Proc. Nat. Acad. Sci. USA
Vol. 69, No. 8, pp. 2122–2124, August 1972

Coboglobins: Heterotropic Linkage and the Existence of a Quaternary Structure Change Upon Oxygenation of Cobaltohemoglobin

(Bohr effect/2,3-diphosphoglycerate)

G. C. HSU, C. A. SPILBURG, C. BULL, AND B. M. HOFFMAN

Department of Chemistry, Northwestern University, Evanston, Illinois 60201

Communicated by Myron L. Bender, May 31, 1972

ABSTRACT **Cobaltohemoglobin prepared from horse hemoglobin retains the full heterotropic linkage properties of the normal iron hemoglobin, including both the Bohr and phosphate effects. From this result, and the known connection between heterotropic linkage properties and hemoglobin conformational change, it is concluded that cobaltohemoglobin undergoes a quaternary structure transition upon oxygenation. Thus, the stereochemical changes that occur upon oxygen binding to cobalt, as well as to iron, must be compatible with the mechanism triggering such a transition.**

Cobalt-substituted hemoglobins and myoglobins can reversibly bind molecular oxygen, and a study of their structure and function yields information about the mechanisms of function of natural oxygen carriers (1). The influence of an apoprotein upon the reactivity of a prosthetic group is observed directly by comparison of oxygen binding to the dimethyl ester of cobaltous protophyrin IX in solution with binding to the cobaltous prophyrin (free acid) incorporated into apomyoglobin (2, 3). Investigations of the role of the prosthetic group in protein-mediated allosteric interactions have been initiated by examination of the linkage properties of cobaltohemoglobin (CoHb) (2).

The present work demonstrates heterotropic linkage between oxygen binding by CoHb and the binding of organic phosphates (phosphate effect), and extends previous observations of heterotropic linkage between oxygen and proton binding (Bohr effect) and of linkage between the four oxygen-binding sites in a CoHb molecule (heme–heme interactions) (2). These linkage properties are compared with those of Hb. From such a comparison, we conclude that CoHb undergoes a quaternary structure change upon oxygenation.

MATERIALS AND METHODS

Horse hemoglobin was prepared from fresh whole blood (Grand Island Biological Co. Madison, Wis.) and stripped of P_2Glr (2,3-diphosphoglycerate) as described (4). Globin was prepared by the method of Rossi-Fanelli *et al.* (5), with the addition of 1 mM dithiothreitol to all globin solutions. Reconstituted Hb was made by the method of Antonini *et al.* (6). CoHb was prepared as described (2).

Met-CoHb (cobaltihemoglobin) was prepared by oxidation of CoHb with excess $K_3Fe(CN)_6$ overnight at room temperature, followed by gel filtration on a column (1.5 × 20 cm) of Sephadex G-25 at 4°. Bis-Tris·HCl [bis-(2-hydroxyethylamino]-tris-[(hydroxymethyl)methane] was used for oxygenation studies at pH values less than 7.4; Tris·HCl buffer was used at higher pH values. These buffers show negligible interference with the binding of organic phosphates to Hb (7). P_2Glr was prepared according to Benesch *et al.* (7), and titrated with dilute NaOH to the pH at which it was to be used in oxygenation measurements.

Optical spectra were recorded on a Beckman Acta III recording spectrophotometer. Ligation or oxidation of CoHb was observed by following spectral changes in the Soret band (see below). Oxygenation of Hb was followed at either the Soret or visible bands. A tonometer of standard design and 1-cm path length was used (8). CoHb solutions were 1–2 μM (tetramer).

RESULTS

Spectral properties

The porphyrin α–β bands for horse CoHb appear as a maximum at 555 nm, with a shoulder at about 515 nm. The appearance of this shoulder is extremely sensitive to the presence of small amounts of met-CoHb. The Soret peak is at 402 nm. Exposure to oxygen forms oxy-CoHb, with the α–β band replaced by new peaks at 539 and 570 nm, and the Soret peak shifted to 421 nm. These spectral changes caused by oxygenation of CoHb are reversible upon evacuation, as are similar changes of Hb, and were used to measure Y, the fractional oxygen saturation. Results reported here were obtained from measurements of the Soret band at 4°. In the course of oxygenation of CoHb, an isosbestic point could be observed at 412 nm, indicating the absence of oxidation or denaturation (Fig. 1). The spectrum of met-CoHb in the 530- to 580-nm region is very similar to that of oxy-CoHb; the Soret maximum of met-CoHb is at 426 nm.

