Nonenzymatic Reduction and Oxidation of Myoglobin and Hemoglobin by Nicotinamide Adenine Dinucleotides and Flavins*

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SUMMARY

The nonenzymatic reduction of metmyoglobin and methemoglobin by NADH or NADPH in the presence of ethylenediaminetetraacetate can occur at rates greater than most of those previously reported for enzymatic reduction.

The reduction rate is considerably enhanced by the mediation of various flavins, including FAD, flavin mononucleotide, and riboflavin. The system is generally more active at pH 6.4 than at pH 7.4. The presence of Amytal is without effect. Coenzyme Q, and menadione do not function as mediators of the reduction. Myoglobins from mammalian and fish sources behave similarly, but tuna metmyoglobin requires a mediator for reduction.

Flavins, particularly flavin mononucleotide, in the presence of ethylenediaminetetraacetate can catalyze the photooxidation of oxymyoglobin, as well as the photoreduction of metmyoglobin. However, the flavin-mediated reduction of metmyoglobin by NADH or NADPH just mentioned takes place equally well in the dark.

Because of the inability of ferric forms of hemoglobin and myoglobin to bind oxygen reversibly, there has been long interest in systems capable of maintaining these hemoproteins in the reduced state. Much more attention has been given methemoglobin than metmyoglobin. Recent reviews (1-3) are available. In general, it has been considered that one or more enzyme systems were involved, utilizing either NADH or NADPH for the reduction, and, indeed, such enzymes have been isolated (4-8). Certain of the methemoglobin reductases require the addition of an electron carrier, and methylene blue frequently has been used for this purpose (1-6).

Much less is known of metmyoglobin reductases; such systems have been studied and appear also to utilize NADH or NADPH (9-12). Little has been reported of a highly definitive nature; consequently, we undertook an investigation with the initial aim of isolation and characterization of metmyoglobin reductase(s) from muscle. During this investigation, it became obvious that, under suitable circumstances, an efficient nonenzymatic reduction of metmyoglobin could occur.

We report herein that NADH alone can reduce metmyoglobin and that this reduction can be considerably enhanced by the mediation of flavins or methylene blue. NADPH effectively replaces NADH. Methemoglobin, in general, behaves similarly to metmyoglobin in the reduction systems. We also report that flavins, particularly flavin mononucleotide, under the influence of light irradiation can catalyze either the oxidation of oxymyoglobin or the reduction of metmyoglobin.

EXPERIMENTAL PROCEDURE

Materials—All biological chemicals and enzymes were obtained from Sigma and were the highest purity available. Other chemicals were reagent grade.

Bovine myoglobin was a gift from Dr. Lowell Satterlee of Iowa State University and had been prepared by a modification of the method of Snyder and Ayres (13) which involves heating to 55° of an aqueous extract of muscle and then fractionation by ammonium sulfate, retaining the precipitate between 85 and 100% saturation which contains virtually all of the myoglobin. This material was subjected to chromatography on carboxymethyl cellulose; the myoglobin used in this study was from the main peak resolved by this method (14). Sperm whale myoglobin was prepared by the same method except that diethylaminoethyl cellulose chromatography was used (15). Yellowfin tuna myoglobin was isolated by ammonium sulfate fractionation, followed by diethylaminoethyl cellulose chromatography (15). Bovine hemoglobin (Sigma type I, twice crystallized) was used as supplied and also purified by the method suggested by Ngesh and Avron (8) and based on that of Hennessy et al. (16). This diethylaminoethyl cellulose adsorption procedure is claimed to remove hemoglobin reductase contaminants from hemoglobin preparations.

