Biochimica et Biophysica Acta 1817 (2012) 389–394

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Plastoquinol is more active than α-tocopherol in singlet oxygen scavenging during high light stress of *Chlamydomonas reinhardtii*

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ARTICLE INFO

Article history:
Received 27 September 2011
Received in revised form 3 December 2011
Accepted 6 December 2011
Available online 13 December 2011

Keywords:
Chlamydomonas reinhardtii
Oxidative stress
Plastoquinone
Plastoquinol
Singlet oxygen
α-tocopherol

ABSTRACT

In the present study, we have performed comparative analysis of different prenyllipids in *Chlamydomonas reinhardtii* cultures during high light stress under variety of conditions (presence of inhibitors, an uncoupler, heavy water). The obtained results indicate that plastoquinol is more active than α-tocopherol in scavenging of singlet oxygen generated in photosystem II. Besides plastoquinol, also its oxidized form, plastoquinone shows antioxidant action during the stress conditions, resulting in formation of plastoquinone-C, whose level can be regarded as an indicator of singlet oxygen oxidative stress *in vivo*. The pronounced stimulation of α-tocopherol consumption and α-tocopherolquinone formation by an uncoupler, FCCP, together with the results of additional model system studies, led to the suggestion that α-tocopherol can be recycled in thylakoid membranes under high light conditions from 8a-hydroperoxy-α-tocopherone, the primary oxidation product of α-tocopherol by singlet oxygen.

1. Introduction

High light is the main abiotic factor responsible for oxidative stress in plants. This results in an enhanced production of singlet oxygen by reaction center chlorophyll in photosystem II [1–3], which significantly contributes to photoinhibition [4,5]. In the prevention of deleterious effects of this reactive oxygen form on chloroplast components, several water-soluble (ascorbate) and lipophilic antioxidants (tocopherols, plastoquinol, plastochromanol) are engaged [6–8]. It was originally proposed that α-tocopherol scavenges singlet oxygen and prevents degradation of D1 and D2 reaction center proteins in *Chlamydomonas reinhardtii* and the loss of photosynthetic activity [9,10]. Recently, it was proposed that the target of singlet oxygen action in chloroplasts is the repair mechanism [11] and the function of α-tocopherol is protection of the repair of photosystem II during photoinhibition [12]. In scavenging of singlet oxygen, α-tocopherol is irreversibly oxidized and its continuous resynthesis must occur to keep its level sufficient for the photoprotection [9]. Later, a similar function for plastoquinol, as one of its diverse functions in plants [13], has been suggested in *C. reinhardtii* [7] and higher plants [14]. Moreover, the kinetic data of singlet oxygen quenching by various prenyllipids *in vitro* showed that besides tocopherols and plastoquinol, also other prenyllipids such as α-tocopherolquinol, plastochromanol, ubiquinol or even plastoquinone to some extent, are effective in this reaction [15]. In these studies, 8a-hydroperoxy-α-tocopherone was found as the primary oxidation product of α-tocopherol, while oxidation of plastoquinol led to plastoquinone and further to plastoquinone-C. The oxidation products of prenyllipids, such as α-tocopherolquinone, plastoquinone-C and hydroxy-plastochromanol are also found often *in vivo* in minor amounts [13,16–20] and can be regarded as indicators of oxidative stress.

In the present study, we have performed comparative analysis of different prenyllipids (Fig. 1) in *C. reinhardtii* during high light stress under variety of conditions (presence of inhibitors, an uncoupler, heavy water) that made possible for estimation of relative activity of tocopherol and plastoquinone/plastoquinol couple in singlet oxygen scavenging activity, as well as identification of their oxidation products and the pathways of prenyllipid oxidation.

2. Materials and methods

2.1. Culture growth and high light exposure

C. reinhardtii was grown photoautotrophically in HS medium at 25 °C under low light (70 μmol photons m^{-2} s^{-1}) conditions as described in Ref. [7]. For experiments, 7–10 day old cultures were used where chlorophyll (Chl) concentration was 20–35 μg/ml. One day before high-light experiments, the culture was set to Chl concentration of 6 μg/ml and pyrazolate (pyr) was added when necessary. The cultures were grown

