Electrochemical Reduction of Methemoglobin Either Directly or with Flavin Mononucleotide as a Mediator*

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Electrochemical reduction of methemoglobin on a platinum electrode is studied by means of thin layer spectroelectrochemistry. For methemoglobin alone in solution, direct reduction is very slow even for potentials close to those of the reduction of the solvent. The reduction of a methemoglobin-oxyhemoglobin mixture with an imposed potential causes the electrochemical reduction of oxygen, the conversion of oxyhemoglobin into deoxyhemoglobin, and a simultaneous transformation of part of the molecules into methemoglobin. When fixed oxygen has disappeared, reduction of methemoglobin takes place. The reduction of methemoglobin and deoxyhemoglobin is catalyzed by the presence of flavin mononucleotide (FMN).

For the oxyhemoglobin-methemoglobin mixture, flavin makes a fast deoxygenation of oxyhemoglobin without a change in the oxidation state of the iron. It also allows the rapid reduction of methemoglobin.

In each case, the resulting deoxyhemoglobin solutions do not show any electrolysis-induced modification of the equilibrium curves for oxygen binding.

Studies on the reduction of methemoglobin are numerous and have two aims. On the one hand, fundamental studies on the mechanism of this reaction might lead to efficient treatments of the methemoglobinemia which appears in cases of intoxication by chemical substances or due to the genetic deficit of the reducing systems.

On the other hand, methemoglobin appears during the separation operations when placenta is being treated in order to extract albumin and some immunoglobulins and during the lyophilization processes of hemoglobin. The possibility of reducing methemoglobin gives value to the residual placenta solutions.

Reduction by chemical means has been considered with dithionite ion (1), ascorbic acid (2, 3), hydroxyanthranilic acid (4), iron, and EDTA complexes (5), with the solvated electron formed by pulse radiolysis (6, 7), ferredoxin, and NADP-ferredoxin reductase used as a catalyst (8), NADH and NADPH associated to complex enzymatic systems (9).

Another possibility for reduction derives from the fact that the process which takes place in the erythrocytes is known. In these cells, methemoglobin is reduced due to a NADPH-flavin reductase or NADPH-methemoglobin reductase. The electrons needed for the reduction are provided by NADPH which transfers them to the enzyme, which then transmits to FMN, the last electronic relay towards methemoglobin.

The global reduction is slow and the rate of the process is controlled by the enzymatic reduction step. This reaction is much slower than the nonenzymatic reduction of methemoglobin by the reduced form of flavin (10, 11).

Kinetic study of this simple reaction, efficient for the reduction of methemoglobin, was carried out by means of the photoreduction of flavin, using the stopped flow technique (12).

With all these reduction methods, hemoglobin and oxidized products formed from the inorganic or biological reducers used have to be separated after reduction.

An alternative process lies in direct electrochemical reduction which allows for the electron to be used as a reagent for the preparation of hemoglobin. Several attempts were carried out on mercury electrodes, with which the half-wave potentials are close to -0.6 V, versus a saturated calomel electrode (13). Electrolysis carried out over periods of time at the imposed potential of -1.0 V leads to a solution whose spectrum is identical to the hemoglobin spectrum. With a transparent tin oxide electrode in the presence of chloride ions, the heterogeneous transfer constant obtained is equal to 5.2 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1} (14). On a gold electrode modified by methylviologen, sperm whale metmyoglobin is reduced with a transfer constant of 3.8 \times 10^{-11} \text{ cm}^2 \text{ s}^{-1} (15).

The high overvoltages necessary for the reduction on mercury electrodes and the low value of the constants obtained even on modified surface electrodes show that the methemoglobin-deoxyhemoglobin oxidoreduction system is slow, and the elaboration of a direct electrochemical reduction process of methemoglobin does not seem to offer much scope.

