Marcus, R. A., & Sutin, N. (1975) *Inorg. Chem. 14*, 213.
Meloon, D. R., & Wilkins, R. G. (1976) *Biochemistry 15*, 1284.
Moore, G. R., & Williams, R. J. P. (1976) *Coord. Chem. Rev. 18*, 125.
Olivas, E., deWaal, D. J. A., & Wilkins, R. G. (1977) *J. Biol. Chem. 252*, 4038.
Phillips, S. E. V. (1978) *Nature (London) 273*, 247.
Rawlings, J., Wherland, S., & Gray, H. B. (1977) *J. Am. Chem. Soc. 99*, 1968.
Salemme, F. R. (1977) *Annu. Rev. Biochem. 46*, 299.
Stenkamp, R. E., Sieker, L. C., & Jensen, L. H. (1976a) *Proc. Natl. Acad. Sci. U.S.A. 73*, 349.
Stenkamp, R. E., Sieker, L. C., Jensen, L. H., & Loehr, J. S. (1976b) *J. Mol. Biol. 100*, 23.
Stenkamp, R. E., Sieker, L. C., Jensen, L. H., & McQueen, J. E., Jr. (1978) *Biochemistry 17*, 2499.
Sutin, N. (1977) *Adv. Chem. Ser. No. 162*, 156.
Ward, K. B., & Hendrickson, W. A. (1977) in *Metal-Ligand Interactions in Organic Chemistry and Biochemistry, Part 2* (Pullman, B., & Goldblum, N., Eds.) p 173, Dordrecht-Holland, Reidel.
Ward, K. B., Hendrickson, W. A., & Klippenstein, G. L. (1975) *Nature (London) 257*, 818.
Wherland, S., & Gray, H. B. (1977) in *Biological Aspects of Inorganic Chemistry* (Cullen, W. R., Dolphin, D., & James, B. R., Eds.) p 289, Wiley, New York, NY.
Wherland, S., & Pecht, I. (1978) *Biochemistry 17*, 2585.
Wu, C.-S. C., Duffy, P., & Brown, W. D. (1972) *J. Biol. Chem. 247*, 1899.

Functional Properties of Human Hemoglobin Bound to the Erythrocyte Membrane†

J. M. Salhany* and Nurith Shaklai*

ABSTRACT: Studies are presented which deal primarily with the ligand binding kinetic properties of human oxyhemoglobin bound to the erythrocyte membrane. Static 90° relative light scattering measurements are also presented as a necessary preliminary to the functional studies. The light scattering measurements suggest that the dimer of oxyhemoglobin binds to the cytoplasmic surface of the membrane. Binding was apparently noncooperative with a constant of about 4 μM in dimer at pH 6, in 5 mM phosphate buffer at 23 °C. Further evidence for enhanced formation of the oxygenated dimer was obtained from kinetic measurements where oxygen was rapidly removed in the stopped-flow and the kinetics of CO binding studied. A substantial increase in the proportion of rapid CO binding component was observed with increasing ghost concentration. Complete reversibility of the increased fraction of rapid CO binding component was demonstrable upon addition of the enzyme glyceraldehyde-3-phosphate dehydrogenase (G3PD) after addition of oxyhemoglobin. This result may indicate that the oxygenated dimer binds reversibly to band 3 since G3PD has been shown to bind to this membrane protein (Yu, J., & Steck, T. L. (1975) *J. Biol. Chem. 250*, 9176). Oxygen release measurements in the absence and presence of CO indicate diminished cooperativity, as expected if the oxygenated dimer is stabilized. However, the initial rate constant for oxygen release from the β chain of the bound dimer was found to be about 20 times slower than in solution.

The interaction of hemoglobin with the erythrocyte membrane is interesting, both as a model system to study how cytoplasmic components may interact with a plasma membrane and as part of the current effort to develop a more integrated physical description of the human erythrocyte. Detailed studies of this interaction have been presented by Fischer et al. (1975) and Shaklai et al. (1977 a,b). First, and most importantly, it was demonstrated that hemoglobin binding was reversible and largely electrostatic in nature (Fischer et al., 1975). Fluorescence quenching studies (Shaklai et al., 1977a,b) showed the stoichiometry and sidedness of binding and values were obtained for the binding constant. The number of sites involved corresponded to the number of band 3 polypeptides (the predominant, integral membrane protein of the human erythrocyte), and competition experiments with G3PD,[1] an enzyme known to bind to band 3 (Yu & Steck, 1975a,b), offered further evidence for the location of the binding site.

As a consequence of these initial physical characterizations, it was natural to inquire about the functional properties of membrane-bound hemoglobin since the activities of certain enzymes have been altered upon binding (Strapazon & Steck, 1976; Karadsheh & Uyeda, 1977). We elected to investigate the functional properties of membrane-bound hemoglobin using kinetic techniques. Besides the kinetic studies, we also present some new static 90° relative light scattering measurements on the binding of oxyhemoglobin to the inner surface of the membrane. This technique allows measurement of oxyhemoglobin binding over a wide heme concentration range and was needed for comparison with the earlier fluorescence studies.

Materials and Methods

Hemoglobin and unsealed ghosts were prepared as previously described (Shaklai et al., 1977a,b). The unsealed ghosts were equilibrated once with 50 mM NaCl at pH 8 to release

† From the Departments of Biomedicinal Chemistry and Internal Medicine and The Cardiovascular Center, University of Nebraska Medical Center, and Veterans Administration Hospital, Omaha, Nebraska 68105 (J.M.S.), and the Department of Biochemistry, The George S. Wise Center for Life Sciences, Tel-Aviv University, Tel-Aviv, Israel (N.S.). *Received August 21, 1978.* This work and Dr. Shaklai's visit to the Omaha laboratories were supported by Research Funds from the Cardiovascular Center of the University of Nebraska Medical Center and by the Medical Research Service of the Veterans Administration.

[1] Abbreviation used: G3PD, glyceraldehyde-3-phosphate dehydrogenase.