Abbreviations: Chl, chlorophyll; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; DGDG, digalactosyldiacylglycerol; DNP-INT, 2′,4′-dinitrophenyl ether of 2-iodo-4-nitro-thymol; FCCP, carbonylcyanide-*p*-trifluoromethoxy-phenyl-hydrazone; MGDG, monogalactosyldiacylglycerol; PG, phosphatidylglycerol; PQ, plastoquinone; PQH_2, plastoquinol; pyr, pyrazolate, 4-(2,4-dichlorobenzoyl)-1,3-dimethylpyrazol-5-yl toluene-4-sulfonate; SQDG, sulphoquinovosyldiacylglycerol; α-Toc, α-tocopherol; α-TQ, α-tocopherolquinone

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doi:10.1016/j.bbabio.2011.12.002

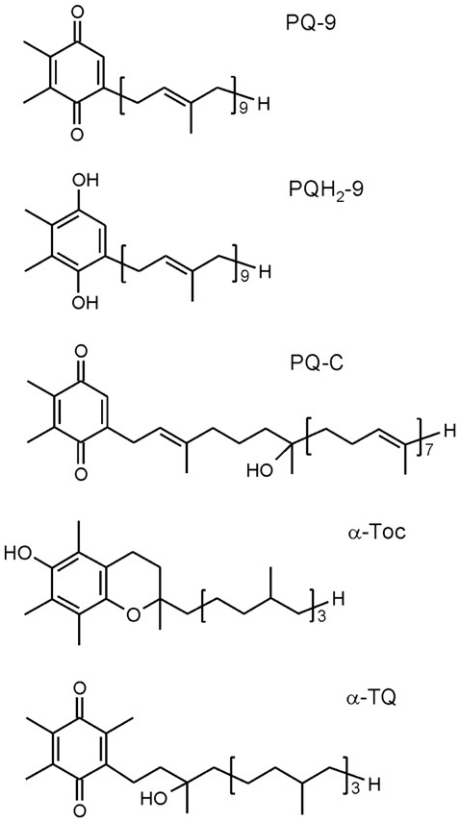

Fig. 1. Structure of the analyzed prenyllipids. PQ-9, plastoquinone-9; PQH_2-9, plastoquinol-9; PQ-C, plastoquinone-C; α-Toc, α-tocopherol; α-TQ, α-tocopherolquinone.

with continuous bubbling with air enriched in CO_2 (5%) for 18 h under 70 μmol photons m^{-2} s^{-1} illumination. Afterwards, the cultures were exposed to high light (2000 μmol photons m^{-2} s^{-1}) for 2 h. Carbonylcyanide-*p*-trifluoromethoxy-phenyl-hydrazone (FCCP), 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU) or 2′,4′-dinitrophenyl ether of 2-iodo-4-nitro-thymol (DNP-INT) were added to the medium 10 min before high-light illumination. For D_2O experiments, the cultures were centrifuged (3 min × 600 *g*) and the cells were resuspended in D_2O-based HS medium.

2.2. Sample extraction and HPLC analysis

For Chl measurements, cell suspension was centrifuged (5 min × 9000 *g*), the pellet was extracted with acetone, centrifuged again (2 min × 9000 *g*) and Chl concentration in the supernatant was determined according to Ref. [21]. For prenyllipids analysis, the culture was centrifuged (5 min × 9000 *g*), the pellet was extracted with acetone, centrifuged again (2 min × 9000 *g*), then the supernatant was evaporated and resuspended in methanol.

HPLC analysis of both redox forms of plastoquinone and α-tocopherol was performed in the following system: C_{18} reverse-phase column, eluent — methanol:hexan (340:20, v/v), flow rate of 1.5 ml/min, absorption detection at 255 nm, fluorescence detection at λ_{ex} = 290 nm, λ_{em} = 330 nm [7]. α-TQ and PQ-C were analyzed using HPLC as described in details in Refs. [22] and [7], respectively, with some modifications, *i.e.* for reduction of the prenyllipid, platinum (5 wt.% on alumina, powder, 325 mesh, Aldrich) post-column was used instead of Zn-column and the eluent was methanol:water (99:1, v/v) or methanol.

