Mechanism of Methaemoglobin Reduction by Human Erythrocytes

Akio TOMODA,*‡ Masayoshi IDA,* Akira TSUJI† and Yoshimasa YONEYAMA*

*Department of Biochemistry, Kanazawa University School of Medicine, and †Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Kanazawa University, Kanazawa 920, Japan

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The time course of methaemoglobin reduction in human erythrocytes treated with nitrite was studied at pH 7.4, 37°C, in the presence or absence of Methylene Blue, and the changes in methaemoglobin, intermediate haemoglobins and oxyhaemoglobin during the reaction were analysed by isoelectric-focusing on Ampholine/polyacrylamide-gel plates. In both cases, with or without the dye, the intermediate haemoglobins were found to be present as $(\alpha^{3+}\beta^{2+})_2$ and $(\alpha^{2+}\beta^{3+})_2$ valency hybrids from their characteristic position on electrophoresis, but amounts changed consecutively with time. The amount of $(\alpha^{3+}\beta^{2+})_2$ was always greater than that of the $(\alpha^{2+}\beta^{3+})_2$ valency hybrid. This result is explained by the differences in redox potentials between α - and β -chains in methaemoglobin tetramer. It was concluded that methaemoglobin was reduced in human erythrocytes through these two different paths: methaemoglobin $\xrightarrow{k_{+1}} (\alpha^{3+}\beta^{2+})_2 \xrightarrow{k_{+2}}$ oxyhaemoglobin; and methaemoglobin $\xrightarrow{k_{+3}} (\alpha^{2+}\beta^{3+})_2 \xrightarrow{k_{+4}}$ oxyhaemoglobin. The reaction rate constants k'_{+1} ($=k_{+1} + k_{+3}$) and k'_{+2} ($=k_{+2} + k_{+4}$) were estimated from the changes in each component methaemoglobin, intermediate haemoglobins [$(\alpha^{3+}\beta^{2+})_2 + (\alpha^{2+}\beta^{3+})_2$] and oxyhaemoglobin.

It is well established that there are two methaemoglobin-reducing pathways involving NADH- and NADPH-dependent methaemoglobin reductases in human erythrocytes (Scott & Hoskins, 1958; Kiese, 1944). The NADH-dependent enzyme, which is characterized as NADH-cytochrome b_5 reductase, plays a significant role in methaemoglobin reduction in erythrocytes (Passon & Hultquist, 1972; Sugita *et al.*, 1971). On the other hand, the contribution of NADPH-dependent methaemoglobin reductase to methaemoglobin reduction in the erythrocytes is small (Scott *et al.*, 1963; Gibson, 1948). This enzyme, however, begins to operate actively in the presence of Methylene Blue (Huennekens *et al.*, (1969).

The detailed kinetic analysis of methaemoglobin reduction, especially for the changes in intermediate haemoglobins, was not, however, carried out by the above workers. We previously showed that $(\alpha^{2+}\beta^{3+})_2$ and $(\alpha^{3+}\beta^{2+})_2$ valency hybrids are present during the reduction of methaemoglobin by NADH-cytochrome b_5 reductase and NADPH-flavin reductase purified from human erythrocytes, and that these intermediate haemoglobins change consecutively with time (Tomoda *et al.*, 1979a,b). However, the actual contribution

of these enzymes to the reduction in the cells was not investigated; we decided therefore to investigate how these enzymes operate for methaemoglobin reduction in erythrocytes.

In the present study we analysed the changes in methaemoglobin, intermediate haemoglobins [$(\alpha^{3+}\beta^{2+})_2$ and $(\alpha^{2+}\beta^{3+})_2$] and oxyhaemoglobin during the reduction of methaemoglobin in the presence or absence of Methylene Blue under physiological conditions (pH 7.4), by using isoelectric-focusing, and thereby measured the actual contribution of NADH- and NADPH-dependent methaemoglobin reductases to methaemoglobin reduction in the erythrocytes. The kinetics of methaemoglobin reduction in the erythrocytes were compared with the results for the isolated enzymes.

Experimental

Materials

Sephadex G-25 (coarse grade) was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), and Ampholine/polyacrylamide-gel plates (pH 3.5–9.5) were from LKB Produkter (Bromma 1, Sweden). Sodium nitrite and Methylene Blue were obtained from Wako Pure Chemicals (Tokyo, Japan).

‡ To whom requests for reprints should be addressed.

