as observed voltage vs. time. Curve 1 shows the observed voltage when ghosts *plus* hemoglobin (2.25 mg/mL and 127 μM heme, respectively) in 5P(6) are mixed with 5P(6). This is also the same final voltage observed when 2.25 mg/mL ghosts were mixed with 127 μM hemoglobin at the same instrument settings. Curve 2 shows the observed time course of light-scattering reversal when the same suspension of ghosts and HbO_2 as in curve 1 was mixed with 50 mM Tris base to a final pH of 9. Curve 3 shows the time course when 2.25 mg/mL ghosts are mixed with 50 mM Tris base. The temperature was 25 °C. Over 60% of the voltage change in curve 2 occurred during the dead time of the stopped-flow apparatus (~3–5 ms).

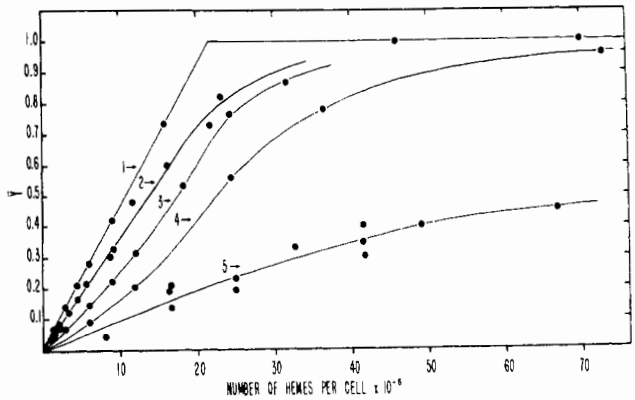

FIGURE 3: Stoichiometry and membrane concentration dependence of hemoglobin binding. Fractional saturation is plotted vs. the total number of hemes added per cell in millions. Experimental conditions were 5P(6) and 25 °C with the following final ghost protein concentrations: (1) 2.0; (2) 1.3; (3) 1.0; (4) 0.25; (5) 0.036 mg/mL. Curve 1 indicates a stoichiometry of ~22 × 10^6 hemes/cell. The lines are drawn and have no theoretical significance (see the text).

The results are shown in Figure 2. Curve 1 is the observed voltage when ghosts and hemoglobin were premixed in 5P(6) and then mixed with 5P(6) 50:50 in the stopped-flow apparatus. The same voltage was attained when hemoglobin-free membranes were mixed with hemoglobin in the stopped-flow apparatus. When membrane-bound hemoglobin was mixed in the stopped-flow apparatus with 50 mM Tris base to a final pH of 9, light scattering was rapidly reversed (curve 2). The base line voltage was the same as that obtained when hemoglobin-free ghosts in 5P(6) were mixed with 50 mM Tris base to pH 9. Similar results were obtained with inside-out vesicles and with NaOH-stripped ghosts which form inside-out vesicles with all peripheral proteins removed (Steck, 1974a,b). Centrifugation measurements with inside-out vesicles confirmed the complete release of hemoglobin. The surprising result of Figure 2 was that over 60% of the reaction was lost in the dead time of the stopped-flow apparatus (~3–5 ms). Binding kinetics under heme-limited conditions were similarly rapid.

Dependence of Hemoglobin Binding on Membrane Concentration. Stoichiometry of Binding. In order to completely describe equilibrium binding, one should determine the probability of binding as a function of total ligand and total site concentration. At the highest site concentration, one may expect to see stoichiometric binding. As the concentration of sites decreases, one of several possible results may then occur. If there is only one type of binding site and binding is noncooperative, a plot of the fraction of saturation, Y, vs. the concentration of total ligand added will deviate from the stoichiometric line hyperbolically. If there is positive cooperativity between sites, sigmoidal curves will develop as the site concentration is decreased. However, if there are two classes of sites which do not interact but have different affinities, one may expect to see pseudopositive cooperativity with the *initial* dilutions owing to the fact that initial binding to the high-affinity site will be stoichiometric. With further dilution, pseudonegative cooperativity will appear if the sites do not differ greatly in affinity. Noncooperative binding would be observed only at the lowest ghost concentrations and only if there is a wide difference in affinity between the two classes of sites such that binding to the second site does not occur. In this latter situation, Hill's constant will decrease with dilution. The experiment may be described by rearranging the Hill equation as (Anderson & Antonini, 1968)

$$X_0/P_0 = Y + (K_d^{1/n}/P_0)[Y/(1 - Y)]^{1/n} \qquad (1)$$

where K_d is the equilibrium dissociation constant $(1 - Y)X^n/Y$ with $K = X_{1/2} = K_d^{1/n}$, X_0 is the total concentration of ligand, and P_0 is the total site concentration.

