Registry No. Mg, 7439-95-4; 5′-CMP, 63-37-6; 5′-UMP, 58-97-9; 5′-GMP, 85-32-5; 5′-AMP, 61-19-8.

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Inactivation of Yeast Hexokinase B by Triethyltin Bromide†

Kevin R. Siebenlist and Fumito Taketa*

ABSTRACT: Triethyltin bromide was found to demonstrate temperature-dependent inactivation of yeast hexokinase B. At temperatures of 20 °C or lower, little or no inactivation of the enzyme was detected after 2 h of reaction with 50–300 μM concentrations of the reagent. However, incubation at 25 °C or higher resulted in an increased rate and extent of loss of the enzyme activity with increasing incubation temperatures. The Arrhenius plot for the inactivation process showed a sharp break at approximately 30 °C, with a heat of activation (ΔH^*) above this temperature of 55.2 kcal, indicating that a triethyltin-induced conformational change occurred at the elevated temperatures. Sugar substrates provided protection against the inactivating effect by reducing the binding of triethyltin to the enzyme. In the absence of glucose, two sites of different affinity for triethyltin exist in the hexokinase monomer. Binding of triethyltin to the enzyme shifted its monomer–dimer equilibrium toward the monomeric form in an early stage of the interaction. Inactivation of the enzyme was associated with a slower subsequent event. Comparative effects of various organotin compounds on the activity of the enzyme indicated that inhibitory potency was associated with increasing hydrophobicity of the alkyl groups attached to the tin.

Hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) is the first enzyme of the glycolytic pathway and, as such, is of critical importance to the overall energy-producing metabolism of the cell. The enzyme isolated from yeast exists as two noninterconvertible isoenzymes, A and B, which at neutral pH are predominantly dimers of molecular weight 102 000, that can be dissociated into identical monomers by increasing the pH or the ionic strength of the medium (Schultze & Colowick, 1969; Derechin et al., 1972; Easterly & Rosemeyer, 1972). In addition to its hexose phosphorylating activity, yeast hexokinase has an ATPase activity that is 5 orders of magnitude lower that can be stimulated 20-fold by addition of lyxose or xylose, nonphosphorylatable analogues of the sugar substrates (Dela Fuente et al., 1970). The lyxose stimulation of ATPase activity of the hexokinase is reportedly due to the fit of this pentose in the hexose binding site which induces a conformational change in the enzyme from a

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"resting" state to an active state with increased affinity for adenosine 5'-triphosphate (ATP).[1] This enzyme complex transfers the terminal phosphate from ATP to a water molecule occupying the position normally held by the hydroxyl group on carbon 6 of the hexose in the hexokinase reactions (Dela Fuente et al., 1970).

Previously, we reported that triethyltin bromide is a specific inhibitor of the hexokinase of red cells from several animal species and from yeast (Siebenlist & Taketa, 1980, 1981). The results of the work with the red cell hexokinases showed that the sensitivity of the enzyme to the selective inhibitory effect of triethyltin depended upon the source of the red cell and the exposure temperature. Rainbow or brook trout red cell hexokinases showed the effect of inhibition at relatively low temperatures (0–20 °C), conditions under which the carp and human enzymes were insensitive. The latter enzymes demonstrated sensitivity to the reagent when the exposure temperature was raised to 25 °C or higher.

In the present work, the effect of temperature on the interaction of triethyltin with yeast hexokinase B was examined. It is shown that the yeast enzyme is inactivated by the reagent at temperatures similar to those required for inactivation of the human red cell enzyme and that protection is provided by the presence of the sugar substrates of the enzyme. The results of studies on the nature of the alkyltin binding site as well as the stoichiometry of triethyltin binding are presented.

Materials and Methods

Trimethyltin bromide, triethyltin bromide, tributyltin chloride, and diethyltin dichloride were obtained from the Ventron Corp. Danvers, MA. (2-[(Dimethylamino)methyl]-phenyl)diethyltin bromide was the generous gift of Dr. J. G. Noltes, Institute of Organic Chemistry TNO, Utrecht, The Netherlands. Stock solutions were prepared as previously described (Siebenlist & Taketa, 1981). Yeast hexokinase B, type C-302 (320–400 units/mg), glucose-6-phosphate dehydrogenase, Bistris, and Tris were pruchased from Sigma Chemical Co., St. Louis, MO. Dithizone was from Eastman Kodak, Rochester, NY. For reaction with the organotins, 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 after appropriate dilution by the spectrophotometric method of Beutler (1975) using a Gilford 240 recording spectrophotometer.

