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ACTION OF OXIDIZED AND REDUCED GLUTATHIONE ON RABBIT RED BLOOD CELL HEXOKINASE

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Summary

Reduced glutathione at 1 mM concentration is able to mantain rabbit red blood cell hexokinase (EC 2.7.1.1) in the reduced state with fully catalytic activity. At higher concentrations a marked inhibition is observed. In contrast, oxidized glutathione is a strong inhibitor of reduced erythrocyte hexokinase at all the concentrations studied.

Inactivation experiments show that some sulfhydryl groups reacting with oxidized glutathione are responsible for the enzyme inactivations. These findings suggest a cellular inter-relationship between redox and energetic metabolism coupled through glutathione at the hexokinase level.

Introduction

Red blood cell hexokinase (EC 2.7.1.1) is a rate-limiting reaction of the erythrocyte glycolysis. This enzyme is markedly modulated by many compounds such as glucose 6-phosphate [1—6], 2,3-diphosphoglycerate [7,8], glucose 1,6-diphosphate [5,9,10] and others [5,6].

Several years ago, by studying the glucose metabolism in human erythrocytes, Eldjarn and Bremer [11] found that oxidized glutathione was also an effective inhibitor of red cell hexokinase, but Mager et al. [12] suggested that the intracellular glutathione oxidation was associated with the accumulation of an inhibitor rather than with an irreversible inactivation of hexokinase. Recently [6], no inhibition of purified human erythrocyte hexokinase by reduced or oxidized glutathione has been reported. On the other hand, oxidized glutathione is known to be able to oxidize the thiol groups of several enzymes [13—15] and hexokinase has been recognized for several years to be

inhibited by sulfhydryl reagents [16,17]. Furthermore, by the use of 5,5′-dithiobis(2-nitrobenzoate) [18,19], two of the sulfhydryl residues of hexokinase have been found to be responsible for the enzyme inactivation.

The present paper shows the effects of reduced and oxidized glutathione on highly purified erythrocyte hexokinase, and suggests that this enzyme could be controlled by the cell's redox state.

Materials and Methods

Materials. Reduced and oxidized glutathione, DL-dithiothreitol, adenosine-5′-triphosphate and glucose-6-phosphate dehydrogenase (from yeast) (EC 1.1.1.49) were obtained from Sigma. All other reagents used were of analytical grade purity.

Enzyme purification. Rabbit erythrocyte hexokinase was purified as reported earlier and used at stage 5 [20]. The preparations at this stage had specific activities of 70—75 units/mg protein. Unless otherwise indicated, the enzyme solution was dialyzed against 5 mM Tris-HCl, pH 7.2, containing 9% (v/v) glycerol, 5 mM glucose and 3 mM mercaptoethanol (standard buffer).

Enzyme assay. The enzyme activity was determined spectrophotometrically at 30°C in a system coupled with glucose-6-phosphate dehydrogenase. The assay mixture contained, in a total volume of 1 ml, 80 mM Tris-HCl (pH 7.2), 5 mM glucose and 5 mM $MgATP^{2-}$ (unless otherwise indicated), 0.5 mM $NADP^+$, 5 mM $MgCl_2$ and 0.05 I.U. of glucose-6-phosphate dehydrogenase. Initial rate measurements were performed by following the reduction of $NADP^+$ at 340 nm with a Beckman spectrophotometer model 25. 1 unit of hexokinase activity is defined as the amount of enzyme which catalyzes the formation of 1 μmol of glucose 6-phosphate/min at 30°C.

Data were analyzed using Lineweaver-Burk plots.

Results and Discussion

Reduced glutathione

When pure rabbit red blood cell hexokinase is dialyzed overnight at 4°C against the standard buffer (see Material and Methods) without mercaptoethanol, in the presence or absence of glucose, it loses most of its activity. Assay of this enzyme performed at non-saturating $MgATP^{2-}$ concentration (0.5 mM) and glucose (5 mM), in the presence of reduced glutathione from 0 to 7.5 mM shows that the catalytic activity lost during the dialysis can be fully recovered if 1 mM reduced glutathione is present in the assay. However, at greater than 1 mM reduced glutathione concentration, a strong inhibition was observed (Fig. 1). A similar recovery of the hexokinase activity was also observed when the concentration of $MgATP^{2-}$ and glucose were, respectively, saturating (5 mM) and non-saturating (0.05 mM). But, in this case, inhibition by reduced glutathione was found only at concentration greater than 5 mM (Fig. 1).

