THE OXIDATION STATE OF NEWLY SYNTHESIZED HEMOGLOBIN*

Herbert M. Schulman, J. Martinez-Medellin** and R. Sidloi

Lady Davis Institute for Medical Research
Jewish General Hospital
Montreal, Quebec, Canada

Received November 12,1973

SUMMARY The oxidation state of newly synthesized hemoglobin in reticulocytes was determined by analyzing the hemoglobin from pulse-labelled cells by isoelectric focusing in acrylamide gels. The newly synthesized material contained oxidized heme groups when either ^{14}C-leucine or ^{59}Fe was used for the radioactive pulse. With both isotopes the radioactive hemoglobin rapidly equilibrated with intracellular ferrohemoglobin during subsequent incubation of the cells in non-radioactive medium.

The results suggest that ferrihemoglobin is an intermediate in the biosynthesis of functional ferrohemoglobin in reticulocytes.

INTRODUCTION

Although much detailed information is available about the biosynthesis of globin chains and of heme in developing erythroid cells, there are still great gaps in our understanding of the final steps in the assembly of hemoglobin. Of particular interest is how heme which is synthesized in mitochondria (1) is transported to globin which is synthesized on polysomes in the cytosol (2). To date, no intracellular heme carrier has been identified (3).

The demonstration that ferri-hemes may undergo exchange between hemoglobin molecules (4) and the suggestion that heme from completed hemoglobin molecules may be incorporated into newly synthesized globin (5) prompted us to examine the oxidation state of newly synthesized hemoglobin for it is conceivable that at least some of the heme associated with newly synthesized globin may originate from pre-existing ferri-hemoglobin.

* Supported by a grant from the Medical Research Council of Canada.

** Permanent address: Facultad de Ciencias, Universidad Nacional Autonoma de Mexico.

METHODS

Reticulocytes from phlebotomized rabbits were purified on dextran gradients (6) and incubated as previously described (7).

Rabbit hemoglobin was purified by chromatography on CM-Sephadex (8). Hemoglobin was auto-oxidized by incubating sterile solutions of the purified protein in 0.01 M Tris-HCl buffer at pH 7.2 at room temperature for periods up to three months.

Rabbit transferrin was purified and labelled with ^{59}Fe by procedures published previously (9).

Ampholines of pH 7 to 8 were prepared by fractionating 8% solutions of pH 3 to 10 ampholine in an LKB 110 ml isoelectric focusing column and pooling the fractions of appropriate pH.

Isoelectric focusing in acrylamide gels was done according to the procedure of Bunn and Drysdale (10). At the conclusion of each run the gels were removed from the tubes under ice water and the bands of ferri- and ferro-hemoglobin were cut from the gels with a razor blade. Protein was eluted from the gels by repeated extrusion of the gel slices through 20 gauge hypodermic syringe needles into 0.01 M Tris-HCl buffer at pH 7.2. The suspensions were then centrifuged and the supernatants removed for spectrophotometry and measurement of radioactivity.

RESULTS

To determine the oxidation state of newly synthesized hemoglobin, reticulocytes were pulse-labelled with ^{14}C-leucine for 15 minutes, after which the cells were washed and then incubated in medium containing non-radioactive leucine. Samples of cells were taken after the pulse and at various intervals during the chase and their hemoglobin was subjected to isoelectric focusing in polyacrylamide gels under conditions which separate ferri- and ferro-hemoglobin (10) (see Figure 1). Figure 2 shows the ratio of specific activities of ferri- and ferro-hemoglobin after the fifteen minute pulse with ^{14}C-leucine and at various times during the cold-chase. Apparently newly synthesized globin is

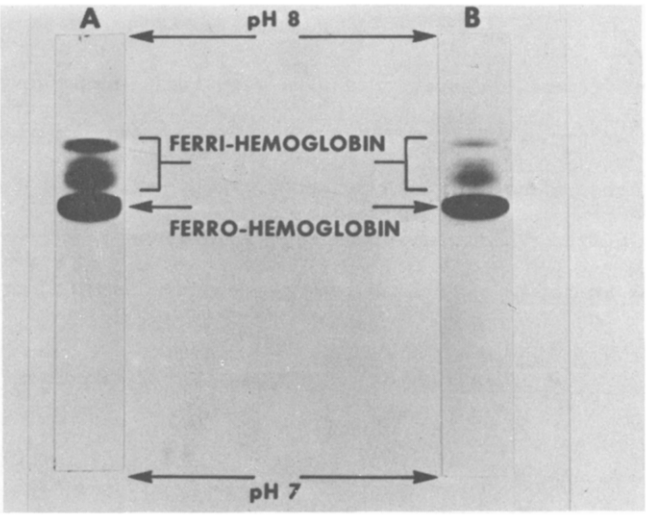

Figure 1: The separation of ferri- and ferro-hemoglobin by isoelectric focusing in acrylamide gels.

