FLUORESCENCE STUDIES ON COMPONENTS OF HUMAN ERYTHROCYTE MEMBRANE

George Wasemiller, Arnold Abrams and Seymour Bakerman

Department of Pathology, Division of Clinical Pathology
Medical College of Virginia, Richmond, Virginia 23219

Received December 27, 1967

The purpose of this investigation was to study the binding characteristics of the two components which have recently been separated from human erythrocyte membranes by Bakerman and Wasemiller (1967). The affinities of the two components for myoglobin and for hemoglobin were measured by the technique of fluorescence quenching. The membrane solutions were irradiated at 290 mμ, and the subsequent fluorescence from the tryptophan residues was measured at 360 mμ. The results were compared to those of Edwards and Criddle (1966) on mitochondrial structural protein.

MATERIALS AND METHODS:

Dispersed erythrocyte membranes, obtained by the method of Bakerman and Wasemiller (1967), were completely solubilized in 1% sodium dodecyl sulfate (SDS) at pH 11.0. The components were separated on a 100 cm x 4 cm column packed with Bio-Gel P-100 polyacrylamide resin (Bio-Red Lab., Richmond, Calif.), Fig. 1. Retarded and excluded components, each comprising one-half of solubilized membrane, were collected separately and dialyzed against water until a white precipitate formed. Next, the lipid of the two components were extracted by repeated washings with 25 vol. of acetone:water (9:1::V:V) at 0°C. The lipid extracted retarded component (ESP-retarded) and the lipid extracted excluded component (ESP-excluded) were dissolved in 50% 2-chloroethanol, pH 9. The membrane dispersions were centrifuged at 90,000g for 15 min., to give an

This work was supported by U.S. Public Health Service Grant He-08445.
Requests for reprints should be addressed to: Seymour Bakerman, Medical College of Virginia. This work was done at the University of Kansas Medical Center, Kansas City, Kansas.

optically clear solution. This treatment would yield protein solutions with concentrations of 0.1 to 0.3 mg/ml. The concentrations were determined by the protein content obtained from hydrolysates, 6 N HCl 20 hr., 105°C., using Beckman Amino Acid Analyzer.

The clear protein solutions were titrated at 25°C ± 0.5°C on the Aminco-Bowman spectrophotofluorimeter, Model 768-D (American Instr. Co., Silver Springs, Md.). One ml aliquots of the ESP-excluded and of the ESP-retarded material were titrated with the addition of sperm whale myoglobin (Mann Research Inc., N. Y., N. Y.) and bovine hemoglobin (Sigma Chem. Co., St. Louis, Mo.). The structural proteins were used at concentrations near 0.1 mg/ml, and the myoglobin and hemoglobin were at concentrations of 0.5 to 1.0 mg/ml. The addition of the myoglobin and the hemoglobin was carried out until a molar ratio of 3:1 moles heme protein/mole ESP was achieved. The addition of the proteins was accomplished by using a Micrometric microburette, Model SB-2 (Micrometric Instrument Co., Cleveland, O.) fitted with a 20 gauge delivery needle. After adding the myoglobin or hemoglobin, the solution was shaken vigorously for about 1 min. to give stable readings, and to insure uniform mixing and binding of the protein solutions.

Fluorescence intensity was plotted against heme protein concentration. When binding was indicated, these plots were corrected for beam attenuation as described by Velick, et al. (1960). The method described by Eisen and Siskind (1964) was used to determine the amount of complex formed and correction for the non-binding protein was employed as described by Edwards and Criddle (1966).

RESULTS AND DISCUSSION

ESP-retarded: The titration of the ESP-retarded component with hemoglobin, Fig. 2, upper pattern, indicated a binding with an endpoint of 2 moles of hemoglobin to one mole of ESP-retarded. This value was obtained by calculating the percent binding (in this experiment, 75%), and correcting the observed endpoint of the titration (here 1.6 moles of hemoglobin to one mole of ESP-retarded). This experiment was repeated several times, and a range of 1.7 to 2.1 mole of

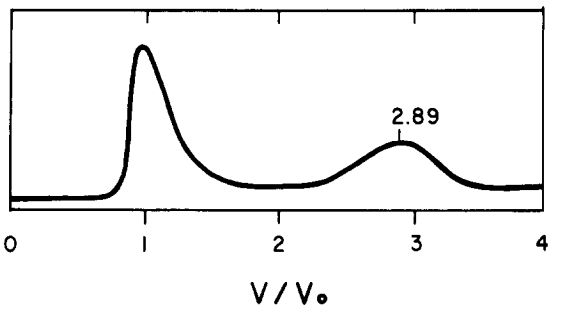

Fig. 1. Gel filtration on polyacrylamide gel-P-100 of human erythrocyte membrane solubilized in 1%, SDS, pH 11 and eluted with 0.5% SDS, pH 9. The effluent from the column was continuously monitored at 280 mμ and the relative optical density is given by the abscissa. The retarded and excluded molecular weight classes were collected separately and used in subsequent experiments.

hemoglobin to one mole of ESP-retarded material was obtained. It was found that the ability of the ESP-retarded material to bind hemoglobin decreased with time when stored at room temp., as is indicated by Fig. 2, middle pattern; this is not a characteristic binding plot. Beside aging, the absolute value for binding is effected by the heterogeneity and by the tryptophan content of the sample and by the strength of binding.

