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Oxygen reduction in chloroplast thylakoids results in production of hydrogen peroxide inside the membrane

Maria Mubarakshina, Sergey Khorobrykh, Boris Ivanov *

Institute of Basic Biological Problems, Russian Academy of Sciences, Pushchino, Moscow Region, 142290, Russia

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Abstract

Hydrogen peroxide production in isolated pea thylakoids was studied in the presence of cytochrome *c* to prevent disproportionation of superoxide radicals outside of the thylakoid membranes. The comparison of cytochrome *c* reduction with accompanying oxygen uptake revealed that hydrogen peroxide was produced within the thylakoid. The proportion of electrons from water oxidation participating in this hydrogen peroxide production increased with increasing light intensity, and at a light intensity of 630 μmol quanta m^{-2} s^{-1} it reached 60% of all electrons entering the electron transport chain. Neither the presence of a superoxide dismutase inhibitor, potassium cyanide or sodium azide, in the thylakoid suspension, nor unstacking of the thylakoids appreciably affected the partitioning of electrons to hydrogen peroxide production. Also, osmolarity-induced changes in the thylakoid lumen volume, as well as variation of the lumen pH induced by the presence of Gramicidin D, had negligible effects on such partitioning. The flow of electrons participating in lumen hydrogen peroxide production was found to be near 10% of the total electron flow from water. It is concluded that a considerable amount of hydrogen peroxide is generated inside thylakoid membranes, and a possible mechanism, as well as the significance, of this process are discussed.

Keywords: Photoreduction of oxygen; Hydrogen peroxide; Thylakoids; Membrane

1. Introduction

Reactive oxygen species, (superoxide radical, hydrogen peroxide, hydroxyl radical, hydroperoxides of organic molecules, and singlet oxygen), collectively known as ROS can be generated in different cell compartments, and their generation is an inevitable part of the metabolism of living organisms. In addition to their destructive effects, ROS have significant importance for redox signaling and this is especially so for hydrogen peroxide [1]. ROS are commonly produced, sometimes in significant concentrations, in chloroplasts, the light-transforming organelles in plants [2]. In general, the photosynthetic electron transport chain (PETC) of chloroplast thylakoids is considered to be the main source of ROS in higher plants [3]. In the past attention has centered on the different possible roles of oxygen reduction in PETC. The first of these roles [2,4] involves the maintenance of electron transport leading to the acidification of the lumen that can drive ATP production (which is not coupled to $NADP^+$ reduction), and which can also trigger processes leading to energy dissipation under excess light. The second possible role involves the direct consequences of the oxidation of PETC carriers, poising the redox-states of PETC carriers to allow the operation of cyclic electron transfer and to prevent photoinhibition. More recently, it has become broadly accepted that the chloroplast redox signals allow the plants to acclimate to natural stress conditions [1,5].

Asada et al. [6] stated that superoxide formation through univalent reduction of dioxygen in Photosystem I (PS I) is the main route of oxygen reduction in the PETC in illuminated thylakoids, and they have shown [7,8] that

Abbreviations: Chl, chlorophyll; cyt *c*, cytochrome *c*; Gr D, gramicidin D; PETC, photosynthetic electron transport chain; PQ, plastoquinone; $PQH^{\bullet}$, plastosemiquinone; PQH_2, plastohydroquinone, plastoquinol; PSI, photosystem I; PSII, photosystem II; ROS, reactive oxygen species; SOD, superoxide dismutase

* Corresponding author. Fax: +7 4967 330532.
E-mail address: ivabor@issp.serpukhov.su (B. Ivanov).