Thus, the enzyme is fully active when maintained in the reduced state and this can be well obtained with 1 mM reduced glutathione or 3 mM mercaptoethanol (results not shown).

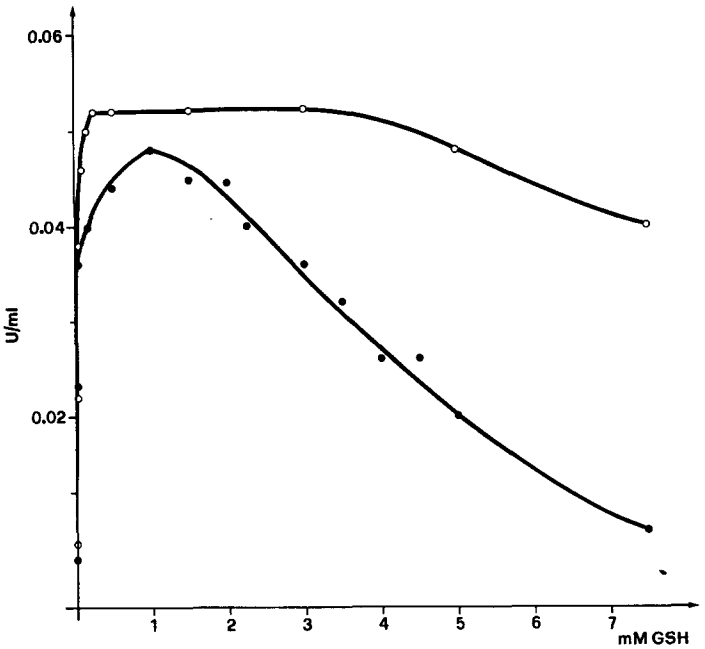

Fig. 1. Effect of reduced glutathione on hexokinase activity. Hexokinase was dialyzed against 5 mM Tris-HCl, pH 7.2, containing 9% (v/v) glycerol and 5 mM glucose when assayed with 0.5 mM $MgATP^{2-}$ and 5 mM glucose (●———●), or against 5 mM Tris-HCl, pH 7.2, containing only 9% (v/v) glycerol when assayed with 5 mM $MgATP^{2-}$ and 0.05 mM glucose (○———○).

The reduced glutathione inhibition seems to be competitive with respect to $MgATP^{2-}$ (K_i = 0.9 mM) and non-competitive with respect to glucose (K_i = 6.5 mM) (Fig. 2). The samples used in these experiments were free of mercaptoethanol so that the baseline was obtained at a reduced glutathione concentration which restored all the hexokinase activity. The K_i values found represent the apparent and not the true dissociation constants of the reduced glutathione-enzyme complex. In fact [21], the true K_i value cannot be derived directly from kinetic plots because the reaction mechanism of rabbit red blood cells hexokinase is, at present, unknown. Furthermore, the reduced glutathione action probably involves more than one binding site.

Since the reduced glutathione erythrocyte concentration is about 2.5 mM [22] an in vivo modulating role of hexokinase can be hypothesized.

The reduced glutathione inhibition of hexokinase is also a pH-dependent phenomenon. As reported in Fig. 3, the percent inhibition greatly increases at pH values lower than the physiological one (pH 7.2).

Hexokinase from human erythrocytes was reported [6] not to be inhibited by reduced glutathione but the experiments were performed at pH 7.7 and, as shown in Fig. 3, the reduced glutathione inhibition at this pH value is very low.