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membrane-bound aldolase and G3PD and the salt was replaced by 5 mM phosphate through dialysis. The concentration of ghosts was calculated based on Lowry protein determinations (Lowry et al., 1951), assuming an average of 4×10^{-10} mg of protein per ghost (Steck, 1974a). Resealed ghosts containing oxyhemoglobin were prepared as described by Salhany & Swanson (1978). Reagents were analytical grade and obtained from J. T. Baker (Phillipsburg, NJ). All gases were from Matheson (East Rutherford, NJ). G3PD was obtained from Sigma (St. Louis, MO) and used without further purification.

Stopped-flow kinetic measurements were performed using a Gibson-Durrum stopped-flow apparatus (Durrum Instrument Co., Palo Alto, CA) interfaced to an On-Line-Instruments-Systems (Olis, Jefferson, GA) data acquisition system as described elsewhere (Demma & Salhany, 1977).[2] Temperature was controlled using a GCA Precision Scientific circulating water bath. Static, 90° relative light scattering measurements were performed with the same instrument equipped with a Durrum 90° light scattering cell. The samples were mixed in the stopped-flow and Δ volts vs. time recorded. The computer also recorded the absolute value of the final voltage and the initial value was always checked by recording the voltage upon mixing the same ghosts with buffer. We used incident light of mean wavelength 650 nm, where the absorbance of oxyhemoglobin is minimal (Antonini & Brunori, 1971).

Results

Static, 90° Relative Light Scattering Measurements for Oxyhemoglobin Binding to Unsealed Ghosts. In order to study the functional properties of membrane-bound hemoglobin, it is necessary to have a knowledge of the number of sites involved in binding as well as the binding constant. A strong 90° relative light scattering change has been discovered upon mixing oxyhemoglobin with unsealed ghosts stripped of aldolase and G3PD at pH 6. The light scattering change was first studied qualitatively since it may be useful to study binding. The light scattering change was reversed by raising the pH of membrane-bound hemoglobin from 6 to 8 and by increasing the ionic strength, in good agreement with the fluorescence results (Shaklai et al., 1977a,b). Reversibility of light scattering also seems to suggest that secondary aggregation of the hemoglobin complexes is probably not occurring, thus allowing measurement of the stoichiometry of binding and the binding constant (Nelsestuen & Lim, 1977).

Figure 1A shows 90° relative light scattering measurements for oxyhemoglobin binding to unsealed and resealed ghosts. The conditions were such that the concentration of sites was high (taking $1-2 \times 10^6$ sites/cell from Shaklai et al., 1977a,b) so that the stoichiometry may be determined. Resealed ghosts showed no significant change in Δ volts as a function of hemoglobin concentration. The plot in Figure 1A did not show the expected theoretical behavior. When Δ volts was plotted vs. the number of dimers added per cell (Figure 1B; see the figure legend for calculations), the expected theoretical curve

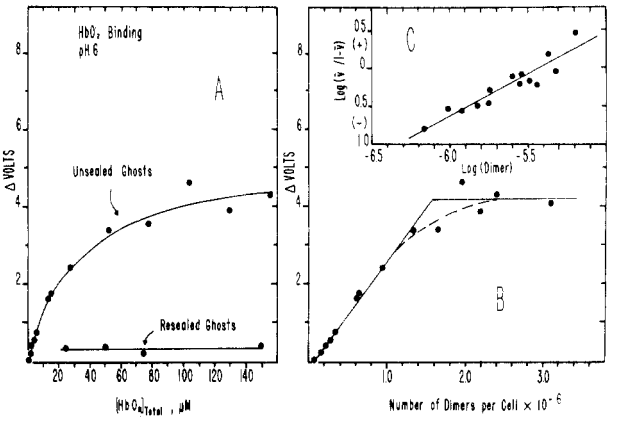

FIGURE 1: Static, 90° relative light scattering measurements for oxyhemoglobin binding to ghosts. The incident λ was 650 nm with slits at 1 mm in all experiments and the measurements were performed in 5 mM phosphate, pH 6, 23 ± 1 °C. (A) Stoichiometry. These measurements were performed under conditions suitable for testing the stoichiometry of binding. The concentration of ghosts was 2.5×10^{12} cells/L, determined as described in the text. The concentration of protein is given as total heme after mixing. Resealed ghosts were prepared as described elsewhere (Salhany & Swanson, 1978) and had 20 mM sucrose on both sides. The curves have no theoretical meaning in this case. (B) Stoichiometry. This is a plot of the same static light scattering data as in A but with Δ volts now plotted vs. the number of dimers (in millions) added per cell. The ordinate was calculated as follows. First, the fraction of dimer at each final hemoglobin concentration in A was calculated using the equation $K_{4,2} = (\text{heme})\alpha^2/(1-\alpha)$, where $K_{4,2}$ is the tetramer to dimer dissociation constant and α is the fraction of dimer (i.e., $\alpha = (D)/((D) + 2(T))$). The value of $K_{4,2}$ used was 3 μM. The value of α determined at each hemoglobin concentration was then multiplied by the total concentration of heme and that number divided by 2. This concentration of dimer was then multiplied by Avogadro's number and divided by 2.5×10^{12} cells/L to yield the number of dimers added, in millions, per cell. The first solid line drawn in the figure comes from linear least-squares fit of the data between 0 and 1.3×10^6 dimers/cell. A correlation coefficient of 0.9976 was obtained. (C) Binding constant. This insert shows a Hill plot of static light scattering measurements for oxyhemoglobin binding to unsealed ghosts under experimental conditions where the ghost concentration was 1/100th (= 0.23 μM sites) of packed ghosts, after the mix. The data fit the following Hill equation: $\log (v/1-v) = 1.09 \log (\text{dimer}) + 5.95$ with a correlation coefficient of 0.9347. Thus, binding was noncooperative over this concentration range since $n = 1.09$. At $\log (v/1-v) = 0$, the value of log (dimer) was computed to be −5.43 and the binding constant (dissociation constant) was 3.7 μM in dimers.

was obtained with a stoichiometry of about 1.4×10^6 dimers per cell.

