

1981

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Ali Mansouri

University of Arkansas for Medical Sciences

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Recommended Citation

Mansouri, Ali (1981) "Oxidation of Native and Modified Hemoglobin and Myoglobin by Sodium Nitrate. Effect of Inositol Hexaphosphate," *Journal of the Arkansas Academy of Science*: Vol. 35 , Article 13.

Available at: <http://scholarworks.uark.edu/jaas/vol35/iss1/13>

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OXIDATION OF NATIVE AND MODIFIED HEMOGLOBIN AND MYOGLOBIN BY SODIUM NITRITE. EFFECT OF INOSITOL HEXAPHOSPHATE

ALI MANSOURI

Division of Hematology and Oncology
Veterans Administration Medical Center and
University of Arkansas for Medical Sciences
Little Rock, Arkansas 72206

ABSTRACT

Native and modified hemoglobin, myoglobin and α and β hemoglobin subunits were oxidized by sodium nitrite at pH 6. The experiments were carried out under oxy and deoxy conditions with and without inositol hexaphosphate (IHP).

It is shown (a) that under oxy condition low concentration of IHP inhibits the oxidation of native hemoglobin only. However, high concentration of IHP inhibits the oxidation of both myoglobin and modified hemoglobin (digested or β -93-SH groups blocked). This inhibition is partially counteracted by high oxygen pressure. (b) Under deoxy condition the oxidation rates of all heme proteins studied are significantly faster than that of native hemoglobin. IHP inhibits the oxidation of all except the myoglobin and hemoglobin subunits.

It is concluded that although the IHP inhibitory effect on hemoglobin oxidation by nitrite can be explained by the shift of the R \rightleftharpoons T conformational equilibrium towards T conformation, some other structural changes such as alteration in molecular surface charges must occur to account for the effect of IHP on the oxidation of heme proteins devoid of heme-heme interaction.

INTRODUCTION

Hemoglobin oxidation by agents such as potassium ferricyanide, hydrogen peroxide and copper is significantly faster in the deoxy (T) than in the oxy (R) conformation. On the contrary, the oxidation of hemoglobin by sodium nitrite is inhibited by both deoxygenation and inositol hexaphosphate (IHP) which shift the molecular conformational equilibrium towards T conformation.

The hemoglobin oxidation by sodium nitrite has been known for over a century (Gamgee, 1869). Although during this time many aspects of the reaction have been studied (Meir, 1925; Jaenig and Jung, 1970), its exact mechanism is not fully understood. The discovery of the effect of organic phosphate on the functional properties of hemoglobin has stimulated more work in this area.

It has been assumed, although not directly proven, that the inhibitory effect of IHP on the oxidation reaction is related to the stabilization of the T conformation (Tomoda, et al., 1977). However, blockage of β -93-SH groups by iodoacetamide which conserves most of the heme-heme interaction (Taylor, et al., 1966), accelerates the oxidation reaction under deoxy condition leaving the inhibitory effect of IHP intact (Mansouri, 1979). This work was undertaken to explore further the mechanism of this reaction.

MATERIALS AND METHODS

All oxidation reactions were carried out at 25°C. All buffers contained 10^{-4} M ethylenediaminetetraacetic acid (EDTA). The hemoglobin samples were stripped of organic phosphates by the method of Antonini (Antonini and Brunori, 1971). The following chemicals were obtained from Sigma Chemical Co., St. Louis, MO: bis (2-hydroxyethyl) iminotris (hydroxymethyl) methane or bis-tris, diisopropylfluorophosphate-treated carboxypeptidase A (CPA), catalase, di-dithiothreitol, 5,5'-dithiobis-(2-nitrobenzoic acid), iodoacetamide, p-chloro-mercuribenzoate (PCMB) and sperm whale myoglobin. Inositol hexaphosphate (IHP) was purchased from P-L Biochemicals, Milwaukee, WI.

Hemoglobin A was purified from freshly drawn human citrated blood by ion exchange chromatography (Winterhalter and Huehns, 1964) (600 \times 25 mm column for 20 ml of blood). Pure fractions were concentrated and dialyzed against 0.05 M tris buffer at pH 8.6.

Hemoglobin α and β subunits were isolated by the method of Bucci and Fronticelli (1965) except that the regeneration of the -SH groups was carried out by incubation of α -PCMB and β -PCMB with 20 mM dithiothreitol under nitrogen for 30 min in the presence of catalase (Ikeda-Saito, 1977). The purity of the subunits was checked by cellulose acetate electrophoresis (Marengo-Rowe, 1965).

