Pentachlorophenol Inhibits Photosynthetic Electron Flow and Quenches Chlorophyll Fluorescence after Preillumination

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The pesticide PCP was shown to inhibit the Hill reaction in broken chloroplasts (I_{50} = 15 μM) and to quench chlorophyll fluorescence. Both effects require preillumination. In contrast to the common "phenol-type" inhibitors, neither inhibition of Hill reaction nor chlorophyll fluorescence quench were affected by pretreatment of chloroplast with trypsin instead of preillumination. An inhibition site differing from the "phenol type" inhibitors is therefore assumed. The results presented indicate that the observed light requirement is due to electron transport through PS II. Measurements of intrinsic tryptophane fluorescence relate the PCP site of binding to a hydrophobic environment.

Introduction

PCP is toxic for most animal and plant cells, as well as for microorganisms. It is used as a fungicide and pesticide in preservatives for wood and industrial protein manufacturing processes [1, 2]. PCP uncouples oxidative phosphorylation [1] and inhibits membrane associated properties such as ATPase activity, as well as purine and sugar transport [3]. In this paper the influence of PCP on photosynthetic electron flow, determined as the inhibition of the DCPIP-Hill reaction in isolated chloroplasts, is examined.

Moreover we analyzed the fluorescence yield of chloroplasts at room temperature which reflects the redox state of the primary electron acceptor and is affected by a large number of parameters, thus probing the primary photochemical reactions of photosynthesis [4, 5]. We found that PCP strongly inhibits the Hill reaction and quenches chloroplast fluorescence after illumination.

Materials and Methods

Broken spinach chloroplasts were prepared from young, freshly harvested spinach leaves by grounding 40 g with purified sand in 120 ml isolation buffer containing 30 mM tricine, 10 mM KCl, 2.5 mM $MgCl_2$, 0.4 M sucrose, adjusted to pH 7.5. The suspension was filtered through cloth and pelleted by brief centrifuging at 900 × *g*. The supernatant was sedimented 10 min at 1500 × *g* and the chloroplasts were osmotically shocked by resuspending in 15 ml isolation buffer minus sucrose. After centrifuging at 1500 × *g* for 10 min the chloroplasts were resuspended in 15 ml isolation buffer; 50% glycerol was added for storage at −20 °C. Chlorophyll concentration was 5 μg/ml in the experiments if not indicated otherwise. Hill reaction was measured using the system H_2O/DCPIP under saturating illumination for one minute with a demmolux 800 white light lamp at room temperature. The reaction was performed in the isolation buffer complemented with 25 μM DCPIP. The reduction of DCPIP was measured at 620 nm with a Gilson spectrophotometer.

All fluorescence measurements were performed in a Perkin Elmer 650-40 fluorescence spectrophotometer at room temperature with 471 nm for excitation and 686 nm for emission. Fluorescence spectra were uncorrected for detector response. PCP was added as ethanolic solution, never exceeding 15 μL ethanol/ml chloroplast suspension. PCP and Ioxynil were from Riedel de Haen, all other chemicals from sigma.

Abbreviations: PCP, Pentachlorophenol; DCPIP, 2,6-dichlorophenolindophenol; PS II, photosystem II; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; HDBMIB, dibromothymochinon; HCB, hexachlorobenzene; IOXYNIL, 4-hydroxy-3,5-diiodobenzonitril.

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Results

Hill activity

PCP inhibits the Hill reaction strongly at μM concentrations (Fig. 1). Remarkably, inhibition requires a preillumination of a few minutes. Although the chloroplast suspension had been illuminated for one

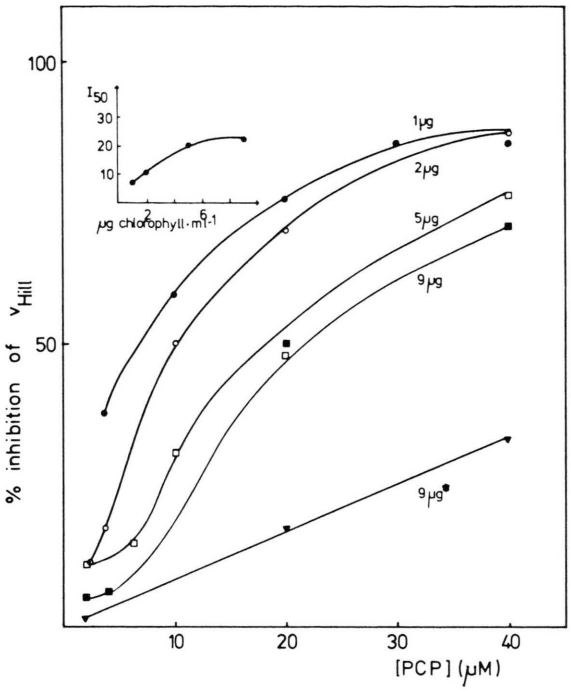

Fig. 1. Inhibition of the H_2O/DCPIP reaction by PCP at different chlorophyll concentrations. Conditions as described in Methods. All samples except (*) were preilluminated for 14 minutes. Extinction difference was measured 1 minute after DCPIP addition. Inset: Influence of the chlorophyll concentration on the I_{50} for the Hill reaction.

