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Characteristics of the Inhibitory Action of Protease Inhibitors on the Glucose-6-phosphate Transporter

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Abstract: The present paper reports characteristics and specificity of the inhibitory action of N^{α}-tosyl-L-lysine-chloromethyl ketone (TLCK) and N^{α}-tosyl-L-phenylalanine-chloromethyl ketone (TPCK) on the glucose-6-phosphate transporter of rat liver microsomes. The TLCK-induced inhibition was pH dependent. The inhibition constants for TPCK were determined by following pseudo-1st order reaction mechanism. The inhibition was protected by preincubation with excess amount of glucose-6-phosphate. The results proved that (a) TLCK inactivates the microsomal glucose-6-phosphate transporter, (b) the inhibition results from the modification of sulfhydryl groups of the transporter.

Key words: D-glucose-6-phosphatase (EC 3.1.3.9), differential labeling, iodoacetamide, N^{α}-tosyl-L-lysine-chloromethyl ketone, protein modification.

Blood glucose level is carefully controlled by glycogenolysis and glycogenesis. The liver stores glycogen in the well-fed state as a reserve fuel for periods of starving and breaks down glycogen in the fasting state. Fasting leads to secretion of glucagon and it stimulates the glycogenolysis cascade to produce glucose-6-phosphate (G6P). Liver has glucose-6-phosphatase (G6Pase, D-glucose-6-P phosphohydrolase; EC 3.1.3.9), which enables mobilization of glucose into the blood stream. The enzyme exists in the endoplasmic reticulum membranes and supposed to be a multicomponent enzyme system (Burchell *et al.*, 1991: Nordlie *et al.*, 1993). G6P is transported into the luminal side of the membrane by the G6P transporter (Arion *et al.*, 1980: Zoccoli *et al.*, 1982a, 1982b) and hydrolyzed by G6Pase. The deficiency of any of the proteins of the G6Pase system impairs the normal glucose level control and leads to fatal diseases. Type 1a glycogen storage disease (Type 1a GSD) is caused by deficiency of G6Pase whereas Type 1b GSD is caused by deficiency of the G6P transporter (T1) (Lange *et al.*, 1980: Schaub *et al.*, 1983: Igarashi *et al.*, 1984); Type 1c GSD is due to a deficiency of the phosphate/pyrophosphate transporter (T2) (Nordlie *et al.*, 1983: Waddell *et al.*, 1989).

It was reported that chloromethyl ketone protease inhibitors inhibit papain by reacting specifically with the sole sulfhydryl group of papain (Whitaker *et al.*, 1968). Speth *et al.* (1992) initially reported that both TLCK and TPCK inhibited G6Pase activity in intact rat liver microsomes with almost the same efficiency and that there was no significant inhibition of the enzyme in detergent-treated microsomes before or after exposure to these reagents. These results strongly show that these reagents react with the G6P transporter specifically (Murataliev *et al.*, 1986). But, it was not clearly shown why the inhibition occurred. In this report, the inhibition kinetics for TPCK and the effects of TLCK on the G6P transporter were investigated and characterized. It was also reported that sulfhydryl reagents inhibit G6P hydrolysis by the enzyme (Wallin *et al.*, 1972: Vakili *et al.*, 1981). We made use of differential labeling technique developed by Kupfer *et al.* (1979). It could be shown from the results that both TLCK and TPCK take part in covalent bonding to the G6P transporter by chemical modification of sulfhydryl groups, causing fast inactivation of G6P hydrolysis activity of the G6Pase system.

Materials and Methods

Materials

Female Wistar rats were obtained from Seoul National University Breeding Laboratories. TLCK was purchased from Boehringer Manheim (Manheim, Germany). TPCK, DTNB, DTT, iodoacetamide, D-glucose-6-phosphate and D-mannose-6-phosphate were purchased from Sigma (St. Louis, USA).

Preparation of microsomes

Microsomes were isolated from the liver of female

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wistar rats fasted for 24 h, as described in (Bergstrand *et al.*, 1969). Intactness of the microsomal preparations was determined on the basis of low-K_m mannose-6-phosphatase activity (Arion *et al.*, 1976) and was routinely ≥ 90%.

