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STUDIES ON THE STRUCTURE OF HEMOGLOBIN

II. PROPERTIES OF RECONSTITUTED PROTOHEMOGLOBIN AND PROTOPORPHYRIN-GLOBIN

A. ROSSI-FANELLI, E. ANTONINI AND A. CAPUTO

Institute of Biochemistry of the University and
"Regina Elena" Institute for Cancer Research, Rome (Italy)

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SUMMARY

Human hemoglobin has been reconstituted from protohematin and pure native globin.

The reconstituted Hb was the same as the natural pigment in its physico-chemical properties (absorption spectra, isoelectric point, electrophoretic behaviour, sedimentation, diffusion, stability to heat and alkali) and in its oxygen equilibrium (heme-heme interaction, BOHR effect, oxygen affinity). The compound of globin with protoporphyrin was also obtained and investigated. The molecular properties of this protoporphyrin-globin are very similar to those of hemoglobin.

INTRODUCTION

In the previous paper[1] of this series, we described the preparation and properties of pure human globin. The study of the properties of reconstituted products of native globin with different prosthetic groups can give important information on the structure of hemoglobin, on the linkages and on the arrangement of hemes in this pigment. In the present paper, the reconstitution of protohemoglobin and protoporphyrin-globin is described and some of the structural properties of these reconstituted pigments are reported.

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The resynthesis of hemoglobin (Hb) from globin and protohematin has been attempted by several authors but the results obtained on such reconstituted Hbs have been equivocal and disconcordant. This is probably due to the fact that the globin employed was not really pure and native, and that only some properties of the reconstituted hemoglobin were studied on the same preparation. The reconstituted hemoglobin was generally found to differ in one or more properties from the natural pigment[2-4]. The recombination of globin with protoporphyrin has been attempted but few data are available[5-7].

MATERIALS AND METHODS

Human Hb was prepared by fractional precipitation with ammonium sulfate[8]. Apohemoglobin was prepared, as before[1], by acid acetone splitting of human HbO_2.

Crystalline protohemin IX was a commercial product from BDH.; protoporphyrin IX was prepared and crystallized according to FISCHER[9]. For the coupling with globin, protohemin and protoporphyrin were dissolved in a minimum amount of 0.05–0.1 *N* NaOH or Na_2CO_3 and the volume made up with water. The electrophoresis, sedimentation and diffusion experiments were performed as before[1].

The absorption spectra were determined with a Beckman D.U. spectrophotometer. The Hb concentration was determined by reduced pyridine hemochromogen. Unless otherwise stated, all experiments were performed with the ferric derivative of natural and reconstituted Hb.

RESULTS

(a) *Reconstituted protoHb*

As previously reported[1], the coupling capacity of globin for protohematin is 3.8 %. Protohemoglobin was reconstituted by mixing at 0° stoichiometric amounts of apoHb in pH 7 phosphate buffer (0.05–0.1 *M*) and protohematin. The slight, coloured precipitate which often appeared after the coupling was removed by filtration. The solution then remained unchanged for weeks when stored at 2–5°. As will be seen later reconstituted Hb was as stable as natural Hb.

The reaction between the hematin and the globin, followed spectrophotometrically in the SORET zone, appeared to be instantaneous; after some seconds the spectrum of ferri-Hb could be recognized.

Absorption spectra

Table I shows the wavelengths of maximum absorption and the extinction coefficients of reconstituted and natural Hb. The absorption spectra of the various derivatives (HbO_2, Hb, Hb^+, HbCO) show that there are no significant differences either in the visible or the near u.v. (SORET zone) range, between the 2 types of Hb.

Oxygen capacity

The ferric derivative obtained from globin and protohematin was reduced to the ferrous compound by the enzyme system consisting of DPNH, DPN-cytochrome reductase and methylene blue[10], following the procedure for the reduction of ferrimyoglobin[11].

