Interaction of Hemoglobin and Its Component α and β Chains with Band 3 Protein†

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ABSTRACT: The equilibrium binding of hemoglobin to isolated band 3 protein exhibited positive cooperativity [Hill coefficient = 1.65 ± 0.1; total number of binding sites at pH 6.6 in 5 mM sodium phosphate buffer = 32 500 ± 940 pmol/mg; K_a = (3.0 ± 0.5) × 10^5 M^{-1}]. The binding was reversible and ionic in nature as the bound hemoglobin was readily displaced by KCl, ATP, and 2,3-diphosphoglycerate, the latter two being more effective than KCl on a molar basis. The ratio of the interaction of hemoglobin to band 3 protein per se was 1:1, whereas the band 3 preparation as a whole (protein + lipids) was 3:1. Saturating levels of glyceraldehyde-3-phosphate dehydrogenase blocked only 33% of the total binding sites which were localized at the cytoplasmic segment; the remaining 67% was localized in lipids by their extraction with acetone. Reconstitution of acetone-extracted band 3 with phospholipid liposomes indicated phosphatidylserine as the binding site. The positive cooperativity in binding to acetone-extracted band 3 was increased (Hill constant = 2.1 ± 0.1) compared to the band 3 preparation. After separation of the α and β chains of hemoglobin, only the α chain binds to band 3 with positive cooperativity to an extent of 45–50% of native hemoglobin with similar affinity. The binding capacity of *p*-(hydroxymercuri)benzoate (HMB) derivatives of hemoglobin and its α chain was less than that of native hemoglobin, whereas HMB–β chain or β chain did not bind. Any nonspecific effects that might actuate the observed positive cooperativity in the binding of hemoglobin or its derivatives were ruled out by its demonstration using varying concentrations of band 3 and hemoglobin. The loss of hemoglobin binding was observed upon enzymatic cleavage of the cytoplasmic segment of band 3. Blocking the free amino groups or thiol groups in band 3 or hemoglobin did not affect the interaction, ruling out their participation; similarly, concanavalin A, which binds to glycoproteins, did not affect the binding. These results suggest that hemoglobin might interact at the cytoplasmic surface of the erythrocyte membrane through its α chain.

Band 3 protein is one of the well-characterized transmembrane proteins of the erythrocyte membrane (Steck, 1978). It accounts for 25% of the total Coomassie blue stainable protein in the red cell membranes, and 1.2 × 10^6 copies are present per cell (Fairbanks et al., 1971). Its role as the anion-exchange mediator has been demonstrated (Cabanatchik & Rothstein, 1974; Ho & Guidotti, 1975; Wolosin et al., 1977; Ross & McConnel, 1977; Knauf, 1979). It has an apparent molecular weight of 90 000–96 000 during sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Steck et al., 1976) and has been shown to be distributed as a dimer in the membrane (Steck, 1972; Yu et al., 1973; Yu & Steck, 1975; Clarke, 1975; Kichm & Ji, 1977; Lieu et al., 1977; Nigg & Cherry 1979; Wang & Richards, 1974). On the cytoplasmic domain of band 3, specific binding of bands 2.1 (Bennett & Stenbuck, 1979) and 4.2, glyceraldehyde-3-phosphate dehydrogenase (Yu & Steck, 1975), aldolase (Strapazon & Steck, 1977), and phosphofructokinase (Richards et al., 1979) has been demonstrated. Numerous reports are available which suggest that hemoglobin binds to the cytoplasmic surface of the erythrocyte ghost membrane (Jacob et al., 1968; Schneider et al., 1972; Lessin et al., 1972; Winterbourn & Carred, 1974; Kimmelberg & Papahadjopoulos, 1971; Clalissano et al., 1972; Kimmelberg, 1976). At low pH (6.0) and low ionic strength, each ghost has (1.2–1.3) × 10^6 sites for the binding of hemoglobin A (Shaklai et al., 1977a,b; Salhany et al., 1980). Studies on fluorescence energy transfer indicate that glyceraldehyde-3-phosphate dehydrogenase competes with hemoglobin in binding to the membranes (Shaklai et al., 1977; Salhany et al., 1980). 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS), an anion transport inhibitor, reduces hemoglobin binding to isolated ghosts (Salhany et al., 1980). In recent reports, hemoglobin has been cross-linked to band 3 protein (Salhany et al., 1981; Sayare & Fikiet, 1981). By using spin-label detection at physiological pH, the hemoglobin–membrane interaction is found to exhibit a K_d of 10^{-4}, with half-saturation of hemoglobin binding occurring at a ratio of 10^8 hemoglobins per cell (Fung, 1981). The association of cytosol hemoglobin with band 3 protein in the intact cell has been studied by using fluorescence energy transfer (Eisinger et al., 1982). In a recent report, both glycophorin and band 3 protein have been identified as probable hemoglobin binding sites at the inner surface of the erythrocyte membrane (Rauenbuehler et al., 1982). While the present work was in progress, quantitative analysis of the association of hemoglobin with an isolated segment of band 3 protein was reported (Cassoly, 1983); also, Walder et al. (1984) reported the interaction of hemoglobin with the synthetic peptide corresponding to the first 11 residues of band 3 and with the entire 43-kilodalton cytoplasmic domain of the protein.

In view of the overwhelming evidence of hemoglobin association with the erythrocyte membrane, we chose to investigate further into this phenomenon under a different set of conditions. In contrast to previous studies, we have used a band 3 preparation which has an inside-out conformation with the lipid and a glycoprotein composition similar to that of intact erythrocyte membrane. The pH chosen is nearer to the physiological condition. Also, α and β polypeptide chains of hemoglobin have been separated, and the characteristics of

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their binding to band 3 protein have been studied. The effect of various cytoplasmic components like 2,3-diphosphoglycerate, ATP (not reported so far), and potassium chloride that affect the hemoglobin–membrane interaction have been investigated. The number of binding sites located in the lipid fraction has been distinguished from those interacting with band 3 protein per se by acetone extraction; further, the site of interaction of hemoglobin with lipids has been investigated by using liposomal reconstitution studies with a lipid-depleted band 3 preparation. The essential findings of the present publication have appeared in abstract form (Premachandra, 1985).

