Inactivation of Yeast Hexokinase B by Triethyltin Bromide and Reactivation by Dithiothreitol and Glucose†

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ABSTRACT: Binding of triethyltin bromide to yeast hexokinase B results in a rapid change in the reactivity of the sulfhydryl groups of the molecule. The change was characterized by an increased rate as well as extent of reaction of the –SH groups, and it preceded the onset of inhibition of the enzyme. Rapid gel filtration of the enzyme–triethyltin complex reversed this change in sulfhydryl reactivity, and when the eluted enzyme was subjected to short incubation periods, the slow inhibition that occurs with the unfiltered enzyme–triethytin complex was no longer manifested. With prolonged incubation, however, the gel-filtered sample demonstrated increased rate of loss of enzyme activity, indicating that the gel filtration step did not completely reverse the effects of triethyltin on the enzyme. Active enzyme was recovered, following the inactivation of yeast hexokinase with triethyltin, by incubation of the inactivated enzyme with a large excess of glucose and dithiothreitol. Near total recovery of enzyme activity with reversion to native enzyme conformation was achieved following incubation at 35 °C of the enzyme with glucose and dithiothreitol each at 0.1 M. The possible involvement of either cysteine or histidine in the binding of triethyltin to the enzyme was probed, and it was concluded that neither of these amino acids are donor ligands for tin.

Much work has focused upon attempts to characterize the biochemical effects of the trialkyltins on nervous tissue components (Brody & Moore, 1962; Rose & Aldridge, 1968; Aldridge, 1977) since the most obvious morphological changes are localized in this tissue. Gross effects on other tissues have not been reported except for observations indicating that these compounds can cause hemolysis of red cells (Yoshikawa & Ishii, 1962; Byington & Forte, 1973; Byington et al., 1974). During our studies on the interaction of triethyltin with the hemoglobins in red cells from different species, we found that its lytic effect was exerted in association with inhibition of red cell glycolytic activity (Siebenlist & Taketa, 1981a). Investigation of the basis for this inhibition resulted in the discovery that triethyltin is a selective inhibitor of red cell hexokinase. Yeast hexokinase was also found to be sensitive to inhibition by the reagent (Siebenlist & Taketa, 1980, 1981b).

In our earlier studies using yeast hexokinase B (Siebenlist & Taketa, 1983), it was noted that the inhibition by triethyltin could be distinguished in terms of a two-step process. There appeared to be an initial rapid shift in the monomer–dimer equilibrium of the protein toward the monomer form, followed by a slow temperature-dependent inactivation of the enzyme. The Arrhenius plot of the effect of temperature on the triethyltin-induced inactivation resembled that of Otieno et al. (1977) for the inhibition of yeast hexokinase B by sulfhydryl-modifying reagents. There was a sharp break in the plot at approximately 30 °C, and the ΔH^*, the heat of activation, above this temperature was consistent with that for protein unfolding. It was also noted that the yeast enzyme can be protected from the inhibitory action of triethyltin by its sugar substrates, apparently because the conformation of the enzyme–substrate complex prevented binding of the organotin.

In the present work, the possible involvement of the sulfhydryl groups of cysteine or of the imidazole groups of histidine residues in yeast hexokinase B in triethyltin binding was examined. In addition, the possibility of reactivating the enzyme following its inhibition by triethyltin was explored. We report that enzyme activity can be recovered and describe conditions to achieve the reactivation.

Materials and Methods

Triethyltin bromide was obtained from the Ventron Corp., Danvers, MA. Stock solutions were prepared as previously described (Siebenlist & Taketa, 1981b). Yeast hexokinase B, type C-302 (320–400 units/mg), glucose-6-phosphate dehydrogenase, dithiothreitol, Bistris,[1] and Tris were purchased from Sigma Chemical Co., St. Louis, MO. 4,4′-Dipyridyl disulfide (PDS) and diethyl pyrocarbonate were purchased from Aldrich Chemical Co., Milwaukee, WI. The hexokinase obtained from Sigma was judged to be 95–98% homogeneous by SDS–polyacrylamide gel electrophoresis (Fairbanks et al., 1971). In those cases where the enzyme was judged to be less than 95% pure, it was repurified by the method of Ågren et al. (1963).