Figure 3 shows the results from such experiments for hemoglobin binding to erythrocyte membranes as a function of ghost concentration. The stoichiometry of binding was found to be ~22 × 10^6 hemes/cell (curve 1). As the concentration of membranes was decreased, apparent positive cooperativity developed. However, with further dilution cooperativity disappeared. This visual appearance was confirmed by computer fitting of the data using eq 1. The value of Hill's constant varied from 2.74 for curve 3 to 1.09 for curve 5. This behavior would seem to indicate that the positive cooperativity was only apparent and the consequence of two or more sites with wide differences in hemoglobin affinity. We have estimated that the low-affinity site has a dissociation constant of greater than or equal to 40 μM expressed in tetrameric units (see Discussion). As we shall see below, this is at least 40 times lower in affinity than the high-affinity site.

Since curve 1 of Figure 3 represents the stoichiometry for all sites, we sought to determine the stoichiometry of those sites related to the binding of hemoglobin to band 3. In order to accomplish this, we used the fact that one G3PD tetramer binds to one band 3 monomer (Yu & Steck, 1975). If one adds enough G3PD to half-saturate and then fully saturate the band 3 sites, stoichiometric hemoglobin binding should be proportionally reduced. The results of such experiments are shown in Figure 4. Increasing the ratio of G3PD tetramer to band 3 monomer caused a total of ~4 × 10^6 hemes (or 1 × 10^6 hemoglobin tetramers) per ghost to titrate.[2]

Inhibition of Hemoglobin Binding by G3PD. We have seen that there are at least two classes of sites, one with a high hemoglobin affinity and the other with a much lower affinity (Figure 3). Under stoichiometric conditions, addition of G3PD titrates a finite number of sites corresponding to ~1 × 10^6

[2] It should be noted that addition of G3PD also causes a light-scattering change. However, the G3PD-induced change is expected to be additive to saturation. Therefore, normalization of the hemoglobin-induced voltage change by the Δ $volts_{total}$ value reached at saturation for that given condition accounts for the additive effect of G3PD.

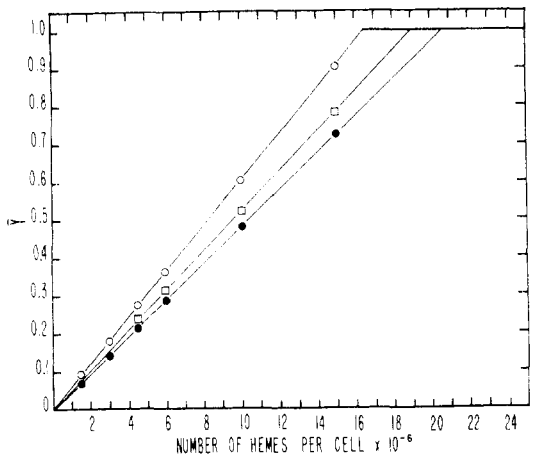

FIGURE 4: Effect of G3PD on the stoichiometry of hemoglobin binding to erythrocyte membranes. The fractional saturation is plotted vs. the total number of hemes added per cell, in millions, for various ratios of G3PD tetramer to band 3 monomer. The ratios of G3PD tetramer to band 3 monomer were (●) 0, (□) 0.5, and (○) 1. The concentration of band 3 was calculated by using the final ghost concentration (2 mg/mL) and assuming 1×10^6 band 3 monomers per cell. G3PD was prepared as described in the text. The temperature was 25 °C, and the buffer was 5P(6). The difference in the stoichiometry between control ghosts and the 1:1 G3PD tetramer/band 3 monomer ghosts was $\sim 4 \times 10^6$ hemes/cell.

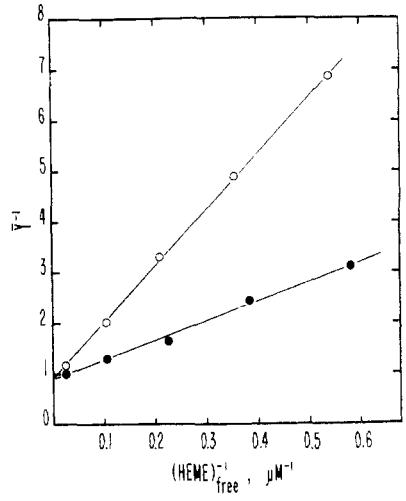

FIGURE 5: Effect of G3PD on hemoglobin binding under affinity conditions. Double-reciprocal plot of the fraction of saturation vs. the concentration of free heme (see text). Control ghosts (●) are salt stripped while those with G3PD (○) contained 0.45 μM tetramer after mixing. The plots can be represented by the equation $Y^{-1} = K_d \cdot [\text{heme}]_{free}^{-1} + 1$ with $K_d = 1.1$ μM (tetramer) for control and 3.1 μM for plus G3PD. Binding was studied at 25 °C and in 5P(6). The ghost concentration in both cases was ~42 μg/mL.