2.3. Model system study

8a-Hydroperoxy-α-tocopherone was obtained as described previously [15]. It was mixed with plant lipids (monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulphoquinovosyldiacylglycerol (SQDG), phosphatidylglycerol (PG); 4:2:1:1, mol/mol), obtained from Lipid Products (South Nutfield, Redhill, Surrey, U.K.), and the ethanol solution of these compounds was injected into a buffer (50 mM Hepes pH 7.5, 20 mM Mes pH 6.0, 50 mM citrate buffer pH 4.5 and 3.2). The final concentrations of plant lipids and of 8a-hydroperoxy-α-tocopherone were 1 mM and 50 μM, respectively. Concentrated stock solution of sodium ascorbate was added to give 30 mM final concentration after taking 'time 0' sample for analysis. For HPLC analysis, 100 μl of liposome suspension was vortexed with 300 μl of ethyl acetate for 60 s and the mixture was centrifuged (30 s × 9000 *g*). Afterwards 150 μl of the acetate layer was evaporated, dissolved in methanol and analyzed on C_{18} reverse-phase column in acetonitrile:methanol:water (72:8:1, v/v), at the flow of 1.5 ml/min using absorption detection at 260 nm and fluorescence detection at λ_{ex} = 290 nm, λ_{em} = 330 nm.

2.4. Fluorescence measurements

Maximum quantum yield of photosystem II (F_v/F_m) and non-photochemical quenching ($NPQ = (F^0_m - F'_m)/F'_m$) [23] was measured with PAM-101 instrument (Walz, Germany) using saturating pulse of 2600 μmol photons m^{-2} s^{-1} intensity and actinic light intensity of 1000 μmol photons m^{-2} s^{-1}. The control and pyrazolate-treated cultures were dark-adapted for 20 min before the measurement.

2.5. Chlorophyll and carotenoid determination

Chlorophylls concentration was determined spectrophotometrically according to Ref. [21] and carotenoids were determined by HPLC as described in Ref. [24].

3. Results

In contrast to the control culture, in the presence of pyrazolate, a biosynthetic inhibitor of *p*-hydroxyphenylpyruvate dioxygenase — an enzyme responsible for producing homogentisate, which is precursor of tocopherol and plastoquinone [10], α-tocopherol level decreased during high light stress (Fig. 2). This was explained by continuous resynthesis of α-tocopherol under stress conditions

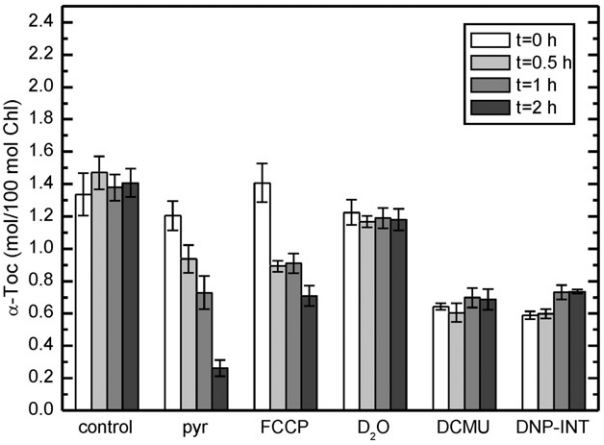

Fig. 2. The effect of high light on α-tocopherol content in the control culture, in the presence of 5 μM pyrazolate (pyr), 1 μM FCCP, 50 μM DCMU, 10 μM DNP-INT or in D_2O. The data are means ± SE (n = 3–4).

when the antioxidant is consumed irreversibly due to oxidation by singlet oxygen [10]. In the presence of FCCP, an uncoupler of pH gradient across the thylakoid membrane [25,26], the pronounced consumption of α-tocopherol was found in the first 30 min of illumination. On the other hand, heavy water, wherein singlet oxygen lifetime is prolonged, as well as inhibitors of PQ-pool reduction (DCMU) and oxidation (DNP-INT) [27] were without any effect on α-tocopherol level during high light stress. Chlorophyll concentration did not change significantly during high light treatment; only in the presence of pyrazolate it decreased about 23% after 2 h of illumination (data not shown).

When total plastoquinone was analyzed, its content increased during high light stress in the control culture, while in the presence of pyrazolate it decreased, as it was expected (Fig. 3). The percentage consumption of both plastoquinone forms was considerably faster than that of α-tocopherol, although the rate of consumption in relation to chlorophyll was similar for both prenyllipids. Moreover, the initial level of total plastoquinone was only about half of the initial α-tocopherol level in the presence of pyrazolate (Figs. 2 and 3). In the case of FCCP, the effect was even more pronounced than that for α-tocopherol. In contrast to α-tocopherol, when the culture was illuminated in D_2O, the total plastoquinone was quickly consumed. In the presence of DCMU, where oxidation of the plastoquinone-pool is expected [27], the pronounced consumption of total plastoquinone was observed while for DNP-INT, which should promote accumulation of plastoquinol [27], there was no clear effect on the total plastoquinone level (Fig. 3). It should be mentioned that DCMU was shown to decrease singlet oxygen production in photosystem II due to change in the redox potential of Q_A/Q_A^- couple [1,2].