Commercial sperm whale ferric myoglobin was dissolved in 2-3 ml of 0.05 M bis-tris buffer at pH 6. Slight molar excess of sodium dithionite/heme was added to reduce the heme iron. The sample was then applied on a G-25 sephadex column (300 \times 12 mm) equilibrated with the same buffer to eliminate the excess of dithionite.

The blockage of β -93 cysteine residues was carried out at pH 7.8 in the presence of 10 fold molar excess of iodoacetamide/heme. Then the sample was incubated in the dark at 20°C (Winterbourn and Carrell, 1977). It was applied on cellulose acetate electrophoresis to prevent significant chain separation. The absence of the free thiol groups was tested by Ellman's method (1959).

Hemoglobin was digested by CPA following exactly the method described by Moffat (1971). The product of digestion was applied first on a G-25 Sephadex column, to free it from small molecules, and then on cellulose acetate electrophoresis, to demonstrate its homogeneity. No amino acid analysis was carried out on the portion containing the carboxy termini.

Two ml of 0.2 mM native or modified hemoglobin, myoglobin or hemoglobin subunits in 0.05 M bis-tris buffer, pH 6, was placed in a 1 cm light path quartz cuvette. Twenty μ l of 20 mM sodium nitrite solution (heme/nitrite molar ratio = 2) was added with a micropipette (Corning, Corning, New York) and mixed immediately. The increase of light absorbance at 631 nm (proportional to methemoglobin formation) was monitored by a double beam Beckman spectrophotometer, model 35, at 25°C with the recorder started when the sodium nitrite was added. All experiments under deoxy condition were carried out in a 1 cm light path tonometer. The hemoglobin solution was deoxygenated using a high vacuum pump by the method of Rosseifanelli and Antonini (1958). Spectrum between 700-500 nm was obtained to ascertain complete deoxygenation. One hundred μ l of 4 mM sodium nitrite solution previously deoxygenated by nitrogen wash was injected into the tonometer with a microsyringe (Hamilton, Reno, NV) but not yet mixed with hemoglobin. Further vacuum was applied to eliminate any oxygen that entered during the injection

process. The tonometer was brought to 25°C, and the two solutions were mixed vigorously while the recorder was started simultaneously. The reaction was followed spectrophotometrically at 631 nm.

RESULTS

Oxidation of Oxyhemoglobin and Oxymyoglobin With and Without IHP.

Hemoglobin and myoglobin were oxidized in air with and without varying concentration of IHP. Figures 1 and 2 show that the oxidation rate of myoglobin is slower than that of hemoglobin. IHP inhibits the oxidation reaction. The inhibition depends on the concentration of IHP for both hemoproteins. However, for the same degree of inhibition a much lower concentration of IHP is needed for hemoglobin.

Effect of High Oxygen Pressure on Oxidation.

Because of marked difference in oxidation rate of hemoglobin under oxy and deoxy conditions, the effect of high concentration of oxygen was tested. Figures 3 and 4 show the oxidation kinetics of hemoglobin and myoglobin in air and under one atmosphere of oxygen. High oxygen pressure not only accelerates the rate of oxidation, but also partially counteracts the inhibitory effect of IHP on the hemoglobin oxidation.

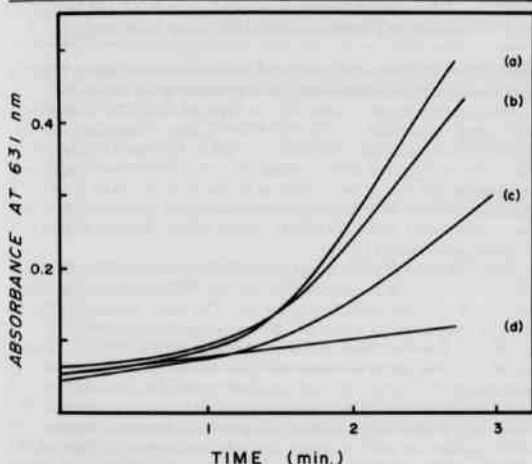


Figure 1. Oxidation of oxyhemoglobin (nitrite/heme molar ratio = 2 in all experiments) with no IHP (a) and in the presence of IHP/heme molar ratio of 0.1 (b); 0.2 (c); and 1.0 (d).

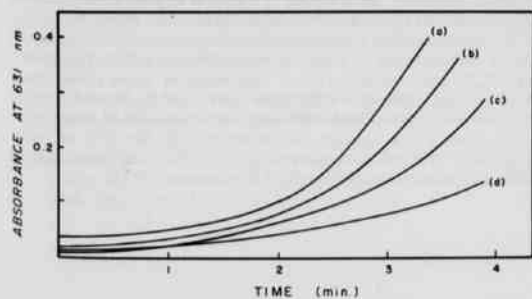


Figure 2. Oxidation of oxymyoglobin with no IHP (a) and in the presence of IHP/heme molar ratio of 0.5 (b); 1.0 (c); and 5 (d).

