Vol. 85, No. 2, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 29, 1978 Pages 526-531

A DECREASED EFFECT OF ORGANIC PHOSPHATES ON HEMOGLOBIN S AT LOW CONCENTRATIONS

Yoshihiro Ueda, Robert M. Bookchin and Ronald L. Nagel
Department of Medicine, Albert Einstein College of Medicine
Bronx, New York 10461 USA

Received September 29,1978

SUMMARY - Hemoglobin (Hb) S, at concentrations well below those required for gelation, has previously been found functionally indistinguishable from HbA. The present communication describes small but consistent differences in the responses of the two hemoglobins to organic phosphates. Inositol hexaphosphate and 2,3-diphosphoglycerate (2,3-DPG) lowered the oxygen affinity of HbS less than that of HbA. This difference was confirmed by studies of the competitive effects of 2,3-DPG and CO_2 on Hb, which showed that the increase in oxygen affinity when 2,3-DPG was displaced by CO_2 (at pH over 7.0) was considerably greater for HbS than for HbA. These findings strongly suggest that the binding constant of organic phosphates for HbS is lower than that for HbA. This is the first functional abnormality detected in native HbS at low concentrations and it suggests that the β6Val substitution induces a small conformational change in the vicinity of the α-amino terminal groups of the β chains.

The functional properties of Hb S vary considerably with the protein concentration. The polymerization of this mutant (which is highly concentration-dependent), appears primarily responsible for the abnormal respiratory functions of red cells in sickle cell anemia (1, 2, 3). With Hb solutions at concentrations much lower than those in red cells, however, the oxygen equilibria, Bohr effect and ligand binding kinetics of HbS and HbA have been indistinguishable (4, 5, 6). This lack of intrinsic functional differences appeared consistent with the failure to observe structural variance between deoxy HbS and A by X-Ray crystallography (7), although recently described differences between the two hemoglobins in surface activity (8) and optical activity (9), and NMR data (10) may reflect some conformational changes resulting from the β6Valine substitution.

Since the effects of 2,3-diphosphoglycerate (2,3-DPG) on the oxygen equilibria of Hb were discovered independently by Chanutin and Curnish (11) and by Benesch and Benesch (12) 11 years ago, the structural basis by which this small molecule binds to Hb

0006-291X/78/0852-0526$01.00/0

has been studied extensively. Findings of differences in effects of 2,3-DPG on normal and mutant Hbs have contributed to both the knowledge of binding mechanisms and to understanding the functional effects of the mutations. Bunn and Briehl compared the effects of 2,3-DPG on HbS and HbA and concluded that there were no differences (5). Carbon dioxide also binds to Hb at the amino termini of the β chains but its binding to HbS has not been studied previously. Since more accurate methods are now available for determining oxygen dissociation curves, which may serve as a sensitive probe of Hb conformation (13), we have reexamined the effects of 2,3-DPG and studied the competitive effects of CO_2 on the oxygen equilibria of hemoglobins S and A.

METHODS

Hemolysates were prepared by the method of Drabkin, with small modifications (14), from red cells of normal donors (HbAA) and persons with sickle cell anemia (HbSS) whose hemolysates contained less than four percent HbF. The hemoglobins were stripped of 2,3-DPG and inorganic phosphate by chromatography on columns of DE-52 (Whatman) developed with 0.05 Tris-HCl buffer, pH 8.2, and after elution from these columns by addition of 0.5 NaCl to the buffer, were equilibrated with 0.05 M Bis-tris, pH 7.5 + 0.1 M NaCl on columns of Sephadex G-25. The eluates were free of 2,3-DPG as measured by the method of Rose (15) using a Sigma 35-UV kit. For final adjustments of pH, the Hb samples were titrated with 0.1 M HCl or NaOH in 0.05 M bis-tris + 0.1 M NaCl.

