Stopped Flow Studies on the Nonenzymatic Reduction of Methemoglobin by Reduced Flavin Mononucleotide*

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The nonenzymatic reduction of methemoglobin by the reduced form of flavin mononucleotide was studied under various conditions by following the reaction with a stopped flow apparatus. The reaction was very fast, compared with the reduction of the flavin by NADPH-flavin reductase of human erythrocytes, and followed a second order rate law: the rate constant (K) for the reduction of methemoglobin by reduced flavin mononucleotide was determined to be 5.5 × 10^6 M⁻¹ s⁻¹ in 50 mM phosphate buffer (pH 7.0) at 25°C. The reaction was not influenced by changing phosphate buffer concentration from 10 to 100 mM. The rate of reduction at the physiological pH, 7.0, was about 95% of the maximal value that observed at around pH 6.4. Formation of deoxyhemoglobin and oxidized form of flavin mononucleotide by the reaction proceeded stoichiometrically in a ratio of unity. These results apparently indicate that the limiting step for the reduction of methemoglobin by the NADPH-flavin reductase system in human erythrocytes is the enzymatic reduction of flavin.

Previously we explained that NADPH-linked reduction of methemoglobin in human erythrocytes is brought about by NADPH-flavin reductase (1), which has been called NADPH-methemoglobin reductase (3-5). Overall reduction of methemoglobin by the enzyme through flavin is, however, a slow reaction, and proceeds in two steps; the enzymatic reduction of flavin, and subsequent nonenzymatic reduction of methemoglobin by the reduced form of flavin. The maximal activity of the reduction of flavin by the enzyme was determined to be 1 μmol/min/mg of protein (2). We also described that the nonenzymatic reduction of methemoglobin by the reduced form of flavin seems to be a very fast reaction (1). In this study we report the determination of the rate of the reduction of methemoglobin by the reduced form of flavin under various conditions. From the second order rate constant for the nonenzymatic reduction of methemoglobin by FMNH₂ obtained in this study, and the maximal enzymatic activity of the reduction of FMNH₂ by the NADPH-flavin reductase (2), the enzymatic reduction of flavin is suggested to be the rate-limiting step for the reduction of methemoglobin by the NADPH-flavin reductase system in human erythrocytes. Comparison of the rate of reduction of methemoglobin by FMNH₂ with that by other low molecular weight compounds is also described.

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MATERIALS AND METHODS
Catalase (EC 1.1.1.4), FMN, FAD, and glucose oxidase (EC 1.1.3.4) were purchased from Boehringer Mannheim, Germany. Other reagents were obtained commercially. Sephadex G-25 was a product of Pharmacia Fine Chemicals, Uppsala, Sweden.

Preparation of Methemoglobin—Freshly obtained human red cells were washed sufficiently with isotonic saline, and finally hemolyzed by adding two volumes of distilled water in the cold. The hemolysate was centrifuged to remove stroma at 10,000 rpm for 15 min. To the supernatant an equal volume of 10 mM potassium ferricyanide was added to oxidize hemoglobin. The solution was applied to a small column (0.9 × 5 cm) of Dowex 1-8 to remove excess of ferricyanide and ferrocyanide. The methemoglobin eluted from the column was passed through a Sephadex G-25 column (1.8 × 50 cm) to equilibrate the solution with 10 mM phosphate buffer (pH 7.0).

Stopped Flow Measurements—Stopped flow measurements were performed with a Union stopped flow apparatus (Union Giken, Osaka, Japan), model RA 401 fitted with a computer system 71 (Union Giken, Osaka, Japan). The stopped flow traces were monitored on an oscilloscope, and a smoothed curve computed from the summation of every 2 to 3 runs was recorded. Rapid mixing of the reaction mixtures from the reservoirs in this apparatus was performed by high pressure nitrogen gas (5 kg/cm²).

Reduction of Methemoglobin—Reduction of methemoglobin by FMNH₂, was determined by following the absorbance change at 630 nm or at 556 nm after mixing the two reaction mixtures which contain methemoglobin or flavin, respectively. The reactions were determined using a 10-mm light path cell at 24 to 26°C. Four milliliters of the reaction mixture in one side of the reservoirs in the apparatus contained 50 mM phosphate buffer (pH 7.0), 40 mg of glucose, 40 μg of glucose oxidase (4.2 units), 20 μg of catalase (1000 units), 5 mM EDTA, and 40 to 90 μm methemoglobin (on heme basis). To the other side of the reservoirs was added 4 ml of the reaction mixture which contained the same components as described above, except that methemoglobin was replaced by flavin, 20 to 200 μM. The concentration of stock solution of methemoglobin was determined by the pyridine hemochromogen method, using a millimolar extinction coefficient of 34.4 at 557 nm (6). The concentration of flavin was determined as described previously (2). These spectrophotometric determinations were performed with a Union spectrophotometer, model SM 401.

