Biochimica et Biophysica Acta, 766 (1984) 209–214
Elsevier

BBA 41532

DIFFERENTIAL INHIBITION OF F_0F_1-ATPase-CATALYSED REACTIONS IN BOVINE-HEART SUBMITOCHONDRIAL PARTICLES BY ORGANOTIN COMPOUNDS

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(Received November 11th, 1983)

Key words: F_0F_1-ATPase inhibition; Organotin; (Bovine heart)

Preincubation of coupled submitochondrial particles with low concentrations of triorganotin compounds results in complete inhibition of the oligomycin-sensitive ATPase activity without any significant effect on the rate of succinate-driven ATP synthesis. The residual ATP synthetic activity is inhibited by oligomycin and uncouplers. The differential inhibition of ATP synthesis and hydrolysis by the triorganotin compounds examined suggests that the two processes are not 'mirror images' of each other, but that they occur through different routes and that the F_1F_0-ATPase is at least bifunctional.

Introduction

Most of the current models for the mechanism of ATP synthesis in the mitochondrial membrane [1–4] are based on the assumption that ATP hydrolysis, catalysed by mitochondrial membrane preparations, occurs by a mechanism which is the exact reverse of that occurring during oxidative phosphorylation:

$$ADP + P_i \rightleftharpoons ATP + H_2O$$

Synthesis and hydrolysis of ATP by the F_0F_1-ATPase complex are assumed to be coupled to H^+ translocation through F_0 [1,2]. The general assumptions are based on the action of inhibitors such as dicyclohexylcarbodiimide, oligomycin and trialkyltin compounds which inhibit both ATP synthesis and ATP hydrolysis [5,6]. These inhibitors have been shown to exert their inhibitory effect by binding to the F_0 part of the F_0F_1-ATPase complex [5,7] and block H^+ translocation through it [8–10].

There are, however, several reports in the literature on the differential inhibition of ATP synthesis and hydrolysis in mitochondria [11–21]. Although explanations for the unidirectional inhibition of the ATP hydrolytic activity have been offered for inhibitors such as adenylimide diphosphate and the inhibitor protein of Pullman and Munroy [14–17], the effects of others, such as the covalent modifiers of F_1 [18–21], still await further explanation.

In this paper, we report on the differential inhibition of the ATP synthetic and hydrolytic reactions of bovine-heart submitochondria particles by triorganotin compounds and their differential reversal by 2,3-dimercaptopropanol. The implications of these findings for the mechanism of inhibition of these energy-linked reactions by triorganotin compounds are discussed.

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Abbreviations: F_0 and F_1, components of the mitochondrial ATPase system; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

Materials and Methods

The source of all the chemicals used in this study has been described previously [6]. Dibutylchloromethyltin chloride was synthesised by Dr. D.E. Griffiths and 2-[(dimethylamino)methyl]phenyldiethyltin bromide (Ve_{2283}) was a gift from Professor B. Beechey, Shell Research Ltd., Sittingbourne, Kent, U.K.

Bovine-heart mitochondria and phosphorylating submitochondrial particles were prepared as described by Griffiths et al. [6]. Protein was determined by the biuret method of Gornall et al. [24].

ATP synthesis was assayed in a glucose hexokinase trap system, containing 250 mM sucrose; 50 mM Hepes-KOH (pH 7.4); 20 mM glucose; 5 mM potassium phosphate; 2 mM magnesium sulphate; 2 mM ADP; 0.5 mM EDTA and 5 units of yeast hexokinase (Sigma type F300). ATP synthesis was measured as the appearance of glucose-6-phosphate as described previously [6]. ATP hydrolysis was measured by the method of Soper and Pedersen [25] in the phosphorylation buffer minus hexokinase, glucose and ADP, and by the release of inorganic phosphate [6]. Assay of ATP and succinate-driven transhydrogenase activities were as described by Beechey et al. [26].