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TABLE I

WAVELENGTHS OF MAXIMUM ABSORPTION AND MOLECULAR EXTINCTION COEFFICIENTS OF NATURAL AND RECONSTITUTED Hbs

Derivative	*Natural Hb*			*Reconstituted Hb*		
HbO_2						
max. mμ	412	541	577	412	541	577
$\varepsilon \cdot 10^{-3}$	135	13.8	14.6	—	13.6	14.2
Hb						
max. mμ	430		555	430	555	
$\varepsilon \cdot 10^{-3}$	119		13.5	120	13.2	
Hb^+						
max. mμ	405	500	630	405	500	630
$\varepsilon \cdot 10^{-3}$	155	9.5	4.1	148	9.2	4.0
HbCO						
max. mμ	419	539	569	419	539	569
$\varepsilon \cdot 10^{-3}$	191	13.4	13.4	183	13.4	13.3

$\varepsilon = \frac{1}{cd} \log \frac{I_0}{I}$; c = molar concn. as hemin.

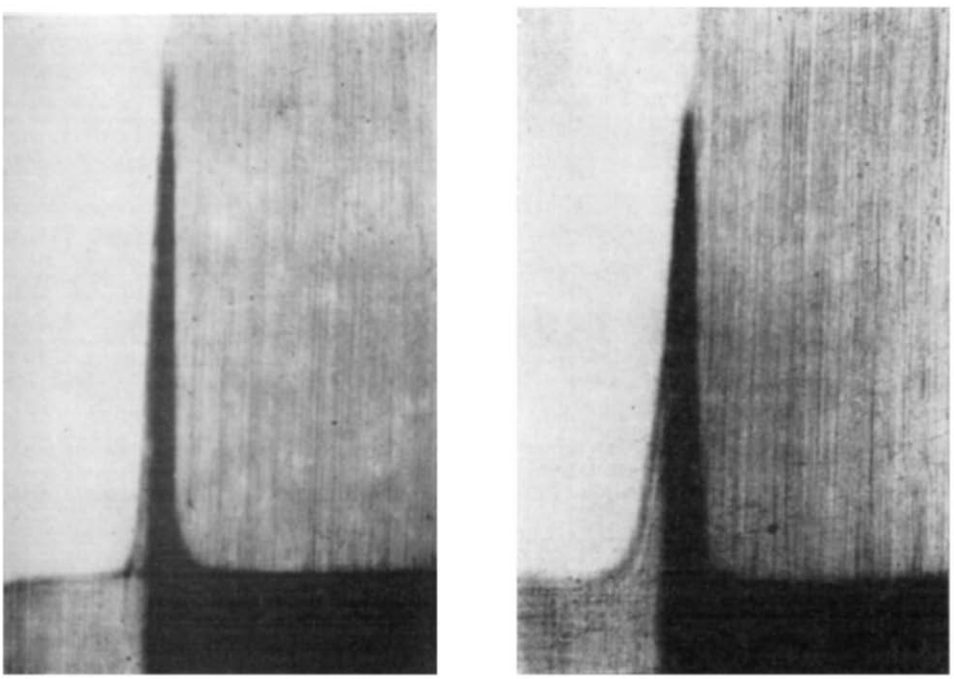

Fig. 1A Fig. 1B

Fig. 1. Ascending electrophoretic patterns of reconstituted Hb (A), and of a mixture of equal amounts of natural and reconstituted Hb (B), MILLER AND GOLDER buffer, pH 6.1, ionic strength 0.1, 80 min, 140 V, 13 mA.

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The oxygen capacity was determined in the standard Warburg apparatus, by measuring the oxygen evolved from a solution of ferrous Hb equilibrated with air after the addition of ferricyanide. The capacity of reconstituted reduced Hb was identical with that of unsplit Hb; the mean value obtained was 0.98 μmole O_2/μmole of heme.

Electrophoretic behaviour

Natural and reconstituted Hbs migrated as homogeneous systems and the electrophoretic pattern was characterized by the presence of a single coloured component (Fig. 1A). Moreover, a mixture of equal amounts of the two Hbs migrated as a single homogeneous component (Fig. 1B). The same mobility values were obtained (Fig. 2) for natural and reconstituted Hbs.