When reduced and oxidized forms of plastoquinone were analyzed in the control culture separately during the experiment (Fig. 4), pronounced increase of the reduced form was observed but only little decrease of the oxidized form. This could be expected taking into account that plastoquinol is the primary biosynthetic form of plastoquinone and that the reduced form shows considerably more pronounced singlet oxygen scavenging activity than the oxidized form [15]. In the presence of pyrazolate, the biosynthetic inhibitor of plastoquinol, mainly the oxidized form was observed. In the presence of FCCP and D_2O, the redox state of plastoquinone was not significantly altered during the high light stress. On the other hand, as expected, in the presence of DCMU mostly the oxidized form of plastoquinone was found that was gradually decreasing during the experiment. It seems that in the presence of DCMU, newly synthesized plastoquinol is rapidly oxidized to plastoquinone in the electron transport chain. When DNP-INT was applied, a similar time course of the level of both plastoquinone forms as in the control culture was observed, although in this case no increase in total plastoquinone occurred (Fig. 3).

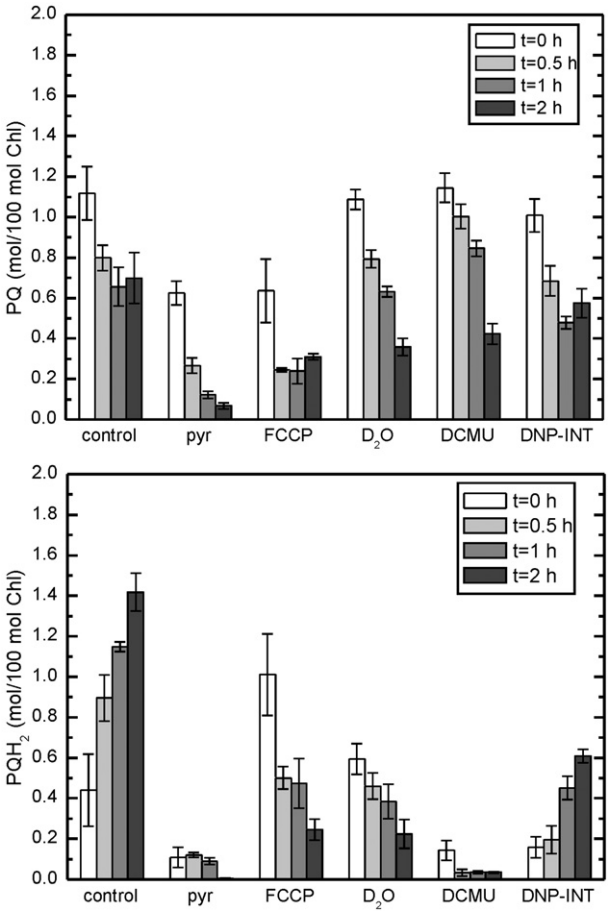

Fig. 4. The effect of high light on the content of oxidized (top) and reduced (bottom) plastoquinone in the control culture, in the presence of 5 μM pyrazolate, 1 μM FCCP, 50 μM DCMU, 10 μM DNP-INT or in D_2O, (n = 3–4).

A valuable information on the antioxidant activity of the investigated prenyllipids can be also obtained from the analysis of their oxidation products. Well known stable product of singlet oxygen scavenging activity of α-tocopherol is α-tocopherolquinone [15]. When it was analyzed during high light stress (Fig. 5), its level did

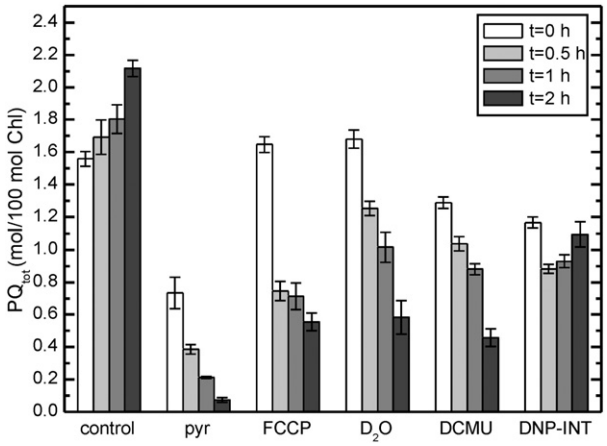

Fig. 3. The effect of high light on total plastoquinone (oxidized and reduced) content in the control culture, in the presence of 5 μM pyrazolate, 1 μM FCCP, 50 μM DCMU, 10 μM DNP-INT or in D_2O, (n = 3–4).

