Vol. 86, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 30, 1979 Pages 415-419

ENZYMATIC METHEMOGLOBIN REDUCTION - EFFECT OF ORGANIC PHOSPHATE

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Received December 18, 1978

SUMMARY: The enzymatic reduction of aquomethemoglobin A, A_1C, fluoromethemoglobin A (high spin) and cyanomethemoglobin A (low spin) by NADH-methemoglobin reductase was studied in the presence and absence of IHP and NaCl. It is shown that at alkaline pH, IHP accelerates the rate of reduction of high spin methemoglobins only. This effect is specific for IHP and cannot be produced by NaCl, although NaCl does exert similar effect as IHP at acid pH. Blocking of the NH_2- termini of β-chains (Hb A_1C) does not alter the effect of IHP on methemoglobin reduction.

INTRODUCTION

Although several methemoglobin reducing systems are present in the red cell, the NADH-methemoglobin reductase system is physiologically the most important. Its functional absence leads to clinical methemoglobinemia (1-4). In the red cell, alkaline pH (7.2) and high concentration of oxyhemoglobin slows down markedly the rate of methemoglobin reduction. Although methemoglobin T conformation is more accessible to reduction (5,6), whether this is physiologically important is not certain. This work was undertaken to evaluate the relationship of organic phosphate (IHP) and methemoglobin molecular spin on methemoglobin reduction.

MATERIALS AND METHODS

Reduced nicotinamide adenine dinucleotide was obtained from Sigma Chemical Company, St. Louis, MO, U.S.A. Inositol hexaphosphate was obtained from PL Biochemicals, Inc., Milwaukee, WI, U.S.A.

Abbreviations: IHP = Inositol hexaphosphate, 2,3-DPG = 2,3-Diphosphoglycerate
β-NADH = Reduced nicotinamide adenine dinucleotide,
Hb = Hemoglobin

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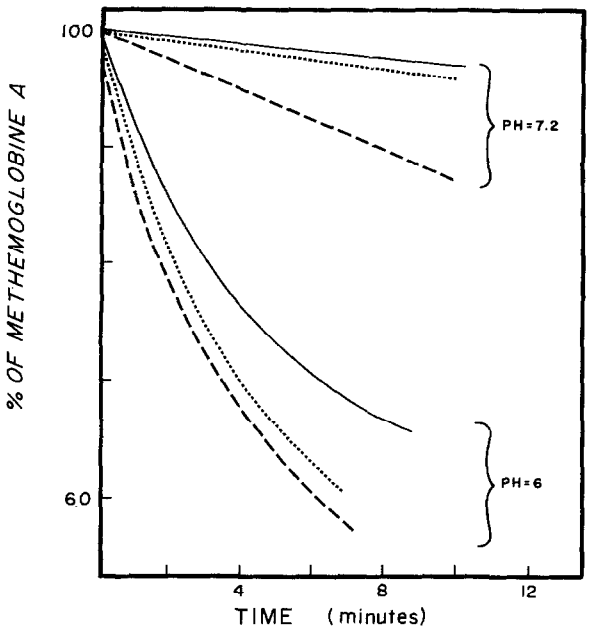

Figure 1.

0.2 mmolar methemoglobin solution in 0.05 bis-Tris buffer was reduced by methemoglobin reductase and potassium ferrocyanide at 25°. The reduction was carried out with stripped methemoglobin (——), methemoglobin + 0.5 mole of IHP/mole of heme (----) and methemoglobin + 0.5 mole of NaCl/mole of heme (....). The effect of IHP on reduction rate is more pronounced at pH 7.2. Two-fold at pH 6 versus seven-fold at pH 7.2. NaCl exerts similar effect at pH 6 but its effect is minimal at pH 7.2. The reduction was measured by the decrease of absorbance at 631 nm.

Hemoglobins A and A_1C were prepared from fresh human blood by CM-Sephadex chromatography (7). All hemoglobin solutions were stripped of 2,3-DPG (8) and equilibrated in 0.05 molar bis-Tris buffer at appropriate pH. Methemoglobin was prepared by oxidation of fresh hemoglobin by potassium ferricyanide (9). NADH-methemoglobin reductase was prepared as previously reported (10,11), except that the purification was not carried out beyond G-75 Sephadex gel filtration. Methemoglobin reduction was carried out as previously described (10,11).

RESULTS

0.2 mmolar methemoglobin solution in 0.05 molar bis-Tris buffer at pH 6 and 7.2 was reduced enzymatically in the presence and absence of IHP as well as NaCl (0.5 mole of IHP or NaCl/mole of heme).

