DITHIOL-SPECIFIC REVERSAL OF TRIPHENYLTIN INHIBITION OF CF_0-CATALYZED TRANSMEMBRANE PROTON TRANSFER IN CHLOROPLASTS

J. Michael GOULD

Program in Biochemistry and Biophysics, Department of Chemistry, University of Notre Dame, Notre Dame, IN 46556, USA

Received 20 June 1978

1. Introduction

The chloroplast transmembrane CF_1–CF_0 complex catalyzes the synthesis of ATP in a reaction coupled to the net movement of protons from the inside of the thylakoid vesicle to the outer (medium) phase:

$$ADP + P_i + nH^+_{in} \rightleftharpoons ATP + H_2O + nH^+_{out}$$

The catalytic site for the ATP synthetase and ATPase reactions per se is located within the CF_1 portion of the complex, whereas the tightly membrane-bound CF_0 portion of the complex is thought to function in the transfer of the charged internal proton(s) across the hydrophobic membrane to CF_1. That is, CF_0 appears to act as an intrinsic membrane proton ionophore whose activity is regulated (gated) by CF_1 (reviewed [1]).

The movement of hydrogen ions through the CF_0 pathway can be blocked by certain inhibitors of photophosphorylation; notably dicyclohexylcarbodiimide (DCCD) [2] and trialkyl- and triaryltin halides (e.g., triphenyltin chloride) [3]. Although R_3SnX compounds exhibit a generally low reactivity with biological materials [4], triphenyltin chloride has been found to be an extremely potent and stoichiometric inhibitor of proton transfer through CF_0, with 50% inhibition of ATP synthesis in chloroplasts occurring at triphenyltin/chlorophyll ratios of about 1/50 [3].

At the moment little is known about the mechanism by which protons are conducted through CF_0 and across the thylakoid membrane to CF_1. There is also very little information concerning the mechanism by which this process is disrupted by inhibitors such as DCCD and triphenyltin. However, there is some evidence suggesting that, in vivo, the regulation of CF_0 proton conduction by CF_1 involves one or more critical SH residues within CF_1 [5–7], possibly located in the γ subunit of the enzyme [8,9]. The data presented here suggest the possibility that SH residues within the CF_0 portion of the complex may also play a critical role in the mechanism of transmembrane proton transport by this component of the ATP-synthesizing complex.

Abbreviations: DCCD, dicyclohexylcarbodiimide; HEPPS, *N*-2-hydroxyethylpiperazine-*N'*-propanesulfonic acid; DTT, dithiothreitol; DLA, dihydrolipoic acid (reduced DL-6,8-thioctic acid)

2. Experimental methods

Chloroplasts (intact, naked lamellae) were isolated from leaves of fresh market spinach as in [3]. CF_1-depleted chloroplasts were prepared as follows: chloroplasts were washed once in 10 mM NaCl, 10 mM HEPPS–NaOH (pH 7.5) and resuspended to 0.1 mg chlorophyll/ml in 0.75 mM EDTA, 5 mM HEPPS–NaOH (pH 7.5) for 15 min at 0°C. The treated chloroplasts were sedimented and taken up in a small volume of suspension medium (0.1 M sucrose, 5 mM HEPPS–NaOH (pH 7.8), 2 mM $MgCl_2$).

Photophosphorylation was determined from the rate of H^+ consumption according to the equation:

$$Mg\text{-}ADP^{1-} + P_i^{2-} + nH^+ \rightarrow Mg\text{-}ATP^{2-} + H_2O$$

Elsevier/North-Holland Biomedical Press

where $n = 0.96$ at pH 8 in the presence of Mg^{2+} [10]. Light-induced pH changes (whether due to ATP synthesis or proton accumulation) were detected with a miniature combination pH electrode connected to a Radiometer 26 pH meter and a strip-chart recorder. The chart was calibrated in H^+ equivalents by titrating the sample in the light with 0.001 M HCl. The overall half-response time for the pH measuring system was ~1 s. Actinic illumination (> 560 nm) was supplied by a 750 W projector lamp focused through a 250 ml round-bottomed flask containing ~2% $CuSO_4$ solution as an infrared filter. Reactions were run in a thermostated, 2 ml reaction vessel at 18°C.