Treatment of intact microsomes with TLCK, TPCK or iodoacetamide

TLCK, TPCK (prepared in DMSO) and iodoacetamde (in water) were added to microsomes in 5 mM Hepes pH 7.4, 5 mM $MgCl_2$, 0.25 M sucrose and the mixture was incubated at 30℃. In all preincubation experiments, the protein concentration was set to be 1.0 mg/ml.

Treatment with DTNB, exposure to TLCK and restoration with DTT

The reaction mixtures (0.75 ml), containing intact microsomes (1.0 mg/ml) and 40 μM DTNB were incubated. Temperature was adjusted to be 30℃ throughout the experiment. After incubation for 10 min, two samples of equal volume were removed from the mixture. One sample received TLCK and to the other was added DMSO. Both samples were treated with 20 mM DTT at 21 min. The percentage of remaining activity was calculated by considering the control mixture that contained DMSO only, neither DTNB nor TLCK.

The pH dependence of TLCK-induced inhibition

The preincubation buffer contained 10 mM imidazole/HCl was prepared. Microsomes were incubated for 10 min with 40 μM TLCK in the imidazole buffer and aliquots were withdrawn and assayed quickly. Control samples were treated at the same condition without treating TLCK.

Treatment of microsomes with TLCK in the presence of G6P

Microsomes (2.0 mg/ml) were pre-equilibrated at 4℃ for 6 min with varying concentrations of G6P (Zoccoli *et al.*, 1980). Then each sample was incubated with 200 μM TLCK for 6 min at 30℃. Aliquots of 30 μl from each mixture were transferred to the solution containing 40 mM G6P in sodium cacodylate buffer (pH 6.4), and the activity was assayed after 10 min at 30℃. Control samples preincubated with G6P received DMSO instead of the reagent and were assayed at the same conditions as above. The activity was calculated by considering the blank values of substrate hydrolysis during the same experimental conditions.

Activity assay of G6Pase

G6Pase activities were assayed in a final volume of 0.3 ml 50 mM sodium cacodylate/HCl, 5 mM EDTA pH 6.4 containing 10 mM G6P. The reaction was stopped by addition of 0.1 ml of 72% trichloroacetic acid to the mixture. Specific activity (nmol × min^{-1} × mg $protein^{-1}$) was expressed as released inorganic phosphorous (Chen *et al.*, 1956).

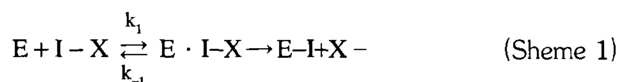
Protein quantification

The microsomal protein quantification was carried out as described (Peterson, 1977) using bovine serum albumin as standard.

Results and Discussion

Kinetics of TPCK-induced inactivation

As reported previously by Speth *et al.* (1992), both TLCK and TPCK showed almost the same efficiency in the respective inhibition profile. We could obtain the same results in our laboratory. We studied the inhibition kinetics for TPCK. The progressive development of inhibition occurred by reaction of intact microsomes with five different concentrations of TPCK, plotted as a semilogarithmic curve (Fig. 1a). The inhibition constants were calculated using a pseudo-first order reaction mechanism (Kitz *et al.*, 1962). The kinetic mechanism is described by Scheme 1 and Eq. (1).



$$E + I - X \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E \cdot I\text{–}X \rightarrow E\text{–}I + X^- \qquad \text{(Sheme 1)}$$

$K_I = k_{-1}/k_1$ (E, enzyme; I–X, TPCK : X, Cl)

$$k_{app} = k_2[TPCK]/(K_I + [TPCK]) \qquad (1)$$

The values of k_{app} and K_I are 1.31 min^{-1} and 65.2 μM respectively at pH 7.4, 33.7℃. A plot of the reciprocal of the pseudo-first order rate constants versus the reciprocal of inhibitor concentration fails to pass through the origin (Fig. 1b), which indicates the formation of a reversible enzyme inhibitor complex prior to covalent modification (Petra, 1971).

Effects of TLCK on G6Pase activity and restoration with DOC

Fig. 2 shows the dependence of G6Pase activity on the time of preincubation with 50 μM TLCK. It was found that G6Pase in intact microsomes was inhibited during incubation with TLCK, and the enzyme activity was restored to about 120% of the original activity by a slight disruption of the microsomal membrane barrier with 0.039% DOC. In using 0.2% DOC, we could observe about 2-fold restoration in activity (data not shown). Because the G6Pase is located inside the microsome, G6P must be delivered by the G6P transporter to produce glucose and inorganic phosphorous.