This study deals with the electrochemical reduction of methemoglobin on platinum electrode with FMN used as oxidoreduction mediator. With the high rate constants obtained with photochemically reduced flavin, one can hope that an efficient and interesting electrochemical process for the reduction of methemoglobin can be elaborated. Besides, this hypothesis implies a fast electrochemical reduction reaction of FMN. This molecule has been the subject of previous electrochemical studies. With classical polarography, reduction takes place via a semi-quinone form, whereas polarography with superimposed sinusoidal potential shows the adsorption of oxidized and reduced forms on the surface of the electrode. This adsorption is confirmed by chronopotentiometry and cyclic voltammetry (16). Similar adsorption phenomena are also seen on gold electrodes (17). Coulometric determination allows the existence of the FMNH-FMNH$_2$ complex to be suggested (18). Potential pH diagrams were drawn after the results of experiments carried out with thin layer electrodes (19).

Experiments with FMN in our laboratory have shown that
the electron exchange on platinum is fast (20), and the exchange takes place with the formation of a semi-quinone intermediate, whose dismutation constant was calculated (21).

MATERIALS AND METHODS

For most experiments, the technique of thin layer spectroelectrochemistry was used. This method allows for the simultaneous acquisition of electrochemical and optical data. The electrolysis cell includes a working electrode made of a platinum grid placed between two glass slides (1.5 × 5 cm) in order to mark off a volume comprised between 20 and 200 μl. The optical beam goes through the solution observed at the level of the platinum grid. This electrode is practically identical to the one described by De Angelis and Heineman (22) and has previously been used for the study of direct electronic transfer between platinum and several biomolecules (20, 21, 23). The end of the glass slides is dipped in a 300-μl beaker in which the auxiliary electrode and the extremity of a Luggin capillary are immersed. This capillary is fitted with an agarose gel containing the electrolytic solution and links the solution in the beaker to a saturated calomel reference electrode in relation to which the potentials are referred. The three electrodes are linked to a potentiostat (Solea Tacussel, Lyon, France, model PRT 20x2X), with which the working electrode potential can be regulated in relation to the reference electrode. This difference in potential between these two electrodes can vary as a linear function of time by means of a signal generator (Solea Tacussel, model Servovit).

Electrolysis current is determined from the potential difference across a standard resistance placed in series with the auxiliary electrode. The current-potential curves are obtained by means of an XY plotter (Sefram, Paris, France, model Luxytrace).

By means of a specially adapted support, the electrolysis cell is put in the cell compartment of a spectrophotometer (Hewlett Packard, type 8451 A) which allows for the storing of optical data. Spectra are drawn on a plotter (Hewlett Packard, type 7090). Several experiments of fixed potential electrolysis were carried out with a paralllelepiped cell in which the working electrode (platinum grid) is parallel to the larger sides of the paralllelepiped. This cell is approximately an extrapolation of the thin layer cell; it is connected to the compartment of the auxiliary electrode by a junction filled with the electrolytic solution. The tightness allows for an easy deoxygenation of the solution by electrochemistry. Depending on the experiment, its volume varies from 1 to 25 cm³. The equilibrium curves for the hemoglobin-oxygenation are determined by means of a tonometer (24).

The different electrolytic solutions were made from twice-distilled water and high purity salts. FMN is provided by Sigma. Hemoglobin and methemoglobin are provided by three lots: lot 1 contains about 45% hemoglobin and 55% methemoglobin, it is of placental origin; lot 2, also of placental origin, contains almost exclusively hemoglobin and a very low percentage of methemoglobin; and lot 3 is made of standard adult hemoglobin.

Methemoglobin is produced by the action of potassium ferricyanide on hemoglobin. Ferricyanide in excess and ferrocyanide produced are eliminated by passage through Sephadex G25 column. The methemoglobin content of a sample is determined by a comparison of absorbance variations at 630 nm between the sample before and after addition of cyanide on the one hand and the sample in presence of ferricyanide, then of cyanide on the other hand.

Voltametry with linear potential sweep and imposed potential electrolysis are the electrochemical methods used. During the tracing of the current-potential curve or during the electrolysis, the absorption spectra are stored on floppy disks.

