Involvement of the Xanthophyll Cycle in Regulation of Cyclic Electron Flow around Photosystem II

W. I. Gruszecki[a], K. Strzałka[b], K.P. Bader[c], A. Radunz[c] and G.H. Schmid[c]

[a] Department of Biophysics, Institute of Physics, Maria Curie-Skłodowska University, 20–031 Lublin, Poland
[b] Department of Plant Biochemistry and Physiology, Institute of Molecular Biology, Jagiellonian University, Al. Mickiewicza 3, 31–120 Kraków, Poland
[c] Fakultät für Biologie, Lehrstuhl Zellphysiologie, Universität Bielefeld, Postfach 10 01 31, D-33501 Bielefeld, Bundesrepublik Deutschland

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In our previous study (Gruszecki *et al.*, 1995) we have postulated that the mechanism of cyclic electron transport around photosystem II, active under overexcitation of the photosynthetic apparatus by light is under control of the xanthophyll cycle. The combination of different light quality and thylakoids having various levels of xanthophyll cycle pigments were applied to support this hypothesis. In the present work photosynthetic oxygen evolution from isolated tobacco chloroplasts was measured by means of mass spectrometry under conditions of high or low levels of violaxanthin, being transformed to zeaxanthin during dark incubation in an ascorbate containing buffer at pH 5.7. Analysis of oxygen evolution and of light-induced oxygen uptake indicate that the de-epoxidation of violaxanthin to zeaxanthin results in an increased cyclic electron transport around PS II, thus dimishing the vectorial electron flow from water. An effect similar to de-epoxidation was observed after incubation of thylakoid membranes with specific antibodies against violaxanthin.

Introduction

The xanthophyll cycle is a process leading to a strong light-induced accumulation of zeaxanthin in thylakoid membranes due to violaxanthin de-epoxidation, which is reversed by light-independent zeaxanthin epoxidation (Pfündel and Bilger, 1994). Between several recently proposed physiological functions of the xanthophyll cycle the most important ones seem to be: (1) regulation of thermal energy dissipation from the photosynthetic apparatus, indirectly (Ruban *et al.*, 1994; Gruszecki *et al.*, 1994) or directly via photophysical interaction of zeaxanthin with singlet-excited chlorophyll a (Demming-Adams, 1990; Frank *et al.*, 1994; Owens, 1994); (2) regulation of thylakoid membrane fluidity (Gruszecki and Strzałka, 1991; Havaux and Gruszecki, 1993) and permeability to small molecules like oxygen (Subczynski *et al.*, 1991) a function which plays an important role in the protection of the lipid core of the thylakoid membrane against strong light-induced peroxidation (Havaux *et al.*, 1991; Sarry *et al.*, 1994). The latter function seems to be the direct consequence of the presence of the xanthophyll pigment zeaxanthin in the lipid phase (see Gruszecki and Krupa, 1993 for a discussion). Relatively new ideas with respect to the physiological functions of the xanthophyll cycle see a possible involvement of zeaxanthin in the photoreception of blue light, having an effect on stomata opening (Srivastava and Zeiger, 1993) and the growth direction of coleoptiles (Quinones and Zeiger, 1994). Recently, we have proposed an explanation for a certain type of blue light-enhanced oxygen evolution in terms of the xanthophyll cycle-controlled cyclic electron flow around photosystem II (Gruszecki *et al.*, 1995). According to the presented molecular model, the pool of violaxanthin localized in minor antenna pigment-proteins and being under control of the xanthophyll cycle, is postulated to play a role in excitation energy transfer from the major antenna to the reaction centre of PS II and is responsible for an efficient flow of excitation energy from antenna carotenoid pigments to the reaction centre β-carotene. Ground state β-carotene, ac-

Abbreviation: PS II, photosystem II.

Reprint requests to Prof. Dr. Georg H. Schmid. Telefax: 0521/1065626.