hemoglobin tetramers/ghost (Figure 4). The stoichiometric results do not directly indicate whether these sites are the high-affinity site or the low-affinity site seen in Figure 3. Although we would expect the high-affinity site to bind G3PD first under stoichiometric conditions, we sought direct evidence for this by studying hemoglobin binding at very low ghost concentrations in the absence and presence of G3PD. Under these conditions, the results of Figure 3 show that binding occurs almost exclusively to the high-affinity site. Figure 5 shows that addition of G3PD lowers hemoglobin's affinity under these conditions, suggesting that both proteins at least compete for the high-affinity site.

Sidedness Effects of Proteolytic Digestion by Chymotrypsin. Evidence That Hemoglobin Binds to a Protein on the Inner Surface of the Membrane. If hemoglobin binds to band 3 at

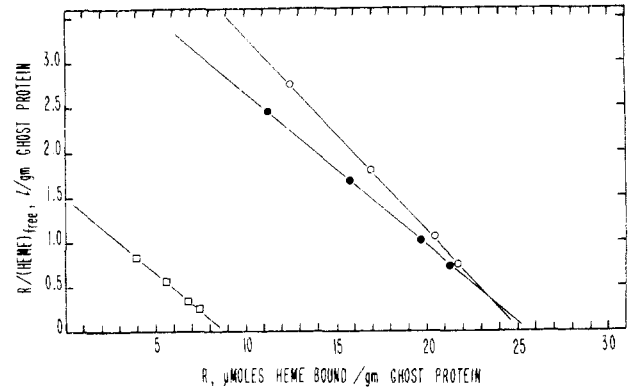

FIGURE 6: Effect of the sidedness of proteolytic digestion with α-chymotrypsin on hemoglobin binding under affinity conditions. Scatchard plots for control, salt-stripped ghosts (●), α-chymotrypsin outside-digested ghosts (○), and ghosts digested on both sides (□). The final ghost protein concentrations were the same in all cases (36 μg/mL). R is the number of moles of heme bound per gram of ghost protein. These plots were calculated by using the *measured* hemoglobin binding capacities for control and chymotrypsin-digested ghosts under stoichiometric conditions (see the text for further details). $T = 25$ °C in 5P(6).

the cytoplasmic portion of that transmembrane protein, proteolytic digestion of the outer surface of the membrane might be expected to have little effect on binding while digestion at the inner surface should have a marked effect. Figure 6 shows Scatchard plots from combined results of both stoichiometric and affinity measurements. Digestion of unsealed ghosts with chymotrypsin cleaved band 3 at both surfaces (Steck et al., 1976) and caused about a 60% reduction in hemoglobin binding capacity when measured under stoichiometric conditions. Digestion of intact erythrocytes with chymotrypsin prior to isolation of the membranes was without significant effect. Under affinity conditions, digestion of both surfaces of the membrane also gave evidence for reduction in the number of sites. This was established by inspection of the raw data. Double-reciprocal plots of Δ volts vs. heme concentration showed no significant difference in slopes or intercepts for outside digestion vs. control but about a 50% reduction in Δ volts_{max} (i.e., the intercept) and no significant change in slope for chymotrypsin digestion at both surfaces, thus supporting the direct measurements made under stoichiometric conditions.

Finally, the 40000-dalton, water-soluble, cytoplasmic "tail" of band 3 was isolated according to the method of Steck et al. (1976) Confirmation of the purity of the material was made by combined gel chromatographic and electrophoretic analysis. Addition of stoichiometric amounts of this protein to hemoglobin solution caused an increase in the fraction of rapid CO binding component similar to that seen with membrane-bound hemoglobin (Salhany & Shaklai, 1979).

Effect of DIDS Binding to Band 3 on Hemoglobin Binding to the Cytoplasmic Portion of the Membrane. Further evidence that hemoglobin binds to band 3 would be to show some specific perturbation of band 3 which changes the mechanism of hemoglobin binding at its cytoplasmic site. Grinstein et al. (1979) have recently provided some evidence that DIDS binding to the anion transport site causes a change in band 3 (and other components of the membrane) which lowers the affinity of the anion NAP-taurine for transport sites at the cytoplasmic surface. These results suggest some DIDS-induced structural change in band 3. If this structural change extends to the hemoglobin binding site, one might expect hemoglobin's affinity at the cytoplasmic surface to change.

