THE EFFECT OF DILUTION ON THE OXYGENATION PROPERTIES OF CAT AND HUMAN HEMOGLOBINS

Bolling Sullivan

From the Department of Biochemistry
Duke University Medical Center
Durham, North Carolina 27706

Received June 23, 1967

Recently, Taketa and Morell (1) reported the oxygen affinity of cat hemoglobin. They found that the low oxygen affinity of cat blood (relative to human blood (2)) was reflected in the low oxygen affinity of cat hemoglobin. Thus, the difference was not attributable to an intracellular modification of the oxygenation properties of cat or human hemoglobins. They also reported that cat hemolysates contain two hemoglobin components. The concentration of the minor component varied from 10-40% in twelve samples. Both components were reported to have similar oxygenation properties.

Prior to this publication we had completed studies on the oxygenation properties of cat hemoglobin. Our primary interest was in the functional changes associated with protein subunit dissociation, but because our results differ somewhat from those of Taketa and Morell (1), they are reported in full here. We find that cat hemoglobin readily dissociates below 0.1% protein concentration, and that this dissociation affects the functional properties of the hemoglobin. The oxygen affinity is increased and the Bohr effect decreased. Human hemoglobin shows a much smaller increase in the oxygen affinity and the Bohr effect is unchanged. The pK's and ΔH's of these hemoglobins are reported. The primary differences between cat and human hemoglobins are the position and magnitude of the deoxy-oxy shift of the acid pK. Only a single hemoglobin band was observed during electrophoresis.

Methods and Procedure

Cat blood was collected by cardiac puncture and human blood was collected by venipuncture. Coagulation was prevented with heparin. After removal of the serum the solutions were washed three times with cold 0.9% NaCl and the cells lysed with 3-5 volumes of cold glass-distilled water. The stroma were separated by centrifugation and the hemoglobin solutions were dialyzed overnight at 4°C against 0.2M NaCl which had been exhaustively bubbled with N_2. Dialysis was continued for 24 hours against N_2 bubbled 0.01M phosphate buffer of pH 8.0.

Oxygen equilibria were determined spectrophotometrically by the method of Riggs and Wolbach (3). Methemoglobin concentrations were determined on the hemoglobin samples before and after the equilibrium measurements (4). The degree of dissociation was measured by Sephadex chromatography (5). Starch-gel electrophoresis was carried out at pH 9.0 according to the method of Smithies (6).

Results

Figure 1 shows the variation in oxygen affinity (plotted as log of the oxygen pressure (mm Hg) required for half saturation versus pH) of cat and human hemoglobins at 20°C, 0.15% Hb concentration, and 0.1M phosphate buffer. The smooth curves are theoretical and derived from calculations of the pK values (7). The major difference between the two hemoglobins at alkaline pH is the affinity for oxygen. The major difference in the acid pH region is in the magnitude of the pH dependence (acid Bohr effect). Heme-heme interaction values fall in the range 2.6-3.1 for cat hemoglobin and 2.7-3.2 for human hemoglobin.

If the assumption is made that two non-interacting ionizing groups per heme are involved in the Bohr effect and that one becomes a weaker and the other a stronger acid upon oxygenation then the pK values may be calculated

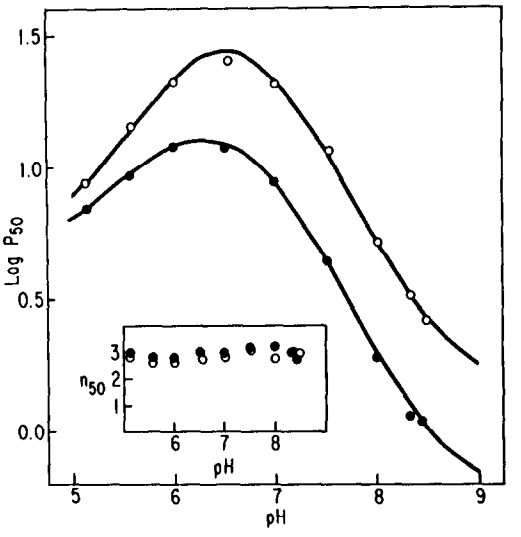

Fig. 1. Oxygenation properties of cat (O) and human (●) hemoglobins (0.15% Hb) at 20°C in 0.1M phosphate buffers. The log of the half saturation value (P_{50}) is plotted versus the pH. The points are experimental, the smooth curves are theoretical. Insert shows the heme-heme interaction values at 50% saturation as a function of pH.