These results indicate that TLCK specifically reacts with the G6P transporter causing fast inactivation and latent activity comes out by G6Pase exposed by detergent. It was also shown that DTT-treatment could not restore G6Pase activity inactivated by TLCK. We already observed that the enzyme activity (calculated as positive control) remained constant for 20 min at pH 7.4, 30°C and DTT did not affect G6Pase activity of the intact microsomes even at 20 mM (data not shown).

Effects of preincubation of iodoacetamide on G 6Pase activity and the TLCK-induced inhibition of the enzyme

Iodoacetamide has been widely used in protein chemistry as a reagent for sulfhydryl groups (Korman *et al.*, 1956; Tomoharu *et al.*, 1982). Even though iodoacetamide is a more reactive alkylating reagent than TLCK, it did not cause any strong inactivation of G6Pase. As shown in Fig. 3, the G6Pase activity in intact microsomes was not affected by iodoacetamide even at 4 mM. The result indicates that chemical modification with a small molecular weight reagent does not lead to significant inactivation of the G6Pase system and that the hydrophobic side chains of alkylating reagents are important in inhibitory effect. Fig. 3 also demonstrates that

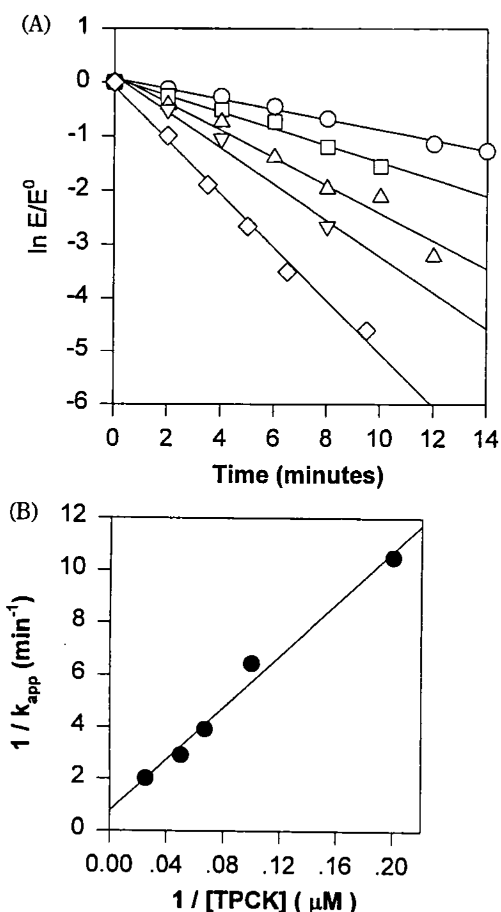


Fig. 1. (A) Intact microsomes (1.0 mg/ml) were incubated at 33.7°C with TPCK at varying concentrations (○, 5 μM; □, 10 μM; △, 15 μM; ▽, 20 μM; ◇, 40 μM). Aliquots were removed at specific time and assayed in the medium (50mM sodium cacodylate buffer, pH 6.4 5mM EDTA, containing 20mM glucose-6-phosphate) at 33.7°C, (B) Double reciprocal plot of apparent pseudo-first order rate constants of inhibition versus TPCK concentrations.

