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Effect of organotin compounds on trout AMP-deaminases

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The comparative toxicity of the organotin compounds tributyltin chloride (TBTC), dibutylin dichloride (DBTC) and monobutyltin trichloride (MBTC) was investigated on 5′ adenylic acid deaminase (AMP-deaminase) purified from trout skeletal muscle and heart. Treatment with TBTC of both enzymatic forms of AMP-deaminase rapidly decreases the enzyme activity, and the organotin derivatives DBTC and MBTC have a significant minor inhibitory effect. Differences were observed in TBTC inhibition rate between trout muscle and heart purified enzymes that could be related to the different cellular localization of the two AMP-deaminases. Copyright © 2001 John Wiley & Sons, Ltd.

KEYWORDS: AMP-deaminase; organotins; trout; pollutant

INTRODUCTION

Organometallic tin compounds (or organotins) have been employed as stabilizers or glass coatings, as catalysts for the formation of polyurethane foam, as biocides for agricultural applications and as preservatives for timber, wood textiles, paper and leather.[1,2] In particular, tributyltin (TBT) compounds are used as biocides in marine antifouling paint formulations. Other derivatives, such as monobutyltin (MBT) and dibutyltin (DBT), are especially used as stabilizers for PVC.[1,2] In addition, the applications of tributyltin chloride (TBTC) as a disinfectant agent in some industries may lead to contamination of sewage effluents.[1,2] Their increasing use has given rise to ubiquitous environmental contaminations, and the study of the biological effects produced by the increase in these pollutants is of considerable interest. Organic derivatives of tin are more toxic than their inorganic analogues; alkyltin compounds are generally more toxic than aryltin ones. In general, the toxicity decreases from tri- to mono-alkyltins.[1,3,4] Their high lipid solubility may give rise to bioaccumulation, with negative consequences on contamination also in the human diet. The rapid expansion and possible large-scale use of organotin derivatives—as antifouling agents in marine paints for protection against microbial or fungal attack, use in disinfection of circulant industrial cooling water, or as agricultural fungicides—have increased the investigations on the presence of organotins in the aquatic environment and on their toxicity for aquatic organisms and survival of fish species. Past investigations on fish have shown that TBT and organotin derivatives induce some cellular and biochemical alterations, such as cytotoxicity, changes in Ca^{2+} homeostasis, inhibition of ion pumps, cell membrane damage, and hemolysis.[1] Organotins can interact with membrane and intracellular proteins by coordinating with the amino groups of α-amino acids such as histidine, and this seems to affect many enzymes, especially those containing the SH group of cysteine that are important for enzymatic activity.[1] Previous investigations in our laboratory on trout erythrocytes have demonstrated that these organotin compounds produce modifications in the physico-chemical features of plasma membranes and changes in the hemolytic rate and stability of trout hemoglobins.[5–8] The above observations stimulated our interest in finding other enzymatic systems that could be selectively modified or that change their enzymatic activity in the presence of organotin derivatives such as TBT, DBT and MBT. In this paper, we have extended this investigation to an important enzyme of purine metabolism, the adenylate deaminase [AMP-deaminase (E.C. 3.5.4.6.)] which is responsible for stabilizing adenylate energy changes and which also plays an important role in cellular energy metabolism.[9,10] This enzyme also catalyzes hydrolytic deamination of the catabolite 5′-adenin-monophosphate (AMP) to its 5′-inosin-monophosphate derivative and ammonia. Deamination of

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Abbreviations used: TBTC, tributyltin chloride; MBTC, monobutyltin trichloride; DBTC, dibutylin dichloride; AMP, adenosine 5′-monophosphate; DTT, dithiothreitol; TBT, tributyltin; DBT, dibutyltin; MBT, monobutyltin; PMB, *p*-chloromercuribenzoic acid; AMP-deaminase, adenylate deaminase; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

