Cytochrome *c* is reduced mainly by plastoquinol and not by superoxide in thylakoid membranes at low and medium light intensities: its specific interaction with thylakoid membrane lipids

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We have found that, at low light intensity (5–10 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), photoreduction of cyt (cytochrome) *c* by isolated thylakoids was not inhibited by dinitrophenylether of iodonitrothymol, an inhibitor of the cyt *b₆-f* complex, and the inhibition was only partial at medium light intensity (50–200 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$). The photoreduction was not significantly influenced by superoxide dismutase. The conclusion that cyt *c* could be reduced directly by the plastoquinone pool was confirmed by the observation that plastoquinol-9 reduced cyt *c* efficiently when it was incorporated into liposome membranes prepared from thylakoid membrane lipids. It was shown that the cyt is specifically bound to thylakoid lipid liposomes owing to the presence of nega-

tively charged lipids, phosphatidylglycerol and sulphoquinovosyldiacylglycerol, and the reduction was stimulated by the presence of monogalactosyldiacylglycerol, an inverted micelles-forming lipid, in the membranes, where the cyt *c* reduction by plastoquinol probably takes place. The results obtained are also discussed in terms of reliability of the method of cyt *c* photoreduction for determining superoxide production by illuminated thylakoids.

Key words: cytochrome *c*, liposome, oxygen evolution, plastoquinol, superoxide, thylakoid lipid.

INTRODUCTION

During photosynthetic electron transport in thylakoids, a significant part of the electron flow can be directed from PSI (photosystem I) to molecular oxygen, forming superoxide and hydrogen peroxide that is further decomposed to water in stroma of chloroplasts by ascorbate peroxidase [1]. Such an electron-transfer pathway is called the water–water cycle [1], and its function is supposed to prevent photoinhibition under excess light conditions [2]. In isolated thylakoids, the main source of superoxide is probably the low-potential iron–sulphur centres (F_x , $F_{A/B}$) [3] or vitamin K_1 , whereas in intact chloroplasts a PSI-associated flavin enzyme MDAR (monodehydroascorbate reductase) is the main source of superoxide radicals [4]. The thylakoid-dependent superoxide generation is saturated at low light intensity (10 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) [1] with the production rates of superoxide over the range 15–25 $\mu\text{mol} \cdot (\text{mg of Chl})^{-1} \cdot \text{h}^{-1}$ (where Chl stands for chlorophyll) [5], whereas this reaction in chloroplasts is saturated at considerably higher light intensities (approx. 300 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) [2] and the rate of superoxide generation may reach as much as 240 $\mu\text{mol} \cdot (\text{mg of Chl})^{-1} \cdot \text{h}^{-1}$ [1]. Among the different methods of determining superoxide production in thylakoids and chloroplasts [5], the photoreduction of cyt (cytochrome) *c* is one of the most widely used methods [3,4,6,7]. This reaction can be conveniently followed spectrophotometrically. Since cyt reduction might be non-specific, inhibition of this reaction by SOD (superoxide dismutase) is regarded as an evidence of superoxide-mediated cyt *c* reduction. However, in many studies where superoxide generation by thylakoids or chloroplasts was studied, this inhibition was between 25 and 80 % [3,4,6,7]. Moreover, it was found

that the inhibition was dependent on the buffer used [6]. These results suggest that cyt *c* can be also reduced directly by some components of the photosynthetic electron-transport chain, such as PQH₂ (plastoquinol).

In the present study, we tried to determine whether there are pathways of cyt *c* reduction, other than superoxide-mediated, in thylakoids and chloroplasts at different light intensities by using electron-transport inhibitors and examining SOD sensitivity of cyt *c* photoreduction. To investigate the possibility of direct cyt *c* reduction by PQH₂ in thylakoid membranes, we measured this reaction in liposome system. The mechanism of cyt *c* production by thylakoids and chloroplasts is discussed in respect to the reliability of the method of cyt *c* photoreduction for the measurement of superoxide generation in thylakoids and chloroplasts.

