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The mechanism of anthracene interaction with photosynthetic apparatus: A study using intact cells, thylakoid membranes and PS II complexes isolated from *Chlamydomonas reinhardtii*

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ABSTRACT

Intact cells of *Chlamydomonas reinhardtii* as well as isolated thylakoid membranes and photosystem II complexes were used to examine a possible mechanism of anthracene (ANT) interaction with the photosynthetic apparatus. Since ANT concentrations above 1 mM were required to significantly inhibit the rate of oxygen evolution in PS II membrane fragments it may indicate that the toxicant did not directly interact with this photosystem. On the other hand, stimulation of oxygen uptake by ANT-treated thylakoids suggested that ANT could either act as an artificial electron acceptor in the photosynthetic electron transport chain or function as an uncoupler. Electron transfer from excited chlorophyll to ANT is impossible due to the very low reduction potential of ANT and therefore we propose that toxic concentrations of ANT increase the thylakoid membrane permeability and thereby function as an uncoupler, enhancing electron transport *in vitro*. Hence, its unspecific interference with photosynthetic membranes *in vitro* suggests that the inhibitory effect observed on intact cell photosynthesis is caused by uncoupling of phosphorylation.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) and their derivatives are widely known as anthropogenic pollutants harmful to plants, animals and humans (Arfsten et al., 1996; Bispo et al., 1999; Xue and Warshawsky, 2005). Many of the PAHs are recommended for investigations by the U.S. Environmental Protection Agency (Dabestani and Ivanov, 1999) because of their high toxicity and wide spread occurrence in the environment. One of these compounds, anthracene (ANT), is known to inhibit many biochemical processes including respiration and photosynthesis (Aksmann and Tukaj, 2004, 2008; Huang et al., 1997; Tripuranthakam et al., 1999). ANT is assumed to influence photosynthesis in nature mainly as its photomodified products. The majority of the ANT photoproducts are quinones (Mallakin et al., 2000) similar to those in living organisms, thus having a potential to replace these components, e.g. in the electron transport chains (Huang et al., 1997; Srivastava et al., 1995; Tripuranthakam et al., 1999). Toxic effects of intact ANT on photosynthetic activity have also been observed (Aksmann and Tukaj, 2004; Gensemer et al., 1999; Huang et al., 1997; Mallakin et al., 2002) but the mechanism of its toxicity remains totally unclear.

Typically, inhibition of photosynthesis by PAHs has been examined by measurements of oxygen evolution (Aksmann and Tukaj, 2004), inorganic carbon fixation (Huang et al., 1997) or chlorophyll fluorescence using PAM methods (Huang et al., 1997; Kummerová et al., 2006). Recently, PAHs toxicity was also determined using fast chlorophyll fluorescence transients analysis (OJIP method) (Aksmann and Tukaj, 2008). This method allowed us to show that intact ANT inhibits photosynthetic activity of *Chlamydomonas reinhardtii* cells due to the lowering of quantum efficiency of electron trapping and transport, decrease in the number of active PS II reaction centres (RCs) and impairment of PS II – PS I cooperation (Aksmann and Tukaj, 2008). The multifaceted action of ANT observed in treated plant cells suggests that hydrophobic PAHs accumulate in thylakoid membranes (Duxbury et al., 1997) and may induce conformational changes in their structures, thus causing disturbance of electron transport at both donor and acceptor site of PS II (Aksmann and Tukaj, 2008; Babu et al., 2001; Kummerová et al.,

Abbreviations: ANT, anthracene; 1,2-dhANTQ, 1,2-dihydroxyanthraquinone; ANTQ, anthraquinone; BBY, PS II membrane fragments; DBMIB, 2,5-dibromo-3-methyl-6-isopropylbenzoquinone; DCBQ, 2,6-dichloro-p-benzoquinone; DMSO, dimethylsulfoxide; FeCy, potassium ferricyanide; MV, methyl viologen; NPQ, non-photochemical fluorescence quenching; OEC, oxygen evolving complexes; OJIP, O-J-I-P-steps chlorophyll fluorescence method; PAHs, polycyclic aromatic hydrocarbons; PAM, pulse-amplitude modulation method; PS I, photosystem I; PS II, photosystem II; Q_a, primary plastoquinone; Q_b, secondary plastoquinone; qEmax, non-photochemical, pH-dependent quenching; RC, reaction center; SCE, saturated calomel electrode.

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2008). Other data indicate (Huang et al., 1997) that PS II disruption may be a secondary effect of PS I inhibition, leading to increased excitation pressure on PS II and subsequent oxidative stress.

Until now neither measurements of photosynthetic oxygen evolution nor analysis of *in vivo* chlorophyll *a* fluorescence have been able to indicate a precise mode of action of ANT. Therefore in the present work the approach was extended to use of both intact cells and thylakoid membranes as well as PS II complexes from *C. reinhardtii* cells. We were able to show that ANT did not directly influence photosystem II. Investigation of the ANT effect on electron transport downstream from PS II revealed that ANT stimulates an oxygen uptake by isolated thylakoids, which suggests that ANT could act as an artificial electron acceptor. However, further investigations with DCBQ and DBMIB as well as literature data about physico-chemical properties of ANT (Vaný sek, 2004) lead us to rule out this possibility. In our opinion, a more plausible explanation for the increased electron transport observed in the presence of ANT is that the hydrocarbon affects thylakoid membrane integrity leading to uncoupling of phosphorylation from electron transport, as indicated by the low values of non-photochemical quenching parameters (NPQ, qEmax) in ANT-treated cells. Thus, we propose that the photosynthesis inhibition caused by ANT starts by a non-specific thylakoid disruption leading to inhibition of photophosphorylation and subsequent photosystems damages.