Incubations with organotin compounds and dithiols

Ethanolic solutions of organotins were added to 1 ml aliquots of submitochondrial particles (10 mg/ml) to give a range of final organotin concentrations and incubated at 4°C for 30 min. Aliquots were then removed and assayed for succinate-driven oxidative phosphorylation (1 mg), ATP hydrolysis (20 μg) and ATP- and succinate-driven transhydrogenation (1 mg). The final concentration of ethanol was less than 1% (v/v). Dithiols were added as ethanolic solution (2,3-dimercaptopropanol) or aqueous solutions (dithiothreitol/dihydrolipoic acid) to the untreated or organotin-treated submitochondrial particles and incubated for 10 min prior to enzymic analysis.

The concentration of dithiols was measured using Ellman's method [27].

Results

The effect of increasing concentrations of organotin compounds on the rate of ATP synthesis and hydrolysis catalysed by submitochondrial particles is shown in Fig. 1. 2-[(Dimethylamino)methyl]phenyldiethyltin bromide, with an I_{50} value of 1.0 nmol/mg protein for oxidative phosphorylation (Table I), is the most potent of the organotin compounds examined. Dibutylchloromethyltin chloride with an I_{50} value of 5.4 nmol/mg protein for oxidative phosphorylation is similar in potency to the other trialkyltin compound examined (Table I). The oligomycin-sensitive ATPase activity was found to be totally inhibited at low concentrations of organotin compounds (2–3 nmol dibutylchloromethyltin chloride/mg protein) which did not significantly affect

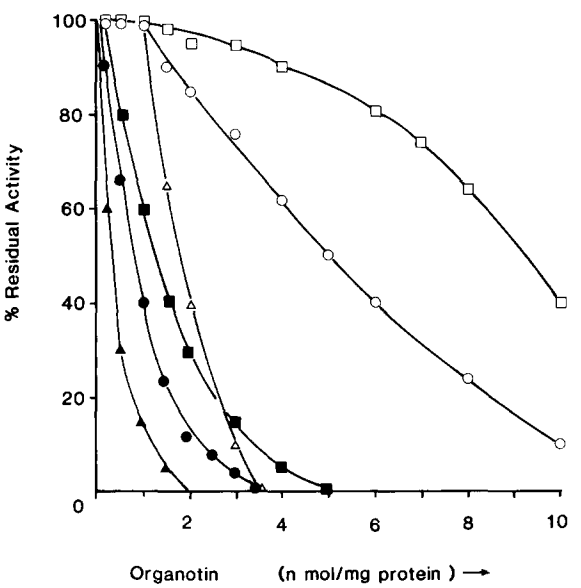

Fig. 1. Inhibition of ATP synthesis and hydrolysis in submitochondrial particles by organotin compounds. Incubation procedures and measurements of succinate-driven ATP synthesis and ATP hydrolysis were performed as described in Materials and Methods. The specific activities of the untreated submitochondrial particles were: ATPase, 2.4 ± μmol/min per mg protein; ATP synthase, 90 ± nmol/min per mg protein. The experimental data are expressed in the Figure as a percent of these original values. (●, ○) Dibutylchloromethyltin; (■, □) tributyltin chloride; ▲, △) 2-[(dimethylamino)methyl]phenyldiethyltin bromide. ATP synthesis (○, △, □); ATP hydrolysis (■, ▲, ●). Each datum point is the mean of duplicates.

TABLE I

SENSITIVITY OF ENERGY-LINK REACTIONS TO INHIBITION BY TRIORGANOTIN COMPOUNDS

Submitochondrial particles suspended at 10 mg protein/ml in 0.25 M sucrose/10 mM Tris-HCl (pH 7.5)/1 mM EDTA, were preincubated with varying concentrations of organotin at 4°C for 30 min. Aliquots were then removed and assayed for ATPase, ATP-dependent transhydrogenase and ATP synthase activities. The concentration of each inhibitor causing 50% inhibition of each reaction was estimated from Fig. 1. Values shown in brackets are the I_{50} values determined in the presence of succinate and TTFB and, in the absence of potassium cyanide; ATP hydrolysis having been followed by monitoring the release of inorganic phosphate. TBT, tributyltin chloride; DBCT, dibutylchloromethyltin chloride; Ve_{2283}, 2[(dimethylamino)methyl]phenyldiethyltin bromide; TTFB, 4,5,6,7-tetrachloro-2-(trifluoromethyl)benziimidazole.