Materials and Methods

Fresh human blood was obtained from healthy volunteers. Glyceraldehyde-3-phosphate dehydrogenase (G3PD), *p*-(chloromercuri)benzoate, and concanavalin A were obtained from Sigma. Hemoglobin was prepared from the hemolysates of ghost preparations (Salhany et al., 1972). The α and β chains of hemoglobin were separated and purified according to the described procedure (Gerau et al., 1969). Since α and β chains are more unstable than their respective *p*-(hydroxymercuri)benzoate derivatives, the latter were reduced by mercaptoethanol, equilibrated with 5 mM sodium phosphate buffer of the indicated pH, and used for binding on the same day. The homogeneity of these hemoglobin derivatives was assessed by their mobility on starch gel electrophoresis (Bucci et al., 1965). To prevent oxidation during binding to band 3, sodium dithionite (4 μM final concentration) was added to the incubation mixture. Ghosts were prepared from freshly drawn blood according to the method of Steck (Steck & Kant, 1974). Band 3 protein was isolated as described by Steck: the ghosts were extracted with water adjusted with 1 M NaOH to pH 12, containing 0.001 M 2-mercaptoethanol, by incubating for 15 min in the cold (5 °C) and then centrifuging at 15 000 rpm in an SS-34 rotor in a Sorvall RC 2B refrigerated (5 °C) centrifuge (Steck & Yu, 1973). The sedimented band 3 protein was further washed twice with 0.5 mM sodium phosphate buffer, pH 8.0, and dialyzed against the buffer used for binding studies. Neuraminidase treatment of band 3 protein was carried out at pH 6.5, following the procedure of Singer and Morrison (1974).

Oxidation of free thiol groups of band 3 was carried out as already described (Steck, 1972). Band 3 (1 mg/mL) was treated with 50 μM *o*-phenanthroline and 10 μM copper sulfate, final concentration (from concentrated stock solutions) in 5 mM sodium phosphate buffer, pH 6.60. At the end of a 10-min incubation at 25 °C, the reaction was stopped by adding ethylenediaminetetraacetic acid (EDTA) (1 mM final concentration) followed by dialysis against the same buffer. For reaction of free amino groups with trinitrobenzenesulfonate (TNBS), band 3 (1 mg/mL) was treated with TNBS (final concentration 1 mM) in 5 mM sodium phosphate buffer, pH 8.0, for 1 h at 5 °C. The reaction mixture was extensively dialyzed against 5 mM sodium phosphate buffer (pH 6.60) to remove the unreacted reagent.

Binding studies were carried out by incubating 0.1 mg of band 3 protein with varying concentrations (0.1–4.0 μM) of hemoglobin or its derivatives; band 3 was mixed with the hemoglobin solution in 5 mM sodium phosphate buffer, pH 6.60 (final total volume 1 mL), incubated at 20 °C for 15 min in Beckman microfuge tubes, and spun for 15 min in a high-speed Savant microfuge refrigerated to 5 °C. Oxidation of hemoglobin to methemoglobin is prevented by adding equimolar amounts of sodium dithionite (4 μM) which did not affect the binding. Under these conditions, hemoglobin-bound band 3 settles as a crisp pellet. In the initial experiments, the optimal conditions (such as the time and speed of the spin) were standardized for quantitative separation of hemoglobin-bound band 3 from the supernatant-free hemoglobin. The concentration of hemoglobin in the supernatant was determined in a Perkin-Elmer spectrophotometer at 415 nm. The amount of bound hemoglobin was estimated from the difference between the total and the free hemoglobin concentrations (Shaklai et al., 1977a,b). The stability of the oxyhemoglobin form was assessed by its spectral absorption characteristics. The data were analyzed by Scatchard plots, extrapolating to infinity for the free hemoglobin concentration, to determine the maximal average number of hemoglobin binding sites per milligram of band 3 protein. The pH of the supernatants of the incubations was routinely determined to make sure that it does not deviate from the incubation conditions (6.60 ± 0.02 units). G3PD and hemoglobin and its derivatives were all dialyzed against 5 mM sodium phosphate buffer, pH 6.60, before the titration of band 3 which was also suspended in the same buffer. Hemoglobin concentrations were measured both by the absorption at 415 nm ($E^{m}_{415} = 5.35 \times 10^5$; Van Assendelft, 1970) and by the estimation of the protein concentration in the stock solutions by Lowry's method.

G3PD was estimated by using $E^{1\%}_{1cm} = 10.0$ at 280 nm (Fox & Dandliker, 1956). Reconstitution of acetone-extracted band 3 or band 3 protein was carried out by using phosphatidylserine (PS) or phosphatidylethanolamine (PE) (20 μg of P_i/mg of protein) liposomal preparations as follows: A solution of phospholipids (PE or PS) in chloroform (20 μg of P_i) was evaporated under vacuum to remove the organic solvent and dispersed in 5 mM sodium phosphate buffer, pH 6.60 (0.5 mL), by incubating at 37 °C for 15 min followed by sonication for 15 min at 37 °C under nitrogen. The dispersion was mixed with band 3 (20 μg of P_i/mg of protein) and incubated with gentle magnetic stirring for 30 min at 37 °C under nitrogen. The preparation was diluted with 10 volumes of the ice-cold 5 mM sodium phosphate buffer, pH 6.60, centrifuged, and resuspended in the same buffer; the protein in the liposomal preparation was estimated by Lowry's method and phospholipids (after resuspension in distilled water) as described by Broekhuyse (1968). The hemoglobin binding was carried out with matched controls (without liposome reconstitution) as described previously. In an alternate set of controls, under identical conditions, binding of hemoglobin to phospholipid dispersions (without reconstitution with band 3 protein) was also carried out.