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cording to this model an element of the cyclic electron flow around PSII, is able to transfer electrons back to $P680^+$ but excited β-carotene cannot participate in such an electron transfer for energetical reasons (Gruszecki *et al.*, 1995). In such a model by regulating the excitation of the reaction centre β-carotene an epoxidation state of the xanthophyll cycle has a decisive influence on the operation of a cyclic electron flow around PSII. Under conditions of low light and "high level of violaxanthin" the cyclic electron flow would not be required and therefore blocked whereas under the condition of high light and "low level of violaxanthin" it would be important to have a highly active cyclic electron flow. In the present work further evidence is presented for the participation of the xanthophyll cycle in the regulation of cyclic electron transfer around PS II.

Material and Methods

Chloroplast membranes were isolated from tobacco (*Nicotiana tabacum*) leaves (var. John William's Broadleaf) as described earlier (Homann and Schmid, 1967). Thylakoid membranes containing different amounts of violaxanthin (due to the action of the enzyme violaxanthin de-epoxidase) were obtained by incubation in 50 mM 2-N(morpholino)ethanesulfonic acid (MES)-NaOH buffer, pH 5.7 containing 0.3 M KCl and 30 mM ascorbate. Incubation was carried out in darkness at room temperature, for various lengths of time (from 30 to 60 min.). Control membranes, containing the highest level of violaxanthin were incubated in the same buffer which did not contain ascorbate. Incubation was terminated by spinning down the membranes (5000 rpm, Biofuge 15, Heraeus, Sepatech). The resulting pellet was resuspended in 0.15 M N-tris(hydroxylmethyl)methylglycine (Tricine)-NaOH buffer, pH 7.5 containing 0.3 M KCl and 1 mM potassium ferricyanide.

Oxygen evolution from thylakoid membranes (300 μg of chlorophyll), illuminated with continuous blue or red light of the same quantum flux density or with series of 50 saturating 5 μs flashes of white light separated by 300 ms intervals, was monitored by the mass spectrometric technique described earlier (Bader *et al.*, 1992).

Violaxanthin determination. In order to determine the content of violaxanthin in the investigated thylakoids, the pigment fraction was extracted with diethyl ether. Individual carotenoids were separated by thin layer chromatography as described by Gruszecki and Sielewiesiuk (1990). The bands of violaxanthin and neoxanthin (internal standard) were eluted with ethanol and their amount determined spectrophotometrically (Gruszecki and Sielewiesiuk, 1990).

Fluorescence life-time kinetics of chlorophyll were measured and analyzed with photosystem II particle preparations from tobacco thylakoids prepared according to Berthold *et al.* (1981), using a *K2-Multifrequency Cross-Correlation Phase and Modulation Fluorometer* (ISS, USA) equipped with a xenon lamp and a Pockels cell light modulator. A diluted solution of glycogen was used as light scattering reference. For excitation, 440 nm monochromatic light modulated at frequencies up to 250 MHz was applied.

Results and Discussion

Blue light absorbed by carotenoid pigments was demonstrated to have a pronounced effect in enhancing photosynthetic oxygen evolution. This was interpreted in terms of a blockage of cyclic electron flow around PS II (Gruszecki *et al.*, 1995). Fig. 1 shows the effect of blue light on mass spectrometry-monitored photosynthetic oxygen evolution from tobacco chloroplasts containing higher or lower levels of the xanthophyll pigment violaxanthin. The effect of red light absorbed exclusively by chlorophylls was measured as a standard in each investigated sample. As expected, due to the transformation to zeaxanthin (Havaux and Gruszecki, 1993), the violaxanthin content in chloroplasts expressed as violaxanthin to neoxanthin ratio (V/N, neoxanthin being the not convertable xanthophyll pigment) dropped by a factor of 1.55 during a prolonged incubation of chloroplasts in the acidic buffer (pH 5.7) which contained 30 mM ascorbate. As may be seen from Fig. 1, the violaxanthin content clearly correlates with the amplitudes of blue light-induced photosynthetic oxygen evolution, when compared to those induced by red light of the same fluence rate (B/R). Fig. 2 represents the summary of several similar experiments, each point being the average from three to five measurements. As may be seen in Fig. 2, the beginning of de-epoxidation of violaxanthin corre-

