Vol. 88, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 13, 1979 Pages 980-987

OXYGEN EQUILIBRIUM OF EMULSIFIED SOLUTIONS OF NORMAL AND SICKLE HEMOGLOBIN

J.G. Pumphrey[1], K. Holroyd[2], and A.P. Minton

Laboratory of Biophysical Chemistry, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, MD, 20014

Received April 30, 1979

Summary: Emulsions containing microdroplets of concentrated solutions of normal or sickle hemoglobin dispersed in a continuous oil phase have been prepared, and the aggregation of sickle hemoglobin within microdroplets in a deoxygenated emulsion demonstrated. The equilibrium oxygen saturation of hemoglobin in the emulsions has been measured as a function of the partial pressure of oxygen by a novel spectrophotometric technique which corrects for the scattering of light in the emulsion. The half-saturation oxygen pressure and cooperativity of oxygen binding are substantially greater in concentrated (27 g/dl) solutions of sickle hemoglobin than in solutions of non-aggregating hemoglobin. The shape of the oxygen equilibrium curve of concentrated sickle hemoglobin is qualitatively discussed in terms of a previously proposed model (1).

INTRODUCTION

Analysis of the difference between the oxygen equilibrium curves of solutions of normal hemoglobin (HbA) and sickle hemoglobin (HbS) can provide substantial information about the thermodynamics of aggregation, or "gelation" of sickle hemoglobin (1,2,3). However, "gelled" solutions of HbS are both highly concentrated and highly viscous, and conventional methods of measuring oxygen equilibrium in hemoglobin solutions are inapplicable. Hence, this powerful analytical method has remained essentially unexploited.

The oxygen equilibrium of whole sickle blood and suspensions of sickle erythrocytes is, however, readily measured (4,5,6,7), indicating that HbS solutions do equilibrate with oxygen when a sufficient surface to volume ratio exists. One technique for attaining an adequate surface volume ratio was utilized by Gill et al. (8), who recently reported the measurement of oxygen equilibrium in concentrated HbS solutions in a specially constructed optical cell, with a thin layer of HbS solution (tens of microns thick) bounded on the

1. Present address: D-477, Abbott Laboratories, North Chicago, Illinois 60064
2. Present address: Box 746, Johns Hopkins University, 34th and Charles Streets, Baltimore, Md 21218

0006-291X/79/110980-08$01.00/0

surface by a transparent oxygen-permeable membrane. We report here another method for measuring the oxygen equilibrium in concentrated solutions of HbS, in which the necessary large surface to volume ratio is obtained by dispersion of the hemoglobin solution as microdroplets in a continuous oil phase.

METHODS

Preparation of HbS and HbA Solutions. Fresh whole blood was obtained from heterozygous (sickle trait) donors. All succeeding steps were carried out in the cold. The erythrocytes were washed three times in 0.9% NaCl and the buffy coat removed. The cells were hemolysed by three cycles of freezing/thawing followed by the addition of an equal volume of distilled water, and the hemolysate dialyzed overnight against 0.05 M Tris, 5 mM EDTA, pH 8.0 (23°C). The hemolysate was centrifuged at 30,000 RPM for 1 hr and the supernatant decanted. This step was repeated until no more precipitate was obtained. The clarified hemolysate was applied to a DEAE-Sephadex column equilibrated in 0.05 M Tris, pH 7.8 (23°C), and eluted with this same buffer. Pooled fractions containing either HbS or HbA were dialyzed against 0.05 M phosphate, 0.1 M KCl, 10 mM EDTA, pH 7.0 (23°C), and concentrated to >20 g/dl by ultrafiltration. The concentrated, purified solutions may be stored in liquid nitrogen for several months without significant increase in the amount of metHb present.

