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The interaction of zinc pyrithione with mitochondria from rat liver and a study of the mechanism of inhibition of ATP synthesis

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The interactions of zinc pyrithione (ZnPT) with rat liver mitochondria were investigated. Since most of the organometals, principally the triorganotin compounds, induce the inhibition of ATP synthesis in rat liver mitochondria, the efficiency of the ATP synthesis was measured in the presence of ZnPT. The results indicate that ZnPT inhibits ATP synthesis. In order to individuate the molecular mechanism responsible for a failure in ATP synthesis, all of the steps involved in ATP synthesis or in its inhibition were investigated separately, i.e. the respiratory chain, the uncoupling effect, the ATPase and the opening of a permeability pore. All of the steps are inhibited by ZnPT, but the crucial one, the one responsible for the inhibition of ATP synthesis, seems to be the opening of a small-size cyclosporine-sensitive pore. The results are different from those obtained using other organometallic compounds, but are similar to those obtained when using methylmercury and Zn^{2+}, both of which also induce the opening of a cyclosporine-sensitive pore. However, although Hg^{2+} and Zn^{2+} would seem to induce the opening of large-size pores, in the case of ZnPT the pores involved are of a small size. This action mechanism seems to exclude the possibility that ZnPT is a deliverer of Zn^{2+}.

KEYWORDS: mitochondria; zinc pyrithione; ATP synthesis; cyclosporine-sensitive pore

INTRODUCTION

The study of the interactions of organometallic compounds with biological structures has received increasing attention over the last few years, since these compounds are involved in many areas of environmental concern.[1] Therefore, the behaviour and toxic effects of organometallic compounds have been widely investigated in animals, in order to study the toxicological effects on these animals, and in cells and subcellular structures, in order to explain the molecular mechanisms responsible for the effects observed in the whole organisms.[1–12]

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In many cases, the results indicate that the preferential target for the organometallic compounds are mitochondria.[2–9] This fact explains the toxicity as being due to an inhibition in ATP synthesis, with consequent cell damage. Being one of the large number of organometallic compounds, zinc pyrithione (ZnPT) is widely used in medicine and cosmetology as an antibacterial, antifungal or antiseborrheic agent.[13] Because of its metal chelating property, it is also employed in the mining industry for the extraction of zinc from metal ore samples.[14] In recent years, following the widespread banning of organotin compounds, ZnPT has been added to the formulation for antifouling paints for boats, as an alternative biocide agent against a broad spectrum of fouling species.[15] This increasing utilization of ZnPT, coupled with the stability of the molecule, gives rise to an environmental problem, as is true for all organometallic compounds. The

interactions of ZnPT with living organisms, both prokaryotic and eukariotic, have already been studied.[16–19] According to the Danish EPA Report 2000, the registered average concentration in antifouling paints for vessels is 7% with an annual consumption of 0.4 t. Since the lowest effect concentrations (EC/LC_{50}) are less than 30 nM on aquatic organisms, the highest exposure concentrations of ZnPT are estimated to be between 1.7 and 5.3 nM for harbours. In this paper, we have studied the interactions of ZnPT with mitochondria from rat liver, in order to compare the behaviour of ZnPT with that of other organometallic compounds.

MATERIALS AND METHODS

The mitochondria from rat liver were prepared following the procedures previously used.[20] The mitochondrial protein content was determined using the Lowry *et al*. method.[21]

The mitochondrial oxygen consumption was measured using a Clark oxygen electrode (Yellow Springs Instruments, OH, USA) fitted in a closed thermostatic chamber, equipped with magnetic stirring. The spectrophotometric experiments for the detection of the mitochondrial swelling were performed using a Jenway 6400 spectrophotometer (cell length 1 cm) at room temperature. The swelling was monitored by means of the absorbance quenching at 540 nm.