The experimental conditions in Figures 1A and 1B are not ideal for determining the binding constant. If 1.4×10^6 sites/cell is taken as a firm number for the binding sites, packed ghosts contain 23 μM of sites (using 10^{10} ghosts/mL as the concentration of packed ghosts). In order to study binding under more favorable conditions, light-scattering measurements were made on 1/100 diluted packed ghosts after the mix. The insert to Figure 1 (C) shows a Hill plot from these independent experiments. The plot of the raw data showed near-saturation, but it was necessary to calculate the value of Δ volts at saturation by using a weighted linear least-squares fit for a double-reciprocal plot (Wilkinson, 1961) of 1/Δ volts vs. 1/(dimer). The calculated value for Δ volts (max) was then used to make the Hill plot shown in Figure 1C, where noncooperative binding is observed over the concentration range studied ($n = 1.09$), with a constant of 3.7 μM in dimer.

Carbon Monoxide Binding Properties of Membrane-Bound Hemoglobin. If the dimer of oxyhemoglobin is bound, then rapid removal of oxygen, followed by CO binding, should show

[2] The optical kinetic results presented in this paper were performed in a stopped-flow with a "head-on" photomultiplier tube allowing substantial portions of the scattered light to be collected. A 20% diminution in absorbance due to the Duysens effect (Duysens, 1956; Latimer & Holmes-Eubanks, 1962; Gordon & Holzworth, 1971) was observed for conditions where hemoglobin was bound to ghosts. In addition, we also could observe a short-lived decay in suspensions of membrane-bound hemoglobin (Frojmovic, 1975). Since the reactions we studied are distinctly wavelength dependent, it was not possible to use available split-beam stopped-flow methods (Salhany & Swanson, 1978).

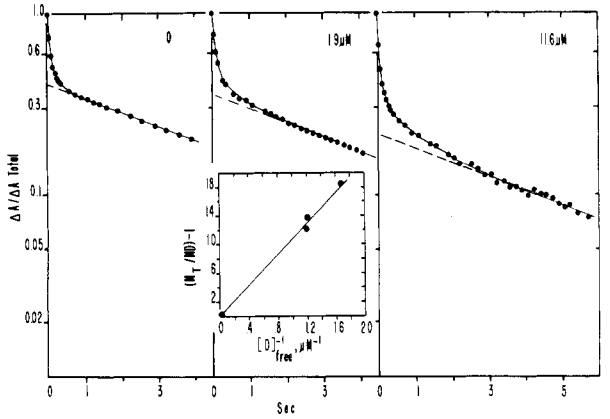

FIGURE 2: Oxygen "off" followed by CO binding to hemoglobin in solution as a function of increasing membrane concentration at pH 6. The concentration of sites before the mix is indicated in the figure and was determined as described in the text using 1.4×10^6 sites/ghost (Figure 1B). The concentration of heme was 3 μM, dithionite, 2 g/L, and CO, 6 μM, all before the mix. The wavelength was 422.5 nm. Temperature was 25 °C, with a 2-cm path length. The rate constants for the fast and slow phases were 3×10^6 and 1×10^5 M^{-1} s^{-1}, respectively. The insert shows a plot of $[(M)_T/(MD)] - 1$ vs. $[D]^{-1}_{free}$. See text for discussion of this insert.

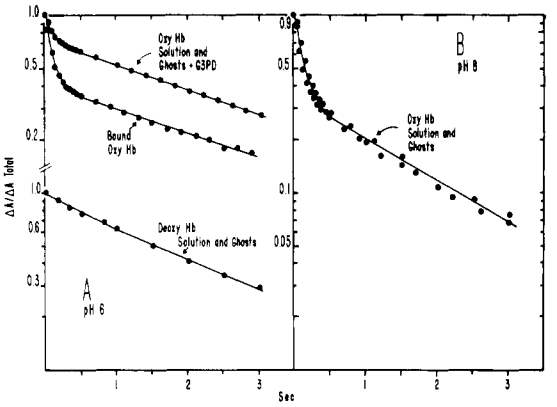

FIGURE 3: Carbon monoxide binding to solution and membrane-bound hemoglobin. The top portion of A shows results from O_2 "off", followed by CO "on" (*not* replacement) experiments for: HbO_2 *solution* at pH 6; membrane-*bound* HbO_2 at pH 6; and membranes plus hemoglobin at pH 6 to which the enzyme G3PD was added. The bottom portion of A shows CO binding to deoxyhemoglobin in solution and in the presence of ghosts. The concentrations are (ghosts) = 2.5×10^{12} cells/L; (heme) = 2.9 μM; (dithionite) = 2 g/L; and (CO) = 6 μM all before the mix (50:50). λ = 419 nm; slits = 0.6 mm; T = 25 °C; (G3PD), where present, 2 μM; 2-cm path length. The rate constant for deoxyhemoglobin was 1×10^5 M^{-1} s^{-1} initially. (B) Shows O_2 "off" CO "on" experiments for HbO_2 solution and HbO_2 in the presence of ghosts at pH 8. Conditions were otherwise the same as in A. The rate constants were 3×10^6 and 1.8×10^5 M^{-1} s^{-1}.

an increase in the rapid CO binding component over that seen in solution (Roughton, 1934; Antonini & Brunori, 1971; Andersen et al., 1971). If we assume the simplest possible model, where the membrane exclusively binds the dimer of oxyhemoglobin, the fraction of fast CO binding component (Andersen et al., 1971) should increase as the concentration of ghosts increases. The scheme may be represented as

$$T \leftrightarrow 2D,\ K_{4,2} \quad (1)$$

$$MD \leftrightarrow D + M,\ K \quad (2)$$

where T and D are the tetramer and dimer of oxyhemoglobin, M is the free membrane site, and MD is the membrane site complexed with the oxygenated dimer. We can define the fraction of fast component observed as α where

$$(MD) + (D)_{soln} = \tfrac{1}{2}(heme)\alpha \quad (3)$$

Then, the concentration of tetramer present is

$$(T) = \tfrac{1}{4}(heme)(1 - \alpha) \quad (4)$$

The concentration of dimer in solution is

$$(D) = [K_{4,2}(T)]^{1/2} = \tfrac{1}{2}[K_{4,2}(heme)(1 - \alpha)]^{1/2} \quad (5)$$

and

$$(MD) = \tfrac{1}{2}(heme)\alpha - \tfrac{1}{2}[K_{4,2}(heme)(1 - \alpha)]^{1/2} \quad (6)$$

With eq 2 in dissociation terms, rearrangement gives

$$[(M)_{total}/(MD)] - 1 = K/(D) \quad (7)$$

where $(M)_{total} = (MD) + (M)$. Thus, a plot of $[(M)_{total}/(MD)] - 1$ vs. $(D)^{-1}$ will have a slope equal to K and intercept the axis at zero. The values of $(D)^{-1}$ and (MD) may be calculated from the corrected[3] value of α; $K_{4,2}$ and (MD) and (D) are from eq 5 and 6. The value of K should be approximately equal to the value determined from the 90° relative light scattering measurements (Figure 1C).

