Vol. 95, No. 2, 1980
July 31, 1980

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 758-764

INHIBITION OF RED CELL AND YEAST HEXOKINASE BY TRIETHYLTIN BROMIDE [$(C_2H_5)_3SnBr$]

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Received June 16,1980

Summary: Triethyltin bromide was found to be a selective inhibitor of red cell hexokinase. When intact red cell suspensions were exposed to the reagent (0.025-0.5 mM), inhibition of hexokinase occurred without any hemolysis and without effects on the activity of other glycolytic enzymes. Yeast hexokinase was also inhibited by triethyltin. The red cell and yeast hexokinases were protected against inhibition by triethyltin when the sugar substrates of the enzymes were included in the incubation mixture. This work identifies hexokinase as another among a limited number of proteins that are known to interact with triethyltin.

A variety of synthetic organotin derivatives have found applications in the past as insecticides, fungicides, wood preservatives and plasticizers (1). Their toxicity in animals has been considered and it has been shown that the central nervous system is particularly sensitive to the effects of trialkyltin compounds (2). Aldridge and his associates (3,4) have found that triethyltin interacts strongly with myelin and with mitochondrial ATPase of the central nervous system; the latter interaction being reflected in inhibition of respiration and of energy conservation. Extensive studies by these investigators (4-7) on the mechanism of interaction of triethyltin with proteins have led to the conclusion that binding is selective and that it requires specific ligands in an appropriate three-dimensional structure. Only a few proteins among several examined were found to bind triethyltin with significant affinity. They included glutathione-S-aryl transferase and cat and rat hemoglobins (7,8). Others examined, including human, rabbit, mouse, horse and guinea pig hemoglobins, myoglobin, cytochrome c, serum albumin, chymotrypsin, phosvitin, salmine and ribonuclease demonstrated no significant binding. Further studies with cat hemoglobin have indicated that two molecules of triethyltin are bound to each hemoglobin tetramer (7) and

0006-291X/80/140758-07$01.00/0

that properly juxtaposed -SH and imidazole groups within each native α-globin subunit serve as ligands in the formation of a pentacoordinate complex (7,9).

We reported earlier that a functional consequence of triethyltin binding to cat hemoglobin is an increase in the oxygen affinity of the protein (9). A similar effect was found when washed cat red cell suspensions or whole blood were exposed to the reagent, indicating that the compound freely entered the red cell. During these studies on the effect of the compound on the intact red cell system, we noted a strong hemolytic action on animal red cells in general. Investigation of the molecular basis for this effect has led to the present identification of hexokinase as another of a limited number of proteins that interact with triethyltin. In this report, we present evidence that triethyltin exerts a selective inhibitory effect on red cell hexokinase with little or no effect on any of the other glycolytic enzymes. We also show that yeast hexokinase is similarly inhibited, and that protection against inhibition is provided by the presence of the sugar substrates for the enzyme.

MATERIALS AND METHODS

Fresh heparinized human blood obtained from the Blood Bank was centrifuged at 5,000 x g and plasma was removed by aspiration. The red cells were washed three times with isotonic saline and then suspended in isotonic phosphate buffer (10) or lysed with an equal volume of water. Stroma was removed from lysates by centrifugation at 20,000 rpm in a Sorvall RC-5 refrigerated centrifuge (SS-1 rotor) for 20 minutes. Protein concentration in intact red cell suspensions or lysates was estimated by measuring hemoglobin by the cyanmethemoglobin procedure (11).

Triethyltin bromide was obtained from the Ventron Corporation (Danvers, MA). Stock solutions were prepared by dissolving appropriate amounts in small volumes of absolute ethanol (10% final volume) and bringing to volume in isotonic phosphate buffer. Yeast hexokinase (370 U/mg) was purchased from Sigma (St. Louis, MO).

Red cells or lysates in appropriate buffers were incubated with the indicated amounts of triethyltin bromide for various periods of time at 37°C. Aliquots were taken for enzyme assays. Yeast hexokinase was incubated in the same manner and then assayed. Assays for glycolytic enzymes were conducted using procedures described by Beutler (12). The required substrates, cofactors and enzymes for these assays were purchased from Sigma.