An experimental approach to this question would be to react DIDS with intact erythrocytes, wash free DIDS away, and

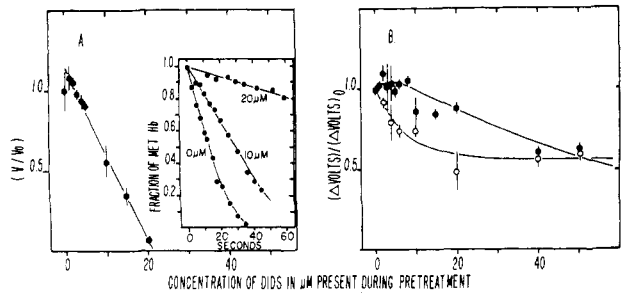

FIGURE 7: Effect of DIDS reaction with intact erythrocytes (A) on the velocity of dithionite–sulfate heteroexchange and (B) on the light-scattering change due to hemoglobin binding to membranes isolated from similarly treated cells. (A) Dithionite flux. Methemoglobin-containing erythrocytes were prepared and reacted with DIDS as described in the text. The reaction with DIDS was at 50% hematocrit. Dithionite–sulfate heteroexchange for intact cells was measured at 34 °C for a 1% hematocrit after mixing according to the method described by Salhany & Swanson (1978). The concentration of dithionite was constant at 50 mM after mixing. The data are plotted as the ratio of the measured velocities over the velocity at zero DIDS. Mean and standard deviations are shown. A linear least-squares fit of all but the point at zero DIDS (see text) gives a good fit to a straight line with a correlation coefficient of 0.9984. The intercept at the DIDS axis was 21.2 μM, which corresponds to $\sim 1.8 \times 10^6$ DIDS binding sites per cell. The insert shows a plot of typical time courses for 0, 10, and 20 μM DIDS. (B) Light scattering due to hemoglobin binding to membranes from DIDS-reacted erythrocytes and DIDS-reacted α-chymotrypsin-treated erythrocytes. Cells were reacted with various DIDS concentrations as described, and membranes were isolated or they were reacted with DIDS and then with α-chymotrypsin, as described in the text, following which membranes were isolated. Total protein present after mixing in each case was 36 μg/mL. Hemoglobin was constant at 20 μM after the mixing. Binding was studied in 5P(6) at 25°C and is presented as the normalized voltage change relative to zero DIDS control or α-chymotrypsin predigested. (Note: predigestion with α-chymotrypsin had no significant effect on light scattering.) The data are presented as mean values ± SE from three separate determinations on different days with different samples of blood. The curves shown are best-fitting curves from the *weighted nonlinear* curve-fitting procedures discussed in the text. The equation which gave the best fit for control (●) was $(\Delta \text{ volts})/(\Delta \text{ volts})_0 = A \exp(B[\text{DIDS}]) + C \exp(D[\text{DIDS}])$. The solution was found after six iterations with a mean residual of 0.25%. The values of the constants plus or minus a linear estimate of the standard deviation were $A = +1.146 \pm 0.051$ (multiple correlation coefficient = 0.99881), $B = -0.013 \pm 0.001$ (multiple correlation coefficient = 0.97487), $C = -0.147 \pm 0.063$ (multiple correlation coefficient = 0.96864), and $D = -0.33 \pm 0.028$ (multiple correlation coefficient = 0.99761). Note that this best-fitting curve describes a dampening equation. Treatment of cells with α-chymotrypsin *after* the usual DIDS reaction and *prior* to isolation of membranes caused the second dampening component to disappear (○). There is now an enhanced effect as a function of DIDS concentration which can be described by a single exponential plus a constant. The equation is $(\Delta \text{ volts})/(\Delta \text{ volts})_0 = A + B \exp(C[\text{DIDS}])$ where $A = 0.604 \pm 0.047$ (multiple correlation coefficient = 0.99925), $B = 0.396 \pm 0.047$ (multiple correlation coefficient = 0.99924), and $C = -0.142 \pm 0.031$ (multiple correlation coefficient = 0.87618). The mean residual was 0.2%. The lines shown in (B) are theoretical lines calculated according to the equations just described.

then study hemoglobin binding to the isolated DIDS-reacted membranes by using light scattering. Since DIDS binding is irreversible, the use of isolated membranes is valid. However, one serious complication to work with DIDS involves secondary binding sites (Cabantchik & Rothstein, 1974a,b). Although ~90% of the DIDS is located on band 3, ~5% is located on glycophorin and less than 1–4% on lipids. In order to see which DIDS sites influence hemoglobin binding, we correlated irreversible DIDS inhibition of anion transport with DIDS effects on hemoglobin binding to similarly reacted membranes. The results are shown in Figure 7. Figure 7A presents the effect of DIDS treatment on the dithionite–sulfate heteroexchange kinetics (Salhany & Swanson, 1978). The insert shows