As shown in Fig. 2 the isoelectric point calculated from the pH-mobility curve was 6.9 for both natural and reconstituted ferri-Hbs.

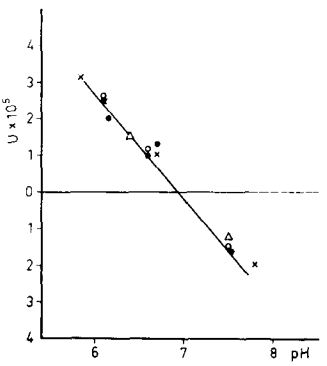

Fig. 2. Electrophoretic mobility as a function of pH for: natural Hb (●); reconstituted Hb (○); mixtures of natural and reconstituted Hb (×) and protoporphyrin globin (△). MILLER AND GOLDER buffers, ionic strength 0.1.

Molecular properties

Reconstituted protoHb showed, in the ultracentrifuge, a sedimentation pattern characterized by the presence of one coloured symmetrical component (Fig. 3) with a sedimentation constant, extrapolated to zero protein concentration, of $S_{20} = 4.69$. This value and the slope of the curve of S versus protein concentration were the same as those obtained for natural Hb and are in good agreement with the data in the literature for human Hb[12]. The stabilities of natural and reconstituted Hb in the ultracentrifuge as a function of pH were also studied*.

The results obtained in these experiments are summarized in Fig. 4; again, there is no appreciable difference between natural and reconstituted Hbs.

The diffusion constant of reconstituted Hb, calculated as the mean of 4 experiments at different protein concentrations, was found to be $D_{20} = 6.4 \cdot 10^{-7}$. This value lies within the range reported in the literature for mammalian Hbs and is practically identical with the value which we obtained under the same conditions for human Hb. On the basis of the partial specific volume, $\bar{V} = 0.75$, the mol. wt. of reconstituted Hb, calculated from S and D, was 66,800, and the frictional ratio $f/f^\circ = 1.2$.

* The proteins were brought to the desired pH with MILLER AND GOLDER buffers immediately before the runs.

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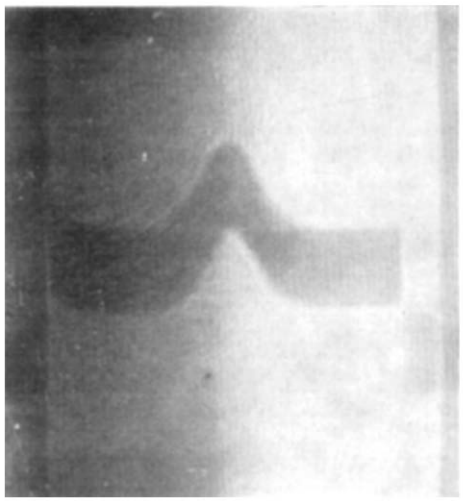

Fig. 3. Sedimentation pattern of reconstituted proto Hb (0.8%) in MILLER AND GOLDER buffer, pH 7.2, ionic strength 0.1. The picture was taken 40 min after reaching full speed of 59,780 rev./min.

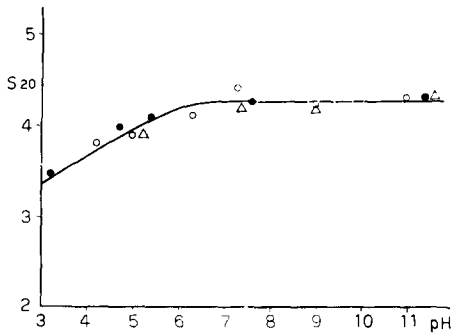

Fig. 4.

