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INHIBITION OF NADH-METHEMOGLOBIN REDUCTASE BY ORGANIC PHOSPHATES

KARLA J. MATTESON and FUMITO TAKETA *

Department of Biochemistry, The Medical College of Wisconsin, Milwaukee, WI 53226 (U.S.A.)

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Summary

The organic phosphate allosteric effectors of hemoglobin, inositol hexaphosphate, 2,3-diphosphoglycerate, and ATP, interact with NADH-methemoglobin reductase (NADH-diaphorase). Significant inhibitory effects on the enzyme were found when dichlorophenolindophenol, or ferricyanide were used as electron acceptors in place of methemoglobin. In contrast, apparent stimulation of enzyme activity was observed when adult human methemoglobin was used as the electron acceptor. The latter is explained on the basis of the larger effect of the organic phosphate on the rate of reaction due to its interaction with the substrate methemoglobin to produce the favored T type of quaternary conformation. The inhibitory effect of inositol hexaphosphate on the enzyme is associated with a perturbation in the reactivity of essential sulfhydryl group(s) on the enzyme. It is suggested that the interaction of the organic phosphate with the enzyme as well as with the substrate is significant in determining the overall rate of methemoglobin reduction.

Introduction

The non-enzymic, as well as enzymic, rate of reduction of methemoglobin is enhanced by organic phosphates such as 2,3-diphosphoglycerate and inositol hexaphosphate [1,2]. This rate-enhancement is explained on the basis of an interaction of the organic phosphate with methemoglobin. This interaction results in a change in quaternary conformation of the protein and an increase in reactivity of the heme iron. That inositol hexaphosphate produces a change in

* To whom requests for reprints should be sent.

conformation of methemoglobin from what appears to be an R to a T type of quaternary structure is now well established [3].

The enzymic reduction of methemoglobin uses NADH as a primary reductant and cytochrome b_5 as an intermediate electron carrier [4]. In such a system, it is not entirely certain that the observed effect of the organic phosphate can be ascribed to its interaction with the methemoglobin alone. It is possible that the activity of cytochrome b_5 as well as the enzyme could also be affected. The possibility of effects on the activity of the enzyme was suggested by the observation [5] that inorganic phosphate influences the ability of the enzyme to catalyze the reduction of the artificial electron acceptor, dichlorophenol indophenol by NADH. In addition, studies in this laboratory using methemoglobin substrates that have little or no ability to bind organic phosphates appeared to indicate that the enzyme was sensitive to the effects of the phosphates. Consequently, the effect of organic phosphates on the enzyme was explored by using a variety of electron acceptors in place of methemoglobin for the assay. In this way, the possible effect of the phosphates on the enzyme was dissociated from their known effect on the methemoglobin substrate. We report here that organic phosphates demonstrate inhibitory effects on the enzyme. Thus, their overall effect on the reaction catalyzed by NADH-methemoglobin reductase is a result of the effects of activation of substrate and inhibition of enzyme.

Experimental procedures

Purification of the enzyme. Methemoglobin reductase was isolated by the method of LeRoux [6] from outdated human red cells obtained from the blood bank. The purified enzyme was stored at −20° C until needed.

Enzyme assay. For most of the work, the method of Scott [7] which uses the dye dichlorophenol indophenol as electron acceptor for NADH diaphorase activity was employed. Activity was measured in a 1.2-ml cuvette with a 1-cm light path in a Gilford recording spectrophotometer at 600 nm and 27° C. The assay solution contained 0.033 mM EDTA, 16 mM bis-Tris-HCl(bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane) buffer (pH 7.5), 0.057 mM dichlorophenol indophenol, 0.067 mM NADH, and appropriate amounts of water and enzyme solution adjusted to a volume of 1.05 ml. The reaction was initiated by the addition of NADH and the observed rate of change in absorbance was converted to units of activity. One unit of activity is the amount of enzyme that reduces $1 \cdot 10^{-9}$ mol dichlorophenol indophenol per min at 27° C. The dye concentration was calculated using the millimolar extinction coefficient of 20.6. Neutralized solutions of inositol hexaphosphate (Sigma), ATP (Calbiochem), or 2,3-diphosphoglycerate (Calbiochem) were added to the cuvette at the time of assay.

