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Research paper

The mitochondrial F_1F_O-ATPase desensitization to oligomycin by tributyltin is due to thiol oxidation

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ABSTRACT

The antibiotic oligomycin is known to inhibit mitochondrial F-type ATP synthases. The antibiotic inhibits both ATP synthesis and hydrolysis by blocking the H^+ translocation through F_O which is coupled to the catalytic activity of F_1. The amphiphilic organotin tri-*n*-butyltin (TBT), a known mitochondrial poison, can penetrate into biological membranes and covalently bind to electron-donor atoms of biomolecules such as sulfur. This study aims at exploring the mechanism(s) involved in the enzyme desensitization to oligomycin which occurs at concentrations >1 μM TBT. This poorly known effect of TBT, which only appeared at temperatures above the break in the Arrhenius plot of the enzyme activity, was found to be accompanied by the oxidation of isolated thiol groups of the mitochondrial complex. The oligomycin sensitivity was restored by the reducing agents glutathione and dithioerythritol and not influenced by antioxidants. The whole of data is consistent with the hypothesis that thiol oxidation is caused by TBT covalent binding to cysteine residues in a low-affinity site on F_O and not by other possible oxidative events. According to this putative model, the onset of tin–sulfur bonds would trigger conformational changes and weaken the oligomycin interaction with F_O.

1. Introduction

The mitochondrial ATP synthase (EC 3.6.1.34) consists of two matched rotary engines; the catalytic portion F_1 which binds adenylate nucleotides and is the seat of ATP synthesis/hydrolysis and the membrane-embedded F_O-ATPase, which exploits the transmembrane protonmotive force (Δp) to translocate H^+ across the inner mitochondrial membrane [1]. Through the central stalk which connects the two rotor movements, the electrochemical energy can be converted into mechanical rotation and *vice versa* [2]. The intimate mechanism of the coupled energy transmission in F_1F_O has been one of the most amazing questions of biology, absolutely justifying the definition of the enzyme as a splendid molecular machine [3]. Both ATP synthesis and hydrolysis coupled to H^+ translocation are strongly inhibited by oligomycins [4]. These natural macrolide antibiotics contain a lactone ring bound to deoxy sugars [5] and slightly differ in the molecular forms. All known molecular species are strong inhibitors of the ATP synthase. Unfortunately the binding site of these compounds on the enzyme complex has been somehow elusive for more than 50 years [6]. Among the potential candidates, the OSCP-subunit (oligomycin sensitivity conferring protein) [7], which is part of the peripheral stalk, and/or the same proton translocator coupling factor (F_O) [8], which contains many subunits required for binding and conferring the oligomycin sensitivity on F_1 [9], have been repeatedly involved. However OSCP lacks oligomycin binding sites and probably acts indirectly by stabilizing F_O and F_1. Multiple mutations under both mitochondrial and nuclear control have been associated with oligomycin-resistance in mammalian cell lines; accordingly crucial aminoacids for oligomycin binding have been localized [10–12]. Recently, the F_1F_O inhibition by oligomycin has been more precisely related to the inhibitor binding to helix 2 of two adjacent subunits of *c*-ring, namely the core of F_O which acts as H^+ channel. This oligomycin binding would cover the deprotonated carboxylates which bind H^+ and block H^+ translocation [13].

Interestingly, clues of the interaction modes between oligomycins and F_O may be provided by recently explored effects on the ATP synthase of the synthetic biocide tributyltin (TBT). This organotin compound was widely exploited in the past in antifouling paints. In

Abbreviations: ASC, L-ascorbate; ARS, sodium (meta) arsenite; BSA, bovine serum albumin; DTE, 1,4-dithioerythritol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NEM, *N*-ethylmaleimide; OI Mg-ATPase, oligomycin-insensitive Mg-ATPase; OS Mg-ATPase, oligomycin-sensitive Mg-ATPase; Tm, melting temperature; TBT, tri-*n*-butyltin; TOC, α-tocopherol.