Oxidation of Modified Hemoglobins Under Deoxy Condition.

Two types of modified hemoglobin, iodoacetamide reacted hemoglobin with normal heme-heme interaction, and carboxypeptidase A digested hemoglobin with little or no heme-heme interaction, were oxidized under deoxy condition. Figure 5 shows that both of these hemoglobins are oxidized rapidly under deoxy condition (unlike native hemoglobin), but the addition of IHP inhibits both reactions (like native hemoglobin).

Oxidation of Myoglobin and Hemoglobin Subunits Under Deoxy Condition With and Without IHP.

Because of similarity of myoglobin and hemoglobin subunits and the lack of heme-heme interaction, these heme proteins were oxidized under deoxy condition in the absence of IHP (Fig. 6) and in the presence of IHP (Fig. 7). Due to precipitation of α PCMB and α chains in the presence of IHP, the oxidation of these subunits could not be carried out with IHP. In both figures the oxidation curve of native hemoglobin is inserted for comparison.

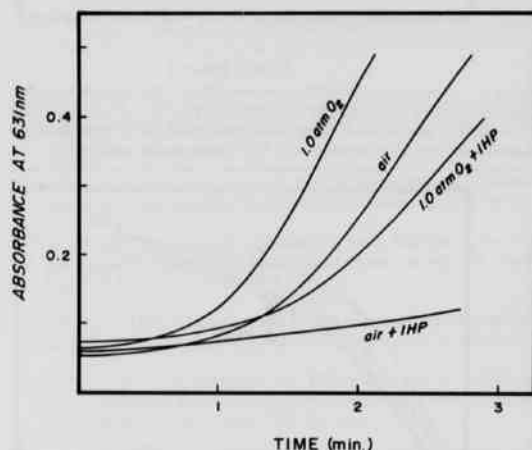


Figure 3. Oxidation of oxyhemoglobin in air and under 1.0 atmosphere of oxygen with and without IHP. IHP/heme molar ratio = 0.5. Note that oxygen increases the rate of the oxidation reaction as well as offsets largely the inhibitory effect of IHP.

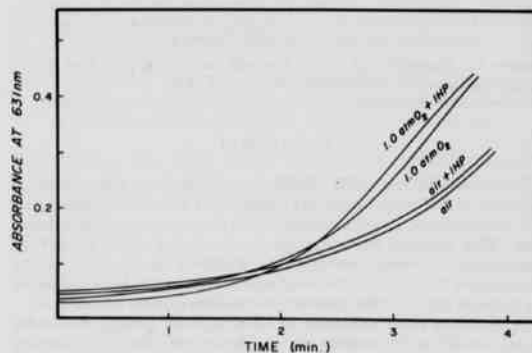


Figure 4. Oxidation of oxymyoglobin under exactly same conditions as in Figure 3.

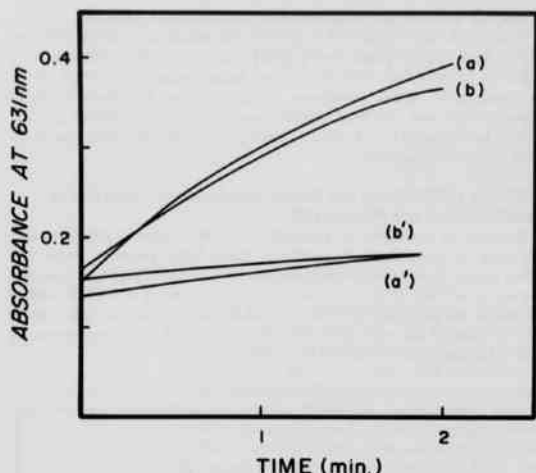


Figure 5. Oxidation of modified hemoglobin (CPA digested) under deoxy conditions without IHP (a) and with IHP (a'). Curves (b) and (b') represent oxidation of hemoglobin with (β -93-SH groups blocked) without and with IHP respectively. IHP/heme molar ratio = 0.5.

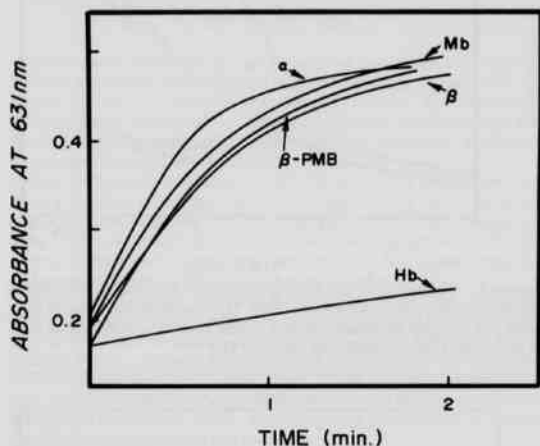


Figure 6. Oxidation of deoxy hemoglobin, myoglobin and hemoglobin subunits (No IHP added). PMB = PCMB.