Other assays for the enzyme used ferricyanide in place of dichlorophenol indophenol as electron acceptor. For the assay of enzyme activity using ferricyanide as substrate [8] the 1.2 ml cuvette contained (in 1 ml) 0.1 mmol Tris-acetate buffer (pH 8.1), 0.25 μmol $K_3Fe(CN)_6$, 0.125 μmol NADH, and the indicated amounts of phosphate compound and enzyme. The rate of reduction was monitored at 340 nm and 27° C in a Gilford recording spectrophotometer.

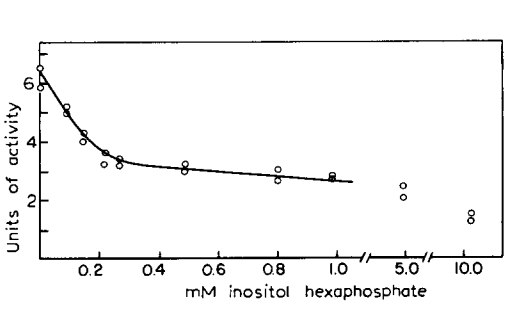
A third method of assay was that of Hegesh [9] in which a ferricyanide-methemoglobin complex served as the electron-accepting substrate.

Reaction with sulfhydryl reagents. The purified enzyme (9 units) was reacted in 0.09 M bis-Tris-HCl buffer (pH 7.5) with *N*-ethylmaleimide, *p*-chloromercuribenzoic acid, or iodoacetamide (0—1 mM) for various periods of time at 25° C. At appropriate times. aliquots were removed from the mixture and the initial velocities of the NADH-diaphorase reaction were measured. When NADH or dichlorophenol indophenol was also included during the reaction of *N*-ethylmaleimide and enzyme, the concentrations were adjusted such that the aliquot taken for the subsequent enzyme assay contained the necessary amount of reagent for the assay procedure.

Protection of enzyme from inhibition by N-ethylmaleimide. To determine whether inositol hexaphosphate has any effect on the rate of *N*-ethylmaleimide inactivation of the enzyme, the activity of the enzyme was monitored at various times in the presence and absence of inositol hexaphosphate and *N*-ethylmaleimide. Enzyme activity was assayed in terms of the NADH-diaphorase activity. Enzyme (19.5 units) 0.09 M bis-Tris-HCl buffer (pH 7.5), ±1 mM inositol hexaphosphate (pH 7), and 0.07 or 0.25 mM *N*-ethylmaleimide were incubated at 27° C for periods up to 1 h. Aliquots of the incubation were removed at various times and assayed for residual enzyme activity.

Results

As was reported by Kuma [5] it was found that red cell NADH diaphorase is inhibited slightly at low concentrations of inorganic phosphate and activated to a small degree at concentrations of phosphate above 0.2 M. The results shown in Figs. 1 and 2 demonstrate that organic phosphates also have an effect on the enzymic rate of dichlorophenol indophenol reduction by NADH. Using 0.02 mg of a partially purified enzyme preparation (6.7 units), 50% inhibition was observed in the presence of 0.2 mM inositol hexaphosphate. A greater degree of inhibition was found at higher concentrations of inositol hexaphosphate, but even at levels as high as 10 mM complete inactivation was not observed. Weaker



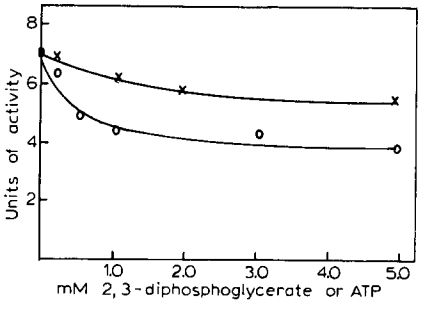


Fig. 1. Inhibition by inositol hexaphosphate of NADH-diaphorase activity of purified human red cell methemoglobin reductase. One unit of activity is defined as the amount of enzyme necessary for the reduction of 10^{-9} mol dichlorophenol indophenol per min.