Methods

Packed erythrocytes were prepared from 1-day-old acid/citrate/dextrose-treated blood obtained from the Kanazawa Red Cross Centre. The erythrocytes were separated by centrifugation at 3000 rev./min (r 10.8 cm) for 10 min at 4°C, and washed twice with iso-osmotic saline (0.9% NaCl). After the removal of the buffy coats, the erythrocytes were suspended in iso-osmotic saline. Then 2.4 ml of 0.5 M-sodium nitrite dissolved in iso-osmotic saline was added to the 60 ml suspension (haematocrit 10%), and this was placed in air for 30 min. By this procedure, oxyhaemoglobin in the cells was converted into methaemoglobin. The erythrocytes were separated by centrifugation at 3000 rev./min for 10 min at 4°C, and were further washed five times with iso-osmotic saline in order to remove nitrite in the cells. The packed cells were suspended in glucose-free Ringer's solution containing 120 mM-NaCl, 5 mM-KCl, 2 mM-NaH₂PO₄ and 1 mM-MgCl₂ so as to give a haematocrit of about 10%.

After adjustment of the suspension pH to 7.4 at 37°C with 0.1 M-NaOH solution, incubation of the erythrocytes was initiated by the addition of final concentrations of 10 mM-glucose. The pH of the suspension was kept constant by a pH-stat (TOA auto-burette TBS-10A) at 37°C by titration with 0.1 M-NaOH solution containing 10 mM-glucose. In the case without the addition of Methylene Blue, streptomycin solution was added to the suspension (50 mg/100 ml of erythrocyte suspension).

(1) *Experiment in the absence of Methylene Blue.* The erythrocytes were incubated at pH 7.4 and 37°C after the addition of final concentrations of 10 mM-glucose for 32 h. During incubation there was little haemolysis. Samples (0.4 ml) were taken out at intervals for analyses, and mixed with 4 ml of ice-cold water. The haemolysates were then applied on Ampholine/polyacrylamide-gel plate (pH 3.5–9.5) and isoelectric focusing was performed. The gel plate was fixed with a solution containing 0.14 M-sulphosalicylic acid, 0.7 M-trichloroacetic acid and 7 M-methanol, and scanned by a Gilford spectrometer with a gel scanner. The percentage of each fraction was estimated by cutting out and weighing the chart paper.

(2) *Experiment in the presence of Methylene Blue.* The incubation of erythrocytes was carried out at pH 7.4 and 37°C after the addition of final con-

centrations of 10 mM-glucose and 10 μ M-Methylene Blue. Samples were taken out at intervals for analyses and haemolysed with 10 vol. of ice-cold water. Then the haemolysates were passed through a column (1.5 cm $\times$ 20 cm) of Sephadex G-25 (coarse grade) equilibrated with 10 mM-potassium phosphate buffer. The effluents were applied to the ampholine/gel plate. The gel plate was then fixed and scanned as described above. The percentage of each component (methaemoglobin, intermediate haemoglobins and oxyhaemoglobin) was estimated by cutting out and weighing the chart paper.

(3) *Simulation of the gel-scanning pattern of methaemoglobin reduction in the presence or absence of Methylene Blue.* The fractions of methaemoglobin, intermediate haemoglobins and oxyhaemoglobin as a function of time were fitted by using eqns. (1)–(3) derived from Scheme 1 through non-linear regression analysis (NONLIN program; Metzler, 1969). The digital computer FACOM M-160 was used at the Data-Processing Centre, Kanazawa University.

$$f_1 = f_1^0 e^{-k'_{+1}t} \quad (1)$$

$$f_2 = f_1^0 \frac{k'_{+1}}{k'_{+2} - k'_{+1}} (e^{-k'_{+1}t} - e^{-k'_{+2}t}) + f_2^0 e^{-k'_{+2}t} \quad (2)$$

$$f_3 = 1.0 + f_3^0 - (f_1 + f_2) \quad (3)$$

where f_1^0 , f_2^0 and f_3^0 are the fractions at zero time, and f_1 , f_2 and f_3 the fractions at time t of $(\alpha^3 + \beta^3)_2$, intermediate haemoglobins $[(\alpha^3 + \beta^2)_2 + (\alpha^2 + \beta^3)_2]$ and $(\alpha^2 + \beta^2)_2$ of total haem respectively. The differential equations for three species of haem molecules can be solved by using appropriate Laplace transformation techniques to yield eqns. (1)–(2) for the fractions of methaemoglobin and intermediate haemoglobins of total haem. The mass balance produces eqn. (3) for the fraction of oxyhaemoglobin.