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superoxides can be generated within thylakoids membranes. Recently, we have found [9] that oxygen reduction occurs also in the plastoquinone pool (PQ-pool) of thylakoid membranes; the characteristics of this process implied that dioxygen is reduced to superoxide by plastosemiquinone. It was proposed in that work that the superoxide produced is reduced by plastohydroquinone to produce hydrogen peroxide [9]. The subsequent finding [10] that PQ-pool participation in oxygen reduction to H_2O_2 increased up to 70% upon increasing the light intensity led to the suggestion that plastohydroquinone could reduce not only the superoxides produced in the PQ-pool, but also those produced by PS I. The proposed scheme [10] implied that hydrogen peroxide could be produced inside the thylakoid membrane.

In the present work, we have found that a considerable percentage of the electrons that have been injected into the PETC from the process of water oxidation are involved in hydrogen peroxide formation within the thylakoid membrane. The amount of electrons participating in hydrogen peroxide formation in the lumen was also evaluated.

2. Materials and methods

Pea (*Pisum sativum* L., variety Bogatir) plants were grown in a greenhouse at 23/17 °C (day/night) under natural illumination in summer months and with additional illumination provided by DRLP-400 high pressure mercury lamps during fall and early spring months. Thylakoids were isolated from pea leaves of 10–14 days old plants as described [9], and were suspended in a medium containing 0.4 M sucrose, 20 mM NaCl, 5 mM $MgCl_2$, 25 mM HEPES–KOH (pH 7.6). The basic reaction medium contained 0.1 M sucrose, 20 mM NaCl, 5 mM $MgCl_2$, 50 mM HEPES–KpOH (pH 7.8), and 1 μM Gramicidin D. Thylakoids were added up to 10 μg chlorophyll in 1 ml. To obtain the unstacked thylakoids, the isolation, suspension and reaction media were prepared without NaCl and $MgCl_2$, and the K^+ concentration in the reaction medium, which in the experiments with these thylakoids contained 25 mM HEPES, was 10–12 mM. The absence of grana in the preparations was verified by measuring the optical density [11] and chlorophyll fluorescence of the thylakoid suspension [12]. The chlorophyll concentration was measured in a 95% ethanolic extract [13].

Light-induced change in the oxygen concentration in the thylakoid suspensions (3.2 ml) was measured in a temperature-controlled (21 °C) vessel with a Clark-type pO_2-electrode connected with a computer through an analogue–digital interface. The rates of catalase-independent oxygen uptake (see [9]) were taken into account in the calculation of the rates of change of the oxygen concentration associated with electron flow from water. The photoreduction of ferricytochrome *c* (cyt *c*) was determined as the absorbance change at 550 nm using the differential absorption coefficient 19 mM^{-1} cm^{-1} [14]. The samples for the spectrophotometric assay were taken from a pO_2-electrode vessel before and after illumination, and it was determined that reduced cyt *c* was not oxidized in the thylakoid suspension after cessation of illumination. The superoxide-dependent cyt *c* reduction rate was determined as the difference between cyt *c* reduction rates in the absence and in the presence of SOD used at a saturating concentration. In the experiment presented in Fig. 2, cyt *c* reduction was measured using a spectrophotometer (Hitachi 553, Japan) in dual-wavelength mode with a reference wavelength of 540 nm; the photomultiplier was shielded from actinic light with a SZS-22 (Russia) filter. Reaction mixtures in both the pO_2-electrode vessel and the spectrophotometric cuvette were illuminated in the same way through a red cut-off filter (λ>600 nm). The light intensity was varied using neutral filters, and was measured as photon flux density, μmol quanta m^{-2} s^{-1}, using a Li-Cor quantum meter. SOD, HEPES, cyt *c* and catalase were purchased from Sigma, and other chemicals were of analytical grade.