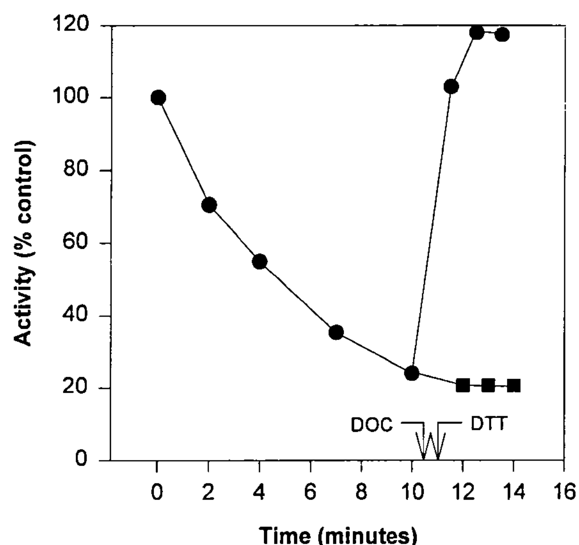


Fig. 2. Effects of 50 μM TLCK on G6Pase in intact and DOC-treated microsomes. The suspension of intact microsomes in buffer A (1.0 mg/ml) was incubated in the absence (as control) or in the presence of TLCK at 30°C. The arrows indicate the addition of 0.039% DOC or 20 mM DTT (■) to TLCK-treated microsomes. Control sample contained neither TLCK nor DOC and was incubated at the same condition.

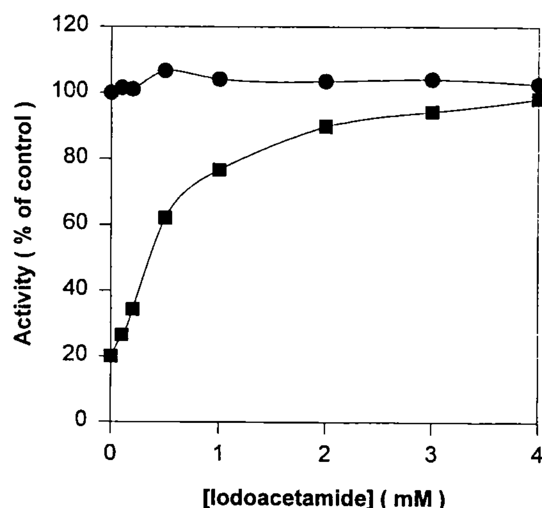


Fig. 3. Prevention of TLCK inhibition by preincubation with iodoacetamide. Intact microsomes (1.0 mg/ml) were preincubated with indicated concentrations of iodoacetamide (●, ■) for 15 min at 30°C in buffer A, and then exposed to 40 μM TLCK (■) in the same condition. After 10 minutes, aliquots were removed and assayed for 15 min in buffer B. The control sample contained neither iodoacetamide nor TLCK.

preincubation of intact microsomes with iodoacetamide protects the TLCK-induced inhibition of the enzyme activity. This result shows that iodoacetamide reacts with the enzyme at millimolar concentration levels preventing the binding of TLCK by pre-occupying the residues, which are essential in enzymatic activity. Considering these results, the inhibition seemed to be closely related to the special features of the chemical modification reagents such as hydrophobicity or steric hindrance. It could be suggested from the results that the reactive thiols are probably located in a hydrophobic micro-environment of the G6P transporter and the hydrophobicity of a reagent increases its affinity for the enzyme.

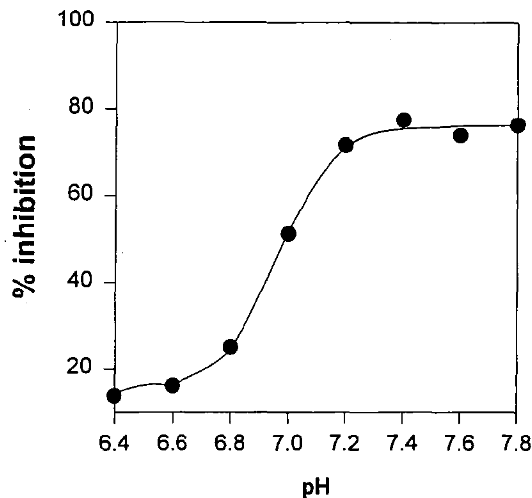


Fig. 4. Effect of pH on TLCK-induced inhibition of the enzyme. The preincubation buffer containing 10 mM imidazole/HCl was prepared. Microsomes were incubated for 10 min with 40 μM TLCK in the imidazole buffer and aliquots were withdrawn and assayed quickly. Control samples were treated at the same condition without treating TLCK.

