# HORSE HEMOGLOBINS CONTAINING DEUTERO- AND MESOHEME: FUNCTIONAL AND STRUCTURAL STUDIES

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Received December 16,1974

## SUMMARY

Preliminary equilibrium, kinetic, and crystallographic investigations are reported for horse hemoglobin reconstituted with deutero- and mesoheme. Both modified hemoglobins exhibit lower n values, higher oxygen affinities, and more rapid rates of CO binding than native horse hemoglobin. Initial crystallographic results indicate that these derivatives are isomorphous with native horse metHb, thus allowing difference Fourier methods to be applied to structure determination.

The structural model of hemoglobin function recently set forth by Perutz et al. (1-3) emphasizes the importance of "tension at the hemes" as a major cause of the low ligand affinity of the deoxy quaternary structure and the manifestation of heme-heme interaction. This model necessitates porphyrin-globin interactions which will anchor the porphyrin group relative to movement of the central iron atom (4); such van der Waals interactions have been identified by Perutz et al. (5,6) from crystallographic studies of horse hemoglobin. We have perturbed these interactions by reconstituting horse hemoglobin with proto-, deutero- and mesoheme, in which the 2- and 4- substituents of the heme are vinyl, hydrogen and ethyl groups, respectively. The use of horse globin throughout enables structural studes to be performed in parallel with the functional studies; previous investigators (7,12,18-20) used human globin.

## MATERIALS AND METHODS

Globin was prepared from horse hemoglobin by the procedure of Rossi-Fanelli et al. (7). Deuterohemin chloride and mesohemin chloride were prepared according

to previously published procedures (8,9). The identity and purity of these modified hemes were determined chromatographically (10) and by their pyridine hemochrome spectra (11). The hemes were recombined with horse globin according to Antonini et al. (12). Samples of the reconstituted hemoglobins for equilibrium and kinetic experiments were reduced with an excess of sodium dithionite and applied to an anaerobic Sephadex G-25 column equilibrated with the appropriate buffer. Native horse hemoglobin which had been "stripped" according to the procedure of Benesch et al. (13) served as a control. The reconstituted hemoglobins were functionally homogeneous as judged by kinetic tests (see below); no further purification was considered necessary.

Oxygen equilibrium curves were determined by the method of Rossi-Fanelli and Antonini (14) at 20°C. Kinetic analyses were performed using the stopped flow apparatus described by Gibson and DeSa (15,16). Crystals of the met form of horse deuteroHb[1] and horse mesoHb were grown according to Perutz (17). Individual crystals were mounted in capillaries and x-ray photographs were recorded with a Supper precession camera using Cu Kα radiation.

## RESULTS

Oxygen Equilibria. The oxygen equilibrium curves for native horse Hb and

Table 1. Oxygen equilibria of reconstituted hemoglobins. The values of the Hill coefficient n and oxygen pressure at half saturation ($P_{0.5}$) were obtained by the method of Rossi-Fanelli and Antonini (14). The units of $P_{0.5}$ are mm Hg. Errors in n and $P_{0.5}$ are estimated to be less than ± 10%.

| | 0.05 M bis-tris pH 7.0 | | 0.10 M phosphate pH 7.0 | |
|---|---|---|---|---|
| Hemoglobin | n | $P_{0.5}$ | n | $P_{0.5}$ |
| Native horse Hb | 2.7 | 2.6 | 2.8 | 11.4 |
| Horse protoHb | 3.0 | 2.4 | 2.6 | 10.0 |
| Horse deuteroHb | 2.4 | 1.2 | 2.1 | 4.6 |
| Horse mesoHb | 1.8 | 0.6 | 1.3 | 1.0 |

[1]Abbreviations: Hb, hemoglobin; metHb, methemoglobin; protoHb, deuteroHb, and mesoHb, horse globin which has been recombined with proto-, deutero- and meso-heme, respectively; bis-tris, 2-2-bishydroxymethyl- 2,2',2"-nitrilotriethanol; IHP, inositol hexaphosphate.