3. Results

3.1. Oxygen uptake and appearance of superoxides outside thylakoids

Oxygen uptake under illumination of thylakoids arises from the reduction of dioxygen by the components of the PETC at the expense of electrons removed from the oxidation of water:

$$2H_2O = 4H^+ + 4e^- + O_2\uparrow \quad - \text{water oxidation in PSII;} \tag{1}$$

$$4O_2 + 4e^- = 4O_2^{\bullet -} \quad - \text{dioxygen reduction;} \tag{2}$$

$$4O_2^{\bullet -} + 4H^+ = 2H_2O_2 + 2O_2\uparrow \quad - \text{disproportionation of superoxides.} \tag{3}$$

The stoichiometry between electrons transferred to oxygen and oxygen uptake is one O_2 molecule consumed per $4e^-$ [15]. If disproportionation of superoxide is substituted by its reduction by components of the PETC (e.g., plastohydroquinone), which themselves are reduced by electrons 'from water':

$$O_2^{\bullet -} + PQH_2 = H_2O_2 + PQ^{\bullet -}, \tag{4}$$

then the stoichiometry remains the same. Cyt *c*, the established superoxide trap, can readily react with superoxide in solution:

$$O_2^{\bullet -} + (\text{cyt } c)^{ox} = (\text{cyt } c)^{red} + O_2\uparrow \quad - \text{oxidation of superoxides.} \tag{5}$$

In the following experiments, cyt *c*, a species unable to penetrate into the membrane, was added in order to trap all of the superoxides outside of the thylakoid membrane. The experiments were conducted at pH 7.8 where the second-order rate constant of disproportionation was low enough (it decreases with increasing pH), to be comparable with the rate constant for the reaction (5). Under these conditions the cyt *c* reduction rate was saturated at concentrations of less than 30 μM; at pH 6.5 it was impossible to reach saturation even at 100 μM cyt *c* (Fig. 1).

Fig. 2 shows the light-intensity dependencies of oxygen uptake rate as well as the superoxide-dependent cyt *c* reduction rate. In earlier studies, the rate of oxygen uptake in the suspension of broken chloroplasts was found to be saturated at approximately 220 μmol quanta m^{-2} s^{-1} with a 0.4 mm light path [16], and the cyt *c* reduction rate was found to be saturated already at 12 μmol quanta m^{-2} s^{-1} when measured in a diluted suspension [17]. The data of these studies are in qualitative agreement with the data of Fig. 2, insofar as the rate of superoxide-dependent cyt *c* reduction was saturated at appreciably lower intensities. The quantitative discrepancies probably arise from differences in the conditions in our experiments (the same for measurements of both rates) compared to those in the cited works; apparently, this is the reason why saturation in Fig. 2 occurred at higher light intensities.

