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FORMATION *IN VITRO* OF HAEMOGLOBIN AND MYOGLOBIN FROM IRON, PROTOPORPHYRIN AND GLOBIN IN THE PRESENCE OF AN IRON-CHELATING ENZYME

YOSHIMASA YONEYAMA, HARUMI OHYAMA, YOSHIKI SUGITA AND HARUHISA YOSHIKAWA

Department of Physiological Chemistry and Nutrition, Faculty of Medicine, University of Tokyo, Tokyo (Japan)

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SUMMARY

1. The formation of haemoglobin has been demonstrated in the presence of iron, protoporphyrin, iron-chelating enzyme and apohaemoglobin from duck erythrocytes. Chromatographic as well as spectrophotometric evidence is presented for this.

2. Apohaemoglobin is effective when added to the incubation mixture either before, or after, the incubation.

3. Spectrophotometric evidence of the formation of myoglobin, has been obtained in the same way using apomyoglobin from the myoglobin of horse heart.

4. The presence of apohaemoglobin or of apomyoglobin inhibits the rate of haem formation.

5. The mechanism of the formation of haemoglobin is discussed.

INTRODUCTION

In previous communications[1,2], the authors described some properties of an iron-chelating enzyme, in the presence of which formation of protohaem (not haemoglobin) from iron and protoporphyrin was demonstrated. Inside cells, however, haem is always found in combination with proteins forming haemoproteins with special functions. The role of the iron-chelating enzyme in the formation of haemoprotein was next investigated. Using the crude enzyme from duck erythrocytes, the authors made a preliminary report on the formation of haemoglobin with the addition of haemolysate supernatant[3]. SCHWARTZ *et al.*[4] also reported the incorporation of ^{59}Fe into carrier haemoglobin by a preparation of chicken particles and globin. Using column chromatography and spectrophotometry, the authors tried to demonstrate directly the formation of haemoglobin and myoglobin from iron, protoporphyrin and globin in the presence of the iron-chelating enzyme. A part of this study was reported previously as preliminary communications[5,6].

MATERIALS AND METHODS

Preparation of iron-chelating enzyme

The iron-chelating enzyme was prepared from a haemolysate of duck erythrocytes according to the method previously reported[1,2]. Haemoglobin-free stroma was extracted with an equal volume of 0.05 M Tris buffer (pH 7.2) containing 1% of sodium cholate for 15 h at 4°. The supernatant of this extract or its 28–50% saturated $(NH_4)_2SO_4$ precipitate was used as the solution of the enzyme.

Preparation of globin

Globin was prepared from the erythrocytes of the duck or from the myoglobin[7] of horse-heart according to ROSSI-FANELLI AND ANTONINI[8]. A fresh preparation was always used.

Determination of the iron-chelating activity of the enzyme

The incubation mixture contained protoporphyrin, ferric chloride (labelled or not), cysteine, Tris buffer (pH 8.0) and the enzyme. Globin (apohaemoglobin or apomyoglobin) was added at the beginning or after the incubation. The mixture was incubated in a Thunberg tube for a definite time under nitrogen at 37° or 30°. When the measurement of protoporphyrin decrease was desired, the reaction was stopped by the addition of acetic acid – ethyl acetate (1:4) and the denatured protein was removed by centrifugation. The ethyl acetate layer was then washed with an aqueous solution of sodium acetate, and the protoporphyrin was extracted with 10% hydrochloric acid. The protoporphyrin content of the acid solution was determined spectrophotometrically at 409 mμ. For the measurement of radioactivity, the reaction was stopped by the addition of acetone, and carrier haemoglobin was added, if necessary. [^{59}Fe]Haemin was extracted and purified according to CHU AND CHU[10]. Haemin was determined according to KING *et al.*[11]. The total radioactivity of purified [^{59}Fe]haemin incorporated with the correction for loss during purification was an index for the formation of haem.

Spectrophotometric measurement

Various measurements of difference spectra were made in order to identify products of reaction, since they contained large amounts of protoporphyrin.

Protein determination

The amount of protein was determined according to the method of LOWRY *et al.*[12].