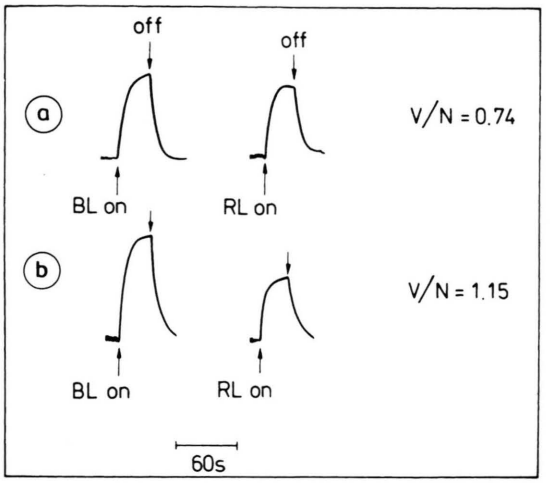

Fig. 1. Original mass spectrometric recording of photosynthetic oxygen evolution from tobacco chloroplasts illuminated with blue light (BL) or red light (RL) of the same fluence rate of 20 μE m^{-2} s^{-1}. Before transfer to the Tricine NaOH buffer, pH 7.5, for the oxygen measurement, chloroplast membranes were (a) incubated for 45 min, (b) for 15 min in 2-(N-morpholino)ethane sulfonic acid (MES)-NaOH buffer, pH 7.5, containing 30 mM ascorbate (see Materials and Methods). The start and the end of the 30 s illumination period are indicated by arrows. Ratios of violaxanthin to neoxanthin (V/N) in the measured membranes are indicated.

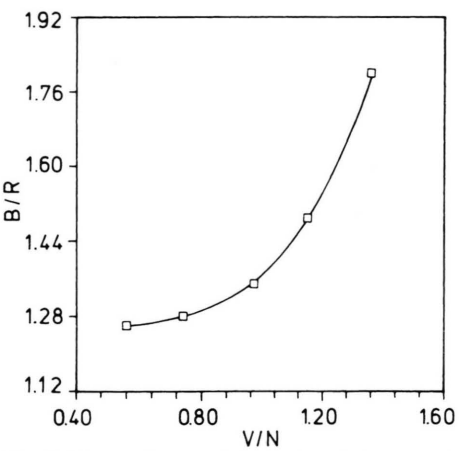

Fig. 2. Dependence of the ratio of photosynthetic oxygen evolution amplitudes obtained by illumination with blue light and red light (B/R) on the violaxanthin to neoxanthin ratio in each sample (V/N). The first four points correspond to the incubation time in the "pH 5.7 buffer" containing 30 mM ascorbate of 60, 45, 30 and 15 min, respectively. The last point corresponds to 30 min incubation in the "pH 5.7 buffer" but without ascorbate. Other conditions as in Figure 1.

lates with the decrease of the B/R ratio. This indicates that the cyclic electron flow around PS II is not linearly dependent on violaxanthin de-epoxidation with time but may be rather considered as being related to the detaching of violaxanthin molecules from antenna proteins, a process which would change the availability of the pigment to de-epoxidation (Gruszecki *et al.*, 1994b; Gruszecki and Krupa, 1993).