Hexokinase ATPase Activity. The ATPase activity of the yeast hexokinase B was determined by the method of Dela Fuente et al. (1970) as outlined below. Hexokinase (3.1 mg/1100 units) was added to test tubes containing 100 μmol of Tris-HCl buffer, pH 7.0, 10 μmol of MgATP, and various concentrations of triethyltin in a total volume of 1.0 mL. The mixtures were incubated at 35 °C, and at various times, aliquots of 0.1 or 0.2 mL were taken and mixed with 1 mL of cold 5% trichloroacetic acid. After the mixtures were allowed to stand on ice for 10 min, the resulting suspensions were centrifuged (10000 rpm), and 0.3 mL of the clear supernatant was taken to measure inorganic phosphate by the method of Chen et al. (1956). When the ATPase activity of yeast hexokinase B was measured in the presence of lyxose, 100 μmol of the sugar was added to the reaction mixtures, and the amount of enzyme was reduced to 0.6 mg (200 units).

Equilibrium Dialysis. Solutions of yeast hexokinase, 20 μM in enzyme monomer in 3 mL of 0.12 M Bistris, pH 7.0, were dialyzed with shaking overnight at 4 °C against 5 mL of the same buffer containing 2.5–25 μM triethyltin. The concentration of triethyltin outside the dialysis bag was then assayed by a direct spectrophotometric procedure to determine the formation of a triethyltin–dithizone complex (Aldridge & Street, 1981). Triethyltin bound to the enzyme within the bag was determined by first adding 0.5 mL of 30% trichloroacetic acid to 2.5 mL of the solution to precipitate the protein and to release the bound triethyltin. The samples were placed on ice for 10 min and then centrifuged at 10000 rpm for 10 min to obtain clear supernatant. Two milliliters of this supernatant was then used in the assay for triethyltin by the dithizone procedure. After appropriate corrections for dilution, the data were analyzed by the method of Scatchard (1949) as modified by Klotz & Hunston (1971). The remaining enzyme solution was used to assay for hexokinase activity.

Sucrose Density Centrifugation. Sucrose density gradients (5–20%) were formed in centrifuge tubes by layering equal volumes (1.2 mL) of 5, 10, 15, and 20% sucrose solutions in either 20 mM Bistris, pH 6.5, or 20 mM Bistris, pH 6.5, containing 0.5 M KCl, and allowing the mixtures to stand at 4 °C for 6 h. After 100 μL of a 3 mg/mL solution of hexokinase was carefully layered onto the gradient, centrifugation was conducted at 4 °C and 47000 rpm for 13 h by using a Beckman SW 50.1 rotor in a Beckman L5-65 ultracentrifuge. The centrifuge tubes were punctured and the contents fractionated by using a Hoeffer Instruments apparatus connected to an LKB 2120 Varioperpex II pump and a Gilford 240 recording spectrophotometer equipped with a flow cell to monitor absorbances at 280 nm. When the effect of triethyltin on the monomer–dimer equilibrium was to be determined, gradients were made up to contain 500 μM triethyltin, and the enzyme was preincubated at either 4 or 35 °C for 5 min with this concentration of the alkyltin immediately prior to loading and centrifugation.

