ANALYSIS OF MET-FORM HEMOGLOBIN IN GLUCOSE-DELETED HUMAN RED CELLS

Akio TOMODA, Masazumi TAKESHITA* and Yoshimasa YONEYAMA

Department of Biochemistry, Kanazawa University School of Medicine and **Department of Medical Technology, Kanazawa University School of Paramedicine, Kanazawa 920, Japan*

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1. Introduction

Though many investigations have been reported on the mechanism of methemoglobin formation in human red cells, complete explanation is not available. Methemoglobin accumulates in human red cells as glucose was consumed during a long period incubation of these cells at 37°C [1]. These glucose-deleted red cells seem to be suitable to investigate the mechanism of hemoglobin oxidation in intact cells because enzymatic methemoglobin reducing systems including NADH and NADPH diaphorases are suppressed due to the deficiency of reproduction of NADH and NADPH.

In this paper, we investigated the methemoglobin formation in intact human red cells, which were incubated without glucose for a long period at various pH values. Especially, we analyzed the nature of methemoglobin derivatives detected in these cells. As a result, fully oxidized hemoglobin (methemoglobin) as scarcely found in these cells, however, met-form hemoglobin was detected as half-oxidized hemoglobin ($\alpha^{3+}\beta^{2+}$). On the basis of these results, the mechanism of hemoglobin oxidation and physiological significance of the oxidation process were discussed.

2. Experimental

Fresh heparinized human blood was centrifuged at 3000 rev/min for 10 min. After removal of buffy coats, red cells were washed with ice cold 0.9% NaCl solution 5 times. These cells were suspended in an incubating solution containing 110 mM NaCl, 30 mM sodium phosphate, 5 mM Kcl, 1 mM $MgCl_2$ and streptomycin (0.5 mg/ml) and incubated at 37°C at various pH values (6.6–7.8) for 22 h without glucose (hematocrit value of the suspension, 30%). During the incubation, little hemolysis was observed. Samples taken out at intervals for analyses of met content were measured as in [2].

The red cells suspension which were incubated at various pH values were hemolysed by the addition 4 vol. ice cold distilled water after 22 h incubation, and then centrifuged at 10 000 rev/min for 10 min. The supernatants thus obtained were used for isoelectric focusing on ampholine plate gel (pH 3.5–9.5, purchased from LKB, Sweden). The isoelectric focusing of the supernatants was carried out on ampholine plate gel within 1.5 at 4°C.

The supernatant at pH 6.6, which was passed through Sephadex G-25 column equilibrated with 10 mM potassium phosphate buffer (pH 6.8), was applied on a CM Sephadex C-50 column and eluted with pH gradient phosphate buffer made up from 10 mM potassium phosphate (pH 6.8) and 20 mM K_2HPO_4 as in [3]. The effluent containing the intermediate hemoglobin was rechromatographed by the same procedure. The intermediate hemoglobin obtained by these procedures was used for the optical measurements, the detailed methods of which are in fig.3,4 legends.

3. Results

3.1. *Changes in met contents during the incubation of red cells without glucose*

Red cells were incubated without glucose at 37°C at various pH values (6.6, 7.0, 7.4, 7.8) for 22 h. Figure 1 shows the changes in met contents during

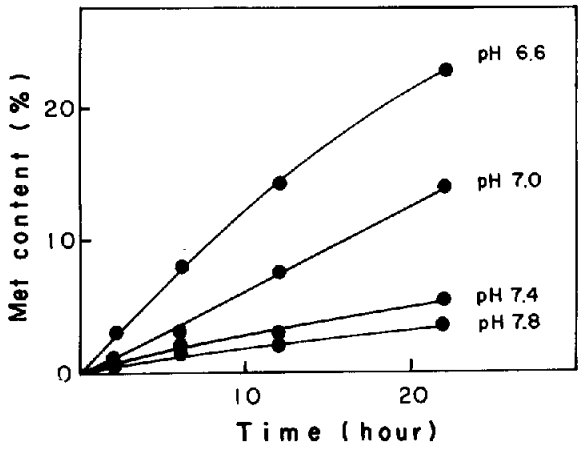

Fig.1. Changes in met contents during incubation of glucose-deleted red cells at various pH values.

the incubation of red cells. Met contents in these cells increased linearly with time and was dependent on hydrogen ion concentration.

