

Photodynamic Action of Hypericin on Cyanobacteria, *Synechocystis* and *Synechococcus* (*Anacystis nidulans*)

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The photodynamic action of hypericin on photosynthesis and respiration were monitored in *Synechocystis* sp PCC 6803 and *Synechococcus* ATCC 6311 (*Anacystis nidulans*). An oxygen electrode was used to measure net oxygen evolution or consumption. The amount of hypericin required to inhibit photosynthesis was quantitatively determined. Photosynthesis was completely inhibited in *Synechocystis* when the molar ratio of chlorophyll/hypericin was about 70:1. Higher concentrations of hypericin did not stop the uptake of oxygen, but rather stimulated the process in the light. Hypericin was readily washed out of the cells forming no permanent associations in the bacterial cell. Hypericin inhibited electron transfer and consequently oxygen production by PSII particles. For half maximum inhibition of oxygen evolution, with PSII membrane particles, the molar ratio of chlorophyll/hypericin was about 10:1.

Introduction

It has long been known that many photosensitizing pigments produce photochemical reactions when exposed to light (Moan, 1986). The photochemical reactions have been well documented, including the production of singlet oxygen which has strong oxidative properties. The plant pigment hypericin was shown to have photodynamic activity (Pace and MacKinney, 1941; Knox and Dodge, 1984; Doran and Soon Song, 1986; Senthil *et al.*, 1994). For example, it is responsible for the photodynamic disease of grazing animals (Giese, 1971). Hypericin, a polycyclic aromatic dianthraquinone, is found in plants of the genus *Hypericum*. It can be obtained in pure form from plant extracts (Meruelo *et al.*, 1988; Knox and Dodge, 1984). In this report we evaluate the photodynamic action of hypericin with two species of cyanobacteria.

Materials and Methods

Synechococcus ATCC 6301 (*Anacystis nidulans*) and *Synechocystis* sp PCC 6803 were cultured in

cotton-plugged 250 ml Erlenmeyer flasks (containing 110 ml culture) in a light- and temperature-controlled orbital incubator. The culture medium was BG11 (Castenholz, 1988) or C_s (Stevens *et al.*, 1973). In either case the incubator was supplied with 5% v/v CO₂ in air. The cultures were illuminated continuously with white fluorescent light (30-w Sylvania Lifeline fluorescent lamps; PPFD (photosynthethetic photon flux density, 53 E.m⁻².s⁻¹) and the temperature of the incubator was maintained at 30 °C. The cyanobacteria were harvested by low speed centrifugation (8 000 x g, 10 min) at the logarithmic growth phase and were resuspended in fresh growth medium.

Photosystem II membrane particles, isolated from spinach according to Kuwabara and Murata (1982), were stored at -80 °C in the presence of 25% v/v glycerol. For the oxygen evolution experiments, the frozen stock was thawed at 0 °C, precipitated by centrifugation at 35,000 x g for 10 min and resuspended, after one washing, in MES (2 (N-morpholino) ethanesulfonic acid) NaOH 0.025 M, NaCl 0.01 M, sucrose 0.3 M, pH 6.5.

Hypericin was extracted from *Hypericum perforatum* plants and was purified by the HPLC method of Liebes *et al.* (1991). The purity of the preparation was about 90%, with traces of carotenes and chlorophylls. The final lyophilized powder was dissolved in absolute ethanol. The absorption spectrum of our preparation in ethanol and C_s (Stevens *et al.*, 1973) is shown in Fig. 1. Hyperic-

Abbreviations: PBQ, p-phenyl-benzoquinone; PPFD, photosynthetic photon flux density.