Additional support may come from our recent study on the light induced oxygen uptake observed during photosynthetic oxygen evolution in tobacco chloroplasts (Gruszecki *et al.*, 1994a). This uptake clearly depends on vectorial electron flow, as demonstrated by the use of several electron acceptors and is saturated at relatively low rates of photosynthetic oxygen evolution (see Fig. 5 in Gruszecki *et al.*, 1994a). Since photosynthetic oxygen evolution obviously depends on the operation of the cyclic electron flow (competition for reduction of $P680^+$) one may design an experiment in which both processes namely light-induced oxygen evolution and oxygen uptake will be observed with conjunction to the epoxidation state of the xanthophyll cycle pigments. In order to carry out such an experiment exogenous electron acceptors were not present in the assay and chloroplasts were illuminated with flashes (Gruszecki *et al.*, 1994a). The relatively high number of 50 white light flashes was applied in order to be sure that light-induced molecular oxygen uptake is saturated with respect to a light dose-dependent oxygen evolution (Gruszecki *et al.*, 1994a). As it is seen in Fig. 3, differences in the violaxanthin content do not correspond to the amplitudes of light-induced oxygen uptake proportional to vectorial electron flow, but are rather related to photosynthetic oxygen evolution. As expected, a high violaxanthin content corresponds to an increase in oxygen evolution, an effect which again might be explained in terms of a blockage of the cyclic flow around PS II.

Inhibition of photosynthetic electron transport on the donor side of photosystem II by an antiserum to violaxanthin reported by Lehmann *et al.* (1979) can reasonably only be discussed within the newly proposed model of cyclic electron flow around photosystem II mediated by β-carotene (Gruszecki *et al.*, 1995). As already discussed earlier (Lehmann *et al.*, 1979), it was difficult to conceive that violaxanthin directly participated in

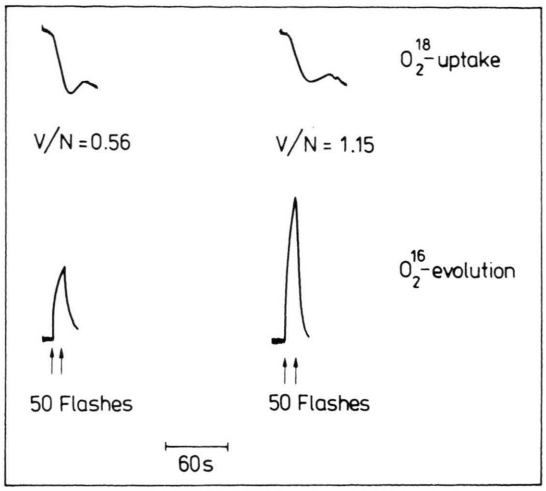

Fig. 3. Original mass spectrometric recordings of oxygen gas exchange in tobacco chloroplasts upon illumination with 50 short (5 μs) saturating flashes of white light spaced 300 ms apart. Photosynthetic oxygen evolution from $H_2^{16}O$ was detected at m/e = 32. In order to monitor oxygen uptake processes at m/e = 36 the assay was supplemented with 20 ml $^{18}O_2$ to the gas phase brought to equilibrium between the aqueous and gas phase. Arrows indicate the start and the end of the train of 50 flashes.

photosynthetic electron transport. The explanation given at that time, however, is still valid and was that antibody binding to violaxanthin affected the molecular structure of photosystem II, namely molecular distances in the antenna system, thus affecting photosynthetic electron transport in the region of photosystem II. In view of the model of cyclic electron transport around photosystem II (Fig. 4 in Gruszecki *et al.*, 1995), the antibody to violaxanthin decreases the efficiency of transfer of excitation energy towards the reaction centre on the energetic level of carotenoids, feeding less energy into the β-carotene of the reaction centre of photosystem II. This in turn inhibits vectorial electron flow as it increases the cyclic one. The analysis of the effect of the antiserum to violaxanthin on the fluorescence behaviour of photosystem II particle preparations clearly speaks in favour of the proposed modulation of the excitation state of the reaction centre β-carotene via the xanthophyll cycle. The antiserum to violaxanthin increases in photosystem II particles the chlorophyll fluorescence emission (Fig. 4). The analysis of the fluorescence emitting chlorophyll species reveals two main components as expected from Figure 4. It appears that the antiserum affects the fluorescence life time of these. As seen from Table I the increase in fluorescence which reflects the observed inhibition of photosynthetic electron transport, reported by Lehmann *et al.* (1979) and Gruszecki *et al.* (1995), corresponds to an increase of the fluorescence life time of only one fluorescing component namely the minor one of the two main components detected. This component is thought to belong to the distal part of the antenna system, i.e. the light harvesting pigment complex II. This experiment is taken as further evidence for the aspect that the antiserum affects the efficiency of the transfer of excitation energy towards the reaction centre.