Fig. 2. Inhibition by 2,3-diphosphoglycerate or ATP of NADH-diaphorase activity of purified human red cell methemoglobin reductase. X, ATP; ○, 2,3-diphosphoglycerate.

but still significant effects were observed when 2,3-diphosphoglycerate or ATP was used in place of inositol hexaphosphate. 50% inhibition was found at 5 mM 2,3-diphosphoglycerate, while a similar concentration of ATP gave approx. 30% inhibition of the enzyme. The activity of the enzyme was essentially unchanged in the presence of analogs such as inositol monophosphate, inositol, and mannitol. Inhibition of NADH-cytochrome b_5 reductase activity assayed using cytochrome *c* as the final electron acceptor has been reported previously with pyrophosphate, citrate and EDTA [10]. Thus, inhibition of the enzyme by organic phosphates might also be expected on the basis of the non-specific effect of anions; however, it should be noted that the much lower concentrations (0.2—5 mM) of organic phosphates are required to produce the same effects. In the case of inositol hexaphosphate, the large number of charges present might lead to the assumption that the inhibition is due to ionic strength effects despite the low concentration. However, at pH 7—7.5 the ionic strength of a 0.2 mM inositol hexaphosphate solution (0.015) is still far below that of the salt solutions found to inhibit methemoglobin reductase [11] or cytochrome b_5 reductase [10]. Furthermore, it is of interest to note that at similar concentrations the same organic phosphates possess specific allosteric effects on methemoglobin, a substrate for the enzyme.

To ascertain whether the inhibition of the enzyme by inositol hexaphosphate is observed when electron acceptors other than dichlorophenol indophenol are used, several other electron acceptors were substituted in the assay. Two other electron acceptors have been used in assay procedures for methemoglobin reductase. They include ferricyanide [8], and ferricyanide-hemoglobin complex [9]. When ferricyanide was used as electron acceptor, a smaller effect due to inositol hexaphosphate was detected than was observed with dichlorophenol indophenol (Table I). In contrast with these results, when the assay used ferricyanide-hemoglobin as the electron acceptor, inositol hexaphosphate produced an apparent activation of the enzyme (Table I). This phenomenon was observed previously and is attributed to the binding of inositol hexaphosphate to the methemoglobin substrate. Such binding produces the more favored quaternary T state in the hemoglobin substrate, which is

TABLE I

EFFECT OF INOSITOL HEXAPHOSPHATE ON RED CELL NADH-METHEMOGLOBIN REDUCTASE ACTIVITY AS A FUNCTION OF THE ELECTRON ACCEPTOR

Electron acceptor		Activity	Change (%)
DCIP *	—inositol hexaphosphate	6.5 units	
	+inositol hexaphosphate	1.8	—67
Ferricyanide	—inositol hexaphosphate	0.066 A_{340}/min	
	+inositol hexaphosphate	0.055	—17
Ferricyanide/-human methemoglobin A	—inositol hexaphosphate	0.065 A_{575}/min	
	+inositol hexaphosphate	0.175	+269
Ferricyanide/-cat methemoglobin B	—inositol hexaphosphate	0.038 A_{575}/min	
	+inositol hexaphosphate	0.022	—40

* DCIP dichlorophenol indophenol.

favored for the reduction of heme iron in the substrate [1,2]. In support of this conclusion is the observation that an actual inhibition by inositol hexaphosphate of the rate of methemoglobin reduction is observed when a methemoglobin substrate incapable of binding organic phosphates is used in the assay (Table I) [12–14].

Human erythrocyte NADH-methemoglobin reductase (NADH diaphorase) has recently been purified to homogeneity [13] and has been reported to be similar, if not identical, to red cell NADH-cytochrome b_5 reductase as well as to soluble and microsomal NADH-cytochrome b_5 reductases of other tissues [6,15,16]. Before this association between methemoglobin reductase and cytochrome b_5 reductase became recognized, Strittmater had reported an extensive series of studies on liver microsomal NADH-cytochrome b_5 reductase showing that the enzyme contained an essential sulfhydryl group involved in the binding of NADH [8]. Since it now seems reasonably certain that microsomal NADH-cytochrome b_5 reductase and erythrocyte NADH-methemoglobin reductase are very similar enzymes, a relationship between essential sulfhydryl groups, NADH, and enzyme activity ought to exist in the latter as well. The data in Table II demonstrate that the red cell NADH-diaphorase is indeed inactivated by the sulfhydryl reagents *p*-chloromercuribenzoic acid, *N*-ethylmaleimide and iodoacetamide. It is more sensitive to *p*-chloromercuribenzoic acid than to *N*-ethylmaleimide and is somewhat less sensitive to iodoacetamide, presumably because of the different rates of reaction of the enzyme thiol group(s) with each of these reagents. Nearly complete inhibition of the diaphorase activity occurred after 15 min of reaction with 0.04 mM *p*-chloromercuribenzoic acid, and after one hour with 1 mM *N*-ethylmaleimide. However, with 1 mM iodoacetamide, about 80% of enzyme activity still remained after 1 h of reaction. The effects of NADH and other substrates of the enzyme on the inhibition by *N*-ethylmaleimide are also shown in Table II. It can be seen that in addition to NADH, NADPH (1 mM) provided complete protection from *N*-ethylmaleimide inhibition under these conditions. On the other hand, the co-substrate for the enzyme, dichlorophenol indophenol was only weakly protective, as was NAD, a product of the diaphorase reaction. The specific protective effects of NADH and NADPH indicate that binding of these substrates perturbs the reactivity of