RESULTS

Chromatographic study of haemoglobin formation

Using ^{59}Fe and $(NH_4)_2SO_4$-fractionated enzyme, two incubation mixtures were prepared; one without apohaemoglobin and the other with it added at the beginning. The incubation mixtures contained 200 mμmoles of $FeCl_3 \cdot 6H_2O$ (^{59}Fe, $5 \cdot 10^5$ counts per min), 163 mμmoles of protoporphyrin, 4 μmoles of cysteine, 200 μmoles of Tris buffer (pH 8.0) and 1 ml of enzyme with, or without, 3.1 mg of apohaemoglobin in a final volume of 5 ml. They were incubated for 2 h under nitrogen. After the reaction

had been stopped by chilling, 2.5 mg of haemin chloride and 100 mg of CO-haemoglobin from duck erythrocytes, in 4 ml solution, were added to each reaction mixture as carriers. The mixtures were each divided into two parts: 0–50 % and 50–80 % $(NH_4)_2SO_4$ saturated fractions. The precipitates were dialyzed against water, and [^{59}Fe]haemin was crystallized as described in MATERIALS AND METHODS. The results are given in Table I. Without globin, the main radioactivity of haem was found in the 0–50 % fraction, while it was found in the 50–80 % fraction with the addition of globin. These fractions were further examined by means of chromatography on columns of CG-50 and the radioactivity of [^{59}Fe]haemin of the effluent in each tube was measured after the crystallization of [^{59}Fe]haemin. The results of these measurements are given in Fig. 1 (0–50 % saturated fraction) and Fig. 2 (50–80% saturated fraction). The 0–50 % fraction without globin showed two subfractions of radioactivity, the first being probably haemin, bound to the iron-chelating enzyme[13], and the second being free haemin attached to the column and only eluted by NaOH. The 0–50 %-saturated fraction with globin possessed the same sub-fractions, but they had much less radioactivity. The 50–80 % saturated fraction with globin showed one peak of radioactivity, the position of which was identical with that of the peak of 540 mμ of carrier CO-haemoglobin, revealing the formation of [^{59}Fe]haemoglobin. In the 0–50 %

TABLE I

RADIOACTIVITY OF [^{59}Fe]HAEMIN AFTER $(NH_4)_2SO_4$ FRACTIONATION

Fraction	*Without globin (counts/min × 10⁴)*	*With globin (counts/min × 10⁴)*
Product	17.2	15.8
Fraction (0–50% saturated)	10.1	1.2
Fraction (50–80% saturated)	3.5	11.0

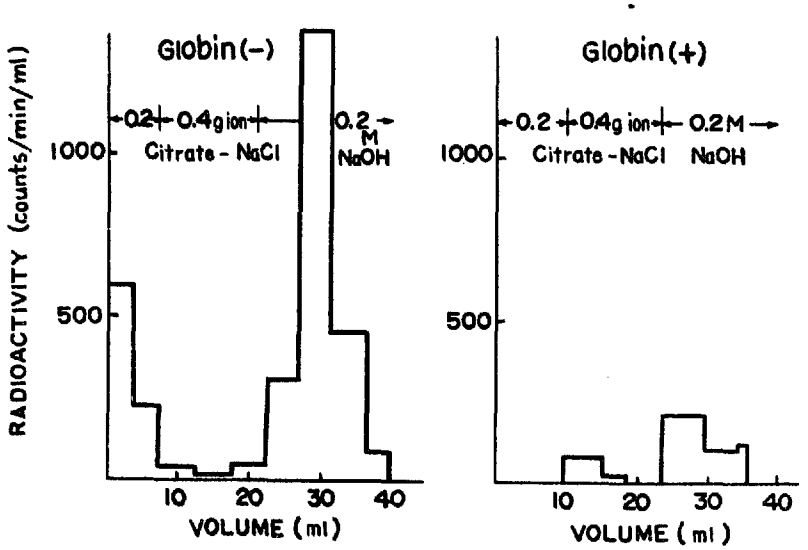

Fig. 1. Elution diagram of 0–50 % saturated $(NH_4)_2SO_4$ fraction from CG-50 column. Fraction 0–50 % in Table I was employed. To a column of 0.7 × 10 cm, buffered with 0.2 M sodium citrate (pH 6.8) the solution of the product was applied. Stepwise elution as indicated. From each fraction of 2 ml, radioactive haemin was crystallized.

fraction without globin the same peak was found also but the radioactivity was much less. These results showed that, without globin, the main product was haem, while, with the addition of globin at the beginning of incubation it was haemoglobin. We found no clear reason for the probable existence of the small amount of haemin in the presence of globin and of haemoglobin in the absence of globin.