MATERIALS AND METHODS

Thylakoids and chloroplasts from 7-day-old wheat plant were isolated by the method described in [8]. All measurements were performed at a Chl concentration of 25 $\mu\text{g/ml}$ in 50 mM Hepes buffer (pH 7.5), containing 20 mM NaCl and 5 mM MgCl₂ in a final volume of 2.5 ml. Photoreduction of cyt *c* was measured using red light (> 630 nm) for illumination. Cyt *c* from pig hearts was obtained from Biomed (Kraków, Poland) and SOD from bovine erythrocytes was from Boehringer Mannheim (Mannheim, Germany). DNP-INT (dinitrophenylether of iodonitrothymol) was a gift from Professor W. Oettmeier (Ruhr University, Bochum, Germany) and PQ (plastoquinone) homologues, PQ-1–4, were gifts from Dr H. Koike (Himeji Institute of Technology, Hyogo, Japan). PQ-9 was obtained as described

Abbreviations used: Chl, chlorophyll; cyt, cytochrome; DBMIB, dibromothymoquinone; DGDG, digalactosyldiacylglycerol; DMBQ, dimethylbenzoquinone; DNP-INT, dinitrophenylether of iodonitrothymol; FeCy, ferricyanide; MDAR, monodehydroascorbate reductase; MGDG, monogalactosyldiacylglycerol; MV, methylviologen; PC, egg-yolk phosphatidylcholine; PG, phosphatidylglycerol; PQ, plastoquinone; PQH₂, plastoquinol; PSI, photosystem I; SOD, superoxide dismutase; SQDG, sulphoquinovosyldiacylglycerol.

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by Kruk [9]. PQH₂s were prepared from the corresponding oxidized forms by the method described in [10]. PC (egg-yolk phosphatidylcholine), type V-E, was obtained from Sigma; the plant lipids MGDG (monogalactosyldiacylglycerol), DGDG (digalactosyldiacylglycerol), SQDG (sulphoquinovosyldiacylglycerol) and PG (phosphatidylglycerol) were obtained from Lipid Products (South Nutfield, Redhill, Surrey, U.K.).

Liposomes were prepared by the injection method [11]. After mixing appropriate volumes of lipid stock solutions in chloroform and PQH₂ in hexane, the solvent was evaporated under a stream of nitrogen and then under vacuum for 10 min. Subsequently, the dried mixture was dissolved in 25 μ l of ethanol and injected into 2 ml of the buffer with a Hamilton syringe under continuous stirring. The total lipid concentration in the ethanol solution used for preparing liposomes was 40 mM, and the final lipid concentration in the buffer was 0.5 mM for all the samples. The detailed lipid composition and PQH₂ content in liposomes are given in the Figure legends. The molecular masses of the lipids used for preparing liposomes were assumed to be 786, 775, 936, 824 and 743 for PC, MGDG, DGDG, SQDG and PG respectively. Anaerobic conditions, used where indicated, were obtained by bubbling the mixture with nitrogen for 5 min. Cyt *c* reduction was measured in a dual-wavelength mode at 550–560 nm using an SLM Aminco DW2000 spectrophotometer equipped with a stirring accessory. Cyt *c* reduction rate was determined using molar absorption coefficient of 19 000 [12]. Oxygen evolution or uptake was measured with a Clark electrode (Hansatech, King's Lynn, Norfolk, U.K.) under saturating white-light illumination.

RESULTS

Figure 1 shows the light intensity dependence of cyt *c* photoreduction by isolated thylakoids and chloroplasts. In both cases, photoreduction occurs at low light intensity and saturates at approx. 100 μ mol photons $\cdot$ m⁻² $\cdot$ s⁻¹. The maximal rates of cyt *c* photoreduction are approx. 18 and 22 μ mol $\cdot$ (mg of Chl)⁻¹ $\cdot$ h⁻¹ for thylakoids and chloroplasts respectively (Table 1). Assuming that all the produced superoxide molecules are trapped by cyt *c*, these values correspond to the superoxide production rates and fit well into the range reported for the isolated thylakoids. To examine the possible sites of cyt *c* reduction in the electron-transport chain, we measured its reduction in the presence of various inhibitors. As expected, 3-(3,4-dichlorophenyl)-1,1-dimethylurea totally inhibited the photoreduction at all the light intensities used (results not shown), indicating that neither superoxide is produced by PSII nor cyt *c* is reduced directly by this photosystem. DBMIB (dibromothymoquinone), the well-known inhibitor of the cyt *b*₆-*f* complex, appeared not to be useful for our measurements since it strongly stimulated cyt *c* photoreduction (results not shown), probably due to the DBMIB autooxidation. The most appropriate inhibitor of the cyt *b*₆-*f* complex was found to be DNP-INT, which acts at the same site as DBMIB but is not redox-active [13]. The initial rates of photoreduction of cyt *c* in the presence of this inhibitor at different light intensities are shown in Table 1. Apparently, the inhibition of cyt *c* reduction was the lowest at dim light and increased with increase in the light intensity, reaching 68 % for thylakoids and 78 % for chloroplasts at 200 μ mol photons $\cdot$ m⁻² $\cdot$ s⁻¹ in terms of the initial photoreduction rate (Table 1). This suggests that at low light intensity and also partially at the highest light intensity applied, cyt *c* is reduced between 3-(3,4-dichlorophenyl)-1,1-dimethylurea and DNP-INT inhibition sites, probably by the reduced PQ of the pool. The initial photoreduction rate of cyt *c* was not sensitive to SOD at 5–50 μ mol photons $\cdot$ m⁻² $\cdot$ s⁻¹ and it was inhibited only by 20 % for thylakoids and 25 % for chloroplasts at 200 μ mol