Lipids were extracted from band 3 by rapidly mixing 1 volume of the preparation (4 mg/mL protein concentration) with 2 volumes of ice-cold acetone for 2 min. Following centrifugation, the supernatant was removed, and the pellet was washed and resuspended in 5 mM sodium phosphate buffer, pH 6.60. The protein content of acetone-extracted band 3 was determined by Lowry's method and its phospholipid composition by two-dimensional thin-layer chromatography (TLC) as described by Broekhuyse (1968).

Results

The equilibrium binding studies of purified hemoglobin A with the band 3 preparation revealed a parabolic curve on Scatchard plot, suggesting positive cooperativity (Figure 1). In 5 mM sodium phosphate buffer at pH 6.60 ± 0.02, the total number of binding sites was found to be 32 500 ± 940 pmol/mg of band 3 protein [$K_a = (3.0 \pm 0.5) \times 10^5$ M^{-1}; $r = 0.970$)]. By the Hill plot of the binding data, the positive cooperativity (Hill coefficient) was found to be 1.65 ± 0.1 ($r = 0.997$). At pH 6.80, the total number of binding sites was 21 180 ± 1100 pmol/mg [$K_a = (3.1 \pm 0.4) \times 10^5$ M^{-1}] with

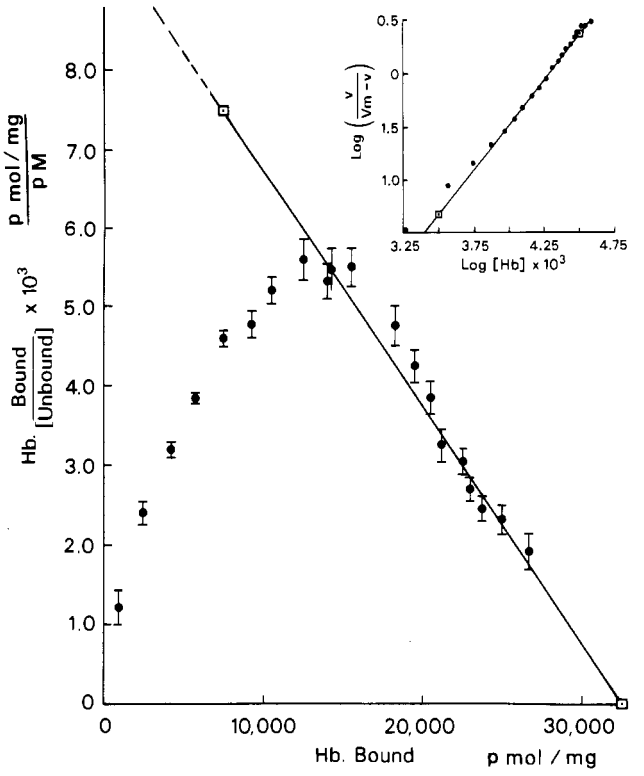

FIGURE 1: Scatchard analysis and Hill plot (insert) of hemoglobin binding to the band 3 preparation. Band 3 (0.1 mg/mL) in 5 mM sodium phosphate buffer, pH 6.60, was incubated with varying concentrations of hemoglobin at 20 °C for 15 min and centrifuged; the bound and the free hemoglobins were estimated as described under Materials and Methods. For regression analysis, only the points on the descending portion of the Scatchard plot were considered, extrapolating the graph for free hemoglobin concentration to infinity to obtain the total number of binding sites and the association constant. The points were chosen for regression analysis based on a trial and error method to get the best fit for a linear curve (i.e., $r \rightarrow 1.0$) using a Hewlett Packard computer. In the figure, each point represents the mean of four separate experimental determinations; the vertical bars represent the standard deviation and are enclosed by circles when not shown; bound = picomoles per milligram, unbound = picomolar. In the insert, v = fractional saturation (picomoles per milligram), V_m = the total number of binding sites (picomoles per milligram, derived from the Scatchard plot), and [Hb] = concentration of hemoglobin A in picomolar.

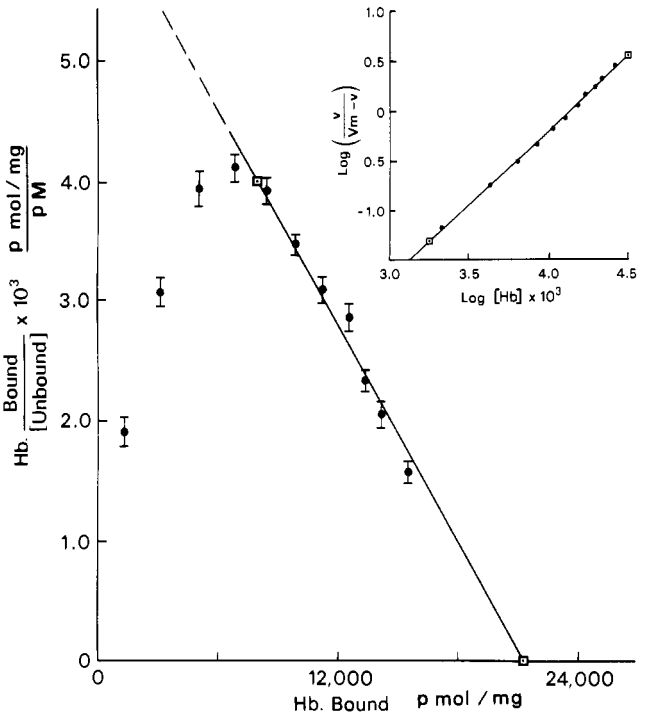

FIGURE 2: Scatchard analysis and Hill plot (insert) of hemoglobin A binding to the band 3 preparation in 5 mM sodium phosphate buffer, pH 6.80 (refer to legend of Figure 1). Each point refers to the mean of three separate determinations; vertical bars represent the standard deviation.