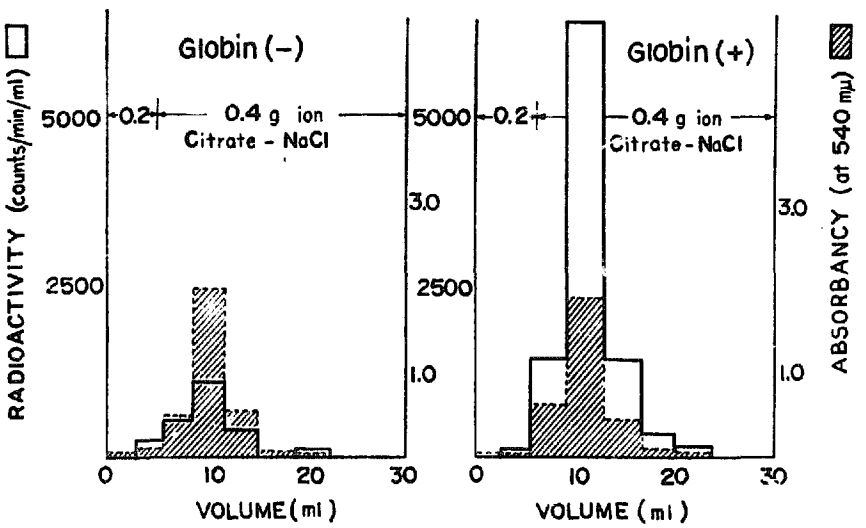

Fig. 2. Elution diagram of 50–80% saturated $(NH_4)_2SO_4$ fraction from CG-50 column. Fraction 50–80 in Table I was used. Other conditions were the same as described in Fig. 1, except that the elution with 0.2 M NaOH was not carried out.

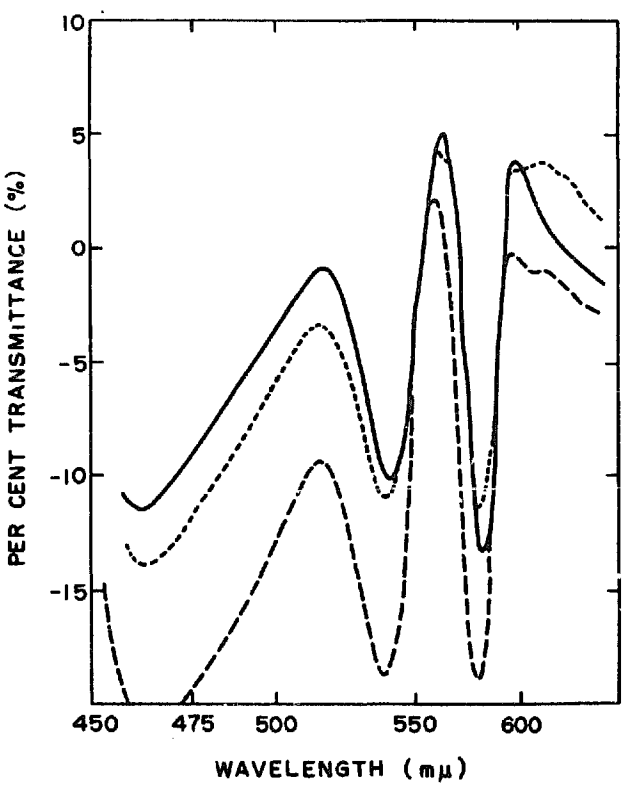

Fig. 3. Difference spectra between oxygenated and reduced forms. ———, haemoglobin; – – – – incubation mixture (apohaemoglobin was added at the beginning); — — — —, incubation mixture (apohaemoglobin was added after the incubation).