(7). Thus,

$$\log P_{50} = C + \log \frac{(H^+ + K_1')\ (H^+ + K_2')}{(H^+ + K_1)\ (H^+ + K_2)}$$

where K_1' and K_2' refer to deoxyhemoglobin and K_1 and K_2 refer to oxyhemoglobin. The pK values and constants for cat and human hemoglobin are shown in Table I. Clearly the main difference lies in the magnitude of the acid pK shift and the position of the pK values. This difference not only accounts for the increased acid Bohr effect, but also contributes strongly to the differences in oxygen affinity at alkaline pH. The heats of

Table I

The pK's and Heats of Oxygenation of Cat and Human Hemoglobins (0.15% Hb) at 20°C in 0.1M Phosphate Buffers

	C	pK_2'	pK_2	pK_1'	pK_1	ΔpKacid	$\Delta pK_{alk.}$	ΔH
cat	.683	5.13	6.15	8.51	6.94	1.02	1.57	-14.9 Kcal
human	.654	5.21	5.86	8.47	6.90	.65	1.57	-13.6 Kcal

oxygenation also are shown in Table I and are very similar. They were derived by averaging values obtained at different pH's (4 for cat and 8 for human hemoglobin) and 15°C.

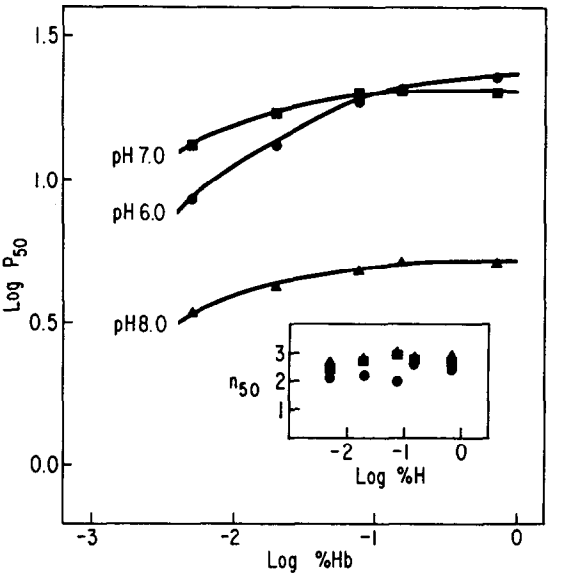

Fig. 2. The effect of dilution on the oxygenation properties of cat hemoglobin at pH 6.0, 7.0, and 8.0. Heme-heme interaction values at 50% saturation as a function of protein concentration are shown in the insert.

In an effort to explain the oxygenation properties of hemoglobin many workers have considered the effects of subunit dissociation (8-14). Svedberg and Hedenius (15) reported years ago that cat hemoglobin dissociates at low protein concentrations. Our data (Fig. 2) confirm the observations of Svedberg and Hedenius. Below 0.1% hemoglobin concentration there is a large increase in the oxygen affinity of cat hemoglobin. The effect is most pronounced at lower pH values. Similar data for human hemoglobin is given in Fig. 3. For human hemoglobin the effect is much smaller and apparently independent of pH. Diluting the protein concentration to 0.005% at pH 6.0 results in a P_{50} change of 2 mm Hg for human hemoglobin and 13 mm Hg for cat hemoglobin. The values of n show a slight decrease at low protein concentrations.

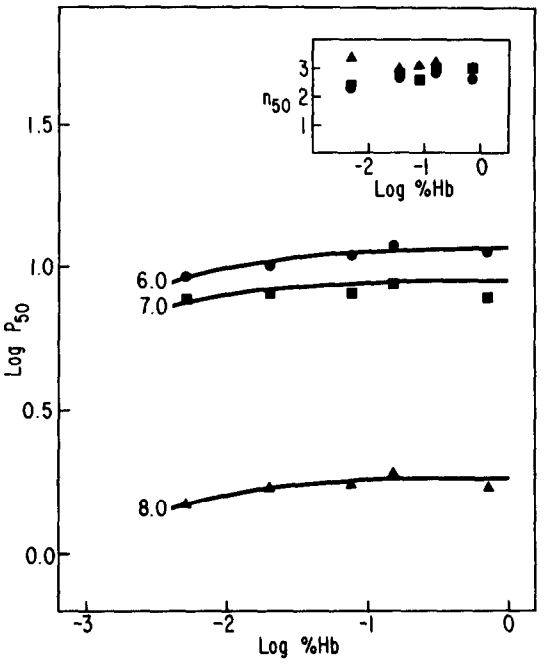

Fig. 3. The effect of dilution on the oxygenation properties of human hemoglobin at pH 6.0, 7.0, and 8.0. Heme-heme interaction values at 50% saturation as a function of protein concentration are shown in the insert.