For reaction with triethyltin the yeast hexokinase was diluted to 0.002–2.0 mg/mL (0.2–800 units/mL) in 0.12 M Bistris buffer, pH 7.0, containing 0.1 M KCl. Hexokinase activity was determined by the method of Beutler (1975) on a Gilford 240 recording spectrophotometer.

Kinetics of Sulfhydryl Reaction. The kinetics of sulfhydryl reactivity and the number of reactive –SH groups on hexokinase were determined with PDS. The concentration of hexokinase was 5×10^{-6} M (M_r 102 000) in 0.12 M Bistris buffer, pH 7.0, and the concentration of PDS in the assay was 1.5×10^{-4} M. The course of reaction was monitored at 324 nm and 25 °C. At completion of the the reaction with the rapidly reacting –SH groups, sodium dodecyl sulfate (0.1% final concentration) was added to the cuvette and the additional reaction monitored to completion. The number of –SH groups per hexokinase dimer was calculated from the change in absorbance at 324 nm by using the millimolar extinction coefficient 1.98×10^4 (Grassetti & Murray, 1967). When included, triethyltin bromide was added to give a final concentration of 500 μM.

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[1] Abbreviations: Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; Tris, tris(hydroxymethyl)aminomethane; PDS, 4,4′-dipyridyl disulfide; DEP, diethyl pyrocarbonate; TET, triethyltin bromide; DTT, dithiothreitol; SDS, sodium dodecyl sulfate.

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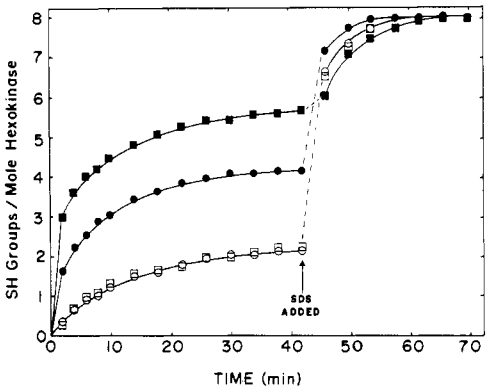

FIGURE 1: Kinetics of reaction of yeast hexokinase B with 4,4'-dipyridyl disulfide at 25 °C. The measurements were made in the absence or presence of 1 mM glucose as described in the text. Arrows indicate time when 0.07 mL of 0.3% SDS was added to the reaction mixtures. (●) Control, (■) 500 μM triethyltin bromide, (○) 1 mM glucose, and (□) 1 mM glucose plus 500 μM triethyltin.

Modification of the Nonessential Sulfhydryl Groups. The nonessential -SH groups of the enzyme were modified with iodoacetamide by the method of Otieno et al. (1977). The modified enzyme was then assayed by PDS titration to determine the extent of -SH modification and analyzed for sensitivity to triethyltin.

Diethyl Pyrocarbonate Modification of Yeast Hexokinase. The histidine residues of yeast hexokinase B were modified with diethyl pyrocarbonate by the method of Grouselle et al. (1973) at pH 6.1. A Gilford 240 recording spectrophotometer was used to monitor the reaction at 242 nm. The number of histidine residues modified was calculated by using the molar extinction coefficient of 3200 cm^{-1} M^{-1} for *N*-carbethoxyimidazole. This modified enzyme was then used to study the effects of triethyltin.

Reversibility of Triethyltin Binding. Yeast hexokinase (0.5 mg/mL) in 0.12 M Bistris, pH 7.0, containing 0.1 M KCl was incubated in the absence or presence of triethyltin (500 μM) for short periods of time at 37 °C and then chilled on ice. Half of each reaction mixture was then transferred to 1-mL pipet tips packed with a 4-cm layer of Sephadex G-25 fine equilibrated with the same buffer and rapidly gel filtered by centrifugation at 5000 rpm in an Internal Clinical centrifuge at 4 °C. The other half of the sample served as the unfiltered control. Samples (controls and gel filtered) were then analyzed for -SH reactivity or incubated further at 37 °C and assayed for hexokinase activity.

