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Superoxide generated in photosystem I is scavenged by plastoquinol and other natural prenyllipids in thylakoid membranes.

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Introduction

The main source of oxygen consumption in chloroplasts is chlororespiration and photoreduction of oxygen by low-potential Fe-S centers in photosystem I (PS I), however, only the superoxide generation by PS I is directly light dependent. Within PS I, superoxide is produced in the aprotic, hydrophobic interior of the thylakoid membrane at Fe-S centers: X and A/B. Because of the aprotic character of the membrane interior, the lifetime of superoxide is extended. After diffusing to the membrane surface, the superoxide undergoes protonation and dismutation to hydrogen peroxide. It was suggested (Takahashi et al. 1980) that superoxide could also reduce oxidized cytochrome f or plastocyanin forming pseudocyclic electron transport around PS I.

In our study, we examined whether the prenyllipids present in thylakoid membranes, like plastoquinone (PQ-9), its reduced form (PQH_2), tocoquinone (TQ) and tocopherol (Toc) react with the superoxide generated in PS I. Such a reaction would inhibit and prevent the formation of the toxic hydrogen peroxide in chloroplasts.

The formation of hydrogen peroxide from oxygen manifests in the oxygen consumption that can be followed directly using an oxygen electrode. Another indirect approach used in many previous studies (Asada et al. 1974, Miyake et al. 1998, Takahashi and Asada 1988) was to observe spectrophotometrically the reduction of the added cytochrome c that is reduced by superoxide. As a result of this reaction, oxygen is released back from superoxide and this process manifests in the inhibition of hydrogen peroxide formation and oxygen consumption. However, our own experiments showed that at low and medium light intensity cytochrome c is mainly reduced by the PQ-pool in thylakoids and not by superoxide generated in PS I (Kruk et al., unpublished results). Therefore, the method based on cytochrome c reduction is not suitable and not specific for the measurements of superoxide generation in thylakoids and direct methods of oxygen consumption measurements seem to be only appropriate for this purpose.

Materials and methods

Spinach thylakoids were isolated according to the method described by Robinson and Yocum (1980). The oxygen consumption measurements were performed in 50 mM Hepes buffer, pH 7.5 containing 10 mM NaCl and 5 mM $MgCl_2$ using a three electrode system (Schmid and Thibault 1979) in the presence of 50 μM DCMU and 10 mM hydroquinone as an electron donor. Saturating light flashes of 5 μs (full width at half-maximum) were provided by a xenon lamp (Stroboscope 1539A from General Radio). The samples were illuminated by 15 flashes spaced 300 ms apart. Hexane extraction of thylakoids was performed by 3-fold extraction of lyophylised thylakoids (1 mg Chl) with 1 ml hexane for 20 min, each extraction. After centrifugation and removing the supernatant, thylakoids were dried in rotatory evaporator and suspended in the buffer giving final conc. of 1 mg/ml Chl and used as a stock solution. 1 μM vit. K_1 was added to all the extracted samples.

Results and Discussion

We used two approaches in the investigation of the inhibition of superoxide generation by PS I. In one case, the prenyllipids were added to fresh, dark adapted thylakoids suspension. Since within thylakoid membranes some of the investigated prenyllipids (PQ-9, Toc, TQ) are already present, we used lyophylised and hexane-extracted thylakoids to obtain the control sample devoid of the prenyllipids. This procedure removes selectively prenyllipids from the membranes preserving their structure and function. Because this extraction removes also partially vitamin K_1 (vit. K_1) from PS I, which is the primary electron acceptor in PSI, we added this vitamin at 1 μM concentration to all extracted samples. First, we measured the influence of the extracted components added back to the thylakoids (Table 1).

Table 1. Oxygen consumption by flash-illuminated, hexane-extracted spinach thylakoids (150 μg/ml Chl) in the presence of 10 mM hydroquinone and 50 μM DCMU. The numbers in '( )' denote the proportion of the added components of the extract in relation to their original concentration in thylakoids; Y_1 and Y_2 correspond to oxygen uptake amplitudes at the first and the second flash, respectively.

sample	oxygen uptake (a.u.)	oxygen uptake (% of control)	Y_1/Y_2 signals ratio
control	15±1	100	1.0
+ extract (1:1)	12±1	80	1.3
+ extract (5:1)	5±1	30	2.0

It can be seen from the Table 1 that components of the extract inhibit the oxygen consumption by PS I. The main prenyllipid components of this extract are PQ-9 and Toc which are probably responsible for the observed effects. With the increase in the added amount of the extract, the ratio of the first two signals (Y_1/Y_2) increases. Similar relationship was observed in the experiments on extracted thylakoids when the increasing amount of vit. K_1 was added

to the extracted thylakoids (data not shown). This indicates that vit. K_1 present in the extract was responsible for the observed changes in the Y_1/Y_2 ratio.

The results of the experiments on the influence of different native and synthetic (PQ-2) prenyllipids on the oxygen uptake by PS I measured on hexane-extracted thylakoids are shown in Table 2.