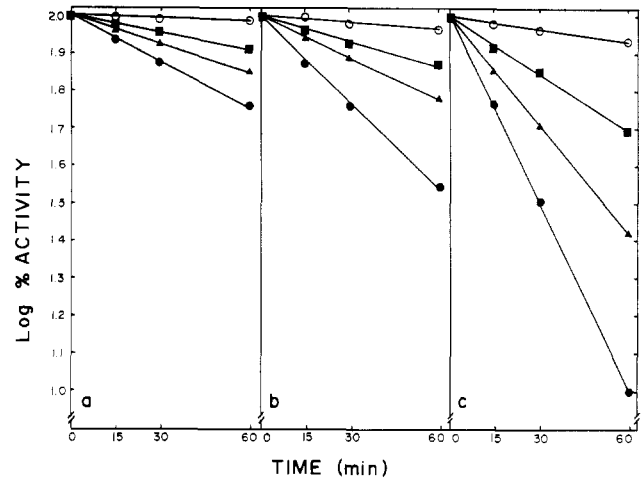

FIGURE 1: Time course of inactivation of yeast hexokinase B with 0–300 μM triethyltin bromide. Yeast hexokinase B (0.2 unit/mL) was incubated at (a) 25, (b) 30, or (c) 35 °C, and at the indicated times, hexokinase activity was assayed as described under Materials and Methods. No triethyltin (○) and 50 (■), 100 (▲), and 300 μM (●) triethyltin.

Results

The time course of inactivation of yeast hexokinase B by triethyltin at 25, 30, and 35 °C is shown in Figure 1a–c. Incubation at temperatures of 20 °C or lower resulted in little or no inactivation of the enzyme, but at higher temperatures, inhibitory effects were evident. The loss of enzyme activity as a function of time of preincubation with triethyltin is clearly

[1] Abbreviations: Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; Tris, tris(hydroxymethyl)aminomethane; ATP, adenosine 5'-triphosphate; ADP, adenosine 5'-diphosphate.

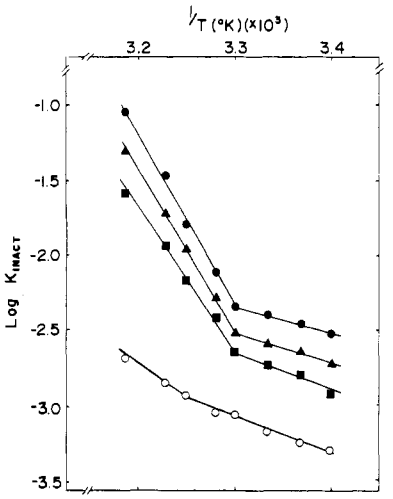

FIGURE 2: Arrhenius plot for the rate of inactivation by triethyltin bromide of yeast hexokinase B. (○) Control; (■) 50 μM triethyltin; (▲) 100 μM triethyltin; (●) 300 μM triethyltin.

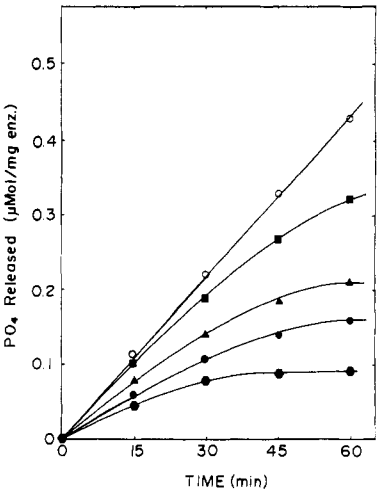

FIGURE 3: ATPase activity of yeast hexokinase B in the presence of 0–500 μM triethyltin bromide. Yeast hexokinase B (1100 units/mL) was incubated with 10 mM MgATP at 35 °C. At the indicated times, aliquots of 0.2 mL were removed and mixed with 1 mL of 5% trichloroacetic acid. The amount of PO_4 released was then assayed for as described under Materials and Methods. No triethyltin (○) and 50 (■), 100 (▲), 250 (●), and 500 μM (solid hexagon) triethyltin.