A: An artificial mixture of rabbit ferri- and ferro-hemoglobin.
B: Hemoglobin prepared from freshly lysed reticulocytes.
In both cases the proteins were precipitated with 10% TCA.

For the determination of protein concentration and radioactivity in the pulse labelling experiments the gels were cut between the ferro-hemoglobin band and the lowest band of ferri-hemoglobin and the protein was eluted as described in Methods. No attempt was made to separate the different bands of ferri-hemoglobin (which are composed of hemoglobin with different numbers of oxidized hemes (10)) because of the rapid diffusion of the proteins in 4% acrylamide.

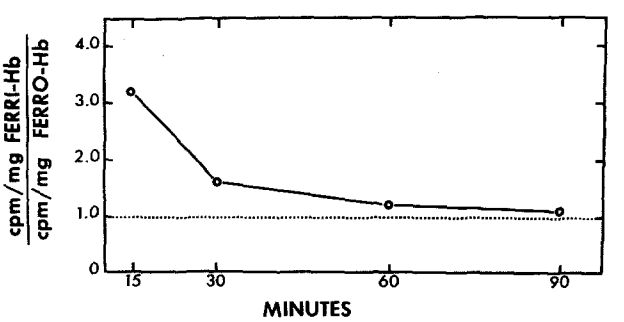

Figure 2: The ratio of specific activities of reticulocyte ferri- and ferro-hemoglobin following a 15 minute pulse with ^{14}C-leucine.

associated with oxidized heme which, during the chase, equilibrates with the bulk of the intracellular ferrohemoglobin through the action of the ferrihemoglobin reductase system in the cells.

Essentially the same experiment as that just described was performed using ^{59}Fe-labelled transferrin instead of ^{14}C-leucine for the radioactive pulse. As can be seen in Figure 3 a similar result was obtained; the radio-

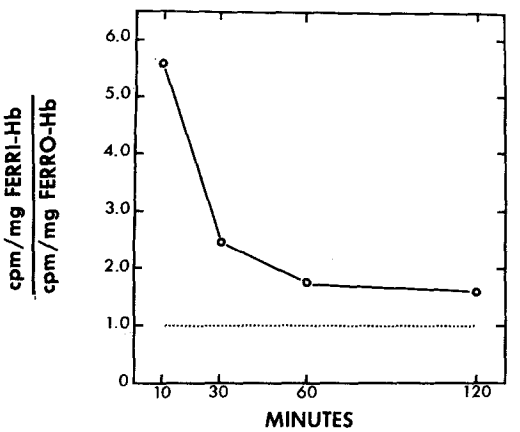

Figure 3: The ratio of specific activities of reticulocyte ferri- and ferrohemoglobin following a 10 minute pulse with ^{59}Fe.

activity was initially associated with hemoglobin in the oxidized or partially oxidized state and during the chase equilibrated with the cells' ferro-hemoglobin.

That newly synthesized heme becomes associated with oxidized or partially oxidized hemoglobin was confirmed by incubating reticulocytes with ^{59}Fe-labelled transferrin for ten minutes and determining the ratio of specific activities of ferri- and ferrohemoglobin in control cell extracts and those which had been incubated with NADH and phenazinemethosulfate to reduce ferrihemoglobin (11). The results shown in Table I would appear to confirm the conclusion drawn from the previous experiment.

TABLE I

	cpm/mg Hb		
	Ferri-Hb	Ferro-Hb	Ferri-Hb/Ferro-Hb
Control	17.6	5.3	3.3
+NADH;PMS*	7.4	6.7	1.1

* phenazine methosulfate

DISCUSSION

To explore the possibility that intracellular oxidized or partially oxidized hemoglobin may act as heme donors for newly synthesized globin the oxidation state of newly synthesized hemoglobin was determined using ^{14}C-leucine and ^{59}Fe as radioactive markers. In both cases, that is whether the radioactivity was present in newly synthesized globin chains or newly synthesized heme groups, the hemoglobin synthesized was in the oxidized or partially oxidized state.