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AMP is the first step in the purine nucleotide cycle, making AMP-deaminase a key enzyme for adenylate salvage in the working skeletal muscle. This enzyme is widely distributed in invertebrate and vertebrate systems and is present in fish tissues in large quantities.[11] AMP-deaminase has been isolated from the skeletal muscle and heart of several fish species,[12] where it has been shown to have particular kinetic properties that could be affected by environmental agents such as variation in water salinity and by the presence of different organic and inorganic anions, pollutants or by changes in the membrane phospholipids composition.[13] Generally, fish are able to adapt their physiological conditions to environmental changes, but adaptation requires an increased demand in energy[14] and the process requires highly efficient enzymes interconnecting the adenylate pool.[15] The main purpose of our investigation was to evaluate how enzymes involved in the synthesis and degradation of purine nucleotides could be affected by the presence of organotin compounds. To this end, we have isolated muscle and heart AMP-deaminase from a freshwater fish, trout *Salmo irideus*, and we have studied the effect of the organotin compounds tributyltinchloride (TBTC), monobutyltin trichloride (MBTC), and dibutyltin dichloride (DBTC) on the activity of these two enzymes. The results obtained suggest that pollutants such as organotin compounds could play a role in the regulation of intracellular AMP deamination and on the energetic metabolism in fish with important consequences on their survival.

MATERIALS AND METHODS

All reagents were of analytic grade purity. TBTC, DBTC, MBTC and AMP were obtained from Aldrich; cellulose phosphate was purchased from Sigma Chem. Co., St Louis, MO (USA).

AMP-deaminase from heart and skeletal muscle of *S. irideus* were purified according to Raffin and Leray[16] and were obtained from fish each weighing 200–300 g. The tissue samples of trout heart and skeletal muscle were each homogenized in 3 vols (v/w) of 0.05 M phosphate buffer pH 6.5, containing 0.18 M KCl, by using a precooled Waring blender set at maximum speed; two 30 s homogenizations were conducted. The homogenate obtained was stirred for 1 h and then centrifuged at 18 000*g* for 30 min. About 2 g of previously equilibrated phosphocellulose was added to the supernatant and the suspension obtained was stirred for 1 h at 4 °C. The slurry was washed twice with 25 ml of the extraction buffer, inserted into a glass column (1 cm × 20 cm) and washed with 26 ml of 0.4 M KCl pH 7.0. Subsequently, the AMP-deaminase adsorbed on the resin was eluted with 1.5 M KCl pH 7.0 at a flow rate of 20 ml h^{-1} and fractions of 2 ml volume were collected.

Samples were stored at −80 °C after adding 10% glycerol. The AMP-deaminase activity was monitored at 265 nm using a Varian Cary 1 spectrophotometer. AMP-deaminase inhibition by organotin derivatives was carried out at 25 °C in 0.1 M phosphate buffer pH 6.5, containing 0.15 M KCl. The reaction was initiated by addition of aliquots of a diluted ethanolic solution of organotin compounds to the enzyme solution. At intervals, samples of the incubation mixture were withdrawn and assayed for catalytic activity in 50 mM phosphate buffer pH 6.5, 0.273 mM of AMP and 0.15 M KCl.

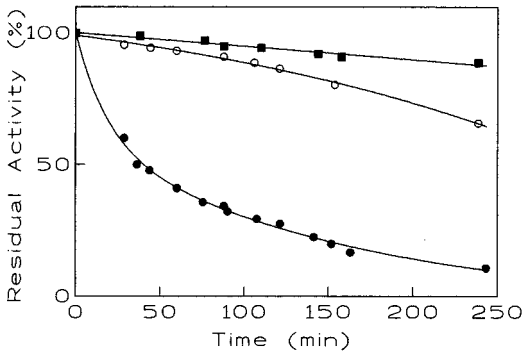

Figure 1. Effect of organotins on the activity of trout skeletal muscle AMP-deaminase. The reaction mixture contains 50 mM phosphate buffer pH 6.5, 0.1 M KCl, skeletal muscle AMP-deaminase (0.06 units about 0.35 μM) and 50 μM of (■) MBTC, (○) DBTC, or (●) TBTC. The reaction mixture was incubated at 25 °C and aliquots were withdrawn at the times indicated and assayed for AMP-deaminase activity.

RESULTS

Samples containing AMP-deaminase enzyme extracted from heart and skeletal muscle of *S. irideus* were incubated in the presence of different concentrations of organotin compounds at 25 °C. The choice of this temperature was decided after evaluation of optimal enzyme stability. Samples were analyzed for 4 h. Figure 1 shows the time course of MBTC, DBTC and TBTC inactivation of AMP-deaminase from trout skeletal muscle. It is possible to observe that the inhibitory effect of TBTC on enzyme activity is more effective compared with that shown by MBTC and DBTC. The enzyme inactivation is directly correlated to the increase in organotin compounds, which can be seen in Fig. 2 for the three compounds considered. The effect of various concentrations of TBTC on trout skeletal muscle can be observed in Fig. 2A. The increase in the amount of this organotin produces an inhibition on AMP-deaminase activity from trout skeletal muscle that seems strongly dependent on the action of this compound. Semi-logarithmic presentation of data (Fig. 2B) clearly shows the biphasic nature of the inactivation observed. This could be related to a multi-step modification process: in a fast reaction, essential groups for enzyme activity are modified, with a consequent loss of 60% of total enzyme activity; changes in other groups, which are probably important for structural assembly of the enzyme,