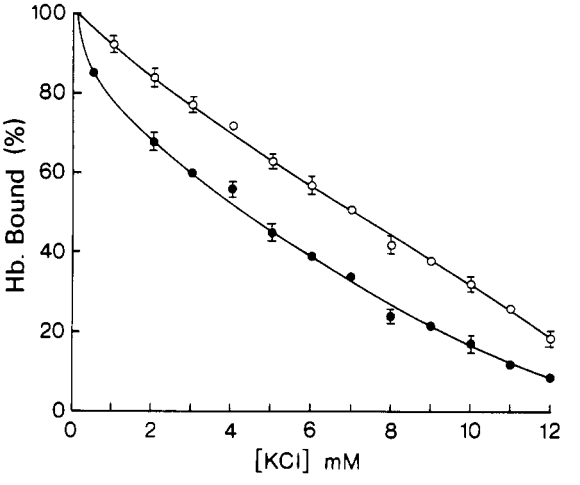

FIGURE 3: Effect of KCl concentration on hemoglobin binding to band 3. Band 3 (0.1 mg/mL) was incubated for 15 min at 20 °C in 5 mM sodium phosphate buffer of the indicated pH containing 4 μM hemoglobin. Varying concentrations of KCl were added from a concentrated stock in the same buffer, and hemoglobin binding was determined as described under Materials and Methods. Values are expressed as the percent of control (without KCl) (○) at pH 6.60 and (●) at pH 6.80. Each point represents the mean of three separate determinations; the vertical bars represent the standard deviation and are enclosed by circles when not shown.

the retention of positive cooperativity (Hill coefficient = 1.6 ± 0.1; Figure 2).

Effect of KCl. The binding of hemoglobin A to band 3 was found to be ionic in nature, as has been observed in studies using unsealed ghost preparation by previous workers (Shaklai et al., 1977a,b; Salhany et al., 1980, 1981). The bound hemoglobin was readily removed by increasing the ionic strength. The effective concentration of potassium chloride required for 50% displacement of bound hemoglobin at pH 6.60 was found to be 7.1 mM, and that at pH 6.80 was 4.25 mM (Figure 3) when saturating levels of hemoglobin (4 μM) were used. It should be noted that under physiological conditions both the hemoglobin concentration and the ionic strength are much larger.

Effect of ATP and 2,3-DPG. To understand the effects of other red cell constituents on hemoglobin binding individually, ATP and 2,3-DPG were studied in particular (Figure 4). The displacement of bound hemoglobin was found to be linear with the increase in concentration of ATP or 2,3-DPG, both being more effective than KCl on a molar basis (compare Figures 3 and 4); at pH 6.6, for example, ATP is 19 times and 2,3-DPG is 26 times more effective than KCl. At pH 6.60, the concentration of ATP required to displace 50% of the bound hemoglobin from band 3 was 0.37 mM; at pH 6.8, it was 0.27 mM; for 2,3-DPG, these values were 0.27 and 0.13 mM, respectively. Since ATP has only four charges (on the phosphate) and 2,3-DPG has five charges per molecule, the extent of inhibition observed is far larger on the molar basis, suggesting their specificity in interacting with the hemoglobin binding site on the membrane.

Effect of G3PD. G3PD, which has been shown to bind specifically to band 3 protein and inhibit hemoglobin binding to unsealed ghosts, was found to block partially hemoglobin binding to the band 3 preparation. Scatchard analysis of the

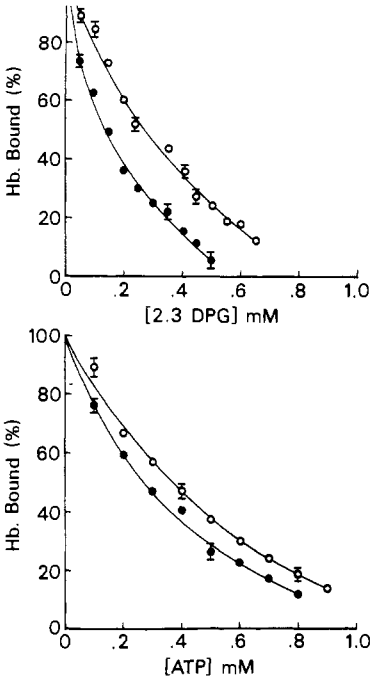

FIGURE 4: Effect of ATP and 2,3-DPG concentrations on hemoglobin binding to band 3 in 5 mM sodium phosphate buffer. Experimental conditions are similar to those described in the legend to Figure 3 [(○) at pH 6.60 and (●) at pH 6.80]. Each point represents the mean ± 1 SD of three separate determinations.