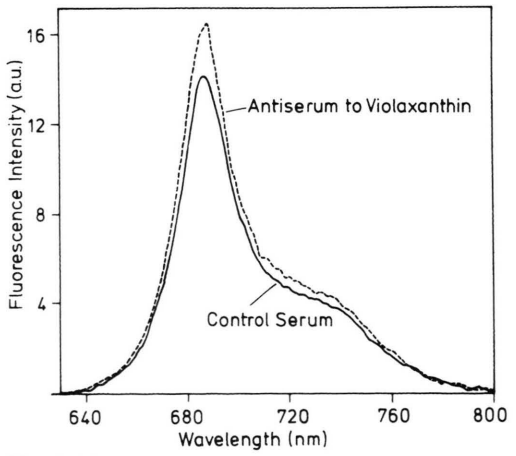

Fig. 4. Fluorescence emission spectrum of photosystem II particle preparations from *N. tabacum* var. Su/su in the presence of the antiserum to violaxanthin. Excitation wavelength 440 nm.

Summarizing, several experiments are presented here which support our hypothesis that excitons absorbed by antenna carotenoid pigments and transferred to the reaction centre β-carotene are, in dependence on the state of the xanthophyll cycle pigments (violaxanthin being a promotor of such a transfer), able to block cyclic electron transfer around photosystem II. Such a mechanism may be considered as an additional aspect of an overall protecting activity of the xanthophyll cycle, being essentially active under the condition of overexcitation by light and other stress situations (Pfündel and Bilger, 1994).

The violaxanthin-controlled effect of the cyclic electron flow on photosynthetic oxygen evolution might explain several phenomena reported in the literature:

Table I. Effect of the antiserum to violaxanthin on the fluorescence behaviour of photosystem II-particles from *N. tabacum*.

	Component I		Component II		Component III	
	Fluorescence life time [ns]	Portion of the comp. in the normalized system	Fluorescence life time [ns]	Portion of the comp. in the normalized system	Fluorescence life time [ns]	Portion of the comp. in the normalized system
Antiserum to violaxanthin	0.214	0.813	1.959	0.166	37.044	0.020
Control serum	0.209	0.822	2.206	0.163	139.06	0.015

Fluorescence life-times were determined with the K-2 Model Fluorimeter from ISS in a three component fit. Measurements were carried out with 200 measuring cycles on each sample. The square of the mean deviation in per cent (x^2) was 5.49 for the life-time values measured in the presence of control serum and 6.13 for those in the presence of the antiserum. It is seen that three fluorescing components occur in the system, one of them (component III) being negligible. PS II-particles from *N. tabacum* var. Su/su corresponding to 20 μg chlorophyll and 70 μl antiserum or control serum were used in 2 ml assay containing 0.15 M Tricine and 0.3 M KCl, pH 7.5. Excitation wavelength was 440 nm.

1. The carotenoid-dependent efficiency of photosynthetic oxygen evolution (Sandmann *et al.*, 1993).

2. The quantum yield of photosynthetic oxygen evolution as dependent on the epoxidation state of the xanthophyll cycle pigments (Thayer and Björkmann, 1990).

3. The effect of antisera to carotenoids on photosynthetic oxygen evolution (Lehmann-Kirk *et al.*, 1979).

4. The correlation of the state of the xanthophyll cycle pigments with the degree of reduction of the plastoquinone pool (Grumbach, 1983).

It looks as if the four phenomena could be explained within our model of a regulation of cyclic electron flow around PS II discussed above and demonstrated in Figs. 1–4.

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