Results and Discussion

Methaemoglobin reduction in the absence of Methylene Blue

Fig. 1(a) shows the analysis of methaemoglobin, intermediate haemoglobins and oxyhaemoglobin in human erythrocytes, which were incubated for 32 h in the absence of Methylene Blue, by isoelectric focusing on ampholine/gel plates. During the incubation of erythrocytes, methaemoglobin was

Scheme 1. Reduction of methaemoglobin in erythrocytes

Fig. 1. Isoelectric-focusing pattern of samples on Ampholine/polyacrylamide-gel plates

(a) Gel photographs relating to samples obtained at intervals during methaemoglobin reduction of erythrocytes in the absence of Methylene Blue. The arrows numbered 1, 2, 3, 4, 5, 6 show methaemoglobin A₂, oxyhaemoglobin A₂, methaemoglobin, ($\alpha^3\beta^2$)₂, ($\alpha^2\beta^3$)₂ and oxyhaemoglobin respectively. (b) Scan at 630nm of the gel obtained for the 16 h-incubated sample.

changed to intermediate haemoglobins and oxyhaemoglobin consecutively. The intermediate haemoglobins were identified as ($\alpha^3\beta^2$)₂ and ($\alpha^2\beta^3$)₂ valency hybrids from their characteristic positions on electrophoresis (Tomoda *et al.*, 1978a). From the results, the course of methaemoglobin reduction in the erythrocytes can be considered as:

Although only ($\alpha^3\beta^2$)₂ and ($\alpha^2\beta^3$)₂ were considered as intermediate haemoglobins because of the presence of these haemoglobins on electrophoresis, the above scheme is a simplification, for intermediate species of haemoglobins that have accepted only one electron per tetramer must in principle exist.

Rein *et al.* (1964) showed that NO-haemoglobin (nitrogenmonoxyhaemoglobin) is significantly detectable by e.p.r. signals when nitrite was added to the erythrocyte suspension. Since NO-haemoglobin is rapidly oxidized to methaemoglobin, probably via intermediate haemoglobins under aerobic conditions (Kiese, 1974), it is possible that NO-haemoglobin, even if present in erythrocytes, may change to intermediate haemoglobins and methaemoglobin during haemolysing treatment of the cells. The intermediate haemoglobins and a part of methaemoglobin observed in the zero-time sample on electrophoresis may therefore be derived from NO-haemoglobin. These intermediate haemoglobins were not present when purified haemoglobin was oxidized completely by nitrite (A. Tomoda & Y. Yoneyama, unpublished work).

The electrophoretic pattern of the sample at 16 h was further analysed by gel scanning (Fig. 1b). Consequently the percentages of each component such as methaemoglobin, ($\alpha^3\beta^2$)₂, ($\alpha^2\beta^3$)₂ and oxyhaemoglobin were estimated to be 32.1, 26.4, 21.6 and 19.9% respectively. The electrophoretic patterns shown in Fig. 1(a) were also analysed by gel scanning. The fractional changes in methaemoglobin, intermediate haemoglobins and oxyhaemoglobin during methaemoglobin reduction in the erythrocytes were measured (Table 1). The amount of ($\alpha^3\beta^2$)₂ was always higher than that of ($\alpha^2\beta^3$)₂ during the reduction. These results are further illustrated in Fig. 2. The observed values of each component such as methaemoglobin, intermediate haemoglobins and oxyhaemoglobin were fitted to the curves obtained by the simulation of the values observed. In this case, the changes in intermediate haemoglobins such as ($\alpha^2\beta^3$)₂ and ($\alpha^3\beta^2$)₂ were depicted together in the

Table 1. Fractions (%) of methaemoglobin, $(\alpha^3\beta^{2+})_2$, $(\alpha^2\beta^{3+})_2$ and oxyhaemoglobin during methaemoglobin reduction in nitrite-treated erythrocytes in the absence of Methylene Blue

The gels shown in Fig. 1 were scanned and the fractions of haemoglobin derivatives such as methaemoglobin, $(\alpha^3\beta^{2+})_2$, $(\alpha^2\beta^{3+})_2$ and oxyhaemoglobin were estimated.

