Mechanism of Inhibition of Purified Leaping Mullet (*Liza saliens*) NADPH-Cytochrome P450 Reductase by Toxic Metals: Aluminum and Thallium

Azra Bozcaarmutlu

Department of Chemistry, Institute of Natural and Applied Sciences, Abant Izzet Baysal University, Bolu, Turkey;
E-mail: *bozcaarmutlu_a@ibu.edu.tr*

Received 23 April 2007; revised 13 August 2007; accepted 13 August 2007

ABSTRACT: Aluminum and thallium may reach life-threatening levels in aquatic systems in the near future because of their extensive use in various industrial fields. It is therefore important to study the mechanism of toxicity of aluminum and thallium on fish enzymes. To this aim, the effects of aluminum and thallium on the activity of purified leaping mullet (*Liza saliens*) cytochrome P450 reductase, an essential component of the important cytochrome P450 system, have been studied. Results indicated that both metal ions strongly inhibited the NADPH-cytochrome P450 reductase. The IC_{50} values of $AlCl_3$ and $TlCl_3$ were estimated to be 34 μM and 3 μM, respectively. The Lineweaver–Burk plot and Dixon plot revealed that both metal ions noncompetitively inhibited the purified mullet cytochrome P450 reductase. The K_i values of Al^{3+} and Tl^{3+} were calculated from Dixon plots as 8.9 and 5.6 μM, respectively. The inhibitory effects of Al^{3+} and Tl^{3+} on purified cytochrome P450 reductase were partially recovered by 1 mM EDTA. Additionally, tin and magnesium were shown to have no apparent effect on purified mullet cytochrome P450 reductase. J Biochem Mol Toxicol 21:340–347, 2007; Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10:1002/jbt.20200

KEYWORDS: **Aluminum; Thallium; Tin; Magnesium; EDTA; NADPH-Cytochrome P450 Reductase; Leaping Mullet (*Liza saliens*); Noncompetitive Inhibition**

INTRODUCTION

Metals, contrary to chemically synthesized organic compounds, are naturally present in the Earth's crust. They are mobilized to aquatic systems by human activities. As a result of rapid increases in industrial development, aquatic life is under the risk of metal pollution. Not all metals are toxic to the organisms. The metals such as selenium, iron, copper, zinc, and manganese are essential metals since they play an important role in a biological system, whereas cadmium, mercury, aluminum, and thallium are nonessential metals, as they are toxic, even in trace amounts.

Aluminum and thallium are the members of group 13 elements. Although their industrial use is increasing, the adverse impacts of these toxic metals on ecosystems are not fully understood. Aluminum is the third most abundant element in the Earth's crust. It has been used in various industrial areas. It is present in drinking water, in foods, and in several pharmaceutical preparations [1,2]. It was considered nontoxic in the past. However, studies have indicated the presence of relationship between aluminum and several human pathologies, such as Alzheimer's disease, haemotopoiesis, osteomalacia, and adynamic bone disease [2–5].

Thallium is a rare but widely dispersed element. It is used as a catalyst in certain alloys, optical lenses, jewelry, low-temperature thermometers, semiconductors, dyes and pigments, and scintillation counters [2,6–10]. Thallium compounds have also been used as rat poison and insecticides [2]. There is an increasing demand for thallium in the high technology and future technology fields because of the role in high-temperature superconductors [2,10–12]. Although thallium pollution does not present major threat at this time, the increased industrial usage might cause unrecoverable effects in the aquatic life in the near future.

Correspondence to: Azra Bozcaarmutlu.
Contract Grant Sponsor: Abant Izzet Baysal University Research Project Fund.
Contract Grant Number: BAP-2005-03-03-238.

