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OXIDATION OF HUMAN HEMOGLOBIN BY SODIUM NITRITE - EFFECT OF β-93 THIOL GROUPS

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Summary: The influence of β-93 sulfhydryl groups on the oxidation of human hemoglobin by sodium nitrite was studied. It is shown that the blocking of these groups by iodoacetamide counteracts the inhibition of the hemoglobin oxidation reaction caused by inositol hexaphosphate. This effect is not present under anaerobic condition. However, in the absence of free oxygen (deoxyhemoglobin), blocking of the β-93 sulfhydryl groups accelerates markedly the rate of oxidation which is otherwise very slow. In the light of these observations, it is concluded that the hemoglobin β-93 free-SH groups play a protective role for the heme iron against oxidation. The rapid oxidation of modified hemoglobin by nitrite under anaerobic condition as well as the abolishment of the effect of IHP under aerobic condition by β-93-SH groups blockage argue against the assumption that R conformation is primarily responsible for the rapid oxidation of oxyhemoglobin by nitrite.

Introduction:

Nitrites are abundant in nature and are frequently used in food and drug. Certain aspects of the interaction of nitrites with hemoglobin leading to its oxidation have been worked out by several authors (1-6), but the role of free-SH groups of the β chains has not been studied in this regard except the study of Kakizaki et al. in which they measured the rate of hemoglobin oxidation in the presence of various concentrations of p-CMB (7). The work of Winterbourn and Carrell (5) demonstrates the role of β-93 cysteine residues in the oxidation of hemoglobin by copper. In view of this finding as well as the large difference between the autoxidation rates of hemoglobin α and β subunits (8), it was postulated that the β-93-SH groups might generally participate more directly in the hemoglobin oxido-reductive reactions which lead to this investigation.

Abbreviations: IHP = inositol hexaphosphate; p-CMB = p-chloromercuribenzoic acid ; bis-Tris= bis (2-Hydroxyethyl) imino-tris (Hydroxy-methyl) methane

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Materials and Methods:

Inositol hexaphosphate (IHP) was obtained from P-L Biochemicals, Milwaukee, WI. Iodoacetamide and 5,5'-dithibis (2-nitrobenzoic acid) were purchased from Sigma Chemical Co., St. Louis, MO. Hemoglobin A was prepared from fresh adult blood by ion exchange chromatography (9). All samples were stripped of organic phosphates by passing them through a column of G-25 Sephadex equilibrated with 0.1 M NaCl solution (10). The samples were then dialyzed against 0.05 M bis-Tris buffer containing 10^{-4}M EDTA at pH 6 or 7 as needed. Reaction of β-93 cysteine residues with iodoacetamide was carried out at pH 7.8 in the presence of 10-fold molar excess of iodoacetamide over heme. The incubation was continued for 3 hours at 20°C in the dark as previously reported (5). The modified hemoglobin was freed from the excess of iodoacetamide by passing it through a G-25 Sephadex column (300 x 8 mm for 2 ml sample) previously equilibrated with 0.05 M bis-Tris buffer pH 6 or 7 according to the experiments planned. The absence of free thiol groups was checked by the method of Ellman (11). Hemoglobin α and β subunits were separated by the method of Bucci and Fronticelli (12).

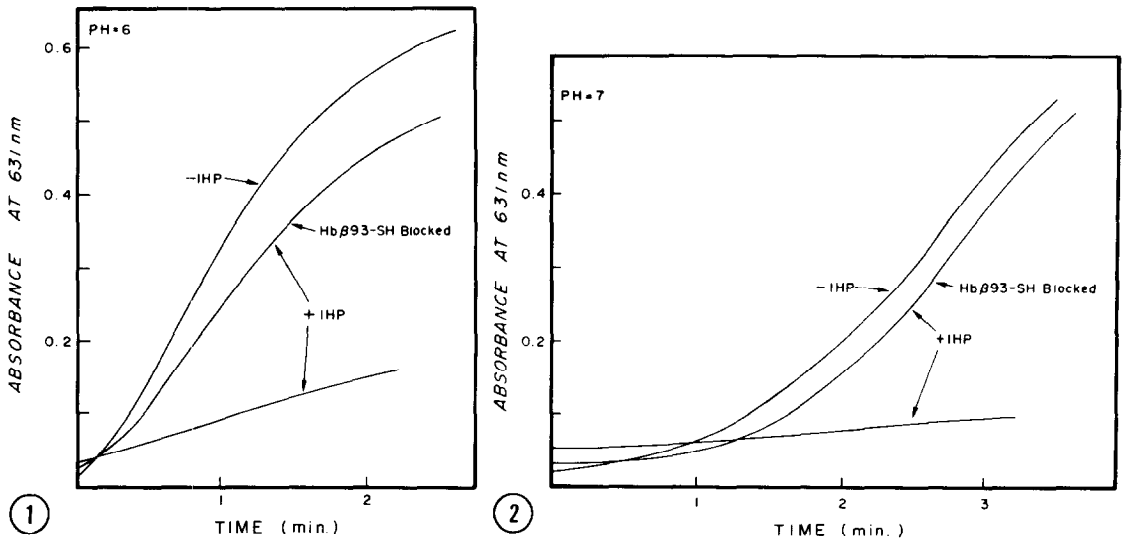
Experimental Procedures:

Two ml of 0.2 mM hemoglobin solution in 0.05 M bis-Tris buffer pH 6 was placed in a 1 cm light path quartz cuvette. Ten μl of 20 mM sodium nitrite solution (heme/nitrite molar ratio = 1) was added by a micro pipette (Corning, Corning, New York) and mixed immediately. The increase of light absorbance at 631 nm (proportional to methemoglobin formation) was monitored by spectrophotometer (Beckman 35) at 25°C with the recorder started simultaneously with the addition of sodium nitrite. Similar experiments were carried out at pH 7 with the only difference that at this pH five times excess of sodium nitrite over heme (50 μl) was added because of the slowness of the reaction. The above experiments were performed also in the presence of IHP with the molar ratio of heme/IHP kept unity. Deoxyhemoglobin oxidation was carried out in a 1 cm light path quartz tonometer. The hemoglobin solution was deoxygenated by the method of Rossi-Fanelli and Antonini (13). Spectrum between 700-500 nm was obtained to ascertain complete deoxygenation. One hundred μl of sodium nitrite solution (heme/nitrite molar ratio = 1) previously deoxygenated by nitrogen wash was injected - but not yet mixed with hemoglobin - with a microsyringe (Hamilton, Reno, Nevada). Further vacuum was applied to exclude any oxygen which might have entered by the injection process. The tonometer was brought to 25°C and the two solutions were mixed vigorously while the recorder was started simultaneously. The increase of light absorbance at 631 nm was monitored. These experiments were performed at pH 6 and 7 with and without IHP. Similar to the oxyhemoglobin oxidation 5 times more nitrite was used at pH 7. All the above experiments were performed with modified hemoglobin under the same conditions. α and β subunits were oxidized by sodium nitrite in the presence of oxygen only (air).

Results:

Oxidation of Native and Modified Oxyhemoglobin

Native and modified hemoglobin (β-93 cysteine residues blocked) in 0.05 M bis-Tris buffer was oxidized as is described in the experimental procedures with nitrite/heme molar ratio of 1 and 5 at pH 6 and 7 respectively with and without IHP (Figs. 1 & 2). The inhibition of oxidation reaction by IHP is largely counteracted when the β-93 cysteine residues are blocked by iodoacetamide.



Figures 1 & 2:

Oxidation of native and modified oxyhemoglobin by sodium nitrite at 25°C. Hemoglobin concentration is 0.02 mM in 0.05 M bis-Tris buffer containing 10^{-4} M EDTA.

Molar ratio of nitrite/heme = 1 at pH 6 and 5 at pH 7. Molar ratio of IHP/heme = 1 at both pH's. Note that the blocking of the β-93-SH groups abolishes the inhibitory effect of IHP on oxidation at both pH's.

This effect is present at pH 6 and 7 although at the latter pH, all reactions are slower.

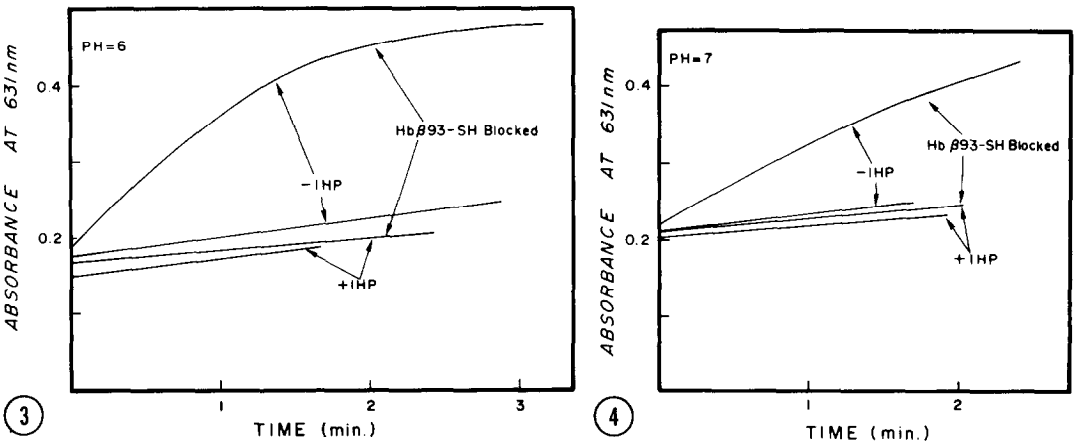
Oxidation of Native and Modified Deoxyhemoglobin

Figure 3 shows that the rate of oxidation of deoxyhemoglobin by nitrite which is usually slow, is accelerated approaching nearly that of oxyhemoblogin oxidation when β-93 cysteine residues are blocked.* Contrary to the oxidation of oxyhemoglobin, the inhibitory effect of IHP persists in native as well as in modified hemoglobin. Figure 4 represents the same reactions at pH 7.