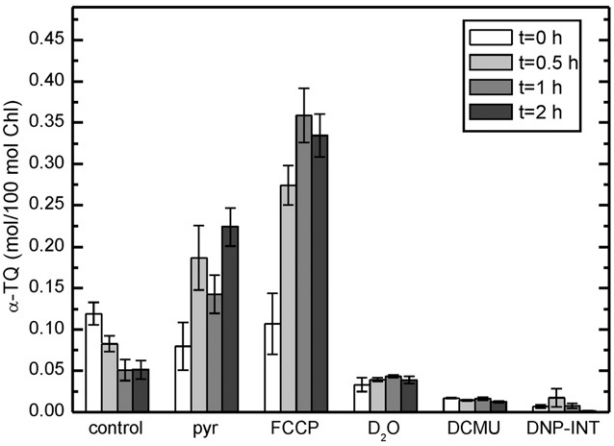

Fig. 5. The effect of high light on α-tocopherolquinone content in the control culture, in the presence of 5 μM pyrazolate, 1 μM FCCP, 50 μM DCMU, 10 μM DNP-INT or in D_2O, (n = 3–4).

not change significantly, like that of α-tocopherol (Fig. 2). In the case of cultures with pyrazolate, the level of α-tocopherolquinone increased evidently during illumination, although not as much as α-tocopherol declined (Fig. 2). Interestingly, the most pronounced increase in α-tocopherolquinone content was found after 30 min of illumination of the culture with FCCP (Fig. 5). This correlated well with the decrease in α-tocopherol level (Fig. 2). The effect of FCCP points to a role of pH gradient in the mechanism of α-tocopherol oxidation.

The level of PQ-C increased during the high light stress most clearly in D_2O-based culture, as well as in the cultures with the addition of electron transport inhibitors (Fig. 6). In these cases, the increase in plastoquinone-C correlated well with the decrease in the level of plastoquinone (Fig. 4), suggesting that plastoquinone is oxidized to plastoquinone-C as a result of singlet oxygen scavenging. On the other hand, the cultures with pyrazolate or FCCP showed no significant changes in the plastoquinone-C level during high light stress.

In order to investigate details of the mechanism of FCCP action in oxidation of the prenyllipids, the combined effects of FCCP and pyrazolate or D_2O were analyzed (Fig. 7). For all the cultures, changes in the levels of the investigated prenyllipids were very pronounced, especially those for α-tocopherolquinone, where the increase approximately corresponded to the consumption of α-tocopherol in these cultures.

In order to examine the possibility of α-tocopherol recycling (Fig. 8) from its primary oxidation product by singlet oxygen, 8a-hydroperoxy-α-tocopherone [15], the latter compound was incorporated into liposomes and the pH dependence of tocopherols and α-tocopherolquinone was measured in the presence of ascorbate (Fig. 9). The results clearly demonstrate that α-tocopherol can be non-enzymatically formed from the peroxide, especially at acidic conditions. At pH close to neutral (7.5), practically only α-tocopherolquinone was formed. In analogical experiments, where the peroxide was replaced by α-tocopherolquinone or its reduced form, no α-tocopherol formation was observed (data not shown).

Taking into consideration that pyrazolate might indirectly affect carotenoid biosynthesis, in subsequent experiments we measured effect of the inhibitor on photosynthetic pigment composition and fluorescence parameters. The most evident effect of pyrazolate was lowering of the proportion of total carotenoids to chlorophylls, especially after high light stress (Table 1). Chlorophyll *a* to *b* ratio was not much affected. In carotenoid composition, the most pronounced change was the increase of neoxanthin content at the expense of lutein and β-carotene. Under pyrazolate action, there was 25% less carotenoids under low light and 36% less under high light stress in respect to chlorophylls. In the control culture under low light conditions, the epoxidation ratio, reflecting