The ATP hydrolysis and synthesis experiments were performed following the pH changes which accompany the reaction

$$ATP + H_2O \rightarrow ADP + \text{phosphate} + H^+$$

using a pH meter (PHM 84 Radiometer, Copenhagen) connected to a Linseis recorder, in a low buffered medium[22] (medium composition: 125 mM KCl, 10 mM $MgCl_2$, 0.5 mM Hepes pH 7.4, 0.5 mM K_2HPO_4). All of the reagents were of analytical grade. Cyclosporine, valinomycin, 2,4-dinitrophenol (DNP), glutamate, malate, ascorbate, tetramethylphenylendiamine (TMPD), Zn PT and oligomycin were purchased from Sigma–Aldrich. ZnPT was first dissolved in dimethylsulfoxide to obtain a 10 mM stock solution.

RESULTS

In mitochondria, the substrates arising from the Krebs cycle are oxidized by molecular oxygen. The oxidation occurs by means of a sequence of cytochromes, i.e. the respiratory chain (RC). The electron flow in the RC is coupled with the extrusion of protons in a stoichiometric ratio.[23] Since the membrane is not permeable to protons, the proton extrusion gives rise both to ΔpH and $\Delta\Phi$. According to Mitchell's chemiosmotic hypothesis,[24] the protonmotive force (p.m.f. $= \Delta pH + \Delta\Phi$) is the high-energy intermediate that stores the free energy arising from the oxidation of the substrates. This energy is further utilized to synthesize the ATP from ADP + phosphate (P_i).

Most of the available evidence suggests that mitochondria are often the preferential target for many toxic compounds. Acute toxicity results from damage to the mitochondria, which produce ATP for the cell, thus giving rise to corresponding cell damage.

Figure 1 shows that ZnPT inhibits ATP synthesis at low concentrations. On the grounds of the mechanism discussed above, ATP synthesis inhibition can result from many causes.

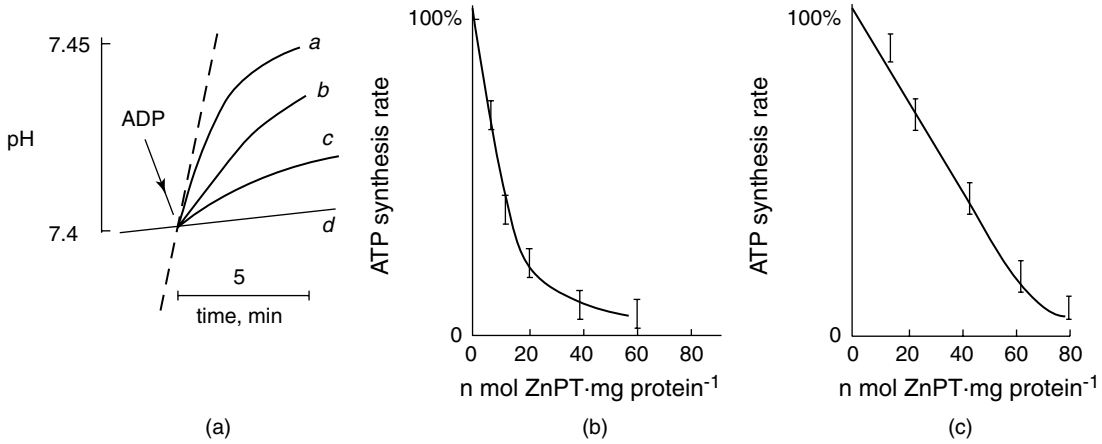

Figure 1. ATP synthesis in mitochondria and its inhibition by ZnPT. The figure reports the ATP synthesis, analysed by studying the pH changes in a low buffered medium (see Materials and Methods) containing 2 mM sodium succinate and 1 mM ADP). The mitochondria (0.5 mg ml^{-1}) were added to the medium (trace a in graph A). In b, c, and d, the medium (4 ml) contained 10 μM, 20 μM, and 40 μM ZnPT respectively. Graph B reports the corresponding value of the initial rate of ATP synthesis assuming as 100% the rate of ATP synthesis in the absence of ZnPT. In graph C the conditions are the same as in B, but the medium contained 1.6 μM of cyclosporine. Points presented are the mean values of four experiments.