NADPH-cytochrome P450 reductase (NADPH: ferrihemoprotein oxidoreductase, EC 1.6.2.4) is an integral membrane flavoprotein that catalyzes the transfer of electrons from NADPH to cytochrome P450. It is an essential component of the microsomal cytochrome P450 monooxygenase system. The monoxygenase system consists of NADPH-cytochrome P450 reductase and a family of heme proteins, cytochrome P450, and is involved in oxidative metabolism of both endogenous (steroids, fatty acids, prostaglandins, biogenic amines, and retinoids) and exogenous compounds (drugs, alcohols, organic solvents, anesthetic agents, dyes, environmental pollutants, and chemicals) [13–19]. In addition to the electron transport role in monooxygenase reactions, cytochrome P450 reductase alone can catalyze the reduction of variety of chemicals including therapeutically important antitumor drugs, antibiotics, and aromatic nitro compounds [20,21]. NADPH-cytochrome P450 reductase has been purified from microsomes of guinea pig liver, pig liver and kidney, sheep lung and liver, rat liver, rabbit liver, human liver, and beef liver [22–27]. Fish cytochrome P450 reductase has been purified and some kinetic and functional properties have been characterized from scup, rainbow trout, and leaping mullet [28–30].

The inhibitory effects of metal ions on the purified cytochrome P450 reductase activity are quite important because of the essential role of this enzyme in the metabolism of xenobiotics such as drugs, steroids, and carcinogens. As a result of improper detoxification, toxic xenobiotics may accumulate and become hazardous to the body. Not all metals affect the cytochrome P450 reductase activity. It has been shown that Cu^{2+} inhibited the purified rat and pig NADPH-cytochrome P450 reductase, whereas Zn^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, and Co^{2+} had no apparent effects on the rat cytochrome P450 reductase activity [31–32]. Recently, Bozcaarmutlu and Arinç [33] examined the in vitro effects of Hg^{2+}, Cd^{2+}, Ni^{2+}, and Cr^{2+} on purified mullet cytochrome P450 reductase and observed that Hg^{2+}, Cd^{2+}, Ni^{2+}, and Cr^{2+} inhibited the purified mullet cytochrome P450 reductase activity, whereas Zn^{2+} had no apparent effects on the P450 reductase activity.

The inhibitory effects of the aluminum have not been studied on the microsomal or purified cytochrome P450 reductase activity. Woods et al. [34] showed the inhibitory effect of thallium in vivo and in vitro on the rat microsomal cytochrome P450 reductase activity and they emphasized the requirement of additional studies with purified cytochrome P450 reductase for understanding the overall mechanism. The major objective of this study was to examine the mechanism of inhibition of purified fish cytochrome P450 reductase by aluminum and thallium. The kinetic analysis was carried out to determine the type of inhibition and kinetic constants. The ability of EDTA, as a strong chelating agent, to recover the inhibitory effects of aluminum and thallium on the cytochrome P450 reductase activity was also studied. In addition to aluminum and thallium, the effects of tin and magnesium on purified mullet cytochrome P450 reductase were examined.

MATERIAL AND METHODS

Chemicals

All of the chemicals used in this study were of analytical grade and were purchased from Sigma Co. (St. Louis, MO) and Merck Co. (Darmstadt, Germany) at the highest grade of purity available. Aluminum chloride ($AlCl_3$), thallium chloride ($TlCl_3$), tin chloride ($SnCl_2$), and magnesium chloride ($MgCl_2$) were dissolved in distilled water.

Purification of Mullet NADPH-Cytochrome P450 Reductase

Leaping mullets (*L. saliens*) ($n = 80$) were collected by fish net in Izmir Bay on the Aegean coast of Turkey. Leaping mullet liver microsomes were prepared as described by Arinç and Sen [35] except that the homogenization solution contained 10 mM EDTA, 0.25 mM phenylmethanesulfonyl fluoride, and 0.25 mM ε-amino caproic acid. The microsomes were resuspended in 10% glycerol containing 10 mM EDTA. Microsomal suspensions containing approximately 25–35 mg protein per milliliter were gassed with nitrogen in small polypropylene plastic bottles and stored at $-70^{\circ}C$ until used. All the procedures involved in the preparations of microsomes and purification of enzyme were performed at 0–4°C. NADPH-cytochrome P450 reductase was purified from mullet liver microsomes according to the procedure described by Sen and Arinç [30]. Briefly, the purification procedure involved anion exchange chromatography of Emulgen 913-cholate solubilized microsomes on two sequential DEAE–cellulose columns. This was followed by affinity chromatography using 2′,5′-ADP Sepharose 4B, then further purified and concentrated on hydroxylapatite column as described in detail by Sen and Arinç [30]. The purified enzyme preparation was gassed with nitrogen, aliquoted, and stored at $-196^{\circ}C$ in liquid nitrogen until use. Purified cytochrome P450 reductase was made metal-free during the preparation of microsomes by resuspending microsomes in 10 mM EDTA, a metal-chelating agent, containing solution. At the purification steps, cytochrome P450 was tightly bound to all chromatography columns and column content was extensively washed with EDTA containing solutions. During