Organotin compounds	Concentrations causing 50% inhibition of maximal activity (nmol/mg protein)		
	ATP synthase	ATP hydrolase	ATP-driven transhydrogenase
TBT	8.6±0.4	1.2 ±0.2 (1.0)	1.0±0.15
DBCT	5.4±0.5	0.8 ±0.12(0.7)	0.8±0.1
Ve_{2283}	1.0±0.2	0.12±0.02(0.1)	0.1

ATP synthesis (Fig. 1, Table II). However, the residual ATP synthetic activity (80–90%) was sensitive to inhibition by oligomycin and the uncoupler 4,6,6,7-tetrachloro-2-(trifluoromethyl)benziimidazole (Table II).

The organotin compounds are also potent inhibitors of the ATP-driven NADH/NADP-transhydrogenase in bovine-heart submitochondrial particles (Fig. 2) and the I_{50} values of this reaction are very similar to those obtained for ATP hydrolysis (Table I). However, all the organotin compounds were found to stimulate the succinate-driven transhydrogenase activity at low concentrations and inhibit it at high concentra-

TABLE II

DIFFERENTIAL INHIBITION OF ATP SYNTHESIS AND HYDROLYSIS BY DIBUTYLCHLOROMETHYLTIN CHLORIDE

Submitochondrial particles suspended at 10 mg protein/ml were preincubated with DBCT at 3.3 nmol/mg protein at 4°C for 30 min. Aliquots were then removed and assayed for ATP synthetic and hydrolytic activity. Oligomycin (2 μg/mg) and TTFB (2 μg) were directly added to the assay systems and incubated for 5 min before the reactions were initiated. The data presented are the means of quadruplicates. Abbreviations, see legend to Table I.

Additions	ATPase (mol/min per mg)	ATP synthetase (nmol/min per mg)
None	2.4 ±0.3	95.0±5.0
DBCT	0.1 ±0.2	90.0±6.0
DBCT + oligomycin	0.0 ±0.1	0.0
DBCT + TTFB	0.15±0.2	0.0

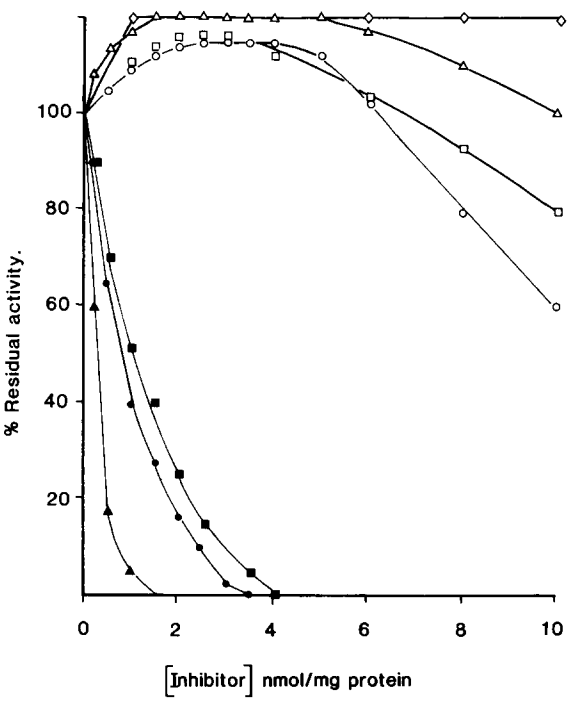

Fig. 2. The effect of organotin compounds on succinate- and ATP-driven transhydrogenase activity in submitochondrial particles. Incubation procedure and the measurement of ATP- and succinate-driven transhydrogenase activities were performed as described in Materials and Methods. The specific activities of the untreated submitochondrial particles were 42.8±3.6 and 60±5 nmol NADPH formed/min per mg protein for the ATP- and succinate-driven reactions, respectively. Experimental data have been expressed as percent of these original values. The non-energy-linked transhydrogenase activity was 4±1 nmol/min per mg. (●, ○) Dibutylchloromethyltin chloride; (▲, △) 2-[(dimethylamino)methyl]phenyldiethyltin bromide; (■, □) tributyltin chloride; (◇) oligomycin. Succinate-driven (□, △, ○, ◇); ATP-driven (■, ●, ▲) transhydrogenase activity.

