INHIBITION OF THE MEMBRANE-BOUND Mg^{++}-ATPase OF CHLOROPLASTS BY LIPOPHILIC CHELATORS

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Received January 31,1975

SUMMARY

The lipophilic iron chelators, bathophenanthroline and TTFA inhibit the membrane-bound light-activated and sulfhydryl-dependent Mg^{++}-ATPase of spinach chloroplasts, but not the trypsin-activated Ca^{++}-ATPase of the solubilized enzyme. Phosphorylation was also inhibited by both chelators. Under these conditions BP acts as an electron transport inhibitor, not an energy transfer inhibitor, i.e. the inhibition was not reversed by uncouplers. TTFA on the other hand showed the classical energy transfer inhibition pattern, the inhibition being reversed by uncouplers. The chelators also make the membranes more leaky toward H^{+} ions. This alone does not seem to account for the inhibition of energy transduction, i.e. the chelators are not simply uncouplers. The data are consistent with the concept that a metal ion may be intimately involved in energy transduction.

INTRODUCTION

The lipophilic chelator, 4,7-diphenyl-1,10-phenanthroline has been shown to inhibit the membrane-bound ATPase activities of mitochondria (1) and E. coli (2). The inhibition was not observed when the enzymes were extracted from the membranes. The inhibition of the membrane-bound ATPase was prevented by certain uncouplers (e.g., CCCP), but not by ionophores (e.g. gramicidin). Based on these results a metalloprotein such as a non-heme iron protein was suggested as a fuctional component in energy transduction. (1,2).

The soluble ATPases were not inhibited as might be expected, since there are no metal atoms in the ATPase protein per se, and when the ATPase is solubilized it is removed from the site of inhibition which is buried in the membrane. The following studies were undertaken to test for metal ion chelator inhibition of

Abbreviations: TTFA, 4,4,4-trifluoro-1-(2-thienyl)-1-3-butanedione; FTFA, 4,4,4-trifluoro-0-(2-furyl)-1,3-butanedione; BP, Bathophenanthroline; MV, methyl violgen; CCCP, carbonyl cyanide 3-chlorophenylhydrazone.

electron transport, phosphorylation and coupling factor ATPase activity in chloroplast membranes.

METHODS

Chloroplasts were isolated from market spinach as described previously (3). The light-activated and sulfhydryl-dependent Mg^{++} ATPase assays were conducted according to McCarty and Racker (4) with a three-minute illumination (5) in saturating light. The inhibitors were added immediately after the light phase of activation so as not to interfere with the pyocyanine-catalyzed light-activating step. The soluble Ca^{++}-ATPase was tested with EDTA extracts containing coupling factor (CF_1), a latent ATPase, as previously described (6). Inorganic phosphate was determined colorimetrically according to the method of Fiske and SubbaRow (7). Protein was determined according to Lowry, et al (8). Inhibitors were added to chloroplasts before the EDTA wash, or to the trypsin-activated enzyme during the incubation with ATP.

Electron transport rates were determined polarigraphically with a Clark-type electrode, at pH 8.0 while simultaneously measuring ATP formation or proton transport by following pH changes of the medium (9).

TABLE 1. Effects of Several Chelators on the Membrane-bound Mg^{++}-ATPase

Additions	%Control
None	100
1.5 µM BP	85
3.0 µM BP	59
15 µM BP	25
0.15 mM BP	5
0.3 mM BP	0
0.1 mM Tiron	91
1.0 mM Tiron	85
2.0 mM Tiron	79
0.5 mM α,α-Dipyridyl	93
1.0 mM α,α-Dipyridyl	111
2.0 mM α,α-Dipyridyl	97
0.1 mM TTFA	88
0.5 mM TTFA	45
0.75 mM TTFA	13
1.0 mM TTFA	12
0.16 mM FTFA	95
0.5 mM FTFA	87
1.6 mM FTFA	44
3.3 mM FTFA	46

The reaction was carried out as described in Methods. The reaction mixture contained in a final volume of 6 ml: 0.05 M Tris-Cl, pH 8.0, 0.05 M NaCl, 0.005 M $MgCl_2$, 0.05 mM Pyocyanine, 0.005 M dithioerythritol, 0.6 mg Chl. After illumination, (10^6 ergs/cm^2·sec) 5 mM ATP was added, and the hydrolytic stage was carried out for 15 min., then stopped with 1 ml 10% TCA. Aliquots of the supernatant were then assayed for Pi with the Fiske-SubbaRow reagent. The specific activity of the control reaction was 125.0 µmoles Pi released· mg Chl^{-1}·hr^{-1}.