Solutions of NADH and NADPH were freshly prepared for each set of determinations. Solutions of flavins were prepared daily and protected from light. These compounds and ATP, ADP, and AMP were dissolved in the buffer to be utilized in a particular experiment. Aqueous stock solutions were used for
Each system contained 0.25 μmole of bovine metmyoglobin, or 0.06 μmole of methemoglobin, and, except as shown, 1.5 μmoles of EDTA in a total volume of 3.0 ml. Other constituents were included, where shown, in the following amounts (micromoles): NADH or NADPH, 1.3; methylene blue, 0.05; FMN, 2.0; FAD, 1.6; riboflavin, 0.1. Final phosphate buffer concentration was 0.03 M, and the temperature was 22–23°C. The increase in absorbance of the (approximately) 575 nm peak of oxyhemoglobin or oxyhemoglobin was followed with time. Oxyhemoglobin values are based on micromoles of iron, and are shown in brackets following values for oxymyoglobin formation.

<table>
<thead>
<tr>
<th>Additives</th>
<th>pH 6.4 μmoles/min × 10^3</th>
<th>pH 7.4 μmoles/min × 10^3</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH (minus EDTA)</td>
<td>Trace</td>
<td>Trace</td>
</tr>
<tr>
<td>NADH</td>
<td>13</td>
<td>3</td>
</tr>
<tr>
<td>NADPH</td>
<td>14</td>
<td>3</td>
</tr>
<tr>
<td>NADH, FMN</td>
<td>66 (28)</td>
<td>42 (21)</td>
</tr>
<tr>
<td>NADPH, FMN</td>
<td>47 (21)</td>
<td>34 (22)</td>
</tr>
<tr>
<td>FMN (minus NADH)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>NADH, FAD</td>
<td>39</td>
<td>25</td>
</tr>
<tr>
<td>NADPH, FAD</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>NADH, riboflavin</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>NADPH, riboflavin</td>
<td>34</td>
<td>16</td>
</tr>
<tr>
<td>NADH, methylene blue</td>
<td>32</td>
<td>21</td>
</tr>
<tr>
<td>NADH, FMN, methylene blue</td>
<td>60</td>
<td></td>
</tr>
</tbody>
</table>

*a* Methemoglobin was purified by the method of Hegesh and Avron (8) said to remove methemoglobin reductase; see "Experimental Procedure" for details.

all materials, except for menadione and coenzyme Q₆, which were dissolved in methanol.

**Methods**—The general procedure for studying the nonenzymatic reduction of metmyoglobin was to place reactants in a cuvette, except for constituents necessary to initiate the reaction (usually NADH). The optical blank contained all components except metmyoglobin. An initial spectrum of metmyoglobin was recorded with a Beckman model DK-2 or a Cary model 11 spectrophotometer. After the addition of NADH, the absorbance of the 575 nm peak of oxyhemoglobin was recorded at 1-minute intervals. The absorbance representing 100% oxyhemoglobin was calculated from known extinction coefficients or determined experimentally by adding methylene blue (0.05 μmole), NADH (1.3 μmoles), and an excess of either diaphorase (dihydrolipoamide dehydrogenase) or cytochrome c reductase to the system to convert metmyoglobin (0.25 μmole) totally to oxyhemoglobin. Cuvettes were kept in a cell holder, but removed from the instrument between scans to avoid being heated. These systems were aerobic and at ambient temperature (22–23°C). Experiments with methemoglobin were done in a completely analogous fashion.

Additional controls for systems containing flavin were held in the dark. Concentrations of reactants are shown in the legends of figures mentioned under "Results." Systems were completely soluble, except for those containing coenzyme Q₆, for studies with this compound, the procedure of Piazzo et al. (17) was used; this involves a preincubation period that stabilizes the light scattering produced by precipitated CoQ. Determinations were done at least in duplicate.
Nonenzymatic Reduction of Metmyoglobin and Methemoglobin

TABLE II
Comparison of nonenzymatic reduction with previously reported methemoglobin reductases

Our system is that containing NADH, or NADPH, with FMN as a mediator as described in Table I; temperature, 23°; pH 7.4. Results with these systems have been recalculated to the units used by earlier investigators. The enzymatic systems reported used NADH and methemoglobin except as indicated by Footnotes a and g. Values for other investigators are based on their average or mean values if reported as such.