Oxygen equilibria were determined using a Cary Model 17D recording spectrophotometer modified to hold a chamber constructed by Dr. Kiyohiro Imai of Osaka University, Japan. This chamber, which has gas ports, temperature control and a magnetic stirring devise, allows continuous monitoring of pO_2 and pH with a Clark oxygen electrode, a radiometer pH electrode, and a Radiometer pH-pO_2 meter Model pH71 MK2. Outputs of the spectrophotometer and pO_2 electrodes were transcribed with a Hewlett Packard X-Y Recorder Model 7044A for continuous recording of oxygen equilibrium curves.

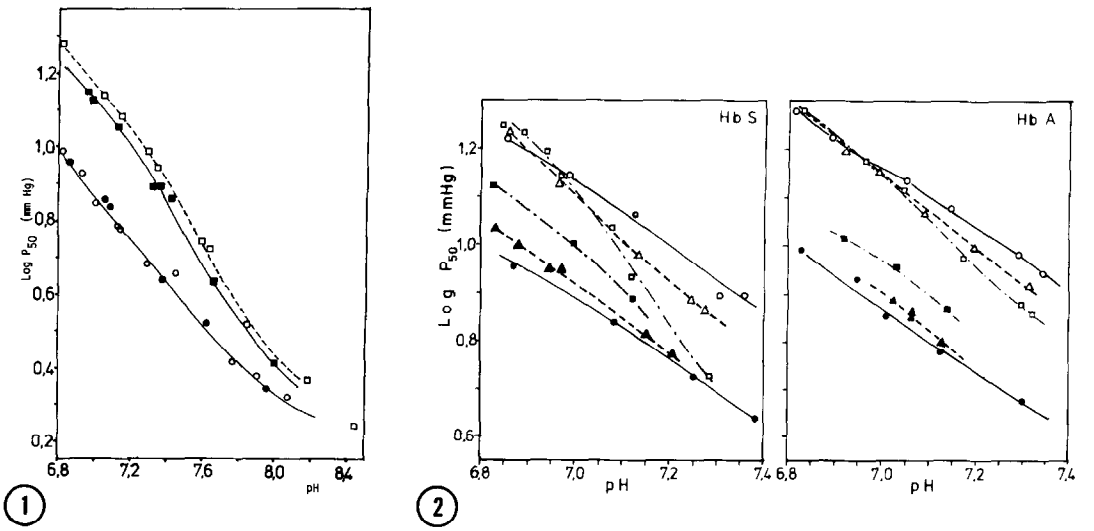

Fig. 1. Effect of 2,3-DPG on the oxygen affinity and Bohr effect of HbS and HbA.

●-● stripped HbS; ○-○ stripped HbA; ■-■ stripped HbS + 2,3-DPG; □-□ stripped HbA + 2,3-DPG; conditions: 0.05 M Bis-tris buffer + 0.1 M NaCl; molar ratio of DPG/Hb_4 = 10; Hb concentration 1.5×10^{-5} M; 25°.

Fig. 2. Effects of 2,3-DPG and CO_2 on the oxygen affinity and Bohr effect of HbA and HbS.

●-● stripped Hb; ▲-▲ stripped Hb, pCO_2 = 41 mmHg; ■-■ stripped Hb, pCO_2 = 85 mmHg; ○-○ stripped Hb + 2,3-DPG; △-△ stripped Hb + 2,3-DPG, pCO_2 = 41 mmHg; □-□ stripped Hb + 2,3-DPG, pCO_2 = 85 mmHg; conditions: 0.05 M Bis-tris buffer + 0.1 M NaCl, molar ratio of DPG/Hb_4 = 10; Hb concentration 1.5×10^{-5} M, 25°.

After equilibration of Hb solutions with O_2 or with prepared gas mixtures of $O_2 + CO_2$, progressive deoxygenation was obtained by switching to N_2 gas or the corresponding mixture of $N_2 + CO_2$. Solution pH values were measured anaerobically at the end of each determination.