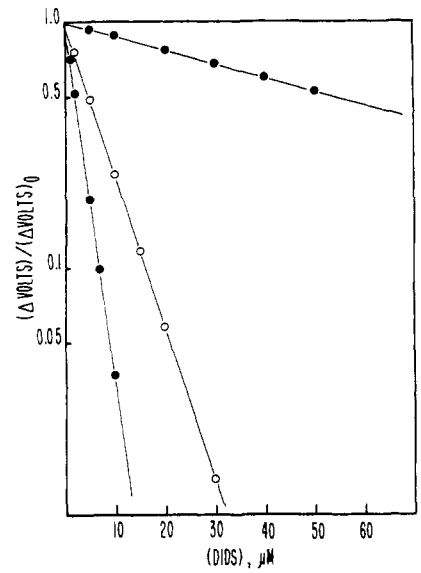

FIGURE 8: Theoretical plot of the individual exponential terms from the equations in Figure 7 describing the effect of externally reacted DIDS on hemoglobin affinity for the cytoplasmic portion of the membrane. The symbols have the same meaning as those in Figure 7.

typical time courses from which the velocities were calculated. Since DIDS reacts irreversibly and since the inhibition shows excellent linear behavior, the intercept on the DIDS axis corresponds to the concentration of DIDS required to completely inhibit transport. The number of DIDS sites from this intercept was calculated to be $\sim 1.8 \times 10^6$ sites/cell. The heteroexchange inhibition results are in excellent agreement with the more conventional experiment where DIDS inhibition of sulfate self-exchange was studied (Lepke et al., 1976; Ship et al., 1977). It is interesting to note that the inhibition line in Figure 7A does not intersect the zero axis at 1. This results could indicate that there is a slight activation of anion transport by DIDS under the conditions of the experiment, following which linear inhibition occurs. Although the previously published results at 25% hematocrit are quite comparable with respect to the DIDS intercept value, the authors did not report observing this type of activation (Lepke et al., 1976; Ship et al., 1977).

Figure 7B (closed circles) shows the effect of DIDS pretreatment on hemoglobin binding as a function of DIDS present during the reaction of intact cells at 50% hematocrit. The results appear to show an initial increase in the magnitude of the hemoglobin-induced light-scattering change followed by a decrease. We have analyzed this biphasic response by using a computer program which performs a *weighted* analysis of either linear or nonlinear equations with up to 20 adjustable parameters so as to find the equation which gives the "best fit" (see Materials and Methods). The empirical equation which gave the best fit was a dampening type consisting of the *difference* between two exponential functions:

$$(\Delta \text{ volts})/(\Delta \text{ volts})_0 = 1.146e^{-0.013[\text{DIDS}]} - 0.147e^{-0.33[\text{DIDS}]} \quad (2)$$

The standard errors and multiple correlation coefficients for these four constants are given in the legend to Figure 7. The line drawn through the closed circles of Figure 7B was calculated by using eq 2. When the two exponential terms were plotted separately (Figure 8), one term correlated with DIDS inhibition of anion transport while the other did not. It should be noted that no light-scattering changes were observed as a

function of DIDS in the absence of hemoglobin.

On the basis of the ratio of the two exponential constants in eq 2, the weak DIDS effect appeared to correspond to ~4% of the larger constant. It seemed likely that the second component was due to secondary DIDS binding sites located on glycophorin or on other parts of band 3 (Cabantchik & Rothstein, 1974a,b). If this were true, then DIDS treatment of intact cells followed by treatment with chymotrypsin *prior to* membrane isolation could experimentally eliminate the influence of the secondary DIDS binding sites (Cabantchik & Rothstein, 1974b). The open circles in Figure 7B show the results. We observed only one process which completely titrated over the DIDS concentration range where anion transport was inhibited. The line in Figure 7B drawn through the open circles was calculated from

$$(\Delta \text{ volts})/(\Delta \text{ volts})_0 = 0.6 + 0.4e^{-0.14[\text{DIDS}]} \quad (3)$$

The exponential term of this equation compares favorably with that determined by the quantitative analysis of the control results (Figure 8).

In order to see how DIDS binding has affected the mechanism of hemoglobin binding, light-scattering measurements were performed at the same ghost concentration for *both* DIDS-treated and DIDS-chymotrypsinized (outside) membranes and their respective DIDS-free controls. The results are shown in Figure 9. DIDS binding converted the hemoglobin binding patterns from noncooperative to "negative cooperative". DIDS-chymotrypsinized (outside) ghosts showed the same effect, thus indicating that the change in the mechanism of hemoglobin binding was *not* related to the presence of secondary DIDS binding sites. The estimate of the first binding constant for DIDS-treated membranes was ~0.4 μM in tetrameric units. This was ~5 times higher in affinity than control. The second hemoglobin binding processes for DIDS-treated membranes had about a factor of 2 lower affinity as compared to the control case (Figure 9).