TABLE 2. Effect of BP and TTFA on the soluble Ca^{++}-ATPase

Additions	%Control
None	100
0.3 mM BP	95
0.6 mM BP	89
0.9 mM BP	92
1.5 mM BP	85
1.0 mM TTFA	78

Chloroplasts having 2 mg of Chl were incubated with 20 ml 0.8 mM EDTA for each sample. After stirring for 20 minutes at room temperature, the chloroplasts were removed by centrifugation at 20,000 x g for 20 minutes. The supernatant was assayed for protein concentration by the method of Lowry, and the Ca^{++}-dependent trypsin-activated ATPase was assayed as described in Methods. The specific activity of the control reaction was 74.0 μmoles Pi released·mg protein^{-1}·hr.

RESULTS AND DISCUSSION

Table 1 shows the inhibition of the membrane-bound light- and thiol-dependent ATPase by BP and TTFA. BP shows 75% inhibition at 15 μM, and complete inhibition at 150 μM. At 0.1 mM TTFA, the ATPase is inhibited 12% and at 1.0 mM, inhibition is virtually complete. FTFA (4,4,4-tri-fluoro-0-(2-furyl)-1-3-butanedione), an analog of TTFA, shows about 50% inhibition at 3 mM. Tiron, a water-soluble ferric iron chelator, and α,α-dipyridyl, a chelator which is less lipophilic than BP, show very little inhibition. The chelator inhibition site (probably a metalloprotein) is likely to be buried in the membrane or masked by a lipophilic layer such that water soluble chelators cannot reach the proposed metal-protein site.

The soluble ATPase is not inhibited by BP or TTFA, as seen in Table 2. This is true whether the chloroplasts are preincubated with the chelator, and the CF_1 then removed and converted to the Ca^{++}-ATPase, or if the chelator is present during the ATP hydrolysis stage. This is consistent with the generally accepted view (11) that the soluble coupling factor Ca^{++}-ATPase activity does not require or involve ions other than Ca^{++}.

If TTFA and BP are acting as energy transfer inhibitors, they should inhibit phosphorylation, while returning the ADP and Pi-stimulated rate of electron transport to the basal level. If the chelators are acting as uncouplers, then

TABLE 3. Effect of BP and TTFA on Electron Transport and Non-cyclic Photophosphorylation

Assay Conditions	$H_2O \rightarrow MV$ Rate $\mu eq\ e^-\ (mg/Chl\ hr)^{-1}$	Phosphorylation $\mu moles\ ATP\ (mg/Chl\ hr)^{-1}$
Experiment 1		
Basal (-Pi)	160	--
+ ADP + Pi	243	60
+ 15 μM BP + ADP + Pi	64	0
+ 30 μM BP + ADP + Pi	46	0
+ 50 μM TTFA + ADP + Pi	165	0
+ 1 mM TTFA + ADP + Pi	174	0
Experiment 2		
Basal (-Pi)	349	0
+ ADP + Pi	552	83
+ 0.01 mM BP + ADP + Pi	308	0
+ 0.1 mM BP + 1 μM CCCP + ADP + Pi	325	--
+ ADP + Pi	437	--
+ 50 μM TTFA + ADP+Pi	460	--
+ 50 μM TTFA + ADP+Pi + 2 μM Gramicidin	644	--
Experiment 3		
Basal (-Pi)	87	--
+ 30 μM BP	54	--
Basal	134	--
+ 1 mM TTFA	150	--

Reaction mixtures contained in 2ml: 0.5 mM methyl viologen, 0.5 mM azide, 0.1 M KCl, 5 mM $MgCl_2$, 1 mM Tricine-NaOH, pH 8.0, 40-60 μg Chl, 1 mM ADP, and 3 mM Pi. Illumination was with saturating light passed through a solution containing 1% $CuSO_4$.

phosphorylation will be inhibited while electron transport will continue at the ADP + Pi stimulated rate, or will increase slightly. Chloroplasts were assayed for $H_2O \rightarrow MV$ electron transport activity with simultaneous measurements of ATP formation and as shown in Table 3, BP and TTFA at low concentrations do inhibit electron transport and phosphorylation.