As a consequence, each of the mechanisms involved in ATP synthesis was analysed separately, in order to find the molecular mechanism that is inhibited by the lowest concentration of ZnPT. This mechanism was then presumed to be the target responsible for the failure in ATP synthesis in mitochondria. To summarize, the inhibition of ATP synthesis can occur by means of: (1) inhibition of the RC; (2) an uncoupling mechanism; (3) inhibition of ATPase and antiporters responsible for the ADP-P_i transport; (4) opening of a permeant pore.

Inhibition of the RC

In this case, a failure in ATP synthesis is due to the fact that the oxidation of the substrates does not take place. This occurrence is illustrated in Fig. 2, which shows that ZnPT actually inhibits the DNP-stimulated RC. DNP is an uncoupler that permeates the membrane to the protons. This phenomenon, which will be discussed in more detail below, stimulates the respiratory rate up to a maximal value. Under these conditions, the addition of ZnPT induces a decrease in the respiratory rate, as shown by the slope of the straight line in the diagram of Fig. 2 regarding the oxygen concentration with respect to time. The corresponding data reported on the percentage inhibition caused by increasing concentrations of ZnPT were obtained using succinate as a substrate for the RC. This allows for the study of the second and third sites of the RC. If glutamate/malate are utilized as substrates, the whole RC can be analysed. In this case, at the same concentrations employed in the experiments using succinate as a substrate, an inhibition of the RC does not occur (data not shown). Analogously, if ascorbate/TMPD are utilized as substrates, no inhibition of the RC by ZnPT occurs. Therefore the results suggest that the action site of ZnPT in the RC is succinic dehydrogenase, but the inhibition of the RC cannot be the step responsible for the inhibition of ATP synthesis, since the latter occurs with lower doses.

Uncoupling mechanism

Uncouplers inhibit ATP synthesis at low doses, since they permeate the membrane to the protons. This phenomenon gives rise to a collapse in ΔpH and $\Delta\Phi$, with a consequent failure in ATP synthesis. All the uncouplers are weak acids, such as DNP ($K_a = 5 \times 10^{-4}$), that enter the membrane as an electroneutral compound, and tend to accumulate inside, since the pH there is alkaline. Once inside, they are extruded as anions (a phenate, in the case of DNP). The whole balance of the cyclic mechanism is the transport, at any cycle, of a proton through the membrane.

The addition of an uncoupler to respiring mitochondria induces a stimulation of the respiratory rate (Fig. 3). Since both ΔpH and $\Delta\Phi$ are forces that oppose proton extrusion during the functioning of the RC, the addition of an uncoupler, which collapses the protonmotive force, stimulates the respiratory rate. Therefore, the presence of an uncoupler can easily be verified in terms of this behaviour, i.e. by means of respiratory rate experiments. Such a stimulation does not occur in the presence of ZnPT (0.5 up to 60 μM; Fig. 3). However, it must be remarked that, as already mentioned, ZnPT inhibits the RC. Consequently, it is not possible to monitor a possible uncoupling effect, which should stimulate the RC, because the RC is inhibited. However, the uncoupling effect, if present, would occur at a higher concentration than that necessary to inhibit the RC.

On the other hand, a stimulatory effect was never observed at a smaller concentration than that necessary to inhibit the RC; consequently, the uncoupling effect, even if present, cannot be the mechanism responsible for the inhibition of ATP synthesis.