We have obtained independent qualitative confirmation of the effect of DIDS pretreatment on hemoglobin binding. During preparation of DIDS-treated membranes, we noticed an increased proportion of hemoglobin-bound ghosts (which are known to settle more rapidly) at the bottom of the centrifuge tube as a function of increasing DIDS at low heme concentration. Thus, DIDS pretreatment increases hemoglobin affinity at low heme concentration in agreement with the light-scattering results. Salt stripping at alkaline pH (see Materials and Methods) completely removes this more tightly bound hemoglobin, as confirmed by spectrophotometric and gel electrophoretic analysis.

Discussion

Light scattering from particles whose largest dimension exceeds ~0.05 the wavelength of light will be dominated by optical interference effects. The amount of interference will be directly related to the distribution of matter in the particle and therefore is a function of particle shape and size (Mie, 1908; Rayleigh, 1911; Debye, 1915; Gans, 1925; Doty & Edsall, 1951; Van De Hulst, 1957; Geiduschek & Holtzer, 1958; Tanford, 1961; Kerker, 1969; Berne & Pecora, 1976). Light scattering has been extensively used to study cellular size and shape. This approach to structure–function relationships at the cellular level has been most recently discussed by Latimer (1979). The technique has been previously applied to the intact erythrocyte to determine cell shape (Latimer et al., 1968; Bryant et al., 1969; Oster & Zalusky, 1974; Frojmovic & Panjwani, 1975) and to follow erythrocyte volume changes (Shaafi et al., 1967) and more recently to investigate the structure of erythrocyte ghosts (Mullaney & Fiel, 1976).

The recent discovery that hemoglobin can produce a large change in 90° light scattering under the specific conditions where hemoglobin binding occurs (Salhany & Shaklai, 1979) directly suggests that hemoglobin binding to some element(s) of the membrane produces an extensive conformational change. Since binding is reversible and dependent on conditions of pH, ionic strength, and hemoglobin and ghost concentrations, it is very probably not due to a significant change in the size of the ghost. Although size changes may occur over very long periods of time due to vesiculization or resealing, they are not the source of our effect. A similarly large and rapid light-scattering change occurs with inside-out vesicles and even with NaOH-stripped ghosts, which are vesicles free of all peripheral proteins (Steck, 1974a). Although the exact nature of the membrane conformational change which leads to light scattering is not presently known, the results of this paper show that the effect can be used empirically to characterize binding.

The results of Figure 3 indicate that the total number of hemoglobin binding sites is ~22 × 10^6 heme or 5.5 × 10^6 tetrameric sites per ghost. Furthermore, the anomalous behavior of binding seen as a function of ghost concentration suggests that the total number of sites is composed of at least two classes with widely differing affinity. About 10^6 tetrameric sites per ghost can be titrated by G3PD, suggesting that they are on the band 3 polypeptide. These band 3 sites may be assigned to the high-affinity class based on the results of Figure 5. In that figure, G3PD is shown to lower hemoglobin's affinity at very low ghost concentrations where binding to the high-affinity site dominates (Figure 3). The dissociation constant for the high-affinity site is about 1 to 2 μM (tetrameric units), while that for the 4.5 × 10^6 low-affinity tetrameric sites was estimated to be about 40 μM.[3]

DIDS was shown to have a biphasic effect on the hemoglobin-induced light-scattering change. One component correlated with the complete inhibition of anion transport while the other did not. The results were interpreted as indicating the existence of two DIDS binding sites, one at the anion transport site and the other at secondary DIDS binding sites (Cabantchik & Rothstein, 1974a). This analysis was confirmed by demonstrating that the secondary DIDS sites could

