Methemoglobin Reduction in Red Cells: Effect of a High Oxygen Affinity Hemoglobin

By F. Taketa, K. J. Matteson, J. Y. Chen, and J. A. Libnoch

Erythrocytes from heterozygous carriers of the high oxygen affinity mutant hemoglobin, Hb Wood, demonstrate lower rates of methemoglobin reduction than normal human red cells when incubated in the in vitro system of Beutler and Baluda. The rate of methemoglobin reduction in red cells from an individual who is heterozygous for both NADH-methemoglobin reductase deficiency and Hb Wood shows the combined effects of the two mutations.

THE RED BLOOD cells of an individual subject to primaquine-induced methemoglobinemia (Libnoch, unpublished observations) were found to be heterozygous with respect to NADH-methemoglobin reductase deficiency and to the presence of a high oxygen affinity mutant hemoglobin. This finding of a highly unusual double mutation, each affecting the functional integrity of hemoglobin, raises questions about effects that could arise from the combination of both mutations. Although the drug-induced methemoglobinemia can be explained on the basis of the enzyme deficiency alone, the properties of the mutant hemoglobin (Taketa, unpublished observations) suggested that the latter might also be a significant factor. The mutant hemoglobin comprises about 50% of the red cell hemoglobin and is characterized as Hb Wood ($\beta^\text{his} \rightarrow \text{leu}$), which has amino acid substitutions at the $\alpha_2\beta_2$ interfaces of the tetrameric structure. As with other “high-affinity” hemoglobins, it demonstrates a shift in its allosteric equilibrium toward the R quaternary conformation. In previous work, it was shown that the rates of enzymatic as well as nonenzymatic reduction of methemoglobin depend on the quaternary structure of the protein, the rate being slower when methemoglobin is in the R than in the T conformation. Thus, it seemed that the rate of reduction of met-Hb Wood might be demonstrably lower than that of normal adult human methemoglobin (met-HbA) and that the presence of significant amounts of the mutant hemoglobin could affect the overall rate of methemoglobin reduction in the intact red cell.

Hb Wood and NADH-methemoglobin reductase deficiency are inherited independently among members carrying mutations for either Hb Wood or NADH-methemoglobin reductase deficiency was available to assess the effect of each mutation on the rate of methemoglobin reduction in the intact red cell. In this article we show that the rates of enzymatic and nonenzymatic reduction of met-Hb Wood are indeed lower than corresponding rates of reduction of met-HbA, and that the occurrence of Hb Wood is a significant factor in the reduced rate of red cell methemoglobin reduction. We also show that the rate of methemoglobin reduction in red cells from family members heterozygous with respect to NADH-methemoglobin reductase deficiency is similarly lower than that of control red cells, and that the corresponding rate in red cells from the propositus demonstrates combined effects of the two mutations.

MATERIALS AND METHODS

Hemoglobin Wood was isolated from the propositus’s red cell lysate by CM-Sephadex chromatography as described previously. Normal adult human red cell lysates were used as the source of HbA. The hemoglobins were converted to methemoglobin by reaction with a fivemolar excess per heme of solid sodium nitrite. The reaction was allowed to proceed for 5 min, at which time the mixture was placed on a column of Sephadex G-25 (fine) equilibrated with 0.1 M NaCl, 1 mM EDTA, 0.005 M bis-Tris buffer (pH 7.5) for removal of organic phosphate, and excess nitrite. The eluate was concentrated by ultrafiltration (Amicon PM10) to about 15% methemoglobin and then dialyzed against four changes of a 100-fold excess of the bis-Tris buffer. Complete conversion to methemoglobin was confirmed by examination of the spectrum between 500 and 600 nm. Inositol hexaphosphate (IHP) was purchased as the sodium salt (Sigma Chemical Co., St. Louis, Mo.). Solutions were neutralized with HCl before use.

Rates of enzymatic reduction of the isolated hemoglobins were measured using the Hegesh method of assay modified for analysis at pH 7.0. Typically, 0.04 mM methemoglobin in 0.1 M NaCl, 0.05 M bis-Tris buffer, pH 7.0, was reacted at 25°C with a standard amount of NADH-methemoglobin reductase in a final volume of 1.0 ml. The enzyme used for these determinations was isolated from normal red cell lysates as a hemoglobin-free eluate from Whatman DE-11 DEAE cellulose. The reaction, started by the addition of 0.1 mM NADH, was monitored at 576 nm in a Giflord 240 spectrophotometer with a recorder attachment. Rates of nonenzymatic reduction by ascorbate were measured as described by Tomoda et al., except that the reaction was monitored at 576 nm. When added, IHP was present at a final concentration of 0.2 mM.