The results of the experiments with ^{14}C-leucine are consistent with the idea that ferriheme from pre-existing hemoglobin may become associated with newly synthesized globin. However, the same result would be obtained if both newly synthesized heme and globin are present in the same molecule and the newly synthesized heme is in the oxidized state.

The results with ^{59}Fe can also be interpreted in two ways. Either newly synthesized heme is in the reduced state but becomes associated with heme-deficient hemoglobin containing ferri-heme; or, again newly synthesized heme is in the oxidized state.

At present it is difficult to distinguish between these possibilities, particularly since we were unable to isolate the separate bands of fully and partially oxidized hemoglobin, however, we are trying to overcome this techni-

cal difficulty. It will also be of interest to prepare heme-deficient hemoglobins (12) and examine the behavior of these molecules on isoelectric focusing gels.

If evidence is obtained which shows that newly synthesized heme is in the oxidized state it may be difficult to reconcile this with the accepted assays for protoheme ferrolyase (the mitochondrial enzyme which inserts iron into protoporphyrin), which utilize Fe(II) as substrate rather than Fe(III) (13). In this connection, it is interesting to note that the iron in transferrin is present as Fe(III) and some workers have concluded that the cells' mechanism for the removal of iron from transferrin does not involve a reduction of the iron (14). It should also be pointed out that in reticulocytes metabolising normally, it has been difficult to identify an iron-containing intermediate in the heme synthetic pathway other than transferrin-bound iron (9). These observations result in a scheme in which transferrin-bound iron would undergo two cycles of oxidation and reduction within the cells during the formation of functional ferrohemoglobin.

The finding that newly synthesized hemoglobin contains oxidized heme may explain the chromatographic heterogeneity which has been reported for newly synthesized hemoglobin from reticulocytes when compared to the bulk of the intracellular hemoglobin. Both newly synthesized hemoglobin (15) and ferrihemoglobin (16) elute from CM-Sephadex at a slightly higher pH than the bulk of intracellular ferrohemoglobin.

In conclusion, although the mechanism remains unknown, it would appear that ferrihemoglobin is a biosynthetic intermediate in the formation of functional ferrohemoglobin in reticulocytes.

REFERENCES

1. Jones, M.S. and Jones, O.T.G., Biochem. J. 113, 507 (1969).
2. Warner, J.R., Knopf, P. and Rich, A., Proc. Natl. Acad. Sci. U.S.A., 49, 122 (1963).
3. Yoda, B. and Israels, L.G., Can. J. Biochem. 50, 633 (1972).
4. Bunn, H.F. and Jandl, J.H., J. Biol. Chem. 243, 465 (1968).

5. Schulman, H.M., Martinez-Medellin, J. and Sidloi, R., Eur. J. Biochem. 37, 178 (1973).
6. Schulman, H.M., Biochim. Biophys. Acta 148, 251 (1967).
7. Schulman, H.M., Biochim. Biophys. Acta 155, 253 (1968).
8. Winterhalter, K.H. and Huehns, E.R., J. Biol. Chem. 239, 3699 (1964).
9. Martinez-Medellin, J. and Schulman, H.M., Biochim. Biophys. Acta 264, 272 (1972).
10. Bunn, H.F. and Drysdale, J.W., Biochim. Biophys. Acta 229, 51 (1971).
11. Kajita, A., Noguchi, K. and Shukuya, R. Biochem. Biophys. Res. Comm. 39, 1199 (1970).
12. Winterhalter, K.H. and Deranleau, D.A., Biochem. 6, 3136 (1967).
13. Barnes, R. and Jones, O.T.G., Biochim. Biophys. Acta 304, 304 (1973).
14. Aisen, P. and Leibman, A., Biochim. Biophys. Acta 304, 797 (1973).
15. Zucker, W.V. and Schulman, H.M., Biochim. Biophys. Acta 138, 400 (1967).
16. Ford, W.H. and Aisnworth, S., Biochim. Biophys. Acta 160, 1 (1968).