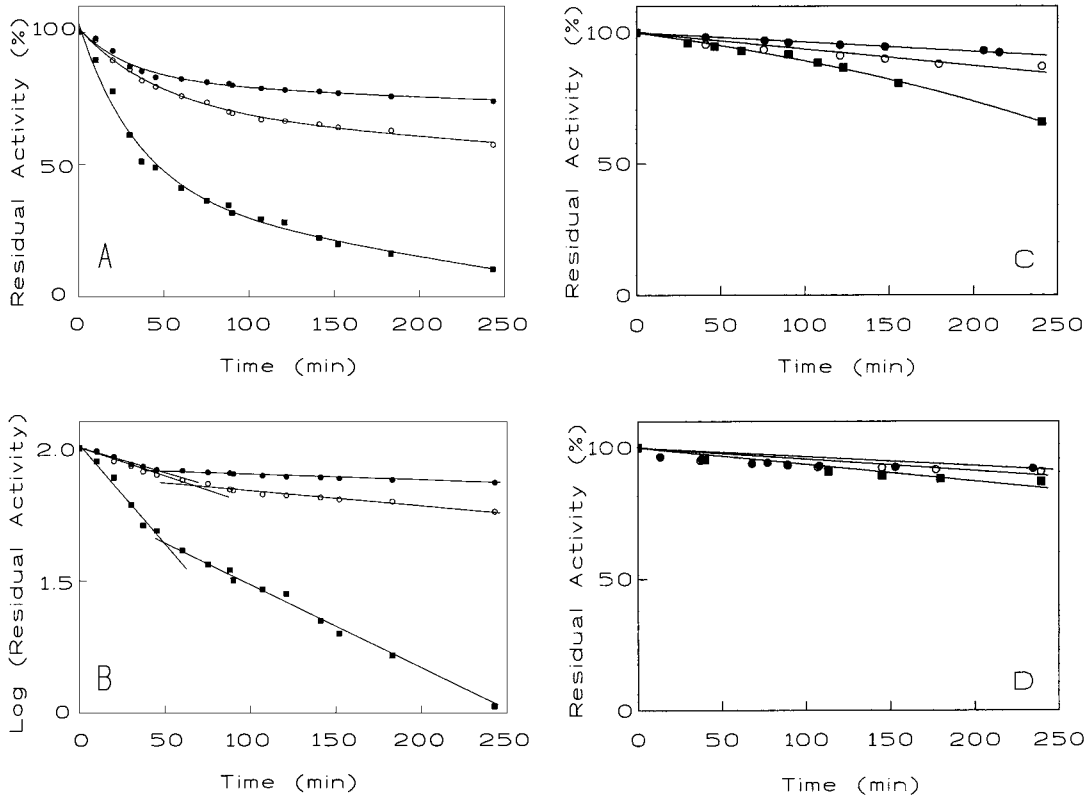

Figure 2. Effect of increasing concentrations of organotins on the activity of skeletal muscle AMP-deaminase: (A) TBTC [(●) 10 μM; (○) 20 μM; (■) 50 μM]; (B) semi-logarthmic presentation of data from (A); (C) DBTC [(●) 10 μM; (○) 20 μM; (■) 50 μM]; (D) MBTC [(●) 10 μM; (○) 20 μM; (■) 50 μM]; The reaction mixture was the same as that indicated in Fig 1.

are modified in a slow reaction, and lead to total inactivation of the enzyme. Since DBTC appears to reduce AMP-deaminase activity remarkably at 240 min (Fig. 2C), we tried to increase the organotin concentration to 70 μM. Unfortunately, the solubility of DBTC decreases, leading to precipitation. MBTC is stable at these concentrations, so as much as 100 μM was analyzed, but no inhibitory activity was observed (Fig. 2D).