Difference Spectra. Difference spectra for enzyme in the presence and absence of triethyltin were recorded on a Cary 210 spectrophotometer interfaced with an Apple II plus computer. A Cary 219/210 UV/vis spectrophotometer wavelength scanning program obtained from Varian was employed.

Results

Our earlier observations that the binding of triethyltin to cat hemoglobin "masks" two of the reactive sulfhydryl groups of the molecule (Taketa et al., 1980) prompted us to examine the -SH reactivity of hexokinase in the presence of triethyltin. Figure 1 shows that in the absence of added triethyltin, four -SH groups per hexokinase dimer were freely reactive as reported by Lazarus et al. (1968). When 500 μM triethyltin was added, a rapid change in reactivity of the -SH groups was observed. However, there was an increase rather than a decrease in the rate as well as extent of reaction of these groups. Five to six sulfhydryl groups per dimer were reactive, and their reactivity was greatly increased over that of -SH groups of the enzyme in the absence of triethyltin. The rapid enhancement of sulfhydryl reactivity occurred within 1 min after addition of triethyltin to the enzyme at room temperature, but it was not accompanied by significant enzyme inhibition. Thiol reactivity of the enzyme was decreased as expected when 1 mM glucose was added to the enzyme solution (Jones et al., 1975), and when triethyltin was added to such a reaction mixture, it produced no further change. When SDS was added as a denaturant a value of eight sulfhydryl groups per dimer was obtained in the presence as well as absence of triethyltin. These results suggest that in the absence of glucose the interaction of triethyltin with hexokinase results in rapid unfolding of the molecule, but in the presence of glucose the conformation of the enzyme–substrate complex either prevents the binding of triethyltin or resists the unfolding influence of the bound reagent. Because of the affinity of tin compounds for sulfur, the possibility that triethyltin was first bound to one of the buried unreactive -SH groups (Otieno et al., 1977) of the native enzyme to induce its unfolding and thereby enhance the reactivity of its remaining thiols was considered. To examine this possibility, the three nonessential reactive sulfhydryl groups on the hexokinase monomer were modified with iodoacetamide. Reaction of an aliquot of the product with PDS confirmed the presence of one remaining -SH group in the enzyme monomer. When this modified hexokinase was incubated with triethyltin, inhibition of enzyme activity similar to that of the unmodified enzyme was observed (Table I). PDS titration of this modified enzyme after reaction with triethyltin revealed that one remaining -SH group of the enzyme monomer was still available for reaction with PDS (results not shown). It therefore appears that the binding site for triethyltin on the molecule does not involve a cysteine residue since the organotin increased the reactivity of the sulfhydryl groups instead of masking it. The reagent was able to inhibit the enzyme when the nonessential -SH groups of the enzyme were modified, and the one remaining sulfhydryl group of the modified enzyme was still reactive toward PDS in the presence of triethyltin.

Table I: Inactivation of Yeast Hexokinase B and Iodoacetamide-Modified[a] Yeast Hexokinase B by Triethyltin

	activity remaining (%)[b]	
[TET] (μM)	control enzyme	modified enzyme
0	100	100
50	72	73
100	50	49
300	31	29

[a] The modification reaction was performed as described in the text. [b] Yeast hexokinase B or the modified enzyme (0.5 unit/mL) was incubated with the indicated concentration of triethyltin at 35 °C for 1 h and then assayed for activity as described under Materials and Methods.

Luijten et al. (1962) showed that trialkyltin complexes are produced with imidazole, and Rose (1969) and Elliot et al. (1979) have implicated involvement of histidine residues in rat and cat hemoglobin from the pH dependence and effects of photooxidation on triethyltin binding. The possibility that histidine residues are likewise involved in binding of triethyltin to hexokinase was considered by conducting experiments to try to protect the enzyme from triethyltin inhibition by prior modification of its imidazole groups. This was attempted with the histidine-modifying reagent, diethyl pyrocarbonate (DEP). Approximately 4.5 of the five histidine residues present on the hexokinase monomer were modified after 30 min with DEP, and the enzyme remained 45% active (results not shown).