Oxidized glutathione

Oxidized glutathione is a strong inhibitor of reduced rabbit erythrocyte

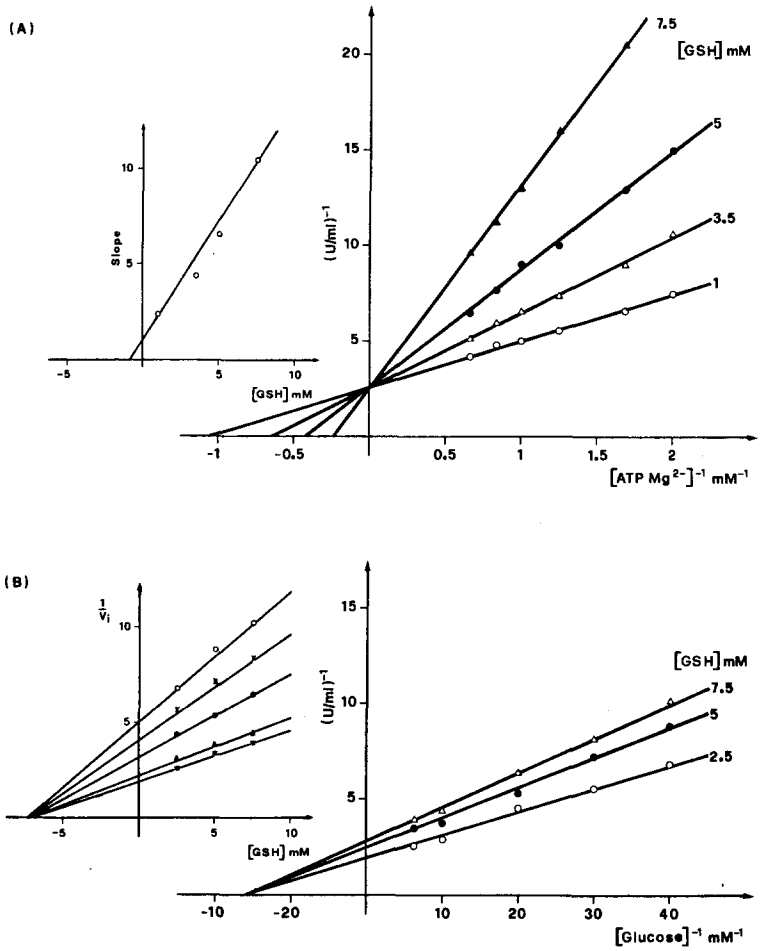

Fig. 2. Lineweaver-Burk plots of hexokinase activity vs. (A) $MgATP^{2-}$ and (B) glucose at several concentrations of reduced glutathione. Inset in A, secondary plot of slope vs. added reduced glutathione; inset in B, Dixon plot. GSH, reduced glutathione.

hexokinase. The enzyme, in the presence of oxidized glutathione varying from 0 to 2 mM, shows a similar behavior if $MgATP^{2-}$ or glucose is maintained at non-saturating concentrations. Furthermore, as shown in Fig. 4, the pattern of inhibition appears to be biphasic with inhibition of about 40 percent of the hexokinase activity at 0.05 mM oxidized glutathione.

The kinetics of this inhibition appear to be competitive with respect to both $MgATP^{2-}$ and glucose with K_i values of 1.5 and 0.8 mM, respectively (Fig. 5). Particularly in this case, the apparent K_i value does not represent the dissociation constants of the glutathione-enzyme complex. In fact, as studies with sulfhydryl reagents have shown [18,19], several hexokinase sulfhydryl groups can react under non-denaturing conditions, but only two of these are required for catalytic activity.

Only binding experiments could give the true dissociation constants for the hexokinase-glutathione complexes.

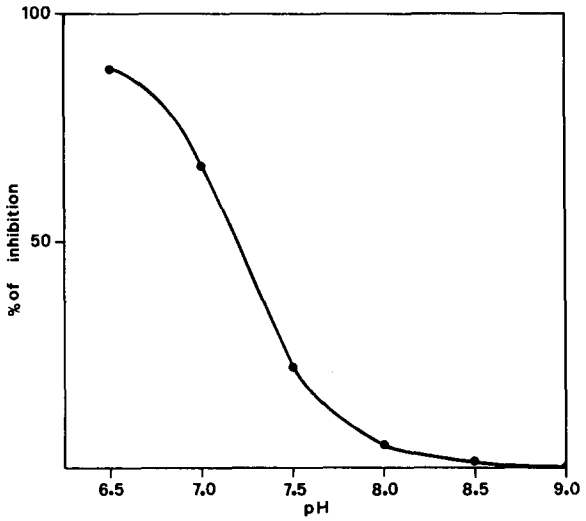

Fig. 3. Effect of pH on hexokinase inhibition by reduced glutathione. Hexokinase samples were dialyzed against 5 mM Tris-HCl, pH 7.2, containing 9% (v/v) glycerol and 5 mM glucose. The percent of inhibition was calculated at 0.5 mM $MgATP^{2-}$ and 3.75 mM reduced glutathione with respect to the 100% of activity measured at 1 mM reduced glutathione at the respective pH.

