STUDIES ON ENERGY-LINKED REACTIONS: INHIBITION OF OXIDATIVE PHOSPHORYLATION AND ENERGY-LINKED REACTIONS BY DIBUTYLTIN DICHLORIDE

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1. Introduction

Recent studies in this laboratory have demonstrated that a new trialkyltin analogue, dibutylchloromethyltin chloride (DBCT), inhibits oxidative phosphorylation in yeast mitochondria by reacting covalently with a non-protein lipid cofactor associated with the mitochondrial inner membrane and the ATP synthase (oligomycin-sensitive ATPase) complex and which has been provisionally identified by thin-layer chromatography (t.l.c.) and bioassay as lipoic acid [1]. In addition dihydrolipoate will specifically reverse the inhibitory effects of DBCT [1]. Other dithiols including dihydrolipoamide are ineffective, indicating that reversal of inhibition is due to replacement of the DBCT-titrated cofactor by dihydrolipoate with consequent restoration of ATP synthesis and ATP-driven energy-linked reactions.

During the course of these studies it was observed that dibutyltin dichloride (DBT), the precursor used for DBCT synthesis, was also a potent inhibitor of the yeast oligomycin-sensitive ATPase [1]. Previous studies of Aldridge and Cremer [2] had indicated that the inhibition of oxidative phosphorylation by dialkyltin compounds was due to the inhibitor reacting with the dihydrolipoate cofactor in the pyruvate and α-ketoglutarate dehydrogenase complexes. This paper describes studies which demonstrate that DBT not only titrates the dihydrolipoate found in the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complexes involved in substrate level phosphorylation, but also the dihydrolipoate cofactor pool found in the membrane-bound oligomycin-sensitive ATPase complex with a consequent inhibition of oxidative phosphorylation and ATP-driven energy-linked reactions. These findings also demonstrate that dialkyltin compounds are very useful dithiol reagents which function at low concentrations and do not suffer from the permeability problems associated with arsenite, the classical dithiol reagent.

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2. Materials and methods

The sources of all the chemicals used in this study have been described previously [1,3]. Dibutyltin dichloride was further purified by fractional distillation as described previously [1].

Ox heart mitochondria (BHM) and submitochondrial particles (BHSMP) were prepared as described by Griffiths et al. [3]. Mitochondrial respiration, ATP-driven transhydrogenase, ATP-driven reduction of NAD^+ by succinate, oxidative phosphorylation and ATPase activity were measured as described previously [3]. Protein and P_i determination were as described by Griffiths et al. [3].

The reaction of DBT with SH compounds was studied essentially as described by Aldridge and Cremer [2] using the dye 2,6-dichlorophenolindophenol (DCPIP) which is decolorised by thiols. The various thiol compounds were incubated in 2 ml 20 mM Tris/HCl, pH 7.5, buffer with and without the

inhibitor for 5 min at 18–20°C before adding 0.5 ml buffer containing 40 μg DCPIP. The rate of decolorisation of the dye was followed at 620 nm in a Unicam SP 1800 spectrophotometer. R is the ratio of inhibitor to –SH compound and T_{50} the time in seconds for 50% decolorisation of the dye.

3. Results

Figure 1a shows that DBT inhibits ADP stimulated respiration of mitochondria utilizing pyruvate + malate as substrates (I_{50} value approx. 3 nmol/mg protein), but unlike DBCT and other trialkyltin compounds, uncoupler stimulated respiration is also inhibited. A similar result was obtained using α-ketoglutarate as a substrate. However, using succinate as a substrate (fig.1b), a different result is obtained in that even when using an excess of DBT (31 nmol/mg protein) only the ADP-stimulated respiration is inhibited and no effect is seen on the uncoupler-stimulated respiration. This finding indicates that DBT has a mode of action similar to the classical inhibitors of oxidative phosphorylation, oligomycin and the trialkyltin compounds. Therefore DBT has two inhibitory sites of action on mitochondrial respiration:

(i) As demonstrated previously [2], an effect on the α-ketoglutarate and pyruvate dehydrogenase complexes leading to the inhibition of pyruvate and α-ketoglutarate oxidation.

(ii) An inhibition of the ADP-stimulated respiration which does not involve a direct action on the respiratory chain and is analogous to the energy-transfer inhibitors, oligomycin and the trialkyltin compounds.