biphasic in nature. Apparently, the rapid initial loss of activity is due to direct inhibition of the enzyme by triethyltin, and the subsequent slower loss of activity is attributable to a slow temperature-dependent conformational change that results in enzyme inactivation, since it was associated with noticeable development of turbidity. The rate of inactivation of the enzyme by triethyltin increased with rising incubation temperatures. Loss of enzyme activity clearly occurred in the range of 50–300 μM triethyltin and was detected within 15 min after addition of the reagent. The semilog plots of the data are linear up to 80% loss of enzyme activity, indicating that the inactivation is a pseudo-first-order process. The Arrhenius plot (Figure 2) obtained from these pseudo-first-order rate constants for inactivation by triethyltin at temperatures from 20 to 40 °C showed a sharp break at approximately 30 °C. The ΔH^*, the heat of activation for this reaction derived from this plot, was 3.8 kcal at temperatures below 30 °C, and 55.2 kcal above 30 °C. The latter very high value, and the sharp transition, indicates that a major conformational change in the molecule occurred at these elevated temperatures. When the data obtained at 35 °C were analyzed in terms of kinetics of inactivation (Aldridge, 1950), they were consistent with that of a second-order reaction with a rate constant of 3.5×10^2 L mol^{-1} min^{-1}.

The buffers used in these experiments are known to undergo changes in pH with temperature. However, pH change, per se, is apparently not the major factor in the observed temperature-dependent effect of triethyltin on the enzyme since the pH of the buffers varied only from 7.25 at 4 °C to 6.82 at 41 °C, the temperature range used in these studies. When the enzyme was preincubated at 35 °C with triethyltin in the presence of buffers ranging from pH 6.0 to 8.0, an increase in the rate as well as extent of the organotin-induced inactivation was found with increasing pH. Therefore, while the decrease in the pH of the buffers due to increasing temperature is significant in the results shown in Figure 2, it does not account for the accompanying loss of enzyme activity since it would tend to diminish rather than enhance the effects of the triethyltin.

In addition to the effects of temperature, the effects of substrates or products of the hexokinase reaction on the interaction of triethyltin with the enzyme were explored. When the enzyme was preincubated for 60 min at 35 °C with 250 μM triethyltin in the presence of these components, 1 mM D-glucose gave 94.8% protection, 1 mM D-mannose 95.6%, and 1 mM D-fructose 86.3%. Other sugars including L-glucose, D-galactose, and D-ribose as well as the product 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. Protection against inhibition by the sugar substrates of yeast hexokinase suggests that a specific interaction with triethyltin occurs only within a particular conformation of the protein. In addition to their protective effects, the sugar substrates of the enzyme also demonstrate the ability to partially restore activity to the triethyltin-inactivated enzyme. Preincubation of hexokinase with 300 μM triethyltin at 35 °C for short periods of time (less than 20 min) followed by dilution and continued preincubation for an additional 10 min in the presence of assay mixture containing glucose but no ATP resulted in significant recovery of active enzyme. Approximately 33% greater activity was found in such a sample compared with samples analyzed immediately after the preincubation with triethyltin alone. Almost 40% greater enzyme activity was recovered in the sample after the 10-min follow-up preincubation in the presence of glucose compared with a 10-min follow-up incubation in the absence of glucose. With longer preincubation times with triethyltin before addition to the glucose-containing assay mixture, the amount of hexokinase activity recovered decreased until at 60 min of preincubation only 5–10% of the activity was regained. For further assessment of the protective effect of the sugar substrates, the effect of triethyltin on the ATPase activity of the enzyme was explored. As can be noted from Figure 3, triethyltin strongly inhibits this activity of the enzyme; 500 μM triethyltin nearly completely inhibited this activity within 15 min of incubation with the enzyme. These curves clearly show the biphasic nature of the triethyltin inactivation of yeast hexokinase in that there is a rapid loss followed by a slower loss of ATPase activity. Addition of 100 mM lyxose, the pentose analogue of mannose, to the incubation medium not only induced the expected stimulation of ATPase activity but also protected this activity of the enzyme from inactivation by triethyltin (results not shown). Under these conditions, incubation with 500 μM triethyltin resulted in only 12% inactivation of the ATPase activity after 60 min of incubation.

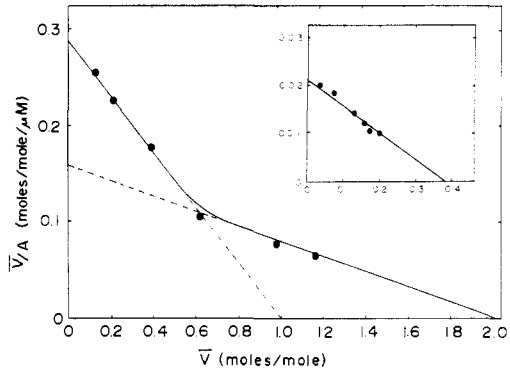

FIGURE 4: Scatchard plot of the binding of triethyltin to yeast hexokinase B. The yeast enzyme (20 μM in terms of monomeric form) was dialyzed overnight at 4 °C against 2.5–25 μM triethyltin. Bound and free triethyltin was then assayed as described under Materials and Methods. The inset depicts the binding of triethyltin to the enzyme in the presence of 5 mM glucose.