TABLE II

EFFECTS OF SULFHYDRYL REAGENTS ON NADH-DIAPHORASE ACTIVITY

Enzyme solutions were pre-incubated with the indicated additions for 60 min before assays for activity were conducted. The dichlorophenol indophenol, NADH, NAD and NADPH additions were made before the addition of *N*-ethylmaleimide.

Additions	Activity remaining (%)
None	100
1 mM iodoacetamide	80
0.04 mM *p*-chloromercuribenzoic acid	0
1 mM *N*-ethylmaleimide	3
1 mM *N*-ethylmaleimide and 0.94 mM dichlorophenol indophenol	15
1 mM *N*-ethylmaleimide and 1 mM NADH	100
1 mM *N*-ethylmaleimide and 1 mM NAD	23
1 mM *N*-ethylmaleimide and 1 mM NADPH	100

functionally essential enzyme-SH group(s), and that enzyme activity and reactivity of this specific sulfhydryl group are interrelated. Titration with *N*-ethylmaleimide or *p*-chloromercuribenzoic acid to determine the number of sulfhydryl groups which are modified in the inhibited enzyme was not performed on the methemoglobin reductase because the enzyme was only partially purified. However, Strittmatter [17] has shown that only one equivalent of these reagents is necessary to inactivate cytochrome b_5 reductase. Since NADH-methemoglobin reductase is apparently identical with NADH-cytochrome b_5 reductase we can assume that modification of one sulfhydryl group is sufficient for total inactivation of the NADH-diaphorase activity of this enzyme.

To further probe the interaction of inositol hexaphosphate with the enzyme, the effect of the organic phosphate on the susceptibility of enzyme sulfhydryl groups towards *N*-ethylmaleimide inactivation was investigated. As can be seen in Fig. 3, inositol hexaphosphate provides a small but significant protective effect against enzyme inactivation of the NADH-diaphorase activity of the enzyme by *N*-ethylmaleimide. This suggests that the binding of inositol hexaphosphate to the enzyme sterically hinders the availability of the -SH group(s) or causes a change in enzyme conformation so that the sulfhydryl group(s) required for enzymic activity are less accessible for reaction with *N*-ethylmaleimide.

To determine if the observed organic phosphate effect was specific for NADH methemoglobin reductase, other diaphorases were also investigated. A NAD(P)H-diaphorase from *Clostridium kluyverii* (Worthington), as well as the NADPH-diaphorase from erythrocytes were assayed in the presence of inositol hexaphosphate using the same diaphorase assay system. The erythrocyte NADPH-diaphorase was obtained in a semipurified form by absorption of non-hemoglobin proteins from a human erythrocyte lysate on DEAE-11 and elution of the enzyme with a citrate buffer [16]. The presence of contaminating

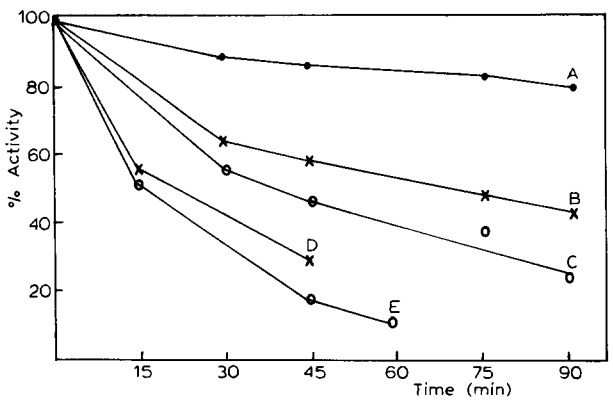


Fig. 3. Effect of inositol hexaphosphate on *N*-ethylmaleimide inactivation of methemoglobin reductase. Enzyme activity measured as NADH-diaphorase activity. Values normalized to percent of initial activity. (A) Control; (B) 0.07 mM *N*-ethylmaleimide and 1 mM inositol hexaphosphate; (C) 0.07 mM *N*-ethylmaleimide; (D) 0.25 mM *N*-ethylmaleimide and 1 mM inositol hexaphosphate; (E) 0.25 mM *N*-ethylmaleimide.