Figure 2 shows the oxygen "off" CO "on" experiment of Roughton (1934) as a function of ghost concentration. As can be seen, the fraction of fast component does increase with increasing ghost concentration. The insert shows a plot of $[(M)_{total}/(MD)] - 1$ vs. $(D)^{-1}$ which gives a value for K in eq 2 of about 10 μM using $K_{4,2}$ = 2.3 μM. This value is in reasonable agreement with the more direct light scattering measurements within experimental uncertainties and seems consistent with the interpretation offered.

Does the increased proportion of rapid CO binding component correspond to the oxyhemoglobin dimer binding to band 3 (Steck, 1974b)? This was investigated in two ways. First, Figure 3B shows that raising the pH to 8 causes the fraction of fast and slow phases present to be the same for solution and ghosts (Shaklai et al., 1977a,b). Second, when G3PD was added to membrane-bound hemoglobin suspensions at pH 6, the kinetics of CO binding showed less fast component as compared with the same suspension without G3PD (Figure 3A). The fraction of fast component in the presence of G3PD was now indistinguishable from that seen for hemoglobin in solution. Finally, the results shown in the lower portion of Figure 3A indicate that there is no significant amount of deoxy dimer present when solution and suspension reactions are compared under the same conditions.

Dissociation of Oxygen from Membrane-Bound Hemoglobin. Release of the "First" Oxygen Molecule and Evidence Concerning Cooperativity in Oxygen Release. Based on the results just presented one would expect membrane-bound hemoglobin to exhibit less cooperativity in the rate of oxygen release, since the current evidence suggests that the dimer is noncooperative (Andersen et al., 1971; Kellett, 1971; Kellett & Gutfreund, 1970; Olson & Gibson, 1972). The reactions in Figure 4 (A–C) show the $HbO_2 \rightarrow$ HbCO replacement reaction with dithionite present. Dithionite serves to keep the oxygen tension at near zero and allows measurement of the kinetics of oxygen release from fully liganded hemoglobin (Gibson & Roughton, 1955, 1959; Olson et al., 1971). The conditions in Figure 4A were arranged so that nearly all of the oxyhemoglobin present would be bound to the membrane. Under these conditions, a substantial portion of a very slow phase in oxygen release is observed at this wavelength. As

[3] We calculate the value for the fraction of fast component, α, according to Andersen et al. (1971): fraction slow = 0.94(percent slow)/[0.94(percent slow) + (percent fast)] with α = 1 − (fraction of slow). The factor 0.94 accounts for the difference in extinction between the deoxy dimer and tetramer at the wavelength used (422.5 nm).

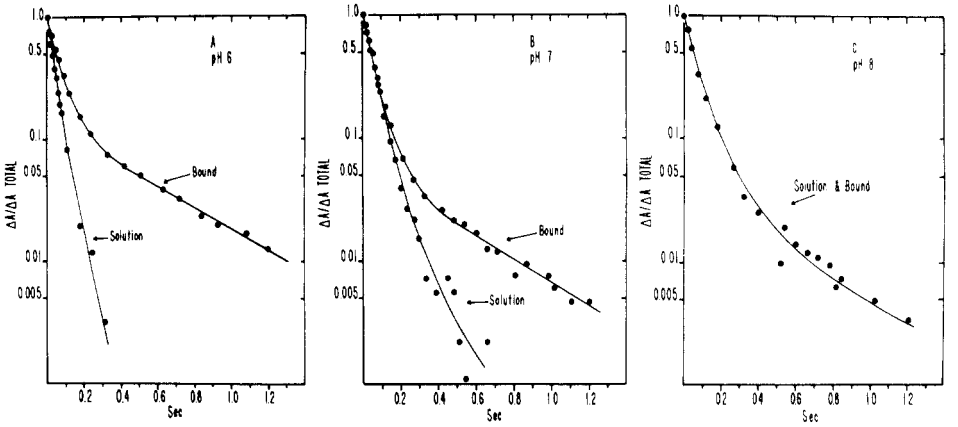

FIGURE 4: $HbO_2 \rightarrow HbCO$ replacement reaction for bound and unbound oxyhemoglobin as a function of pH. Ratio mixing syringes (1:10) were used with ghosts and hemoglobin in the large syringe: (ghosts) = 5×10^{12} cells/L; (heme) = 11 μM, both before the mix and with CO and dithionite in the small syringe such that (CO) = 100 μM and (dithionite) = 1 g/L after the mix. Temperature was 23 ± 1 °C; λ = 420 nm; slits = 0.4 mm; 2-mm path length. (A) pH 6; (B) pH 7; and (C) pH 8. The buffer was 5 mM phosphate. The points shown in A at pH 6 are experimental. The lines are theoretical fits to the equation $\Delta A/\Delta A_{tot} = f_{fast}e^{-k_f t} + f_{slow}e^{-k_s t}$. The fraction of slow phase for the solution results was 0.5 with k_f = 40 s^{-1} and k_s = 16 s^{-1} and with a mean residual (i.e., the mean percentage of discrepancy between observed and calculated absorbance values, based on a total change in absorbance of 0.1–0.2) of 0.82%. The fraction of slow phase for hemoglobin bound to the membrane, at this wavelength, was 0.14 with k_f = 16 s^{-1} and k_s = 1.9 s^{-1}, and with a mean residual of 0.32%.

