Changes in Intermediate Haemoglobins during Methaemoglobin Reduction by NADPH–Flavin Reductase

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The changes in intermediate haemoglobins produced during methaemoglobin reduction by NADPH–flavin reductase were analysed by an isoelectric-focusing method. The $\alpha^3\beta^{2+}$ and $\alpha^2\beta^{3+}$ valency hybrids were observed as intermediate haemoglobins and changed consecutively with time during the reaction. On the basis of the analyses, the course of methaemoglobin reduction was found to involve two different pathways: (1) methaemoglobin $\xrightarrow{k_{+1}} \alpha^3\beta^{2+} \xrightarrow{k_{+2}}$ oxyhaemoglobin; (2) methaemoglobin $\xrightarrow{k_{+3}} \alpha^2\beta^{3+} \xrightarrow{k_{+4}}$ oxyhaemoglobin. The reaction rate constants of each phase (k_{+1} – k_{+4}) were also estimated. The addition of inositol hexaphosphate to the reaction mixture did not affect the overall reaction. The mechanism of methaemoglobin reduction by NADPH–flavin reductase is discussed on the basis of these results.

Though the characterization of methaemoglobin reduction in human erythrocytes has been well established (Huennekens *et al.*, 1968; Kuma *et al.*, 1976; Sugita *et al.*, 1971; Yubisui *et al.*, 1977), there are few reports on the changes in intermediate haemoglobins produced during the reaction. Previously we showed that the $\alpha^3\beta^{2+}$ valency hybrid was detected during the oxidation of haemoglobin in glucose-depleted erythrocytes (Tomoda *et al.*, 1978a) and during the reduction of methaemoglobin by ascorbic acid (Tomoda *et al.*, 1978b). These results showed that the isoelectric-focusing fractionation method is very useful for distinguishing between four haemoglobin derivatives: oxyhaemoglobin, $\alpha^3\beta^{2+}$ and $\alpha^2\beta^{3+}$ valency hybrids and methaemoglobin.

The present paper deals with analysis of the changes in intermediate haemoglobins produced during the enzymic reduction of methaemoglobin by NADPH–flavin reductase, which was purified as a methaemoglobin reductase from human erythrocytes by Yubisui *et al.* (1977), using the isoelectric-focusing method. Since the two intermediate haemoglobin $\alpha^3\beta^{2+}$ and $\alpha^2\beta^{3+}$ valency hybrids were detected during the reaction, the kinetic behaviour of these intermediate haemoglobins was studied. On the basis of the results, the mechanism of methaemoglobin reduction by the enzyme is discussed.

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Experimental

Materials

NADP⁺, glucose 6-phosphate, FMN, glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and catalase (EC 1.11.1.6) were purchased from Boehringer Mannheim (Mannheim, West Germany). Inositol hexaphosphate was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sephadex G-25 (fine and coarse), DEAE-cellulose DE-32 and Dowex 1–8 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), Whatman (Maidstone, Kent, U.K.) and Muromachi Chemicals (Tokyo, Japan) respectively. Ampholine polyacrylamide-gel plates (pH 3.5–9.5) were from LKB Produkter (Bromma 1, Sweden).

Preparation of methaemoglobin

Freshly obtained erythrocytes were haemolysed with 3 vol. of ice-cold distilled water and centrifuged at 10000 rev./min for 20 min. By passing the supernatant through a column (3 cm × 10 cm) of DEAE-cellulose DE-32 that had previously been equilibrated with 5 mM-potassium phosphate buffer (pH 7.0), NADPH-flavin reductase-free haemoglobin was obtained. The haemoglobin solutions were oxidized to methaemoglobin by addition of a slight excess (2 vol.) of K₃Fe(CN)₆ solution and passed through a Dowex 1–8 column. Then the solutions of methaemoglobin were desalted by passing them through a

column (2.4cm×50cm) of Sephadex G-25 (fine) previously equilibrated with 0.05M-Bistris buffer (2-[bis-(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol) (pH 7.0) containing 0.1M-NaCl.