Figure 1 Photoreduction of cyt *c* by wheat thylakoids and chloroplasts at different light intensities

Measurements were performed at a Chl concentration of 25 μ g/ml in 50 mM Hepes buffer (pH 7.5), containing 20 mM NaCl and 5 mM MgCl₂. Photoreduction of 20 μ M cyt *c* was measured using red light (> 630 nm). Arrows denote 'light on'.

Table 1 Initial rates of cyt *c* photoreduction by wheat thylakoids and chloroplasts at different light intensities and the effect of SOD and DNP-INT

Chl concentration, 25 μ g/ml; 50 mM Hepes (pH 7.5), containing 20 mM NaCl and 5 mM MgCl₂; 20 μ M cyt *c*; 20 μ M DNP-INT; 50 μ g/ml SOD. Means $\pm$ S.D are from five replicates.

Light intensity (μ mol photons $\cdot$ m ⁻² $\cdot$ s ⁻¹)	Addition	Photoreduction rate of cyt <i>c</i> [μ mol $\cdot$ (mg of Chl) ⁻¹ $\cdot$ h ⁻¹]	
		Thylakoids	Chloroplasts
200	—	17.8 $\pm$ 1.0	21.8 $\pm$ 1.3
200	SOD	14.4 $\pm$ 0.8	16.3 $\pm$ 0.9
200	DNP-INT	5.7 $\pm$ 0.5	4.9 $\pm$ 0.5
100	—	15.5 $\pm$ 0.9	20.0 $\pm$ 1.0
50	—	12.6 $\pm$ 0.7	15.9 $\pm$ 0.9
50	SOD	12.9 $\pm$ 0.6	—
50	DNP-INT	4.37 $\pm$ 0.30	—
10	—	4.4 $\pm$ 0.2	6.6 $\pm$ 0.3
10	DNP-INT	3.8 $\pm$ 0.1	6.1 $\pm$ 0.3
5	—	1.94 $\pm$ 0.20	3.1 $\pm$ 0.2
5	SOD	1.90 $\pm$ 0.10	3.0 $\pm$ 0.1
5	DNP-INT	1.90 $\pm$ 0.20	2.2 $\pm$ 0.1

photons $\cdot$ m⁻² $\cdot$ s⁻¹ (Table 1). This may suggest that superoxide-mediated cyt *c* reduction in our system is insignificant even at the highest light intensities used. To exclude the possibility

Table 2 Effect of DNP-INT on the oxygen evolution or uptake by wheat thylakoids in the presence of different electron acceptors

Chl concentration, 25 $\mu\text{g}/\text{ml}$; 50 mM Hepes (pH 7.5), containing 20 mM NaCl and 5 mM MgCl_2 . The reaction was measured with the Clark electrode under saturating white-light (WL) illumination ($1000 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) or red light (RL) illumination ($200 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, $> 630 \text{ nm}$). The numbers in parentheses denote percentage of the corresponding control without the inhibitor. Means $\pm$ S.D. are from five replicates.