Experiment 2 of Table 3 shows that the BP inhibition of coupled electron transport is not reversed by the uncoupler CCCP (nor by gramicidin, data not shown), while the TTFA inhibition is reversed by the ionophore gramicidin. These effects are unlike those found in the mitochondrial and E. coli systems (where

TABLE 4. Effects of Uncouplers and Bathophenanthroline on the Proton Gradient and Mg^{++}-ATPase

	% Control ATPase		H^+ Transport parameter	
	Light	Dark	k_d	$\frac{H^+}{}$ μmoles (mg chl)$^{-1}$
Control	100	100	$0.075s^{-1}$	0.105
1.0 μM CCCP	65	100	$0.307s^{-1}$	0.045
3.0 μM CCCP	57	92	$0.460s^{-1}$	0.020
0.5 mM NH_4Cl	68	95	$0.270s^{-1}$	0.043
1.0 mM NH_4Cl	49	52	$0.503s^{-1}$	0.015
10 μM BP			$0.149s^{-1}$	0.052
25 μM BP			$0.205s^{-1}$	0.035
50 μM BP			$0.462s^{-1}$	0.008
75 μM BP			--	0

Reaction mixtures contained in 2ml:0.1 M KCl, 5 mM $MgCl_2$, 1 mM tricine-NaOH, pH 8.0, 30 μM phenazine methosulfate, and 40-60 μg Chl. Uncouplers or inhibitors were added during activation (light column) or in the following dark period immediately after illumination (dark column). The specific activity of the control reaction was 121.3 μmoles Pi released·mg Chl $^{-1}$·hr^{-1}.

the chelator inhibition was reversed by CCCP, but not gramicidin). As indicated in Table 3, Experiments 1 and 3, BP inhibits the basal electron flow while TTFA has little effect.

The BP inhibition shown here is in the manner of an electron transport inhibitor, while the TTFA inhibition is analogous to an energy transfer inhibitor. However, the BP effect is not simply due to inhibition of electron transfer of the type given by DCMU (12) since DCMU-type inhibition has no effect on the light activated ATPase when given in the dark stage (13).

BP and TTFA are both strong iron chelators. BP also acts as an ionophore, collapsing the proton gradient, i.e. increasing the rate of H^+efflux (Table 4). We were unable to measure the effect of TTFA on proton transport due to an acid drift induced by the reagent. However, it seems unlikely that the sole mechanism of inhibition of the ATPase by these chelators is due to dissipation of the proton gradient Compounds such as gramicidin, and NH_4Cl at concentrations which completely dissipate the proton gradient by increasing the k_d have been shown to have little or no inhibitory effect on the ATPase (Table 4) (14). As seen in Table 1, α,α-Dipyridyl is not an inhibitor of the ATPase. However it increased the k_d from a control value of $0.075s^{-1}$ t

$0.207 s^{-1}$ at 1.0 mM. The uncouplers NH_4Cl and CCCP were tested for inhibition of the Mg^{++}-ATPase at concentrations which inhibit proton accumulation. The data in Table 4 indicate that although the proton gradient is abolished, the ATPase is not completely inhibited.

The exact mechanisms of BP and TTFA inhibition of the Mg^{++}-ATPase are not known at this time. Several sites of BP inhibition in the chloroplast electron transport chain have been suggested (15). Although our data show that these chelators can induce changes in membrane permeability in addition to inhibitory effects, it appears that the inhibitions indicate a chelator effect on a membrane bound metalloprotein that is intimately involved in energy transduction.

This work was supported by NIH grant GMI 959503 and NSF grant BMS 7107348 and a Public Health Service Career Development Award to RAD and by NSF grant no. BMS 74-19689 to FLC.

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