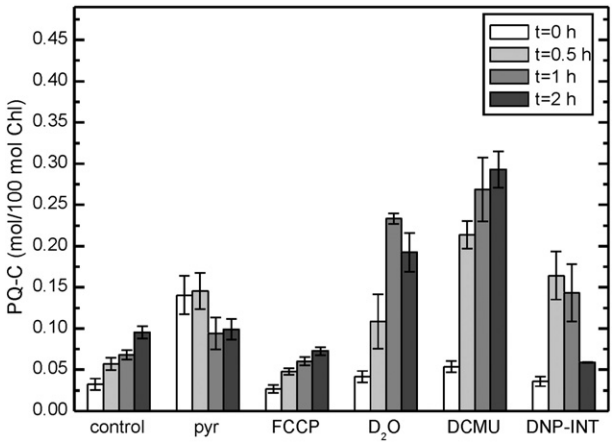

Fig. 6. The effect of high light on plastoquinone-C content in the control culture, in the presence of 5 μM pyrazolate, 1 μM FCCP, 50 μM DCMU, 10 μM DNP-INT or in D_2O, (n = 3–4).

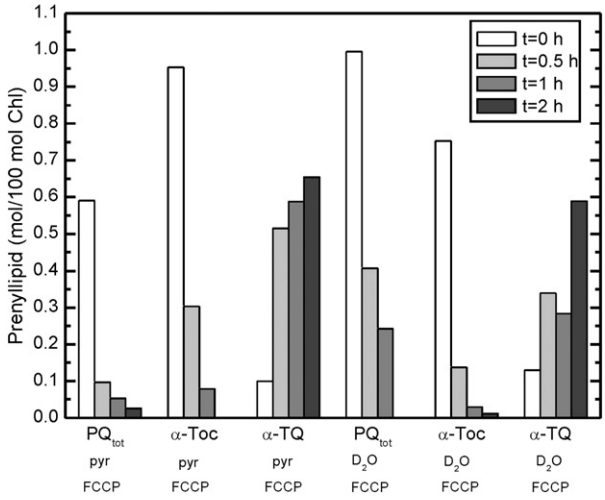

Fig. 7. The effect of high light on the content of prenyllipids in cultures containing both 1 μM FCCP and 5 μM pyrazolate or 1 μM FCCP and D_2O, (n = 3–4).

activation of the xanthophyll cycle, was close to one, while under pyrazolate treatment the ratio dropped below 0.9 (Table 1), indicating activation of the cycle. The maximum yield of photosystem II was similar in both cases (Table 1), however non-photochemical quenching was lower during high light illumination in inhibitor-treated cultures (Fig. 10).

4. Discussion

Deactivation of singlet oxygen is a process of physical quenching and chemical scavenging [6] the latter manifesting in oxidation of an antioxidant that can be followed in our study as a decrease in concentration of the prenyllipids. In the case of tocopherol and plastoquinol their oxidation *in vitro* proceeds at similar rates although the physical quenching rates are different [15]. The literature data indicate that physical quenching of singlet oxygen dominates over chemical scavenging for tocopherols by at least 2 orders of magnitude [6]. It has been calculated that one tocopherol molecule is able to deactivate 40–120 molecules of singlet oxygen before it is oxidized [28]. Similar data can be expected for plastoquinol. Thus, the decomposition kinetics of both prenyllipids observed in the present study can reflect their detoxification activity against singlet oxygen. The effects of pyrazolate (a biosynthetic inhibitor of tocopherols and plastoquinol), as well as of D_2O (where the lifetime of singlet oxygen is considerably increased [29]) indicate that plastoquinol/plastoquinone is consumed more efficiently than α-tocopherol under high light stress and these data also suggest that plastoquinol/plastoquinone is more active than α-tocopherol in singlet oxygen deactivation. The kinetic data of singlet oxygen quenching indicate that plastoquinol is over 5 times more active than plastoquinone in this process in polar solvents, while this ratio is 3.5 in hydrophobic environment [15]. These data suggests the reduced form of plastoquinone (plastoquinol) is mainly responsible for the observed effects *in vivo*. Moreover, the results obtained for DCMU-treated cultures, where only oxidized plastoquinone is found, indicate that also this form shows singlet oxygen scavenging activity. As a result of this reaction, plastoquinone-C is formed, the prenyllipid previously shown to be the main product of plastoquinone oxidation by singlet oxygen *in vitro* [15]. The present results are also in agreement with those obtained *in vitro* showing that the order of oxidation reactions by singlet oxygen is plastoquinol → plastoquinone → plastoquinone-C, *i.e.* the latter is not formed directly from plastoquinol. The obtained data point also to the suggestion that plastoquinone-C can be regarded as an indicator of singlet oxygen oxidative stress *in vivo*.