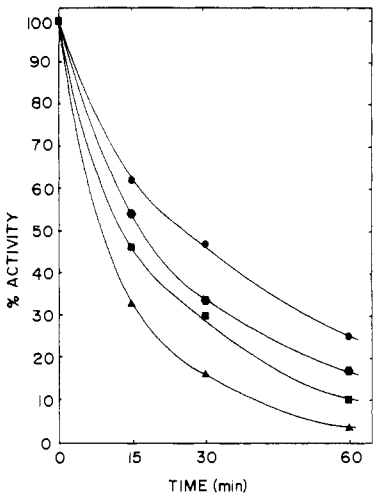

FIGURE 2: Time course of diethyl pyrocarbonate modified yeast hexokinase B with 0–300 μM triethyltin bromide. The modified enzyme (0.5 unit/mL) was incubated at 30 °C, and at the indicated times hexokinase activity was assayed as described in the text. No triethyltin (●) and 50 (●), 100 (■), and 500 μM (▲) triethyltin.

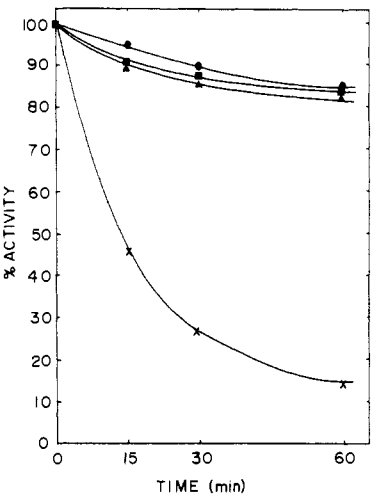

FIGURE 3: Time course of inactivation of yeast hexokinase B by triethyltin before and after gel filtration to remove the organotin. Yeast hexokinase B (0.5 mg/mL) was incubated at 35 °C for 5 min in the presence or absence of 500 μM triethyltin and then chilled on ice, and half of the sample was rapidly gel filtered. The column effluents and remaining portion of the original mixtures were then incubated further at 35 °C, and at the indicated times, samples were removed and assayed for activity as described under Materials and Methods. (●) Control, (×) 500 μM triethyltin, (■) control gel filtered, and (▲) 500 μM triethyltin gel filtered.

However, when this modified enzyme was incubated at 30 °C, a decrease in the stability of the enzyme was observed, in agreement with results obtained previously by Grouselle et al. (1973). The modified enzyme was even more unstable when exposed to triethyltin (Figure 2). Incubation of the modified enzyme with 50 μM triethyltin resulted in 50% inhibition within 15 min, whereas the unmodified enzyme was inhibited to the same extent only after 90 min of incubation. Thus, the ability of the enzyme to interact with triethyltin does not depend upon the availability of its imidazole groups.

Following the initial rapid conformational change, which is not accompanied by loss of enzyme activity, continued exposure of the enzyme to triethyltin results in a slow loss of activity. In attempts to determine whether the loss of enzyme activity can be prevented or reversed, experiments were conducted to try to remove triethyltin after various periods of reaction with the enzyme. Figure 3 shows the result of an experiment in which the triethyltin–enzyme complex was subjected to a rapid gel filtration procedure after various short periods of incubation. The enzyme recovered by gel filtration following its incubation with 300 μM triethyltin for 5 min was fully active, and it no longer manifested the triethyltin-induced loss of activity when subjected to subsequent reincubation. Analysis of the thiol groups in the recovered enzyme showed that their kinetics and stoichiometry of reaction with PDS had reverted to those of the native enzyme (results not shown). Thus, the gel filtration procedure apparently resulted in the removal of triethyltin from the enzyme, and the initial change in enzyme conformation resulting from the interaction with triethyltin is reversible. However, the process was not completely reversible when the enzyme was exposed to triethyltin for longer periods of time, and significant loss of enzyme activity had already occurred. Under these conditions, the fraction of active enzyme recovered after gel filtration was lowered in proportion to the length of incubation. The gel-filtered enzyme fraction demonstrated increased thiol reactivity and continued to lose enzyme activity at an accelerated rate upon subsequent incubation, indicating that recovery of the full complement of native enzyme by the gel filtration procedure does not occur after the inactivation by triethyltin had commenced. Furthermore, these results suggest that the continued loss of enzyme activity occurs either because triethyltin was no longer stripped from the enzyme complex by gel filtration or because the conformation of the enzyme was altered to the extent that the enzyme had become predisposed to denaturation. Clearly, a two-step process, at least, is involved in the interaction of triethyltin with hexokinase. The first step involves a rapid conformational change in the protein indicated by the increased thiol reactivity, a step that is reversible if the organotin is removed. A second step ensues with continued interaction of the enzyme with triethyltin in which a further conformational change is involved, and which cannot be reversed by the removal of the organotin. The latter step results in an apparent irreversible inactivation of the enzyme.