3.2. *Isoelectric focusing of hemolysates on ampholine plate gel*

The samples which were obtained after 22 h incubation of red cells at various pH values were applied on ampholine plate gel with a control (oxyhemoglobin and methemoglobin) and the isoelectric focusing was performed (fig.2). In accordance with a decrease in pH value, intermediate hemoglobin appeared in large amounts besides oxyhemoglobin. However, the band which corresponded to that of methemoglobin was scarcely detected by this experiment, though the met content in red cells increased.

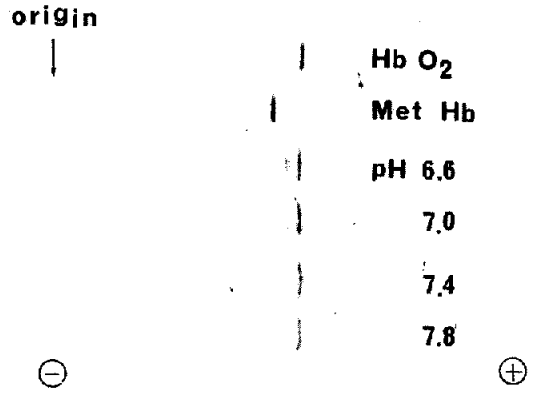

Fig.2. Isoelectric focusing profiles of hemolysates on ampholine plate gel.

3.3. *Purification of the intermediate hemoglobin by CM Sephadex C-50 column chromatography*

In order to isolate the intermediate hemoglobin, the hemolysates of the samples at pH 6.6 were applied on CM Sephadex C-50 column. Oxyhemoglobin was eluted between pH 7.45 and pH 7.52 and the intermediate hemoglobin was eluted between pH 7.52 and pH 7.6. Methemoglobin, which is expected to be eluted between pH 7.65 and pH 7.75, was not detected in the effluent.

The samples containing the intermediate hemoglobin, were rechromatographed on a CM Sephadex C-50 column. The major portion of hemoglobin was eluted between pH 7.5 and pH 7.55 as intermediate form of hemoglobin, while small amounts of contaminated oxyhemoglobin were separated between pH 7.3 and pH 7.5. The samples obtained by this procedure were used for the optical measurements.

3.4. *Absorption spectra of intermediate hemoglobin*

Figure 3 shows the absorption spectra of the intermediate hemoglobin under aerobic and anaerobic conditions. Under aerobic conditions, A_{545} is higher than A_{578} and a small peak at A_{630} and a single narrow peak in the Soret region (406 nm) were observed.

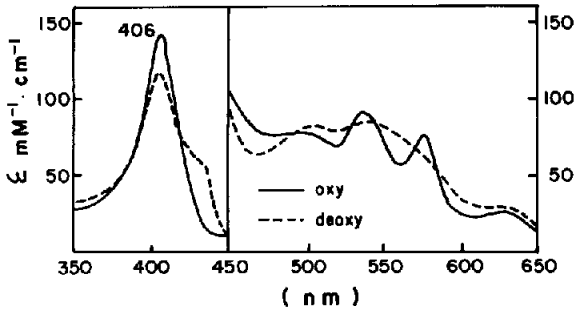

Fig.3. Absorption spectra of the intermediate hemoglobin under aerobic and anaerobic conditions. A_{350} and A_{650} of intermediate hemoglobin were followed under aerobic and anaerobic conditions. The experiment under anaerobic conditions was carried out after replacement of air with Q gas (helium/isobutane, 99.05 : 0.95) in a Thunberg-type quartz cell.

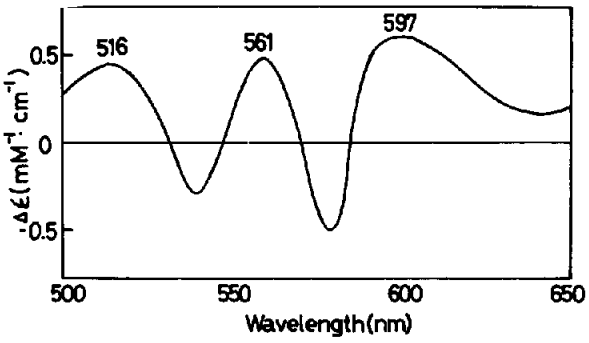

Fig.4. Difference spectra of the intermediate hemoglobin induced by the addition of inositol hexaphospate. $\Delta A_{500-600}$ between the original solutions containing intermediate hemoglobin and solutions containing interminate hemoglobin to which increasing amounts of inositol hexaphosphate have been added were measured.