Spectrophotometric study of haemoglobin and myoglobin formation

The incubation mixture, containing 200 mμmoles of $FeCl_3 \cdot 6H_2O$, 100 mμmoles of protoporphyrin, 4 μmoles of cysteine, 200 mμmoles of Tris buffer (pH 8.0), 1.8 mg of apohaemoglobin and 1 5 ml of cholate extract in a final volume of 5 ml, was incubated in a Thunberg tube at 37° for 1 h under nitrogen. After incubation, the reaction was stopped by chilling, and the solution of the product was divided in two parts, one of which was aerated for 5 min and the other reduced with dithionite. The difference spectrum between them is shown in Fig. 3. The other incubation mixture was composed of the same substances except apohaemoglobin in a main compartment of a Thunberg tube, while globin was placed in a side arm. The incubation was carried out as described above. After incubation and chilling, the reaction mixture was mixed well with globin solution at 4°, and then allowed to stand for 10 h. The difference spectrum of this solution between the oxygenated and reduced forms as well as the spectrum of duck haemoglobin are shown in Fig. 3. The difference spectrum of the same kind after incubation without globin as well as that of 1.8 mg of horse-serum albumin added at the beginning are given in Fig. 4 for comparison. The three curves in Fig. 3 showed the same characteristics, which were quite different from those in Fig. 4 revealing that, in the presence of the iron-chelating enzyme, iron, protoporphyrin and globin combined together to form haemoglobin.

Instead of globin from duck haemoglobin, apomyoglobin from the myoglobin

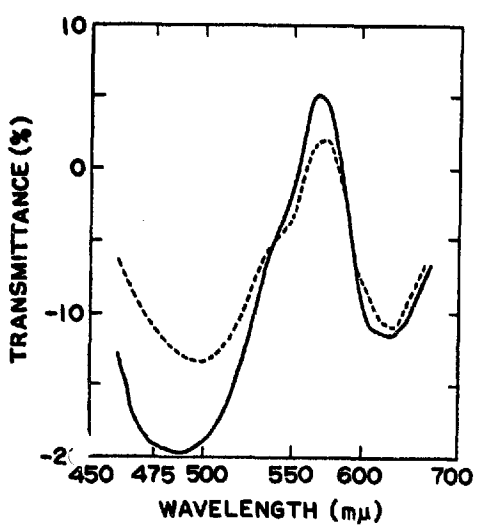

Fig. 4. Difference spectra between oxygenated and reduced forms. ———, incubation mixture without globin; – – – –, incubation mixture with horse-serum albumin added at the beginning.

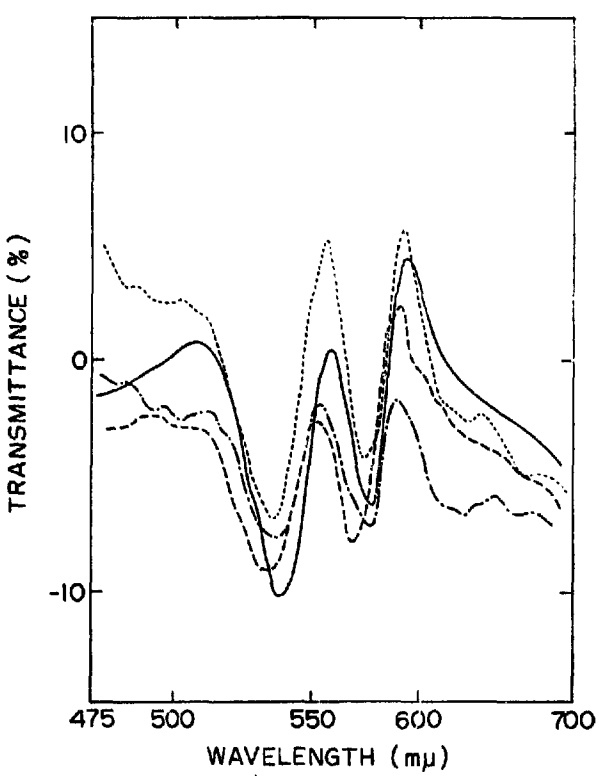

Fig. 5. Difference spectra between CO and, reduced forms. ———, myoglobin; – – – – haemoglobin; –·—·–, incubation mixture (apomyoglobin added at the beginning); -------, incubation mixture (apomyoglobin added after incubation).