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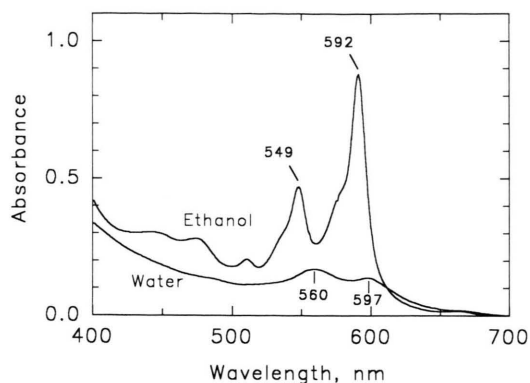


Fig. 1. Absorption spectra of hypericin: (a) in ethanol; (b) in phosphate-free Stevens *et al.* (1973) medium. Concentration 18.7 μM .

cin has a formula weight of 504.5 and composition of $\text{C}_{30}\text{H}_{16}\text{O}_8$. Concentrations of ethanolic stock solutions were determined spectrophotometrically, using the molar absorption coefficient of $\epsilon_{592} = 46,000 \text{ M}^{-1}\cdot\text{cm}^{-1}$ (Liebes *et al.*, 1991). To form the hypericin reagent for the titration of photosynthetic O_2 evolution activities, water was added to the ethanolic solutions of hypericin to reduce the ethanol content to about 1% v/v. During the oxygen evolution assays, the maximum ethanol concentration was about 0.1% v/v.

Respiration and photosynthesis were measured with an oxygen electrode (Hansatech Instruments Ltd., Norfolk, UK) that was thermostated at 25 °C with circulating water. Reaction mixtures, in a total volume of 1 ml, were illuminated as follows: Actinic light provided by a high intensity tungsten-halogen lamp (Model LS2, Hansatech Instruments Ltd., Norfolk, UK). A Corning C. S. 3-69 glass filter (transmission threshold at 510 nm) was used between the actinic light and the oxygen electrode assembly. PPFD at the axis of the sample cylinder was $4 \text{ mE}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Oxygen uptake (respiration) was measured for 1 min in the dark, before and after irradiation. Samples were irradiated for 1 min to measure oxygen evolution. These measurements were corrected for (the averaged) oxygen uptake (measured before and after irradiation).

In the case of cyanobacteria, the typical assay sample (1 ml) was made in fresh growth medium (BG11, pH 7.3, or C_s , pH 8.0) and contained 6–8 μg Chl *a* (chlorophyll *a*) and 0.6 μmol PBQ (*p*-phenyl-benzoquinone) as electron acceptor. In the

case of spinach photosystem II membrane particles, the assay sample (1 ml) was made in MES-NaOH 0.025 M, NaCl 0.01 M, sucrose 0.3 M, pH 6.5, and contained 4 $\mu\text{g}/\text{ml}$ Chl and 0.3 μmol PBQ. These samples were incubated in the dark for 3 min to allow for temperature equilibration before the actinic light was turned on.

Chl concentration of samples was quantitated as in Patterson and Myers (1973). Light intensities were determined with a Li-Cor quantum radiometer (Lambda Instruments Corp., Lincoln, NE).

Results and Discussion

A suspension of *Synechocystis* or *Synechococcus* was placed in an oxygen electrode. The cyanobacteria was titrated with hypericin and illuminated. The results for *Synechocystis* and *Synechococcus* are shown in Fig. 2. While photosynthesis is 'killed' by hypericin, the cells continue to consume oxygen (respire). In Fig. 2 it can be seen that photosynthesis is completely inhibited in *Synechocystis*, when about 1.6 nM of hypericin is added to the 1 ml sample. Further addition of hypericin to the suspension resulted in enhanced respiration in the light. The origin of this enhanced respiration may result from the cells attempting to repair the effects of singlet oxygen. The photodynamic action of hyper-