Attempts were made to recover activity from the triethyltin-inactivated enzyme by extensive dialysis at 4 °C against a variety of buffers containing glucose, or glucose plus a thiol reagent. Some activity was recovered from the completely inhibited form of the enzyme when the dialysis buffer contained 0.5 M concentrations of glucose and dithiothreitol and when the dialysis was carried out over a period of 48 h. However, at best, it was possible to recover only about 25% of the initial activity by this procedure. When the inactivated enzyme was incubated at 30–35 °C with glucose and a thiol, a method similar to that developed by Murakami & Rose (1974) for the reactivation of mammalian hexokinase II, improved recovery of active enzyme was achieved. Figure 4 shows that incubation of a triethyltin-inactivated enzyme preparation with 0.1 M glucose and 0.1 M β-mercaptoethanol at 35 °C resulted in good recovery of enzyme activity. More than 45% of the initial activity was recovered after 90 min of incubation. When the β-mercaptoethanol was replaced with an equal concentration (0.1 M) or dithiothreitol, a more rapid recovery of enzyme activity was observed. Approximately 50% of the original enzyme activity was recovered within 15 min after the addition of the glucose and DTT, and greater than 85% was recovered at 90 min. It was possible to recover between 95 and 100% of the initial enzyme activity if the glucose and DTT were added after 30 min of incubation with 300 μM triethyltin. When glucose and DTT were tested individually for their ability to reverse the triethyltin inhibition, little recovery of enzyme activity was observed. When di-

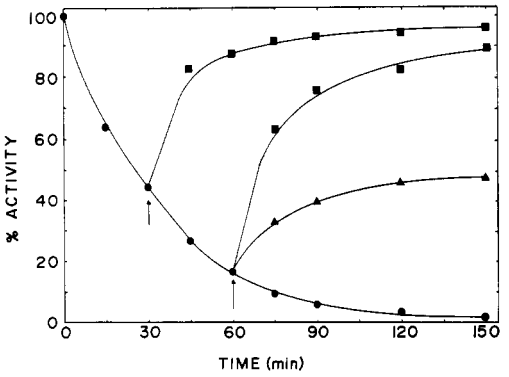

FIGURE 4: Time course of reactivation of yeast hexokinase B inactivated by 300 μM triethyltin. Yeast hexokinase B (0.5 unit/mL) was incubated with 300 μM triethyltin at 35 °C, and at the indicated times, aliquots were removed and assayed for hexokinase activity as described under Materials and Methods. Arrows indicate where either 0.1 M glucose and 0.1 M β-mercaptoethanol (▲) or 0.1 M glucose and 0.1 M dithiothreitol (■) were added to the incubation mixture. Triethyltin-treated enzyme with no subsequent additions (●).

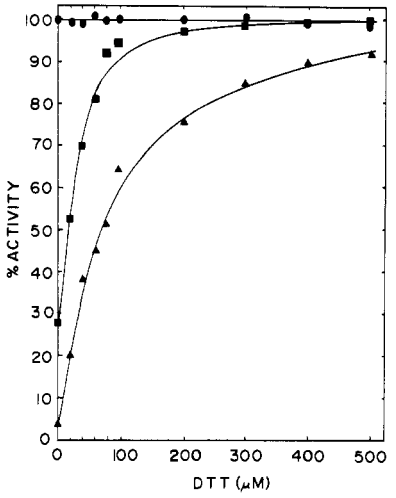

FIGURE 5: Protection by dithiothreitol of yeast hexokinase B from triethyltin inactivation. Yeast hexokinase B (0.5 units/mL) and the indicated concentrations of dithiothreitol were incubated at 35 °C for 1 h in the presence of (●) 0, (■) 100, and (▲) 300 μM triethyltin and then assayed for activity as described in the text.