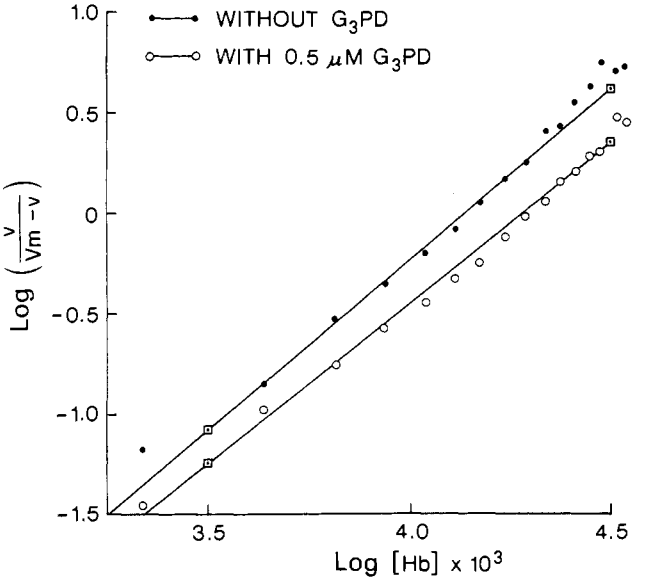

FIGURE 5: Effect of 0.5 μM G3PD on hemoglobin binding to band 3: Hill plot. [Refer to legend of Figure 1 for details: (●) without G3PD; (○) with 0.5 μM G3PD.] Each point represents the mean of duplicates; the individual values vary from 1% to 6% of the mean. The total number of binding sites were derived from Scatchard analysis (not shown).

saturation isotherms similar to Figure 1 (data not shown) in the absence of G3PD revealed the total number of binding sites = 33 314 ± 1010 pmol/mg and K_a = (2.9 ± 0.2) × 10^5 M^{-1} and in the presence of 0.5 μM G3PD, the total number of binding sites = 23 336 ± 920 pmol/mg and K_a = (4.10 ± 0.3) × 10^5 M^{-1} (the values represent the mean ± SD of two separate analyses in each case). The positive cooperativity was found to be retained in the presence of G3PD as shown by Hill plots [Figure 5; Hill coefficients of 1.6 (r = 0.996) in the presence of G3PD and 1.7 (r = 0.994) in the absence of G3PD, respectively]. Thus, nearly one-third of the available (10 000 pmol/mg) binding sites were totally blocked by G3PD, and

Table I: Phospholipid Composition of Acetone-Extracted Band 3[a]

phospholipid	band 3	acetone-extracted band 3
phosphatidylethanolamine	6.4	2.4
phosphatidylserine	4.8	1.5
sphingomyelin	8.0	2.7
phosphatidylcholine	7.8	2.4
total	33.0 ± 1.6	13.2 ± 3.6

[a] The values (micrograms of P_i per milligram of protein) of total phospholipids represent the mean ± SD of triplicates; the data for individual phospholipids are the mean of duplicates.

we can assume that these sites correspond to G3PD binding sites (i.e., these are common for both hemoglobin and G3PD). By Scatchard analyses, the results were found to be similar if G3PD concentrations were increased to 0.75 μM (10 460 pmol/mg) of 1 μM (11 020 pmol/mg), respectively. In a separate experiment, using varying concentrations of G3PD (0–1 μM at 0.1 μM intervals), it was found that 0.5 μM G3PD represented the minimal saturation concentration for maximal inhibition of hemoglobin binding (data not shown).

Effect of Acetone Extraction of Lipids. Assuming that 96 000 is the molecular weight of band 3 protein, the molar ratio of the interaction of hemoglobin to band 3 (estimated on a heme basis as a tetramer at 415-nm absorption) is 3:1, and the extent of binding due to interaction with the cytoplasmic surface of band 3 protein per se is only 33%. The remaining 67% of the binding sites could be due to interaction with lipids and/or glycoproteins (in the present preparation of band 3, the compositions of lipids and glycoproteins are similar to those of unsealed ghosts; Steck & Yu, 1973). To locate the probable binding sites in the lipid fraction, band 3 was depleted of lipids by acetone extraction. The composition of phospholipids of acetone-extracted band 3 is given in Table I. Analysis of total phospholipids indicated a 60 ± 10% loss; individual phospholipids were also reduced by comparable amounts. It should be noted that the PS content is reduced by 70%. The protein content of the extracted band 3 was found to be similar to that of the unextracted band 3, as estimated by Lowry's method. Scatchard and Hill plots of binding of hemoglobin A to band 3 and acetone-extracted band 3 are represented by Figure 6. The total number of binding sites for acetone-extracted band 3 was 11 590 ± 920 pmol/mg of band 3. Also, the association constant was found to be significantly increased [(6.6 ± 0.3) × 10^5 M^{-1}] as compared to that of unextracted band 3 [(3.0 ± 0.4) × 10^5 M^{-1}]. The Hill coefficient was also increased from 1.65 ± 0.1, r = 0.984 (unextracted), to 2.0 ± 0.1, r = 0.990 (extracted). The stoichiometry in these studies reveals a 1:1 interaction of band 3 (lipid depleted) with hemoglobin on a molar basis. To locate these sites at the cytoplasmic segment of acetone-extracted band 3, hemoglobin binding was studied in the presence of 1 μM G3PD. Under these conditions, the total number of binding sites was reduced by 80% with a loss in cooperativity (data not shown) with the Hill coefficient = 1.1 ± 0.1, total number of binding sites = 2500 ± 500, and K_a = (4.5 ± 0.5) × 10^5 M^{-1}.

Reconstitution of Acetone-Extracted Band 3 and Band 3 with Phospholipids. The above results suggest that lipids account for 67% of the total hemoglobin bound to band 3. Phosphatidylethanolamine (PE) and phosphatidylserine (PS) constitute the phospholipids situated at the cytoplasmic surface of the erythrocyte membrane. Hence, both band 3 and acetone-extracted band 3 were enriched with PE or PS individually by using their respective liposomal preparations, and