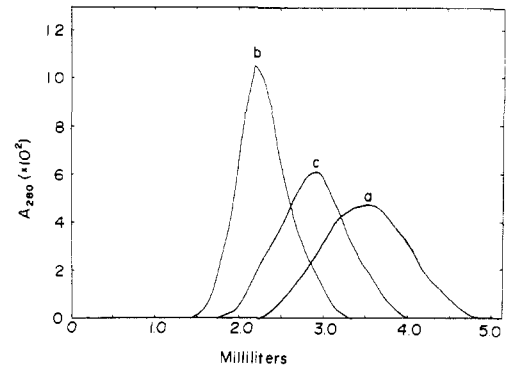

FIGURE 5: Sucrose density centrifugation of yeast hexokinase B. Yeast hexokinase B (0.3 mg) was incubated for 5 min at 35 °C and then carefully layered on top of a 5–20% sucrose gradient made up in 20 mM Bistris, pH 6.5, or 20 mM Bistris, pH 6.5, and 0.5 M KCl. Centrifugation and fractionation were performed as described under Materials and Methods. When the dissociative effects of triethyltin were tested, the enzyme was incubated with 500 μM triethyltin for 5 min at 35 °C before being layered on top of the gradient. Peak a is the dimeric form of the enzyme, peak b is the monomeric form obtained at high ionic strength, and peak c is the form of the enzyme obtained in the presence of 500 μM triethyltin.

When assayed for hexose phosphorylating activity, between 80 and 100% of this activity was recovered in these mixtures containing lyxose. These results, therefore, indicate that the sugar-induced change in conformation of the enzyme either prevents the binding of triethyltin or prevents the change in enzyme conformation that results in inactivation after triethyltin is bound.

The method developed by Aldridge & Street (1981) to measure triethyltin by a spectrophotometric procedure was employed to study the binding of triethyltin to yeast hexokinase. It was used in conjuction with equilibrium dialysis to explore the binding of the reagent to the enzyme in the presence and absence of the sugar substrates. The Scatchard plot of the data obtained from equilibrium dialysis of hexokinase B against triethyltin at 4 °C is presented in Figure 4. When calculated in terms of the M_r 50000 subunit of the enzyme, it appears from the data that the enzyme monomer has two binding sites for triethyltin, the first site having an affinity constant of 2.4×10^5 M^{-1} and the second with affinity constant of 8.1×10^4 M^{-1}. When this triethyltin-treated enzyme was assayed for hexokinase activity, more than 90% of the original enzyme activity was recovered, indicating either that the bound organotin did not produce significant inactivation of hexokinase during preincubation at 4 °C or that it was dissociated from the enzyme during the assay in the presence of glucose. Figure 4 (inset) also shows that the addition of 5 mM glucose to the system decreased the binding of triethyltin to the enzyme. In the presence of glucose, only about 0.39 mol of triethyltin was bound per hexokinase monomer, and the binding constant was reduced to 5.0×10^4 M^{-1}. Therefore, the protective effects afforded the hexokinase by the presence of sugar substrates appear to be due to lack of binding of triethyltin to the enzyme–sugar complex.