the purification steps, the concentration of EDTA was gradually decreased. The final concentration of EDTA was negligible (0.075 μM) in the cytochrome P450 reductase activity assay mixture. The protein concentration of purified cytochrome P450 reductase was determined according to the method of Lowry et al. [36] using bovine serum albumin as a standard.

Measurements of the Mullet NADPH-Cytochrome P450 Reductase Activity

The NADPH-cytochrome P450 reductase activity was assayed by measuring the rate of cytochrome c reduction at 550 nm, spectrophotometrically, according to the method of Master et al. [37], with some modifications. The assay mixture contained 0.3 M HEPES, pH 7.7 containing 80 nmoles cytochrome *c*, 130 nmoles NADPH, and appropriate amounts of enzyme preparation in a final volume of 0.8 mL. The reaction was carried out at room temperature (24–25°C). Purified enzyme preparation was diluted 1:50 with 0.3 M HEPES (pH 7.7) containing 20% glycerol solution daily. The NADPH-cytochrome P450 reductase activity was found to be stable at 4 °C at least up to 9 h in the presence of glycerol. The enzyme activity was calculated using the extinction coefficient of 19.6 mM^{-1} cm^{-1} for the difference in absorbance between reduced and oxidized cytochrome c at 550 nm [38]. One unit of reductase was defined as the amount of enzyme catalyzing the reduction of 1 μmole cytochrome c per minute under the described conditions.

Inhibition of the NADPH-Cytochrome P450 Reductase Activity by Metal Ions

The effects of aluminum, thallium, tin, and magnesium ions were studied by adding various concentrations of metal ions on the reaction mixture containing 0.3 M HEPES, pH 7.7 buffer, and appropriate amount of purified enzyme preparation. After the addition of metal ions, the reaction mixture was immediately mixed and 80 mM cytochrome c was added. Finally the reaction was initiated with the addition of NADPH and followed for 2 min at room temperature in the Hitachi U-2800 double-beam spectrophotometer. The enzyme activity was calculated using the extinction coefficient of 19.6 mM^{-1} cm^{-1} for the difference in absorbance between reduced and oxidized cytochrome c at 550 nm as described for the control activity.

Kinetic analysis was done to determine the type of inhibition of purified mullet cytochrome P450 reductase in the absence and presence of various concentrations of aluminum and thallium. Both Lineweaver–Burk plot and Dixon plot were drawn to determine the type of inhibition. K_i, dissociation constant of enzyme-inhibitor complex, values of aluminum and thallium were calculated from the Dixon plot.

The ability of EDTA to recover the cytochrome P450 reductase activity was studied at two different concentrations of aluminum and thallium. 1 mM EDTA was added into the reaction mixture after the addition of metal ions. The reaction mixture was incubated at room temperature for 5 min. Finally the reaction was initiated with the addition of NADPH and followed for 2 min.

RESULTS

The effect of aluminum on the purified cytochrome P450 reductase activity was studied by adding various concentrations of metal ions into the assay system. Aluminum showed an inhibitory effect on the cytochrome P450 reductase in a concentration-dependent manner (Figure 1). The IC_{50} value (namely, the concentration giving 50% inhibition) of $AlCl_3$ was estimated to be 34 μM. In the determination of the inhibition mechanism of aluminum on NADPH-cytochrome P450 reductase, the concentration of the aluminum was held at three different values (5, 7.5, and 10 μM) at the activity assay mixture, while the substrate concentration was varied (10, 20, 40, and 80 μM). When 1/v

