ACTIVATION OF THE NADH-METHEMOGLOBIN REDUCTASE REACTION BY INOSITOL HEXAPHOSPHATE

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Received January 31, 1977

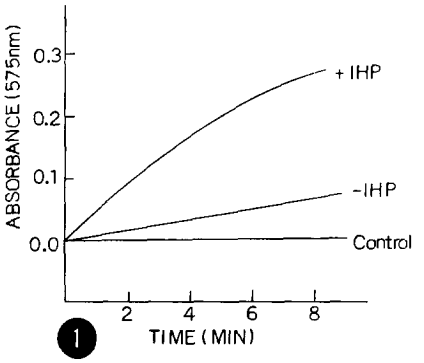
SUMMARY: Inositol hexaphosphate (IHP)[1] increases the rate of the NADH-methemoglobin reductase reaction at neutral pH. This effect is apparently associated with the change in conformation of methemoglobin induced by its interaction with IHP. It is consistent with the suggestion that IHP stabilizes the quarternary T type of structure in methemoglobin.

INTRODUCTION

The NADH-methemoglobin reductase (Diaphorase) reaction is the major mechanism for methemoglobin reduction in mature human red cells (1). However, the reaction proceeds very slowly in vitro and it is difficult to follow spectrophotometrically unless it is measured using the artificial electron acceptor, dichlorophenolindophenol (2), or a methemoglobin-ferrocyanide complex as "activated" substrate under the acidic conditions described by Hegesh et al. (3,4,5). The possibility that the rate of methemoglobin reduction at neutral pH might be enhanced by using inositol hexaphosphate to form an activated substrate arose from a series of recent observations. When IHP is bound to methemoglobin, changes in conformation of the protein that affect the reactivity of the ferric hemes are noted (6,7). The properties of the complex suggest that a shift in the allosteric equilibrium of the methemoglobin tetramer from R towards T (8) has occurred. Because of the formal analogy between the oxygenation and oxidation reaction (8,9) such a shift should facilitate the reduction of ferrihemoglobin to ferrohemoglobin. That IHP indeed enhances the rate of the NADH-methemoglobin reductase reaction is reported in this work.

[1]Abbreviations used are as follows: IHP, inositol hexaphosphate; bisTris, N,N-bis-(2-hydroxyethyl)iminotris(hydroxy)methane.

ISSN 0006-291X



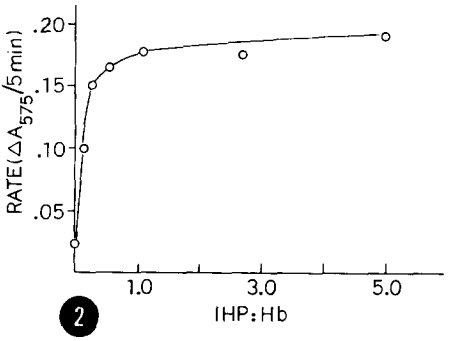


Figure 1. Effect of IHP on the rate of methemoglobin reduction by NADH-methemoglobin reductase. Hemoglobin was converted to methemoglobin with potassium ferricyanide (molar ratio Hb:$K_3Fe(CN)_6$ = 1:4). The lysate was then diluted to appropriate concentrations in a system that contained the following in a final volume of 1 ml: lysate containing 0.04 mM Hb; 0.05 mM bisTris buffer pH 7.0, 0.2 mM IHP when added. The reaction was started by addition of 0.1 mM NADH. No NADH was added to the control cuvet.

Figure 2. Dependence of NADH-methemoglobin reductase activity on IHP concentrations. Reactions were performed as described in the legend to Figure 1.

MATERIAL AND METHODS

Pre-weighed vials of NADH and IHP (sodium salt) were purchased from Sigma Chemical Co. (St. Louis). Solutions of IHP were neutralized to pH 7.0 with HCl and the concentration of IHP was verified by phosphate analysis according to Ames and Dubin (10).

Hemolysates were prepared from fresh normal human red cells. Hemoglobin was converted to methemoglobin with potassium ferricyanide (molar ratio Hb:$K_3Fe(CN)_6$ = 1:4 as described by Hegesh and Avron (4)) or with a slight molar excess of sodium nitrite. The lysate was then diluted to appropriate concentrations and assayed for endogenous NADH-methemoglobin reductase in a system that contained the following in a final volume of 1 ml: lysate containing 0.04 mM Hb; 0.05 M bisTris buffer pH 7.0; 0.1 mM NADH; and the indicated concentrations of IHP when added. The reaction was initiated by the addition of NADH and monitored at 575 nm and 25° in a Gilford recording spectrophotometer.