Triphenyltin chloride (Alpha Inorganics) was recrystallized twice from ethanol before use.

3. Results

As reported, triphenyltin chloride is a potent inhibitor of photophosphorylation in chloroplasts (fig.1A). The amount of triphenyltin necessary to cause a given level of inhibition is stoichiometrically dependent upon the chloroplast concentration, with about 1 molecule triphenyltin/50 molecules chlorophyll required for 50% inhibition [3]. The inhibition of ATP formation by 1.5 μM triphenyltin in fig.1A could be effectively reversed, however, by the subsequent addition of small amounts of certain dithiol compounds, such as dithiothreitol (DTT; fig.1B). Further addition of triphenyltin (to final 4.5 μM), even in the presence of 15 μM DTT, restored the inhibited state. Similar results were also obtained using dihydrolipoic acid (table 1).

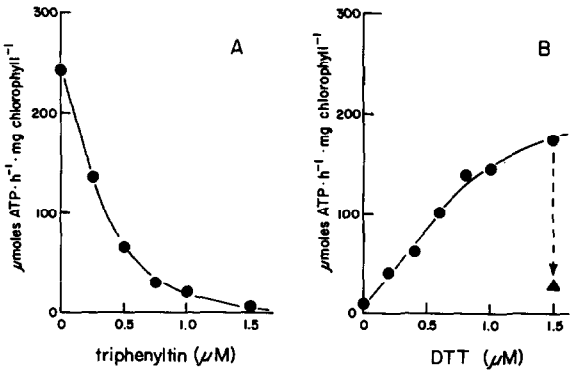

Fig.1. (A) Inhibition of ATP formation in chloroplasts by triphenyltin chloride. The reaction mixture (2.0 ml) contained 0.1 M sucrose, 2 mM $MgCl_2$, 1 mM ADP, 5 mM Na_2HPO_4, 100 μM methylviologen, 1 mM tricine–NaOH (pH 8.0) and chloroplasts equivalent to 28 μg chlorophyll/ml. (B) Reversal of triphenyltin–inhibited ATP formation in chloroplasts by dithiothreitol (DTT). The reaction mixture was as in (A), except that 1.5 μM triphenyltin was also present. The triangle represents the rate of ATP formation obtained in the presence of 15 mM DTT when triphenyltin was raised to 4.5 μM.

Removal of CF_1 from the thylakoid membrane results in a large increase in transmembrane proton permeability, and the familiar light-induced pH rise associated with net proton uptake by the chloroplasts is abolished [11]. We have previously shown ([3] and fig.2A) that triphenyltin chloride will effectively restore net proton uptake to CF_1-depleted chloroplasts by blocking the ungated flux of protons through the CF_0 channel. Again this effect by triphenyltin can be effectively reversed by the subsequent addition of dithiol compounds such as DTT or DLA (fig.2B). Further addition of triphenyltin overcomes the reversing effect of the dithiol, however. Interestingly, monothiols such as β-mercaptoethanol and oxidized dithiols (disulfides) such as lipoic acid and lipoic acid amide are very much less effective in reversing the effects of triphenyltin, requiring ~100-fold higher concentrations than the reduced dithiols tested (fig.3).

Trialkyl- and triaryltin compounds have also been

Table 1
Reversal of triphenyltin chloride-inhibited photophosphorylation by dihydrolipoic acid (DLA)

Additions	ATP formation (μmol ATP · h^{-1} · mg $chlorophyll^{-1}$)
1.5 μM triphenyltin	0
1.5 μM triphenyltin + 2.5 μM DLA	29
1.5 μM triphenyltin + 12.5 μM DLA	109
3.0 μM triphenyltin + 12.5 μM DLA	31