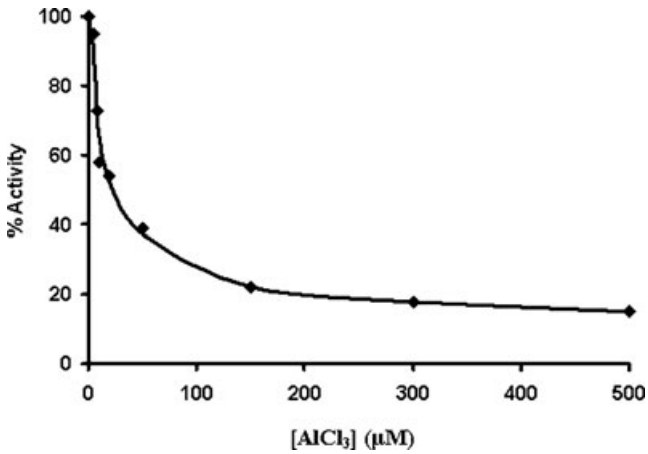

FIGURE 1. The effect of $AlCl_3$ on purified mullet NADPH-cytochrome P450 reductase. Enzyme activity was measured at 5–500 μM concentrations of aluminum. Metal ion was added on the reaction mixture containing 0.3 M HEPES, pH 7.7 buffer and appropriate amount of purified enzyme preparation. After the addition of aluminum, the reaction mixture was immediately mixed and 80 mM cytochrome *c* was added. Finally the reaction was initiated with the addition of NADPH and followed for 2 min at room temperature in the Hitachi U-2800 double-beam spectrophotometer. The points are the average of duplicate determinations. The average values varied less than 5%.

(1/μmole/min/mg protein) was plotted against 1/[S] (1/[cytochrome c]) (Lineweaver–Burk plot) at four different substrate concentrations, the four lines crossed apparently at a point on the *X*-axis. The Lineweaver–Burk plot indicated that the Michaelis constant (K_m) remained unchanged by the presence of different concentrations of aluminum, while V_{max} (maximum velocity) decreased with increasing aluminum concentrations (Figure 2A). 1/v was also plotted against inhibitor concentration [I] (the Dixon plot) since the Dixon plot is used frequently to identify the type of inhibition and to determine the K_i value. Both of these plots suggest the inhibition manner to be apparently noncompetitive. From the intersecting point of the four lines, the K_i value was determined to be 8.9 μM (Figure 2B).

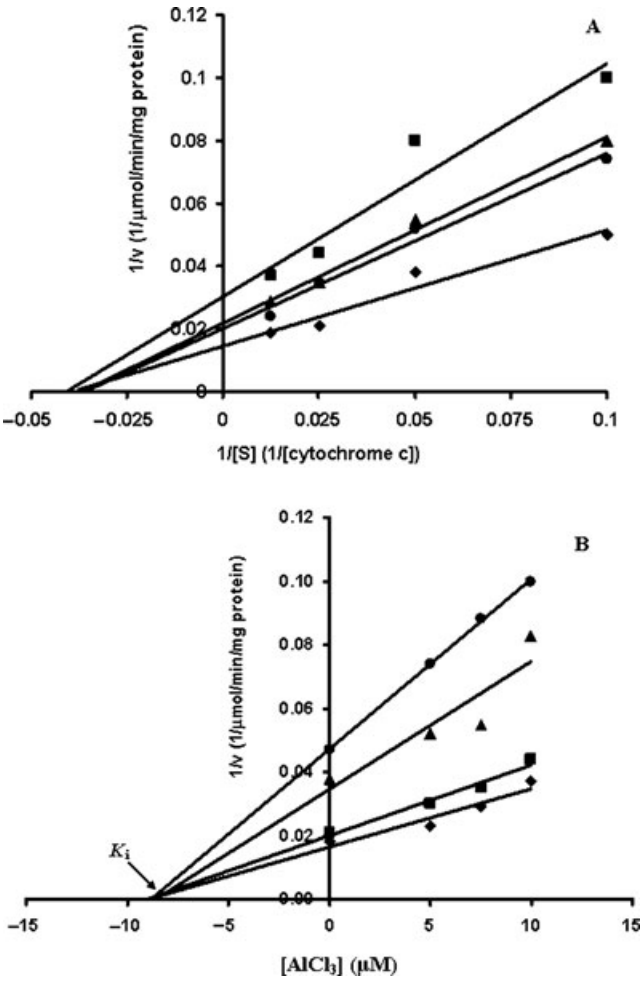

FIGURE 2. Determination of the type of inhibition and inhibition constants for aluminum-induced inhibition of purified mullet cytochrome P450 reductase. (A) Lineweaver–Burk plot of purified mullet NADPH-cytochrome P450 reductase at 5.0 μM (●), 7.5 μM (▲), and 10 μM (■) concentrations of aluminum. (B) Dixon plot of purified mullet NADPH-cytochrome P450 reductase at different concentrations of aluminum using substrate concentrations of 10 μM (●), 20 μM (▲), 40 μM (■), and 80 μM (♦).