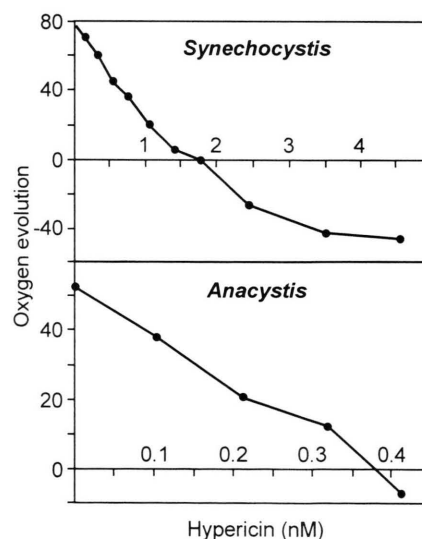


Fig. 2. Photodynamic action of hypericin on the ability of *Synechocystis* and *Synechococcus* (*Anacystis nidulans*) to produce oxygen in the light. The sample size was 1 ml, chlorophyll concentration was 65 $\mu\text{g}/\text{ml}$ and the hypericin stock solution was 42 μM .

icin probably arises mainly from its production of singlet oxygen in the light (Senthil *et al.*, 1994). This singlet oxygen could well be responsible for the 'inhibition' of photosynthesis, probably by the oxidation of areas of the reaction center.

In the case of *Synechocystis* it was found that a molar ratio of chlorophyll/hypericin of about 70/1 completely inhibits or 'kills' photosynthesis. In the case of *Synechococcus* a ratio of chlorophyll/hypericin of about 320/1 was obtained for inhibition of photosynthesis. We have not yet determined whether these ratios depended on culture conditions, type of organism, purity of the hypericin, etc. In all cases controls were run to ascertain the effect, if any, of the ethanol on the cyanobacteria. Ethanol concentrations up to 30 times higher than used in any experiment had no effect on either respiration or photosynthesis.

According to Sherman *et al.* (1994), in *Synechococcus*, the photosynthetic apparatus is not evenly distributed throughout the thylakoids. Both the photosystem I (PSI) reaction center and the ATP synthase proteins are located mostly in the outermost thylakoid and the cytoplasmic membrane, toward the periphery of the cell. That hypericin inhibits photosynthesis before inhibiting respiration might be related to the location of these reaction centers in the cyanobacter.

Fig. 3 presents an experiment in which we examined whether hypericin, which is readily absorbed by cyanobacterial cells, can be subsequently washed out by resuspending the cells in hypericin-free medium. All treatments were performed in the dark. The relevant question here was whether hypericin must bind irreversibly to substrates in order for its photodynamic effect to be expressed, or whether such binding is not necessary. The experiment was performed with *Synechococcus* grown and resuspended in the C_s medium of Stevens *et al.* (1973) medium. Oxygen evolution traces are shown for 'control' cells (i.e. without hypericin). With 'hypericin'-containing cells it can be seen that there is very little oxygen evolution. On the other hand, hypericin-containing cells that had been washed twice by centrifugation and resuspension in fresh C_s medium (marked 'Hypericin+wash') show almost normal oxygen evolution. These results prove that hypericin forms no permanent associations within the bacterial cell, since it can be readily washed out of the cells.

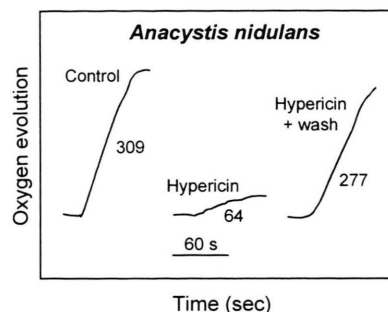


Fig. 3. Oxygen evolution kinetics of *Synechococcus* (*Anacystis nidulans*) in relation to the hypericin content of cells. All treatments were performed in the dark, except for the illumination periods required for recording the oxygen evolution traces shown. Trace labelled 'Control' was recorded with hypericin-free suspension; trace 'Hypericin' recorded with hypericin-containing cells; trace 'Hypericin+wash' recorded with cells that were first treated with hypericin, then subsequently washed twice and finally resuspended in fresh C_s medium. The reaction mixtures (1 ml) contained *Synechococcus* equivalent to 12 μg of Chl, 0.6 μmol *p*-benzoquinone as electron acceptor and, when present, 5.5 μg hypericin. Numbers (309, 64, 277) next to the kinetic traces indicate rates of oxygen evolution in $\mu\text{mol}/\text{mgChl.h}$