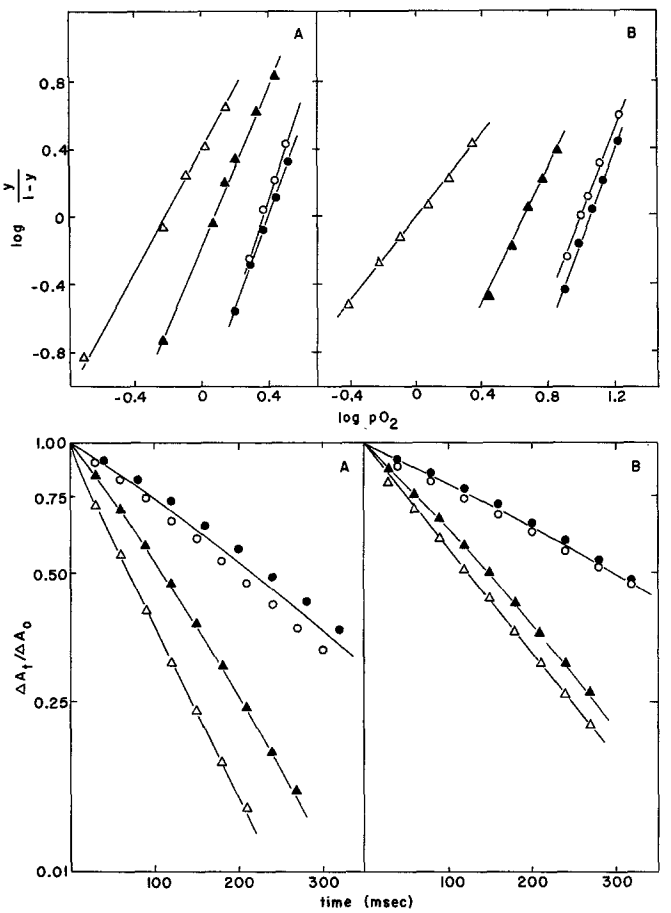


Figure 1. (Top) Oxygen equilibrium curves for reconstituted horse hemoglobins. All curves were determined at 20°C with hemoglobin concentrations of 0.25-0.30 mM. Conditions were:

A, 0.05 M bis-tris, pH 7.0; B, 0.10 M phosphate, pH 7.0. • native horse Hb, o horse protoHb, ▲ horse deuteroHb, Δ horse mesoHb.

Figure 2. (Bottom) Normalized time courses for the reaction of reconstituted horse deoxyhemoglobins with CO. Reactions were carried out in 0.05 M bis-tris, pH 6.0 at 20°C using a 2 cm path length cell. The reactions for native horse and protoHb were followed at 432 nm; the reactions for deuteroHb and mesoHb were observed at 421 nm and 422 nm, respectively. Concentrations after mixing were 2.5 μM hemoglobin and 23 μM CO.

A, reactions of stripped hemoglobins; B, same reactions with 25 μM IHP added. • native horse Hb, o horse protoHb, ▲ horse deuteroHb, Δ horse mesoHb.

reconstituted protoHb are identical (Fig. 1 and Table 1). Horse deuteroHb exhibits n values of 2.4 and 2.1 in bis-tris and phosphate, respectively, which are somewhat higher than the values for human deuteroHb (18,19); horse deuteroHb

also shows a higher oxygen affinity than the corresponding human derivative. However, horse and human globin contain sequence differences which are sufficient to preclude direct comparisons. Horse mesoHb exhibits very low n values and high oxygen affinity in both bis-tris and phosphate, in qualitative agreement with the data for human mesoHb (18,19).

Carbon Monoxide Binding. The binding of CO to the reconstituted horse deoxyhemoglobins was studied at pH 6 in bis-tris. Figure 2 shows a comparison of the normalized time courses for the CO binding reaction in the presence and absence of IHP. The curves demonstrate that horse globin which has been reconstituted with protoheme yields a product which is kinetically homogeneous and identical with native horse Hb; deutero- and mesoHb exhibit rates of CO binding which are two to three times faster than for native Hb. The time courses for native, proto-, and deuteroHb are all autocatalytic, but the time course for mesoHb is pseudo first order, in agreement with earlier observations on human deutero- and mesoHb (20). IHP addition slows the overall CO binding reaction of each derivative by about the same factor, without substantially altering the shape of the progress curve. In contrast, the time course of CO binding to such hemoglobins as deoxyHb Hiroshima (21), des-his (146)β deoxyHb (22) and deoxyHb Chesapeake (23) is converted from a biphasic to an essentially monophasic form by the addition of IHP.

Crystallographic Results. A preliminary comparison of x-ray precession photographs of the met forms of deutero- and mesoHb with photographs of native horse metHb indicated that all three are isomorphous. A difference Fourier projection was calculated at 2.8 Å resolution from the hOℓ zone for both deutero- and mesoHb using the photographic amplitudes for the derivatives, and the amplitudes and signs obtained for native horse metHb (5). A portion of the difference Fourier projection of deuteroHb minus native metHb is shown in Figure 3, superimposed on the projected porphyrin skeletons of the α and β hemes, as obtained from the atomic coordinates (5). Four large negative peaks coincide with the projected positions of the 2- and 4-vinyl substituents of the α and β heme groups