Samples having undergone electrolysis in relatively thick cells are taken, and the equilibrium curve corresponding to the reaction of hemoglobin oxygenation \( \text{Hb} + O_2 \rightarrow \text{HbO}_2 \) is determined by tonometry. These experiments are carried out at 37 °C in phosphate medium at pH 7.0.

Work was carried out successively on methemoglobin solutions and on methemoglobin-oxyhemoglobin mixture without added FMN and with FMN as mediator in the electronic transfer.

RESULTS AND DISCUSSION

Direct Electrochemical Reduction

**Solution of Methemoglobin**—Prior to the experiment, an electrolysis is carried out at a potential of 0.0 V in order to reduce dissolved oxygen. The current-potential curve corresponds to the reduction of the solvent (Fig. 1). During oxidation, peak A is due to the reoxidation of the adsorbed hydrogen formed during the first phase of the experiment. The evolution of the absorption spectra shows a very slight reduction of methemoglobin.

This experiment enables us to choose the potential range needed for an electrolysis to be carried out without evolution of hydrogen due to the reduction of the solvent. Spectra obtained during an electrolysis at −0.75 V are shown on Fig. 1. The reduction is slow; approximately 20 h are needed for a methemoglobin concentration of 10 μM. If the case of more concentrated solutions, not all the molecules present in the thin layer can be reduced, probably due to adsorption phenomena which caused a strong resistance to the platinum-biomolecule electronic transfer. However, this type of experiment proves that the direct electronic transfer between methemoglobin and platinum is possible even if the current-potential curve shows no distinct peak or plateau. The contribution of spectroelectrochemistry must be underlined here, without which methemoglobin could be understood to be electroinactive.

**Methemoglobin and Oxyhemoglobin Mixture**—When −0.65 V is applied to the working electrode, reduction of the oxygen dissolved in the thin layer and reduction of the oxygen fixed on the oxyhemoglobin take place. This reduction displaces the \( \text{HbO}_2 = \text{Hb} + O_2 \) equilibrium to the right, and the formation of methemoglobin can be observed simultaneously. The transitory formation of methemoglobin is deduced from the absorbance (at 630 nm) increase. When all the fixed

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**TABLE 1**

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<th>No.</th>
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<td>1</td>
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Electrochemical Reduction of Methemoglobin

Electrochemical Reduction in Presence of FMN

Methemoglobin Solution—An example of current-potential curve associated to absorption spectra obtained at different potentials is shown on Fig. 2.

The quantity of FMN corresponds to a quarter of the heme groups in solution. The experiment begins at a potential of 0.0 V and during the first cycle plateau A can be observed due to the reduction of dissolved oxygen. There is also a peak B centered on the potential of −0.65 V with a much higher amplitude than the one obtained with the same concentration of FMN alone in solution. This phenomenon can be accounted for by the presence of a chemical reaction between methemoglobin and a reduced form of flavin FMNs. The following mechanism can be put forward:

\[
4 \text{FMN} + 4 e^- \rightarrow 4 \text{FMN}^2+ \text{at the electrode.}
\]

\[
4 \text{FMN}^2+ + \text{MetHb} \rightarrow 4 \text{FMN} + \text{Hb} \quad \text{in solution.}
\]

Absorption spectra on Fig. 2 show that up to a potential of −0.65 V, only methemoglobin appears in reduced form, which confirms the mechanism of electrochemical catalysis proposed.

Between points B and C, the spectra show the simultaneous reduction of methemoglobin and flavin. Reduction of methemoglobin is complete at point C.

For potentials below −0.70 V, the increase in current intensity is due to the beginning of solvent reduction. Simultaneously, the end of the reduction of flavin can be observed (the absorbance decreases at 460 nm and the spectra are constant beyond 520 nm).

After reversal of the direction of the potential sweep, peak D is due to the reoxidation of hydrogen formed at potentials lower than −0.70 V and peak E is assigned to the oxidation of the previously totally reduced flavin, according to the following reaction:

\[
\text{FMNH}_2 \rightarrow \text{FMN} + 2 \text{H}^+ + 2 e^-
\]

During the second cycle, the solution no longer contains any dissolved oxygen, peak F corresponds to the reduction of FMN alone.