The effect of the same organotin compounds on the activity of AMP-deaminase from trout heart was studied. As shown in Fig. 3, the enzyme is inhibited by all compounds tested, and in this case MBTC and DBTC have a stronger inhibitory effect on enzyme activity compared with that reported for the enzyme purified on skeletal muscle (see Fig. 1). The dependence of inhibition rate on the increased concentrations of organotin compounds is shown in Fig. 4, where the inhibitory effect of organotins follows the sequence: TBTC > MBTC > DBTC. The biphasic mechanism of the organotin TBTC on the inactivation of heart AMP-deaminase can also be observed in this form of enzyme (Fig. 4B). The values of IC_{50} (which represent the amount of compound that determines a 50% inhibition of enzyme activity) for the organotin compounds tested on AMP-deaminase from trout skeletal muscle and heart can be calculated from Figs 1 and 3 and are comparatively reported

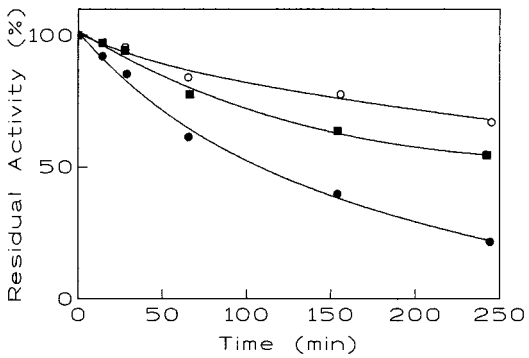

Figure 3. Effect of organotins on the activity of AMP-deaminase purified from trout heart. The reaction mixture contains 50 mM phosphate buffer pH 6.5, 0.1 M KCl, trout heart AMP-deaminase (0.04 units, about 0.4 μM) and 50 μM of (■) MBTC, (○) DBTC, or (●) TBTC. The reaction mixture was incubated at 20°C and aliquots were withdrawn at the times indicated and assayed for AMP-deaminase activity.

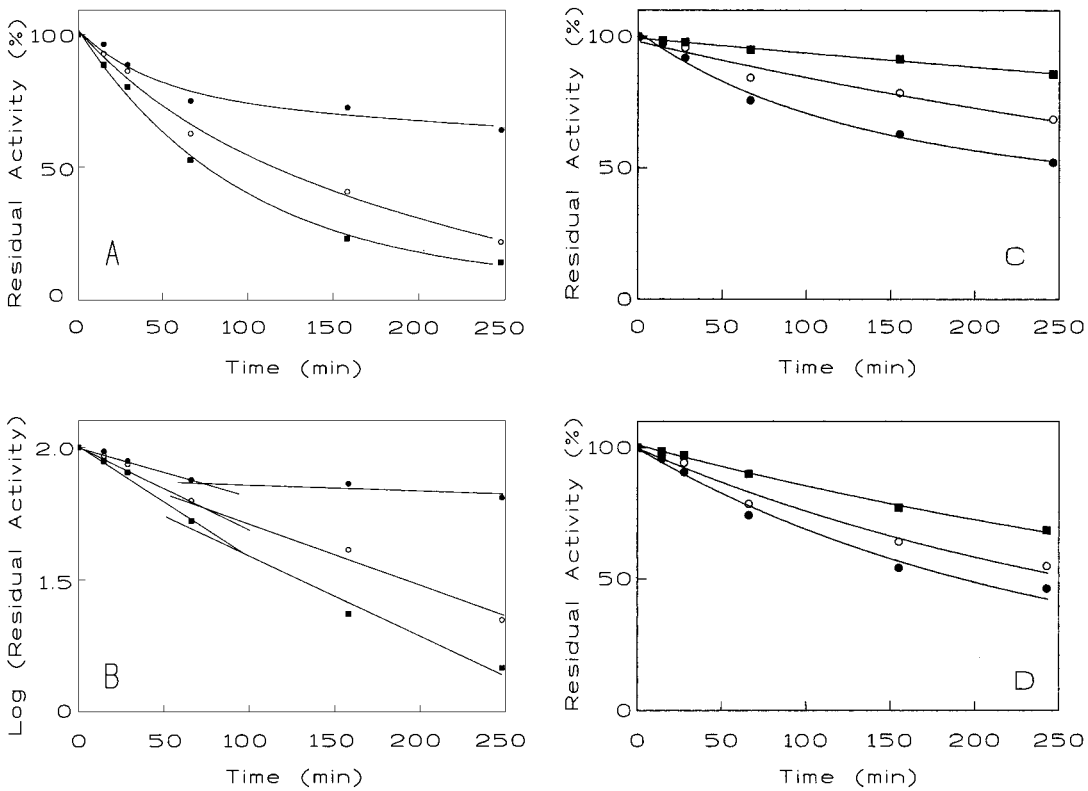

Figure 4. Effect of increasing concentrations of organotins on the activity of purified AMP-deaminase from trout heart: (A) TBTC [(●) 10 μM; (○) 50 μM; (■) 70 μM]; (B) semi-logarithmic presentation of data from (A); (C) DBTC [(■) 10 μM; (○) 50 μM; (●) 70 μM]; (D) MBTC [(■) 10 μM; (○) 50 μM; (●) 70 μM]. The reaction mixture was the same as that indicated in Fig. 1.