thiothreitol was added to the enzyme before the addition of triethyltin, a pronounced protective effect was observed (Figure 5). It can be seen that the inhibitory effect of 300 μM triethyltin was decreased by preincubating the enzyme with increasing concentrations of DTT. At equimolar concentrations of DTT and triethyltin, only about 10–20% of the enzyme activity was lost after 60 min of preincubation. That this was due to complex formation between DTT and triethyltin was demonstrated by carrying out PDS titrations of dithiothreitol (40 μM) in the presence of various concentrations of triethyltin (0–4 mM) (results not shown). The DTT–triethyltin complex has an apparent dissociation constant of about 2×10^{-4} M; therefore, it appears that part of the process leading to the reactivation of the hexokinase by DTT and glucose is due to removal of triethyltin by complexation with DTT. The role of glucose in the reactivation process is uncertain, but it is probably associated with the stabilization of the renatured active enzyme conformation.

The recovery of enzyme activity appears to coincide with a reversion of the enzyme to its native conformation. Difference spectra taken during the course of the interaction of triethyltin with the enzyme showed a significant decrease in absorbance in the 270–290-nm region (Figure 6a). An initial

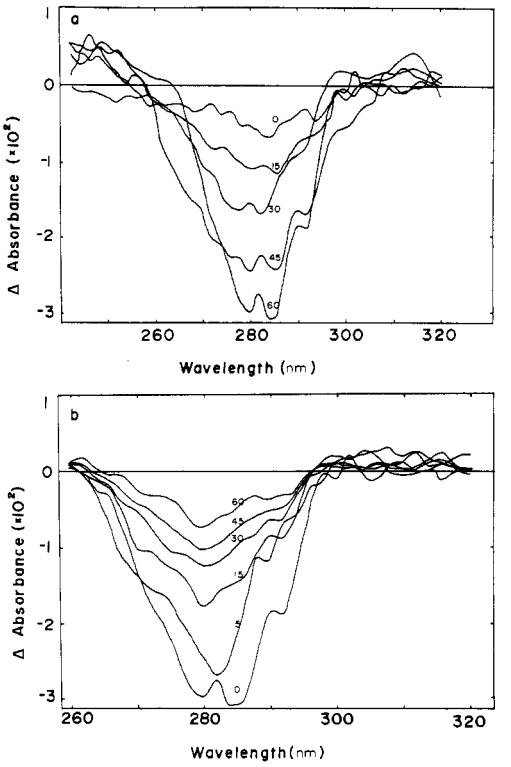

FIGURE 6: (a) Difference spectra of yeast hexokinase B. Yeast hexokinase B (0.25 mg/mL, 10 mL final volume) was incubated with and without 300 μM triethyltin at 35 °C, and at the indicated times, samples (1.5 mL) were removed and their difference spectra recorded. (b) Difference spectra of yeast hexokinase B. Yeast hexokinase B (0.25 mg/mL, 12 mL final volume) was incubated with and without 300 μM triethyltin for 60 min at 35 °C (zero time), a sample (1.5 mL) was removed, and the spectra were recorded. Dithiothreitol (0.1 M) and glucose (0.1 M) were then added to the reaction mixtures. the solutions were incubated at 35 °C, and at the indicated times, samples (1.5 mL) were removed and their difference spectra recorded.

decrease in absorbance is noted immediately after the addition of the organotin, along with exposure of the -SH groups of the enzyme as noted above. Additional changes in absorbance were observed with continued incubation at 35 °C which paralleled the loss of enzyme activity. When DTT and glucose was added to the inactivated enzyme, these changes in absorbance were reversed (Figure 6b), and under conditions in which nearly complete recovery of enzyme activity was obtained, little difference in the spectra from that of the native enzyme was observed. The sulfhydryl reactivity of this reactivated enzyme also indicated that the native enzyme conformation had been restored.