TABLE 1. *Effect of pH on oxygen binding to CoHb and Hb*

		log $P_{0.5}$		
		pH 7.3*	pH 9.4†	Δlog $P_{0.5}$
CoHb	[P_2Glr] = 0.2 mM	2.04	0.96	1.08
	[P_2Glr] = 0	1.56	0.96	0.60
Hb	[P_2Glr] = 0.2 mM	0.63	−0.47	1.10
	[P_2Glr] = 0	0.21	−0.47	0.68

* 0.05 M Bis-Tris; T = 4°.
† 0.05 M Tris; T = 4°.

Abbreviations: CoHb, cobaltohemoglobin; met-CoHb, cobaltihemoglobin; P_2Glr, 2,3-diphosphoglycerate; Y, fractional oxygenation; $P_{0.5}$, oxygen pressure at Y = 0.5; *n*, slope of Hill plot [log (Y/1 − Y) against log P].

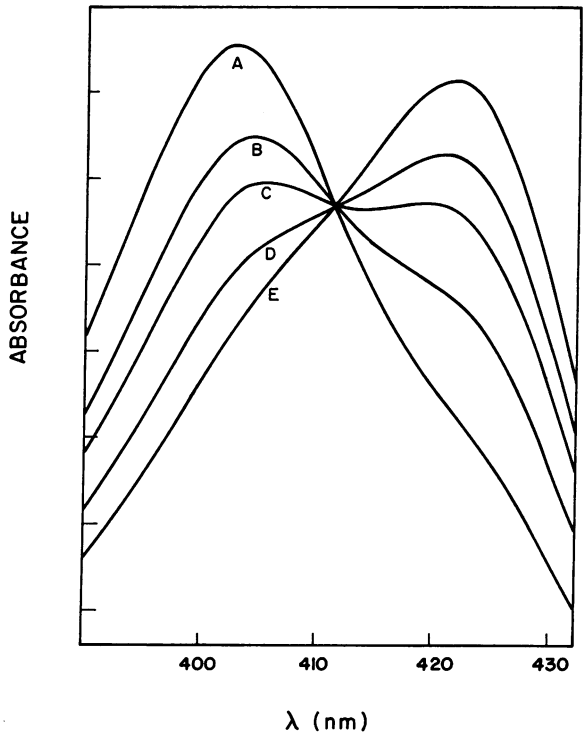

FIG. 1. Soret spectrum of horse CoHb at 20° in 0.05 M Tris buffer (pH 8.8), and Y between 0 (*A*) and 0.75 (*E*).

Linkage Properties of CoHb

Fig. 2 gives representative oxygen-binding curves for horse Hb and CoHb. For both proteins $P_{0.5}$, the oxygen pressure at which Y = 0.5, is *relatively* high at pH 9.2, and independent of the presence of P_2Glr. If the pH is lowered to 7.2 in the absence of P_2Glr, $P_{0.5}$ is increased; the addition of 0.2 mM P_2Glr causes a further increase. Doubling the P_2Glr concentration at pH 7.2 has no significant effect on $P_{0.5}$.

Although the shapes of the oxygenation curves of CoHb and of Hb are different, these shapes do not change measurably with either pH or P_2Glr concentration (Fig. 2).* In such a case, the Bohr and phosphate effects for each protein can be obtained by measurement of the changes in its log $P_{0.5}$ with pH and phosphate concentration, respectively (9). Table 1 gives the values of log $P_{0.5}$ for CoHb and Hb measured at pH 7.3 and 9.4 in the absence of phosphate and in the presence of 0.2 mM P_2Glr. Although the affinity of CoHb for oxygen is about 25 times lower than that of Hb (higher $P_{0.5}$), the changes in log $P_{0.5}$ with pH for both the cobalt and iron proteins are essentially the same. Thus, CoHb exhibits a full alkaline Bohr effect, and substitution of cobalt for iron does not inhibit the heterotropic linkage between oxygen and proton binding.

Table 2 gives the values of log $P_{0.5}$ for CoHb and Hb at pH 7.3 in the presence and absence of P_2Glr. The change in log $P_{0.5}$ caused by the binding of P_2Glr is essentially the same for the two proteins; thus, the linkage between oxygen and phosphate binding is also retained in full upon substitution of cobalt for iron. Although P_2Glr is the normal effector molecule

* This apparent shape invariance is being examined in experiments with higher precision.