When yeast hexokinase B was subjected to sucrose density centrifugation at neutral pH and low ionic strength, a single peak of protein with an *s* value approaching 6.0 S was found (Figure 5, peak a), and when the ionic strength of the centrifugation medium was increased, a shift in the peak to an *s* value of approximately 3.9 S occurred (Figure 5, peak b), consistent with a dimer–monomer dissociation of the enzyme. Centrifugation of the enzyme exposed to 500 μM triethyltin at 35 °C for 5 min under conditions of neutral pH and low ionic strength resulted in a single peak with an intermediate *s* value, indicating an average molecular weight for the enzyme that is between that of the dimer and monomer forms. The single peak and its symmetry and intermediate position suggest that the monomeric and dimeric forms of the enzyme were in rapid equilibrium, with a significant shift in the equilibrium toward the monomeric form. Incubation of the enzyme with 500 μM triethyltin at 4 °C for 5 min prior to centrifugation produced an identical change in the monomer–dimer equilibrium. This change in the monomer–dimer equilibrium of the enzyme induced by triethyltin is similar to the shifts in equilibrium observed by Hoggett & Kellett (1976) when they centrifuged hexokinase in buffers of varying ionic strength. Addition of triethyltin to the enzyme in high ionic strength medium produced a peak identical in position with that seen when the enzyme is in the monomeric form (results not shown), suggesting that the organotin does not cause a gross change in molecular shape. Thus, it appears that triethyltin exerts a dissociative effect on the hexokinase dimer in a temperature-independent manner.

Table I: Effects of Various Alkyltins on Hexokinase Activity

organometallic compd concn (μM)	% hexokinase activity remaining[a]				
	trimethyltin	diethyltin	triethyltin	SnC[b]	tributyltin
0	100	100	100	100	100
10	99	99	95	90	84
25	97	89	83	79	69
50	94	77	51	50	32
100	89	58	34	32	5
250	79	39	23	20	0
500	69	27	10	11	0
1000	58	10	0	0	0

[a] Yeast hexokinase (0.5 unit/mL) was incubated with the indicated concentration of organometallic compound for 1 h at 35 °C and then assayed for activity as described under Materials and Methods. [b] SnC = (2-[(dimethylamino)methyl]phenyl)diethyltin bromide.

The results shown in Table I demonstrate the comparative effects of different organotin compounds on the activity of yeast hexokinase B. Among the indicated limited number of compounds tested, tributyltin was the most effective, triethyltin and (2-[(dimethylamino)methyl]phenyl)diethyltin were about equipotent and next in activity, and trimethyltin was the least active. Comparable effects on enzyme activity were found, for example, when 100 μM tributyltin, 500 μM triethyltin, and 1000 μM diethyltin were used. Clearly, the potency was

associated with the hydrophobicity of the alkyl groups attached to tin.

Discussion

In their study of the inactivation of yeast hexokinase B by iodoacetate, mercurials, and the affinity reagent *N*-(bromoacetyl)galactosamine, Otieno et al. (1977) found evidence for a major structural transition in the enzyme at temperatures above 31 °C, in association with an increase in the reactivity of two of the four SH groups in the hexokinase monomer. The fact that the reactivity of the two remaining SH groups of the protein did not change appreciably suggested to them that the conformational change did not involve a general unfolding of the protein but rather a more limited change in structure that exposed the catalytically essential sulfhydryl group in a relaxed enzyme structure (Otieno et al., 1977). This relaxed enzyme conformation appears to be the form with which triethyltin preferentially interacts, since a similar break in the Arrhenius plot at approximately 30 °C was observed for the organotin-induced inactivation of the enzyme. The increased rate of inactivation at the elevated temperatures is not due to reaction of triethyltin with the sulfhydryl groups of the enzyme, since in preliminary studies we have observed triethyltin to cause an increase rather than a decrease in SH reactivity. It may be due either to the increased accessibility of the organotin binding site in this form of the enzyme or to the susceptibility of the triethyltin-bound enzyme in this conformation to undergo further temperature-dependent changes to an inactive state. Of these two possibilities, the latter appears to be the more likely explanation. Williams & Jones (1976) noted that at low protein concentrations, yeast hexokinase underwent a temperature-dependent loss of activity, consistent with the interpretation that increased dissociation of the dimeric form of the enzyme was followed by a slow inactivating conformational change in the resulting monomer. Triethyltin likewise causes the dissociation of the hexokinase B dimer together with a conformational change in the structure of the protein, characterized by a rapid exposure of the sulfhydryl groups of the molecule as measured by their reaction with sulfhydryl-modifying reagents such as dithionitrobenzoic acid or 4,4′-dipyridyl disulfide. A similar rapid exposure of sulfhydryl groups has been reported to occur as a first step in the thermal inactivation of mammalian hexokinase type II (Murakami & Rose, 1974; Rose & Warms, 1982). However, the dissociation of the hexokinase dimer and the concomitant change in conformation due to triethyltin appear to be temperature-independent phenomena. The triethyltin-induced inactivation of yeast hexokinase B is envisioned to occur by the following sequence of events. Triethyltin binds to the enzyme, causing a shift in the monomer–dimer equilibrium of the enzyme toward the monomeric form with a simultaneous conformational change and exposure of the sulfhydryl groups. This step is independent of incubation temperature, and the enzyme to this point remains fully active. With continued incubation, the enzyme–triethyltin complex then undergoes further temperature-dependent conformational changes resulting in its inactivation. The inactivation is at least a two-step process since at short times of preincubation some enzyme activity is recoverable by the addition of glucose, whereas at longer times the inactivation appears irreversible.