Rates of methemoglobin reduction in intact red cells, in vitro, were measured at 37°C using the system of Beutler and Baluda, except that methylene blue was omitted from the reaction mixture.
In this assay, red cells containing nitrite-oxidized methemoglobin are incubated with glucose in buffered isotonic solution, and the rate of methemoglobin reduction is measured using the spectrophotometric method of Evelyn and Malloy.\textsuperscript{13}

**DISCUSSION**

The results shown in Fig. 1 demonstrate that met-Hb Wood and met-HbA differ with respect to rates with which they are reduced by ascorbate or NADH and NADH-methemoglobin reductase. Not only is the rate of reduction of met-Hb Wood slower than that of met-HbA, but the rate enhancement produced by addition of the allosteric effector IHP is much less when met-Hb Wood is used as the substrate. It appears, therefore, that the allosteric equilibrium of met-Hb Wood is biased more toward R than in met-HbA and that it is not switched as far toward T with IHP compared with met-HbA.

This observation raises questions about the significance of the Hb Wood in the overall rate of methemoglobin reduction within the intact red cell. Thus, it was of interest to examine the relative effects of Hb Wood, NADH-methemoglobin reductase deficiency, and the combination of the two mutations on methemoglobin reduction in red cells from members of the propositus's family. Table 1 presents relevant analytical data for red cells from a normal individual and from members of the propositus's family used in the present work.

Hemoglobin Wood was detected by isoelectric focusing, and the relative amounts of Hb Wood and HbA were determined by spectrophotometric scanning of the developed gels as described previously.\textsuperscript{2} NADH-methemoglobin reductase and NADPH-diaphorase were assayed as described by Beutler.\textsuperscript{14} The propositus's (A.D.) red cells contain about 50% Hb Wood and about one-half of the normal NADH-methemoglobin reductase activity. Cells from O.D. and D.D. lack Hb

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**Table 1. Red Cell Methemoglobin Reductase Activities and Content of Hb Wood**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex</th>
<th>NADPH Diaphorase (U/g Hb)</th>
<th>NADH Met-Hb Reductase (U/g Hb ± SD)</th>
<th>Hb Wood (% Total Hb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.D.</td>
<td>M</td>
<td>1.33</td>
<td>1.85 ± 0.1</td>
<td>54.2</td>
</tr>
<tr>
<td>M.D.</td>
<td>F</td>
<td>1.34</td>
<td>3.16 ± 0.07</td>
<td>56.2</td>
</tr>
<tr>
<td>O.D.</td>
<td>M</td>
<td>1.70</td>
<td>1.97 ± 0.1</td>
<td>0</td>
</tr>
<tr>
<td>D.D.</td>
<td>F</td>
<td>1.19</td>
<td>2.11 ± 0.07</td>
<td>0</td>
</tr>
<tr>
<td>J.L.</td>
<td>M</td>
<td>1.38</td>
<td>3.33 ± 0.04</td>
<td>0</td>
</tr>
</tbody>
</table>

Enzyme activities were measured as described by Beutler.\textsuperscript{14}

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Fig. 1. (A) Enzymatic reduction of methemoglobin A and methemoglobin Wood in the absence and presence of 0.2 mM IHP. The rates of reduction were measured using NADH and NADH-methemoglobin reductase as described in the text. (B) Nonenzymatic reduction of methemoglobin A and methemoglobin Wood in the absence and presence of 0.2 mM IHP. The rates of reduction were measured using ascorbic acid as reductant according to Tomoda et al.\textsuperscript{12}

Fig. 2. Rates of methemoglobin reduction in erythrocytes from individuals described in Table 1. Erythrocytes were incubated at 37°C in the presence of buffered isotonic solution of glucose (pH 7.4) and the rate of methemoglobin reduction measured as described by Beutler and Baluda.\textsuperscript{7} The rates shown were normalized to an initial concentration of 100% methemoglobin. Actual initial methemoglobin concentrations ranged from 94% to 99%, and the results shown were obtained from replicate analyses of samples obtained from the various individuals on several different occasions. (○) J.L.; (●) M.D.; (▲) O.D.; (■) D.D.; (×) A.D.
Wood and contain only about one-half of the normal enzyme activity, whereas M.D.’s cells contain about 50% Hb Wood and the normal level of NADH-methemoglobin reductase activity. All specimens contained nearly the same levels of NADPH-diaphorase.

The results shown in Fig. 2 demonstrate that methemoglobin reduction in intact red cells containing 50% Hb Wood proceeds at a slower rate than in normal red cells. This low rate is comparable to that found in red cells with about one-half of the normal levels of NADH-methemoglobin reductase activity. Thus, the presence of Hb Wood, as well as the reduced level of the enzyme, appear to be significant factors in the rate of methemoglobin reduction by normal physiologic mechanisms prevailing within intact red cells. This conclusion is supported by the fact that the rate of methemoglobin reduction is even lower in red cells from A.D. who bears both mutations; the effects of the enzyme deficiency and the presence of a 50% level of Hb Wood appear to be additive.

It is concluded that the presence of a “high-affinity” type of mutant hemoglobin can affect the rate at which methemoglobin is reduced within the red cell. Clearly, the redox equilibrium of the hemoglobin within the erythrocytes of a Hb Wood carrier is perturbed, and this can be explained on the basis of changes in the quaternary conformation of the methemoglobin. Whether this phenomenon is a significant factor in the propositus’s drug-induced methemoglobinemia is uncertain, but the present results suggest this possibility.

REFERENCES

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