Table 2. Inhibition of oxygen consumption by flash-illuminated, hexane-extracted spinach thylakoids in the presence of different prenyllipids at the prenyllipid/chl ratio 1:5 and 1:10 (mol/mol). Other conditions as in Table 1.

	prenyllipid/chl ratio 1:5 (mol/mol)		prenyllipid/chl ratio 1:10 (mol/mol)	
sample	oxygen uptake (a.u.)	oxygen uptake (% of control)	oxygen uptake (a.u.)	oxygen uptake (% of control)
control	14 ±1	100	-	-
Toc	8 ±1	57	12 ±2	86
TQ	11 ±1	78	12 ±1	86
PQ-2	7 ±0.5	50	9.5 ±0.5	68
PQ-9	5.5 ±0.5	40	9 ±1	64
TQH_2	6 ±1	43	8.5 ±1	61
PQH_2-2	6 ±1	43	8 ±1	57
PQH_2-9	4 ±0.5	29	7.5 ±0.5	53

The presented data show that all the investigated prenyllipids inhibited the measured oxygen consumption by PS I. The most effective in this respect was PQH_2-9 followed by PQ-9, PQH_2-2 and TQH_2. The well-known antioxidant, Toc was relatively poorly active whereas TQ showed nearly no effect in the investigated reaction. All the three reduced forms of prenylquinones were more active than the corresponding oxidised forms. Since PQ-2 and PQ-9 have the same redox potentials, the differences observed for both the reduced and oxidized forms of these two plastoquinones are probably caused by their different localization in thylakoid membranes. PQ-2, TQ and Toc are supposed to be located close to the membrane surface, while the reduced and especially the oxidized form of PQ-9 are supposed to reside close to the interior of the thylakoid membrane. Since the superoxide is generated in the hydrophobic, internal part of the membrane, both PQ-9 forms, which are located close or at the site of superoxide generation, have the highest activity in the reaction with superoxide

among the investigated prenyllipids. The influence of the investigated prenyllipids on the oxygen consumption for the untreated thylakoids (not extracted) is presented in Table 3.

Table 3. Inhibition of oxygen consumption by flash-illuminated spinach thylakoids in the presence of different prenyllipids at the prenyllipid/chl ratio 1:2.5 and 1:5 (mol/mol). Other conditions as in Table 1. The sample with ferredoxin (Fd) was measured additionally in the presence of 30 mM $MgCl_2$.

	prenyllipid/chl ratio 1:2.5 (mol/mol)		prenyllipid/chl ratio 1:5 (mol/mol)	
sample	oxygen uptake (a.u.)	oxygen uptake (% of control)	oxygen uptake (a.u.)	oxygen uptake (% of control)
control	65 ±3	100	-	-
Toc	41 ±2	63	51 ±1	78
TQ	32 ±1	49	41 ±1	63
PQ-2	55 ±2	85	63 ±2	97
PQ-9	45 ±1	70	60 ±2	92
TQH_2	47.5 ±2	73	-	-
PQH_2-2	47.5 ±2	73	-	-
PQH_2-9	25 ±1	38	-	-
5 μM Fd + 0.25 mM NADPH	20 ±4	30		

The results show that similar pattern of the inhibition for the reduced prenylquinones and Toc can be observed as in the case the extracted thylakoids. However, the influence of both plastoquinones on the oxygen uptake is considerably less pronounced and that of TQ is more evident than in the case of extracted thylakoids. Generally, higher concentration of the prenyllipids are required to obtain similar extend of inhibition for the reduced prenylquinones or Toc. This is probably caused by only partial incorporation of the added compounds into the lipid bilayer because of the presence of native prenyllipids, as well as peripheral membrane proteins that are partially removed in the case of extracted thylakoids. The PQH_2-9 formed in thylakoids by enzymatic reduction of PQ-9 with ferredoxin-PQ reductase, after addition of ferredoxin and NADPH, was even more active in the inhibition of oxygen consumption than the externally added PQH_2-9 (Table 3).

Our results indicate that the PQH_2-9/PQ-9 couple, as well as other natural membrane prenyllipids, such as α-Toc or α-TQH_2, play an important role in scavenging superoxide

radical formed in PS I. This reaction reduces the level of superoxide diffusing towards membrane surface and inhibits formation of the toxic hydrogen peroxide in chloroplasts.

The model presented below shows the protective function of the investigated prenyllipids against the superoxide generated by PS I in thylakoid membranes.

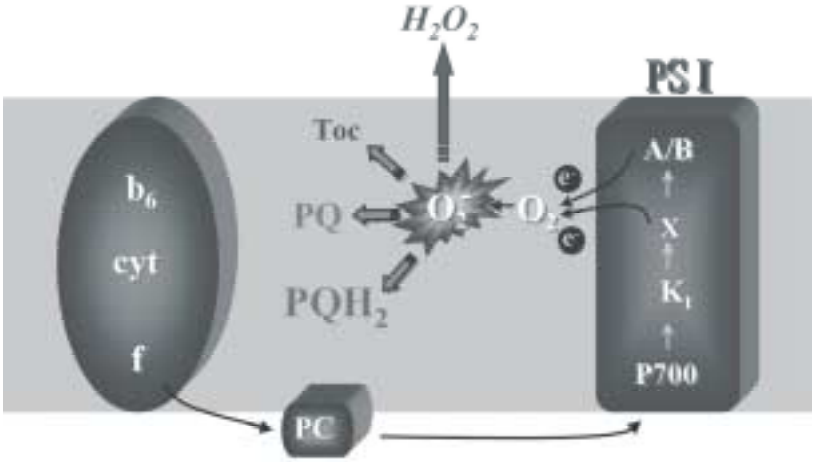

Fig. 1. The model shows generation of superoxide radical in PS I and its scavenging by membrane prenyllipids. The size of the prenyllipid symbols reflects their reactivity with superoxide (based on the results from Table 2).

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