RESULTS

Figure 1 shows the enhanced rate of the NADH-methemoglobin reductase reaction that is observed at pH 7.0 when a 5-fold molar excess of IHP to methemoglobin tetramer is included in the reaction mixture. The initial velocity of the reaction is stimulated about 5-fold under the conditions used.

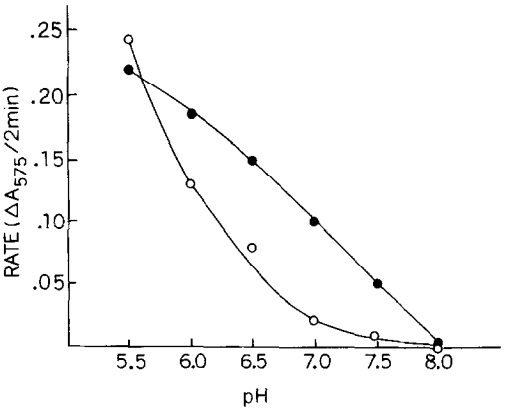


Figure 3. Effect of pH on the rate of methemoglobin reduction. Buffers used were 0.05 M in bisTris. ●—●, a 5-fold molar excess of IHP was added. o—o, no IHP present. Reactions were performed as described in the legend to Figure 1.

Figure 2 shows the relationship between the molar ratios of IHP to methemoglobin substrate and the activation of the reductase reaction. The results indicate that a strong interaction occurs. Saturating effects are approached at about a molar ratio of 1.0, indicating a 1:1 stoichiometry for the formation of a IHP-methemoglobin tetramer complex.

Figure 3 shows the effect of pH on the IHP activated reaction. Maximal effects are observed at pH near neutrality. At pH 5.5 or lower, the rate becomes slower in the presence of IHP because of instability in the IHP-methemoglobin complex. This is indicated by turbidity in the reaction mixture containing IHP, probably associated with increased tetramer-dimer dissociation at acid pH and a correspondingly increased IHP-dimer interaction (11). The diminished IHP effect at higher pH can be ascribed to weaker binding of the effector.

The results shown in Table I demonstrate that the IHP effect is associated with the NADH-methemoglobin reductase reaction. The effect is observed only in the presence of an unheated enzyme source and is associated with a preferential requirement for NADH as reductant. A requirement for ferro-

TABLE I

Component Study of the IHP Stimulated NADH-methemoglobin Reductase System

Reaction Mixture	Initial Rate of Ferrihemoglobin Reduction (% of Complete System)
Complete system	100
-NADH	0
-IHP	21.1
-Enzyme	0
+NADPH instead of NADH	4.5
Nitrite oxidized Hb	0
Heated enzyme (lysate was heated at 50°C for 1 hr)	0

Reaction mixture in complete system was the same as described in the legend to Figure 1. In nitrite oxidized Hb sodium nitrite instead of $K_3Fe(CN)_6$ was used to prepare the methemoglobin substrate.

cyanide-methemoglobin as "activated" substrate is indicated by the fact that the IHP effect was not observed when nitrite oxidized hemoglobin was used as substrate.

DISCUSSION

As with the Hegesh-Avron assay (3), the enhancement of the rate of the NADH-methemoglobin reductase reaction by IHP requires a ferrocyanide-methemoglobin complex as substrate. Apparently, the intrinsic rate of reaction is so slow in the absence of ferrocyanide that the IHP effect cannot be demonstrated under these conditions. The mechanism of activation by ferrocyanide is not understood. Hegesh and Avron conclude that it does not participate as an electron carrier, but that "it induces a change in ferrihemoglobin structure which permits the enzyme to approach the site of re-

duction more freely." The additional activating effect produced by IHP can be rationalized in more detail. It is apparently due to the well recognized conformational change in hemoglobin structure produced by IHP binding at the β chains of the methemoglobin tetramer (8). The consequent increase in ferric heme reactivity toward enzymatic reduction is consistent with the suggestion of a resulting shift in the allosteric equilibrium of methemoglobin toward the T or deoxyhemoglobin-like state (8). According to this view, the R state is favored in oxidation or oxygenation and the T state is favored in reduction or deoxygenation reactions of hemoglobin. A report on the enhancement by IHP of the nonenzymatic (ascorbic acid) reduction of methemoglobin has just appeared (12). In this system, however, the IHP effect is apparently demonstrable on methemoglobin alone, without any concomittant requirement for activation by ferrocyanide.

Whatever may be their mechanism of action, the combined activating effects of ferrocyanide and IHP may provide the basis for a convenient spectrophotometric assay for NADH-methemoglobin reductase at a pH that is more nearly physiological than that which is most commonly used (4).

ACKNOWLEDGEMENT

This work was supported by a grant from the National Institutes of Health (AM-15770).

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