<table>
<thead>
<tr>
<th>Investigator(s)</th>
<th>Temperature</th>
<th>Units</th>
<th>Activity</th>
<th>Our nonenzymatic system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Huennekens et al. (5)</td>
<td>-b</td>
<td>moles/ml/min</td>
<td>3.6 x 10^-2</td>
<td>19 x 10^-7</td>
</tr>
<tr>
<td>Huennekens et al. (6)</td>
<td>-b</td>
<td>A/min</td>
<td>0.020</td>
<td>0.037</td>
</tr>
<tr>
<td>Ross (18)</td>
<td>&quot;Room&quot;</td>
<td>A/min</td>
<td>0.0045</td>
<td>0.050</td>
</tr>
<tr>
<td>Scott et al. (7)</td>
<td>38°</td>
<td>k, min^-1</td>
<td>0.002</td>
<td>0.115</td>
</tr>
<tr>
<td>Gutmann et al. (19)</td>
<td>38</td>
<td>μmoles/min</td>
<td>0.0002</td>
<td>0.006</td>
</tr>
<tr>
<td>Gutmann et al. (19)</td>
<td>38</td>
<td>μmoles/min</td>
<td>0.0023</td>
<td>0.006</td>
</tr>
<tr>
<td>Rossi-Fannelli et al. (9)</td>
<td>18</td>
<td>μmoles/min</td>
<td>0.013</td>
<td>0.042</td>
</tr>
</tbody>
</table>

- NADPH replaces NADH.
- Value not given in reference.
- Absorbance measured at 576 nm.
- Based on mean value shown in Fig. 1 of Reference 18; absorbance measured at 600 nm.
- Based on highest value shown in Fig. 6 of Reference 7 for methemoglobin reduction by "DPNH diaphorase."

Details of the procedure for studying the photocatalytic reduction of metmyoglobin are given in the legend for Fig. 1. The formation of oxyhemoglobin was determined as just outlined.

Oxymyoglobin used in the oxidation experiments was prepared by dissolving 100 mg of metmyoglobin in 1 to 2 ml of H₂O and adding 10 mg of Na₂S₂O₃. The reduced material was placed on a column (2 x 20 cm) of mixed bed ion exchange resin (Bio-Rad AG 501-X8) and eluted with water at a flow rate of about 3 ml per min. Emerging oxymyoglobin fractions were stored in ice water until their USC within a few minutes. Details of the oxidation procedure are given in the legend for Fig. 2.

RESULTS

Reduction of Metmyoglobin by NADH and NADPH—From the results shown in Table I, it is clear that NADH or NADPH can reduce metmyoglobin or methemoglobin if EDTA is present. The reduction is substantially enhanced by flavins. This system is pH dependent, being more active at pH 6.4 than at 7.4. All the flavins tested appear able to mediate this reaction. Riboflavin was not tested at amounts larger than 0.1 μmole because of its insolubility. Methylene blue was about as effective as the flavins and was effective at lower concentrations.

Methemoglobin was reduced at a lower rate than was metmyoglobin. As indicated in Table I, Footnote a, the methemoglobin used was purified by a method said to remove methemoglobin reductase contaminants. Results with methemoglobin not so treated did not differ from those given in the table.

The data presented in Table I are based on the initial rate of reduction, i.e. up to 5 min. During this time, the reaction is first order, and in the most efficient reducing systems studied, the reaction is virtually complete within this period. There is the possibility of the reverse, or autoxidation, reaction taking place. This was particularly noted in systems to which was added GSH; this material did not appreciably enhance the reduction when it was added to systems containing NADH and flavins, but it tended to increase the autoxidation reaction after a few minutes. GSH was ineffective alone as a reductant.

The addition of ATP, ADP, or AMP in micromolar quantities to the systems described in Table I was without effect. The incorporation of the inhibitor Amytal in like amounts was similarly without effect. Possible physiological electron carriers tested were coenzyme Q₆ and menadione; neither would function in this system.

Because of subsequent findings with photocatalytic reactions, we also did controls with various flavins by using systems identical with those reported in Table I, but held in the dark. These results did not differ significantly.