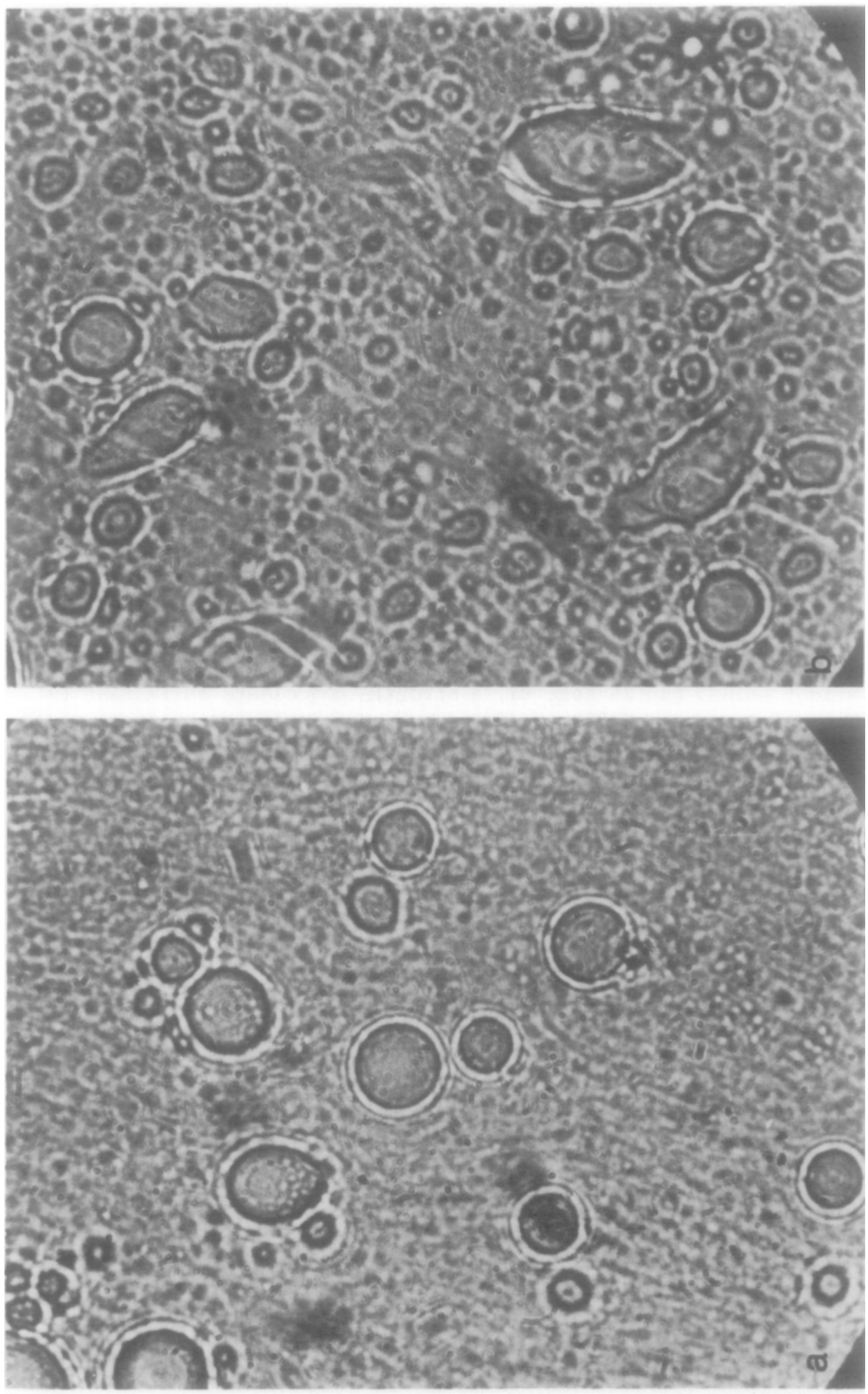
Preparation of Emulsions. An emulsion-forming liquid was prepared by mixing 95% S100N, a high molecular weight paraffin oil, 3% ENJ-3029, a high molecular weight amine, and 2% Span 85, a sorbitan monooleate surfactant (9). The solubility of oxygen in S100N was found by gas chromatography to be roughly 4 times greater than its solubility in water at room temperature and atmospheric pressure.

Four parts of cold emulsion-forming liquid were added to one part of hemoglobin solution and the mixture emulsified by milling in a chilled tissue homogenizer for ca. 2 min. The resulting emulsion is stable for at least several weeks. However, it was found that oxyHbS autooxidizes more rapidly than usual when emulsified, so the emulsions were generally prepared the evening before their use, deoxygenated immediately, and stored under nitrogen until used.

Measurement of Oxygen Equilibrium. Aliquots of deoxygenated emulsion were equilibrated with N_2:O_2 mixtures of known composition (Lif-O-Gen, Cambridge, MD.) by passing the gas over the gently agitated emulsion. It was found that oxygen equilibrium was achieved within 15 minutes at room temperature (23±1°C). The sample to be measured was then transferred with a gas-tight syringe (previously flushed with the gas mixture being used) to a 0.1mm optical cell (also flushed with the gas mixture being used). The cell was sealed with no gas space over the emulsion.