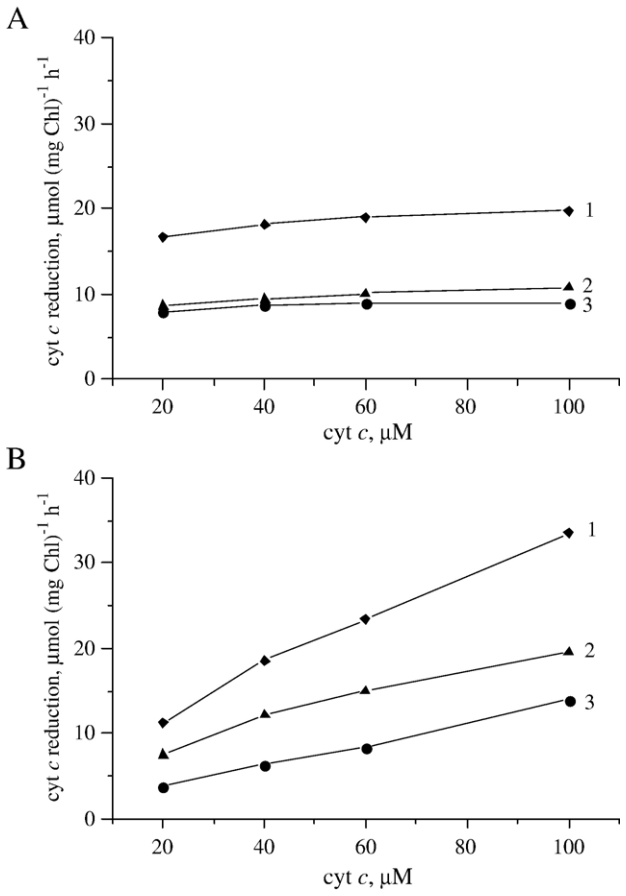

Fig. 1. The effect of cyt *c* concentration on the cyt *c* reduction rate in thylakoids at pH 7.8 (A) and at pH 6.5 (B). Thylakoids, 10 μg Chl ml^{-1}; cyt *c*, 40 μM. Light intensity, 450 μmol quanta m^{-2} s^{-1}. 1—cyt *c* reduction rates in the absence of SOD; 2—cyt *c* reduction rates in the presence of SOD; 3—superoxide-dependent cyt *c* reduction rates.

If all of the superoxide that is disproportioned in the absence of cyt *c* were able to reduce cyt *c* when it was present, then the rate of superoxide-dependent cyt *c* reduction should be equal to the rate of generation of the superoxide. The latter rate is four times the rate of oxygen uptake (see above), and Fig. 2 shows that it is higher than superoxide-dependent cyt *c* reduction. The difference between these rates shows that some superoxides did not leave the thylakoid (membrane plus lumen). This difference is higher at higher light intensities, and this implies that the amount of superoxide that does not leave the thylakoid increased at higher intensities.

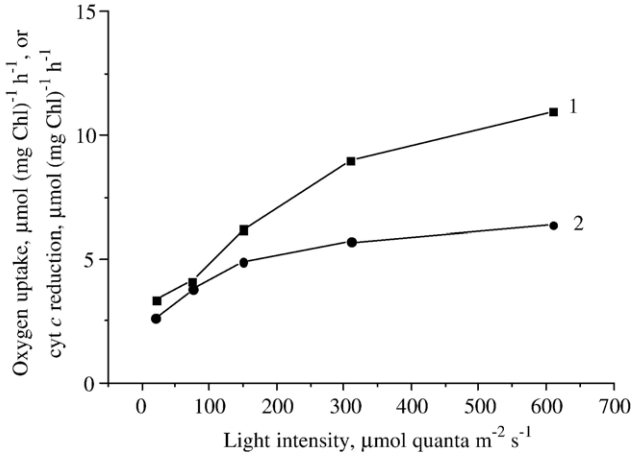

Fig. 2. The dependencies of the oxygen uptake rate (1) and the superoxide-dependent cyt *c* reduction rate (2) in isolated thylakoids on light intensity. Thylakoids, 10 μg Chl ml^{-1}; cyt *c*, 40 μM; pH 7.8.

3.2. Hydrogen peroxide formation in the presence of cyt c and calculation of the amount of electrons participating in this process

In response to catalase addition after illumination in the absence of cyt *c*, the amount of oxygen evolved was equal to the amount consumed in the light (Fig. 3A). This confirmed that, in the absence of cyt *c*, oxygen was the sole electron acceptor in the thylakoids, and that hydrogen peroxide was the final product of O_2 reduction (see reaction (1)–(3)). Thus, all of the superoxide, including that which we suggested above did not leave thylakoids was converted into hydrogen peroxide.

The presence of cyt *c* in the thylakoid suspension under illumination caused a decrease in the oxygen uptake rate (Fig. 3B vs. Fig. 3A). The decrease of oxygen uptake resulted from two routes: (a) a "return" of O_2 into the medium, due to superoxide oxidation by cytochrome (see reaction (5)) and (b) photosynthetic oxygen evolution from Photosystem II induced by the electron flow due to direct reduction of cyt *c* by components of the PETC, i.e. PSI acceptors situated on the