Reaction conditions were essentially as in fig.1b legend

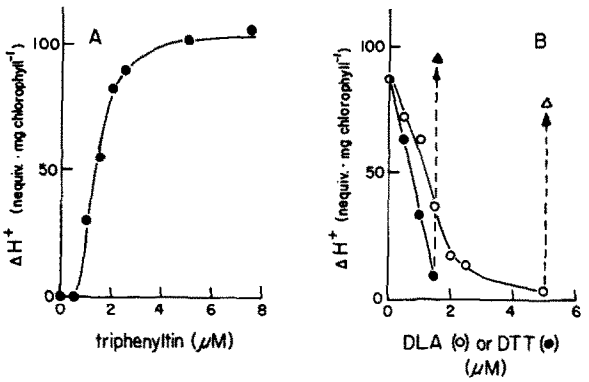

Fig.2. (A) Restoration of light-induced proton uptake in CF_1-depleted chloroplasts by triphenyltin. CF_1-depleted chloroplasts were prepared as in section 2. The reaction mixture (2.0 ml) contained 0.1 M sucrose, 25 mM NaCl, 2 mM $MgCl_2$, 100 μM methylviologen, 0.5 mM HEPPS–NaOH (pH 8.1) and chloroplasts equivalent to 48 μg chlorophyll/ml. (B) Reversal of triphenyltin-reconstituted proton uptake in CF_1-depleted chloroplasts by dihydrolipoic acid (DLA) and dithiothreitol (DTT). The reaction mixture was as in 2 (A) except 3 μM triphenyltin was also present. The triangles represent the extent of proton uptake when triphenyltin was raised to 6 μM.

reported to react with amines to form stable complexes [12,13], and the formation of a tin–nitrogen linkage between the imidazole nitrogens of adjacent, paired histidine residues has been postulated as the mechanism of triethyltin binding to rat hemeoglobin

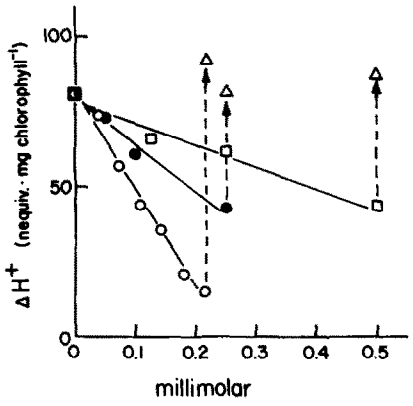

Fig.3. Reversal of triphenyltin-reconstituted proton uptake in CF_1-depleted chloroplasts by β-mercaptoethanol (○), lipoic acid (●), and lipoic acid amide (□). Reaction conditions were as in fig.2b legend.

[14]. However, addition of histidine in large (16-fold) excess over the triphenyltin concentration had no detectable reversing effect upon the triphenyltin-restored proton uptake reaction in CF_1-depleted chloroplasts (table 2).

Finally, it was found that despite the stoichiometric nature of triphenyltin's inhibitory actions, the interaction of triphenyltin with CF_0 is apparently not covalent, since the restoration of the proton uptake reaction in CF_1-depleted chloroplasts by triphenyltin could be reversed by repeated washings of the chloroplasts (table 3).

4. Discussion

The mechanism by which CF_0 catalyzes the net movement of protons across the thylakoid membrane is largely unknown. Inhibitors of CF_0 function such as DCCD and triphenyltin can provide insight into the molecular requirements for normal CF_0 activity, however, provided sufficient information about the mechanism of the inhibition can be obtained. For example, it has been found that DCCD is linked covalently to a low molecular weight proteolipid component within CF_0, although the precise role of this proteolipid remains to be established [15].

Triphenyltin is ~100-times more potent than DCCD as an inhibitor of CF_0, the level of inhibition

Table 2
Lack of reversal by histidine of triphenyltin-stimulated net proton uptake in CF_1-depleted chloroplasts

Additions	Proton uptake (nequiv. H^+/mg chlorophyll)
None	12
5 μM triphenyltin	262
5 μM triphenyltin + 40 μM histidine	265
5 μM triphenyltin + 80 μM histidine	273

Reaction conditions were as in section 2 and table 3 legend. The reaction mixture (pH 8.1) contained CF_1-depleted chloroplasts equivalent to 75 μg chlorophyll/2 ml

Table 3
Reversal of triphenyltin-stimulated net H^+ uptake in CF_1-depleted chloroplasts by washing

Chloroplasts	Additions	Proton uptake (μequiv. H^+/mg chlorophyll)
Untreated (control)	none	1.63
	5 μM triphenyltin	3.07
CF_1-depleted	none	0.58
	5 μM triphenyltin	4.05
CF_1-depleted + triphenyltin (4 × washed)	none	0.83
	5 μM triphenyltin	2.97