NADH-diaphorase in the preparation posed no problem since each enzyme has a specific requirement for the appropriate pyridine nucleotide. The crude preparation of partially purified NADH-diaphorase (methemoglobin reductase) (5.7 U) was inhibited 70% by 1 mM inositol hexaphosphate, as was the purified enzyme (6.5 U). In contrast, the crude NADPH-diaphorase (2.8 U) showed little sensitivity to inositol hexaphosphate. Similarly, the bacterial NAD(P)H-diaphorase (11 U) also showed no sensitivity to inositol hexaphosphate.

Discussion

The present results suggest that organic phosphates with known allosteric effects on hemoglobin also have direct effects on NADH-methemoglobin reductase. The inhibitory effects of organic phosphates on enzyme activity occur at the same concentration range in which activation of methemoglobin as a substrate is observed. The overall result is an enhancement in rate of methemoglobin reduction since the activating effect produced from conformational change in the structure of methemoglobin is generally greater than the inhibitory effect on the enzyme. That the effect on the enzyme is significant with respect to the overall rate of reaction is indicated by the fact that rate enhancement by inositol hexaphosphate is less for the enzymic compared to the non-enzymic reduction of methemoglobin [1,2]. Furthermore, an inhibition rather than a stimulation of the rate of methemoglobin reduction is found when the methemoglobin substrate does not bind organic phosphates.

While most of these studies were carried out using partially purified enzyme preparations the same degree of inhibition due to inositol hexaphosphate was observed for an enzyme preparation of greater purity (5000 U/mg). The earlier preparations may have contained reductases other than methemoglobin reductase, as indicated by an assayable cytochrome *c* reductase activity. However, the enzymic activity observed with the dichlorophenol indophenol substrate was due to a single enzyme identifiable by staining of disc electrophoresis gels in the dichlorophenol indophenol-containing system of Kaplan and Beutler [19].

The mechanism of the enzymic reduction is not thoroughly understood but it appears from recent work [4,20] that the reaction proceeds in two steps. The first step involves an enzyme-catalyzed reduction of cytochrome b_5 by NADH, which is then followed by the second reduction of methemoglobin by reduced cytochrome b_5. Apparently, the inositol hexaphosphate inhibition of the enzyme is associated with the first of these steps and the overall rate of methemoglobin reduction is determined by the steady state concentration of reduced cytochrome b_5. Although the effect of inositol hexaphosphate on the levels of reduced cytochrome b_5 has not yet been examined directly, its inhibitory effect on the rate of enzymic reduction of dichlorophenol supports such a mechanism. Clearly, the protective effect afforded by inositol hexaphosphate on the *N*-ethylmaleimide inactivation of the enzyme shows that there is an interaction between enzyme sulfhydryl groups and the organic phosphate. In this connection, it is of interest to note that Strittmatter [8] has shown that a reactive sulfhydryl group is involved in NADH binding and electron transfer in

liver microsomal NADH-cytochrome b_5 reductase. The results of our work suggests that inositol hexaphosphate interacts with the red cell enzyme and may directly or indirectly (by conformational change) affect the accessibility of the functional -SH group toward reaction with either NADH or *N*-ethylmaleimide. That there is a specific interaction between the enzyme and inositol hexaphosphate is suggested by our recent unpublished observation that the enzyme is bound to a Blue-Dextran Sepharose affinity column and eluted with low concentrations of inositol hexaphosphate or NADH.

The major organic phosphate in most mammalian red cells is 2,3-diphosphoglycerate [21]. Red cell phosphofructokinase [22] and 2,3-diphosphoglycerate mutase [23] are two enzymes that have been reported to be regulated by an inhibitory action of 2,3-diphosphoglycerate. Red cell NADH-cytochrome b_5 reductase may be yet another of these enzymes. Since it is known that methemoglobin has a higher affinity for organic phosphates than oxyhemoglobin [24], it is conceivable that under conditions in which the level of methemoglobin is raised, binding of 2,3-diphosphoglycerate to methemoglobin would lower the concentration of free 2,3-diphosphoglycerate and have an effect of relieving the inhibition of the enzyme. Consequently, under this condition, the overall effect could be an enhancement in the rate of methemoglobin reduction resulting from the synergistic effect of activation of substrate as well as the enzyme.

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