RESULTS

As seen in Fig. 1, the oxygen affinities of HbA and HbS, at low concentration (1.5×10^{-5}M) and stripped of phosphates, appear indistinguishable over the pH range 6.8 to 8.1. The addition of 2,3-DPG, however, resulted in a small but consistent difference: the extent to which 2,3-DPG lowered the oxygen affinity of HbS was slight-

ly less than with HbA, particularly below pH 7.7 where the binding of 2,3-DPG to Hb is greater. Similar differences in the oxygen affinities of HbA and HbS were observed in the presence of another allosteric effector, inositol hexaphosphate (IHP). With other experimental conditions the same, addition of IHP (3 moles per mole hemoglobin tetramer) to stripped Hb solutions at pH 7.34 raised the p50 of HbA from 4.5 to 28.2 mmHg and that of HbS to a lesser extent, from 4.7 to 26.0 mmHg.

The effects of carbon dioxide on the oxygen affinity of HbA and HbS, in the presence and absence of 2,3-DPG, are shown in Fig. 2. Over the pH range studied (pH 6.9 to 7.2), CO_2 appeared to lower the oxygen affinities of the two stripped hemoglobins to about the same extent. In the presence of 2,3-DPG however, the effects of CO_2 on Hb and HbS were quite different at pH $\geq$ 7.1. By competing with and displacing 2,3-DPG from binding sites at the amino termini of the β chains, CO_2 counteracts the effect of 2,3-DPG, producing a net increase in the oxygen affinity. At pH 7.10 the effects of CO_2 on the oxygen affinities of stripped HbA and HbS were quite similar, but as shown in Table I and Fig. 2, at the same pH, CO_2 counteracted the effects of 2,3-DPG more with HbS than with HbA. As the pH was increased further (thereby lessening the binding of 2,3-DPG and increasing carbamate formation), the differences between HbS and HbA were even greater in the presence of both DPG and high levels of CO_2 (Fig. 2, Table I). Thus at pH 7.3 addition of CO_2 (pCO_2 = 85 mmHg) lowered the p50 of HbA by 1.9 mmHg and that of HbS by nearly twice that amount, 3.7 mmHg.

DISCUSSION

Carbon dioxide and 2,3-DPG decrease the oxygen affinity of hemoglobin, facilitating the delivery of O_2 to the tissues (8, 9). The effect of 2,3-DPG is larger than that of CO_2 (Fig. 1) but both effects result from the stronger binding of these compounds to deoxyHb than to the oxyHb. Both small molecules bind to a common site on the Hb molecule. CO_2 binds to the α amino groups of the β chains (16, 17, 18) and probably also

Table I. Comparison of the effects of 2,3-DPG and CO_2 on the oxygen affinities of Hb A and Hb S at pH 7.10 and 7.30. Conditions were as shown in the legend of Fig. 2. The column designated $\Delta p50$ shows the difference in p50 values without CO_2 and with CO_2 at the concentrations shown.

Conditions	pH 7.10		pH 7.30	
	p50 (mmHg)	$\Delta p50$	p50 (mmHg)	$\Delta p50$
Hb A + 2,3-DPG	12.5		9.4	
+ 2,3-DPG, pCO_2 = 41 mmHg	11.4	1.1	8.2	1.2
+ 2,3-DPG, pCO_2 = 85 mmHg	11.2	1.3	7.5	1.9
Hb S + 2,3-DPG	11.6		8.6	
+ 2,3-DPG, pCO_2 = 41 mmHg	10.1	1.5	7.0	1.6
+ 2,3-DPG, pCO_2 = 85 mmHg	9.5	2.1	4.9	3.7

the α chains (16), while 2,3-DPG binds in the central cavity of the hemoglobin tetramer involving several residues including the α amino groups of Val 1β (19). Therefore, binding at the common β chain sites are competitive (16).