Oxidation of FMNH₂.—Oxidation of FMNH₂ by the reaction with methemoglobin was determined by following the absorbance change at 445 nm, an isobestic point for deoxyhemoglobin and methemoglobin.

Photoreduction of Flavin—Before mixing the two reaction mixtures, the solutions in the reservoirs were allowed to stand for at least 15 min to make them oxygen-free by the action of the glucose-glucose oxidase system. Flavin in the reaction mixture was then photoreduced by illumination with a tungsten lamp (300 watts) at a distance of 35 cm from the reservoirs. EDTA (5 mM) was used as an electron donor for the photoreduction of flavin by the method of McCormick et al. (7). Anaerobiosis by the glucose-glucose oxidase system used in this study was very effective, and did not cause any denaturation of methemoglobin. Bubbling of nitrogen gas through the reaction mixture to remove oxygen was not performed, because it caused denaturation of methemoglobin (precipitation of the protein), and moreover it required a much longer time to accomplish anaerobiosis than that by the glucose-glucose oxidase system. When the anaerobiosis by the system was monitored with hemoglobin, deoxygenation was completed within 10 min.
RESULTS

Photoreduction of Flavin—Fig. 1 shows the photoreduction of FMN as a function of illumination time under anaerobic conditions. The results show that after 15 min of illumination, approximately 85% of the flavin was constantly photoreduced. Based on these results, all experiments for the reduction of methemoglobin were done assuming that always 85% of FMN added to the reaction mixture was photoreduced form.

Reduction of Methemoglobin—Examples of the stopped flow measurements on the reduction of methemoglobin by FMNH$_2$ are shown in Fig. 2. By the analyses of the traces obtained both at 630 nm and 556 nm, the reaction was found to follow a typical first order. Oxidation of FMNH$_2$ by the reaction was also a first order reaction as described below.

Fig. 3 shows the effect of pH on the reduction of methemoglobin. Although optimal activity was found at around pH 6.4, as the activity obtained at the physiological pH, 7.0, was close to the optimal value (about 95%), the other experiments in this study were carried out at the physiological pH, 7.0.

The effect of salt concentration was examined by changing the phosphate buffer concentration at pH 7.0. The rates of reduction of methemoglobin were not at all influenced by increasing the buffer concentration from 10 mM up to 100 mM.

In Fig. 4, the apparent first order rate constants for the reduction of methemoglobin obtained are plotted against flavin concentrations. The rate constants obtained were increased linearly by increasing flavin concentration up to 100 μM. As shown in the figure, the rate constants obtained, both with 26 and 45 μM methemoglobin (cell concentration), were very similar to each other at every flavin concentration. The second order rate constant was calculated from the slope in the figure to be 5.5 x 10$^6$ M$^{-1}$ s$^{-1}$ in 50 mM phosphate buffer (pH 7.0) at 25°C. The effect of methemoglobin concentration on the rate of reduction of methemoglobin by FMNH$_2$ was examined at 84 μM of the flavin (cell concentration) by changing the methemoglobin concentration from 11 to 52 μM (cell concentration). The rate constants obtained were all very similar values, indicating that the results obtained in this study are reliable.

Stoichiometry of the reduction of methemoglobin by FMNH$_2$ was examined by following the formation of products. As shown in Table I, the rate of deoxyhemoglobin formation and that of FMN formation examined at various substrate concentrations coincided well with each other. The results indicate that methemoglobin (heme) reacts with FMN in a ratio of 1:1.
Methemoglobin Reduction by FMNH₂