Similar results take place with a FMN concentration 10 times lower than methemoglobin one.

Methemoglobin-Oxyhemoglobin Mixture—In this case electrolysis at a fixed potential causes both a reduction of dissolved and fixed oxygen. Contrary to the case of flavin in solution, the presence of methemoglobin is not related to the reduction of fixed oxygen (Fig. 3). As soon as electrolysis begins, absorbance at 630 nm decreases.

Simultaneously, reduced flavin in the intermediate form catalyzes the reduction of methemoglobin present in solution. As soon as flavin is reduced, the reduction of methemoglobin according to the probable reactions:

\[
2 \text{H}^+ + \text{FMN} + 2 e^- \rightarrow \text{FMNH}_2
\]

\[
\text{O}_2 + \text{FMNH}_2 \rightarrow \text{FMN} + \text{H}_2\text{O}_2
\]

\[
\text{FMN} + e^- \rightarrow \text{FMN}^2+
\]

\[
4 \text{FMN}^2+ + \text{MetHb} \rightarrow 4 \text{FMN} + \text{Hb}
\]

The electrode potential is sufficiently negative for the hydrogen peroxide produced to be reduced electrochemically.

On the other hand, the reaction of flavin semi-quinone with oxygen is thermodynamically easy, and the reaction of the oxidation of flavin catalyzes the oxyhemoglobin deoxygenation reaction and the reduction of methemoglobin according to the probable reactions:

\[
2 \text{H}^+ + \text{FMN} + 2 e^- \rightarrow \text{FMNH}_2
\]

\[
\text{O}_2 + \text{FMNH}_2 \rightarrow \text{FMN} + \text{H}_2\text{O}_2
\]

\[
\text{FMN} + e^- \rightarrow \text{FMN}^2+
\]

\[
4 \text{FMN}^2+ + \text{MetHb} \rightarrow 4 \text{FMN} + \text{Hb}
\]

The electrode potential is sufficiently negative for the hydrogen peroxide produced to be reduced electrochemically.

On the other hand, the reaction of flavin semi-quinone with oxygen is thermodynamically easy, and the reaction of the
flavin semi-quinone with methemoglobin is very fast (11, 25). So no increase of absorbance at 630 nm is observed.

The equilibrium curves for the oxygenation of deoxyhemoglobin were determined by tonometry for the different lots before and after electrolysis. The electrolyzed solution was either passed through a Sephadex G25 column for the elimination of flavin or was used directly after dilution in the tonometer. The equilibrium curves depend on the hemoglobin lot considered, each lot being characterized by a Hill coefficient and a value of $P_50$ (pressure when 50% of the molecules are oxygenated). The curves and parameters deduced are not influenced by the electrochemical treatment, which suggests negligible denaturation of hemoglobin by the action of the electrode.

This study shows the utility of electrochemistry in the reduction of methemoglobin. With simple material, the potential of the electrode can be controlled and flavin can be used as a mediator in the platinum-methemoglobin electronic transfer.

The method of thin layer spectroelectrochemistry has proved to be particularly well adapted as it allows for the acquisition of simultaneous electrochemical and optical data. The fact of being able to reduce dissolved oxygen independently of flavin allows for the transformation of methemoglobin into deoxyhemoglobin to be observed.

Given these results, the elaboration of an electrochemical indirect reduction process of methemoglobin can be considered. Using FMN as a mediator has several advantages. This molecule acts as a catalyst for the platinum-methemoglobin electronic transfer. Overvoltage is lowered to about $-0.4$ V, and the electrolysis current is much higher. Moreover, the presence of oxyhemoglobin is not inconvenient to reduced methemoglobin. As FMN and hemoglobin are naturally associated in the electron exchange in the red cell, the use of FMN does not lead to the denaturation of hemoglobin. However, a separation phase will have to be associated to the electrochemical treatment, particularly in order to recycle FMN in the electrolyzer.

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REFERENCES