In the absence of sugars, the yeast hexokinase monomer appears to have two sites available for interaction with triethyltin. The association constant for the site with higher affinity is of the same order of magnitude as that determined for the binding of triethyltin to cat hemoglobin (Elliot et al., 1979). In the presence of glucose, binding of triethyltin to the enzyme is greatly reduced, apparently because of the conformational change induced by the binding of glucose. This decreased binding of triethyltin accounts for the protection from inactivation afforded the enzyme by its sugar substrates and explains our observation that triethyltin added to the enzyme in the presence of glucose does not result in enhanced reactivity of the sulfhydryl groups of the enzyme.

The nature and location of the donor ligand(s) on the hexokinase molecule are not known. Nor is it known whether both binding sites of the enzyme for the organotins contain the same type of ligand(s). Some insights into the nature of the organotin binding sites on yeast hexokinase can be gained by comparing the observed effects of these compounds on the enzyme with available information on their binding to cat hemoglobin. Binding of triethyltin to cat hemoglobin involves the formaton of a pentacoordinate tin complex in which the three ethyl groups occupy the equatorial positions and a cysteine and a histidine residue located within the native hemoglobin molecule donate the fourth and fifth axial ligands to the tin (Rose & Aldridge, 1968; Taketa et al., 1980). (2-[(Dimethylamino)methyl]phenyl)diethyltin does not bind to cat hemoglobin. It is 5-coordinate, but it contains only one axial site on the tin available for binding to a ligand donated by the protein; the other axial site is internally coordinated to an amino group within the molecule. From studies using this compound and similar internal pentacoordinate organotin compounds containing various alkyl or aryl side groups, Aldridge et al. (1981) concluded that the internal pentacoordinate compounds will bind to a protein only if a suitable ligand for tin is located in an appropriate hydrophobic environment, or in a region of the molecule having restricted access to hydroxyl ions. Since (2-[(dimethylamino)methyl]phenyl)diethyltin is as active as triethyltin in inhibiting yeast hexokinase, binding of the organotins to the enzyme evidently involves a single ligand for tin in a hydrophobic area of the protein.

Registry No. Hexokinase, 9001-51-8; triethyltin bromide, 2767-54-6; trimethyltin bromide, 1066-44-0; diethyltin dichloride, 866-55-7; tributyltin chloride, 1461-22-9; SnC, 86422-36-8; D-glucose, 50-99-7; D-mannose, 3458-28-4; D-fructose, 57-48-7.

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An Enzyme with Ubiquitin Carboxy-Terminal Esterase Activity from Reticulocytes†