Because of the high turbidity of even thin films of these emulsions, a novel spectrophotometric technique was used to measure fractional absorbance changes. The spectrophotometer employed (Aminco-Chance, American Instrument Co., with Beam-Scrambler accessory) has separate monochromators for the sample and reference beams, which pass alternately at 60 Hz through the optical cells. The reference beam is fixed at 548.9 nm, an isosbestic point for oxy- and deoxyhemoglobin. The sample beam scans from 535-565 nm, producing a difference spectrum: $\Delta A(\lambda)$ [$\equiv A(\lambda) - A(548.9)$]. An apparent fractional oxygen saturation is defined as follows:

$$Y_{app}(\lambda,p) \equiv \frac{\Delta A(\lambda,p) - \Delta A(\lambda,0)}{\Delta A(\lambda,1\ atm) - \Delta A(\lambda,0)}$$



1. a. Oxygenated emulsion of concentrated solution of HbS (c > 20 g/dl). Original photograph (10.2x12.7 cm) is 1000x magnification.
   b. Same emulsion after one day under deoxygenating conditions.

where $\Delta A(\lambda,p)$ refers to the differential absorbance recorded at wavelength λ at oxygen partial pressure p. Because of scattering, $y_{app}(\lambda,p)$ may vary with λ. However, the true fractional change in absorbance of the emulsified hemoglobin, independent of scattering effects, should be given by

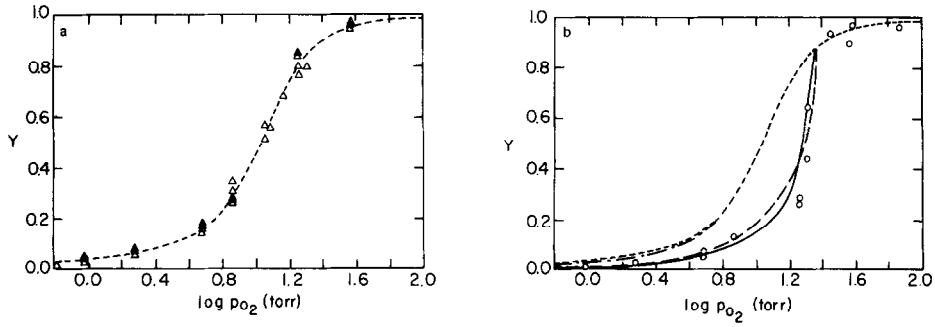
$$y(p) = \lim_{\lambda \to 548.9} y_{app}(\lambda,p)$$

In our experiments, $y_{app}(\lambda,p)$ was observed to vary essentially linearly with wavelength (with a small slope) over the region 535-565 nm. Experimental noise increased, as expected, in the regions immediately adjacent to 548.9 nm (i.e., as $\Delta A(\lambda,p)$ goes to zero for all p). The value of y(p) was obtained by calculating the best least-squares straight line through the data for $y_{app}(\lambda,p)$ for a single value of p, excluding a region 2.5 nm on either side of the isosbestic point, and interpolating to the isosbestic point.

RESULTS AND DISCUSSION

Figure 1a shows a 1000x magnification of an oxygenated emulsion. The largest microdroplets observed were several microns in diameter, roughly comparable in size to red blood cells. Although the majority of the microdroplets present are much smaller than these, the larger microdroplets contain the bulk of the aqueous phase. In oxygenated and deoxygenated emulsions containing HbA or dilute HbS solutions, almost all microdroplets observed appeared to be spherical. However, when an emulsion of sufficiently concentrated (>20 g/dl) HbS solution was deoxygenated, we observed the slow deformation of microdroplets (Figure 1b) and concomitant appearance of weak birefringence. These observations indicate that the deoxygenated HbS is aggregating within the microdroplets in a manner comparable to that observed in red blood cells (10).

The oxygen equilibrium data summarized in Figures 2a and 2b were obtained from a number of homogeneous and emulsified hemoglobin solutions. All of the data points shown in Figure 2a fall, to within experimental error, on the same oxygen equilibrium curve (short dashed line), which was obtained by least-squares fitting of an Adair-type equation to the combined data. The values of the Hill coefficient ($n \simeq 2.6$) and half-saturation oxygen pressure ($p_{50} \simeq 11$ torr) of the calculated curve are in good agreement with the literature values for normal hemoglobin solutions under similar conditions (11). The significance of these results is threefold:



2. a. Fractional saturation plotted vs. log p (oxygen) for the following samples: HbA solution, 4 g/dl (n=6); HbS solution, 5 g/dl (n=7); HbS solution #2, 5 g/dl (n=7); emulsified HbS solution, 13 g/dl in aqueous phase (n=6); emulsified HbS solution, 17 g/dl in aqueous phase (n=5). T = 23.5±0.5, aqueous phase in .05 M PO_4, .1 M Cl^-, .01 M EDTA, pH 6.95±.05. Dashed line is least-squares fit of Adair-type expression to the combined data (see text).
   b. Fractional saturation plotted vs. log p (oxygen) for the following samples: emulsified HbS solution, 27% in aqueous phase (n=9); emulsified HbS solution #2, 27% in aqueous phase (n=7). Conditions as above. Continuous and long dashed curves are simulations of 27 g/dl data, and long-short dashed curve is simulation of 17 g/dl data (see text). Short dashed curve same as in Fig.2a.

(1) They confirm the utility of the method described above for measuring the oxygen equilibrium of hemoglobin, both in homogeneous and emulsified solution. At present the method is not sufficiently precise to estimate reliably the "interior" Adair constants k_2 and k_3. However, these quantities are not essential to the analysis discussed below and in reference 1.

(2) The results support previous observations (8,12,13) indicating that, below a critical HbS concentration, the oxygen binding isotherms of HbS and HbA are identical.

(3) They show that the technique of emulsification of HbS solutions employed here does not itself significantly affect the oxygen equilibrium of hemoglobin.

Shown in Figure 2b are data points from two emulsified solutions of HbS at a concentration of 27 g/dl. These data sets have a larger scatter than the other data sets and do not admit of a thorough quantitative analysis. Nonetheless, certain qualitative and semi-quantitative observations may be made and conclusions drawn.

The oxygen equilibrium curve of the 27 g/dl HbS solutions is substantially right-shifted and steepened relative to those of more dilute HbS and HbA solutions. Gill et al. (8) recently reported a qualitatively similar, but smaller right shift and steepening of the oxygenation curve in a 33 g/dl HbS solution. The origin of the quantitative discrepancy between the two sets of data may be due to differences in the composition of the hemoglobin samples, differences in the experimental conditions, and/or experimental artifacts.

In Figure 2b are also shown curves representing attempts to simulate the data using modifications of a model previously proposed by Minton (Method ii of reference 1). In this model, the equilibrium oxygen saturation, y, of an aggregating solution of HbS at oxygen pressure, p, is taken as the mass average of the oxygen saturations of non-aggregating (monomeric) and aggregated (polymeric)hemoglobins. According to this model, the hemoglobin polymer has a lower affinity for oxygen than does the monomer, leading to a decrease in the overall fractional oxygen saturation when polymer is present. However, increasing the partial pressure of oxygen increases the solubility of hemoglobin monomer, thereby decreasing the mass fraction of hemoglobin present as polymer. At some critical value of the oxygen partial pressure, the solubility of monomeric hemoglobin will be equal to the total hemoglobin concentration, and at pressures above the critical value no polymer can exist in equilibrium with the monomer. One would expect to observe a discontinuity in the slope of the oxygen equilibrium curve at this critical value of the oxygen pressure.

In order to obtain semiquantitative agreement between the calculated curves and the data points shown it was necessary to make several additional assumptions:

(1) Aggregated HbS becomes significantly saturated with oxygen ($y_p \rightarrow 0.3$) as p approaches $p_{critical}$.[3]

3. In contrast to this result, Hofrichter (14) found that polymeric HbS binds little or no carbon monoxide over the range of CO concentrations in which monomeric and polymeric HbS coexist in equilibrium.

(2) The mass fraction of hemoglobin polymer, f_p, is substantially greater than that calculated using equation (2) of reference 1 when the concentration of monomeric hemoglobin present, c_e, is substantially less than the total hemoglobin concentration, c_T. A detailed examination of the derivation of the equation indicates that it may be valid only for small values of f_p (Minton, unpublished observations).

(3) The solubility of deoxy HbS was assumed to be approximately 14 g/dl under the conditions of these experiments. This value is not well defined by our results, but is in fair agreement with direct solubility determinations under similar conditions (15).[4]

We also attempted to measure the oxygen equilibrium of HbS emulsions using a commercially available apparatus for the measurement of whole blood oxygenation curves (Hem-O-Scan, American Instrument Co., Silver Spring, MD). We obtained reproducible oxygen equilibrium curves for "dilute" (<12 g/dl) solutions and emulsions of HbA and HbS, which agreed well with the above data (Hill n $\simeq$ 2.6 and $p_{50} \simeq$ 12-13 torr). However, with concentrated HbS solutions or emulsions, reproducible data could not be obtained. We suggest that values of p_{50} and the shapes of the oxygen "equilibrium" curves obtained using the Hem-O-Scan with concentrated (i.e., aggregating) HbS solutions cannot be relied upon.