of horse heart was employed in investigating the formation of myoglobin. The incubation mixture contained 300 mμmoles of $FeCl_3 \cdot 6H_2O$, 300 mμmoles of protoporphyrin, 12 μmoles of cysteine, 400 mμmoles of Tris buffer (pH 8.0), 5.8 mg of apomyoglobin and 1 ml of the cholate extract in a final volume of 7 ml. It was incubated in a Thunberg tube for 1.5 h under nitrogen. After incubation, the solution was divided into two parts and difference spectrum between the carbon monoxide (CO) and the reduced forms were recorded (Fig. 5), for spectral difference between haemoglobin and myoglobin is most evident in their carbon monoxide (CO) forms. The other incubation mixture without apomyoglobin was incubated in the same way. After incubation and chilling, the resulting solution was mixed with 5.8 mg of apomyoglobin and was then allowed to stand for 1 h. That the difference spectrum is of the same kind is shown in Fig. 5. Similar difference spectra of duck haemoglobin and of horse-heart myoglobin are also given in Fig. 5 for comparison. Thus the formation of myoglobin, clearly distinct from haemoglobin and haem, was observed in the system containing iron, protoporphyrin, apomyoglobin and iron-chelating enzyme.

TABLE II

EFFECT OF APOHAEMOGLOBIN ON HAEM FORMATION

The incubation mixture contained 50 mμmoles of $FeCl_3 \cdot 6H_2O$, 20 mμmoles of protoporphyrin, 2 μmoles of cysteine, 100 μmoles of Tris buffer (pH 8.0), and 0.34 mg of apohaemoglobin in a final volume of 1.5 ml. Incubation was carried out at 30° under nitrogen.

Globin	*Time of incubation (min)*	*Decrease of protoporphyrin (mμmoles)*
—	30	4.75
+*	30	3.36
+	30	2.06
—	60	5.56
—	120	5.83
+	120	3.40
+**	120	5.93

* Preincubated with protoporphyrin and globin in the presence of buffer at 30° for 30 min.
** Globin was added 60 min after incubation.

Effect of globin on the rate of formation of haem

The influence of apohaemoglobin on the rate of haem synthesis was examined under various conditions. The decrease of protoporphyrin was measured after the incubation with the cholate extract. The results are given in Table II and it will be seen that the presence of globin inhibited the rate of formation of haem. Similar results were obtained in the experiment on the incorporation of ^{59}Fe as well as with apomyoglobin. Furthermore, no activation was found on addition of apohaemoglobin after incubation for 1 h, when the rate of haem synthesis was decreased. These results were contrary to those of SCHWARZ *et al.*[4], who observed the stimulation of haem synthesis by apohaemoglobin.

DISCUSSION

Concerning the mechanism of haemoglobin formation, two possibilities are, at present, being discussed[4,14]; (a) the combination of iron with protoporphyrin–globin complex,

(b) the formation of haem from iron and protoporphyrin and then the combination of globin with the preformed haem. The authors demonstrated spectrophotometrically the formation of haemoglobin in both cases. The former way was also proved with column chromatography. Myoglobin formation may take place in the same way. Our results, however, do not mean that there exist two parallel courses of haemoglobin formation in the same immature red cells. Our earlier experiment[13] suggested that the latter way is more likely. Recent findings in our laboratory[15], concerning the preferential use of particle-bound protoporphyrin for the synthesis of haem, also suggested the latter pathway, for water-insoluble particle-bound protoporphyrin might be different from water-soluble protoporphyrin–globin complex. But the results of SCHWARTZ *et al.*, *viz.*, the stimulation of haem synthesis by apohaemoglobin, suggested the former way to be more likely. We could not, however, confirm their observations, and some inhibitory effect of globin was obtained instead. Our results might further indicate that the latter way is more plausible. Globin is, however, one of the proteins which are easily subject to denaturation. We tried every effort to avoid the denaturation of apohaemoglobin and apomyoglobin, but the possibility of slight denaturation could not be excluded. The reason for the discrepancy between our results and those of SCHWARTZ *et al.* may be decided after further purification of the iron-chelating enzyme.

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