[3] The presence of two classes of hemoglobin binding sites had been suggested in previous fluorescence and centrifugation studies of hemoglobin binding (Shaklai et al., 1977a,b). The stoichiometry of hemoglobin binding determined by fluorescence at very low ghost concentration (Shaklai et al., 1977a) was consistent with the currently accepted value for the number of band 3 monomers present in the membrane (Cabantchik et al., 1978). However, the earlier fluorescence measurements were performed under conditions where hemoglobin would be nearly 100% dimeric (Shaklai et al., 1977a). The functional properties of membrane-bound hemoglobin suggested that either the high-affinity dimer of oxyhemoglobin or the R state tetramer was stabilized with binding (Salhany & Shaklai, 1979). Since there was no way to decide between these alternatives from functional studies alone, the previously discovered light-scattering effect was used in the hope of making some preliminary discrimination. When binding was studied under conditions thought to be suitable for band 3 stoichiometry, hyperbolic binding was observed. Band 3 stoichiometry could be obtained when the data were plotted assuming that only the oxyhemoglobin dimer was binding. However, we now see that sigmoidal and not hyperbolic curves should have been observed (Figure 3) at the ghost concentrations used by Salhany & Shaklai (1979). We have found that this difference results from incomplete stripping of G3PD when 50 mM NaCl is employed (Shaklai et al., 1977a,b). In the present study, we have employed a method similar to that of Steck et al. (1976) using 200 mM NaCl at pH 8 to strip G3PD from the membrane. There is now no need to require dimer binding to explain the present results. Furthermore, Salhany did show that deoxyhemoglobin could bind (Salhany & Shaklai, 1979), suggesting that the hemoglobin tetramer can bind.

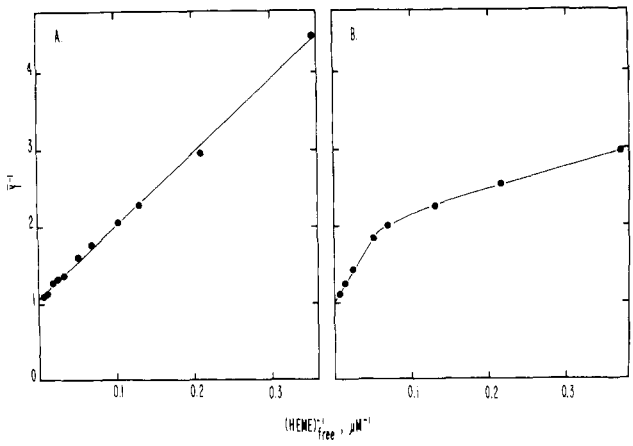

FIGURE 9: Effect of DIDS pretreatment on the mechanism of hemoglobin binding. The conditions were 5P(6) and 25 °C, with all ghost concentrations at 36 μg/mL after the mixing. Double-reciprocal plots of the fraction of saturation vs. the concentration of free heme are shown for control (A) and DIDS-reacted (B) membranes. The concentration of DIDS during pretreatment of cells was 50 μM. The value of K_d for the control was 2 μM (tetramer). K_d values were estimated for the two components of the DIDS-treated membranes and found to be 0.4 μM for the high-affinity component and 4.3 μM for the low-affinity component.

be experimentally removed by treatment of DIDS-reacted cells with chymotrypsin prior to the isolation of membranes. The component of the DIDS effect which remained showed a lowering of hemoglobin affinity at relatively high heme concentration which correlated with the complete inhibition of anion transport. Although we cannot come to a definite conclusion about the exact location of the secondary DIDS binding site, it is clear that it does not influence the affinity of hemoglobin for the membrane since the change in the mechanism of hemoglobin binding to the membrane seen in Figure 9 was independent of the presence or absence of secondary DIDS bound to the outer surface (Figure 7).

The primary DIDS effect on hemoglobin binding (Figure 9) is clearly related to DIDS binding to band 3. The effect titrates completely over a DIDS concentration range which directly corresponds to DIDS inhibition of anion transport (Figure 7A) and therefore to DIDS coverage of band 3 with a 1:1 stoichiometry (Lepke et al., 1976; Ship et al., 1977). The change in the mechanism of hemoglobin binding due to DIDS binding to band 3, presumably at the anion transport site, could be explained as either a change in affinity at one or both classes of hemoglobin binding sites on the cytoplasmic surface of the membrane. The high-affinity site could change its mechanism of hemoglobin binding from noncooperative to apparent negative cooperative if DIDS binding produced a transmembrane conformational change which leads to steric hindrance in the binding of two hemoglobin tetramers to the cytoplasmic segments of the band 3 dimer (Steck, 1974b). Alternatively, the apparent negative cooperativity could result from a relatively small but uniform change at the high-affinity site, with a comparatively large change in affinity at the 4.5×10^6 sites. These latter sites would have to increase their affinity by about a factor of 20–100 to be observed at the low ghost (i.e., site) concentrations used to study the effect of DIDS binding. It is not possible to decide between these two alternatives at present since we do not know where the 4.5×10^6 sites are located or if they could be affected by a specific perturbation at the anion transport site on band 3. Until further information is available, our tendency would be to favor the view that DIDS binding to band 3 causes a transmembrane effect which is localized at the cytoplasmic domain of the band 3 dimer.