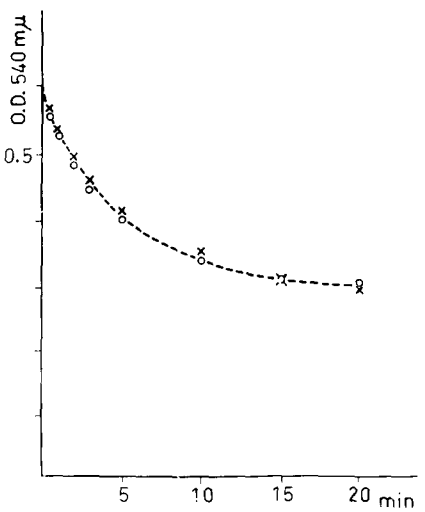

Fig. 6.

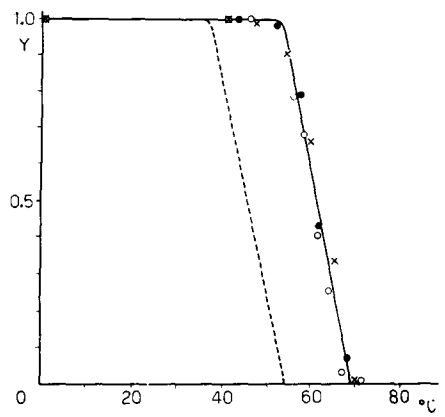

Fig. 5.

Fig. 4. Sedimentation constants as a function of pH of natural Hb (●) reconstituted Hb (○), and protoporphyrin-globin (△). Protein concentrations 0.5%.

Fig. 5. Heat stability curves for natural Hb (●), reconstituted Hb (○), and protoporphyrin globin (×). 0.2% protein in phosphate buffer 0.02 *M* pH 7.2. Y = fraction of protein remaining in solution after 15-min heating. The dashed line represents the heat stability of globin in the same conditions.

Fig. 6. Alkaline denaturation of natural (×) and reconstituted HbO_2 (○) (0.8‰). Borate–NaOH buffer, 0.05 *M*, pH 12.

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Heat stability and alkali denaturation

Fig. 5 shows the heat stability curves of natural and reconstituted ferri-Hbs. There was no significant difference between the 2 hemoglobins; both began to coagulate after heating for 15 min at 55°. It is interesting to note the great difference in the behaviour of the Hbs and that of globin, which appears to be much less resistant to heat denaturation. The rate of alkaline denaturation of HbO_2 determined as previously described[13] at pH 12 was also the same for natural and reconstituted Hbs (Fig. 6).

(b) *Protoporphyrin-globin*

When globin was coupled with protoporphyrin in neutral solution, a compound (protoporphyrin-globin) was obtained; this was clearly shown by the colour change in the solution, the brownish colour of the aqueous solution of porphyrin becoming red-violet. Spectrophotometrically, the diffuse absorption bands of the porphyrin in the visible range were replaced by defined peaks which were displaced toward the blue end of the spectrum (Fig. 7). A strong and large band with a maximum at 400 mμ appeared in the near u.v.

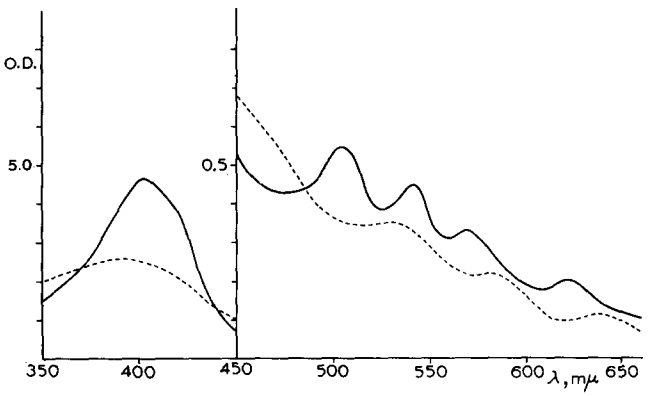

Fig. 7. Absorption spectra of protoporphyrin (dashed line) and protoporphyrin plus globin (full line), in phosphate buffer 0.05 *M*, pH 6.8. Porphyrin concentration 30 μg/ml; globin concentration 0.9 mg/ml. The spectra were taken 1 h after mixing the globin with the porphyrin.