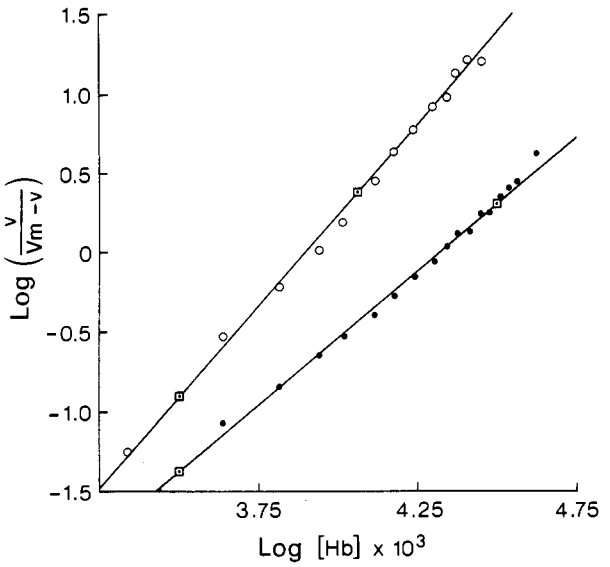

FIGURE 6: Hill plot of binding of hemoglobin A to (●) band 3 and (○) acetone-extracted band 3. One volume of band 3 (4 mg/mL) was rapidly mixed with 2 volumes of ice-cold acetone for 2 min and centrifuged; the pellet was washed with 5 mM sodium phosphate buffer, pH 6.60, and resuspended in the same buffer. In the control set, acetone was substituted by the same buffer. Conditions of binding and analysis were as in the legend for Figure 1. Each point represents the mean of triplicates; the individual values varied from 1% to 6% of the mean; the total number of binding sites were derived from Scatchard analysis as described for Figure 1.

Table II: Reconstitution of Band 3 with Phospholipids

sample	hemoglobin bound (% control)	total phospho-lipid
band 3 (control)	100[a]	31.8
acetone-extracted band 3	32–35	12.7
band 3 + PE	97–102	49.1
band 3 + PS	147–158	50.2
acetone-extracted band 3 + PE	30–36	28.8
acetone-extracted band 3 + PS	79–93	26.5
PE	0–3	20.0
PS	44–59	20.0

[a] Arbitrarily chosen as 100 by using saturating levels of hemoglobin; the values (% control) define the lowest and the highest values observed in four separate measurements; the total phospholipid (micrograms of P_i per milligram of protein) is the mean of duplicates.

binding was carried out by using saturating levels of hemoglobin in 5 mM sodium phosphate buffer, pH 6.60. The results in Table II indicate that enrichment of band 3 or acetone-extracted band 3 with PS (and not PE) enhanced their hemoglobin binding capacity. Under similar conditions, hemoglobin was also found to bind to a lipid dispersion of PS and not PE to an extent comparable to its enhancement of the binding capacity of the band 3 preparation. These experiments suggest that PS may be a potential binding site for hemoglobin among the lipids at the cytoplasmic surface.

Binding Characteristics of Separated α and β Hemoglobin Chains. Treatment of hemoglobin (Hb) with *p*-(chloromercuri)benzoate resulted in a mixture of *p*-(hydroxymercuri)benzoate (HMB) derivatives of α and β chains which were separated and reduced with mercaptoethanol to yield the free chains. The purity of hemoglobin chains and their HMB derivatives was assessed by starch gel electrophoresis (Bucci et al., 1965) in which they exhibited single homogeneous bands. Since the free α and β chains are more unstable than their HMB derivatives, binding to band 3 was carried out immediately after their regeneration, adding equimolar amounts of sodium dithionite.

Table III: Effect of Band 3 Concentration on Hemoglobin Binding to Band 3 Preparation[a]

band 3 concn (mg/mL)	no. of binding sites (pmol/mg)	association constant (M^{-1})	Hill coefficient
0.10	32596 (0.970)	3.3×10^5	1.65 (0.997)
0.25	31222 (0.965)	2.24×10^5	1.60 (0.965)
0.50	33807 (0.961)	1.86×10^5	1.60 (0.994)
1.0	32267 (0.960)	7.8×10^5	1.75 (0.990)
2.0	29800 (0.970)	9.8×10^5	1.95 (0.970)

[a] Values in parentheses refer to the correlation coefficient (*r*). The binding was carried out as described under Materials and Methods. The total number of binding sites and the association constant were derived from Scatchard analysis, and the Hill coefficient was derived from the Hill plot. The values represent the mean of duplicate experiments in each case, and individual values differed ±1–8% of the mean value.

The binding characteristics of Hb, HMB–Hb, HMB–Hb α and β chains, and Hb α and β chains were studied.[1] The binding parameters are as follows (note that each value represents the mean of triplicates and the individual values varied to 1–7% of the mean; H.Q. = Hill quotient): Hb, 33 150 pmol/mg, $K_a = 3.1 \times 10^5$ M^{-1} ($r = 0.986$), H.Q. = 1.6 ($r = 0.996$); HMB–Hb, 25 800 pmol/mg, $K_a = 2.3 \times 10^5$ M^{-1} ($r = 0.978$), H.Q. = 1.5 ($r = 0.995$); HMB–Hb α chain, 11 625 pmol/mg, $K_a = 3.4 \times 10^5$ M^{-1} ($r = 0.972$), H.Q. = 1.6 ($r = 0.986$). HMB–Hb β chain and Hb β chain did not bind to band 3 under these conditions. An inspection of these values suggests (a) the total number of binding sites in HMB derivatives and the free chains is reduced) (b) the affinity constants of all the derivatives are comparable, and (c) the cooperative binding is retained in all the derivatives. As observed with HbA, the interaction of all the derivatives with band 3 was reversible and ionic, being quantitatively displaced by increasing the ionic strength or by raising the pH to 7.4.

Effect of Band 3 Concentration on Cooperativity. Even though the cooperative binding of hemoglobin to inside-out erythrocyte membrane vesicles was reported in a preliminary communication (Premachandra & Mentzer, 1980), the observation had been questioned, attributing it to a nonspecific effect (Salhany et al., 1980). However, in the present studies, we have consistently observed cooperativity in binding to lipids and/or to protein in the band 3 preparation. To rule out any nonspecific effect due to ghost concentration, the saturation isotherms were studied by using 0.1, 0.25, 1.0, and 2 mg/mL band 3 and appropriate hemoglobin concentrations. The data are summarized in Table III. No significant differences could be observed in the number of binding sites, association constants, or Hill coefficients, suggesting that the observed cooperativity is not due to any nonspecific interactions that are dependent on protein concentration (Salhany et al., 1980). Interestingly, even though the number of binding sites remained the same at 1.0 and 2.0 mg of band 3/mL, there was a marked increase in the association constant and cooperativity (Table III).

