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THE INFLUENCE OF INOSITOL HEXAPHOSPHATE ON THE REDUCTION OF ISOLATED METHEMOGLOBIN SUBUNITS AND RECONSTITUTED MOLECULES

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SUMMARY: Enzymatic reduction of α and β methemoglobin subunits was studied both in the isolated and tetrameric (reconstituted) forms. It is shown that the isolated forms are more accessible to reduction. The reduction rates of the subunits in the tetrameric form approaches those of the isolated forms only in the presence of IHP. However, the ratio of reduction rates of β/α is greater in the tetrameric molecule than in the isolated subunits (5 fold (-IHP), 7 fold (+IHP) versus 3 fold-isolated subunits) implying a greater IHP effect on the reduction of β subunits. IHP does not affect the reduction rates of isolated subunits indicating that participation of quaternary structure is necessary for this effect.

INTRODUCTION

Hemoglobin α and β subunits are non-equivalent in many of their reactions such as autoxidation (1) and 2,3-DPG binding (2). As it has been shown previously, they are also non-equivalent in their reduction, β chains being reduced preferentially (3). The recent report that reduction of methemoglobin by ascorbic acid leads to $\alpha^{3+}\beta^{2+}$ compound is of great interest (4,5) and confirms the preferential reduction of β subunits. However, direct enzymatic reduction of methemoglobin subunits in the isolated form or in valency hybrids has not been tested. In this work, the enzymatic reduction of isolated methemoglobin subunits and reconstituted tetramers in the presence and absence of IHP is reported.

Abbreviations: IHP, inositol hexaphosphate; β-NADH, reduced nicotinamide adenine dinucleotide; 2,3-DPG, 2,3-diphosphoglycerate; p-CMB, p-chloromercuribenzoic acid.

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MATERIALS AND METHODS

β-NADH and p-CMB were obtained from Sigma Chemical Company, St. Louis, MO, U.S.A. IHP was purchased from P-L Biochemicals, Inc., Milwaukee, WI, U.S.A.

Hemoglobin A was purified from fresh human red cells by ion exchange chromatography (6). α and β subunits were prepared by the method of Bucci and Fronticelli (7). The pure subunits were then reacted with 1.5 molar excess of KCN and potassium ferricyanide/heme in order to transform the subunits to cyanometform. Two ml of the cyanometform of α or β subunits was applied to columns of G-25 300 x 1 mm equilibrated with 0.05 M bis Tris, pH 6 or 7, as necessary. γ chains were prepared as has been described by Nobel et al (8). NADH methemoglobin reductase was purified in the same way as previously reported with the difference that purification was not carried out beyond G-75 gel filtration (9,10). Valency hybrids were prepared by the method previously reported (11). Methemoglobin was reduced enzymatically in the presence of ferrocyanide (9,10).

RESULTS

Cyanometform of α and β hemoglobin subunits were reduced by NADH-methemoglobin reductase with and without IHP 1 mole/mole of heme at pH 6. It is shown that the rate of reduction of β subunits is three times greater than that of α subunits. IHP does not have any effect on the reduction rate of either subunits (Fig. 1).

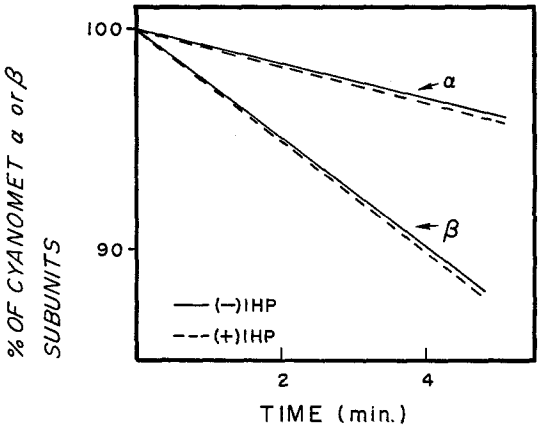

Figure 1: Reduction of cyanomet α and β subunits (0.2 mM) by NADH-methemoglobin reductase and potassium ferrocyanide with and without IHP (equimolar) at 22°C in 0.05 M bis-Tris buffer, pH 6. The percent reduction is calculated from:

$$\frac{\Delta E \times 100}{E_c - E_o} \text{ at 576 nm } (E_c = \text{complete reduction})$$