These findings suggest that DBT may have a direct effect on the ATP synthase complex. This conclusion was examined by studying ATP synthesis in submitochondrial particles. ATP synthesis using NADH, succinate and ascorbate + *NNN'N'-tetra*-methyl-*p*-phenylenediamine as substrate is inhibited by DBT (I_{50} value approx. 6 nmol/mg protein) demonstrating that ATP synthesis utilising all three segments of the respiratory chain is inhibited and indicating that there is a common site of action, i.e., the energy-transfer reaction in the ATP synthase complex.

This conclusion is supported by the experiments shown in fig.2. which show that DBT is also a potent inhibitor of the ATP-driven reduction of NAD^+ by succinate (I_{50} value 2.3 nmol/mg protein). The inhibitory effects are unusual in that a 'lag phase' is produced which is proportional to the amount of inhibitor added. These findings are similar to those found for DBCT [4]. Figure 2 also shows that the ATP-driven transhydrogenase reaction is also inhibited by DBCT (I_{50} value 6.3 nmol/mg protein). In contrast

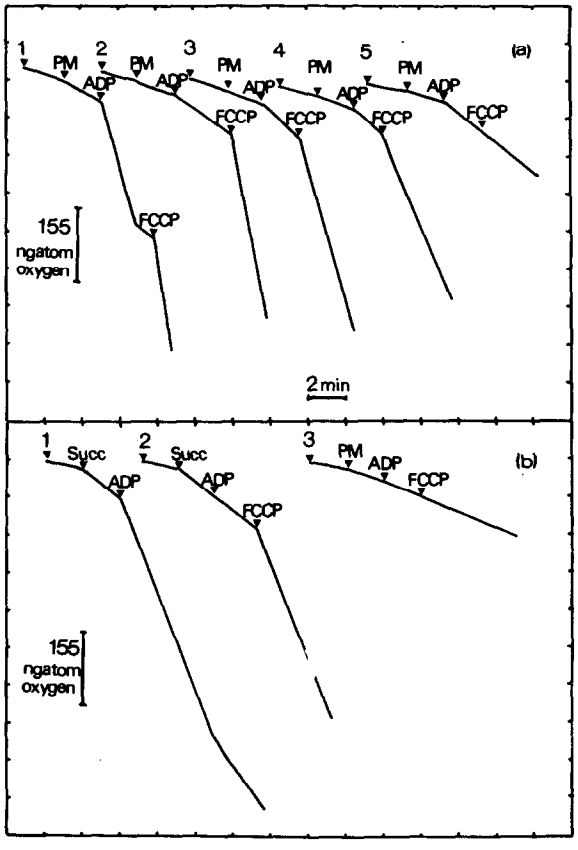

Fig.1. Inhibition of mitochondrial respiration by DBT. Mitochondrial respiration was recorded as described by Griffiths et al. [3]. Inhibitors were added as indicated in maximum vol. 5 μl ethanol. (a) The reaction medium contained 2.25 mg BHM to which the following additions were made: PM, 10 μmol pyruvate + 1 μmol malate; ADP, 900 nmol; FCCP, 10 nmol. In addition, 5 μl ethanol was added at (1), control; 6.3 nmol of DBCT at (2); 3.3, 6.6 and 13.2 nmol DBT added at (3), (4), (5). (b) The following additions were made to the reaction medium which contained 1.6 mg BHM; Succ, 10 μmol succinate; PM, 10 μmol pyruvate + 1 μmol malate; ADP, 600 nmol; FCCP, 10 nmol. In addition 5 μl ethanol was added at (1); 70 nmol DBT at (2), (3).