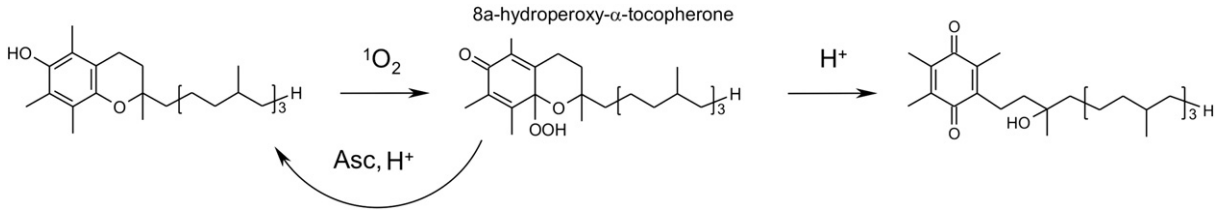

Fig. 8. Singlet oxygen-initiated oxidation pathway of α-tocopherol and its recycling under acidic conditions. Asc, ascorbate; α-Toc, α-tocopherol; α-TQ, α-tocopherolquinone.

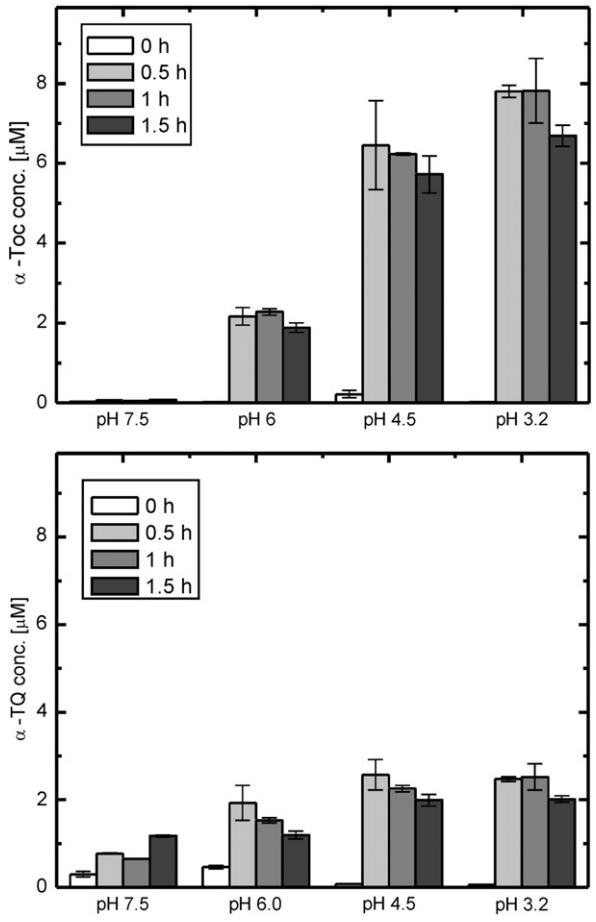

Fig. 9. The effect of pH on α-tocopherol (top) and α-tocopherolquinone (bottom) formation from 8a-hydroperoxy-α-tocopherone (50 μM) in plant lipid liposomes, in the presence of 30 mM ascorbate, (n = 2–3).

One of the most intriguing findings of the present study is the strong stimulatory effect of an uncoupler, FCCP, on α-tocopherol and plastoquinone/plastoquinol oxidation. This effect could be partially due to inhibition of non-photochemical quenching which requires proton gradient for activation. The concentration of FCCP used in our study was not affecting photosystem II activity since no redox change of plastoquinone was found. The reason why the uncoupler was effective only during the first 30 min of illumination could be its low concentration used and its photodegradation in the course of the experiment. The effect of FCCP was considerably more pronounced on accumulation of α-tocopherolquinone, the final oxidation product of α-tocopherol, than on plastoquinone-C, the product of plastoquinone oxidation. This points to the possible mechanism of α-tocopherol oxidation and recycling in thylakoid membranes. It is known that α-tocopherol can be recycled from α-tocopherolquinone in methanol in the presence of ascorbic acid at acidic conditions [30]. However, when the reaction was performed in liposomes prepared from plant lipids or phosphatidylcholine we have observed no α-tocopherol formation (data not shown). On the other hand, when α-tocopherolquinone was replaced by 8a-hydroperoxy-α-tocopherone, α-tocopherol was formed efficiently at low pH. This observation points to the suggestion that such a non-enzymatic reaction could take place *in vivo* in thylakoid membranes at high light conditions, where low pH is supposed to be generated in the lumen of thylakoids. In the presence of FCCP, pH gradient is uncoupled and formation of α-tocopherolquinone from the peroxide dominates. This hypothesis would explain why little α-tocopherolquinone is formed under high light stress in the control culture and the stimulatory effect of FCCP on α-tocopherolquinone formation during the stress. However, it was shown that pH of the lumen is not as acid as suggested before under high light conditions and remains moderate (between 5.8 and 6.5) [31]. Moreover, it could be unclear why the recycling mechanism is not active in the presence of pyrazolate where α-tocopherolquinone accumulates. As