in Table 1. In addition, the influence of organotin compounds on the reactivity of sulfhydryl groups on trout skeletal muscle AMP-deaminase was examined. Figure 5 shows that the enzyme is rapidly inactivated by *p*-chloromercuribenzoic acid (PMB, compound that is a powerful reagent for the thiol groups of cysteine in proteins) and that its activity can be completely restored by addition of dithiothreitol (DTT) or *β*-mercaptoethanol (two reducing agents used to remove PMB from SH groups), confirming that SH groups are important for enzyme activity. Incubation of PMB-treated AMP-deaminase (with residual activity of about 20%) with TBTC for 40 min increases enzyme inhibition, but the PMB–TBTC-treated enzyme was not reactivated by DTT or *β*-mercaptoethanol. Figure 6 shows the time course of the enzyme treated with TBTC for 40 min followed by treatment with PMB to block the residual free SH groups. Addition of DTT to the TBTC–PMB-treated

Table 1. Inhibition constant (IC_{50}) for the inhibition of AMP-deaminase from different sources treated with organotin compounds (50 μM). Data were calculated from Figs 1 and 3

Enzyme	IC_{50} (min)		
	TBTC	DBTC	MBTC
Skeletal muscle	46	386	>500
Heart	118	296	265

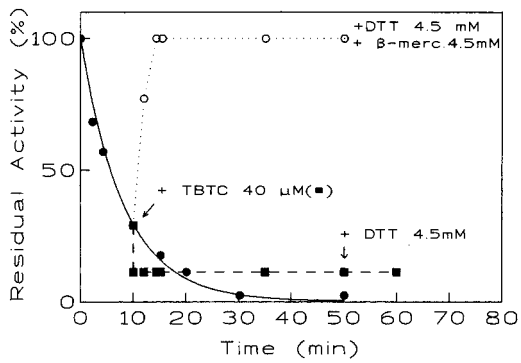

Figure 5. Effect of PMB and TBTC on the activity of trout skeletal muscle AMP-deaminase: (●) effect of PMB titration on the enzyme activity; (○) reactivation of the PMB-inhibited enzyme after incubation with DTT and *β*-mercaptoethanol; (■) effect of TBTC on the PMB-treated enzyme and reactivation by incubation with DTT.

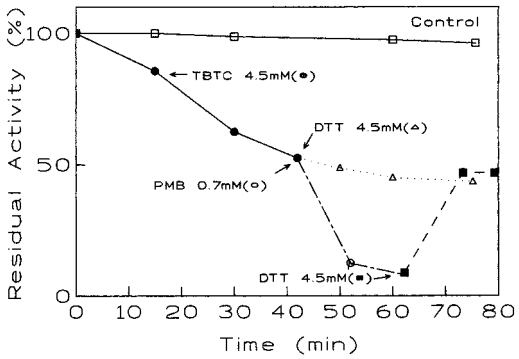

Figure 6. Effect of TBTC (●) on the activity of trout skeletal muscle AMP-deaminase. The TBTC-inhibited enzyme was incubated for 10 min with PMB (○) and subsequently with DTT (■); reactivation of the TBTC-treated enzyme with DTT (△); control (□), the native enzyme was incubated alone for the same time.

enzyme restores only the activity that had been blocked after treatment with PMB, without further recovery of the total enzyme activity. Treatment of TBTC-treated enzyme with DTT did not alter the enzyme reactivity. Similar results were obtained by treatment of AMP-deaminase from heart with PMB and TBTC (data not shown).