Cat hemoglobin elutes after human hemoglobin from a 2 x 40 cm column of Sephadex G-100. It appears to be approximately 15-30% more dissociated. Electrophoresis for 4 hours at room temperature and 400 volts showed a single band moving slightly slower than human hemoglobin.

Discussion

The oxygen affinity data of Taketa and Morell (1) are similar to those presented here. However, their data for cat hemoglobin are 0.11 and 0.18 log units lower than ours. The salt concentrations used in their experiments were higher (0.14M versus 0.10M), but these should decrease, not increase the oxygen affinities (16). They also reported lower heme-heme interaction values (1.8, 2.0) for cat hemoglobin. Both of these results would be expected if their cat hemoglobin was partially oxidized (17). Our sample initially contained 0.77% methemoglobin; Taketa and Morell (1) did not

report methemoglobin levels. Oxidation could also explain their finding of two hemoglobin components in cat hemolysates. Lessard et al. (28) reported that cat hemoglobins contain 8 "reactive" sulfhydryl groups per tetramer. They located three of these on the alpha chain and one on the beta chain. Therefore it is possible that oxidation caused polymerization by disulfide formation between tetramers (32). This would account for the varying amounts of the minor component observed by Taketa and Morell (1) and also might account for their observation of a single peptide difference between the major and minor components. Although hemoglobin polymorphism is widespread among vertebrates (18), actual quantitative variation in a minor component is unusual. Other workers have reported only a single component in cat hemolysates (19-21).

In Wyman's equation, C represents a constant which reflects the oxygen affinity of the hemoglobin molecule under a given set of conditions, irrespective of pH effects. On dissociation of the protein into subunits under these same conditions, the value of C may change, and/or any or all of the values of the four pK's may change. In the case of human hemoglobin, apparently only C changes. For cat hemoglobin C is changing, but also the acid pK shift is increasing in magnitude. It appears doubtful that the basic pK values are changing.

This pK change implies that the basic pK group, presumably a histidine in the hydrophobic pocket in which the heme is located (7) or one or more α amino groups (27, 33), is changed very little as the molecule dissociates. This is in agreement with the recent findings of Steinhardt et al. (22). The acid pK, presumed to be a carboxyl, is changed in the cat, but not in human hemoglobin as dissociation occurs. It is difficult to say quantitatively what is happening to the acid pK's of cat hemoglobin. In order to do so, we need data at low protein concentrations and low pH. The formation of methemoglobin under these conditions is prohibitive.

Antonini et al. (23, 24) have qualitatively described the dependence

upon protein concentration of human adult and human fetal hemoglobins. Similar data have been presented for the lamprey (9), bullfrog and tadpole (25), and various turtle hemoglobins (34). There has been some disagreement as to whether sheep hemoglobins dissociated upon dilution (25, 26), but recently Gilbert (29) has shown that sheep hemoglobins do dissociate, and that there are differences in the dissociation properties of the multiple sheep hemoglobin components. Chiancone et al. (30) have shown that the sedimentation coefficients of mammalian hemoglobins are lower than those of hemoglobins from lower vertebrates. They interpreted these data to mean that mammalian hemoglobins are more dissociated and that the degree of dissociation decreases as one goes from mammals toward fish. Cat hemoglobin appears to be one of the most easily dissociated of mammalian hemoglobins. With attention focused on the subunit dissociation and exchange reactions as possible models to explain the oxygenation properties of hemoglobin (8-14), the existence of hemoglobins with different subunit dissociation properties should facilitate the design and execution of critical experiments to support or negate these models.

It should be mentioned that the close similarity in the pK's and constants for cat and human hemoglobins is probably not a good indication of close structural relationships. Hemoglobins from primates, which are presumed to be more closely related structurally to human hemoglobin, often show oxygenation properties more dissimilar to those of human hemoglobin than does cat hemoglobin (31).

Acknowledgements

This work was supported by NIH grants to Dr. Robert L. Hill. The author is a NIH postdoctoral fellow (5-F2-HE-31,481-02). Dr. Austen F. Riggs called our attention to the early report on the dissociation properties of cat hemoglobin.