Oxidation of Isolated α and β Hemoglobin Subunits

α and β subunits were oxidized by nitrite at pH 6 and 7 (Fig. 5). It is evident that the time needed for β subunits to reach 50% oxidation level is about 3-fold greater than that for α subunits.

*This is not immediately apparent from comparison of Figures 1 and 3 because the increase in light absorbance at 631 nm is smaller for deoxy than oxyhemoglobin oxidation.



Figures 3 & 4:

Oxidation of native and modified deoxyhemoglobin by sodium nitrite. All other conditions are the same as in Figures 1 and 2. Note that the blocking of the β-93-SH groups does not abolish the IHP effect. However, it accelerates the rate of deoxyhemoglobin oxidation. Deoxyhemoglobin has greater absorbtion than oxyhemoglobin at 631 nm.

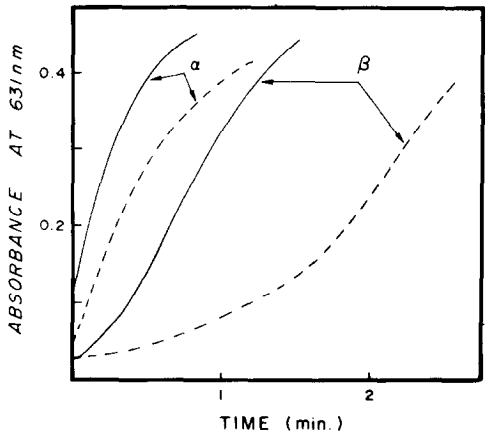


Figure 5:

Oxidation of isolated hemoglobin α and β subunits by sodium nitrite. The molar ratio of nitrite/heme is kept unity at both pH's.

Discussion

Contrary to the hemoglobin oxidation by ferricyanide, the oxidation by nitrite is faster in the oxy than in the deoxy form and is inhibited to a

great extent by the presence of IHP. For these reasons it has been claimed that the hemoglobin R conformation (oxy) is more easily oxidized by nitrite (6).

Figures 1 and 2 show that the blocking of the β-93 cysteine residues by iodoacetamide counteracts largely the inhibitory effect of IHP on the oxidation reaction indicating that the effect of IHP may not necessarily be due to the change in conformation. Figure 3 demonstrates that the rate of the oxidation of deoxyhemoglobin (T conformation) by nitrite is very slow and similar to that of oxyhemoglobin oxidation in the presence of IHP. One could then easily assume that the IHP effect on oxidation is the result of a shift in the conformational equilibrium. But, it is evident from Figures 3 and 4, that the rate of oxidation of modified deoxyhemoglobin is much faster and is inhibited by IHP as well. These results are suggestive of another factor being operative rather than simply conformational change being uniquely responsible for this effect.

Incidentally, it was shown that 2,3-DPG, NADP and ATP at concentrations of 2 and 5 molar excess over heme did not affect the rate of native or "modified" hemoglobin oxidation by nitrite not shown here which strengthens the assumption that another factor rather than conformational change must be at work. The rapid oxidation of modified hemoglobin under anaerobic conditions indicates the independence of this reaction on free oxygen. The lack of oxidation of carboxyhemoglobin by nitrite (4) does not seem to be due to the lack of oxygen but rather to the stronger binding of carbon monoxide. This is similar to myoglobin oxidation by ferricyanide in which the dissociation of the ligand must occur before ferricyanide can react (14). However, the lack of effect of β-93 blockage on the inhibitory effect of IHP under anaerobic conditions suggests that oxygen might be necessary for the IHP effect on the oxidation reaction. The exact mechanism of the effect of β-93-SH groups on the oxidation of heme iron by nitrite is not clear. The work of Winterbourn and Carrell on copper oxidation has shown that copper binds to β-93 residues and the

oxidation of heme iron follows. In fact blocking of β-93 residues inhibits hemoglobin oxidation by copper.

In the present study, the blocking of β-93 residues actually facilitates the oxidation of deoxyhemoglobin as well as removes the inhibitory effect of IHP under aerobic conditions. It is unlikely that either IHP or nitrite bind to β-93 residues. However, it is conceivable that blocking of these residues facilitates the transfer of electron by heme iron similar to binding of copper which facilitates electron transfer from iron to copper.

It has been concluded from the study of hemoglobin autoxidation that one of the mechanisms influencing the rate of oxidation of hemoglobin subunits is their intrinsic rate of oxidation which is lower for β chains (8). This is also evident in hemoglobin oxidation reaction by nitrite (Fig. 5). The above findings are highly suggestive that the low intrinsic oxidability of β subunits is related at least in part to the presence of free β-93 cysteine residues. In fact preliminary data obtained by the autoxidation of modified hemoglobin is in favor of this hypothesis which emphasizes the protective role of β-93 free-SH groups against heme iron oxidation. Lastly, it seems that conformational changes alone do not explain adequately the behavior of hemoglobin in its reaction with nitrite and the inhibition of this reaction by IHP.

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