**TABLE I**

**Stoichiometry of the nonenzymatic reduction of methemoglobin with FMNH₂**

<table>
<thead>
<tr>
<th>Methemoglobin</th>
<th>FMNH₂</th>
<th>Deoxyhemoglobin</th>
<th>FMN</th>
</tr>
</thead>
<tbody>
<tr>
<td>μM</td>
<td>μM</td>
<td>kₛ⁻¹</td>
<td>kₛ⁻¹</td>
</tr>
<tr>
<td>52</td>
<td>19.9</td>
<td>95.3</td>
<td>96.9</td>
</tr>
<tr>
<td>52</td>
<td>39.8</td>
<td>150.4</td>
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<td>52</td>
<td>59.7</td>
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<tr>
<td>26</td>
<td>10.0</td>
<td>60.6</td>
<td>64.5</td>
</tr>
<tr>
<td>26</td>
<td>39.8</td>
<td>184.8</td>
<td>179.6</td>
</tr>
<tr>
<td>26</td>
<td>59.7</td>
<td>266.3</td>
<td>263.8</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Previously, we stated that the reduction of methemoglobin by NADPH-flavin reductase proceeds in two steps; the enzymatic reduction of flavin, and subsequent nonenzymatic reduction of methemoglobin by the reduced form of flavin (1, 2). Maximal activity for flavin reduction by the purified NADPH-flavin reductase of human erythrocytes was 1 μmol/min/mg of enzyme protein (2). Present results revealed that the nonenzymatic reduction of methemoglobin by the reduced form of flavin is a very fast reaction with a second order rate constant of 5.5 × 10⁷ M⁻¹ s⁻¹ (3.3 × 10⁷ M⁻¹ min⁻¹) at 25°C in 50 mM phosphate buffer (pH 7.0). When the concentrations of methemoglobin and flavin in human erythrocytes were assumed to be 50 μM and 1 μM, respectively, the rate of methemoglobin reduction by flavin could be 1.65 × 10⁻⁸ M/min, based on the second order rate constant obtained in this study. In the previous paper we reported that the overall rate of methemoglobin reduction in human erythrocytes would be about 50% of that at pH 6.4, and was not influenced by changing the salt concentration up to 100 mM phosphate buffer. These findings suggest that FMNH₂ is a very effective reductor of methemoglobin. In Table II the rate of methemoglobin reduction by FMNH₂ obtained in this study is compared with that by other low molecular weight compounds such as glutathione (9), ascorbic acid (10), or 5-hydroxyanthranilic acid (11). The second order rate constant for nonenzymatic reduction of methemoglobin by FMNH₂ is 10⁻² to 10⁻³-fold higher than those rate constants by other reductants. These findings indicate that flavin is one of the most effective reductants of methemoglobin in human erythrocytes. Gibson and Hastings (12) have reported that FMNH₂ is a very effective reductor of oxygen, and also of potassium ferricyanide. They suggested that the rate of reduction of ferricyanide by FMNH₂ must be of the order of 10⁴ M⁻¹ s⁻¹.

The reduction product of methemoglobin by FMNH₂ was studied by following the absorbance changes at various wavelengths. Fig. 5 shows the plots of the absorbance changes at various wavelengths obtained by the stopped flow studies; and those were compared to the difference spectrum, dithionite-reduced minus methemoglobin, which was obtained statically with an ordinary spectrophotometer. As the whole absorbance change is not detected by the stopped flow studies due to the dead time (5 ms) of the apparatus, two times the absorbance changes at various wavelengths obtained by the stopped flow apparatus were plotted, and those fit well the curve by chance. These results indicate that the product is the typical deoxyhemoglobin, and no intermediate product was found with the apparatus within the range from 500 to 650 nm.

In this study the glucose-glucose oxidase system was used to make the reaction mixtures oxygen-free. As the glucose oxidase is a flavin-containing enzyme, the effect of the enzyme on the reduction of methemoglobin was examined under the same conditions used in this study without the free flavin. No detectable reduction of methemoglobin, however, was found by the glucose oxidase contained in the reaction mixtures used in this study.

Reduction of methemoglobin by FADH₂ was also examined in the present study. As was described by McCormick et al. (7), photoreduction of FAD using EDTA as an electron donor required a much longer time to complete than that of FMN. The photoreduction of FAD carried out under the same conditions used for the photoreduction of FMN revealed that only about 50% of FAD added to the reaction mixture was reduced after 30 min, and the flavin was still continuously present.

**TABLE II**

**Rate constants (K_{red}) for nonenzymatic reduction of methemoglobin by naturally occurring low molecular weight compounds**

<table>
<thead>
<tr>
<th>Reductant</th>
<th>K_{red} (μM⁻¹ min⁻¹)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMNH₂</td>
<td>3.3 × 10⁸</td>
<td>This paper</td>
</tr>
<tr>
<td>Glutathione (reduced)</td>
<td>5.6 × 10⁻⁹</td>
<td>(7)</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>1.8 × 10⁻¹³</td>
<td>(8)</td>
</tr>
<tr>
<td>5-Hydroxyanthranilic acid</td>
<td>2.8 × 10⁻¹³</td>
<td>(9)</td>
</tr>
</tbody>
</table>

*Calculated from data in Fig. 6 in Ref. 7.  
*Calculated from data in Table III and methemoglobin concentration in Ref. 8.
Methemoglobin Reduction by FMNH₂

Reducing. As the quantitative estimation of FADH₂ was difficult, and the results of methemoglobin reduction under these conditions were not reproducible, results of the reduction of methemoglobin by FADH₂ are not presented here. A remaining or leaking trace amount of oxygen is suspected under the conditions employed in this study, although there was no problem in obtaining the typical deoxyhemoglobin as shown in Fig. 5. Improvement of the experimental conditions is required for the quantitative determination of methemoglobin reduction by FADH₂.

Previously we observed that the effect of inositol hexaphosphate on the overall reduction of methemoglobin by the NADPH-flavin reductase system is not inhibitory with a high concentration of flavin (100 µM) (13), but is inhibitory with a low concentration of flavin (1 µM) (8). In this study the effect of inositol hexaphosphate on the nonenzymatic reduction of methemoglobin by FMNH₂ was examined. Addition of the organic phosphate (1 mM) to the reaction mixture did not have any significant effect on the reduction of methemoglobin (46 µM) by FMNH₂ (60 µM), but was slightly inhibitory. To clarify these problems, further studies must be awaited.

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REFERENCES