When the intermediate hemoglobin was deoxygenated, the absorption spectrum with a shoulder at 430 nm was observed in the Soret region. These absorption spectra were in good agreement with those obtained by the reconstituted $\alpha^{3+}\beta^{2+}$ valency hybrid [4].

3.5. *Difference spectra of intermediate hemoglobin induced by inositol hexaphosphate*

Figure 4 shows the difference spectra of the intermediate hemoglobin induced by the addition of inositol hexaphosphate between 500 nm and 650 nm. The peaks were observed at 516 nm, 561 nm and 597 nm, and trough at 540 nm and 578 nm. These spectra were in good agreement with those obtained by the reconstituted $\alpha^{3+}\beta^{2+}$ valency hybrid. (The difference spectra of the reconstituted $\alpha^{2+}\beta^{3+}$ valency hybrid were much smaller in intensity with peaks at 557 nm and 593 nm [4]).

4. Discussion

It has been shown that the rate of oxidation of isolated hemoglobin is increased as pH of the medium is decreased [5,6]. The rate of oxidation of hemoglobin in the red cells also was increased as pH is decreased as shown in fig.1. Since the reducing systems which are mainly occupied by NADH and NADPH dependent diaphorases in the red cells do not operate under glucose deleted conditions, because of the deficiency of the reproduction of NADH from NAD and NADPH from NADP, the accelerated oxidation of hemoglobin in the acidic pHs in these cells will be due to the direct effect of hydrogen concentrations.

Therefore, the mode of hemoglobin oxidation in intact red cells seems to be very similar to that of isolated hemoglobin.

The oxidation rate of α chains in hemoglobin tetramers is much faster than that of β chains [6]. This result suggests that the intermediate hemoglobin will be present as $\alpha^{3+}\beta^{2+}$ rather than $\alpha^{2+}\beta^{3+}$ valency hybrid in intact red cells. Our results, fig.2–4, clearly demonstrate this heterogeneous distribution of the intermediate hemoglobin in the red cells, since the intermediate hemoglobin was identified to be $\alpha^{3+}\beta^{2+}$ valency hybrid. This result, therefore, supports the process of hemoglobin oxidation in intact red cells is similar to that observed in the isolated hemoglobin.

However, we would stress that fully oxidized hemoglobin (methemoglobin) is scarcely found in the glucose deleted red cells, though it is known that methemoglobin is formed during the autoxidation of isolated hemoglobin [6]. Our results strongly suggest that oxidized hemoglobin in red cells circulating in the body is present not as fully oxidized hemoglobin (methemoglobin) but as half-oxidized hemoglobin ($\alpha^{3+}\beta^{2+}$). Furthermore, met content theoretically will not exceed 50% total heme contents as far as met-form hemoglobin exists as $\alpha^{3+}\beta^{2+}$ valency hybrid without further oxidation to methemoglobin. Met contents of red cells of patients of hereditary methemoglobinemia due to deficiency of NADH diaphorase (about 150 cases reported) do not exceed 40% total hemoglobin [7] and some mechanism in addition to NADH diaphorase is operating to reduce methemoglobin in the patients, otherwise the methemoglobin level would be considerably higher [7]. Our hypothesis above may be a plausible explanation for their indications, and the process of hemoglobin oxidation shown in the present paper may serve for protection against the formation of methemoglobin which is no longer capable of binding oxygen.

Acknowledgements

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References

[1] Jandl, J. H., Engle, L. K. and Allen, D. W. (1960) J. Clin. Invest. 39, 1818–1836.
[2] Evelyn, K. A. and Malloy, H. T. (1938) J. Biol. Chem. 126, 655–662.
[3] Banerjee, R. and Cassoly, R. (1969) J. Mol. Biol. 42, 337–349.
[4] Tomoda, A., Takeshita, M. and Yoneyama, Y. (1978) submitted.
[5] Kikuchi, G., Shukuya, R., Suzuki, M. and Nakamura, C. (1955) J. Biochem. 42, 3–20.
[6] Mansouri, A. and Winterhalter, K. H. (1973) Biochemistry 12, 4946–4949.
[7] Harris, J. W. and Kellermeyer, R. W. (1974) The Red Cell, 3rd edn, p. 487, Harvard University Press, Cambridge, MA.