The protoporphyrin-globin used in our experiments was obtained by mixing at 0° the globin in pH 7 phosphate buffer (0.05–0.1 *M*) with the porphyrin in the proportion 100:3.5, in accordance with data on the coupling capacity (see later). The solution was left in the cold room for at least 24 h. As in the reconstitution of protohemoglobin, a small precipitate often appeared after the coupling; this was removed by filtration. The protoporphyrin-globin obtained was as stable at 2–5° as the natural and reconstituted Hbs.

Coupling capacity

The coupling capacity of globin for protoporphyrin was determined by mixing at 0° a fixed quantity of globin with increasing amounts of porphyrin and measuring the O.D. of the solutions at 400 mμ after 20 h. The data shown in Fig. 8 indicate that

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the spectrophotometric changes attained a maximum when 3.5–4 % porphyrin was added; thus, the coupling capacity of globin for the porphyrin is 4 moles/66,000 g of protein. The rate of reaction between globin and porphyrin was measured spectrophotometrically by the changes in the O.D. between 490 and 500 mμ. The reaction appeared to be slower than that of hematin with globin; the $t_{\frac{1}{2}}$ was in the range of minutes.

It is interesting to note that after globin had reacted with porphyrin it no longer had a coupling capacity for protohematin.

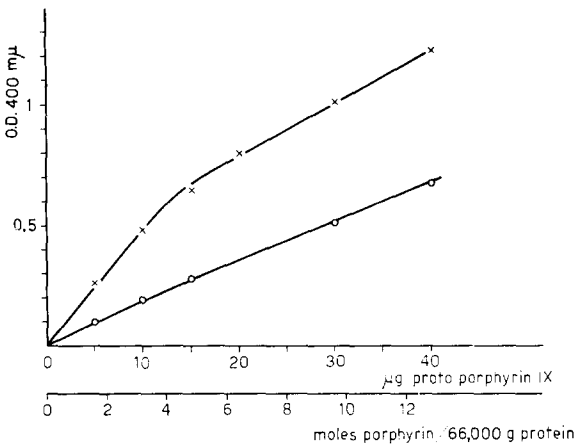

Fig. 8. Coupling capacity of globin for protoporphyrin. Lower curve: protoporphyrin alone. Upper curve: protoporphyrin plus 335 μg of globin. Phosphate buffer 0.05 *M* pH 7.

Electrophoretic behaviour

The electrophoretic properties of protoporphyrin-globin were very similar to those of hemoglobin (Fig. 2); the electrophoretic pattern was characterized by the presence of a single coloured component.

Molecular properties

The protoporphyrin-globin was stable in the ultracentrifuge and sedimented as a homogeneous and symmetrical coloured component (Fig. 9) with $S_{0,20} = 4.61 \cdot 10^{-13}$. The pH-stability diagram of protoporphyrin-globin was similar to that observed for natural and reconstituted Hbs (Fig. 4).

The mean diffusion constant of protoporphyrin-globin was found to be $6.5 \cdot 10^{-7}$. The mol. wt. obtained from sedimentation, diffusion and partial specific volume ($\bar{V} = 0.75$) determinations was calculated to be 69,000. The frictional ratio of the molecule was $f/f_0 = 1.19$.

Heat stability

The heat stability of protoporphyrin-globin was practically identical to that of natural and reconstituted ferri-Hbs under the same conditions. As can seen from Fig. 5, protoporphyrin-globin began to coagulate after heating for 15 min at 56°.