Figure 1 shows that low concentration of IHP increases the rate of methemoglobin reduction. This increase is only marked at alkaline pH although the intrinsic reduction rate is low at this pH. The pH dependance of the effect of

TABLE I

pH	5	6	6.7	6.9	7.2
CyanometHb	-	4.5 ± 0.62	-	-	1 ± 0.16
FluorometHb	-	1.8 ± 0.52	-	-	6.1 ± 1.20
AquometHb	1 ± 0.12	2 ± 0.59	4 ± 0.58	7 ± 1.10	8.2 ± 1.15

Table representing the ratios of reduction rates in the presence and the absence of 0.5 mole of IHP/mole of heme.

IHP on reduction reaction is also apparent for fluoromethemoglobin (high spin) but not for cyanomethemoglobin (low spin) (Table I). NaCl exerts similar effect as IHP at acid but not at alkaline pH. This was shown to be the case for all methemoglobins studied. Besides, the combined effect of NaCl and IHP at both pHs is the same as the effect of IHP alone (no additive effect). The rate of reduction of methemoglobin A_1C (β NH_2-termini blocked) is similarly affected by the presence of IHP under the same experimental conditions (Fig. 2).

DISCUSSION

Methemoglobin reduction by methemoglobin reductase is maximal at acid pH. The ratio of reduction rates at pH 4.8/7.2 equals 7-fold. At intracellular pH and in the presence of large amounts of oxyhemoglobin, this enzyme functions suboptimally. It has been suggested that methemoglobin T conformation favors its reduction (5,6). In this case organic phosphates most likely play an important role in facilitating the reduction reaction. Figure 1 shows that the accelerating effect of IHP is much more pronounced at alkaline pH. This is in agreement with the work of Taketa and Chen (12) although they used red cell hemolysate. It is shown here that this effect is only manifested by aquo and fluoromethemoglobin (high spin). Cyanomethemoglobin (low spin) reduction is not affected by IHP at alkaline pH (Table I). As is shown in Figure 1, NaCl has similar effect as IHP at acid pH and no effect at alkaline pH, implying the necessity of specific binding

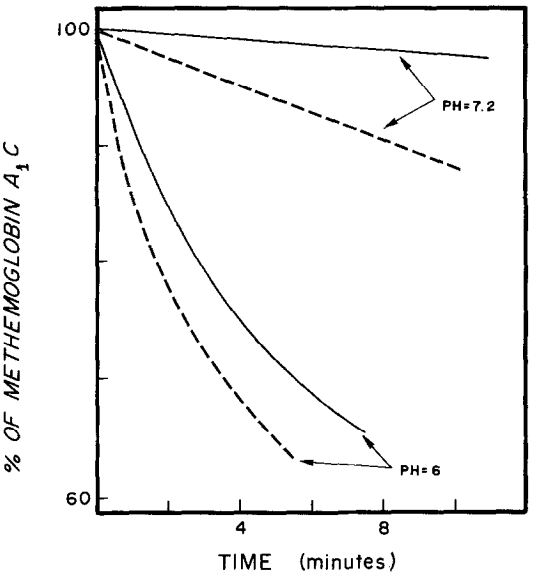

Figure 2.

Methemoglobin A_1C reduction under the same conditions as in Fig. 1. Reduction was carried out in the absence (——) and the presence (----) of IHP. The effect of IHP on methemoglobin A_1C (β-NH_2 termini blocked) reduction is similar to its effect on methemoglobin A reduction.

of IHP to methemoglobin and stabilization of the T conformation. Although NaCl is known to exert qualitatively similar effect on hemoglobin molecule as organic phosphates, its concentration should be sufficiently high which in this case would be inhibitory for the reduction reaction. However, the binding of IHP to hemoglobin is stronger and more specific which would explain the difference between IHP and NaCl effect at alkaline pH. The effect of IHP on the reduction of methemoglobin A_1C (β-NH_2 termini- sites of 2,3-DPG binding) is similar to its influence on methemoglobin A reduction (Figure 2).

This work demonstrates that at alkaline pH, IHP accelerates the rate of reduction of high spin methemoglobin derivatives only, by favoring the T conformation. This is in agreement with the work of Perutz, et al (5) who showed that IHP does not affect the R⇌T transition of low spin derivatives but not with the work of Hensley, et al (13) who reported the independence of this transition on spin state. The similar response of methemoglobin A and A_1C to the presence of IHP

suggests strongly the presence of binding sites other than β-NH_2 termini. These sites might be the α-NH_2 termini which have been shown by Benesch, et al (14) to be responsible for binding of certain pyridoxal derivatives. In view of alkaline intracellular pH, the presence of organic phosphates is probably important in reduction of methemoglobin.

ACKNOWLEDGEMENT

The author wishes to thank Ms. Carol A. Perry for her technical assistance and Ms. Diane Butler for her secretarial assistance. This work was supported by NIH grant #1 RO1 AM20181-02.

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