The reaction mixture (2.0 ml) contained 0.1 M sucrose, 25 mM NaCl, 2 mM $MgCl_2$, 200 μM methylviologen, 0.5 mM HEPPS–NaOH (pH 7.3), and chloroplasts containing 200 μg chlorophyll. Triphenyltin-treated, washed chloroplasts were prepared by adding triphenyltin to CF_1-depleted chloroplasts at a ratio of 100 nmol triphenyltin/2 mg chlorophyll. A small aliquot (containing 200 μg chlorophyll) was assayed for proton uptake, and the remainder diluted to 40 ml with the chloroplast suspending medium (see section 2). The treated chloroplasts were sedimented at 7000 × *g* for 5 min (4°C), resuspended in 40 ml fresh medium, and again sedimented. This washing procedure was repeated 2 additional times. The final pellet was resuspended in a small volume of medium and an aliquot (200 μg chlorophyll) assayed for proton uptake

being stoichiometrically dependent upon the triphenyltin/CF_0 ratio [3]. This degree of potency is somewhat surprising considering the normally very unreactive chemical character of trialkyl- and triaryl-derivatives of the group IVb elements tin, lead and germanium. However, it is known that trialkyltin compounds can form polymeric coordination complexes with certain heterocyclic nitrogen-containing ring complexes such as imidazole [12,13]. Nevertheless, triethyltin has been shown to complex with very few biological materials [4]. Rose [14] has postulated that the mechanism of triethyltin binding to rat hemeoglobin involves the formation of tin–nitrogen linkages between the triethyltin and paired histidine residues, and Davidoff and Carr [16] have postulated a similar mechanism for the binding of triethyltin to pyruvate kinase.

In this paper we have shown that the inhibition of CF_0 catalyzed trans-thylakoid proton transport by triphenyltin can be effectively reversed by low levels of dithiol compounds. Monothiols and oxidized dithiols (disulfides) are ⩾ 100-times less effective in reversing the inhibition by triphenyltin. Although these results do not provide conclusive proof, they can nevertheless be taken as suggestive evidence for a role of vicinal dithiol residues in the mechanism of triphenyltin inhibition of CF_0, and hence in CF_0 function. Numerous investigations have established the importance of SH residues within CF_1 in the mechanism of ATP formation [5–8] and in the regulation (gating) of CF_1-mediated proton transport [9]. Thus it is not unreasonable to assume that SH residues within CF_0 may also play an important role in the mechanism of H^+ transfer catalyzed by this component.

The possible involvement of vicinal SH residues in the mechanism of triphenyltin binding to CF_0 is consistent with the fact that the inhibition by triphenyltin cannot be reversed by large excesses of exogenous histidine, but can be reversed by washing in aqueous buffer. The lack of involvement of tin–nitrogen linkages in the mechanism of triphenyltin inhibition of CF_0 is also consistent with the observation that triphenylgermanium, which unlike triphenyltin does not

form 5-coordination compounds in imidazole model systems, nevertheless exhibits inhibitory properties identical to those seen with triphenyltin (not shown).

The reactive trialkyltin compound dibutylmethyltin chloride has been found to inhibit the mitochondrial ATP synthetase complex by attaching covalently at the triethyltin-binding site within F_0 [17]. The isolated dibutylmethyltin-labelled component was found to be distinct from the DCCD-binding proteolipid [18], and was postulated to consist of a conjugated form of dihydrolipoic acid. The data presented here are not inconsistent with this view, although they do not rule out the possibility that in chloroplasts another sulfhydryl containing component or components is involved in the binding of triphenyltin.

Recently Kanner has found that triphenyltin is an extremely potent inhibitor of γ-aminobutyric acid transport in membrane vesicles prepared from rat brain [19], as well as in the purified γ-aminobutyric acid transporter reconstituted into phospholipid vesicles [20]. In both cases the inhibition by triphenyltin could be completely reversed by the addition of dithiothreitol.

Acknowledgements

This work was supported by a Biomedical Sciences Support Grant (RR07033-12) from the National Institutes of Health, and by a grant in Aid from Miles Research Laboratories. I would like to thank Dr J. Labinger for helpful discussions, and Dr B. Kanner for providing a manuscript prior to publication.

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