Comparison of Nonenzymatic with Enzymatic Systems—In Table II are presented comparisons of our nonenzymatic rates of reduction of metmyoglobins and methemoglobins with those reported by other enzymatic systems. The values for methemoglobin reduction in terms of micromoles per min differ by a factor of 4 from the data shown in Table I. This is because the values in Table I are based on iron molarity rather than that of hemoglobin to enable direct comparison of methemoglobin with metmyoglobin reduction.

These comparative data clearly show the significance of the nonenzymatic reduction because it is more efficient than any of the enzyme systems shown. The range of pH values used by the various investigators was 7.35 to 7.55. We have seen reports of one methemoglobin reductase that is considerably more effective than those in Table II (8, 20). However, it is effective under substantially different conditions. It has an optimum pH of 5.2 and is not active at neutral pH. In addition, it requires ferrocyanide ion activation. Consequently, the physiological significance of this enzyme may be subject to question.

Reduction of Metmyoglobin from Other Species—The results presented to this point were all obtained with bovine metmyoglobin or methemoglobin. Table III shows some comparative rates, utilizing tuna and whale metmyoglobins as well. The tuna met-
myoglobin was reduced at a slower rate than those from mammalian sources, either with flavin or methylene blue mediation. It also was reduced very little by NADH alone, although EDTA was present. Both mammalian metmyoglobins showed a pH effect, being reduced somewhat more rapidly at 6.4 that at 7.4, whereas the tuna metmyoglobin was not similarly affected.

Effect of Varying Concentrations of NADH and FMN: The effects on the rate of reduction of sperm whale metmyoglobin of varying concentrations of NADH or FMN are shown in Table IV.

Photocatalytic Reduction of Metmyoglobin: From Fig. 1, it can be seen that illumination of systems containing metmyoglobin and FMN (no NADH present) results in reduction of metmyoglobin. The slight drop in percentage oxymyoglobin during the dark periods presumably is caused by autoxidation. The amount of FMN present is not highly critical. For example, increasing the amount 4 times increased the reduction rate only slightly.

If FMN was illuminated alone and subsequently added to metmyoglobin, there was no reduction, unless the final system was illuminated. There was formed, however, a compound with an absorption maximum at 545 nm and a shoulder at about 575 nm. This same compound could be produced by illumination of the solution at the end of a typical reduction run such as those reported in Table I, i.e. containing oxymyoglobin, FMN, and EDTA.

This reduction is quite pH dependent, being much more rapid at 6.4 than at 7.4. The presence of EDTA markedly enhanced the reaction.

If FAD was used, rather than FMN, the extent of reduction was greatly lowered; similarly, reduction of yellowfin tuna methmyoglobin (with FMN) was much slower than that of bovine preparations.

Photocatalytic Oxidation of Oxymyoglobin by Flavins—Figs. 2 and 3 illustrate the oxidation of oxymyoglobin by flavins when irradiated. Samples held in the dark were unaffected by the flavins. When illuminated, however, both flavins, but particularly FMN, catalyzed the oxidation reaction.

The data in Fig. 3 indicate that in this system tuna oxymyoglobin does not differ markedly from bovine samples. The presence of EDTA in the control (no flavin) inhibited the autoxidation reaction slightly; however, in the presence of FMN, it enhanced the oxidation rate considerably.

**DISCUSSION**

The results show that a nonenzymatic system for the reduction of methemoglobin or metmyoglobin by NADH or NADPH can be very efficient. In fact, we have obtained rates of reduction greater than those reported by most workers for enzymatic systems (Table II). In the reports of enzymatic reductase activity, there are indications that nonenzymatic controls were "an order of magnitude slower" (6), "extremely slow" (9), and "incapable of accomplishing the direct reduction" (21). We cannot offer an explanation for the discrepancy between our findings and those of others. One possibility is the role of EDTA in our systems. Metal ions are known to be potent catalysts for the autoxidation of hemoproteins, and the presence of EDTA will effectively pre-

**TABLE III**

Comparative study of nonenzymatic reduction of metmyoglobins

Experimental conditions were like those shown in Table I, except that different metmyoglobins were used as indicated. All systems contained 1.3 pmoles of NADH and 1.0 pmole of EDTA.