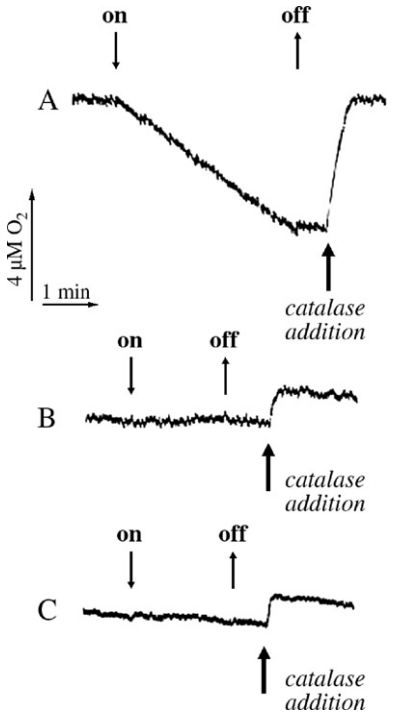

Fig. 3. The light-induced changes of oxygen concentration in a suspension of isolated thylakoids. A—basic reaction medium; B and C—with 60 μM cyt c in the reaction mixture; B—the usual (stacked) thylakoids, and C—"unstacked" thylakoids. Where it is specified, catalase was added up to 500 ū per ml. Thylakoids, 10 μg Chl ml^{-1}. Light intensity, 450 μmol quanta m^{-2} s^{-1}.

outer surface of thylakoid membrane and PQ-pool components [9,18], in other words, a route not involving superoxide. Thus, after addition of cyt *c* to suspension, some of electrons coming from water produce superoxide, which is then reoxidized by cyt *c*. This process is reported as the superoxide-dependent cyt *c* reduction in Table 1. Since cyt *c* has a redox-potential of +0.26 V, while that of the dioxygen/superoxide redox couple is −0.16 V, cyt *c* is expected to be directly reduced by electrons from two sources: (a) part of those which in the absence of cyt *c* reduced dioxygen, and (b) those from the PETC that are able to reduce cyt *c*, but not dioxygen. The occurrence of the last route is greater at lower light intensities (see the difference in total electron flows with and without cyt *c* in Table 1). All electrons that reach cyt *c* result in stoichiometric oxygen evolution. The oxygen uptake observed in some experiments in the presence of cyt *c* (e.g. in high light in Table 1) showed that not all electrons from water end up reducing the cytochrome and that a fraction of them can produce H_2O_2 instead. In various experiments either oxygen uptake or oxygen evolution were observed in the light; this depended on the proportion of electrons that were accepted by cyt *c*.

Fig. 3B shows that when catalase is added to the thylakoid suspension in the presence of cyt *c* then oxygen is evolved. This clearly demonstrates that prevention of superoxide disproportionation outside thylakoids (by cyt *c*) did not prevent H_2O_2 production in the light. The amount of electrons participating in such "intrathylakoid" H_2O_2 production in the presence of cyt *c* was evaluated as follows. From the rate of cyt *c* reduction (V_{Cyt}, see Table 1), considering that injection of 4 electrons into the PETC from the water is accompanied by $1O_2$ evolved (this standard stoichiometry was proved under our experimental conditions in the course of cyt *c* reduction), the rate of oxygen evolution coupled to cyt *c* reduction was determined. Subtracting this rate from the rate of oxygen concentration change (V_{O2}), the rate of consumption of O_2 molecules incorporating into H_2O_2 could be found, and, considering the stoichiometry between this oxygen consumption and electron flow from water ($1O_2$: $4e^-$, see above), the rate of flow of electrons participating in H_2O_2 production ($V_{e,\ H2O2}$) was calculated. The proportion of this electron flow producing "intrathylakoid" H_2O_2 in the presence f cyt *c*, in total electron flow along the PETC, P_{th}, increased from 22.5% at 75 μmol quanta m^{-2} s^{-1} to 57% at 630 μmol quanta m^{-2} s^{-1} (Table 1).