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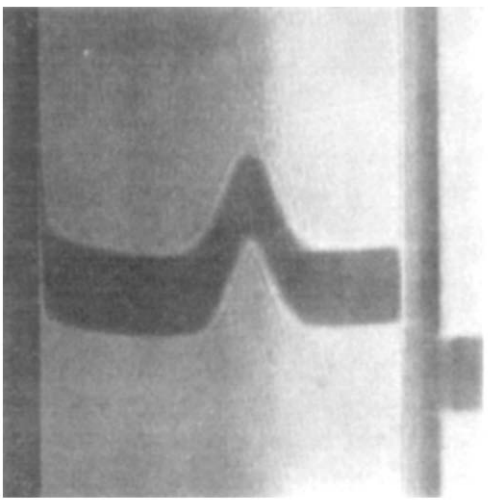

Fig. 9. Sedimentation pattern of protoporphyrin globin (0.8%) in MILLER AND GOLDER buffer, pH 7.2; ionic strength 0.1. The picture were taken 40 min after reaching the full speed of 59,780 rev./min.

DISCUSSION

The results obtained show that our globin preparation can bind heme to give a recombination product indistinguishable from natural Hb. The absorption spectra, electrophoretic and molecular properties, heat stabilities and rates of alkaline denaturation were found to be the same for the natural and reconstituted Hbs. Moreover, with an enzymic system we obtained the ferrous derivative of the reconstituted Hb which had the same oxygen capacity as the natural Hb. We have already reported[14] that the oxygen equilibrium of our reconstituted Hb is the same as that of natural Hb; the 2 proteins have the same type of oxygen dissociation curve, and the same oxygen affinity and BOHR effect.

These results are very different from those obtained by previous workers, who found that reconstituted Hb differed from natural Hb in its isoelectric point, absorption spectra, oxygen equilibrium, heat and alkali denaturation[2-4]. These discrepancies are most probably due to the state of the globin employed for the reconstitution of the pigment; we believe that the state of the globin can best be judged by the properties of the reconstituted Hb. The present experimental results indicate that our globin is really native apoHb. The stoichiometry of the reaction of hematin with globin and the identity of some particular properties of natural and reconstituted Hb (*e.g.* the BOHR effect) indicate that hematin reacts with globin at the specific sites where the hemes are joined in the unsplit protein. These results open the way for investigations on the mechanism and kinetics of the reaction between globin and hematin. Research along these lines is in progress in our laboratory.

Another interesting finding is the difference in some molecular and physicochemical properties of globin and reconstituted Hb. The increase in S, molecular weight, and heat stability indicate that a profound change occurs in the configurational structure of the globin when it reacts with hematin. The increase in molecular

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weight shows that the hemes play a primary role in the establishment of the dimeric structure of Hb.

Further information about the structure of Hb has been obtained from a study of the properties of protoporphyrin-globin. The spectral changes occurring when porphyrin is added to the globin are similar to those observed when porphyrin is treated with organic solvents or with some purine bases[15] (caffeine or pilocarpine). In the latter cases, however, the effect is probably only "solvation" action which causes depolymerization of the porphyrin aggregates*.

In the case of the reaction between globin and porphyrin, there is evidence for the formation of a true compound of the same type as that between the globin and the hematin; this is shown by the stoichiometry of the reaction and by the properties of the compound obtained. The physico-chemical and molecular properties of protoporphyrin-globin are very similar to those of natural and reconstituted Hb and profoundly different from those of globin. Protoporphyrin-globin and Hb contain equivalent quantities of porphyrin or ferroporphyrin, have the same sedimentation and diffusion constants, the same molecular weight and, even more remarkably, the same heat stability. Thus, all the properties analysed indicate that the molecular architecture of protoporphyrin-globin is the same as that of Hb despite the absence of the hematinic iron. This fact suggests that in the Hb molecule the porphyrin part of the heme plays a primary role in binding the hemes to the globin and in the acquisition of particular structural properties (*i.e.* dimerization); linkages of globin to the hematinic iron would confer on the Fe^{++} atom the unequivocal property of reversible combination with oxygen.

ACKNOWLEDGEMENT

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* Porphyrin in aqueous solution exists in a polymerized form[4].