The present data demonstrate that there are indeed abnormalities in the functional properties of HbS in dilute solutions, which cannot be attributed to polymerization. The slightly decreased extent to which the oxygen affinity of HbS is lowered by 2,3-DPG indicates that it binds less strongly to HbS than to HbA. Consistently, the finding that a high concentration of CO_2 counteracts the effect of 2,3-DPG with HbS much more than with HbA indicates that CO_2 displaces 2,3-DPG from the β^S amino-termini more readily than from the normal β chains. These differences must reflect conformational alterations in the 2,3-DPG binding regions of the β^S chains, which result from the β6 Glu → Val substitution.

With the present data it is not possible to exclude the possibility that in the higher pH regions at which this difference is largest (pH 7.3), CO_2 might bind to β^S chains more readily than to β^A chains even in the absence of 2,3-DPG. This latter possibility requires further experimental testing.

The failure to find differences in the conformation of Hb S and Hb A by X-ray crystallography thus far may be attributed to the levels of resolution achieved and to the poor definition of the β chain NH_2 - terminal regions by this method, particularly at β2His. We predict that higher resolution studies in the future will demonstrate small conformational alterations in HbS, involving the initial portion of NH_2-terminal sequence, either at β2His, the beginning of the A helix, or both.

ACKNOWLEDGEMENT - This work was supported by Grants HL 21016-02 and AM 12597 from the NIH and a Grant-in-Aid (77-1065) from the American Heart Association. We are indebted to Joyce Johnson and Vera Neske for competent technical assistance.

REFERENCES

1. May, A. and Huehns, E.R. Haematol. Bluttranfus. 10:279-283, 1972.
2. Seakins, M., Gibbs, W.N., Milner, D.F. and Bertles, J.F. J. Clin. Invest. 52:422-432, 1973.
3. Bookchin, R.M., Ueda, Y., Nagel, R.L. and Landau, L.C. in Molecular and Clinical Aspects of Hemoglobin Abnormalities. Ed. W. Caughey. Acad. Press, N. Y. 1978.
4. Charache, S., Grisolia, S. Fiedler, A.J. and Hellegers, A.E. J. Clin. Invest. 49:806-812, 1970.
5. Bunn, H.F. and Briehl, R.W. J. Clin. Invest. 49:1088-1092, 1970.
6. Noble, R. in Molecular and Clinical Aspects of Hemoglobin Abnormalities, Caughey, W.S. ed., Academic Press, New York. 1978.
7. Wishner, B.C., Ward, K.B., Lattman, E.E., and Love, W.E. J. Mol. Biol. 98:179-186, 1975.
8. Elbaum, D., Harrington, J. and Nagel, R.L. in Erythrocyte Structure and Function, Brewer, G.J. ed., Alan R. Liss, Inc., New York, pp. 311-324.
9. Fronticelli, C. J. Biol. Chem. 253:2288-2291, 1978.
10. Ho, Ch. and Russu, I. in Molecular and Clinical Aspects of Hemoglobin Abnormalities, Caughey, W.S. ed., Academic Press, New York. 1978.
11. Chanutin, A. and Curnish, R.R. Arch. Biochem. Biophys. 121:96-99, 1967.
12. Benesch, R. and R.E. Benesch. Biochem. Biophys. Res. Commun. 26:162-164, 1969.
13. Caldwell, P.R.B., Nagel, R.L. J. Mol. Biol. 74:605, 1973.
14. Drabkin, D.L. J. Biol. Chem. 164:703-723, 1946.
15. Rose, Z.B. and Liebowitz, J. Anal. Biochem. 35:177-180, 1970.
16. Kilmartin, J.V. and Rossi-Bernardi, L. Physiol. Rev. 53:836-890, 1973.
17. Mathews, J.B., Morrow, J.S., Wittebort, R.J. and Gurd, F.R.N. J. Biol. Chem. 252:2234-2244, 1977.
18. Arnone, A.V. Nature. 247:143-145, 1974.
19. Arnone, A.V. Nature. 237:146-148, 1972.