In the Fig. 4 experiment, we examined the photodynamic effects of hypericin on isolated photosystem II membrane particles from spinach using two experimental protocols. The effects of

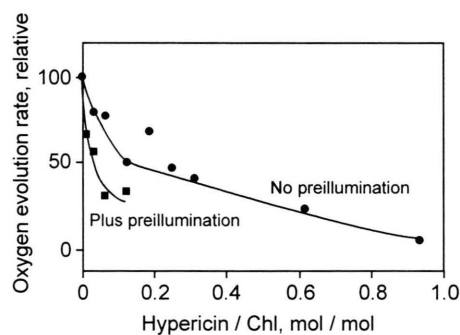


Fig. 4. The dependence of the inactivation of photosynthesis oxygen evolution by purified photosystem II particles on the hypericin/Chl molar ratio and on actinic preillumination. Illumination protocols: (a) Curve labelled "no preillumination". Samples containing hypericin and PBQ were given actinic light to initiate photosynthetic oxygen evolution. The rate of oxygen evolution at '100' on the vertical axis, is 370 $\mu\text{mol}/\text{mg Chl.h}$. (b) Curve labelled "plus preillumination". Samples containing hypericin were given actinic light for 1 min, then given 3 min in dark and PBQ and finally given actinic light again to initiate oxygen evolution. The rate of oxygen evolution at '100', is 310 $\mu\text{mol}/\text{mg Chl.h}$

hypericin on oxygen production by PSII particles were assayed with and without preillumination. The results are shown in Fig. 4. In the case of samples corresponding to the curve denoted as "no preillumination" hypericin and PBQ were introduced into the oxygen electrode sample cell, during the dark equilibration period. Then the mixture was incubated in the dark for 3 min for temperature equilibration. Actinic light was turned on and the oxygen evolution recorded.

In the experiment with "preillumination", all reactants except for PBQ, were also mixed into the oxygen electrode. Following 3 min in the dark for temperature equilibration, actinic light was turned on for 1 min. The mixture was then returned to dark for 3 min and PBQ added, before recording the production of oxygen in the light. Oxygen production by the PSII particles was measured as a function of the molar ratio of hypericin/Chl. It is apparent from Figure 3 that hypericin inhibits the electron transfer in PSII. For half maximum inhibition of oxygen evolution the molar ratio of hypericin/Chl is about 1:10. Approximately 30 Chl *a* molecules serve 1 RCII (reaction center) in *Synechococcus*. Roughly then the stoichiometry is 3 hypericin/RCII. Apparently, this is a rather specific effect.

As demonstrated elsewhere (Papageorgiou *et al.*, 1996) oxygen is indispensable for the photodynamic inhibition of photosynthetic oxygen evolution by hypericin and that photosynthetically evolved oxygen made an important contribution to this effect. Is it possible, however, for hypericin to photoinactivate photosystem II independently of any oxygen evolution activity? We sought to answer this question by the experiment displayed in Fig. 4. The experiment compares the effects of hypericin on oxygen evolution (i) by purified photosystem II particles that were subjected to actinic illumination in the presence of both hypericin and PBQ (labelled as "no preillumination") and (ii) particles that were subjected to actinic preillumination in the presence of hypericin but in the absence of PBQ (labelled as "plus preillumination"). In the absence of an electron sink, the latter particles could not photoevolve oxygen. However, they became oxygen evolution-active after the addition of PBQ during a subsequent dark period. The experiment indicates that photosynthetic oxygen evolution is not a requirement for the photodynamic inactivation of this activity by hypericin.

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