The addition of myoglobin to ESP-retarded is shown in Fig. 2, lower pattern. This curve demonstrated an initial increase in fluorescence, followed by the normal decrease due to beam attenuation. The initial increase was most likely due to the fluorescence of the myoglobin. This curve was interpreted to indicate the virtual absence of binding. This hypothesis was supported by the observation that when the ESP remained at room temp. for several days, no residual binding could be observed with hemoglobin, Fig. 2, middle pattern; however, the same hemoglobin inert material showed no change whatsoever in its behavior toward myoglobin.

ESP-excluded: The results of titration of the protein moiety of the excluded material appear in Fig. 3. With hemoglobin, Fig. 3, upper pattern, a straight line with a slight negative slope and no evidence of significant fluorescence

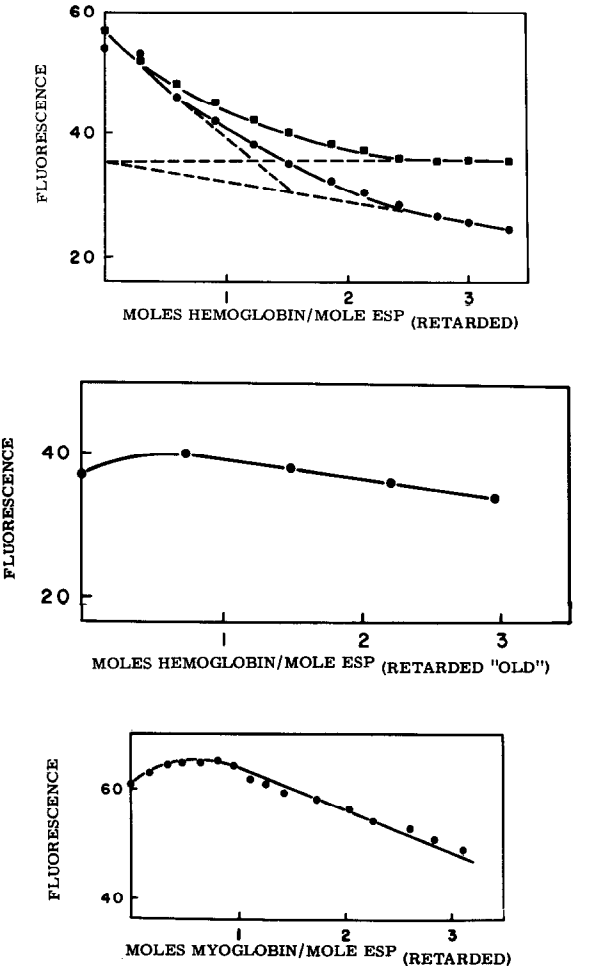

Fig. 2. Titration of ESP-retarded with hemoglobin and myoglobin as measured by fluorescence quenching. (Upper pattern-hemoglobin): The curve is corrected for beam attenuation as described by Velick, et al., 1960. The circles were the experimentally obtained data; the squares were the values corrected for the absorption of the incident beam by the protein. The endpoint, determined by the intersection of the extrapolation of the initial and final slopes of the experimental data yielded a value of approximately 1.6 moles hemoglobin/mole ESP. When corrected for percent of protein bound, a ratio of 2 moles hemoglobin:1 mole ESP was obtained. (Middle pattern-hemoglobin): The lack of fluorescence quenching indicates lack of binding, as does the slight initial rise in fluorescence, which was most likely due to the fluorescence of the hemoglobin. (Lower pattern-myoglobin): No binding was indicated. The initial rise was probably due to myoglobin fluorescence.