2. Materials and methods

2.1. Chemicals

The three-ring polycyclic aromatic hydrocarbon used, anthracene (ANT), of high purity (Sigma–Aldrich Chemical Co., USA) was dissolved in dimethylsulfoxide (DMSO) and supplied to the buffers after dilution to the required concentrations. Chemicals of standard analytical grade were used for growth medium and buffers preparation.

2.2. Cultures

The *C. reinhardtii* (Chlamydomonas Culture Collection at Duke University, Durham, NC, USA) cc-125 strain used in the present work is the wild-type. The cw92 strain is its cell wall-deficient mutant, commonly chosen for thylakoid and PS II complex preparation (Harris, 2008) and regarded as the standard wild type in photosynthetic studies (Palmqvist et al., 1990).

The algae were stored on slants (Watanabe, 2005) containing TAP (Gorman and Levine, 1965) medium solidified with 2% agar. From the slants algae were transferred into liquid HSM medium (Sueoka, 1960) and pre-cultured for several days. After reaching the exponential growth phase, the material was used to set up bath cultures.

The initial cell density of cc-125 cultures was 0.5×10^6 cells per cm^3 in 200 cm^3 of the liquid culture media. Cultures were grown in glass test tubes submersed in a thermostated (30 °C) water bath under continuous fluorescent light (TLD58W/54, Philips), providing 80 $\mu mol\ m^{-2}\ s^{-1}$ of photosynthetically active radiation (380–690 nm) measured inside the culture vessels (QRT1, Hansatech Ltd., UK). Cultures were aerated with a gas mixture containing 2.5% CO_2, which was passed through a bacteriological filter (Sartorius 2000; 0.2 μm PTFE). ANT was delivered to the culture medium to final nominal concentration of 0.89 mg dm^{-3}, which corresponds to EC_{50} value estimated previously for this strain (data not shown). Experiments were repeated three times.

Cultures of cw92 strain, used for PS II complex and thylakoid isolation, were grown according to Villarejo et al. (2002). Algae were grown in 5 dm^3 glass vessels under continuous fluorescent light of 150 $\mu mol\ m^{-2}\ s^{-1}$ of photosynthetically active radiation (380–690 nm) measured at the surface of the culture vessels. The cultures were aerated with a gas mixture containing 5% CO_2, which was passed through a bacteriological filter (Sartorius 2000; 0.2 μm PTFE).

2.3. Isolated thylakoids and PS II complexes

Thylakoids were isolated as described by Allen and Staehelin (1994) and the PS II membrane fragments (BBY) were isolated according to Berthold et al. (1981). Chlorophyll concentration in both thylakoid and BBY fractions was determined spectroscopically after extraction in 100% acetone (Lichtenthaler, 1987). ANT was delivered to the buffer after dilution in DMSO. The effect of ANT on the oxygen evolution (BBY) or uptake (thylakoids) was examined. A DMSO control sample was prepared for each experiment. All experiments were repeated at least three times.

2.4. Measurements

Chlorophyll *a* fluorescence was measured using a Handy Pea fluorometer (OJIP test, continuous excitation fluorescence method) or using a FMS1 fluorometer (PAM method) (Hansatech Ltd., UK). For PAM measurements, the cells suspension was diluted to $OD_{680} = 0.15$ (±0.02) and dark-adapted for 20 min to complete re-oxidation of PS II electron acceptor molecules. Next, aliquots with a volume of 2.8 cm^3 were placed in the measuring chamber. Measurements were carried out at room temperature. For OJIP test, the cell suspension was diluted to $OD_{680} = 0.05$ (±0.005) and dark-adapted for 15 min. Aliquots with a volume of 2 cm^3 were placed in the measuring chamber using vials of 2 cm^3 capacity. Measurements were carried out at room temperature.

Based on the original fluorescence data, non-photochemical quenching parameters were calculated. The NPQ parameter (PAM method), collecting all non-photochemical processes that dissipate excitation energy in photosystems, was calculated as NPQ = (Fm – Fm')/Fm', where Fm is maximum chlorophyll fluorescence at a saturating pulse in the dark-adapted state and Fm' is the maximum chlorophyll fluorescence in the light-adapted state (Lichtenthaler et al., 2005). The capacity of non-photochemical, pH-dependent quenching (qEmax = [Fm – F_{6s}]/Fm) was calculated from OJIP test measurements, according to Strasser et al. (1999).

The photosynthetic oxygen production (consumption) was determined using a Clark-type oxygen electrode (Chlorolab 1, Hansatech Ltd., UK), equipped with a stirrer bar and thermostated by circulating water. To determine the electron transport rate in thylakoid membranes, samples of thylakoids stored in −80 °C were thawed in an ice-bath and kept in the darkness. Aliquots of thylakoids were suspended in buffer "A" containing 50 mM HEPES-KOH (pH 7.5), 100 mM sucrose, 20 mM KCl and 5 mM $MgCl_2$, to a chlorophyll concentration of 0.6 mg cm^{-3}. Diluted samples were immediately used for measurements or incubated with ANT on ice and in the darkness. After incubation, aliquots of thylakoids were taken from the incubation mixture and placed, in darkness, in the measurement chamber containing buffer "A". The final chlorophyll concentration in the chamber amounted to 25 $\mu g\ cm^{-3}$. Measurement of oxygen consumption was performed at 25 °C and 3000 $\mu mol\ m^{-2}\ s^{-1}$ of light intensity with 1 mM methyl viologen (MV) as an artificial electrons acceptor. Influence of the incubation and DMSO (1%, v/v) on the electron transport rate was also determined. Since the incubation itself without additions caused a 15% decrease in oxygen consumption, thylakoids incubated for 15 min in the buffer were considered as control. DMSO had no influence on the rate of oxygen consumption (data not shown).