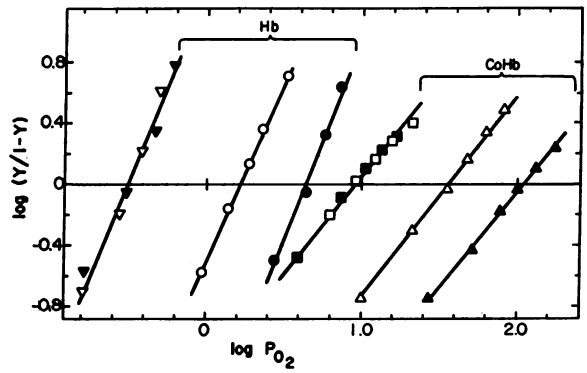

FIG. 2. The effect of pH and P_2Glr on oxygen binding to Hb and CoHb. *Filled symbols* indicate 0.2 mM P_2Glr, *open symbols*, the absence of phosphate: (ΔA) and (O, ●), 0.05 M Bis–Tris(pH 7.3), T = 4°; (□■) and (▽▼), 0.05 M Tris (pH 9.4), T = 4°. Oxygen pressure (P_{O_2}) is measured in mm of Hg.

in mammals *in vivo*, inositol hexaphosphate can also bind to mammalian hemoglobins and alter their oxygen affinity (10). At pH 7.3, the presence of 0.2 mM inositol hexaphosphate causes the same increase in log $P_{0.5}$ as does P_2Glr (Table 2).

The binding curves of CoHb do not indicate a high degree of homotropic linkage. Hill plots [log (Y/1 − Y) against log P] are straight lines, with slopes that give Hill's constant, *n*, of about 1.4; Hill plots for horse Hb give *n* about 2.7 (Fig. 2). Thus, the homotropic linkage, or heme–heme interaction exhibited by these samples of CoHb is smaller than that of Hb. Hb reconstituted from the same globin preparations generally showed *n* between 2.3 and 2.6; thus, the low value of *n* for CoHb is not caused by artifactual changes in the globin. As discussed previously, the Hill's constant is much more sensitive to sample heterogeneity, the presence of met-CoHb, and other factors than is the value of $P_{0.5}$ (2). Thus, the values of *n* for CoHb reported here can only be considered to be lower limits.

Met-CoHb

In observations on the ligation and redox properties of CoHb, we found that the spectrum of met-CoHb was unaffected by the presence or absence of oxygen. Anaerobic dithionite reduction of freshly prepared met-CoHb at room temperature for 12–24 hr regenerated the spectrum of CoHb. Dithionite could be removed by Sephadex G-25 gel filtration at 4°. The protein recovered was primarily oxy-CoHb, as shown by its ability to be reversibly deoxygenated.

DISCUSSION

The ligated and unligated forms of Hb differ in both tertiary and quaternary structure, and the linkage effects exhibited

TABLE 2. *Effect of phosphate on oxygen binding to CoHb and Hb at pH 7.3**

	log $P_{0.5}$		
	[P_2Glr] = 0.2 mM	[P_2Glr] = 0	Δlog $P_{0.5}$
CoHb	2.04	1.56	0.48
Hb	0.63	0.21	0.42

* 0.05 M Bis–Tris; T = 4°.

by Hb arise from the reversible transition between these two forms (11). X-ray crystallographic studies of hemoglobin and more than a dozen mutant and chemically modified hemoglobins have shown that proteins exhibiting substantial heterotropic linkage effects also exhibit a ligation-induced transition of their quaternary structure. Where such a transition is found to be absent, heterotropic linkage is also absent (11–13). On this basis, it was concluded that a change in quaternary structure is both necessary and sufficient for the occurrence of heterotropic-linkage effects (11). Thus, CoHb, which exhibits both Bohr and phosphate effects, must undergo a quaternary structure transition upon oxygenation. This conclusion is unaffected by the decreased heme–heme interaction ($n \sim 1.4$) observed with the horse CoHb studied here; ligand binding can be accompanied by heterotropic linkage and the normal quaternary structure transition, even though the homotropic linkage or heme–heme interaction is weak or absent, as is shown by studies with hemoglobins Kansas (14) and Chesapeake (15).

Moreover, the stereochemical changes that occur as the five-coordinate, low-spin Co(II) protoprophyrin IX of CoHb (1) binds oxygen must be compatible with the mechanism that triggers the postulated quaternary structure transition caused by oxygenation. It has been suggested that this mechanism in hemoglobin involves a motion of the proximal histidine upon oxygenation (11, 16). The iron atom is believed to be about 0.75 Å out of the mean porphyrin plane in deoxy-Hb, and to move into the plane upon oxygenation. This motion, coupled with a possible decrease in iron–imidazole distance of 0–0.2 Å, could shift the proximal histidine as much as 0.75–0.95 Å. This shift is considered to force the protein conformational changes.

Electron paramagnetic resonance data for Co(Hb) (1) can be used to show that the low-spin Co(II) does not lie coplanar with the porphyrin ring (Hoffman, B. M., manuscript in preparation). As in Hb, ligation of CoHb should move the metal into the plane. However, low-spin Co(II) exhibits a smaller covalent radius than does the high-spin Fe(II) of Hb, and in deoxy-CoHb should lie no more than 0.1–0.3 Å out of the porphyrin plane; thus, it would appear that the above mechanism can only apply to CoHb if ligation not only moves metal, but also causes a large decrease in the cobalt–imidazole bond distance. The possibility of such a decrease has been suggested and is subject to crystallographic confirmation in model compounds (Hoard J. L., & Scheidt W. R., manuscript in preparation). Thus, retention of the Hb linkage properties upon cobalt substitution places additional constraints upon any proposed allosteric mechanism.