In Fig. 6 the effect of pH on hexokinase inhibition by oxidized glutathione is shown. As for reduced glutathione, a previous report [6] has shown non-inhibition of this enzyme by oxidized glutathione. These discrepances can be explained by the strong pH dependence of the oxidized glutathione action.

Inactivation and reactivation experiments

When mercaptoethanol was absent, pure rabbit red blood cell hexokinase

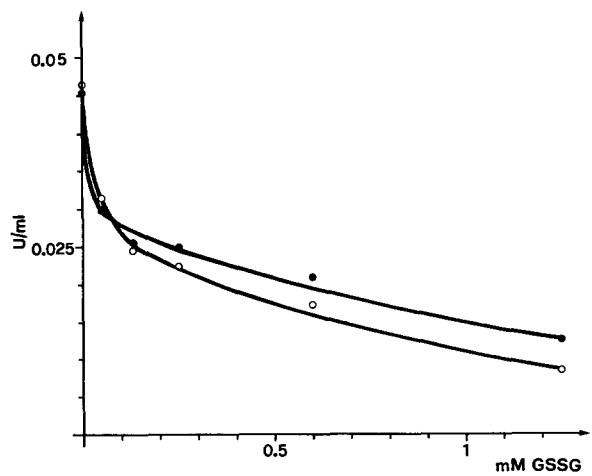

Fig. 4. Effect of oxidized glutathione on hexokinase activity. Assays were performed at 0.5 mM $MgATP^{2-}$ and 5 mM glucose (●———●); or 0.05 mM glucose and 5 mM $MgATP^{2-}$ (○———○) in the presence of various amount of oxidized glutathione (GSSG).

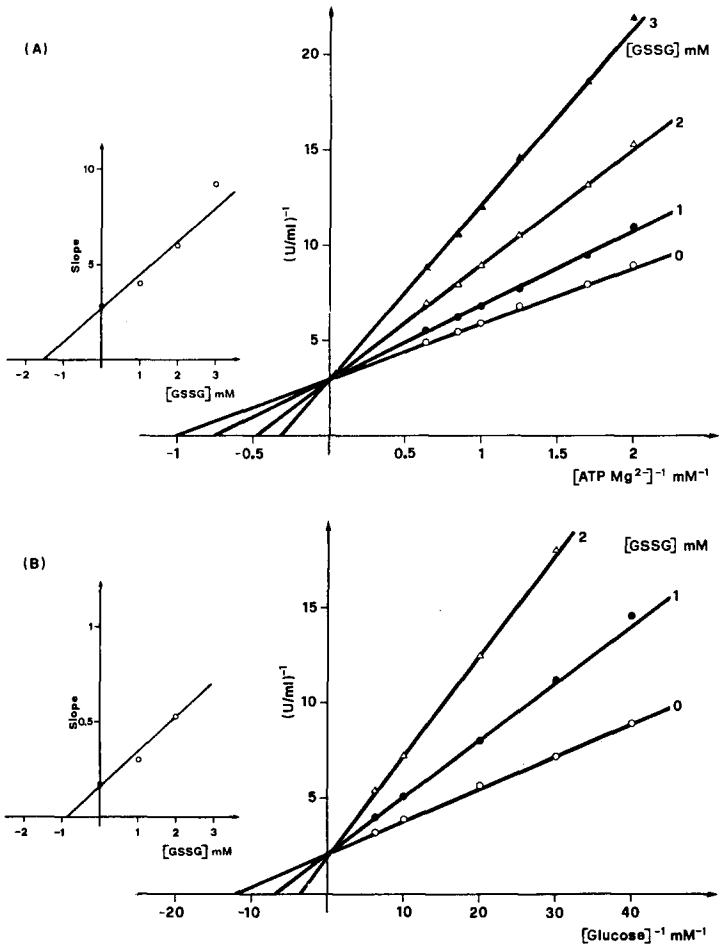

Fig. 5. Lineweaver-Burk plots of hexokinase activity vs. (A) $MgATP^{2-}$ and (B) glucose at several concentrations of oxidized glutathione. Inset plots of slope vs. added oxidized glutathione (GSSG).