TABLE III

DITHIOL REVERSAL OF DIBUTYLCHLOROMETHYLTIN CHLORIDE-INHIBITED ENERGY-LINKED REACTIONS IN SUBMITOCHONDRIAL PARTICLES

Submitochondrial particles suspended at 10 mg/ml were preincubated with DBCT (15 nmol/mg). The submitochondrial particles were then incubated with 2,3-dimercaptopropnal (BAL) at 400 nmol/mg for 30 min at 4°C. Aliquots were then removed and assayed for ATP synthesis, ATP hydrolysis and ATP-driven transhydrogenase activities. in the presence and absence of oligomycin or TTFB. The latter inhibitors (2 μg/mg) were added as methanolic solutions directly to the assay system. Each value is the mean ± S.D. of triplicates. Abbreviations, see legend to Table I.

Additions	ATP synthetase (nmol/min per mg)	ATPase (mol/min per mg)	Transhydrogenase (nmol/min per mg)
None	90.0 ± 5	2.4 ± 0.2	42.5 ± 2.0
DBCT	12.0 ± 3.2	0.0	0.0
DBCT + BAL	92.0 ± 6.0	1.44	43.0 ± 1.5
DBCT + BAL + TTFB	0.0	1.5 ± 0.3	0.0
DBCT + BAL + oligomycin	0.0	0.0	0.0

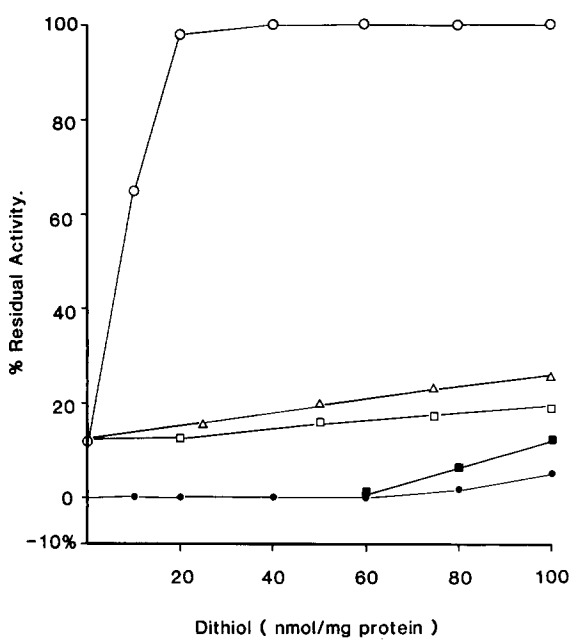

Fig. 3. The differential reversal of dibutylchloromethyltin chloride inhibition of ATP synthesis and hydrolysis by dithiols. Submitochondrial particles (10 mg/ml) pretreated with dibutylchloromethyltin chloride (15 nmol/mg) were preincubated with varying concentrations of 2,3-dimercaptopropanol (○, ●, ■), dihydrolipoic acid (△), and dithiothreitol (□), for 10 min at 4°C. Aliquots were then removed and assayed for ATP synthetic (○, △, □), ATP hydrolytic (●) and ATP-driven transhydrogenase (■) activities. The specific activities of the untreated submitochondrial particles were: ATPase, 2.4 ± 0.2 μmol/min per mg; ATP synthase, 90 ± 5.0 nmol/min per mg; and ATP-driven transhydrogenase 42.5 ± 2.0 nmol/min per mg. The experimental data are expressed in the figure as percent of these original values. Each datum point represents the mean of duplicates.

tions (Fig. 2). Increased succinate-driven transhydrogenase activity was also obtained when oligomycin was used as the inhibitor, but no inhibition was found at high concentrations of oligomycin.

