SHORT COMMUNICATIONS

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Carbonylcyanide *m*-chlorophenylhydrazone as an inhibitor of coupled electron transport in trypsin treated spinach chloroplasts*

Trypsin digestion of chloroplasts has been shown initially to increase electron flow (uncoupling) followed by a slower decrease in photoreductive capacity (measured as 2,6-dichlorophenolindophenol (DCIP) reduction). Addition of the uncoupler carbonylcyanide *m*-chlorophenylhydrazone (CCCP) to trypsin-treated chloroplasts resulted in a dramatic inhibition of electron transport. None of the several other uncouplers tested (methylamine–HCl, NH_4Cl, atebrin or Gramicidin D) showed this effect[1]. This paper presents further data on the CCCP inhibition effect.

Examination of the kinetics of the three reactions (trypsin uncoupling, CCCP inhibition and trypsin inhibition) revealed that all three were first order reactions with respect to incubation time, but with considerably different rate constants. The ratios of the half-times ($t_{1/2}$) of the three reactions are about 1:17:40. The trypsin

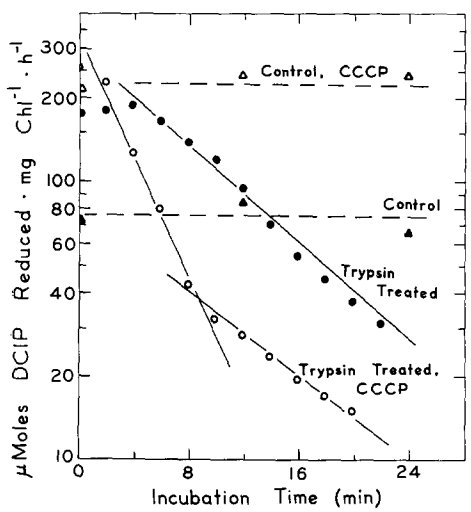

Fig. 1. The effect of CCCP on electron transport in trypsin-treated chloroplasts. Reaction mixture consisted of: sucrose, 0.4 M; KCl, 15 mM; Tricine, 50 mM (pH 7.6); DCIP, 33 μM and chloroplasts containing 48 μg chlorophyll in a total volume of 3.0 ml. CCCP was added to give a final concentration of 16 μM. Enzyme digestion was carried out at 25° in sucrose–KCl–Tricine solution at a chlorophyll concentration of 0.5 mg/ml. Actinic illumination was white light filtered through 5-cm water bath and an infrared absorbing filter. Intensity was about $7.2 \cdot 10^5$ ergs·cm^{-2}-sec^{-1}.

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; DCIP, 2,6-dichlorophenolindophenol.

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uncoupling reaction is very fast in comparison to the other two. In Fig. 1 the uncoupling reaction was completed in the time between addition of the enzyme and removal of the zero-time sample (about 30 sec). As shown in Fig. 1, the trypsin-treated *plus* CCCP inhibition curve is made up of two first order components; one with relatively rapid kinetics and the other with kinetics similar to the trypsin inhibition. Trypsin did not completely uncouple chloroplasts. Addition of uncouplers other than CCCP (methylamine–HCl, atebrin, NH_4Cl or Gramicidin D), or addition of CCCP after a very short incubation with trypsin, resulted in considerable further stimulation of electron transport[1] (see also below). After the trypsin uncoupling has reached its maximum there follows a relatively long period of stable Hill activity (Fig. 1) although the trypsin digestion is proceeding, as evidenced by increasing inhibition of electron transport by added CCCP. At about the time the inhibition by CCCP becomes maximal, a general decrease in photoreductive capacity (paralleled by a decrease in quantum yield)[1] begins. This continues with first order kinetics until all activity is lost.

CCCP at higher concentrations has been reported[2] to inhibit electron transport from water to $NADP^+$ or $Fe(CN)_6^{3-}$ (measured as oxygen evolution). However, the inhibition by CCCP in trypsin digested chloroplasts is concentration independent. As seen in Table I, concentrations as high as 33 μM stimulated electron transport in untreated chloroplasts under the conditions employed while with trypsin digested chloroplasts decreasing the CCCP concentration lessened the amount of inhibition but stimulation of electron transport was never noted.

TABLE I

EFFECT OF CCCP CONCENTRATION ON RATE OF DCIP REDUCTION IN CONTROL AND TRYPSIN-TREATED SPINACH CHLOROPLASTS

Conditions as in Fig. 1. Values expressed as μmoles DCIP reduced/mg chlorophyll per h.

	CCCP concn. (μM)								
	0.00	*0.16*	*0.67*	*3.3*	*6.7*	*16.0*	*33.0*	*67.0*	*99.0*
Control	72.7	71.8	—	128.6	—	199.2	205.5	178.3	149.2
Trypsin-treated	119.0	99.8	94.8	88.2	60.8	55.8	38.0	30.4	25.4

CCCP appears to inhibit only the phosphorylating, or coupled, electron transport pathway. This is suggested by examining the second part of the trypsin treated *plus* CCCP curve in Fig. 1. The rate constant for the slow component is about the same as that for the general trypsin inhibition itself and back-extrapolation gives a zero-time rate of DCIP reduction approximately equal to that in untreated chloroplasts. This portion of the curve, therefore, probably represents only the basal electron flow which is being inhibited by the general trypsin effect.

It is not known why trypsin did not uncouple completely. The degree of uncoupling by trypsin digestion was about half of that elicited by chemical uncouplers, this fraction being quite reproducible. In most experiments, back extrapolation of the general trypsin inhibition curve and the trypsin-treated *plus* CCCP inhibition curve (fast component) gave zero-time values equal to the control *plus* CCCP rate

(usually between 3 and 4 times the control rate), although in the experiment shown in Fig. 1 the control *plus* CCCP value was somewhat lower.

The lag period between the point when uncoupling by trypsin reaches a maximum and the general inhibition first becomes evident is puzzling. During this period trypsin is affecting the chloroplasts in some manner which causes CCCP to inhibit electron flow but the trypsin action itself does not affect the rate of DCIP reduction. After very short incubation times, CCCP still acts only as an uncoupler (Fig. 1). If the decrease in activity is interpreted as a "recoupling", or a lessening of uncoupling ability, it would be expected that with increasing incubation with trypsin the activity after addition of CCCP would drop only to the level observed with trypsin-treated chloroplasts in the absence of CCCP. Instead, activity decreases to that of the basal rate. Addition of other uncouplers to trypsin-treated chloroplasts did not reverse CCCP inhibition. Therefore, it seems likely that CCCP is inhibiting electron transport at a site different from the one where uncoupling takes place.

The most reasonable conclusion is that CCCP, besides acting as an efficient uncoupler, can also react under some conditions with another site in the coupled electron transport pathway in such a way as to block electron flow.

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