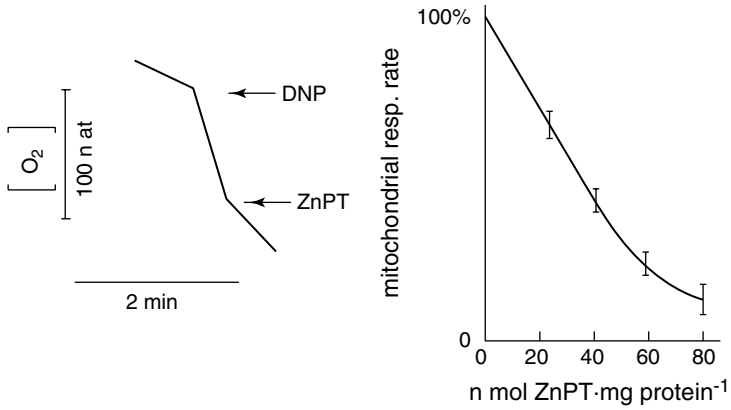

Figure 2. Inhibition of the respiratory rate of mitochondria by ZnPT. The figure reports the inhibition of the respiratory rate by means of ZnPT. Medium (2 ml) composition: 125 mM KCl, 10 mM Hepes pH 7.4, 2 mM sodium succinate, 1.6 μM cyclosporine, 10 mM $MgCl_2$. The mitochondria were added to the medium (0.5 mg ml^{-1}, final concentration) and 50 μM DNP, and successive additions of ZnPT induce inhibition of the respiratory rate. In the graph, the 100% value corresponds to the respiratory rate in the presence of DNP and in the absence of ZnPT. Points presented are the mean values of four experiments.

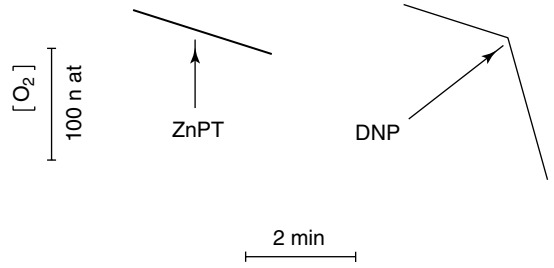

Figure 3. ZnPT does not induce respiratory rate stimulation in respiring mitochondria. Medium composition: 125 mM KCl, 10 mM Hepes pH 7.4, 10 mM $MgCl_2$, 2 mM sodium succinate, 1.6 μM cyclosporine; mitochondria, 0.5 mg ml^{-1}. The addition of 0.5, 1, 2, 10, 20 or 60 μM ZnPT does not stimulate the respiratory rate, whereas the addition of the uncoupler DNP (50 μM) induces an increase in the respiratory rate.

Furthermore, it is difficult to support the hypothesis that ZnPT is an uncoupler of oxidative phosphorylation, since an uncoupler must be a weak acid,[24] and ZnPT is not a weak acid.

For the same reasons discussed above, the possibility that ZnPT induces a detergent-like effect must be excluded, since a detergent is a chemical compound that enhances membrane permeability of all ions (the protons are included) and, consequently, stimulates the respiratory rate.

Inhibition of ATPase and of antiporters responsible for the ADP-P_i transport

ATPase catalyses the transformation of ADP into ATP ($ADP + P_i + H^+ \rightarrow ATP$) and, consequently, the inhibition of ATPase prevents ATP synthesis.

The inhibition of ATPase by ZnPT can be due to the inhibition of the ATPase enzyme (as a whole), or to the inhibition of the antiporters that are needed for the synthesis (Fig. 4). These antiporters are either the ATP/ADP or P_i/OH^- exchanger. They are both essential regarding the transport of the P_i and ADP into the mitochondrial matrix, where ATP synthesis occurs, since the membrane is not permeable to ADP and P_i.

Figure 5 shows the experiment of ATP hydrolysis. The efficiency of the ATPase, or of the ADP/P_i transport, is measured by means of the rate of ATP hydrolysis in a low buffered medium (see Materials and Methods). The corresponding diagram on the right side of Fig. 5 reports the corresponding rates of ATP hydrolysis at increasing concentrations of ZnPT, up to 300 nmol per milligram of protein.

The data show that the inhibition of ATPase, or of ADP/P_i transport, occurs at doses much higher than that necessary

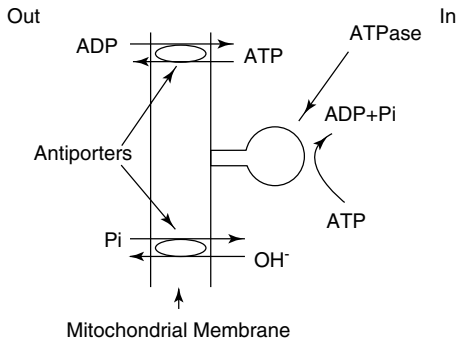

Figure 4. A schematic representation of ATP transport in mitochondria. The ATP synthesis and hydrolysis occur by means of the ATPase in the mitochondrial matrix and the transport of ADP and P_i in the matrix occurs by means of antiporter systems.