	Oxygen evolution or uptake [$\mu\text{mol of O}_2 \cdot (\text{mg of Chl})^{-1} \cdot \text{h}^{-1}$]	
	WL	RL
Sample + 1 mM DMBQ	117.6 ± 6.4	57.7 ± 3.0
Sample + 1 mM DMBQ + 20 μM DNP-INT	76.2 ± 5.1 (65 %)	32.2 ± 2.8 (56 %)
Sample + 1 mM FeCy	54.6 ± 3.9	54.0 ± 3.1
Sample + 1 mM FeCy + 20 μM DNP-INT	7.4 ± 0.8 (14 %)	6.5 ± 0.9 (12 %)
Sample + 50 μM MV	-59.2 ± 4.1	-57.1 ± 2.9
Sample + 50 μM MV + 20 μM DNP-INT	-6.3 ± 0.9 (11 %)	-4.8 ± 0.7 (8 %)

that the low inhibition by the added SOD is due to the presence of endogenous thylakoid-bound SOD, the experiments with SOD were also performed in the presence of 1 mM KCN (inhibitor of SOD), but there were no significant differences observed in the cyt *c* reduction rates (results not shown).

The partial inhibition of the cyt *c* photoreduction by DNP-INT in thylakoids and chloroplasts (Table 1), especially at higher light intensities applied, may suggest that PSI could be responsible for cyt *c* reduction to a certain degree. The results presented in Table 2 show that DNP-INT at the concentration used considerably inhibits electron transfer to PSI in the presence of MV (methylviologen), as expected, and partially inhibits electron transfer to a Q_B site where DMBQ (dimethylbenzoquinone) is supposed to accept electrons from PSII. However, when FeCy (ferricyanide) is used as an electron acceptor, a significant inhibition of electron transport in the presence of DNP-INT takes place. Since in the isolated thylakoids, FeCy accepts electrons from the reduced PQ pool, one can presume that DNP-INT affects PQ-pool diffusion and/or modifies its localization in thylakoid membranes. This, in turn, can slow down the reduction of cyt *c* by the reduced molecules of the PQ pool.

If cyt *c* is able to accept electrons directly from the reduced PQ pool, it should drive the electron transport in the presence of DNP-INT, and this in turn should manifest in increased oxygen evolution. As shown in Figure 2, oxygen evolution found for the sample without cyt *c* is low, and it stops relatively soon owing to the lack of any electron acceptor. However, when cyt *c* is present, the initial reaction rate is increased more than 2-fold and is close to linear for nearly 2 min and the extent of oxygen evolution is increased 3-fold. These observations strongly support the idea that cyt *c* accepts electrons directly from the PQ pool.

To test this hypothesis further, we performed measurements on the reduction of cyt *c* by PQH_2 s with different lengths of the side chain in the buffer, as well as when incorporated into PC liposomes. It can be seen (Figure 3) that PQH_2 s are capable of reducing cyt *c* directly in both systems. The shorter the side chain of a PQH_2 molecule, the higher is the cyt *c* reduction rate in the buffer. The reason for this effect is the increased solubility of short-chain PQH_2 homologues in water as compared with the long-chain homologues. Based on the results for ubiquinone homologues [14], it can be deduced that, in our system, PQH_2 -1 and -2 are in the monomeric state (below their critical micelle concentration), whereas the other PQH_2 homologues are above their critical

Figure 2 Effect of 20 μM cyt *c* on the rate of oxygen evolution by wheat thylakoids in the presence of 20 μM DNP-INT without the addition of any artificial electron acceptor

The initial oxygen evolution rates were 27.9 ± 3.7 and $44.4 \pm 3.6 \mu\text{mol of O}_2 \cdot (\text{mg of Chl})^{-1} \cdot \text{h}^{-1}$ for the control and cyt *c*-treated samples respectively. Means $\pm$ S.D. are from three independent experiments. The arrow denotes 'light on'. Other conditions are as in Table 1.