	Time (h) ...	Fraction (%)								
		0	4	8	10	16	20	24	28	32
MetHb		89.0	75.4	56.9	53.7	32.1	19.4	10.6	11.3	5.9
$(\alpha^3\beta^{2+})_2$		8.2	15.9	23.0	21.8	26.4	26.9	28.8	26.9	20.0
$(\alpha^2\beta^{3+})_2$		2.8	7.6	16.1	17.2	21.6	20.1	11.4	12.0	10.0
HbO ₂		0	1.1	4.0	7.3	19.9	33.6	49.2	49.8	64.1

Fig. 2. Fractional changes in methaemoglobin, intermediate haemoglobins and oxyhaemoglobin during the reduction of methaemoglobin in the absence of Methylene Blue

The results in Fig. 1 (a) were analysed by scanning at 630nm and the fractions of methaemoglobin, intermediate haemoglobins and oxyhaemoglobin were plotted against time. ●, Methaemoglobin; ●, intermediate haemoglobin involving $(\alpha^3\beta^{2+})_2$ and $(\alpha^2\beta^{3+})_2$; ○, oxyhaemoglobin. The curves drawn through the experimental points were obtained by the computer analysis.

Figure because it was difficult to determine all the reaction rate constants (k_{+1} – k_{+4}), owing to the experimental errors, which are probably generated by the conversion of NO–haemoglobin to methaemoglobin during the haemolysing procedure. The reaction rate constants of the two phases involving methaemoglobin $\xrightarrow{k'_{+1}}$ intermediate haemoglobins, and intermediate haemoglobins $\xrightarrow{k'_{+2}}$ oxyhaemoglobin, which were obtained by computer analysis, are listed in Table 2.

Methaemoglobin reduction in the presence of Methylene Blue

The fractions of each components, such as methaemoglobin, $(\alpha^3\beta^{2+})_2$, $(\alpha^2\beta^{3+})_2$ and oxy-

Table 2. Rate constants estimated by non-linear least-squares analysis for the reduction of methaemoglobin in the presence or absence of Methylene Blue

The values represent the rate constant $\pm$ s.d. The rate constants k'_{+1} and k'_{+2} fitting best to all experimental points were obtained by use of NONLIN computer program (Metzler, 1969) according to eqns. (1)–(3).

	$10^3 \times$ Rate constants (min^{-1})	
	Methylene Blue absent	Methylene Blue present
k'_{+1}	1.138 ± 0.038	37.47 ± 0.36
k'_{+2}	0.802 ± 0.030	29.29 ± 0.27

haemoglobin to total haemoglobin during methaemoglobin reduction in the presence of Methylene blue are shown in Table 3. As a result, the changes in these haemoglobin derivatives were much accelerated compared with the case without the dye shown in Table 1. The amount of $(\alpha^3\beta^{2+})_2$, however, was always higher than that of $(\alpha^2\beta^{3+})_2$. These results show that methaemoglobin reduction in the presence of Methylene Blue proceeds by two different pathways: methaemoglobin $\rightarrow (\alpha^3\beta^{2+})_2 \rightarrow$ oxyhaemoglobin; and methaemoglobin $\rightarrow (\alpha^2\beta^{3+})_2 \rightarrow$ oxyhaemoglobin in the erythrocytes.

The results shown in Table 3 were further treated by digital computer, as shown in Fig. 3. The observed values of each of the haemoglobin derivatives such as methaemoglobin, intermediate haemoglobins and oxyhaemoglobin were fitted to the curves obtained by the computer simulation. The reaction rate constants (k'_{+1} and k'_{+2}) of two phases involving methaemoglobin $\xrightarrow{k'_{+1}}$ intermediate haemoglobins, and intermediate haemoglobins $\xrightarrow{k'_{+2}}$ oxyhaemoglobin are listed in Table 2.

Some considerations concerning methaemoglobin reduction in the erythrocytes

It has been shown that there are two main

Table 3. Fractions (%) of methaemoglobin, $(\alpha^{3+}\beta^{2+})_2$, $(\alpha^{2+}\beta^{3+})_2$ and oxyhaemoglobin during methaemoglobin reduction in the nitrite-treated erythrocytes in the presence of Methylene Blue

After isoelectric focusing of the samples obtained during the reduction of methaemoglobin in the presence of Methylene Blue, the gel plate was fixed and was then scanned at 630nm. By this procedure the fractions of methaemoglobin, $(\alpha^{3+}\beta^{2+})_2$, $(\alpha^{2+}\beta^{3+})_2$ and oxyhaemoglobin were estimated.