Table 1
Photosynthetic pigment composition and F_v/F_m parameter of *Chlamydomonas* control and pyrazolate-treated cultures, (n = 3). Chl — chlorophyll, Car — carotenoids, HL, high light, epoxidation ratio = (V + 1/2A) / (V + A + Z) where V, A, Z are violaxanthin, antheraxanthin and zeaxanthin, respectively.

	Control	Pyrazolate
Chl *a*/*b*	2.03 ± 0.03	1.89 ± 0.02
Car/Chl (μg Car/10 μg Chl *a* + *b*)	3.46 ± 0.01	2.60 ± 0.04
Car/Chl (μg Car/10 μg Chl *a* + *b*) + 2 h HL	4.05 ± 0.03	2.60 ± 0.01
Percent content of total carotenoids		
Neoxanthin	32.6 ± 3.0	40.7 ± 0.5
Violaxanthin	22.8 ± 0.5	19.2 ± 0.3
Antheraxanthin	0.8 ± 0.0	3.7 ± 0.1
Lutein	29.5 ± 3.8	24.5 ± 0.3
Zeaxanthin	0.0 ± 0.0	1.3 ± 0.1
β-Carotene	14.1 ± 0.3	10.5 ± 0.1
Epoxidation ratio	0.98 ± 0.01	0.87 ± 0.01
F_v/F_m	0.704 ± 0.004	0.692 ± 0.013

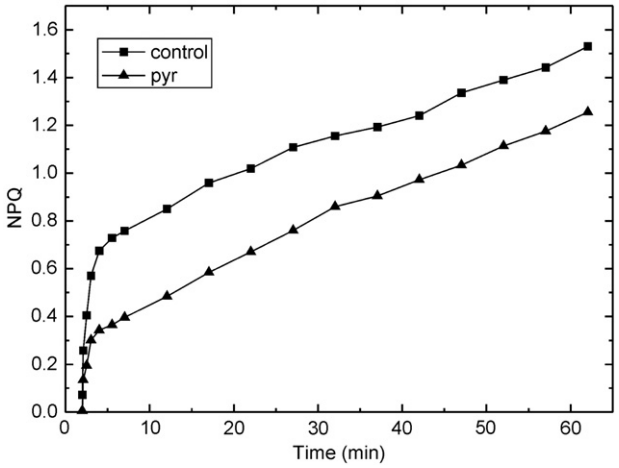

Fig. 10. Non-photochemical quenching (NPQ) during illumination of control and pyrazolate-treated cultures. Actinic light of 1000 μmol photons $m^{-2} s^{-1}$ intensity was applied.

we have shown, pyrazolate also affects indirectly carotenoid biosynthesis by decreasing the level plastoquinone that is required for phytoene desaturation [32]. The lowered carotenoid content may cause structural defects in reaction center and antenna complexes, which results in increased singlet oxygen production and impaired non-photochemical quenching mechanisms. Moreover, carotenoids are important singlet oxygen quenchers in chlorophyll–protein complexes. Under such conditions photosynthetic apparatus does not function properly and the recycling mechanism is not able to overcome the photooxidative effects of increased singlet oxygen generation and keep the required level of prenyllipids.

It cannot be also excluded that a still hypothetical, enzymatic reaction regenerating α-tocopherol directly from α-tocopherolquinone occurs in *Chlamydomonas*, as recently suggested for *Arabidopsis* [33].

Acknowledgements

This work was supported by grant NN303 809440 from the Polish Ministry of Science and Higher Education.

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