Effect of Chymotrypsin, Trypsin, and Papain Treatment of Band 3 on Binding. Band 3 was treated with chymotrypsin, trypsin, and papain as described by Steck (Steck et al., 1976): the enzymes were inhibited after the described time periods; the extent of proteolytic cleavage was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and the patterns observed were similar to the previous

[1] These Scatchard and Hill plots have been submitted to the scrutiny of the reviewers and will be furnished to the interested reader by writing to the author.

report (Steck et al., 1976); the proteolytic digestion of 96K (band 3) was found to be complete in all three enzymatic digestions as found by its total disappearance in the electrophoretic patterns. The loss in hemoglobin binding to band 3 upon enzymatic cleavage is as follows: chymotrypsin, 75 ± 4%; trypsin, 55 ± 3%; and papain, 69 ± 6% (the values represent the mean ± SD of triplicate measurements in each set).

Effect of Oxidation of Free Thiol Groups and Blocking of Free Amino Groups. Both the hemoglobin solution and the band 3 preparation were separately treated with copper sulfate and *o*-phenanthroline to oxidize the free thiol groups, and the binding was carried out. No significant effect on the binding was observed, ruling out the involvement of free thiol groups in the binding of hemoglobin to band 3. Similarly, no effect on the hemoglobin–band 3 interaction was observed when the amino groups were blocked by treatment with trinitrobenzenesulfonic acid as described under Materials and Methods.

Discussion

The interaction of hemoglobin with the cytoplasmic surface of the erythrocyte and its physiological significance are still little understand. Due to experimental limitations in direct measurements under physiological conditions, model systems have been described that use low ionic strength at pH 6.0 (Shaklai et al., 1977). The physiological pH values for erythrocytes have been reported in the range of 7.0–7.3, and under pathological conditions, the pH values are reported to be lower than the normal by 0.1 pH unit. Studies of previous workers have indicated the probable site of interaction as band 3 protein. However, the total amount of hemoglobin bound is 4–5 times more than the available band 3 binding sites. Hence, in the present studies, the interaction of hemoglobin A with the isolated band 3 preparation [described by Steck et al. (1974)] is investigated; it has a similar composition as the native erythrocyte membrane with respect to lipids and glycoproteins, except that it has been stripped of the rest of the membrane proteins and has inside-out configuration. Even though low ionic strength has been used as in the previous studies, the pH condition has been chosen nearer to the physiological value without compromising the stoichiometry of binding. The conditions have been purposely selected to measure the binding stoichiometry and affinity, and the techniques used are similar to those used by the previous workers (Shaklai et al., 1977; Salhany et al., 1980, 1981).

The equilibrium binding studies of hemoglobin with band 3 in 5 mM sodium phosphate buffer, pH 6.60, exhibited positive cooperativity which was demonstrable at pH 6.80. Salhany and co-workers using unsealed ghost preparations at pH 6.0 under similar ionic strength reported that the cooperativity effect is apparent as it disappeared at higher ghost concentration (Salhany et al., 1980). However, in the present studies, no significant changes were observed in the association constant, in the total number of binding sites, and in the positive cooperativity with increase in band 3 concentration; in fact, a significant increase in association and Hill constants is observed (Table II), suggesting that under physiological conditions this effect cannot be ruled out. The effect has been consistently observed at pH 6.80 after depletion of membrane lipids by acetone extraction in the presence of G3PD. The origin and significance of this cooperativity still remain speculative. The affinity constants of hemoglobin and all its derivatives are comparable and are of the order of 10^5 M^{-1}; also, the affinity for band 3 protein per se is comparable to that of lipids in the band 3 preparation. These values are 100-fold lower than those observed at pH 6.0 (Shaklai et al., 1977) and are comparable to the value reported by Fung (1981).

Increasing the ionic strength and pH decreased hemoglobin binding in the present model system as well as in the previous studies; however, this does not mean that the results do not have any physiological significance. The binding characteristics observed under low ionic strength and pH delineate and reflect the preferential interaction of hemoglobin with specific components of the cytoplasmic surface in the intact erythrocytes. The actual physiological affinity may be considerably less than found in these studies (in which experimental conditions are purposely chosen to magnify the affinity for experimental measurement; Shaklai et al., 1977). Since the concentration of hemoglobin used here is micromolar and that under physiological conditions is millimolar (1000-fold higher) and the mode of interaction is charge titration, the binding under physiological conditions cannot be ruled out; this has been supported by recent studies using spin-labeling of hemoglobin in the intact erythrocyte (Fung, 1981).

Since the nature of the interaction of hemoglobin with lipids or proteins is essentially ionic, it is possible to have similar affinity for these two different sites. These discrepancies from previous studies could be due to the different kinds of ghost preparations and/or higher pH conditions used here which might affect the disposition and availability of participant functional groups (which in turn influences the affinity and pattern of interaction with hemoglobin). Characterization of the specific functional groups involved could explain these observations.