Preparation of NADPH-flavin reductase

NADPH-flavin reductase was purified by the method of Yubisui *et al.* (1977) from human erythrocytes.

Methaemoglobin reduction by NADPH-flavin reductase

The reaction was initiated by the addition of 40 μ l of NADPH-flavin reductase (6.3mg/ml) to 6ml solutions containing 200 μ M-methaemoglobin (as haem), 90 μ M-FMN, 75 μ M-NADP⁺, 7mM-glucose 6-phosphate, glucose 6-phosphate dehydrogenase (0.013 unit) and catalase (8 units) in the presence or absence of 1mM-inositol hexaphosphate. Then, 0.5ml samples were removed at intervals and passed immediately through a Sephadex G-25 (coarse) column (1cm×30cm) at 4°C. These samples were applied to Ampholine polyacrylamide-gel plates.

In parallel with this experiment, the overall reaction was followed by measuring the decrease in the A_{630} . The reaction proceeds according to the following electron flows, where the steady-state was approximated for the enzymic process at the early stage of the reaction:

Fractionation of the samples by isoelectric focusing on ampholine polyacrylamide-gel plates

Methaemoglobin reduction by the enzyme systems was carried out as mentioned above, and the isoelectric focusing of the samples on Ampholine polyacrylamide-gel plates was performed at 4°C for 1.5h. The gel was then fixed with the fixing solutions containing 0.14M-sulphosalicylic acid, 0.7M-trichloroacetic acid and 7M-methanol, and scanned at 630nm with a Gilford 2400-S spectrometer with a gel scanner. The percentage of each fraction containing methaemoglobin, intermediate haemoglobins ($\alpha^3\beta^{2+}$ and $\alpha^2\beta^{3+}$) and oxyhaemoglobin was estimated by cutting out and weighing the chart paper.

Simulation of the gel-scanning pattern of methaemoglobin reduction by NADPH-flavin reductase

The plots obtained by gel scanning as described above were analysed by using eqns. (1)–(4) derived in Scheme 1. The computer analysis was carried out on a FACOM 230-35 digital computer at the Data Processing Center, Kanazawa University.

Scheme 1. Reduction of methaemoglobin by NADPH-flavin reductase

$$f_1 = e^{-(k_{+1}+k_{+3})t} \quad (1)$$

$$f_2 = \frac{k_{+1}}{k_{+1}+k_{+3}-k_{+2}} [e^{-k_{+2}t} - e^{-(k_{+1}+k_{+3})t}] \quad (2)$$

$$f_3 = \frac{k_{+3}}{k_{+1}+k_{+3}-k_{+4}} [e^{-k_{+4}t} - e^{-(k_{+1}+k_{+3})t}] \quad (3)$$

$$f_4 = 1.0 - (f_1 + f_2 + f_3) \quad (4)$$

where f_1 – f_4 are the fractions of each component, namely methaemoglobin, $\alpha^3\beta^{2+}$, $\alpha^2\beta^{3+}$ and oxyhaemoglobin, to total haem. In the reduction of methaemoglobin the steady-state condition can be applied to the concentration of reduced flavin, and the rate-limiting steps will be as indicated in the Scheme. The differential equations for four species of haem molecules can be solved by using appropriate Laplace transformation techniques to yield eqns. (1)–(3) for the fractions of methaemoglobin, and the two intermediate haemoglobins to total haem. The mass balance produces eqn. (4) for the fraction of oxyhaemoglobin.

Results

Methaemoglobin reduction by NADPH-flavin reductase

Fig. 1 shows the semi-logarithmic plots of the changes in absorbance at 630nm during methaemoglobin reduction by NADPH-flavin reductase. The reaction proceeded by apparent first-order kinetics and no effect of inositol hexaphosphate on the reaction was observed.