References

1. Taketa, F., and Morell, S. A., Biochem. Biophys. Res. Commun., 24, 705 (1966).
2. Bartels, H., and Harms, H., Pflugers, Arch. Ges. Physiol., 268, 334 (1959).
3. Riggs, A. F., and Wolbach, R. A., J. Gen. Physiol., 39, 585 (1956).
4. Dubowski, K. M., in Sunderman, F. W., and Sunderman, Jr., F. W., Hemoglobin, Its Precursors and Metabolites, J. B. Lippincott Co., Philadelphia, p. 49 (1964).
5. Sullivan, B., and Riggs, A., Comp. Biochem. Physiol., in press.
6. Smithies, O., Biochem. J., 71, 585 (1959).
7. Wyman, J., Adv. Protein Chem., 4, 407 (1948).
8. Briehl, R. W., J. Biol. Chem., 238, 2361 (1963).
9. Briehl, R. W., in Goodwin, T. W., Harris, J. I., and Hartley, B. S., Structure and Activity of Enzymes, Symp. No. 1, Fed. Europ. Biochem. Soc's., London Academic Press, New York, p. 171 (1964).
10. Guidotti, G., in Goodwin, T. W., Harris, J. I., and Hartley, B. S., Structure and Activity of Enzymes, Symp. No. 1, Fed. Europ. Biochem. Soc's., London Academic Press, New York, p. 157 (1964).
11. Benesch, R. E., Benesch, R., and Macduff, G., Proc. Natl. Acad. Sci., 54, 535 (1965).
12. Benesch, R., Benesch, R. E., and Tyuma, I., Proc. Natl. Acad. Sci., 56, 1268 (1966).
13. Douglas, C., Haldane, J. S., and Haldane, J. B. S., J. Physiol., 44, 275 (1912).
14. Schejter, A., Adler, A. D., and Glauser, S. C., Science, 141, 784 (1963).
15. Svedberg, T., and Hedienius, A., Biol. Bull., 66, 191 (1934).
16. Rossi-Fanelli, A., Antonini, E., and Caputo, A., J. Biol. Chem., 236, 397 (1961).
17. Darling, R. C., and Roughton, F. J. W., Amer. J. Physiol., 137, 56 (1942).
18. Gratzer, W. B., and Allison, A. C., Biol. Rev., 35, 459 (1960).
19. Rodnan, G. P., and Ebaugh, F. G., Jr., Proc. Soc. Exp. Biol. Med., 95, 397 (1957).
20. Fine, J. M., Uriel, J., Faure, J., Bull. Soc. Chim. Biol., 38, 649 (1956).
21. Manwell, C., and Baker, C. M. A., in Smith, R. T., Miescher, P. A., and Good, R. A., Phylogeny of Immunity, University of Florida Press, Gainesville, p. 3 (1966).
22. Steinhardt, J., Polet, H., and Moezie, F., J. Biol. Chem., 241, 3988 (1966).
23. Antonini, E., Wyman, J., Brunori, M., Fronticelli, C., Bucci, E., Reighlin, M., and Rossi-Fanelli, A., Arch. Biochem. Biophys., 108, 569 (1964).
24. Rossi-Fanelli, A., Antonini, E., and Caputo, A., Adv. Protein Chem., 19, 73 (1964).
25. Riggs, A., Physiol. Rev., 45, 619 (1965).
26. Kernohan, J. C. and Johnson, P., Biochem. J., 99, 37P (1966).
27. Tanford, C., and Nozaki, Y., J. Biol. Chem., 241, 2832 (1966).
28. Lessard, J. L., Smits, M. R., Dibona, F. J., and Taketa, F., Fed. Proc., 26, 829 (1967).
29. Gilbert, G. A., Nature, 212, 296 (1966).
30. Chiancone, E., Vecchini, P., Forlani, L., Antonini, E., and Wyman, J., Biochim. Biophys. Acta, 127, 549 (1966).
31. Sullivan, B., in preparation.
32. Riggs, A., Sullivan, B., and Agee, J. R., Proc. Natl. Acad. Sci., 51, 1127 (1964).
33. Hill, R. J., and Davis, R. W., J. Biol. Chem., 242, 2005 (1967).
34. Sullivan, B., and Riggs, A., Biochim. Biophys. Acta 140, 274 (1967).