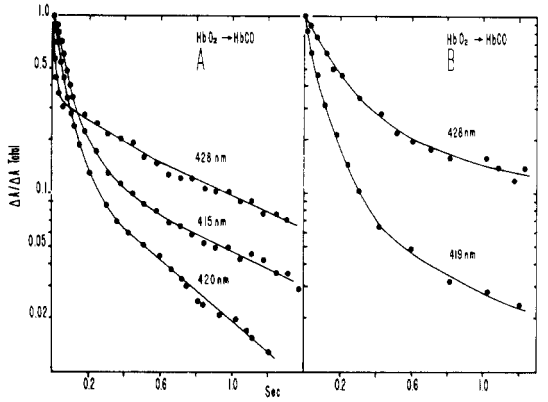

FIGURE 5: $HbO_2 \rightarrow HbCO$ replacement reaction in the presence (A) and absence (B) of dithionite as a function of wavelength, for membrane-bound oxyhemoglobin at pH 6. The conditions in A are the same as in Figure 4A. In B, there was no dithionite present and the samples were mixed 50:50 with (ghosts) = 5×10^{12} cells/L and (heme) = 10 μM, both before the mix with (CO) which was at 1 mM before the mix. Slits were at 0.1 mm and the path length was 2 mm in both cases.

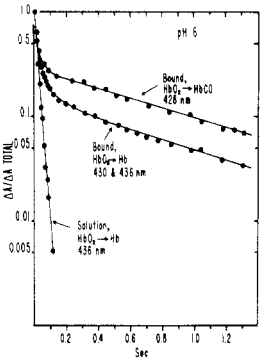

FIGURE 6: Oxygen release from membrane-bound hemoglobin in the absence and presence of carbon monoxide. The reactions were performed with ratio mixing syringes (1:10) with (ghosts) = 5×10^{12} cells/L and (heme) = 11 μM before the mix (large syringe); and (CO) = 100 μM and (dithionite) = 1 g/L after the mix (small). Three reactions are shown. The first is membrane-bound hemoglobin, where the $HbO_2 \rightarrow HbCO$ replacement reaction is measured at 428 nm; the second is the same sample with no CO present measured at 430 and 436 nm (i.e., $HbO_2 \rightarrow Hb$). Note that the rates are the same for the two types of reaction. The third reaction shows hemoglobin deoxygenation in solution at 436 nm for comparison. This latter reaction is about four times faster than the $HbO_2 \rightarrow HbCO$ replacement reaction in solution shown in Figure 4A.

expected from the pH dependence of oxyhemoglobin binding (Shaklai et al., 1977a,b), raising the pH to 8 does cause the kinetics in solution to be indistinguishable from the kinetics with membranes present. It is noted that there is a detectable fraction of slow phase at pH 7 (Figure 4B). The two time courses in Figure 4A can be fit to an equation representing the sum of two exponentials (Olson et al., 1971). For hemoglobin in solution, each phase accounted for 50% of the total reaction at this pH and wavelength, with rate constants of 40 and 16 s^{-1}. The time course for hemoglobin plus membranes could be represented well (see legend to Figure 4) with 86% fast phase and 14% slow. A fast phase rate constant of 16 s^{-1} and a slow phase rate constant of 1.9 s^{-1} were obtained.

Olson et al. (1971) first showed that this replacement reaction was strongly wavelength dependent and they interpreted the wavelength dependence in terms of a difference in the rate of oxygen release between the α and β chains of tetrameric hemoglobin. We investigated the wavelength dependence of this reaction in the absence and presence of dithionite for membrane-bound hemoglobin (Figures 5A,B). As can be seen in both figures, the reaction is strongly wavelength dependent, with the slow component appearing to the red of 420 nm. The wavelength dependence of the 1.9-s^{-1} component for membrane-bound oxyhemoglobin corresponds to the wavelength dependence of the 40-s^{-1} component in solution, while the wavelength dependence of the 16-s^{-1} component corresponds to the 16-s^{-1} component in solution.

The reason for showing the $HbO_2 \rightarrow HbCO$ replacement reaction in the absence of dithionite (Figure 5B) is to indicate that the wavelength dependence seen in Figure 5A is not due to reduction of any methemoglobin present in the system. The difference in rate for the two figures comes from the fact that the oxygen tension is lower with dithionite present and there is less oxygen to compete with CO for available hemes (Gibson & Roughton, 1955). Further checks on the influence of methemoglobin formation in Figures 2–6 were performed by studying these same types of reactions using 100% methemoglobin prepared according to the method of Gibson et al. (1969). For reactions like those in Figures 2 and 3, addition of methemoglobin produced an increase in the fraction of slow

phase at the same wavelengths, not a decrease as was always observed for oxyhemoglobin. Furthermore, the static isosbestic determined by comparing the absorbance at $t = 0$ with the absorbance at $t = \infty$ in Figure 2 always was that for HbO_2 vs. HbCO (~430 nm for solution and ~432 nm with membranes present). This was not the case when methemoglobin was used. For experiments like those in Figures 4–6, addition of 100% methemoglobin to membranes at pH 6 did cause the rate of reduction by dithionite to become slower as compared with an otherwise identical experiment with methemoglobin solution. However, the pseudo-first-order rate constant at the same dithionite concentration was about sixfold slower than the first-order rate constant for the slow phase of membrane-bound oxyhemoglobin. Finally, we have checked the fraction of methemoglobin formed at pH 6 in the absence and presence of membranes by reacting with 1 mM sodium azide in the stopped-flow after conversion of HbO_2 to HbCO. The results indicated that there was less than 1% methemoglobin present.

It is well known that conversion of oxyhemoglobin to deoxyhemoglobin in the presence of dithionite proceeds with an autocatalytic time course (Dalziel & O'Brien, 1961; Salhany et al., 1970; Gibson & Gray, 1970). The observed rate constant should be about four times greater, in a fully cooperative hemoglobin, than the rate for the $HbO_2 \rightarrow HbCO$ replacement reaction with dithionite present, where the molecule remains in the R quaternary structure (Hopfield et al., 1971; Shulman et al., 1975). Figure 6 shows a similar comparison for membrane-bound oxyhemoglobin at pH 6. The solution results reproduce the known effect (compare the solution curve of Figure 6 ($HbO_2 \rightarrow Hb$) with Figure 4A ($HbO_2 \rightarrow HbCO$)). Unlike the results in solution, membrane-bound hemoglobin showed no evidence for cooperativity in oxygen release as indicated by the identity of the slopes for the time courses shown in Figure 6. It will be noted that the rate constants for deoxygenation of membrane-bound hemoglobin were the same at 430 nm vs. 436 nm, unlike hemoglobin solution (Gray, 1974).