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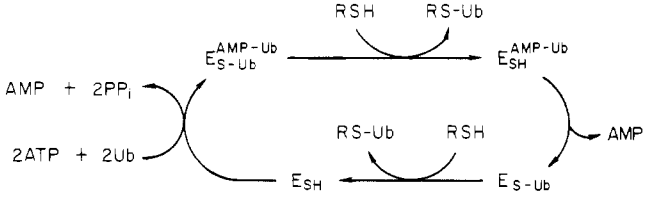
ABSTRACT: Thiols such as dithiothreitol (DTT) are known to allow recycling of the ubiquitin activating enzyme presumably due to attack by thiol on E–ubiquitin forming E + DTT–ubiquitin. It is now reported that the resulting ubiquitin thiol ester is extremely susceptible to hydrolysis, giving rise to free ubiquitin that can then also recycle in the activating enzyme reaction. The instability of ubiquitin thiol esters in this system is caused by a ubiquitin carboxy-terminal thiolesterase activity found as a minor contaminant of the activating enzyme. This activity of rabbit reticulocytes has been extensively purified, and some of its properties are reported. The enzyme, which also cleaves carboxy-terminal adenosine 5′-phosphate–ubiquitin, is inhibited by free ubiquitin at micromolar concentrations. The ubiquitin-specific esterase probably functions to hydrolyze glutathione and other thiol esters of ubiquitin that would be formed spontaneously from activated ubiquitin in cells.

The ubiquitin activating enzyme reaction causes two kinds of activation of the 76 amino acid polypeptide ubiquitin (Ub):[1]

$$E + 2ATP + 2Ub \rightarrow E_{S-Ub}^{AMP-Ub} + 2PP_i + AMP$$

Covalent binding to the enzyme is through a thiol ester bond formed from one round of AMP–Ub formation and followed by a second round (Ciechanover et al., 1981, 1982; Haas et al., 1982). The linkage to ubiquitin in both chemical states is through ubiquitin's carboxyl-terminal glycine group (Hershko et al., 1981). Thiol ester activated ubiquitin is used most immediately for the formation of conjugates with target proteins (Haas et al., 1982). A major fate of such conjugates in reticulocytes is the complete degradation of the target protein with regeneration of the ubiquitin (Hershko et al., 1980; Haas & Rose, 1981). In the presence of pyrophosphatase, all of the activating enzyme is found in the E_{S-Ub}^{AMP-Ub} form, which is sufficiently stable to be isolated by gel filtration. When a thiol compound such as DTT is present, the rapid formation of 2 enzyme equiv of PP_i is followed by additional ATP breakdown (Haas et al., 1982). The thiol is presumed to cause the regeneration of free enzyme by a chemical transacylation:



As is reported here, under limiting concentrations of ubiquitin, the extent of ATP breakdown can exceed by many fold the total amount of ubiquitin present, an observation not explained by the above scheme. Three hypotheses may be considered to explain the catalytic role of ubiquitin: (1) the activating enzyme has an additional hydrolytic activity that is activated by –SH compounds; (2) thiol esters of ubiquitin are unstable; (3) a separate enzyme, a thiolesterase, is present in the preparation of activating enzyme previously believed to be pure. The present study reports a new thiolesterase reaction for ubiquitin carboxy-terminal thiol esters. Possible roles of the enzyme are considered.

Materials and Methods

Ubiquitin activating enzyme was prepared by the reaction affinity method of Ciechanover et al. (1982) with activated CH-Sepharose 4B to which Ub is bound. The enzyme forms a thiol ester linkage to the bound Ub in the presence of ATP and is eluted with AMP plus PP_i. The amount of functional enzyme was determined from the counts of [^{3}H]ATP made acid insoluble by formation of 1 enzyme equiv of [^{3}H]AMP–Ub. Treatment of the activating enzyme with iodoacetamide renders it unable to form E_{S-Ub} but has no effect on formation of E·AMP–Ub (Haas et al., 1982). This enzyme form was prepared by incubation for 15 min at 37 °C with 0.5 mM iodoacetamide in the presence of 50 mM Tris-HCl, pH 7.6, and 0.5 mg/mL BSA, followed by DTT.

Standard Assay for Thiolesterase Activity. A final volume of 50 μL contained 40 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 0.2 mg/mL BSA, 5 pmol of ubiquitin, 20 mM DTT, 100–200

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[1] Abbreviations: Ub, ubiquitin; Ub_{74}, C-terminal des-Gly-Gly-ubiquitin; DTT, DL-dithiothreitol; DTT–Ub, thiol ester of DTT with the carboxyl-terminal glycine of ubiquitin; BSA, bovine serum albumin; Tris, tris(hydroxymethyl)aminomethane; TCA, trichloroacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid.

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