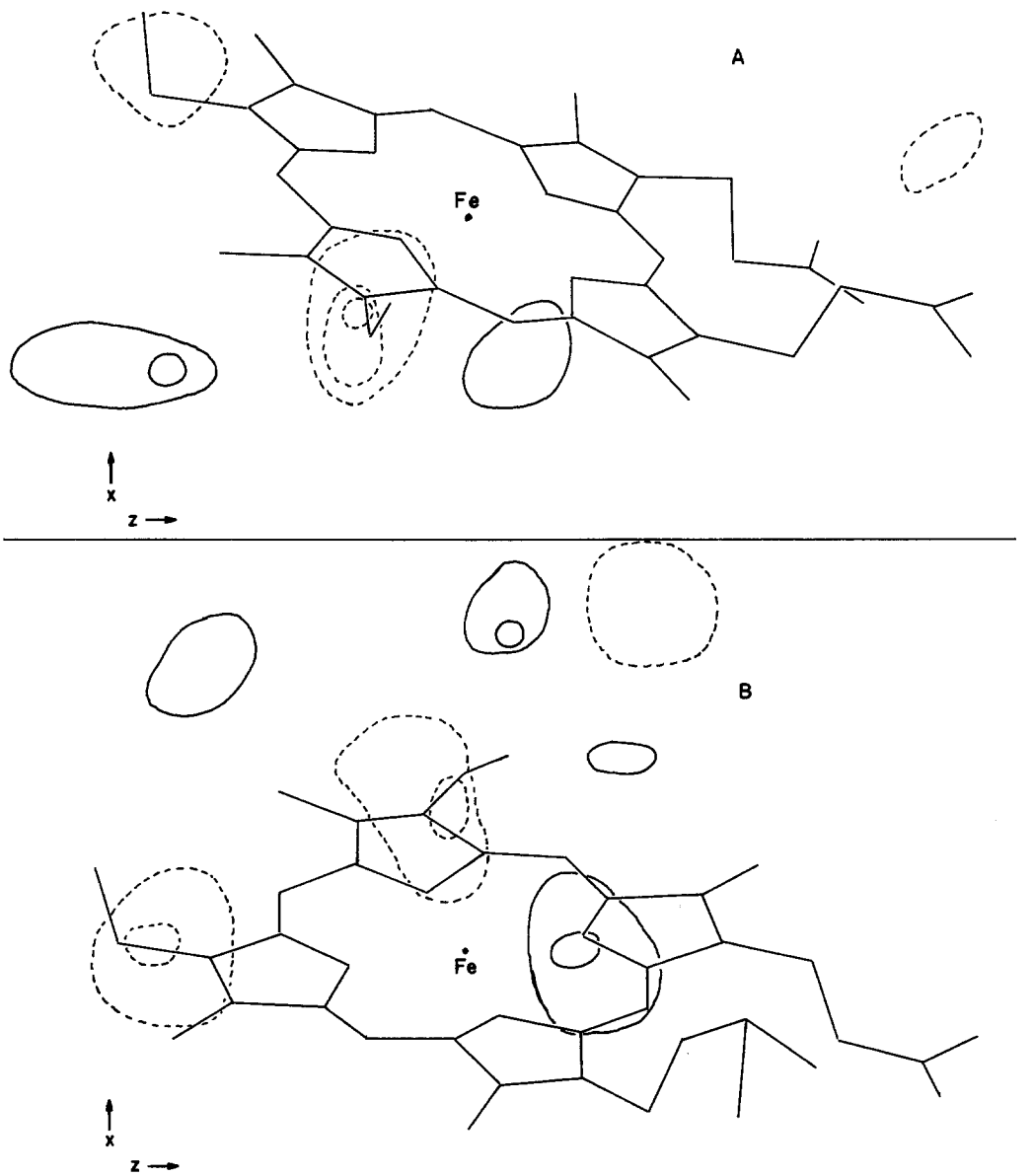


Figure 3. Difference Fourier projection of the met form of horse deuteroHb minus native horse metHb superimposed on the projected porphyrin skeleton of the α and β hemes. Solid contours indicate regions of positive electron density and broken contours represent areas of negative electron density.

A, α heme region; B, β heme region.

## DISCUSSION

These results indicate that reconstituted protoHb is functionally identical with native horse Hb. Hence, the alterations in properties found for deutero-

and mesoHb are a result of the modifications at the 2- and 4- positions of the heme groups, not of artifacts of the preparative procedure. The CO binding studies suggest that the principal effect of the modification is on the tertiary structure of the chains, rather than the quaternary structure as in Hb Chesapeake (23). In both equilibrium and kinetic properties, mesoHb exhibits more extreme behavior than does deuteroHb. Apparently, complete removal of the vinyl substituents produces less drastic alterations than reduction to ethyl groups. This suggests that the orientation of the substituents on the 2- and 4- positions may be more important than their chemical nature. The vinyl groups in Hb are expected to be coplanar with the tetrapyrrole ring, but the ethyl groups are likely to be considerably out of plane. Further, the substituents on the 2- and 4- positions may not be functionally equivalent (24). Three dimensional structure analysis will test these expectations, and provide a structural basis for the functional studies.

In principle, two modes of binding of deuteroheme to globin are possible: binding in the same orientation as protoheme, and binding upside down, in which the 2- and 4- substituents interact with the regions of the globin normally in contact with the 1- and 3-methyl substituents. If such were the case, one would expect negative peaks in the difference Fourier projection at the 1-, 2-, 3-, and 4- positions. The fact that negative peaks are only observed at the 2- and 4- substituents is consistent with deuteroheme being bound by the globin in the same relative orientation as protoheme.

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