Discussion

The triethyltin-induced inhibition of yeast hexokinase B appears to proceed by a mechanism resembling that proposed for the thermal inactivation of mammalian hexokinase type II (Murakami & Rose, 1974; Rose & Warms, 1982). Inactivation is temperature dependent and can be prevented by incubation of the enzyme with its sugar substrates, which appear to exert their effects by stabilizing the enzyme against denaturation. The mechanism of denaturation of the mammalian enzyme involves unfolding of its tertiary structure with exposure of sulfhydryl groups and subsequent oxidation of these groups to their metastable inactive disulfide form (Murakami & Rose, 1974; Rose & Warms, 1982). The recovery of activity by incubation with high concentrations of mercaptoethanol and glucose suggested to Murakami & Rose (1974) that the inactive disulfide form of the enzyme was readily formed from the unfolded state and that glucose was

required to facilitate the refolding of the transiently formed reduced unfolded enzyme. In the absence of reducing thiols and glucose, the inactive disulfide form of this enzyme eventually undergoes further conformational changes that result in irreversible inactivation of the enzyme. As shown here, incubation of yeast hexokinase with triethyltin likewise causes a conformational change in the enzyme to expose the sulfhydryl groups of the molecule. This form of the enzyme can be maintained by lowering the incubation temperature and can be converted back to its native form by rapid removal of the organotin. Furthermore, partial recovery of activity from the inactivated enzyme using mercaptoethanol and glucose suggests that a disulfide form of the yeast enzyme may also be involved during the process of inactivation by triethyltin. Our observations that dithiothreitol and glucose were more effective in restoring activity than mercaptoethanol and glucose and that dithiothreitol binds triethyltin indicate that, in addition to its role in reducing the putative metastable disulfide bond, DTT participates in removing the organotin from the enzyme to facilitate complete recovery of active enzyme.

Rose & Warms (1982) have also shown that the mammalian hexokinase II is stabilized from heat inactivation by physiological concentrations of potassium ion and by the NADPH–thioredoxin reductase system of the cell. In control experiments they noted that potassium ion had no effect on the heat stability of mammalian hexokinase I or of yeast hexokinase. In similar studies we found that potassium has no effect on the inactivation of yeast hexokinase B in the presence or absence of triethyltin. Thus, in this respect, the triethyltin inhibition of yeast hexokinase differs from that of the thermal inactivation of mammalian hexokinase II.

The binding site for triethyltin on yeast hexokinase is still an open question. From data presented here it appears that the amino acid residues identified in the binding of triethyltin to cat hemoglobin (Taketa et al., 1980) are not involved in the interaction with yeast hexokinase. A pentacoordinate complex, involving a thiol group of cysteine and an imidazole group of a histidyl residue of hemoglobin as axial donor ligands, has been proposed for the triethyltin–cat hemoglobin complex (Taketa et al., 1980). Our previous studies of (2-[(dimethylamino)methyl]phenyl)diethyltin, a compound with a single coordination site available on the tin, suggest that the yeast enzyme monomer donates only one ligand to form the hexokinase–triethyltin complex. Current efforts to determine the nature of this ligand are focused on the lysine residues of the enzyme, since it has been reported that lysine residues are involved in the association of the hexokinase dimer (Rossi et al., 1975) and, as noted previously (Siebenlist & Taketa, 1983), triethyltin exerts a dissociative effect on the hexokinase dimer.

The morphological changes observed in the brain cells of triethyltin-intoxicated animals suggest an impairment in the ability of the cell to maintain ion gradients (Torack et al., 1970; Reed et al., 1964; Lee & Bakay, 1965). In agreement with this hypothesis, other investigators have shown that triethyltin inhibits a (Na^+–K^+)-ATPase of the brain (Wassenaar & Kroon, 1973). A similar breakdown in ion gradients might also be expected if triethyltin inhibited brain hexokinase and caused depletion of the cellular ATP. The organotin-induced denaturation of the enzyme with attendant increase in the rate of oxidation of its –SH groups could result in its increased turnover. Oxidation has been proposed as a first step in the degradation of intracellular proteins (Maness & Orengo, 1975, 1976), and it is possible that depleted hexokinase levels could retard cellular recovery mechanisms even after the organotin has been metabolized.

Registry No. TET, 2767-54-6; DTT, 3483-12-3; glucose, 50-99-7; hexokinase, 9001-51-8.

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