	Time (min) ...	Fraction (%)							
		0	6	10	20	40	60	70	80
MetHb		85.5	77.3	63.8	47.1	26.8	0.5	1.3	0
$(\alpha^{3+}\beta^{2+})_2$		9.4	16.6	26.2	35.1	32.9	20.3	17.5	5.7
$(\alpha^{2+}\beta^{3+})_2$		4.9	5.7	7.8	13.3	15.4	7.0	5.4	3.2
HbO ₂		0.2	0.4	2.2	4.5	24.9	72.2	75.8	91.1

pathways for reducing methaemoglobin, which linked to NADH produced in the glycolytic pathway, and to NADPH produced in the pentose phosphate pathway. The former operates mainly for the reduction of methaemoglobin in erythrocytes in the absence of Methylene Blue. The latter, however, does not (Scott & Hoskins, 1958; Kiese, 1944). These are illustrated as follows:

NADPH-dependent methaemoglobin reductase in the erythrocytes. Scott *et al.* (1963) estimated the contribution of NADH- and NADPH-dependent methaemoglobin reductase as 73 and 6% respectively of total methaemoglobin reducing activity in normal erythrocytes. Present results showed that NADPH-dependent methaemoglobin-reducing systems are activated by Methylene Blue by as much as

Therefore the results shown in Figs. 1 and 2 reflect the actual reduction activity of NADH-dependent methaemoglobin reductase (NADH-cytochrome b₅ reductase) in the erythrocytes. In this case the possibility that the reduction rate was apparently modified by many hours incubation of the erythrocytes may be excluded, because the autooxidation of haemoglobin is suppressed at pH 7.4 and 37°C in erythrocytes (Tomoda *et al.*, 1978b). On the other hand, since NADPH-dependent methaemoglobin-reducing systems are much stimulated in the presence of Methylene Blue, and NADH-dependent systems are not (Rosen *et al.*, 1971), the reduction of methaemoglobin shown in Table 3 and Fig. 3 expresses the actual contribution

of 34-fold compared with NADH-dependent methaemoglobin-reducing systems (Table 2). Thus NADPH-dependent methaemoglobin-reducing systems are activated about 400-fold by the addition of Methylene Blue compared with the case without the dye. It has been uncertain whether the intermediate haemoglobins are produced during the reduction of methaemoglobin in erythrocytes. Present results clearly show that methaemoglobin is reduced by way of intermediate haemoglobins such as $(\alpha^{3+}\beta^{2+})_2$ and $(\alpha^{2+}\beta^{3+})_2$ to oxyhaemoglobin by NADH- and NADPH-dependent reducing systems in the cells. It is likely that there is no difference between methaemoglobin and intermediate haemoglobins as a

Fig. 3. Fractional changes in methaemoglobin, intermediate haemoglobins and oxyhaemoglobin during the reduction of methaemoglobin in the presence of Methylene Blue

The results in Table 3 were plotted against time. ●, Methaemoglobin; ●, intermediate haemoglobins; ○, oxyhaemoglobin. The curves drawn through the experimental points were obtained by the computer analysis.

substrate for either NADH- or NADPH-dependent reducing systems, because the reaction rate constants, k'_{+1} (of the first phase) and k'_{+2} (of the second phase), were almost equal in each case for the presence or absence of Methylene Blue. However, there seem to be differences in the reaction with the electron carriers such as cytochrome b_5 and Methylene Blue between α - and β -chains of methaemoglobin tetramer, because the amount of $(\alpha^{3+}\beta^{2+})_2$ was higher than that of $(\alpha^{2+}\beta^{3+})_2$ during the reduction of methaemoglobin in the presence and absence of the dye. These results are consistent with the previous results (Tomoda *et al.*, 1979a) that the proportion of $(\alpha^{3+}\beta^{2+})_2$ is always higher than that of $(\alpha^{2+}\beta^{3+})_2$ during methaemoglobin reduction by the systems including a purified erythrocyte NADH-cytochrome b_5 reductase and cytochrome b_5 , and with the results of Yubisui and colleagues (T. Yubisui, A. Tomoda & Y. Yoneyama, unpublished work) that the proportion of $(\alpha^{3+}\beta^{2+})_2$ is much higher than that of $(\alpha^{2+}\beta^{3+})_2$ during the reduction of

methaemoglobin by the systems including a purified erythrocyte NADPH-flavin reductase and Methylene Blue. The reason for the differences in the proportion of $(\alpha^{3+}\beta^{2+})_2$ and $(\alpha^{2+}\beta^{3+})_2$ may be explained by the differences in the redox potentials between α - and β -chains in the methaemoglobin tetramer. The redox potentials of α - and β -chains are 0.052 and 0.113 V respectively (Banerjee & Cassoly, 1969). Therefore cytochrome b_5 and Methylene Blue, whose redox potentials are 0.02 (Velick & Strittmatter, 1956) and 0.011 respectively, may reduce β -chains of methaemoglobin preferentially.

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