minute during the Hill reaction assay, this time was not sufficient to display the maximal effect at the given temperature. Inhibition increased with decreasing chlorophyll concentration. 50% inhibition (I_{50}) occurs between 8–20 µM, depending on the chlorophyll concentration (inset). These results suggest that PCP, due to its chemical constitution, belongs to the phenol-type herbicides [6, 7] which are known to exhibit their action gradually upon illumination [8–10]. Trypsin treatment was shown to abolish this preillumination effect [8]. We tested this property with PCP. It is known that even slight trypsin treatment leads to a loss in Hill activity. Therefore we used a trypsin concentration which caused by itself a 10% inhibition of the Hill reaction. Table I shows that, under these conditions, trypsin+PCP result in a mere addition of inhibition. However, this effect is not comparable to the inhibition exhibited by preillumination. This leads us to conclude that, at least using the H_2O to DCPIP reaction, trypsin-sensibilisation is not detectable.

Table I. Effect of trypsin treatment and PCP on Hill reaction. Conditions were described in Methods. All samples were treated at 40 °C for 2.5 minutes with or without trypsin. Where indicated, the sample subsequently was preilluminated for 2 min. The Hill reaction was started by addition of 25 µM DCPIP and 5 µg trypsin inhibitor (soybean) and extinction difference was measured after one minute.

(PCP) µM	0	0	20	20	20
Trypsin 0.15 µg/ml	−	+	−	+	−
Preillumination 2 minutes	−	−	−	−	+
dE_{620}/min	0.104	0.093	0.050	0.041	0.015

Chlorophyll-fluorescence measurements

PCP is an effective quencher of chloroplast fluorescence. Illumination was a prerequisite for this effect, also. The quench could not be reversed after extended storage in the dark. Two observations indicate that the functionally intact membrane is necessary for the light-induced fluorescence quench:

a) Extraction of chlorophyll from chloroplasts with acetone and addition of 1 mM PCP did not result in any quench.

b) Incubation of chloroplasts for 2 min at 60 °C abolishes the quench effect.

The necessity of preillumination for both the quench and inhibitory effects on electron flow favours the view that intact electron transport is essential for PCP action. This is supported by the observation that the quench effect was inhibited by 2 µM DCMU, an inhibitor of PS II electron transport (Fig. 2). However, 1 mM KCN, an inhibitor of PS I

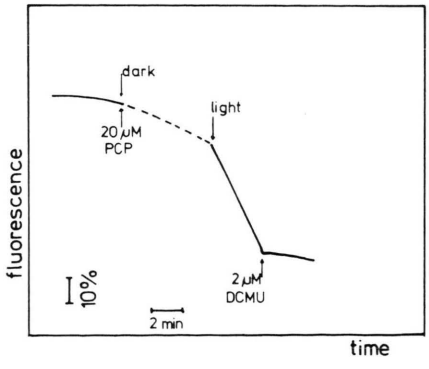

Fig. 2. Effect of DCMU on PCP caused chlorophyll fluorescence quenching. Conditions as described in Methods. The exciting beam was sufficient to induce the quench effect. Exciting beam was shut off where dashed line appears.

electron transport, did not interfere with the quench effect (data not shown), suggesting that electron transport of PS II is needed to induce PCP effects.

Chlorophyll fluorescence could not be quenched totally. Residual fluorescence always remained (Fig. 3), depending on the quality of the chloroplast preparation. Older preparations have a higher residual fluorescence. In the experiment shown, residual fluorescence was 50% of the initial value. The PCP concentration which leads to half maximal quench is in the same range as the PCP concentration for which a half maximal inhibitory effect is observed (approx. 10 μM PCP). This result and the fact that both the quench and inhibitory effects require preillumination for expression indicates a same site of action.

Similar to the inhibitory effect on electron transport, trypsin treatment did not abolish the illumination effect on fluorescence (Fig. 4). In this experiment, 10 μg trypsin/ml was added to a chloroplast suspension. Fluorescence decreased slowly. Then, 4 μM PCP was added and the solution kept dark. Only further slow quenching due to the action of trypsin continued. When the light was turned on again, the fluorescence quench increased rapidly (the exciting beam of the spectrophotometer proved to be sufficient to induce the PCP quench effect), documenting that trypsin treatment could not substitute for the illumination effect.

For comparison, fluorescence quench was tested with DBMIB and IOXYNIL, two phenol type inhibitors. DBMIB is known to quench chlorophyll fluorescence [11, 12]. However, illumination was not necessary for the effect. Quench occurred immediately after addition of the chemical; therefore no light-induced quench enhancement was detectable. Ioxynil shows only a slight quench during prolonged illumination (data not shown).