Figure 3 Dark reduction of cyt *c* by plastoquinols with a different length of the side chain in the buffer and incorporation into PC liposomes

(A) Dark reduction of 20 μM cyt *c* by 20 μM PQH_2 s with a different length of the side chain in the buffer (solid lines) and incorporation into PC liposomes; 0.5 mM PC, 50 μM PQH_2 (broken line). The arrows denote injection of PQH_2 or PQH_2/PC mixture into the buffer. The maximal rates of cyt *c* reduction in the buffer were 13.7 ± 0.7 , 12.2 ± 0.2 , 2.2 ± 0.2 and $0.85 \pm 0.2 \mu\text{mol} \cdot (\mu\text{mol of } \text{PQH}_2\text{-9})^{-1} \cdot \text{h}^{-1}$ for PQH_2 -1, -2, -4 and -9 respectively and $2.0 \pm 0.3 \mu\text{mol} \cdot (\mu\text{mol of } \text{PQH}_2\text{-9})^{-1} \cdot \text{h}^{-1}$ for PQH_2 -2 in liposomes. (B) Dark reduction of 20 μM cyt *c* by PQH_2 -9 incorporated into PC liposomes at different PQH_2 -9/PC molar ratios; 0.5 mM PC. The maximal rates of cyt *c* reduction were 1.65 ± 0.07 , 2.2 ± 0.1 , 3.0 ± 0.2 and $3.8 \pm 0.2 \mu\text{mol} \cdot (\mu\text{mol of } \text{PQH}_2\text{-9})^{-1} \cdot \text{h}^{-1}$ for PQH_2 -9/PC molar ratios of 1:10, 1:20, 1:50 and 1:100 respectively. Means $\pm$ S.D. are from three independent experiments.

micelle concentrations in the buffer and are, therefore, poorly accessible for the reaction with cyt *c*. The non-saturating kinetics observed for PQH₂-4 is probably due to slow dissolution of large aggregates formed after PQH₂-4 injection into the buffer. As a result of PQH₂ incorporation into liposomes, the rate of cyt *c* reduction by PQH₂-2 decreases, whereas for PQH₂-9, an evident increase is observed at the same PQH₂-9/PC ratio, i.e. 1:10 (Figure 3B). The reason for this is disaggregation of PQH₂-9 in liposome membranes in contrast with PQH₂-9 micelles formed in the buffer. For PQH₂-2, the membrane forms a barrier that limits the contact of PQH₂-2 with cyt *c* as compared with the buffer system.

The question arises as to whether cyt *c* reduction rate observed in PC liposomes may be responsible for the observed cyt *c* photoreduction rate in thylakoid membranes. Taking into account that there are approx. 14–20 photoreducible PQ-9 molecules per 1000 Chl molecules in thylakoid membranes [15,16], full reduction of the PQ pool will correspond to the effective PQH₂-9 concentration of approx. 0.55 μM at 25 μg of Chl/ml. Thus, if we recalculate the initial cyt *c* photoreduction rate, e.g. at 5 μmol photons $\cdot \text{m}^{-2} \cdot \text{s}^{-1}$, in terms of μmol cyt *c* photoreduced (μmol of PQH₂-9) $^{-1} \cdot \text{h}^{-1}$, then we obtain the value of 88 $\mu\text{mol} \cdot (\mu\text{mol}$ of PQH₂-9) $^{-1} \cdot \text{h}^{-1}$, which is considerably higher than the value for PQH₂-9/PC liposomes of 1:10 molar ratio which is 1.65 $\mu\text{mol} \cdot (\mu\text{mol}$ of PQH₂-9) $^{-1} \cdot \text{h}^{-1}$ (Figure 3B). Since it is known that the PQ-9/membrane lipid ratio is approx. 1:140 [16] and PQH₂-9 distribution within membranes depends on its concentration [10,17], we measured cyt *c* reduction in PQH₂-9/PC liposomes with decreasing PQH₂-9 content (Figure 3B). Although the maximal cyt *c* reduction rates increased up to $3.8 \pm 0.02 \mu\text{mol} \cdot (\mu\text{mol}$ of PQH₂-9) $^{-1} \cdot \text{h}^{-1}$ for the PQH₂-9/PC ratio of 1:100, it is still far from those observed for thylakoids.