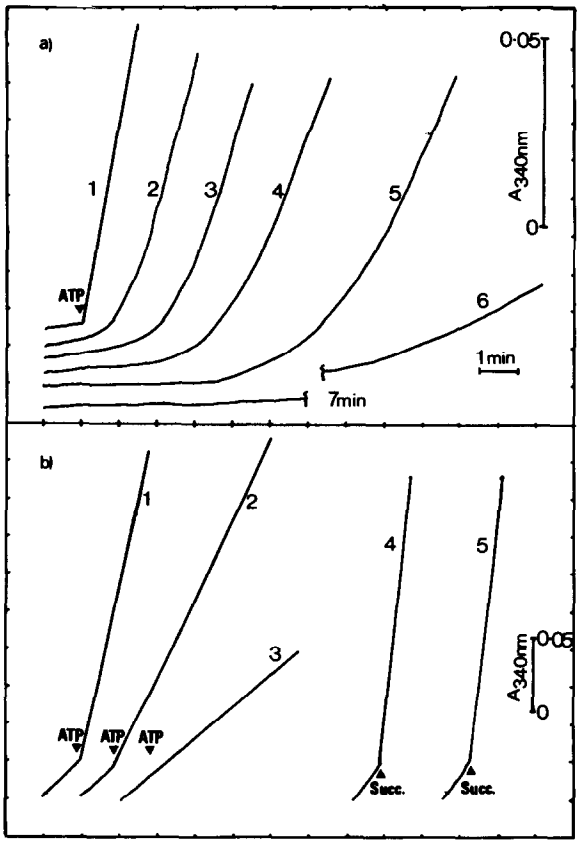

Fig.2. Inhibition by DBT of the ATP-driven reduction of NAD^+ by succinate. (a) The ATP-driven reduction of NAD^+ by succinate was assayed as described by Griffiths et al. [3] with 1 mg BHSMP. The following additions were made: (1) no addition; (2) 0.52 nmol DBT; (3) 1.24 nmol DBT; (4) 1.8 nmol DBT; (5) 3.1 nmol DBT; (6) 12.5 nmol DBT. (b) ATP-driven and succinate (Succ.)-driven transhydrogenase were assayed as previously described [3] with 1 mg BHSMP. The following additions were carried out: (1) no additions; (2) 8.3 nmol DBT; (3) 16.4 nmol DBT; (4) no additions; (5) 18.5 nmol DBT.

the succinate-driven (i.e., respiratory chain-driven) transhydrogenase reaction is unaffected by DBT even at a concentration of 13.5 nmol/mg protein.

These results clearly show that DBT is acting on the ATP synthase (oligomycin-sensitive ATPase) complex and a direct assay of ATPase activity in BHSMP revealed that DBT was a potent inhibitor (I_{50} value 2.8 nmol/mg protein). However, DBT had no effect on the purified heart F_1-ATPase [7] even at a concentration of 82 nmol/mg protein, demonstrating that the effect of DBT is on the membrane components of the oligomycin-sensitive ATPase complex. This result is supported by the finding that the purified oligomycin-sensitive ATPase preparation from yeast mitochondria is also sensitive to DBT (Cain, K. and Griffiths, D. E., unpublished results).

These studies clearly show that DBT has a mode of action which is similar to DBCT and is a direct inhibitory effect on the ATPsynthase (oligomycin-sensitive ATPase) complex resulting in the inhibition of ADP-stimulated respiration, oxidative phosphorylation, ATP-driven energy-linked reactions and ATPase activity. In the case of DBCT, the inhibitory actions are specifically reversible by dihydrolipoate due to the dithiol compound apparently replacing a cofactor which has been titrated by the DBCT. The identity of the cofactor has been provisionally characterised as lipoic acid.

Consequently, we have investigated the effects of dithiol compounds on the inhibitory actions of DBT on ATP synthesis and ATPase activity (tables 1 and 2). The inhibitory effects can be reversed by a variety of dithiol compounds including dihydrolipoate, and even the monothiol glutathione. In addition lipoate or lipoamide, under appropriate preincubation conditions, will also produce some reversal of the inhibitory effects. presumably due to the oxidised compound being slowly reduced by the respiratory chain. Table 3 shows that DBT rapidly reacts with dithiols but not with monothiols such as glutathione. The reversal of the inhibitory actions of DBT by a variety of dithiol compounds is probably a 'scavenging' effect resulting from a displacement of the cofactor from the DBT–cofactor complex. by dithiol interchange reactions analogous to those involved in reversal of arsenite inhibition of dithiols [8].

4. Discussion

The original work by Aldridge and Cremer [2] demonstrated that dialkyltin compounds inhibit the α-ketoglutarate and pyruvate dehydrogenase complexes by reacting with the protein-bound dihydrolipoate cofactor; the inhibitory effects of the dialkyltins could be reversed by the addition of dithiols. The results presented here for DBT confirm these con-

Table 1
Reversal of DBT-inhibited oxidative phosphorylation by various thiol compounds

Additions	ATP synthesised (μmol/20 min)	Activity (% control)
No DBT (control)	2.83	100
DBT	1.1	39
DBT + reduced glutathione (20)	2.7	95
DBT + dihydrolipoate (20)	2.65	94
DBT + dithiothreitol (20)	2.8	99
DBT + 2,3-dimercaptopropanol (20)	2.83	100
DBT + dihydrolipoamide (20)	2.60	92
DBT + lipoate (20)	1.1	41
DBT + lipoamide (20)	1.1	39