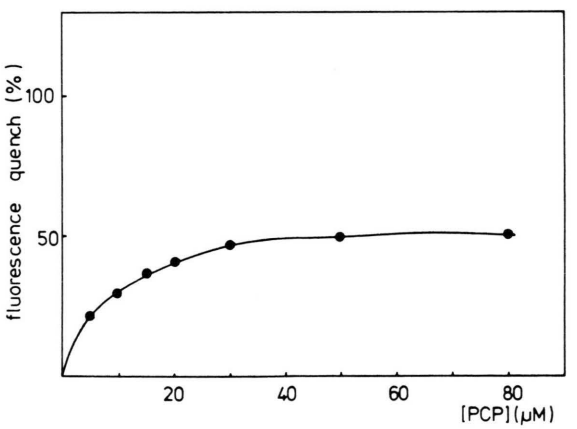

Fig. 3. Quenching of chlorophyll fluorescence by PCP. Conditions as described in Methods. All samples were preilluminated for 8 minutes.

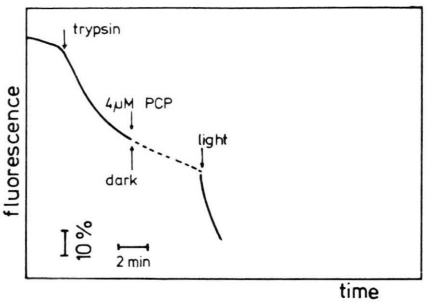

Fig. 4. Effect of trypsin treatment on chlorophyll fluorescence quench by PCP. Conditions as described in Methods. Trypsin concentration was 10 μg/ml. Dashed line indicates exciting beam shut-off.

Site of binding

HCB differs from PCP in the phenolic group and showed no comparable effects when used in the Hill and quench assays (data not shown). It seems that the phenolic group is essential for PCP binding and action. From measurements of intrinsic protein fluorescence which derives primarily from tryptophan, it is possible to obtain information about the hydrophobicity of its binding sites. The protein fluorescence maximum is at about 350 nm, which overlaps with the absorption spectrum of PCP. Therefore, PCP quenches protein fluorescence by energy transfer. The remaining fluorescence spectrum is shifted towards longer wavelengths as can be seen from the difference spectrum which reflects wavelengths where most quenching occurred (Fig. 5). The red shift of the residual fluorescence indicates a domination of fluorescence resulting from tryptophan residues in a more hydrophilic environment. Thus, tryptophan residues in a more hydrophobic environment seem to interact with PCP. The spectrum is not altered after intense preillumination, suggesting that no new binding sites that contain tryptophan were exposed to PCP. Otherwise, these would have been quenched as well.

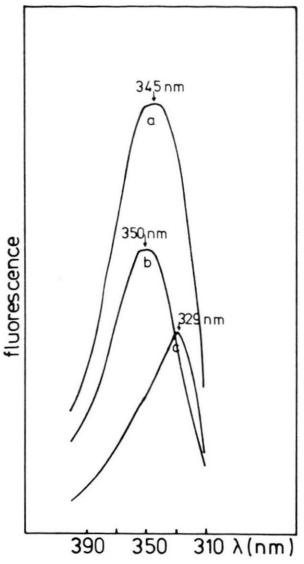

Fig. 5. Quenching of protein fluorescence of chloroplasts by PCP. λ excitation = 293 nm. Conditions as described in Methods.
a) Fluorescence spectrum of untreated control, λ_{max} = 345 nm;
b) after addition of 10 μM PCP, λ_{max} = 350 nm;
c) difference spectrum, λ_{max} = 329 nm.
PCP addition followed by preillumination did not alter spectrum b).

Discussion

PCP is a potent inhibitor of photoelectron transport with an I_{50} of about 15 μM, depending on the chlorophyll-concentration. It is similar to the phenol-type herbicides in so far as preillumination induces an inhibitory effect. However, it lacks trypsin sensitivity. Moreover, it quenches chlorophyll fluorescence after preillumination, whereas this could not be shown for two phenol-type inhibitors, suggesting a different mechanism of action. The quench of fluorescence appears to be related to the inhibitory effect upon electron flow: both effects are initiated by illumination and lead to quantitative proportional effects at equal inhibitor concentration. It is therefore tempting to assume the same site of action.

The effect of illumination on chlorophyll fluorescence quench is due to intact electron transport, presumably of PS II, since DCMU inhibits the quench effect, whereas KCN does not. Assuming the same site of action, electron transport initiates both the inhibitory and quenching effects. The site of action is related to a hydrophobic environment, as indicated by a maximum shift of the quenched protein fluorescence spectrum upon addition of PCP. It remains unknown, how photoelectron transport of PS II and development of PCP action are related. Possibly a conformational change of a PS II protein during electron transport allows PCP to enter its site of action.

Clearly more investigations have to be performed to obtain a final model for the interaction of PCP with the photosystem.

Acknowledgements

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