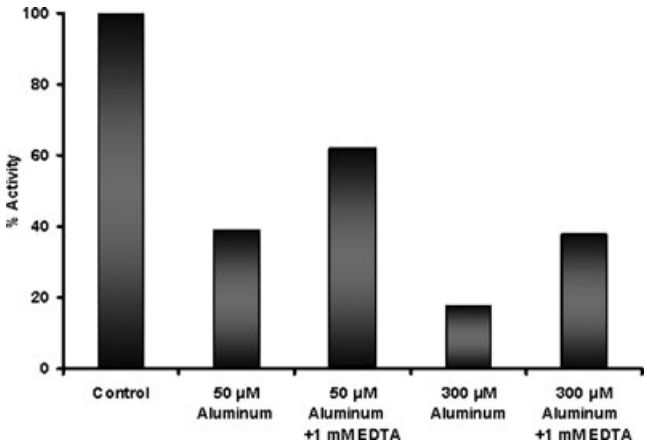

FIGURE 3. Effect of EDTA on the aluminum-induced inhibition of purified mullet NADPH-cytochrome P450 reductase. EDTA (1 mM final concentration) was added to the reaction mixture after the addition of aluminum ions. The reaction mixture was incubated at room temperature for 5 min. Finally the reaction was initiated with the addition of NADPH and followed for 2 min. The values are the average of duplicate determinations. The average values varied less than 5%.

The effect of EDTA was determined at 50 and 300 μM concentrations of aluminum. 1 mM EDTA was added after the addition of aluminum into the enzyme containing reaction mixture and the enzyme activity was measured after the 5 min incubation period. The activity of cytochrome P450 reductase was partially recovered with the addition of 1 mM EDTA. The percentage of recovery was 23 at 50 μM aluminum concentration and it was 20 at 300 μM concentrations of aluminum (Figure 3).

The effect of thallium on purified mullet cytochrome P450 reductase was measured at various concentrations of thallium. The effect of thallium was also concentration dependent (Figure 4). When the effects of thallium and aluminum on purified cytochrome P450 reductase were compared, the enzyme activity was dropped more sharply with increasing thallium concentrations compared to aluminum. At 25 μM metal concentration, Tl^{3+} inhibited the cytochrome P450 reductase activity completely (100%). At this concentration, aluminum inhibited 46% of the initial cytochrome P450 reductase activity (Table 1). The IC_{50} value of $TlCl_3$ was estimated to be 3 μM. In the determination of the inhibition mechanism of thallium on NADPH-cytochrome P450 reductase, the concentration of the thallium was held at three different values (1.0, 3.12, and 6.25 μM) at the activity assay mixture, while the substrate concentrations were varied (10, 20, 40, and 80 μM). Analysis of both Lineweaver–Burk plot and Dixon plot indicated that the mode of inhibition of the purified mullet liver cytochrome P450 reductase by thallium was noncompetitive (Figures 5A and 5B). From the intersecting point of the four lines in the Dixon