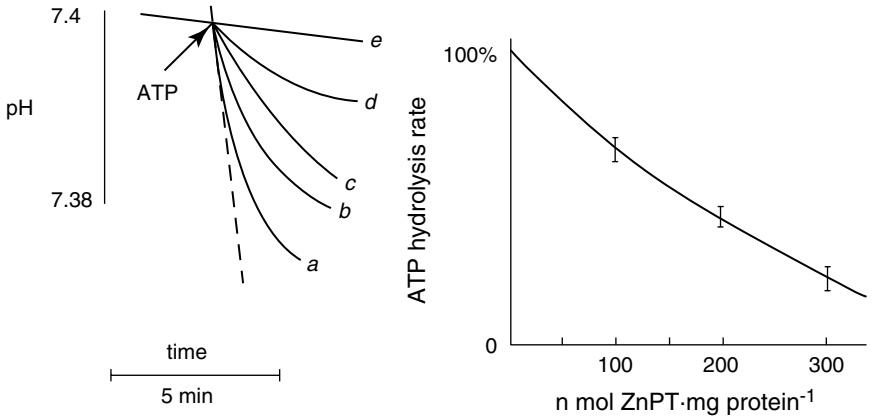

Figure 5. ATP hydrolysis in mitochondria. The figure shows the ATP hydrolysis analysed by examining a pH change in a low buffered medium (see Materials and Methods). The mitochondria (final concentration 0.5 mg ml^{-1}) and 1 mM ATP were added to the medium (a). (b) The medium contained 50 μM ZnPT; (c) 100 μM ZnPT; (d) 150 μM ZnPT; (e) 2.5 μM oligomycin. The corresponding graph reports the inhibition of ATP hydrolysis assuming as 100% the rate of ATP hydrolysis in the absence of ZnPT. Points presented are the mean values of four experiments.

to inhibit ATP synthesis. This procedure does not allow the individuation of the limiting step, i.e. the step inhibited with the lowest concentrations in the whole process, the ATPase or the antiporters for the transport of ADP and P_i. Nevertheless, it may be concluded that this step involving the ATPase or the antiporters is not responsible for ATP synthesis inhibition, since the concentrations are much higher than those shown in Fig. 1.

Opening of a permeant pore

It has been demonstrated that many metals and alkylmetals induce the opening of large- and small-sized membrane pores.[25–29] In the case of the opening of the large-sized membrane pores (MTP pore), this phenomenon is evidenced by means of swelling experiments, since the opening of the MTP pore which collapses all ion gradients, and therefore inhibits ATP synthesis, induces swelling by means of a colloid–osmotic mechanism, in a sucrose medium.[30] The opening of small-sized permeant pores cannot be monitored by means of swelling experiments, but can be evidenced by the fact that such pores, being permeable to protons, collapse the protonmotive forces ($\Delta pH + \Delta\Phi$) and consequently the ATP synthesis.

The opening of the large- and small-sized pores is inhibited by cyclosporine.[25–29]

Figure 6 shows the swelling induced in mitochondria by the addition of P_i or ZPT. The swelling occurs if the concentration of ZnPT is above 10 μM (or 20 nmol·mg^{-1} of protein). Below this concentration, no swelling occurs.

The extent of the swelling increases approximately exponentially upon the addition of the ZnPT inducer and, when the swelling occurs (Fig. 6c), it is time delayed. Therefore, it is impossible to illustrate graphically the rate of swelling versus the ZnPT concentration. However, even if the response of the swelling induced by the ZnPT only undergoes small changes with dosage, owing to the endogenous concentrations of Ca^{2+} and P_i, the swelling is cyclosporine-sensitive and, above all, occurs with higher concentrations than those necessary to inhibit ATP synthesis. In this regard, if one compares the experiments of Fig. 6 with those of Fig. 1B, then a concentration of 10 μM ZnPT does not induce swelling (Fig. 6b), whereas the same concentration of ZnPT gives rise to a strong inhibition of ATP synthesis (about 70%; see Fig. 1B).