DISCUSSION

The bioavailability of environmental pollutants, such as organotin compounds, is related to their accumulation in sediments, which may induce a storage reservoir and lead to food-chain accumulation.[2] Availability also depends on environmental variables, including temperature, pH and ionic composition of water.[1] The adsorption of these compounds onto cellular structures plays a pivotal role for the determination of the degree of uptake.[1] Changes in the hydrophobicity of these compounds are related to their toxicity toward aquatic organisms,[2,17] and in water the organotins undergo a pH-dependent hydrolysis[18] that regulates the concentration of the dominant species present. TBTC seems to be one of the most important organotin environmental pollutants and is a food-chain accumulator, as previously reported for marine organisms.[1] In water, its concentration depends on the pH and Cl^- concentration. In conditions where the pH water is <7, the dominant species are TBT and TBTC, whereas at pH >8 a mixture of TBTC and TBTOH are present.[1] High Cl^- concentrations in sea water and a pH >8 favor the formation of TBTC and of TBTOH respectively, as reported in the literature.[1] The formation of neutral hydroxides in aqueous solution can also be observed for mono-, di- and tri-substituted organotins.[1] TBT degradation in water depends on temperature: during summer it has a half-life on the order of 7 to 30 days; in winter the degradation rate is slower, being about 2 months or more.[1] This, in addition to its very limited volatilization, negligible hydrolysis and stability of TBT, leads to its accumulation. Moreover, the degradation process of TBT gives MBT and DBT species that increase environmental contamination.[19] The toxic effects of organotin compounds on biological structures are known for some organisms, where the lipophilicity of these compounds produces damage at the membrane level and inhibition of Na^+/K^+-ATPase and calcium pumps. Furthermore, their presence may destabilize the membrane and deplete cellular ATP.[20,21] The active ion pump present in fish has been demonstrated to be an ATP-dependent process and, therefore, justifies a better knowledge of the properties of the enzymes involved in the synthesis and degradation of purine nucleotides and nucleosides present in different tissues. The high levels of deaminase activity, the effective irreversibility of the catalyzed reaction and the requirement of adenine nucleotide in energy metabolism indicate that the regulation of AMP-deaminase in vertebrates and fish may, in general, be of special importance in skeletal muscle and heart cells. These observations are interesting in light of the results that we have obtained, because several compounds that were found to inhibit muscle and heart AMP-deaminase in fish could be responsible for serious physiological diseases. Treatment of trout AMP-deaminases with different organotin derivatives shows a very marked decrease in their activity. After treatment with TBTC the activity of both enzymes decreases rapidly, and slight inhibition in the presence of DBTC and MBTC (see Figs 2 and 4) was observed. Comparison of the time-dependent inhibition rate by organotin compounds on the two forms of AMP-deaminase (Table 1) shows that the skeletal muscle enzyme is more sensitive to TBTC inhibition, with an IC_{50} three times lower than that of heart AMP-deaminase. The results obtained for TBTC inactivation could be related to the different accessibility of this compound to TBTC-titratable groups in both enzymes. Many enzymes, especially those containing SH groups, could be affected by organotin compounds *via* the amino or S^- group interaction.[1] In particular, sulfhydryl groups in AMP-deaminase have been reported to participate in the promotion of various ligand-induced regulatory effects. The dependence of AMP-deaminase activity (from different sources) by thiols has also been reported by inhibition studies using 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB, a thiol-modification reagent). These studies showed that the decrease in enzyme activity observed, as a result of chemical modification of thiol residues, is largely due to secondary structure changes. Although the molecular mechanism by which organotin compounds influence trout AMP-deaminase is still unknown, a possible involvement of reactive sulfhydryl groups on the organotin inhibition process could be hypothesized.[22] From our results, AMP-deaminase reacts with PMB rapidly, but the PMB-treated enzyme incubated with TBTC was no longer sensitive towards reactivation by DTT and β-

mercaptoethanol. In human and other mammalian AMP-deaminases, it has been reported that some of the SH groups on the native enzyme are accessible to inactivation by PMB or DTNB, but large quantities of these groups were detectable only on urea-denaturated enzymes, suggesting that the SH groups present in the hydrophobic core of the enzyme are not accessible to the thiol reagents.[23] The internal SH groups could be inactivated preferentially by TBTC (owing to its high hydrophobic properties), which might induce modifications in the enzyme structure with irreversible inactivation. Studies on the structural changes after binding of organotin compounds using different spectroscopic techniques, such as fluorescence and circular dichroism, will be performed to confirm these assumptions. At present, considering the marked effect of organotin compounds on the activity of both muscle and heart AMP-deaminase, it seems that the inactivation of these enzymes could be important for evaluating the environmental risks deriving from pollution of organotin compounds in water. More work is in progress to evaluate the inhibition rate of organotins on other important enzymes of purine metabolism in fish, and to test the potential interference of these compounds on the physiological process in which purine intermediates are involved.

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