It is known that owing to the strongly basic character of cyt *c*, its affinity for different lipids depends on the lipid structure [18]. This interaction is relatively poor with the zwitterionic PC but strong with the negatively charged lipids, such as PG or phosphatidylserine due to ionic interaction [18]. Since, among thylakoid lipids, a negatively charged PG and SQDG are found in significant amounts, one can suspect that thylakoid lipids may facilitate cyt *c* interaction with the membranes. Therefore we performed studies of cyt *c* reduction by PQH₂-9 incorporated into liposomes of a lipid composition resembling that of thylakoid membranes. The results obtained show (Figure 4A) that cyt *c* reduction is considerably faster than that found for PC liposomes (Figure 3B) and the initial reduction rates are the highest at low PQH₂-9 content in liposomes, probably due to closer localization of PQH₂-9 molecules to the membrane surface in these liposomes [10]. Owing to the strong aggregation of thylakoid lipid liposomes in the presence of 20 μM cyt *c*, the reduction was measured in the presence of 5 μM cyt *c*. From Figures 3(B) and 4(A), different cyt *c* reduction kinetics can be observed in thylakoid lipid liposomes compared with those for PC liposomes. Owing to the low affinity of cyt *c* for PC liposomes, local cyt *c* concentration at the liposome surface is low and it is in equilibrium with cyt *c* in the solution; therefore, the reaction kinetics is close to linear. The initial low reaction rate for PC liposomes (Figure 3B) probably reflects a gradual, slow binding of cyt *c* to the surface of liposomes. On the other hand, in thylakoid lipid liposomes, cyt *c* being mostly membrane-bound by the negatively charged lipids is easily accessible for reduction and, hence, the reaction proceeds with second-order kinetics.

If we recalculate the initial reduction rate of cyt *c* for the PQH₂-9/lipid ratio of 1:100 (Figure 4A) for the 20 μM cyt *c* used for thylakoid experiments (Table 1), then we obtain a cyt *c* reduction rate of 140.8 $\mu\text{mol} \cdot (\mu\text{mol}$ of PQH₂-9) $^{-1} \cdot \text{h}^{-1}$ (4×35.2), which

Figure 4 Dark reduction of cyt *c* by PQH₂-9 incorporated into thylakoid lipid liposomes at different PQH₂-9 content and different lipid composition of liposomes

(A) Dark reduction of 5 μM cyt *c* by PQH₂-9 incorporated into thylakoid lipid liposomes (MGDG/DGDG/SQDG/PG ratio = 50:25:12.5:12.5, mol/mol) at different PQH₂-9/lipid molar ratios; final thylakoid lipid concentration was 0.5 mM. The initial rates of cyt *c* reduction were 8.6 ± 1.1 , 7.65 ± 1.2 , 18.5 ± 1.1 and $35.2 \pm 4.9 \mu\text{mol} \cdot (\mu\text{mol}$ of PQH₂-9) $^{-1} \cdot \text{h}^{-1}$ for PQH₂-9/lipid molar ratios of 1:10, 1:20, 1:50 and 1:100 respectively. (B) Effect of different lipid compositions of liposomes on dark reduction of 5 μM cyt *c* by PQH₂-9 incorporated into liposomes of the following lipid compositions: 'complete', as in (A); '-MGDG' (broken line), DGDG/SQDG/PG ratio = 75:12.5:12.5, mol/mol; '-PG', MGDG/DGDG/SQDG ratio = 50:37.5:12.5, mol/mol; '-SQDG', MGDG/DGDG/PG ratio = 50:37.5:12.5, mol/mol; 'DGDG only', DGDG = 100 mol %. Final thylakoid lipid concentration was 0.5 mM; PQH₂-9 concentration was 50 μM . The initial rates of cyt *c* reduction were 8.6 ± 1.1 , 2.00 ± 0.18 , 0.68 ± 0.02 , 0.67 ± 0.03 and 0.042 ± 0.004 for 'complete', '-MGDG', '-PG', '-SQDG' and 'DGDG only' samples respectively. Means $\pm$ S.D. are from three independent experiments. For DGDG liposomes, the initial increase in absorbance was not taken into account in the calculations. Note the doubly expanded ordinate axis compared with (A).

corresponds to the cyt *c* photoreduction rate over the range 5–10 μmol photons $\cdot \text{m}^{-2} \cdot \text{s}^{-1}$.