DISCUSSION

The results of this study clearly demonstrate that although small amounts of IHP only inhibit the oxidation of native hemoglobin and not of myoglobin, larger amounts inhibit the oxidation of the latter as well. This suggests that although the inhibitory effect of IHP on hemoglobin oxidation seems to be mediated by the shift of the quaternary $R \rightleftharpoons T$ conformational equilibrium as has been suggested by Tomoda (1977), other possible mechanisms can not be ruled out.

In fact, the rapid oxidation of CPA digested hemoglobin under deoxy condition by sodium nitrite emphasizes that the molecular conformation is an important rate determining factor. It is disturbing that under deoxy condition, a small amount of IHP inhibits the oxidation of this modified hemoglobin as well. In this regard one can only

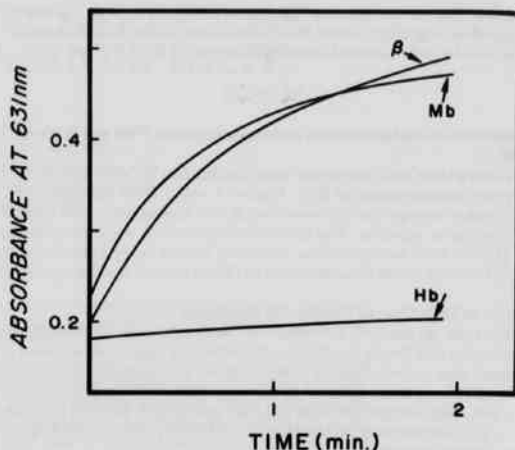


Figure 7. Oxidation of deoxyhemoglobin, myoglobin and hemoglobin β -subunits in the presence of IHP. IHP/heme molar ratio = 0.5.

assume that in spite of digestion of the β -C-termini, there remains perhaps some heme-heme interaction accounting for this effect.

Another important element also in favor of the $R \rightleftharpoons T$ equilibrium shift being responsible for the IHP effect is that oxygen not only significantly increases the oxidation rate but it also partly counteracts the inhibitory effect of IHP. It should be noted, however, that oxygen can modify the rate of the oxidation by mechanism other than $R \rightleftharpoons T$ equilibrium shift, such as direct participation in the oxidation as has been reported by Kakizaki (1964), Smith (1970), Rodkey (1976) and Wallace and Caughey (1975).

Although several factors suggest mechanisms other than the $R \rightleftharpoons T$ equilibrium shift to be responsible for the IHP effect on the oxidation, none of them exclude the former. The most important among these is the inhibitory effect of IHP on myoglobin oxidation. It is to be noted, however, that significantly larger amounts of IHP are needed to bring about the same effect on myoglobin oxidation as that on hemoglobin oxidation. The insensitivity of the oxidation rate of iodoacetamide reacted hemoglobin (β -93-SH groups blocked) to IHP under oxy condition is another factor which does not support the $R \rightleftharpoons T$ equilibrium shift as being the only mechanism explaining the inhibitory effect of IHP on hemoglobin oxidation. Finally, it seems that the addition of a large amount of IHP to the oxidation reaction can be a nonspecific salt effect. But this is not the case because an equivalent amount of NaCl does not change the rate of the oxidation reaction. However, the presence of 5 units of heparin in the reaction mixture inhibits significantly the oxidation rate (not shown here).

It is concluded that although the inhibitory effect of IHP on hemoglobin oxidation can be explained mostly by the shift of the quaternary $R \rightleftharpoons T$ equilibrium towards T conformation, some subtle structural changes other than $R \rightleftharpoons T$ shift must occur to account for the IHP effect on the oxidation of heme proteins devoid of heme-heme interaction. These changes are rather specific for IHP or other negatively charged molecules suggesting that the alteration of molecular surface charges is a likely mechanism.

ACKNOWLEDGMENTS

The author wishes to thank Drs. Karl D. Straub and Rudolf Flueckiger for their constructive criticism as well as Ms. Carol A. Perry for her technical assistance, and Ms. Wanda Stewart for her secretarial assistance. This work was supported by Veterans Administration Research funds, project #5545-002 and NIH Grant No. 5-RO1-AM20181-03. This work was presented at the American Society of Biological Chemists Meeting, New Orleans, LA, June 1980.

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