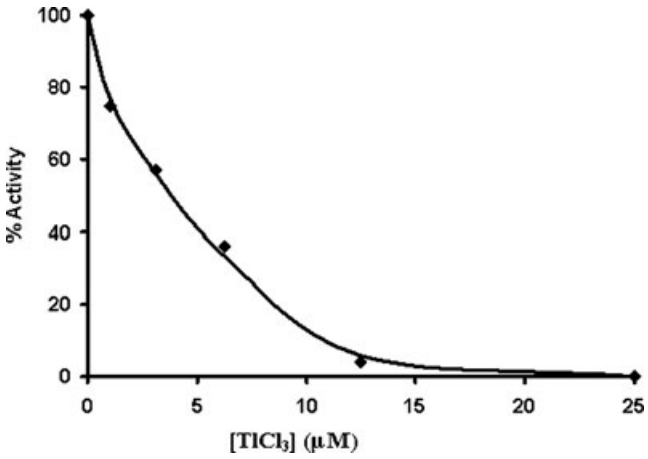

FIGURE 4. The effect of $TlCl_3$ on purified mullet NADPH-cytochrome P450 reductase. Enzyme activity was measured at 1–25 μM concentrations of thallium. Enzyme assay conditions were as shown in Figure 1. The points are the average of duplicate determinations. The average values varied less than 5%.

plot, the K_i value was determined to be 5.6 μM. The effect of 1 mM EDTA on thallium-induced cytochrome P450 reductase was examined at two different concentration of thallium. The cytochrome P450 reductase activity was partially recovered with the addition of 1 mM EDTA. The percentage of recovery was 28 at 3.12 μM thallium concentration and 26 at 12.5 μM thallium concentration (Figure 6).

In addition, effects of tin and magnesium on purified cytochrome P450 reductase were measured at 50 and 100 μM metal concentrations. Both of these metal ions had neither activator nor inhibitory effect at these concentrations on the purified cytochrome P450 reductase.

DISCUSSION

NADPH-cytochrome P450 reductase is an essential component of the microsomal cytochrome P450 monooxygenase system which is involved in the oxidative metabolism of both endogenous and exogenous compounds. The inhibition of NADPH cytochrome P450 reductase may cause insufficient synthesis of endogenous compounds such as steroids, fatty acids, prostaglandins, biogenic amines, and retinoids which in turn may change the growth rate and the reproductive capacity of organisms. Inhibition of NADPH cytochrome P450 reductase may also cause insufficient detoxification of xenobiotics such as drugs, alcohols, organics solvents, dyes, environmental pollutants, and chemicals, the accumulation of which may become hazardous to the body. The present results show that aluminum and thallium inhibits purified mullet NADPH-cytochrome P450 reductase.