lost 30—37% of its activity in 5 min at 30°C. The inactivation is a time-dependent process and semilogarithmic plots of the percent of original activity vs. time show first-order kinetics with an apparent first-order rate constant (K) of 0.03—0.04 min^{-1} (evaluated from the slopes). At 0.5 mM, reduced glutathione is able to prevent this inactivation and to reduce the enzyme protein so that after 5 min of incubation the catalytic activity is 134% of the mercaptoethanol-free hexokinase. When the reduced glutathione concentration was increased to 2.5 mM a rapid inactivation was observed and the semilogarithmic plots also show first-order kinetics with a K value of 0.34 min^{-1}. When both 2.5 mM reduced glutathione and 5 mM $MgATP^{2-}$ were added during the incubation of hexokinase at 30°C, a new phenomena was observed. $MgATP^{2-}$ was able to stabilize the enzyme. In addition, it was an activator, if reduced glutathione was present at concentrations which previously caused inhibition. This can be explained by the conversion of hexokinase to the reduced state by reduced glutathione and, at the same time, with a conformational change

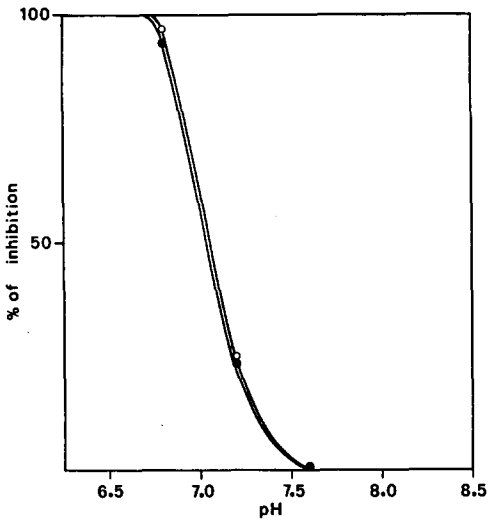

Fig. 6. Effect of pH on hexokinase inhibition by oxidized glutathione. The percent of inhibition was measured at 0.1 mM glucose and 5 mM $MgATP^{2-}$ (○———○), or 0.5 mM $MgATP^{2-}$ and 5 mM glucose (●———●) at 0.5 mM oxidized glutathione with respect to the percent of activity measured without oxidized glutathione at the respective pH.

induced by $MgATP^{2-}$. The latter effect may contribute to the protection of some groups involved in the catalytic process. These experiments are summarized in Table I.

The effects of oxidized glutathione are easier to explain, in fact the inactivation is a time-dependent process with a first-order rate constant (in the semilogarithmic plots) which increases with oxidized glutathione concentration. In Table I are reported typical experiments which show that the inactivation of hexokinase is a function of the oxidized glutathione concentration. In order to start these experiments with the enzyme in the reduced state, 3 mM 2-mercap-

TABLE I

EFFECT OF GLUTATHIONE ON HEXOKINASE ACTIVITY

Compound added	% activity remaining
None	63—70
0.5 mM GSH	134
2.5 mM GSH	2
2.5 mM GSH + 5 mM $MgATP^{2-}$	158
5 mM $MgATP^{2-}$	107
3 mM MSH	93
3 mM MSH + 0.5 mM GSSG	80
3 mM MSH + 1 mM GSSG	63
3 mM MSH + 3 mM GSSG	0

Hexokinase (2 units/ml) was dialyzed against 5 mM Tris-HCl, pH 7.2, containing 5 mM glucose and 9% (v/v) glycerol. The values are expressed as the percent of activity remaining after incubation for 5 min at 30°C with the compounds listed. In the experiments with 2-mercaptoethanol (MSH), the 100% activity was calculated in the presence of 3 mM mercaptoethanol. GSH, reduced glutathione; GSSG, oxidized glutathione.

toethanol was included. On the whole, these results suggest that probably several sulfhydryl groups react with oxidized glutathione and some of these are responsible for the hexokinase inactivation.

Preliminary results show that hexokinase oxidized by oxidized glutathione can be fully recovered in the reduced state. We have obtained this both at 4°C and more rapidly at room temperature by dithiothreitol. Reduced glutathione and mercaptoethanol are much less effective in this sense. In the same way, the loss of hexokinase activity observed at 30°C in the absence of sulfhydryl compounds is completely reversible when the enzyme is treated with reducing agents. Also, in this case, dithiothreitol is more effective than reduced glutathione and mercaptoethanol.

Physiological levels of oxidized glutathione (5.6 μM [23]) are not able to oxidize hexokinase if reduced glutathione is present at physiological concentrations (2.5 mM [21]).

Experiments on the binding of glutathione to hexokinase and studies on the role of -SH groups are now necessary to understand fully the interactions of redox and energetic metabolism coupled through glutathione at the hexokinase level.

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