It is clear that a comprehensive quantitative analysis of the relations between oxygenation and aggregation of HbS will require, at a minimum, additional oxygenation data obtained over a wide range of HbS concentrations exceeding the solubility of deoxy HbS. The objectives of the present communica-

4. It may be recalled that one of the "dilute" HbS emulsions exhibiting an oxygenation curve indistinguishable from that of non-aggregating hemoglobin had a concentration of 17 g/dl. If the solubility of deoxy HbS is really as low as 14 g/dl, some fraction of the HbS in a deoxygenated 17 g/dl solution should be aggregated, resulting in a decrease in the average oxygen affinity. In Figure 2b is plotted a simulated oxygenation curve for a 17 g/dl HbS solution using the same parameter values that were used to generate one of the simulated curves for the 27 g/dl solutions. It may be seen that while the oxygen affinity is in fact decreased at low oxygen pressures, the decrease is so small in absolute terms as to be within the scatter of measurements of the oxygenation curve for non-aggregating HbS solutions.

tion have been (a) to demonstrate that emulsified concentrated solutions of HbS provide a realistic yet analytically tractable physical model for the oxygen binding properties of hemoglobin in sickle erythrocytes, and (b) to describe a technique for measuring the oxygenation equilibria of the emulsified solutions.

ACKNOWLEDGEMENTS

We thank J. Kurlansik of the Naval Medical Research Institute, Bethesda, MD, for assistance in the gas chromatographic determination of the solubility of oxygen in emulsifying oil, N.M. Rumen of the Uniformed Services University of the Health Sciences, Bethesda, MD, for making a Hem-O-Scan available to us, N. Li and J. Frankenfeld of Exxon Research and Engineering Co. for generous gifts of S100N and ENJ-3029, and ICI America, for the gift of the Span 85.

REFERENCES

1. Minton, A.P. (1976). In Proceedings of the Symposium on Molecular and Cellular Aspects of Sickle Cell Disease, Hercules, J.I., Cottam, G.L., Waterman, M.R., and Schechter, A.N., eds. (DHEW-NIH, Bethesda, Md.) pp.257-273.
2. Minton, A.P. (1976) J.Mol.Biol. 100, 519-542.
3. Minton, A.P. (1977) J.Mol.Biol. 110, 89-103.
4. Seakins, M., Gibbs, W.N., Milner, P.F., and Bertles, J.F. (1973) J.Clin.Invest. 52, 422-432.
5. May, A. and Huehns, E.R. (1976) Brit.J.Haemotol. 30, 317-335.
6. Rossi-Bernardi, L., Luzzana, M., Samaja, M., Rossi, F., Perrella, M., and Berger, R. (1975) FEBS Letters 59, 15-19.
7. Winslow, R.M. (1976). In Proceedings of the Symposium on Molecular and Cellular Aspects of Sickle Cell Disease, Hercules, J.I., Cottam, G.L., Waterman, M.R., and Schechter, A.N., eds. (DHEW-NIH, Bethesda, Md.) pp.235-251.
8. Gill, S.J., Sköld, R., Fall, L., Shaeffer, T., Spokane, R. and Wyman, J. (1978) Science 201, 362-364.
9. May, S.W., and Li, N.N. (1972) Biochem.Biophys.Res.Comm. 47, 1179-1185.
10. Harris, J.W. (1950) Proc.Soc.Exp.Biol.Med. 75, 197-201.
11. Antonini, E., and Brunori, M. (1971) Hemoglobin and Myoglobin in Their Reactions with Ligands, (North-Holland, Amsterdam).
12. Wyman, J., and Allen, D.W. (1954) Rev.Hemat. 9, 155-157.
13. Noble, R.W. (1978).In Clinical and Biochemical Aspects of Abnormal Hemoglobins, Caughey, W., ed. (Academic Press, N.Y.).
14. Hofrichter, J. (1978).In Clinical and Biochemical Aspects of Abnormal Hemoglobins, Caughey, W., Ed. (Academic Press, N.Y.).
15. Ross, P.D., Hofrichter, J., and Eaton, W.A. (1977) J.Mol.Biol. 115, 111-134.