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Thiol oxidation is crucial in the desensitization of the mitochondrial F_1F_O-ATPase to oligomycin and other macrolide antibiotics

Salvatore Nesci, Vittoria Ventrella, Fabiana Trombetti, Maurizio Pirini, Alessandra Pagliarani *

Department of Veterinary Medical Sciences, University of Bologna, via Tolara di Sopra 50, 40064 Ozzano Emilia, Bologna, Italy

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ABSTRACT

Background: The macrolide antibiotics oligomycin, venturicidin and bafilomycin, sharing the polyketide ring and differing in the deoxysugar moiety, are known to block the transmembrane ion channel of ion-pumping ATPases; oligomycins are selective inhibitors of mitochondrial ATP synthases.
Methods: The inhibition mechanism of macrolides was explored on swine heart mitochondrial F_1F_O-ATPase by kinetic analyses. The amphiphilic membrane toxicant tributyltin (TBT) and the thiol reducing agent dithioerythritol (DTE) were used to elucidate the nature of the macrolide–enzyme interaction.
Results: When individually tested, the macrolide antibiotics acted as uncompetitive inhibitors of the ATPase activity. Binary mixtures of macrolide inhibitors I_1 and I_2 pointed out a non-exclusive mechanism, indicating that each macrolide binds to its binding site on the enzyme. When co-present, the two macrolides acted synergistically in the formed quaternary complex (ESI_1I_2), thus mutually strengthening the enzyme inhibition. The enzyme inhibition by macrolides displaying a shared mechanism was dose-dependently reduced by TBT $\geq$ 1 μM. The TBT-driven enzyme desensitization was reversed by DTE.
Conclusions: The macrolides tested share uncompetitive inhibition mechanism by binding to a specific site in a common macrolide-binding region of F_O. The oxidation of highly conserved thiols in the ATP synthase *c*-ring of F_O weakens the interaction between the enzyme and the macrolides. The native macrolide-inhibited enzyme conformation can be restored by reducing crucial thiols oxidized by TBT.
General significance: The findings, by elucidating the macrolide inhibitory mechanism on F_O, indirectly cast light on the F_1F_O torque generation involving crucial amino acid residues and may address drug design and antimicrobial therapy.

1. Introduction

The mitochondrial F_1F_O-ATPase (EC 3.6.3.14) is a bi-powered enzymatic engine that exploits the transmembrane proton motive force Δp to clockwise rotate the membrane portion F_O, which channels H^+ and drives ATP synthesis from ADP and inorganic phosphate (P_i) by the hydrophilic catalytic sector F_1 through a torque generation mechanism. Conversely, through a counterclockwise rotation driven by ATP hydrolysis by F_1, the same enzyme complex converts the Gibbs free energy of hydrolysis in uphill proton transport through F_O, thus re-energizing mitochondria [1–3]. The bi-functional capability of the mitochondrial enzyme complex, a unique example of chemo-mechanical coupling in biological systems, is long known to be specifically inhibited by oligomycins (OLIGs) [4]. Accordingly, OLIG inhibition has been widely exploited to study the ATPase/synthase functionality in eukaryotic cells. Conversely OLIG, generally produced by *Streptomyces diastatochromogenes* and other *Streptomyces* species [5] as a mixture of structurally closely related compounds, is ineffective in bacterial F_1F_O-ATPase [6]. OLIG belongs to the polyketide class of macrolide antibiotics whose basic structure consists of polymers of ketide units, featured by a macrocyclic lactone ring bound to one or more deoxy-sugars. Some natural macrolides, such as apoptolidin and ossamycin produced by *Nocardiopsis* and various *Streptomyces* strains, are also known as potent inhibitors of F_O. Apparently, the macrolide ring confers the inhibition power, while the deoxysugar moiety is not crucial for ATPase inhibition [7]. After 50 years of studies, the OLIG binding site has been localized on F_O and precisely on the *c* subunits, which by forming a sort of cylindrical palisade constitutes the *c*-ring, a key element in the torque generation mechanism [2]. The *c*-subunit number, constant for a given species but variable among species, determines the *c*-ring size, in turn related to the bioenergetic efficiency, being small rings associated with low bioenergetic cost of ATP. This advantageous condition is apparently favored from an evolutionary standpoint [1,8,9]. Proton flux through the *c*-ring involves a carboxyl group which, approximately at the midpoint of each C-terminal α-helix, transfers protons by switching from the proton-locked conformation to the deprotonated open conformation [10]. From a close insight, OLIG, by binding to two adjacent *c*-subunits, would shield this carboxyl residue, block proton flux and inhibit the F_1F_O-ATPase/synthase activities [11]. The OLIG molecular arrangement

* Corresponding author. Tel.: +39 0512097017; fax: +39 0512097037.
E-mail address: alessandra.pagliarani@unibo.it (A. Pagliarani).