Table 1 presents also the quantitative data showing an increase in the difference between the rate of superoxide generation (equal to total electron transport rate) measured in the absence of cyt *c*, and the rate of superoxide-dependent cyt *c* reduction with increasing light intensity, 11.9 μeqv (mg Chl)$^{-1}$ h^{-1} vs. 5.4 μmol (mg Chl)$^{-1}$ h^{-1} at 75 μmol quanta m^{-2} s^{-1} and 39.1 μeqv (mg Chl)$^{-1}$ h^{-1} vs. 8.9 μmol (mg Chl)$^{-1}$ h^{-1} at 630 μmol quanta m^{-2} s^{-1}. The increase of both P_{th} and the above difference could obviously be for the same reason (see Discussion).

The total electron transport even in the presence of cyt *c* was appreciably less than the potential input of electrons into the PETC; the electron transport rates with methyl viologen as electron acceptor in the presence of Gramicidin D, at light intensities as in Table 1, were almost 8 times higher (not shown). Thus, all potential routes of electron transfer both in the presence and in the absence of cyt *c* were saturated. So, the flow of electrons participating in H_2O_2 production inside thylakoids in the absence of cyt *c* is, at least, not less than that measured in its presence. Using the value of the latter flow, it is possible to estimate the minimal proportion of the flow producing 'intrathylakoid' H_2O_2 in the absence of cyt *c* (P_{th} with an asterisk in the Table 1). This proportion turned out to be higher than in the presence of cyt *c*, and this was more marked in weak light. In high light it reached 62%.

3.3. Hydrogen peroxide formation in the presence of cyt c is not an extraneous process

Some possible reasons for H_2O_2 production in the presence of cyt *c* were tested. First, the space between the adjacent thylakoid membranes in grana might be the location of H_2O_2 production, if cyt *c* entering this space and/or its reaction with superoxide there were hampered. The long axis of cyt *c* is 34 Å [19], and the gap between the thylakoids in grana was estimated as 45 Å [20]; yet that did not exclude the above situation. It was, however, found that H_2O_2 production in the presence of cyt *c* occurred also in unstacked thylakoids (Fig. 3C). Being calculated as described above, the proportion of electrons participating in this H_2O_2 production decreased by a maximum of 10% as compared with the situation in the stacked thylakoids. This decrease is most likely the consequence of a change in the interactions between electron carriers caused by the absence of Mg^{++} in the media and/or the unstacking itself [21,22]. In fact,

Table 1
The influence of light intensity on the rate of electron flows in the absence and the presence of cyt *c* and on the flow of electrons participating in H_2O_2 formation inside thylakoids (P_{th})

Light intensity μmol quanta m^{-2} s^{-1}	cyt *c*, 40 μM	V_{O2}, μmol (mg Chl)$^{-1}$ h^{-1}	V_{Cyt}, μmol (mg Chl)$^{-1}$ h^{-1}	$V_{sup\rightarrow Cyt}$, μmol (mg Chl)$^{-1}$ h^{-1}	$V_{e,\ H2O2}$, μeqv (mg Chl)$^{-1}$ h^{-1}	V_e, μeqv (mg Chl)$^{-1}$ h^{-1}	P_{th}, %
75	–	−2.98±0.40	–	–	11.92	11.92	36*
	+	2.62±0.07	14.77±0.44	5.41±0.12	4.28	19.05	22.5
630	–	−9.78±0.92	–	–	39.10	39.10	62*
	+	−4.76±0.16	18.10±0.28	8.86±0.47	24.28	42.40	57

V_{O2}, V_{Cyt}, $V_{sup\rightarrow Cyt}$—the measured rates of oxygen concentration change, total cyt *c* reduction, and superoxide-dependent cyt *c* reduction in illuminated thylakoids, respectively; $V_{e,\ H2O2}$, V_e—the calculated rates of electron flow participating in H_2O_2 production, and the total electron flow along the PETC, respectively; P_{th}—the flow of electrons participating in H_2O_2 production, % of total electron flow, *—the values were calculated using $V_{e,\ H2O2}$ in the presence of cyt *c*, and V_e in the absence of cytochrome. Minus indicates oxygen uptake.