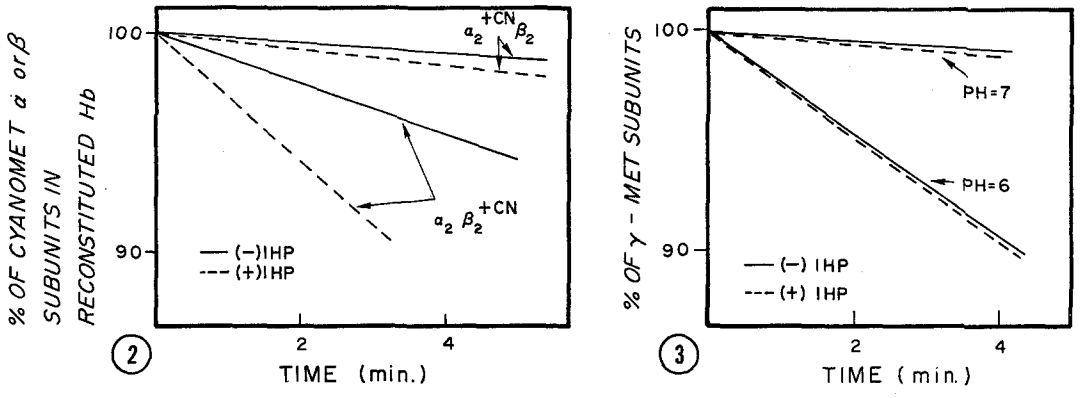

Figure 2: Reduction of $\alpha_2^{+CN}\beta_2$ and $\alpha_2\beta_2^{+CN}$. The percent of reduction is calculated from the increase in light absorbance at 576 nm by:

$$\frac{\Delta E \times 100}{E_c - E_o}$$

Figure 3: Reduction of γ subunits in the metform. Experimental conditions are the same as in Fig. 1. The percent of reduction is calculated from:

$$\frac{-\Delta E \times 100}{E_o}$$ Sign (-) signifies decrease in absorbance.

Artificial valency hybrids $\alpha_2^{+CN}\beta_2$ and $\alpha_2\beta_2^{+CN}$ were reduced enzymatically with and without IHP under similar experimental conditions as in Figure 1. It is shown that β subunits (in tetrameric molecule) reduce faster as well. The ratio of reduction rate of β/α subunits is 5 fold (-IHP) and 7 fold (+IHP). IHP accelerates the reduction of both subunits but its effect is more pronounced on the reduction rate of β subunits (Fig. 2).

Figure 3 shows the reduction of γ subunits from fetal hemoglobin with and without IHP. As the metform of these subunits is relatively stable, it was possible to reduce these subunits directly from the metform rather than cyanometform. It is shown that the reduction of the metform is not affected by IHP as in the case of the cyanometform.

DISCUSSION

In hemoglobin autoxidation the α subunits have about 8-10 times faster oxidation rate (1). The effect of organic phosphates is mostly borne by the

α subunits (12). One would expect that the α subunits should be preferentially reduced. In fact, it has been shown that in methemoglobin reduction reaction (enzymatic or chemical), β subunits reduce preferentially (3,4,5). Figure 1 shows that this is the case even when isolated α and β subunits are reduced. Because of unstability of these subunits in the metforms, the cyanometforms are used. Although the reduction rate of the isolated subunits is faster than those of the tetrameric form, the relative ratio of reduction rate of β/α increases in the whole molecule (3 fold versus 5 fold, respectively).

The organic phosphate (IHP) which accelerate the rate of methemoglobin reduction does not affect the reduction of isolated subunits, which implies that the IHP effect is mediated through some changes in the quaternary structure. The presence of CN^- ion does not interfere with the effect of IHP because the latter does not affect the reduction rate of γ chains in the metform (Fig. 3). Figure 2 shows that the effect of IHP is largely borne by the β chains, although Tomoda et al did not observe any IHP effect on α subunits during the methemoglobin reduction by ascorbic acid (4). It seems that as β subunits have evolved from α subunits, they have acquired the evolutionary advantage of oxidizing more slowly, being reduced faster than the α subunits, but the exact biological role of this difference is to be determined.

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