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in the *c*-ring is thought to be shared by other macrolide antibiotics such as venturicidin (VENT) and bafilomycin (BAF) and to constitute the molecular basis of the F_1F_O complex inhibition. VENT strongly inhibited H^+-translocation and both ATP synthesis and hydrolysis in mitochondrial and in bacterial F_1F_O-ATPases [6]. BAF selectively inhibited V-type ATPases by a similar mechanism to that of eukaryotic F_1F_O-ATPase inhibition by OLIG [12,13]. Since the *c*-rings in the F- and V-ATPases are thought to stem from a common ancestor [14], the possibility that the two enzyme rotary complexes contain a similar antibiotic binding site seems not remote [12].

Antibiotic drug resistance can be achieved by a variety of mechanisms. Accordingly, the events involved in the mitochondrial F_1F_O-ATPase sensitivity loss to natural compounds such as OLIG, can be related to structural changes, in turn involving mitochondrial bioenergetics [15]. Interactions of macrolide antibiotics with the F_1F_O complex are an emerging field, up to now poorly explored [11]. Accordingly, by investigating through a kinetic approach the mutual behavior of OLIG, VENT and BAF in the mitochondrial F_1F_O-ATPase inhibition and the mechanisms of enzyme desensitization to these macrolide inhibitors, we primarily aimed at casting light on the antibiotic binding sites within F_O and on the energy transduction mechanisms involved in ATP synthesis. However, the results can also provide useful information for drug design and to exploit natural compounds as new therapeutic agents.

2. Materials and methods

2.1. Chemicals

Na_2ATP, oligomycin (a mixture of oligomycins A, B and C, in the proportion 64:15:17 respectively), tri-*n*-butyl chloride (TBT), 1,4-dithioerythritol (DTE) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were of the highest purity available and obtained from Sigma–Aldrich (Milan, Italy). Venturicidin A and bafilomycin B_1 were of the highest purity available and obtained from Vinci-Biochem (Vinci, Italy). All other chemicals were reagent grade. Protein Assay kit II was purchased by Bio-Rad (Milan, Italy). Quartz double distilled water was used for all reagent solutions.

2.2. Preparation of the mitochondrial fractions

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) [16]. 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 [17] 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 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% (v/v) aqueous solution of 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 [18].

In all experiments the ATPase activity was calculated as μmoles Pi mg protein^{-1} h^{-1}. The data represent the mean ± SD (shown as vertical bars in the figures) of at least three replicates carried out on distinct tissue preparations.

2.4. Preincubation procedures

In selected experiments, to favor incorporation of the compounds within the mitochondrial membranes and avoid the direct interference between different reagents, enzymatic assays were carried out on mitochondria preincubated in vials for 30 min on ice with the compounds to be tested. Accordingly, the preincubation of mitochondria with selected TBT doses aimed at ensuring TBT incorporation within the membranes and at evaluating the effect of the thiol reagent dithioerythritol (DTE), ruling out a direct interaction between DTE and TBT. In detail, mitochondria were preincubated with 4 μL DMSO (control) or 4 μL of appropriate TBT solutions in DMSO to yield the final 35 μM TBT concentrations in the reaction system. To prevent possible chemical interactions between TBT and DTE, 100 μM DTE were added in the TBT-preincubated mitochondrial suspensions only when were acclimated at 30 °C. After this incubation time, the ATPase reaction was carried out as described in the previous section. The DTE concentrations were previously tested [19].

2.5. Kinetic analyses

Kinetic studies on the mutual interactions between two inhibitors (double inhibitors) of the same enzyme activity (mitochondrial ATPase) (Segel, 1975) were carried out to cast light on the possible binding site of macrolide antibiotics. In all kinetic analyses the enzyme specific activity was taken as the expression of the reaction rate (v).

The mechanism of the enzyme inhibition by OLIG, VENT and BAF was explored by the aid of the graphical methods of Dixon [20] and Cornish-Bowden [21] which complement one another.

A series of parallel lines at the different ATP concentrations tested is typical of uncompetitive inhibition. In this case, according to the Dixon equation:

$$\frac{1}{v} = \frac{1}{K_i' V_{max}}[I] + \frac{1}{V_{max}}\left(1 + \frac{K_m}{[S]}\right) \quad \text{(I)}$$

the slope corresponds to $1/K'iV_{max}$, where $K'i$ is the dissociation constant of the enzyme–inhibitor–substrate complex (*EIS*), and the intercept on the y axis corresponds to $(1 + K_m/[S])V_{max}{}^{-1}$.