We are indebted to Prof. E. Margoliash for assistance in preparing this manuscript. B.M.H. acknowledges a stimulating conversation with Profs. M. F. Perutz and R. W. Scheidt. This work was supported by the National Institutes of Health under Grant HL 13531 and by the Advanced Research Projects Agency through the Center for Materials Research at Northwestern University. B.M.H. is an Alfred P. Sloan Foundation Fellow. A preliminary account of this work was presented at the FASEB meeting, Atlantic City, N.J., April 10–14, 1972.

1. Hoffman, B. M. & Petering, D. H. (1970) "Coboglobins: Oxygen-Carrying Cobalt-Reconstituted Hemoglobin and Myoglobin," *Proc. Nat. Acad. Sci. USA* **67**, 637–643.
2. Hoffman, B. M., Spilburg, C. A. & Petering, D. H. (1971) "Coboglobins: Cobalt Substitution and the Nature of the Prosthetic Group-Apoprotein Interaction in Hemoglobin and Myoglobin, "*Cold Spring Harbor Symp. Quart. Biol.* **36**, 343–348.
3. Spilburg, C. A., Hoffman, B. M. & Petering, D. H. (1972) "Coboglobins: Influence of Apoprotein on Oxygen Binding to Cobaltomyoglobin," *J. Biol. Chem.* in press.
4. Benesch, R., Benesch, R. E. & Yu, C. I. (1968) "Reciprocal Binding of Oxygen and Diphosphoglycerate by Human Hemoglobin," *Proc. Nat. Acad. Sci. USA*, **59**, 526–532.
5. Rossi-Fanelli, A., Antonini, E. & Caputo, A. (1958) "Studies on the Structure of Hemoglobin I. Physicochemical Properties of Human Globin," *Biochim. Biophys. Acta* **30**, 608–615.
6. Antonini, E. (1964) "Studies on the Structure of Hemoglobin III. Physicochemical Properties of Reconstituted Hemoglobins," *Biochim. Biophys. Acta* **79**, 284–292.
7. Benesch, R. E., Benesch, R. & Yu, C. I. (1969) "The Oxygenation of Hemoglobin in the Presence of 2,3-Diphosphoglycerate. Effect of Temperature, pH, Ionic Strength, and Hemoglobin Concentration," *Biochemistry* **3**, 2567–2571.
8. Spoek, G., Bakker, H. & Wolvekamp, H. (1964) "Experiments on the Hemocyanin-Oxygen Equilibrium of the Blood of the Edible Snail," *Comp. Biochem. Physiol.* **12**, 209–214.
9. (*a*) Wyman, J. (1948) "Heme Proteins," *Advan. Protein Chem.* **4**, 407–484; (*b*) Wyman, J. (1964) "Linked Functions and Reciprocal Effects in Hemoglobin: A Second Look," *Advan. Protein Chem.* **19**, 224–286.
10. Benesch, R. & Benesch, R. E. (1969) "Intracellular Organic Phosphates as Regulators of Oxygen Release by Hemoglobin," *Nature* **221**, 618–621.
11. Perutz, M. F. & TenEyck, L. F. (1971) "Stereochemistry of Cooperative Effects in Hemoglobin, "*Cold Spring Harbor Symp. Quant. Biol.* **36**, 295–310.
12. Kilmartin, J. V. & Hewitt, J. A. (1971) "The Effect of Removal of C-Terminal Residues on Cooperative Interactions in Hemoglobin," *Cold Spring Harbor Symp. Quant. Biol.* **36**, 311–314.
13. Greer, J. (1971) "Three-Dimensional Structure of Abnormal Mutant Human Hemoglobins," *Cold Spring Harbor Symp. Quant. Biol.* **36**, 315–323.
14. Greer, J. (1972) "Three-Dimensional Structure of Abnormal Human Hemoglobin Kansas and Richmond," *J. Mol. Biol.* **59**, 99.
15. Greer, J. (1972) "Three-Dimensional Structure of Abnormal Human Hemoglobins Chesapeake and J. Capetown," *J. Mol. Biol.* **62**, 241.
16. Hoard, J. L. (1966) in "Stereochemistry of Porphyrin," *Hemes and Hemoproteins*, eds. Chance, B., Estabrook, R. W. & Yonetani, T. (Academic Press, New York), pp. 9–25.