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Acetylcholine Receptors from Torpedo and Electrophorus Have Similar Subunit Structures†

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ABSTRACT: Previously, acetylcholine receptor purified from the electric organs of electric eels (*Electrophorus electricus*) and electric rays (*Torpedo californica*) (torpedo) had appeared to differ in subunit structure. Receptor from torpedo has the subunit structure $\alpha_2\beta\gamma\delta$, but subunits corresponding only to α, β, and γ had been observed in receptor from eel. Here we report that if membrane fragments of eel electric organ are prepared and detergent extracted in the presence of iodoacetamide, then receptor purified from the extract contains a fourth subunit. Using monoclonal antibodies as well as conventional antisera, we show that the newly recognized subunit of receptor from eel corresponds to the δ subunit of torpedo. A monoclonal antibody to the δ subunit of torpedo cross-reacts with the γ subunit and shows a similar cross-reaction between the δ' and γ' subunits of receptor from eel, indicating the presence of an unexpected structural similarity. Although the function of the β, γ, and δ subunits remains unknown, these results support the concept that receptors from the electric organs of several species and probably also from muscle share a similarly complex subunit structure.

The subunit structure of acetylcholine receptor (AcChR)[1] has been debated (Sobel et al., 1978), but it is now clear that AcChR purified from the electric organ of the marine ray *Torpedo californica* (torpedo) is composed of four subunits (Weill et al., 1974; Raftery et al., 1975; Hucho et al., 1976; Chang & Bock, 1977; Lindstrom et al., 1978) in the mole ratio $\alpha_2\beta\gamma\delta$ (Damle & Karlin, 1978; Lindstrom et al., 1979a; Reynolds & Karlin, 1978). The subunits of *T. californica* are four similarly acidic glycopeptides (Lindstrom et al., 1979a; Vandlen et al., 1979) which can be distinguished by apparent molecular weight (38×10^3, 50×10^3, 57×10^3, and 64×10^3 for α, β, γ, and δ, respectively), peptide maps (Froehner & Rafto, 1979; Lindstrom et al., 1979a; Nathanson & Hall, 1979), and antisubunit sera (Claudio & Raftery, 1977; Lindstrom et al., 1978, 1979b). At least part of each subunit is exposed on the extracellular surface of the membrane (Lindstrom et al., 1978, 1979b). *T. californica* AcChR's, perhaps unlike AcChR from other species, exist primarily as dimers bound by disulfide bonds between their δ subunits (Chang & Bock, 1977; Suarez-Isla & Hucho, 1977; Hamilton et al., 1977, 1979; Weitzman & Raftery, 1978). α subunits contain the acetylcholine binding site (Karlin et al., 1976). The functions of β, γ, and δ subunits are unknown. However, since highly purified membrane fractions containing only AcChR retain full ion channel activity (Neubig et al., 1979; Wu & Raftery, 1979) as does AcChR solubilized and purified under conditions which prevent denaturation of the ion channel and then reconstituted into artificial membranes (Anholt et al., 1980; Lindstrom et al., unpublished experiments; Nelson et al., 1980), one or more of these subunits must be involved in the structure of the ion channel whose opening is regulated by acetylcholine binding.

AcChR purified from the freshwater teleost *Electrophorus electricus* (eel) by the same affinity chromatography procedure used to purify AcChR from torpedo appears to contain three subunits (Karlin & Cowburn, 1973) corresponding to α', β', and γ' (Lindstrom et al., 1979b). α' has an apparent molecular weight similar to that of torpedo α and also contains the acetylcholine binding site (Karlin et al., 1976). β' and γ' have apparent molecular weights similar to those of β and γ (Lindstrom et al., 1979b). Antisera to torpedo subunits have been used to show that α', β', and γ' correspond immunochemically to the α, β, and γ chains of torpedo AcChR (Lindstrom et al., 1979b). No component cross-reacting exclusively with antibodies to torpedo δ chains was observed in these experiments.

Here we report that this apparent difference in structure between AcChR from the electric organs of torpedo and eel results from the loss of δ' chains during normal purification procedures. We found that when we included iodoacetamide (IAA) during the initial phases of purification of AcChR from eel, a procedure which helps protect disulfide bonds between the δ chains of torpedo from interchange (Chang & Bock, 1977), we observed a fourth subunit in the purified AcChR similar in molecular weight to δ. This procedure with eel organ did not result in the preservation of AcChR dimers linked by disulfide bonds between δ subunits, as it does with torpedo electric organ. But we were able to use monoclonal antibodies

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[1] Abbreviations used: AcChR, acetylcholine receptor; [^{125}I]-α-BGT, ^{125}I-labeled α-bungarotoxin; torpedo, *Torpedo californica*; eel, *Electrophorus electricus*; $NaDodSO_4$, sodium dodecyl sulfate; IAA, iodoacetamide; MG, myasthenia gravis; EAMG, experimental autoimmune myasthenia gravis.

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