Discussion

The evidence just presented suggests that the dimer of oxyhemoglobin binds to band 3, the integral membrane protein of the human erythrocyte thought to be involved in passive anion transport (Rothstein et al., 1978). Although the relative light scattering measurements and the CO binding kinetic results of this paper support the proposal and are in good qualitative agreement with the previous fluorescence and centrifugation studies (Shaklai et al., 1977a,b), there is an apparent quantitative disagreement which requires some discussion. Shaklai et al. (1977a,b) reported two apparent binding sites, one determined by fluorescence and both apparently seen with the centrifugation method. The fluorescence measurements showed a high affinity binding constant of about 1.2×10^{-8} M (given here as a dissociation constant) on the assumption that oxyhemoglobin remained tetrameric under the conditions of the experiment. However, at the concentrations required for the fluorescence studies ($\sim 10^{-7}$ M), oxyhemoglobin would be about 97% dimeric, taking a value of $K_{4,2}$ (the tetramer–dimer dissociation constant) of 3 μM for oxyhemoglobin at pH 6 (Edelstein et al., 1970). Since binding was apparently noncooperative, their binding constant may be corrected for dimer binding according to the expression $(K_{obsd} \times K_{4,2})^{1/2}$ or $(1.2 \times 10^{-8}\ M \times 3 \times 10^{-6}\ M)^{1/2}$ which gives about 0.2 μM. The centrifugation measurements showed nonlinear Scatchard plots and were interpreted to indicate the existence of two classes of binding sites. One class had a high affinity ($\sim 1.2 \times 10^{-8}$ M in tetramer or 0.2 μM in dimer), while the other had an affinity of $\sim 6 \times 10^{6}\ M^{-1}$. However, this latter value must also be corrected for dimer binding based on the present results and the fact that the centrifugation studies were conducted with at most 10^{-6} M heme (Shaklai et al., 1977b), where very little tetramer would be present if 3 μM is used for $K_{4,2}$ (Edelstein et al., 1970). When this constant is similarly corrected, a value of 0.7 μM is obtained (i.e., $[1.67 \times 10^{-7}\ M \times 3 \times 10^{-6}\ M]^{1/2}$). The two apparent constants are not widely different and other explanations for the nonlinear Scatchard plots must be tested, especially the assumption that the system is always at equilibrium as the ghost concentration changes with centrifugation. Despite these potential problems, there is still an apparently significant quantitative difference in the dimer binding constant determined by fluorescence as compared with that determined by the light scattering measurements and confirmed by the more indirect kinetic results in Figure 2. Until more direct comparisons can be made between the three techniques and the assumptions implicit in the centrifugation technique tested, we must view the quantitative value for the dimer binding constant as tentative and somewhere between 0.2 and 4 μM.

The CO binding kinetic measurements show that oxyhemoglobin tends to be stabilized in a high affinity state when bound to the membrane. Although this result alone does not prove that the oxygenated dimer is stabilized, the light scattering results and the results of Figure 2 make this the simplest and most consistent view to propose. One important result which may indicate the site of binding was shown in Figure 3, where G3PD addition to ghosts, already containing bound oxyhemoglobin, caused the fraction of fast CO binding component to return to the value seen in solution. This would suggest that G3PD either directly or indirectly displaced the membrane-bound oxyhemoglobin dimer. Direct competition between hemoglobin and G3PD for band 3 seems a likely suggestion based on the stoichiometry of hemoglobin binding and the demonstration that G3PD binds to band 3 (Yu & Steck, 1975a,b). However, more direct evidence for hemoglobin binding to band 3 would be desirable.

Stabilization of the oxyhemoglobin dimer on band 3 is somewhat reminiscent of the interaction of the oxyhemoglobin dimer with haptoglobin (Nagel & Gibson, 1966; Chiancone et al., 1966, 1968; Alfsen et al., 1970). Deoxyhemoglobin apparently does not react with haptoglobin (Nagel & Ranney, 1964; Nagel et al., 1965; Nagel & Gibson, 1971, 1972). The important differences between the two reactions are that oxyhemoglobin binding to band 3 is reversible, while binding to haptoglobin is irreversible. In addition, recent preliminary light scattering measurements by one of us (J.M.S.) show that deoxyhemoglobin *does* bind to the membrane noncooperatively ($n = 1.2$) with an apparent binding constant of about 3.5 μM in tetramer at pH 6, in 5 mM phosphate plus 2 mM dithionite. Although more work on the functional properties of membrane-bound deoxyhemoglobin is required, the results in Figure 3A suggest that deoxyhemoglobin may be bound as the tetramer and not as the high affinity dimer.

Oxygen release measurements indicate unusual functional properties for the bound dimer. There are two types of oxygen release experiments which need to be considered. One is the initial rate of oxygen release, measured by replacement with CO in the presence of dithionite (Gibson & Roughton, 1955; Olson et al., 1971). The second reaction involves measurement of the overall rate of oxygen release upon mixing with dithionite in the absence of CO. This reaction is usually autocatalytic due to cooperativity in oxygen release (Dalziel &