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spite of bans, TBT still represents an environmental threat due to its persistency and widespread toxicity. TBT emerges as the most toxic organotin and particularly as a mitochondrial toxin [14]. Among the variegated mitochondrial effects, the ATP synthase inhibition by organotins has been known since the second half of the last century [15] and in some cases related to deleterious effects on biota at various biological levels [14]. In recent decades, TBT has been shown to have a wide range of targets which embrace biochemical pathways, cell membranes and biomolecules. Its toxic power seems to be tightly related to the chemical versatility [14,16]. Since TBT has an electrophilic central tin atom, and a lipophilic moiety, formed by the three covalently bound butyl arms, it may onset with biomolecules both dative bonds between tin and electron-donor atoms, such as sulfur, nitrogen and oxygen, as well as non-covalent interactions which connect the butyl chains to hydrophobic residues [17,18]. Accordingly, TBT, known as membrane-active toxicant, exploits its butyl arms to penetrate the lipid bilayer and modify the membrane chemical–physical state and function. Once incorporated in mitochondria, TBT perturbs the membrane thermotropic transition and decreases its overall polarity [19]. Consistently, the butyl chains of TBT intercalated between the initial methylene groups of phospholipids decrease the hydration of the interfacial region of membrane [18,20] and unpack the unsaturated phosphatidylethanolamines by promoting the formation of non-lamellar phases in liposomes [21]. When binds covalently to biomolecules, TBT may onset various geometrical arrangements. It generally maintains its tetrahedral arrangement if the Sn(IV) binds to single atoms such as sulfur of thiol groups or the oxygen of carboxylates of amino acid residues [22]. Alternatively, if TBT binds to two or three nucleophilic ligands, it forms a trigonal bipyramidal pentacoordinate complex [23] or an octahedral hexacoordinate complex [24]. In the presence of vicinal thiols (dithiols) the central tin may form a S–Sn–S bridge after debutylation and the dealkylated toxicant may rearrange in a distorted tetrahedral structure bound to the protein [25].

At present, the interaction modes of the toxicant with the ATP synthase are not fully understood [26,27]. Recent findings from our lab [28] lead to the hypothesis that oligomycins and TBT, which both act on the intramembrane portion F_O, can somehow interfere with each other. Accordingly, other than a strong and quite expected F_1F_O-ATPase inhibition, an unexpected enzyme desensitization to the F_O inhibitors oligomycin and N,N′-dicyclohexylcarbodiimide has been pointed out [28,29]. In this paper on swine heart mitochondria we demonstrate that the F_1F_O-ATPase desensitization to oligomycin, initially pointed out in invertebrate mitochondria [28,30], represents an overall mechanism of the action of TBT, due to the toxicant capability to interact with critical thiols within F_O.

2. Materials and methods

2.1. Chemicals

Na_2ATP, oligomycin mixture (A:B:C 64:15:17%), tri-*n*-butyltin (TBT) chloride quercetin, 1,4-Dithioerythritol (DTE), L-Glutathione reduced form (GSH), 5,5′-Dithiobis (2-nitrobenzoic acid) (DTNB), *N*-Ethylmaleimide (NEM), sodium (meta)arsenite (ARS), L-Ascorbic acid (ASC) and butylated hydroxytoluene (BHT) were obtained from Sigma–Aldrich (Milan, Italy). (±)-α-tocopherol (TOC) was purchased by Fluka. All other chemicals were reagent grade. Quartz double distilled water was used for all reagent solutions except when differently stated.