Isoelectric-focusing pattern of methaemoglobin reduction by NADPH-flavin reductase

Since it is difficult to deduce the changes in intermediate haemoglobin concentrations produced during the reduction of methaemoglobin by the enzyme from the decrease in absorbance shown in Fig. 1, we studied these changes by isoelectric focusing. As shown in Fig. 2, the two intermediate haemoglobins were detected during the reduction of methaemoglobin by NADPH-flavin reductase.

Fig. 3 shows the gel-scanning pattern of the sample

Fig. 1. Absorbance changes during methaemoglobin reduction by NADPH-flavin reductase

The overall reaction was followed by measuring the decrease in A_{630} in the presence (●) or absence (○) of inositol hexaphosphate after the addition of NADPH-flavin reductase.

Table 1. Rate constants estimated by non-linear least-squares analysis for the reduction of methaemoglobin by NADPH-flavin reductase

The values represent the rate constants $\pm$ s.d. s.d. of the i th rate constant (k_i) was estimated from $s.d. = (S^2 \cdot a^{ii})^{1/2}$, where S^2 is the sum of the weighed squared deviations divided by the degree of freedom, and a^{ii} is the entry for the i th diagonal element in the matrix, A^{-1} , where A is the matrix of sums of cross-products of certain partial derivatives.

	Rate constants (min^{-1})	
	- Inositol hexaphosphate	+ Inositol hexaphosphate
$10^2 \times k_{+1}$	10.02 ± 0.51	9.20 ± 0.61
$10^2 \times k_{+2}$	5.66 ± 0.38	5.53 ± 0.50
$10^2 \times k_{+3}$	3.07 ± 0.45	3.48 ± 0.56
$10^2 \times k_{+4}$	2.89 ± 0.88	4.35 ± 1.13

at 20min. The two intermediate haemoglobins (IB_{11} and IB_1) were identified as $\alpha^3+\beta^{2+}$ and $\alpha^2+\beta^{3+}$ valency hybrids by their distances from the band of methaemoglobin. [Previously we showed that the second and third bands from the cathode correspond to $\alpha^3+\beta^{2+}$ and $\alpha^2+\beta^{3+}$ valency hybrids respectively (Tomoda *et al.*, 1978b).]

Fig. 2. Electrophoretic patterns on Ampholine polyacrylamide-gel plates of samples of methaemoglobin during reduction by NADPH-flavin reductase

The samples were obtained at intervals (the numbers indicate minutes after start of reaction) for analyses during the reduction of methaemoglobin by NADPH-flavin reductase in the absence of inositol hexaphosphate. Abbreviations: MetHb, methaemoglobin; HbO_2 , oxyhaemoglobin.

Fig. 3. Electrophoretic mobility of the partially reduced methaemoglobin solutions (sampled at 20 min)

The relative mobility of each component, namely methaemoglobin, intermediate haemoglobins (IB_{11} and IB_1) and oxyhaemoglobin, is shown, compared with that of the reconstituted $\alpha^3+\beta^{2+}$ and $\alpha^2+\beta^{3+}$ valency hybrid (dotted lines), the details of which are described by Tomada *et al.* (1978b).

Fig. 4. Gel-scanning pattern of methaemoglobin reduction by NADPH-flavin reductase

Haem contents (%) of each of the components, namely methaemoglobin, intermediate haemoglobins and oxyhaemoglobin, which were analysed by isoelectric focusing on Ampholine polyacrylamide-gel plates, were plotted against time. The continuous lines show the curves generated from eqns. (1)–(4) in Scheme 1 by using the rate constants listed in Table 1. ●, Methaemoglobin; ●, $\alpha^3\beta^{2+}$; △, $\alpha^2\beta^{3+}$; ○, oxyhaemoglobin.