O'Brien, 1961; Salhany et al., 1970; Gibson & Gray, 1970; Hopfield et al., 1971). However, comparison of these two reactions is not entirely straightforward, since both reactions can show a substantial wavelength dependence and it is important to consider this in measuring and interpreting the results. The wavelength dependence for the $HbO_2 \rightarrow HbCO$ replacement reaction in solution has been studied in detail by Olson et al. (1971). They found that the reaction was composed of two phases: a slow component which dominated the reaction at around 415 nm and a fast component whose HbO_2–HbCO difference spectrum was relatively red-shifted with respect to the slow component (see Figure 5 of Olson et al. (1971)). These two components were identified as arising from oxygen release from the α chain (slow) and β chain (fast) of the tetramer, on the assumption that these were the only two spectral components present. The conditions in Figure 5A were arranged so that almost all of the oxyhemoglobin present would be bound. However, only about 35% slow phase was seen at 428 nm (Figure 5A). This may indicate that only one chain in the dimer changes its rate of oxygen release over that in solution. The 40-s^{-1} phase seen in solution has the β chain difference spectrum, and this difference spectrum corresponds to the 1.9-s^{-1} phase for the membrane-bound dimer. The 16-s^{-1} phase in solution has the α chain spectrum, and the rate constant does not change with binding. These findings would suggest that the β chain of the oxy dimer slows its rate constant for O_2 release by a factor of 20 when bound to the membrane, while the α chain of the membrane-bound dimer remains unaffected. It is interesting to note that Hb Rainier, which is a β chain mutant where a disulfide bridge forms between the new sulfhydryl at HC2 (145) β and the F9(93) β cysteine, has an overall rate of oxygen release in solution which is extremely slow ($\sim$6 s^{-1} at pH 7 and 23 °C) (Salhany, 1972) and comparable to the slow β chain off rate seen here for the membrane-bound dimer of oxyhemoglobin. The absence of cooperativity in oxygen release was indicated in Figure 6, where the two rates for the $HbO_2 \rightarrow HbCO$ and $HbO_2 \rightarrow Hb$ reactions were seen to be the same. This contrasted with hemoglobin solution. Under identical conditions, the replacement reaction was about four times slower than the overall rate of oxygen release.

The significance of the present results for erythrocyte function is uncertain until binding studies can be performed under physiologic conditions. The advantage of the relative light scattering method is that such studies can be performed at hemoglobin concentrations approaching that present within the erythrocyte. There are two points which are worth considering. The first concerns the general assumption that there are no oxyhemoglobin dimers present at red cell concentrations of heme. This assumption may not be true. If 3 μM is used as the value for $K_{4,2}$ at physiologic salt, pH, and temperature (to our knowledge $K_{4,2}$ has not been measured under *exact* physiologic conditions for oxyhemoglobin), a value for the fraction of dimer at 20 mM heme would be 0.01. Thus, the concentration of dimer at equilibrium would be 0.1 mM. This would be enough to saturate the band 3 sites if the binding constant were on a similar order or smaller under physiologic conditions. Secondly, if the oxyhemoglobin dimer does not bind under physiologic conditions, the demonstration that deoxyhemoglobin can bind (see above) makes the interactions of this species with the membrane seem more likely within the erythrocyte due to (a) the somewhat greater positive charge of the deoxy tetramer and (b) the relatively enormous concentration of this species within the erythrocyte of venous blood. If deoxyhemoglobin binds and is oxygenated *on* band 3, the tendency for the oxygenated dimer to be energetically stabilized over the oxygenated tetramer could make it an important, albeit transient, species within the cell.

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References

Alfsen, A., Chiancone, E., Wyman, J., & Antonini, E. (1970) *Biochim. Biophys. Acta 200*, 76.

Andersen, M. E., Moffat, J. K., & Gibson, Q. H. (1971) *J. Biol. Chem. 246*, 2796.

Antonini, E., & Brunori, M. (1971) in *Frontiers in Biology* (Neuberger, A., & Tatum, E. L., Eds.) Vol. 21, North-Holland Publishing Co., Amsterdam.

Chiancone, E., Wittenberg, J. B., Wittenberg, B. A., Antonini, E., & Wyman, J. (1966) *Biochim. Biophys. Acta 117*, 379.

Chiancone, E., Alfsen, A., Ioppolo, C., Vecchini, P., Finazzi Agro, A., Wyman, J., & Antonini, E. (1968) *J. Mol. Biol. 34*, 347.

Dalziel, K. & O'Brien, J. R. P. (1961) *Biochem. J. 78*, 236.

Demma, L. S., & Salhany, J. M. (1977) *J. Biol. Chem. 252*, 1226.

Duysens, L. N. M. (1956) *Biochim. Biophys. Acta 19*, 1.

Edelstein, S. J., Rehmar, M. J., Olson, J. S., & Gibson, Q. H. (1970) *J. Biol. Chem. 245*, 4372.

Fischer, S., Nagel, R. L., Bookchin, R. M., Roth, E. F., Jr., & Tellez-Nagel, I. (1975) *Biochim. Biophys. Acta 375*, 422.

Frojmovic, M. M. (1975) *Biorheology 12*, 193.

Gibson, Q. H., & Roughton, F. J. W. (1955) *Proc. R. Soc. London, Ser. B 143*, 310.

Gibson, Q. H., & Roughton, F. J. W. (1959) *J. Physiol. 145*, 32P.

Gibson, Q. H., & Gray, R. D. (1970) *Biochem. Biophys. Res. Commun. 41*, 415.

Gibson, Q. H., Parkhurst, L. F., & Geraci, G. (1969) *J. Biol. Chem. 244*, 4668.

Gordon, D. J., & Holzworth, G. (1971) *Arch. Biochem. Biophys. 142*, 481.

Gray, R. D. (1974) *J. Biol. Chem. 249*, 2879.

Hopfield, J. J., Shulman, R. G., & Ogawa, S. (1971) *J. Mol. Biol.* 61, 425.

Karadsheh, N. S., & Uyeda, K. (1977) *J. Biol. Chem. 252*, 7418.

Kellett, G. L. (1971) *Nature (London) 234*, 189.

Kellett, G. L., & Gutfreund, H. (1970) *Nature (London) 227*, 921.

Latimer, P., & Holmes-Eubanks, C. A. (1962) *Arch. Biochem., Biophys. 98*, 274.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem.* 193, 265.

Nagel, R. L., & Ranney, H. M. (1964) *Science 144*, 1014.

Nagel, R. L., & Gibson, Q. H. (1966) *J. Mol. Biol. 22*, 249.

Nagel, R. L., & Gibson, Q. H. (1971) *J. Biol. Chem. 246*, 69.