DISCUSSION

Given the above situation, the step that inhibits ATP synthesis at the lowest concentration seems to be the opening of a pore, as shown by the experiments of Fig. 1 (ATP synthesis) being cyclosporine-sensitive.

In this regard, it must be remarked that only the opening of the large-sized pores induces swelling, since sucrose is not transported by the small-sized pores. Therefore, since with doses below 10 μM ZnPT the ATP synthesis is inhibited, and swelling does not occur, the only way of explaining ATP synthesis inhibition is that ZnPT induces the opening of small-sized pores.

The behaviour of ZnPT, therefore, seems to be very different from that observed when using other organometallic compounds, such as the trialkyltin and trialkyllead types of compound.[2–9] Regarding the latter, both the interaction and consequent toxicity seem to be due either to an uncoupling effect[5–7] or to a Cl^-/OH^- exchange in the mitochondrial membrane.[2–4] In the case of ZnPT, and likewise with methylmercury,[9] the prevailing effect seems to be the opening of the cyclosporine-sensitive pore, which is probably responsible for the apoptosis in the cell.[25–28]

The behaviour of ZnPT appears similar to that shown by using Zn^{2+} in liver mitochondria, since Zn^{2+} induces opening of large-sized pores at a concentration of about 10 μM,[7] but swelling occurs only in the presence of Ca^{2+}, a potent co-inducer of the opening of pores in mitochondria.[25,26,27,29,31] In the absence of Ca^{2+}, as in our experimental conditions,

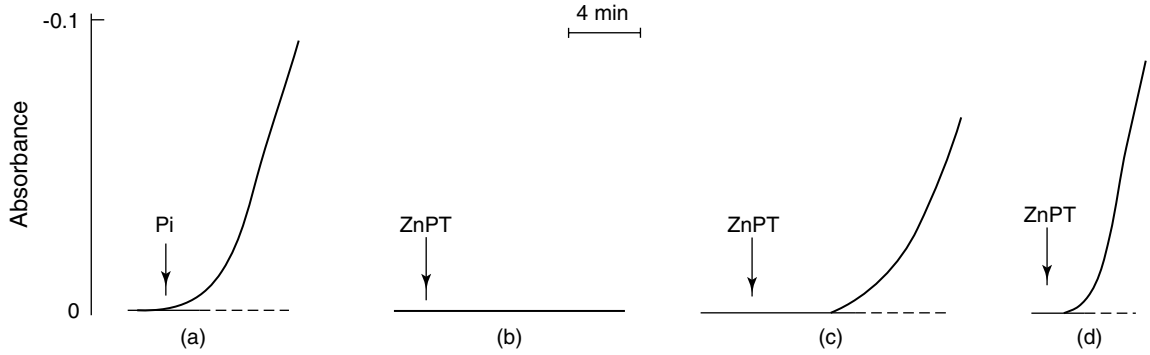

Figure 6. Swelling of mitochondria. Medium (2.5 ml) composition: 0.25 M sucrose, 10 mM Hepes pH 7.4, 10 mM $MgCl_2$, 2 mM sodium succinate; mitochondria: 0.5 mg · ml^{-1}. The swelling can be induced either by addition to the medium of 2 mM (P_i) (a), or by addition of 10 μM ZnPT (b), 15 μM ZnPT (c) and 20 μM ZnPT (d). The dotted line represents the experiment performed after addition to the medium of 20 μM ZnPT in the presence of 1.6 μM of cyclosporine.

Zn^{2+} never induces the opening of the large- and small-cyclosporine-sensitive pores.[7,31]

In conclusion, ZnPT seems to induce the opening only of a small conductance channel; such a response is not known for Zn^{2+}, since the ATP synthesis measurements have not yet been performed. Therefore, at the present time, the only possible conclusion is that ZnPT and Zn^{2+} interact with mitochondria by means of two different mechanisms and at the very least ZnPT does not seem to act as a deliverer of Zn^{2+}.

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