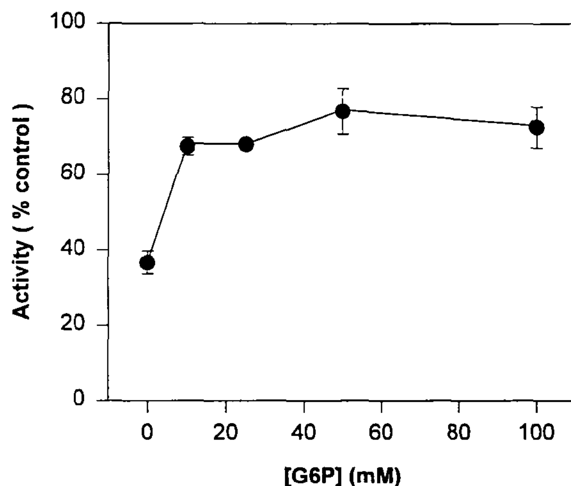


Fig. 5. Effect of substrate pre-equilibration on TLCK inhibition. The experiment was done with three different microsomal preparations and the error bars represent the deviation from the mean. Details are described under Materials and Methods.

pH dependence of TLCK inhibition

Fig. 4 shows TLCK-induced inhibition was dependent upon the pH of the preincubation buffer. The maximal TLCK inhibition was found at pH $\geq$ 7.2, which implies the involvement of some thiol anion residues of the G6P transporter in the inactivation. The local microenvironment around the active site might have changed the pK value of the cysteine residue in the enzyme to about 7.0 seeing that 50% inhibition effect was observed at this point. No investigations were carried out at pH values higher than 8.0 because such conditions lead to membrane disruption (Arion *et al.*, 1972). We observed the same result as above in using TPCK.

Effects of substrate pre-equilibration on the inhibition of G6Pase by TLCK

As shown by Fig. 5, pre-equilibration with G6P reduced the efficiency of inactivation. An increase in the G6P concentration to 50 mM caused no further decrease in TLCK-induced inhibition. It could be suggested that decreased inhibition results from a conformational change of the enzyme due to recognition and/or transporting substrates. In consequence, it can be assumed that TLCK reacts with a specific functional site of the G6P transporter of the G6Pase system.

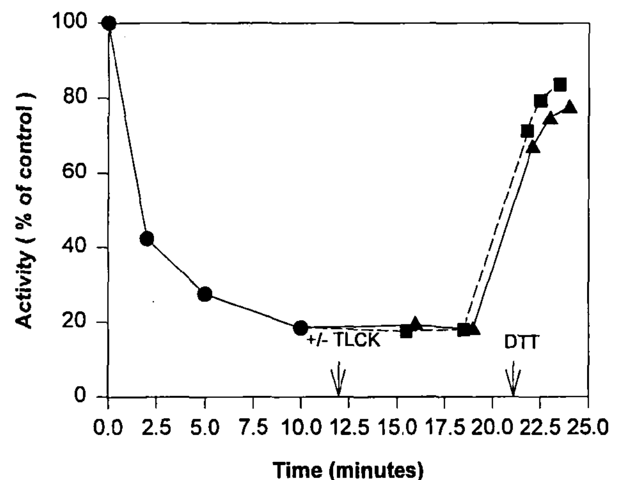


Fig. 6. Differential labeling technique shows the involvement of sulfhydryl groups in TLCK-induced inactivation. After incubation for 10 min with the reagent, two samples of equal volume were removed from the mixture. One sample (▲) received TLCK and to the other (■) DMSO was added. Both samples were treated with 20 mM DTT at 21 min. The percentage of remained activity was calculated by considering the control mixture that contained DMSO only, neither DTNB nor TLCK.

Determination of the involvement of sulfhydryl groups in TLCK-induced inhibition using differential labeling technique

Pre-incubation of G6Pase in intact microsomes with DTNB resulted in significant loss of enzyme activity. Reversal of the inactivation process by treatment with DTT indicates that inhibition is associated with sulfhydryl group modification. The enzyme treated with DTNB first was incubated with TLCK and subsequently exposed to DTT (Fig. 6). The enzyme regained around 80% of its original activity. The result strongly indicates that TLCK also modifies the sulfhydryl groups of the enzyme. The same results could be obtained when TPCK was used instead of TLCK (data not shown). It could be thought from the results that the chloromethyl ketone group of these reagents participates in covalent bonding to the essential cysteine residue(s) of the enzyme system, especially to the G6P transporter.

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