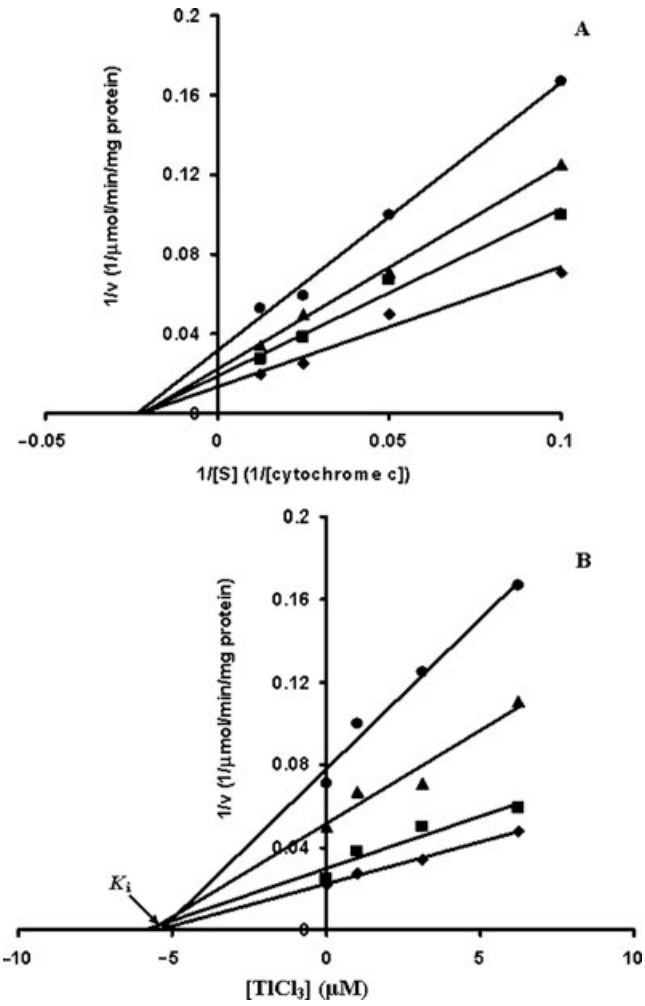

FIGURE 5. Determination of the type of inhibition and inhibition constants of thallium-induced inhibition of purified mullet cytochrome P450 reductase. (A) Lineweaver–Burk plot of purified mullet NADPH-cytochrome P450 reductase at 1.0 μM (■), 3.12 μM (▲), and 6.25 μM (●) concentrations of thallium. (B) Dixon plot of purified mullet NADPH-cytochrome P450 reductase at different concentrations of thallium using substrate concentrations of 10 μM (●), 20 μM (▲), 40 μM (■), and 80 μM (♦).

TABLE 1. The inhibitory properties of aluminum and thallium on NADPH-cytochrome P450 reductase

Parameters	Al^{3+}	Tl^{3+}
% inhibition at 25 μM	46	100
IC_{50} (μM)[a]	34	3
K_i (μM)[b]	8.9 ± 0.24	5.6 ± 0.24
Type of inhibition	Noncompetitive	Noncompetitive
% Recovery with 1 mM EDTA	% 20 (at 300 μM Al^{3+})	% 26 (at 12.5 μM Tl^{3+})

[a] IC_{50} as μM represents the metal ion concentration exhibiting 50% inhibition of the initial NADPH-cytochrome P450 reductase activity.

[b] K_i values were calculated from the Dixon plots of Al^{3+} and Tl^{3+} from Figures 2 and 5.

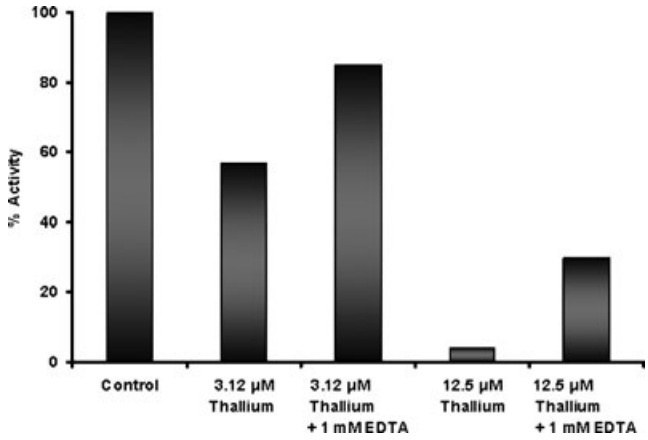

FIGURE 6. Effect of EDTA on the thallium-induced inhibition of purified mullet NADPH-cytochrome P450 reductase. EDTA (1 mM final concentration) was added to the reaction mixture after the addition of thallium ions. The reaction mixture was incubated at room temperature for 5 min. Finally the reaction was initiated with the addition of NADPH and followed for 2 min. The values are average of duplicate determinations. The average values varied less than 5%.

Metals are stable and persistent environmental contaminants and they tend to accumulate. The accumulation of aluminum and thallium may cause the worldwide problem in the near future due to extensive use of both metal ions in various industrial fields. Both metal ions are known to be toxic to living organisms. However, the mechanism of the toxic effect has not been well defined yet. Their presence in environment has increased in some areas to levels which threaten the health of aquatic and terrestrial organisms, including man [39–45]. In some area, the level of thallium exceeded the level of the other toxic metals, such as lead and cadmium [9,44,45]. High concentration of thallium has been found in lake trout from the Great Lakes [44]. Xiao et al. [43] and Lin and Nriagu [44] have drawn attention to the fact that thallium may enter the food chain and may cause unrecoverable effects on the ecosystem. Extensive usage and acidification of the water environment increased the level of aluminum in seawater and caused a decrease in fish populations in many countries [41,46–47]. Although fish is a main food source in many part of the world, the studies of the effects of metal ions on fish enzyme are limited.