ATP and 2,3-DPG (the latter in particular) were found to be more effective than KCl in displacing the bound hemoglobin on a molar basis; this could be partly due to the increased net charge on their molecules. For example, ATP carries four charges (on the phosphate) but is 19-fold more effective than KCl, and 2,3-DPG has five charges per molecule and is 26 times more effective than KCl in displacing hemoglobin bound to band 3. The difference observed in hemoglobin binding inhibition by ATP vs. 2,3-DPG may be explained as the ionic strength effect as can be shown by using the equation $I = {}^1/_2\sum_i C_i Z_i^2$ [Z (the charge) = 4 for ATP and Z = 5 for 2,3-DPG; I = ionic strength and C = concentration]. However, in view of the established physiological role of these components, as well as the observed K_d of 9.9×10^{-6} M for 2,3-DPG for the 11-peptide residue isolated from the cytoplasmic segment of band 3 (Walder et al., 1984), we feel that this observed competitive inhibition is specific, and it may be pertinent to reveal the stoichiometry of these binding studies. Also, we should keep in mind that the equation shown holds for low ionic strengths and under physiological concentrations, the activity coefficients of these individual components in competing for a binding site might be different.

Competitive inhibition studies with G3PD reveal 1:1 (mol/mol) interaction of hemoglobin with band 3 protein (assuming a molecular weight of 96K), in agreement with previous studies (Shaklai et al., 1977; Salhany et al., 1980, 1981); acetone extraction does not seem to affect the binding characteristics of these sites, and we derive this conclusion by comparing results from Figures 1, 5, and 6. Since the number of binding sites inhibited by G3PD in the band 3 preparation and acetone-extracted band 3 are similar (10 000–11 000 pmol/mg), accounting for 33% of the total binding capacity with the remaining 67% localized in the lipid fraction, it could be concluded that no appreciable binding of hemoglobin to other proteins besides band 3 protein is occurring (as there is no significant loss in protein content upon acetone extrac-

tion). A little binding (2500 pmol/mg) found in acetone-extracted band 3 in the presence of G3PD could be attributed either to other proteins like glycophorins (Rauenbuehler et al., 1982) and/or to residual PS remaining after acetone extraction. In this connection, it is interesting to note that concanavalin A and neuraminidase treatment did not affect hemoglobin binding to band 3; only the isolation of glycophorin and direct demonstration of its interaction with hemoglobin might answer this question.

Enrichment of band 3 or acetone-extracted band 3 with PS and PE liposomal preparation suggests that PS can be a potential binding site for hemoglobin among lipids. It should be noted that these two phospholipids span the cytoplasmic surface of the intact erythrocyte, and considered along with the G3PD competitive inhibition studies described above, the results indicate that hemoglobin is binding to the cytoplasmic segment of the band 3 preparation under our experimental conditions. However, the molar proportion of the interaction between hemoglobin and PS was difficult to determine due to experimental variations; this suggests the specific orientation of PS in the liposome may be necessary for effective interaction. Besides PS, other minor lipid components that compose the membrane (Broekhuyse, 1968) which might bind hemoglobin have not been studied here.

A recent paper (Walder et al., 1984) indicates that hemoglobin interacts with the membrane through the β chain. The authors have used the synthetic peptide corresponding to the first 11 residues of band 3 and 43K cytoplasmic domain. It should be noted here that the conditions used in these experiments (as with all other studies) are not physiological. By cross-linking experiments, using unsealed ghost preparations at pH 6.0 in 5 mM sodium phosphate buffer, Sayare and Fikiet (1981) have shown that hemoglobin binds to band 3 protein by the β chain; the limitation of this technique is that only the nearest neighbor, reactive, thiol groups, favorably oriented on both band 3 and hemoglobin for reaction, form the cross-link. In contrast to these results, our studies suggest that the α chain, and not the β chain, binds band 3 with positive cooperativity. The reduced capacity of the α chain and its HMB derivative to bind to band 3 could be due to the difference in the availability and/or participation of the functional group on hemoglobin (which may depend on the pH condition and any effects due to chain separation) involved in the interaction. The lack of binding of the β chain may be due to its reduced positive charge and/or due to its lack of suitably charged and oriented functional group(s) with which it can interact with the band 3 preparation. In view of all these observations, further experiments need to be designed to reveal which of these observations truly reflects the physiological situation.

Treatment with chymotrypsin, trypsin, or papain decreased hemoglobin binding to band 3 by cleaving the cytoplasmic segment of band 3 (Steck et al., 1976); however, the decrease in binding is more than is accounted by cleavage of the protein moiety alone (which binds only 33% of the total). Hence, the enzymatic cleavage might be associated with substantial loss in phospholipids (which could bind to hemoglobin) surrounding the segment cleaved. This is indicated by the considerable loss in the size of the pellet observed after the enzymatic digestion.

Oxidation of the free thiol groups, or blocking the free amino groups by TNBS, either on hemoglobin or on band 3 protein, did not affect hemoglobin binding, ruling out their direct participation. The effect of pH and ionic strength suggests a saltlike linkage between the two. The groups that can participate in such saltlike links are guanidino, arginino, and carboxylate groups on the protein and phosphate groups on the phospholipids. The fact that SS hemoglobin with an increased positive charge binds more (Premachandra & Mentzer, 1980; Walder et al., 19844) suggests that probably hemoglobin is in a cationic state and band 3 is in an anionic state under the experimental conditions of binding. Further studies are in progress to determine the nature of the functional group(s) participating in the hemoglobin–membrane interaction.

When these studies were in progress, a manuscript appeared in which the interaction of hemoglobin with the isolated cytoplasmic segment of band 3 protein in 5 mM sodium phosphate buffer at pH 6.0 has been demonstrated (Cassoly, 1983). The present studies differ from this as well as the previous studies (Shaklai et al., 1977; Salhany et al., 1980, 1981) which have used unsealed ghost preparations in that the band 3 preparation as a whole (as inside-out vesicles, with all the lipids of the erythrocyte membrane intact) has been used. Further, Cassoly's paper (Cassoly, 1983) does not shed light on the interaction with the lipids.

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Registry No. ATP, 56-65-5; 2,3-DPG, 138-81-8; G3PD, 9001-50-7; hemoglobin A, 9034-51-9.

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