BHSMP were preincubated in 0.25 M sucrose, 20 mM Tris–HCl, pH 7.5, 1 mM EDTA buffer at 10 mg/ml with DBT (6 nmol/mg protein). 100 μl (1 mg) was removed and added to 1 ml phosphorylation buffer and then shaken for 5 min with the indicated additions at 30°C. The phosphorylation assay was then started by the addition of 10 μmol succinate and then carried out as described by Griffiths et al. [3]. The figures in brackets refer to nmol indicated compound/mg protein

Table 2
Reversal of DBT-inhibited ATPase activity by various thiol compounds

Additions	μnol P_i/min/mg	Activity (% Control)
No DBT (control)	2.5	100
DBT	0.75	30
DBT dihydrolipoate (10)	2.35	94.4
DBT + reduced glutathione (10)	2.16	86.7
DBT + dithiothreitol (20)	2.39	95.9
DBT + 2,3-dimercaptopropanol (20)	2.47	99
DBT + lipoate (20)	0.75	30
DBT + dihydrolipoamide (20)	2.41	97

ATPase was assayed at 30°C by release of P_i [3]. BHSMP were preincubated with DBT (4.9 nmol/mg protein) at 10 mg/ml for 30 min at 4°C. A 10 μl aliquot was then removed to the ATPase assay and the indicated thiol compound added, followed immediately by ATP. Figures in brackets refer to the final concentration (nmol) in 1 ml ATPase buffer

Table 3
Reaction of DBT with various thiol compounds

SH compound	SH compound (μM)	DBT (μM)	R	T_{50} (s)	
				− DBT	+ DBT
Dihydrolipoate	250	58	0.23	15	60
	250	87	0.34	15	410
	250	145	0.6	15	not reached after 600
Dithiothreitol	81	109	1.34	24	84
	81	182	2.24	24	120
Glutathione	163	364	2.23	120	100
Dihydrolipoamide	77	58	0.75	42	7% in 360
2,3-Dimercaptopropanol	119	36.5	0.3	12	24
	119	72.7	0.61	12	60
	119	218	1.83	12	3% in 360

The reaction of the inhibitor with SH groups was determined by the method of Aldridge and Cremer (1955), using the dye 2,6-dichlorophenolindophenol which is decolourised by SH groups. The indicated compounds were incubated in 2 ml 20 mM Tris–HCl, pH 7.5, buffer with and without the inhibitor for 15 min at 18–20°C before adding 0.5 ml buffer containing 40 μg 2,6-dichlorophenolindophenol. The rate of decolourisation of the dye was followed at 620 nm in a Unicam SP 1800 spectrophotometer

clusions; thus uncoupler-stimulated respiration in BHM is inhibited when pyruvate + malate and α-ketoglutarate are used as substrates. However, the results with mitochondrial respiration utilising succinate clearly show that there is another site of action involving energy-transfer reactions in the ATP synthase complex. This is confirmed by the inhibition of ATP-driven energy-linked reactions and mitochondrial ATPase activity which show that the ATP synthase complex is inhibited by DBT. The reversal of the inhibitory effects of DBT on the ATP synthase complex by dithiol compounds demonstrates that DBT inhibition of ATP-dependent reactions also involves a dithiol residue, presumably the lipoate cofactor pool present in the inner mitochondrial membrane and the ATP synthase complex.

The inhibitory effects of DBT on the ATP synthase complex are similar to those described for DBCT [1,4–6] which has been shown to inhibit ADP-stimulated respiration, ATP synthesis, ATP-driven energy-linked reactions and ATPase activity by titrating a lipoic acid cofactor in the inner mitochondrial membrane and ATP synthase complex. The concentration of this cofactor is around 6–9 nmol/mg protein, therefore the inhibitory effects of DBT which occur at I_{50} values around 3–6 nmol/mg protein and maximal inhibition approx. 10–15 nmol/mg protein are produced by concentrations of the inhibitor which would be expected to titrate the lipoic acid cofactor 'pool'. Titration of this 'pool' with DBCT results in inhibition which can only be reversed by the replacement of the titrated cofactor with dihydrolipoate. In the case of DBT a variety of dithiols are effective and the mechanism of reversal is probably a 'scavenging' action due to a displacement of the cofactor from the DBT–cofactor complex by the dithiol reagent.

These experiments further reinforce the previous conclusions [1] that DBCT is a unique inhibitor for titrating the lipoic acid cofactor present in the mitochondrial inner membrane and that DBT is also an extremely useful dithiol reagent with a similar mode of action but lacks the specificity exhibited by DBCT. As DBT is a lipophilic inhibitor and freely permeable to membranes, it is a useful dithiol reagent for investigating membrane-bound enzymes and offers experimental advantages over arsenite and the arsenical-type dithiol reagents.

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