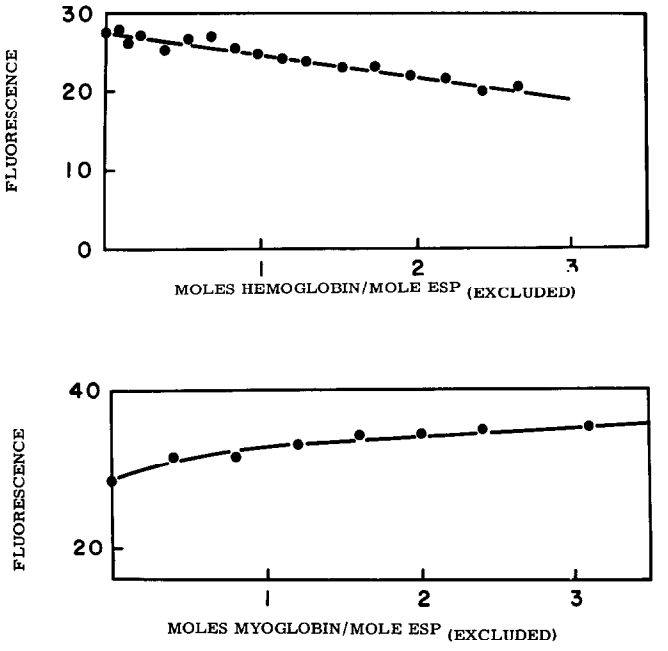

Fig. 3. Titration of ESP-excluded with hemoglobin and myoglobin as measured by fluorescence quenching. (Upper pattern-hemoglobin): The linear slope of the curve was due to beam attenuation and no binding was indicated. (Lower pattern-myoglobin): No binding was indicated. The initial rise was due to the fluorescence of the myoglobin. Beam attenuation has not become fully apparent at this low molar concentration.

quenching was obtained, indicating that there was no significant binding. With myoglobin, Fig. 3, lower pattern, there was a slight initial rise in the curve, followed by a gradual leveling off. Again, as was the case with the retarded ESP, the initial rise appeared due to the myoglobin fluorescence while the gradual leveling off was the beginning of beam attenuation effects. Our dilution tests have shown that the negative slope produced by beam attenuation does not become fully apparent at the low molar concentrations utilized in this particular experiment. We interpreted this curve to indicate the essential absence of binding, a conclusion supported by the essential similarity of this curve to the initial portion of the myoglobin plus ESP-retarded (hemoglobin inert) curve. It was in this portion of that particular curve that equiva-

lently low molar concentrations were to be found.

Our results are in contrast to the binding characteristics observed for the structural protein of mitochondrial membranes, as obtained by Edwards and Criddle (1966). Utilizing fluorescence quenching they observed that mitochondrial structural protein bound with myoglobin in a molar ratio of 1:1 moles myoglobin/moles structural protein, whereas no binding at all was observed with hemoglobin. This behavior of erythrocyte and mitochondrial membranes was not wholly unexpected, since in vivo beef heart mitochondria membranes are "contaminated" with myoglobin, (Edwards and Criddle, 1966) whereas erythrocyte membranes are bound to hemoglobin.

Nevertheless, we cannot as yet explain the observed differences. Both structural proteins are of approximately the same molecular weight (22,000), and have similar distribution of polar and non-polar amino acids as well as relative quantities of the amino acids. The variations in the two systems cannot be excluded as a contributing factor. The mitochondrial preparations were fairly soluble in Tris buffer, pH 9.0, while our protein was insoluble in Tris buffer, and required 50% 2-chloroethanol, pH 9.0, for even partial solubilization. Also, Edwards and Criddle's preparation (1966) was originally dispersed and solubilized in cholate-deoxycholate, whereas we solubilized in 1% SDS. In addition, they obtained their structural protein with $(NH_4)_2SO_4$ precipitation, while we obtained our components with molecular sieve column chromatography. Furthermore, the amino acid analyses of the proteins were different.

In conclusion, we should like to relate the above results to our present hypothesis of membrane architecture. We hypothesize that the ESP-retarded is a basic repeating unit of the membrane. Hence, it might be expected that it would bind with hemoglobin if it retains its binding sites after preparation as the membrane is normally so bound in vivo. In the same way, Edwards and Criddle's structural unit (1966) may well be the basic repeating unit of mitochondrial membrane, and it would hence be expected to bind to at least myoglobin, as well as to the cytochromes which it apparently does in vivo.

On the other hand, the component of the erythrocyte membrane excluded on Bio-Gel P-100 is heterogenous on further fractionation and is hypothesized to contain both membrane and membrane associated material (enzymes, etc.). Hence, if its binding sites are already occupied it would not be surprising for it to be inert to binding by hemoglobin or myoglobin.

SUMMARY: The binding of hemoglobin and myoglobin to two protein components from the human erythrocyte membrane obtained by gel filtration, have been followed by fluorescence titration. Hemoglobin binds to the smaller of the components and not to the larger. Myoglobin does not bind to either component. The results have been compared to the binding of hemoglobin and myoglobin with mitochondrial structural protein.

References

1. Bakerman, S. and Wasemiller, G.: Biochemistry, 6, 1100 (1967).
2. Edwards, D. L., Criddle, R. S.: Biochemistry, 5, 583 and ibid. 588 (1966).
3. Eisen, H. N. and Siskind, G. W.: Biochemistry, 3, 996 (1964).
4. Velick, S. F., Parker, C. W. and Eisen, H. N.: Proc. Natl. Acad. Sci. U.S., 46, 1470 (1960).