The inhibition of oxidative phosphorylation by tributyltin chloride and dibutylchloromethyltin chloride was found to be readily reversible by 2,3-dimercaptopropanol, but not by dithiothreitol, β-mercaptoethanol or cysteine (Table III). Addition of 2,3-dimercaptopropanol equivalent to 2 molar proportion of dibutylchloromethyltin chloride was found to cause total reversal of the ATP synthetic activity without any significant reversal of the ATP hydrolytic or ATP-driven transhydrogenase activity (Fig. 3). However, the ATPase- and ATP-driven transhydrogenase activities could be significantly reversed by 30–50-times the concentration of the dithiol used to reverse ATP synthesis (Table III). The dithiol-reversed inhibition of ATP synthesis, ATP-driven transhydrogenase activity and ATP hydrolysis were sensitive to inhibition by oligomycin. Pretreatment of the submitochondrial particles with low concentration of 2,3-dimercaptopropanol was found to prevent inhibition of oxidative phosphorylation by tributyltin chloride and dibutylchloromethyltin chloride.

Discussion

Organotins have been shown to act as F_0F_1-ATPase inhibitors and uncouplers of oxidative

phosphorylation [6,29–35]. The results presented (Figs. 1 and 2) are not inconsistent with these findings [6,24–35] and indicate that the two modes of inhibition are dependent upon the concentration of organotin. The oligomycin-like action of these inhibitors at low concentrations is a result of the strong affinity of the F_0F_1-ATPase for the organotins ($K_d = 3 \cdot 10^{-7}$ M for dibutylchloromethyltin chloride; Ref. 30), and is responsible for the inhibition of ATP hydrolysis, ATP-driven transhydrogenation and the stimulation of succinate-driven transhydrogenase activity. Stimulation of the latter (Fig. 2) is possibly due to the inhibition of proton leakage through F_0 [9,28]. However, at higher concentrations of organotin, the uncoupler-like action of these inhibitors, possibly caused by their ability to catalyse transmembrane chloride/hydroxyl exchange [35], results in the inhibition of ATP synthesis and succinate-driven transhydrogenation (Figs. 1 and 2). The ready reversal of ATP synthesis by dithiols (Fig. 3) is possibly caused by the inhibition of chloride/hydroxyl exchange, through the formation of penta-coordinated complex between the organotins and the dithiols [31–34]. The differential reversal of ATP synthesis and hydrolysis thus lends support to the proposal that ATP synthesis and hydrolysis are inhibited by two different mechanisms and that intramembrane dithiols might play a part in the mechanism of inhibition of the F_0F_1-ATPase [6,28,29].

Although the differential inhibition obtained could be due to a peculiarity of the assay system, we feel that this is unlikely, since the membrane particles were treated under identical conditions and the systems used to assay ATP synthesis and hydrolysis were very similar. Support for this view was obtained from the findings that the presence of succinate and glucose did not affect the I_{50} values for the ATP hydrolytic reaction (Solani, G., unpublished results) neither did removal of the ATPase inhibitor protein (Emanuel, E., unpublished results). Furthermore, when the ATPase activity was followed by the release of inorganic phosphate [6], the presence of succinate in the absence of potassium cyanide did not affect the I_{50} value for ATP hydrolysis (Table I).

Since organotins are non-covalent F_0 inhibitors, the unidirectional inhibition of the ATPase activity obtained is best explained by the induction of a conformational change in the F_1-ATPase, which results in either the stabilisation of the phosphorylation conformation according to the alternating site model [36,37] or the inhibition of ATP hydrolysis at a site which is intrinsically different from that for ATP synthesis as in the separate-site model [12,13]. However, it is difficult to rationalise our present results in terms of the alternating-site model, since inhibition of ATP hydrolysis should lead to inhibition of ATP synthesis, if these reactions occur by simple dynamic reversal. The differential inhibition obtained is consistent with a model in which separate sites, exist on the F_1-ATPase for ATP synthesis and hydrolysis.

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