2.2. Preparation of the mitochondrial fraction

Swine hearts (*Sus scrofa domesticus*) were collected at a local abattoir and transported to the lab within 2 h in ice buckets at 0–4 °C. After removal of fat and blood clots as much as possible, approximately 30–40 g of heart tissue were rinsed in ice-cold washing Tris–HCl buffer (medium A) consisting of 0.25 M sucrose, 10 mM Tris(hydroxymethyl)-aminomethane (Tris), pH 7.4 and finely chopped into fine pieces with scissors. Each preparation was made from one heart. Once rinsed, tissues were gently dried on blotting paper and weighted. Then tissues were homogenized in the homogenizing buffer (medium B) consisting of 0.25 mM sucrose, 10 mM Tris, 0.2 mM EDTA (free acid), 0.5 mg/mL BSA, pH 7.4 with HCl. After a preliminary gentle break up by Ultraturrax T25, the tissue was carefully homogenized by a motor-driven teflon pestle homogenizer (Braun Melsungen Type 853202) at 450 rpm with 5 up-and-down strokes. The mitochondrial fraction was then obtained by stepwise centrifugation (Sorvall RC2-B, rotor SS34) [30]. Briefly, the homogenate was centrifuged at 1000 *g* for 5 min, thus yielding a supernatant and a pellet. The pellet was re-homogenized under the same conditions of the first homogenization and re-centrifuged at 1000 *g* for 5 min. The gathered supernatants from these two centrifugations, filtered through four cotton gauze layers, were centrifuged at 10,500 *g* for 10 min to yield the raw mitochondrial pellet. The raw pellet was resuspended in medium A and further centrifuged at 10,500 *g* for 10 min to obtain the final mitochondrial pellet. The latter was resuspended by gentle stirring using a Teflon Potter Elvejehm homogenizer in a small volume of medium A, thus obtaining a protein concentration of 20–25 mg/mL. All steps were carried out at 0–4 °C. The protein concentration was determined according to the colorimetric method of Bradford [31] by Bio-Rad Protein Assay kit II with bovine serum albumin (BSA) as standard. The mitochondrial preparations were then stored in liquid nitrogen until the evaluation of ATPase activities.

2.3. Assay of the Mg-ATPase activity

The thawed mitochondrial fractions were immediately used for the Mg-ATPase activity assays. The capability of enzymatic ATP hydrolysis was assayed in a reaction medium (1 mL) containing 75 mM ethanolammine–HCl buffer pH 8.9, 6.0 mM Na_2ATP, 2.0 mM $MgCl_2$ and 0.15 mg mitochondrial protein. After 5 min preincubation at 30 °C, the reaction, carried out at 30 °C, was started by the addition of the substrate ATP and stopped after 5 min by the addition of 1 mL of ice-cold 15% (w/w) aqueous solution trichloroacetic acid. Once the reaction was stopped, vials were centrifuged for 15 min at 5000 rpm (ALC 4225 Centrifuge). In the supernatant, the concentration of inorganic phosphate (P_i) hydrolyzed by known amounts of mitochondrial protein, which is an indirect measure of ATPase activity, was spectrophotometrically evaluated according to Fiske and Subbarow [32].

The F_1F_O-ATPase activity, here defined as oligomycin-sensitive Mg-ATPase activity (OS Mg-ATPase), was routinely measured by subtracting, from the inorganic phosphate (P_i) hydrolyzed by known amounts of mitochondrial protein (which indirectly indicates the total Mg-ATPase activity), the P_i hydrolyzed in the presence of 2 μg/mL oligomycin, defined as oligomycin-insensitive Mg-ATPase (OI Mg-ATPase). To this aim 1 μL from a mother solution of 2 mg/mL oligomycin in dimethylsulfoxide (DMSO) was directly added to the reaction mixture before starting the reaction. The total Mg-ATPase activity was calculated by detecting the P_i in control tubes run in parallel and containing 1 μL DMSO per mL reaction system. In each experimental set, control tubes were alternated to the condition to be tested. The employed dose of oligomycin, a specific inhibitor of F-ATPases which selectively blocks the F_O subunit [4] ensured maximal enzyme activity inhibition and was currently used in Mg-ATPase assays [33,34].

TBT effects were routinely tested by directly adding TBT solutions to the ATPase reaction media as in previous works [27,33,34].