RESULTS

In preliminary studies it was found that when whole blood or red cell suspensions were incubated for 1 hour or longer at 37° in the presence of 1-5 mM triethyltin bromide, extensive hemolysis occurred (13). When the

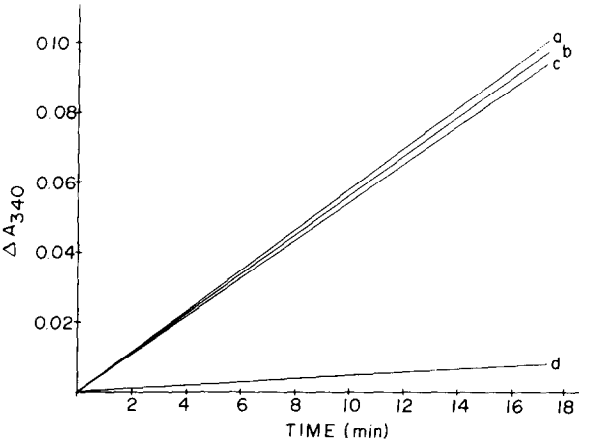

Fig. 1. Hexokinase activity in red cells. a) Control red cell suspension, b) red cell suspension incubated with 0.5 mM NaBr, c) red cell suspension incubated with 0.5 mM $SnCl_2$, d) red cell suspension incubated with 0.5mM triethyltin. Incubations were for one hour at 37°C. Aliquots were taken for assay of hexokinase activity as described by Beutler (12). The assay involved coupling the hexokinase with glucose-6-phosphate dehydrogenase reaction and measuring the increase in absorbance at 340 nm due to NADPH generation. A Gilford 240 spectrophotometer with recorder attachment was used to continuously monitor the progress of the reaction at 37°.

incubation was carried out at lower concentrations (0.025-0.5 mM) of the reagent and for shorter periods, little or no hemolysis was observed. However, analysis of lysates prepared from these cells revealed that hexokinase was selectively inhibited. All other glycolytic enzymes, as well as glucose-6-phosphate dehydrogenase remained fully active. The results shown in Figure 1 demonstrate that hexokinase activity was nearly completely inhibited in red cell suspensions (45% hematocrit) exposed to 0.5 mM triethyltin bromide for 1 hour and 37°. Cell suspensions exposed to 0.5 mM $SnCl_2$ or 0.5 mM NaBr showed the same hexokinase activity as control suspensions incubated in the absence of added triethyltin bromide. Cells incubated with higher concentrations of the reagent (1-5 mM) and for longer periods (6 hours) showed essentially the same results except that there were indications of slight inhibition of phosphofructokinase, phosphoglycerate kinase, and pyruvate kinase. The inhibitory effect of triethyltin on hexokinase was not reversed when the inactivated enzyme was subjected to extensive dialysis or gel filtration. Thus, either the binding occurred with high affinity or the enzyme was irreversibly inactivated.

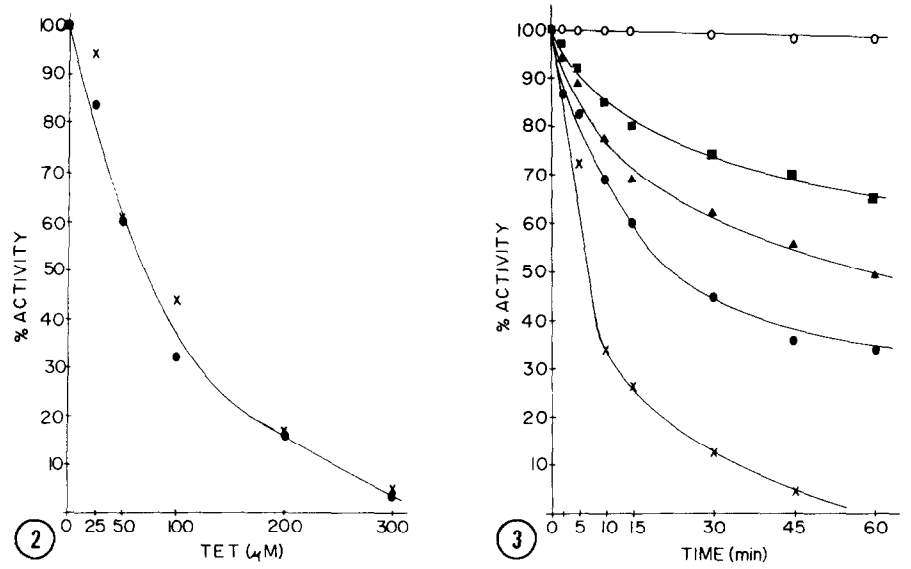

Fig. 2. Comparison of the sensitivity of yeast and red cell hexokinases to triethyltin. Red cell stroma free lysate (X) was incubated with the indicated concentrations of triethyltin (TET) for 1 hour at 37°C; similarly the purified yeast hexokinase (●) (diluted to 0.2 units/ml) was incubated under the same conditions. The hexokinase activity was then assayed as described above.