Analysis of changes in methaemoglobin, intermediate haemoglobins and oxyhaemoglobin

Fig. 4 shows plots of each component, namely methaemoglobin, $\alpha^3\beta^{2+}$, $\alpha^2\beta^{3+}$ and oxyhaemoglobin, in the absence of inositol hexaphosphate that were obtained from gel-scanning analysis of the data shown in Fig. 2. By non-linear curve-fitting analysis of each plot by using the equations in Scheme 1 (f_1 – f_4), it is possible to estimate the pseudo-reaction-rate constants of each phase, i.e.

methaemoglobin $\xrightarrow{k_{+1}} \alpha^3\beta^{2+} \xrightarrow{k_{+2}} \text{oxyhaemoglobin}$ and

methaemoglobin $\xrightarrow{k_{+3}} \alpha^2\beta^{3+} \xrightarrow{k_{+4}} \text{oxyhaemoglobin}$. The reaction rate constants (k_{+1} – k_{+4}) were almost unchanged when measured in the presence of inositol hexaphosphate (results not shown). This result is in good agreement with the result shown in Fig. 1.

Discussion

Though the intermediate haemoglobins have been demonstrated to be present in partially oxidized haemoglobin solutions by different methods (Huisman, 1966; Chanutin & Curnish, 1966; Bunn & Drysdale, 1971), there are some conflicting results. Bunn & Drysdale (1971) indicated that the intermediate bands (IB_I and IB_{II}) between oxyhaemoglobin and methaemoglobin obtained on isoelectric focusing on polyacrylamide gel were probably those of $\alpha^3\beta^{2+}$ and $\alpha^2\beta^{3+}$ valency hybrids. We have shown that the $\alpha^3\beta^{2+}$ valency hybrid appears during oxidation of haemoglobin in glucose-depleted

erythrocytes (Tomoda *et al.*, 1978a) and during reduction of methaemoglobin by ascorbic acid (Tomoda *et al.*, 1978b). Since the present results demonstrate that the $\alpha^3\beta^{2+}$ and $\alpha^2\beta^{3+}$ valency hybrids were present during reduction of methaemoglobin by NADPH-flavin reductase, the following two different pathways for methaemoglobin reduction are considered: (1) methaemoglobin $\xrightarrow{k_{+1}} \alpha^3\beta^{2+} \xrightarrow{k_{+2}} \text{oxyhaemoglobin}$; (2) methaemoglobin $\xrightarrow{k_{+3}} \alpha^2\beta^{3+} \xrightarrow{k_{+4}} \text{oxyhaemoglobin}$.

From the reaction rate constants k_{+1} and k_{+3} , the contribution of the two different pathways of methaemoglobin reduction were deduced. Pathway (1) is predominant (76%), with pathway (2) contributing 24% in the absence of inositol hexaphosphate. In the presence of inositol hexaphosphate, the contributions of the two pathways are almost unchanged [pathways (1) 73% and pathway (2) 27%]. The differences in the contributions of the two pathways may be explained by the functional non-equivalence between the α - and β -chains in the methaemoglobin tetramer indicated by some authors (Gibson *et al.*, 1969; Klapper & Uchida, 1971; MacQuarrie & Gibson, 1971).

We have previously found that methaemoglobin reduction by ascorbic acid is accelerated as much as 10 times by the presence of inositol hexaphosphate (Tomoda *et al.*, 1976), and have showed that this stimulatory effect is restricted to β -chains of the protein (Tomoda *et al.*, 1978a). Furthermore, it has been observed that reduction of methaemoglobin by reduced glutathione and by cysteine is much accelerated in the presence of inositol hexaphosphate (A. Tomoda, unpublished work). Taketa & Chen (1977) reported that reduction of methaemoglobin by NADH-methaemoglobin reductase is accelerated as much as 5 times by the presence of inositol hexaphosphate. Therefore the reduction mechanism of NADPH-flavin reductase may well be different from that of various reductants and NADH-methaemoglobin reductase, because there was little stimulatory effect by inositol hexaphosphate on methaemoglobin reduction by the former enzyme.

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