The influence of different thylakoid lipids on the efficiency of cyt *c* reduction in liposomes by PQH₂-9 was also examined (Figure 4B). It can be noticed that, in the absence of negatively charged lipids (PG and SQDG), the reaction is inhibited severely and it stops after 2–3 min, probably due to aggregation of liposomes. The reaction is also considerably inhibited in the absence of MGDG only, but the kinetics resembles those of control liposomes. The reaction in liposomes prepared exclusively of DGDG is almost completely inhibited. These observations suggest that the presence of charged thylakoid lipids (PG and SQDG) is indispensable for cyt *c* adsorption to thylakoid membranes, and MGDG probably stimulates the reaction by forming hexagonal phases (inverted micelles) in membranes where cyt *c* preferably localizes [18]. Moreover, we have found that, in the presence of cyt *c*, the turbidity of liposomes composed of all thylakoid lipids increases with an increase in PQH₂-9 content

Figure 5 Effect of PQH₂-9 and cyt *c* on turbidity of PQH₂-9/thylakoid lipid liposomes

The turbidity of PQH₂-9/thylakoid lipid liposomes (MGDG/DGDG/SQDG/PG ratio = 50:25:12.5:12.5, mol/mol) was followed at 680 nm at different PQH₂-9 content in the absence (●) and presence (■) of 5 μM cyt *c* and measured 1 min after the time of preparation of liposomes. The corresponding values for samples with 10 mol % PQ-9 are marked with open symbols. The absorbance of 5 μM cyt *c* only is also shown (△). Means ± S.D. are from three independent experiments.

in liposomes (Figure 5). This effect is specific for the cyt *c*-liposomes interaction because the presence of PQH₂-9 by itself in liposomes does not cause such an effect. However, a similar effect is observed for cyt *c* interacting with liposomes containing PQ-9 instead of the reduced form. This indicates that the presence of PQ(H₂)-9 in liposomes changes the membrane structure so that the interaction and/or localization of cyt *c* in the membrane is also affected, especially at higher prenyl-lipid content in liposomes.

The results obtained from the measurements of cyt *c* reduction by PQH₂ in liposomes, together with the lack of effect of SOD on these reactions (results not shown), strongly suggest that cyt *c* can be directly reduced by PQH₂ of the pool in thylakoid membranes without mediation of superoxide.

DISCUSSION

The results of the present study show that, at least at the light intensities used in our experiments, the main pathway of cyt *c* photoreduction in thylakoids proceeds via a direct cyt *c* reduction by the reduced PQ pool and not by superoxide. The light intensity dependence of cyt *c* photoreduction measured for thylakoids and chloroplasts (Figure 1) shows that the rate of cyt *c* photoreduction at saturating light intensities is nearly the same in both cases and it is within the range of superoxide production rates reported in the literature for the isolated thylakoids [15–25 μmol · (mg of Chl)⁻¹ · h⁻¹] [5] that was inferred mainly from cyt *c* photoreduction [3,4,6]. Although chloroplasts undergo lysis under our experimental conditions, any enzymic system that could be responsible for superoxide production and is loosely membrane-bound, such as MDAR [4], should be at least partially preserved in the chloroplast preparation used for the measurements. The rates of superoxide production for intact leaves or cells measured mainly by oxygen isotope methods were shown to be considerably higher [up to 240 μmol · (mg of Chl)⁻¹ · h⁻¹], especially under stress conditions [1]. However, it is not known whether chloroplasts were the only source of superoxide production in these systems. On the other hand, the experiments

performed on intact chloroplasts using isotopic methods showed that the superoxide production rate due to the Mehler reaction extends from that observed for the isolated thylakoids [19,20] up to approx. 80 μmol · (mg of Chl)⁻¹ · h⁻¹ [21,22]. The saturation of cyt *c* reduction rate at the highest light intensities used in our measurements for both thylakoids and chloroplasts is similar to that reported for chloroplasts rather than for thylakoids [2]. It seems that these discrepancies result from the fact that the saturation of cyt *c* reduction by thylakoids depends not only on the light intensity but also on the concentration of thylakoids at a given light intensity. The higher the concentration of thylakoids (Chl), the higher is the light intensity required to saturate the reaction. Chl concentrations used in cyt *c* photoreduction experiments, described in the literature [3,4,6], were usually lower than the Chl concentration in our case (25 μg/ml). Under our experimental conditions, we observed nearly no SOD inhibition of the cyt *c* photoreduction in contrast with many reports in the literature [3,4,6,7]. The reason for that could be considerably higher light intensities used in those experiments together with the low thylakoid concentrations, as low as 0.14 μg/ml [4]. Such conditions, which are often far from physiological, may lead to overexcitation of photosynthetic pigments, their photo-oxidation and non-specific superoxide generation in thylakoid membranes, although physiological superoxide generation in PSI at high light intensity conditions is also possible. Regarding the thylakoid concentration used for the experiments, those used in our measurements (25 μg of Chl/ml) would correspond closely to Chl concentration in leaves (10–30 μg of Chl/cm²) [23,24].