Nagel, R. L., & Gibson, Q. H. (1972) *Biochem. Biophys. Res. Commun. 48*, 959.

Nagel, R. L., Rothman, M. C., Bradley, T. B., & Ranney, H. M. (1965) *J. Biol. Chem. 240*, PC4543.

Nelsestuen, G. L., & Lim, T. K. (1977) *Biochemistry 16*, 4164.

Olson, J. S., & Gibson, Q. H. (1972) *J. Biol. Chem. 247*, 3662.

Olson, J. S., Andersen, M. E., & Gibson, Q. H. (1971) *J. Biol. Chem. 246*, 5919.
Rothstein, A., Grinstein, S., Ship, S., & Knauf, P. A. (1978) *Trends Biochem. Sci. 3*, 126.
Roughton, F. J. W. (1934) *Proc. R. Soc. London, Ser. B 115*, 495.
Salhany, J. M. (1972) *Biochem. Biophys. Res. Commun. 47*, 784.
Salhany, J. M., & Swanson, J. C. (1978) *Biochemistry 17*, 3354.
Salhany, J. M., Eliot, R. S., & Mizukami, H. (1970) *Biochem. Biophys. Res. Commun. 39*, 1952.
Shaklai, N., Yguerabide, J., & Ranney, H. M. (1977a) *Biochemistry 16*, 5585.
Shaklai, N., Yguerabide, J., & Ranney, H. M. (1977b) *Biochemistry 16*, 5593.
Shulman, R. G., Hopfield, J. J., & Ogawa, S. (1975) *Q. Rev. Biophys. 8*, 325.
Steck, T. L. (1974a) *Methods Membr. Biol. 2*, 245.
Steck, T. L. (1974b) *J. Cell Biol. 62*, 1.
Strapazon, E., & Steck, T. L. (1976) *Biochemistry 15*, 1421.
Wilkinson, G. N. L. (1961) *Biochem. J. 80*, 324.
Yu, J., & Steck, T. L. (1975a) *J. Biol. Chem. 250*, 9170.
Yu, J., & Steck, T. L. (1975b) *J. Biol. Chem. 250*, 9176.

Characterization of Protein S, a γ-Carboxyglutamic Acid Containing Protein from Bovine and Human Plasma†

Richard G. DiScipio and Earl W. Davie*

ABSTRACT: Protein S is a vitamin K dependent protein of unknown function, which is present in mammalian plasma. It was isolated from bovine plasma by barium citrate adsorption and elution, ammonium sulfate fractionation, and column chromatography on DEAE-Sephadex, heparin-agarose, and polyhomoarginine-Sepharose. Bovine Protein S (M_r 64 200) is a single-chain glycoprotein with an amino-terminal sequence of Ala-Asn-Thr-Leu-Leu-. It contains 7.0% carbohydrate and 10 residues of γ-carboxyglutamic acid per mol of protein. Human Protein S (M_r 69 000) is also a single-chain glycoprotein with an amino-terminal sequence of Ala-Asn-Ser-Leu-Leu-. It contains 7.8% carbohydrate and 10 residues of γ-carboxyglutamic acid per mol of protein. These results indicate that Protein S from bovine or human plasma shows many similarities to the other vitamin K dependent proteins present in plasma.

Three of the four vitamin K dependent proteins in plasma which are involved in blood coagulation have been shown to contain γ-carboxyglutamic acid (Stenflo et al., 1974; Magnusson et al., 1974; Nelsestuen et al., 1974; Bucher et al., 1976). These proteins include prothrombin, factor IX (Christmas factor),[1] and factor X (Stuart factor). γ-Carboxyglutamic acid is formed by the carboxylation of specific glutamic acid residues in the amino-terminal region of these proteins in a series of reactions requiring vitamin K. Furthermore, a cell-free system that carries out these carboxylation reactions has been described (Esmon et al., 1975; Suttie et al., 1975; Mack et al., 1976; Esmon & Suttie, 1976; Jones et al., 1977).

In 1976, Stenflo identified and characterized another vitamin K dependent protein from bovine plasma which was called Protein C. This protein is converted to activated Protein C by thrombin and may play a regulatory role in blood coagulation (Kisiel et al., 1976; Esmon et al., 1976; Kisiel et al., 1977). Activated Protein C is probably identical with an inhibitor which interferes with the intrinsic pathway of blood coagulation (Marciniak, 1970, 1972; Seegers et al., 1976). It differs, however, from a bovine plasma protein, called Protein Z, which has been described in a preliminary report (Prowse & Esnouf, 1977). Protein Z contains γ-carboxyglutamic acid, but its amino-terminal sequence is not homologous with either Protein C or the other vitamin K dependent proteins of plasma.

More recently, we have described another protein from human plasma which was homologous to the other known vitamin K dependent proteins (DiScipio et al., 1977). This protein, called Protein S, was only partially characterized since rather small amounts were available. Its biological function was not established. In the present paper, we present a more thorough characterization of Protein S from human plasma, as well as its isolation and characterization from bovine plasma. The γ-carboxyglutamic acid content of human and bovine Protein S was quantitated along with that of prothrombin, factor VII, factor IX, factor X, and Protein C.

Experimental Section

Materials. 4-Morpholineethanesulfonic acid (Mes),[2] 4-morpholinepropanesulfonic acid (Mops), thiobarbituric acid, galactose, *N*-acetylneuraminic acid, and poly(L-lysine) (type I-B) were obtained from the Sigma Chemical Co., St. Louis, MO. Cyclohexanone, *O*-methylisourea, and benzamidine hydrochloride were purchased from Aldrich Chemical Co., Milwaukee, WI. Barium chloride and cyanogen bromide were obtained from Baker Chemical Co., Phillipsburg, NJ. DEAE-Sephadex A-50 and Sepharose 4B were products of

† From the Department of Biochemistry, University of Washington, Seattle, Washington 98195. *Received November 1, 1978.* This work was supported in part by Grant HL 16919 from the National Institutes of Health.

[1] The nomenclature for the various coagulation factors is that recommended by an international nomenclature committee (Wright, 1959).

[2] Abbreviations used: Mes, 4-morpholineethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; Gla, γ-carboxyglutamic acid.

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