Fish have the capacity to accumulate metals. Leaping mullet is an economically important fish and the liver microsomal CYP1A-dependent ethoxyresorufin O-deethylase (EROD) activity of mullet has been used in biomonitoring studies in Izmir Bay, Turkey [48]. A recent in vitro study showed that Zn^{2+}, Ni^{2+}, Cd^{2+}, and Hg^{2+} inhibited the EROD activity significantly in mullet liver [49]. The previous study on purified mullet cytochrome P450 reductase has shown that Hg^{2+}, Cr^{3+}, Cd^{2+}, and Ni^{2+} ions are strong inhibitors of purified mullet NADPH-cytochrome P450 reductase [33]. In this study, the information about the effects of heavy metals on purified mullet cytochrome P450 reductase was extended with aluminum, thallium, tin, and magnesium. The results indicated that aluminum and thallium were also strong inhibitors of the purified mullet cytochrome P450 reductase activity. The IC_{50} values of $AlCl_3$ and $TlCl_3$ were estimated to be 34 and 3 μM, respectively. Tl^{3+} exhibited much higher inhibitory effect at lower concentrations and it was evidently more potent inhibitor than aluminum. When the potency of aluminum and thallium to inhibit the purified NADPH-cytochrome P450 reductase activity was compared with previously tested metal ions in mullet, thallium was more toxic than nickel, cadmium, and chromium, but less toxic than mercury. The toxicity of aluminum was similar to cadmium [33].

The inhibitory effect of thallium has been studied in the rat liver [34]. It has been shown that the in vivo treatment of the rat with 10–200 mg/kg concentrations of thallium significantly inhibited the cytochrome P450 reductase activity. In the same study, it has been shown that thallium produced a dose-related inhibition on the rat microsomal cytochrome P450 reductase activity in vitro. When the concentration was 25 μM, thallium caused 61% inhibition on the cytochrome P450 reductase activity [34]. However, the purified mullet cytochrome P450 reductase was completely inhibited at the 25 μM concentration of thallium. Additionally, the results of this study indicated that EDTA was unable to completely restore the thallium- and aluminum-induced inhibition of the purified mullet cytochrome P450 reductase activity.

Both Lineweaver–Burk plot and Dixon plot of the kinetic data showed that the mechanism of inhibition for tested metal ions were noncompetitive. The K_i values of Al^{3+} and Tl^{3+} were calculated from the Dixon plot as 8.9 and 5.6 μM, respectively. The noncompetitive mode of inhibition of cytochrome P450 reductase suggests that the affinity of NADPH-cytochrome P450 reductase to its substrate, cytochrome c, does not change in the presence of these metal ions. The presence of metal ions causes reduction in the maximum activity of NADPH-cytochrome P450 reductase, which indicates an alteration in the structure of the enzyme.

In addition to aluminum and thallium, the effect of tin on purified mullet cytochrome P450 reductase was tested in this study. Tin has been used in various industrial fields including food industries, glass, and plastic industries and in manufacturing of tin chemicals, color pigments, and pharmaceutics [50]. It was considered as low-risk metal in the past [51]. However it has been shown that tin causes pathological changes in the rat and rabbit liver, and tin chloride is toxic and mutagenic

in yeast and induces DNA damage [50,52–53]. In an attempt to determine the mechanism of toxicity of tin, the effect of tin on purified cytochrome P450 reductase was tested in this research. However, tin at 50 and 100 μM metal concentrations had neither activator nor inhibitory effect on the purified cytochrome P450 reductase.

The other metal tested in this study was magnesium. Magnesium is the third most commonly used structural metal. It is an essential metal. Many enzymes require magnesium ions for their activity. The results of this study indicated that magnesium had neither activator nor inhibitory effect at 50 and 100 μM concentrations on purified mullet NADPH-cytochrome P450 reductase.

In conclusion, this study indicated that aluminum and thallium ions were strong inhibitors of the mullet cytochrome P450 reductase activity, whereas tin or magnesium had no apparent effect on the purified cytochrome P450 reductase. EDTA, a strong chelating agent, partially recovered the inhibition of cytochrome P450 reductase caused by aluminum and thallium. Addressing the mechanism of inhibition for the first time, this study demonstrated that aluminum and thallium induced noncompetitive inhibition on purified cytochrome P450 reductase. The effects of noncompetitive inhibition are much severe and may not be reversed by increasing substrate concentration. The observation of low IC_{50} values and noncompetitive inhibition clearly indicates that aluminum and thallium have serious toxic effects on cytochrome P450 reductase catalyzed reactions in mullet.

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