Fig. 3. Time course of inactivation of hexokinase with 0-300 μM triethyltin at 37°C. Red cell stroma free lysate was incubated with (○) No triethyltin; (■) 25 μM; (▲) 50 μM; (●) 100 μM; (X) 300 μM triethyltin, and at the indicated times hexokinase was assayed as described above.

The inhibition of hexokinase is not restricted to the red cell enzyme; yeast hexokinase is similarly inhibited. Figure 2 shows the results that were obtained when equivalent amounts (0.2 units/ml) of yeast and red cell enzymes were incubated for 1 hour at 37°C with various concentrations (0-0.3 mM) of triethyltin bromide and then diluted for assay. The two enzymes appear to be equally sensitive to the reagent. Significant inhibition was observed at concentrations of triethyltin in the range of 25-50 μM in each case. The inhibitory effect of the reagent was found to occur relatively slowly; the effects were observed after a pre-incubation period of several minutes(Figure 3).

To determine whether substrates or products of the hexokinase reaction have any effect on the interaction of triethyltin with the enzyme, the effects of pre-incubating red cell lysates with triethyltin in the presence of various sugar substrates, analogues,ATP, MgATP, ADP and glucose-6-phosphate were examined

Table I
Protection Against Triethyltin Inhibition by Substrates or Products of the hexokinase reaction

Sample	% Hexokinase Activity Remaining
Control	100.0
TET	5.4
" + D-Glucose	92.7
" + L-Glucose	6.7
" + D-Xylose	6.3
" + D-Ribose	7.3
" + D-Mannose	87.2
" + D-Galactose	5.4
" + D-Glucose-6-P	9.0
" + D-Fructose	6.3
" + ATP	5.0
" + ADP	4.4

Red cell suspensions were incubated with 250 μM triethyltin and the indicated components at 1 mM final concentration for 1 hour at 37°C. The samples were then assayed for hexokinase activity as described above.

The results shown in Table 1 indicate clearly that significant protection is afforded only by the sugar substrates of the enzyme. Under the conditions used, 1mM D-glucose gave 92.7% protection and 1 mM D-mannose, 87.2%. The apparent lack of protection by 1 mM D-fructose can be explained by the fact that the affinity of the enzyme for this sugar is much less than that for D-glucose or D-mannose (14) Non-substrate sugars including L-glucose, D-galactose, D-ribose, D-xylose and D-glucose-6-phosphate were without effect. Similarly, ATP, MgATP or ADP did not give any protection. As little as 50 μM D-glucose provided significant protection and concentrations higher than 0.5 mM gave nearly complete protection. Both yeast and red cell hexokinase demonstrated this property.

DISCUSSION

It is intriguing that none of the other glycolytic enzymes of the red cell are effected by triethyltin under conditions where hexokinase is completely

inhibited. This selective action of triethyltin is in accord with the conclusion of Aldridge and his associates that the reagent does not react with any particular group in a protein but only with appropriately juxtaposed ligands within its three-dimensional structure. It is noteworthy that despite the affinity of tin for sulfur, triethyltin has no apparent effect on other glycolytic enzymes containing functionally important -SH groups. It might have been expected to interact, for example, with the catalytically reactive -SH groups of glyceraldehyde-3-phosphate dehydrogenase, but the enzyme was insenstive to its effects even when relatively high concentrations were used (5 mM).

Protection of hexokinase against inhibition by triethyltin by the sugar substrates of the enzyme also suggests that specific interaction of triethyltin with ligands within a particular conformation of the protein is involved. Since, in the catalytic mechanism, binding of the sugar precedes the binding of MgATP to the enzyme (]5), and protection against triethyltin is given by the sugar but not by ATP or MgATP, the inhibitory effect of the reagent is apparently exerted at an early step of the hexokinase reaction.

The observations that yeast hexokinase is likewise sensitive to inhibition by triethyltin provides the basis to explore the mechanism further. Since the enzyme is available commercially and its structure and mechanism of catalysis are understood in some detail (16-20), it should be a useful model to investigate not only the nature of the interaction of triethyltin with the protein, but the catalytic mechanism of the enzyme as well.

ACKNOWLEDGEMENT:

This work was supported by grant AM-15770 from NIAMDD and by a NIEHS Freshwater Biomedical Center Grant 1 P30 ES-01985.

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