<table>
<thead>
<tr>
<th>Metmyoglobin source and additives</th>
<th>Oxymyoglobin formed</th>
<th>pH 6.4</th>
<th>pH 7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmoles/min × 10³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine</td>
<td>13</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Bovine, FMN</td>
<td>63</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>Yellowfin tuna, FMN</td>
<td>Trace</td>
<td>Trace</td>
<td></td>
</tr>
<tr>
<td>Sperm whale, FMN</td>
<td>14</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Bovine, methylene blue</td>
<td>42</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>Yellowfin tuna, methylene blue</td>
<td>21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sperm whale, methylene blue</td>
<td>7</td>
<td>19</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE IV**

Effect of varying levels of NADH and FMN on reduction of metmyoglobin

Experimental conditions were like those shown in Table I, except that sperm whale metmyoglobin was used, and the amounts of NADH and FMN were varied as indicated.

<table>
<thead>
<tr>
<th>Amount of NADH</th>
<th>Oxymyoglobin formed</th>
<th>Amount of FMN</th>
<th>Oxymyoglobin formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>µmoles</td>
<td>µmoles/min × 10³</td>
<td>µmoles</td>
<td>µmoles/min × 10³</td>
</tr>
<tr>
<td>2.6</td>
<td>72</td>
<td>4.0</td>
<td>62</td>
</tr>
<tr>
<td>1.3</td>
<td>43</td>
<td>2.0</td>
<td>41</td>
</tr>
<tr>
<td>0.65</td>
<td>25</td>
<td>1.0</td>
<td>27</td>
</tr>
<tr>
<td>0.32</td>
<td>13</td>
<td>0.5</td>
<td>17</td>
</tr>
</tbody>
</table>

* FMN, 2.0 µmoles, in every case.

b NADH, 1.3 µmoles, in every case.
present. This would seem to be particularly true for the enzymatic systems under similar conditions and might be capable of reducing in vivo the normally small amounts of ferric proteins and metmyoglobin in vivo. Nonetheless, our nonenzymatic systems reported here. Others, who believe the reduction under our conditions, however, is considerably greater than reported in the past.

Of more interest is the demonstration that flavins can function well as mediators of the nonenzymatic reduction of metmyoglobin and methemoglobin. Earlier, it had been reported that riboflavin, FMN, and FAD would not replace methylene blue in NADPH-dependent methemoglobin reductase systems (5, 6). Again, the reason for the discrepancy is not clear. Because the effectiveness of the flavins in our systems was about the same when reactions were carried out in the dark, a photocatalytic process is not involved.

The comparative studies indicate that fish and mammalian metmyoglobins behave similarly. The slower nonenzymatic reduction of tuna metmyoglobin may be due to its much greater susceptibility to autoxidation (25).

There have been numerous reports of photochemical reductions of flavins (e.g. References 23 and 24). The results of our study indicate that flavins, particularly in the presence of EDTA, can catalyze the photooxidation of oxyhemoglobin as well as the photoreduction of methemoglobin.

There are known to be species differences in rates of methemoglobin formation and reduction (26, 27). In general, reduction is stimulated by methylene blue, but not always. It may be that the limiting factor is the amount of NADH or NADPH. This could be the case for either enzymatic systems or the nonenzymatic systems reported here. Others, who believe the reduction is enzymatic, have previously suggested that NADH is the limiting factor in methemoglobin reduction in erythrocytes (28).

It is not suggested that the present data prove that nonenzymatic systems are responsible for reduction of methemoglobin and metmyoglobin in vivo. Nonetheless, our nonenzymatic system works as efficiently or better than most previously studied enzymatic systems under similar conditions and might be capable of reducing in vivo the normally small amounts of ferri proteins present. This would seem to be particularly true for the reduction of metmyoglobin, because the nonenzymatic system is even more effective with metmyoglobin than with methemoglobin.

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REFERENCES

Nonenzymatic Reduction and Oxidation of Myoglobin and Hemoglobin by Nicotinamide Adenine Dinucleotides and Flavins
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