The limitation of the use of cyt *c* photoreduction for the determination of superoxide generation in thylakoid membranes has been illustrated in the work of Miyake et al. [4], who have shown that thylakoids, in the presence of MDAR, generated approx. 60 μmol of superoxide · (mg of Chl)⁻¹ · h⁻¹ at saturating light conditions when determined on the basis of the oxygen consumption measurements, but this value reached as much as 320 μmol of superoxide · (mg of Chl)⁻¹ · h⁻¹ when calculated from the cyt *c* photoreduction rate. For the reasons outlined above, these discrepancies could result from differences in thylakoid concentrations used in both experiments, as it was 70 times less in the cyt *c* photoreduction experiment.

The efficient reduction of cyt *c* by PQH₂s incorporated into liposomes of natural lipid composition indicates that cyt *c* specifically binds to lipids of thylakoid membranes and can be directly reduced by PQH₂-9 of the pool without mediation of superoxide. The rates of cyt *c* reduction obtained for liposomes of natural lipid composition at the lowest PQH₂-9 content used (1 mol%) correspond well to the rates observed for thylakoids at the lowest light intensities used. Judging from the evident correlation between the cyt *c* reduction rate and the PQH₂-9 content in liposomes (Figure 4A), probably the rate of cyt *c* reduction would be even higher at the PQH₂-9/lipid ratio of 1:140 in liposomes, the value corresponding to that found in natural thylakoid membranes [16]. Undoubtedly, the presence of proteins in natural membranes and their interaction with cyt *c* may influence the cyt photoreduction rate. Moreover, apart from PQH₂-9, probably other PSII-reducible PQs (PQ-B and PQ-C) found in thylakoids [25,26] may also reduce cyt *c*.

The results on liposomes with a different thylakoid lipid composition clearly show specificity of the cyt *c* interaction with thylakoid lipids, such as anionic PG, SQDG and MGDG. Cyt *c* is known to bind electrostatically to charged phospholipids, to penetrate lipid bilayers and induce hexagonal phases of cardiolipin in the presence of calcium ions [18]. It was suggested that cyt *c* in membranes containing hexagonal-phase-forming lipids may be present in intra-bilayer structures like inverted

Scheme 1 Model showing photoreduction sites of cyt *c* in thylakoids

The photoreduction site under low and medium light intensities (black 'cyt *c*' symbol) and that occurring additionally at high light intensities (white 'cyt *c*' symbol) are shown. The action sites of the electron-transport inhibitors used are also shown. DCMU, 3-(3',4'-dichlorophenyl)-1,1-dimethylurea.

micelles. It is known that MGDG is a hexagonal-phase-forming lipid and such structures were already suggested to occur in thylakoid membranes [27]. The requirement of MGDG for the optimal rate of cyt *c* reduction in liposomes suggests that cyt *c* may preferentially reside in the inverted micelles of MGDG-containing membranes and such structures are probably the site of efficient cyt *c* reduction by PQH₂-9 within membranes.

In summary, our results indicate that cyt *c* can be reduced directly by the reduced PQ pool in thylakoid membranes, without mediation of the superoxide, and this cyt *c* reduction pathway could be dominating in the isolated thylakoids at low and medium light intensities (Scheme 1). Cyt *c* can be efficiently reduced by PQH₂-9 within membranes due to its specific interaction with thylakoid membrane lipids. Our results also indicate that when the method of cyt *c* photoreduction is used for the determination of superoxide generation in thylakoid membranes, one should bear in mind its limitations.

This work was supported by the Committee for Scientific Research (KBN) of Poland (grant no. 6 P04A 028 19). We are grateful to Professor W. Oettmeier for the gift of DNP-INT and Dr H. Koike for the gift of PQ homologues PQ-1–4. We thank Robert N. Corning for correcting the manuscript.

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Received 21 November 2002/18 June 2003; accepted 1 July 2003

Published as BJ Immediate Publication 1 July 2003, DOI 10.1042/BJ20021820