Table 2
Effects of cyanide, azide, lumen volume and lumen pH on the flow of electrons participating in H_2O_2 production in the presence of cyt *c*

Exp.	Changes in basic reaction medium	$V_{O2.}$, μmol (mg Chl)$^{-1}$ h^{-1}	V_{Cyt}, μmol (mg Chl)$^{-1}$ h^{-1}	$V_{e,\ H2O2}$, μeqv (mg Chl)$^{-1}$ h^{-1}	V_e, μeqv (mg Chl)$^{-1}$ h^{-1}	P_{th}, %
I	+ cyt *c*	1.34±0.25	22.20±5.16	16.8	39.0	43
	+ cyt *c*, NaN_3, 5 mM	1.57±0.08	21.90±6.47	15.6	37.5	42
	+ cyt *c*, NaN_3, 10 mM	1.17±0.39	21.00±6.68	16.3	37.3	44
II	+ cyt *c*	1.90±0.20	24.01±1.10	16.4	40.4	40
	+ cyt *c*, KCN, 1 mM	2.40±0.34	24.90±0.01	15.2	40.1	38
III	+ cyt *c*, no Suc	2.20±0.40	22.90±1.10	14.0	36.9	38
	+ cyt *c*	1.90±0.21	24.00±1.12	16.4	40.4	40
	+ cyt *c*, Suc, 0.4 M	1.61±0.70	23.50±0.20	17.2	40.7	42
IV	+ cyt *c*, no Gr D	2.50±0.42	23.80±0.30	13.6	37.4	36
	+ cyt *c*	2.30±0.30	22.50±1.10	13.2	35.7	37

Light intensity, 450 μmol quanta m^{-2} s^{-1}.
Column headings are as in Table 1. Where specified, cyt *c* was added to the basic reaction medium up to 40 μM; Gr D, Gramicidin D; Suc, sucrose.

the amount of superoxide in the space between the adjacent thylakoids in grana should not be high, because its generation outside the membrane must be negligible due to the near absence of PSI in grana membranes [23], while the superoxide generated in the plastoquinone pool leaves the membrane at a low rate [9].

Second, neither KCN nor NaN_3, inhibitors of all types of superoxide dismutase (SOD) in plants [24,25], influenced the amount of electrons participating in H_2O_2 production in the presence of cyt *c* (Table 2, experiments I, II). These data show that H_2O_2 production is not the result of superoxide disproportionation catalyzed by a SOD that could have been co-isolated with the thylakoids.

3.4. Evaluation of the amount of electrons participating in hydrogen peroxide formation in the lumen

The absence of an influence of cyanide on H_2O_2 production (Table 2) (note that at the pH used cyanide is mainly in its non-ionised form and thus it could easily penetrate the membrane [26]) implies that the H_2O_2 produced in the thylakoid lumen from the disproportionation of any superoxide that may have diffused there did not depend on the presence of SOD. The presence of SOD in the lumen was proposed earlier [7], however, immuno-gold labelling showed that the amount there was negligible [27], and also amino-terminal sequencing of lumenal proteins did not reveal SOD [28].

In order to calculate the amount of electrons participating in production of H_2O_2 in the lumen, the rates of oxygen uptake in the presence and in the absence of ascorbate were measured when SOD was present in the medium. Being a trap for superoxide radicals, ascorbate can prevent their disproportionation [15], so reaction (3) is replaced with reaction (6):

$$O_2^{\bullet -} + AscH + H^+ = MDHA + H_2O_2 \quad - \text{reduction of superoxide.} \qquad (6)$$

This reaction should result in an increase in the oxygen uptake rate when ascorbate is present. Theoretically a threefold increase in the rate is expected since the stoichiometry between electrons and oxygen uptake becomes three O_2 molecules consumed per 4e- transferred to oxygen from the water (from Eqs. (1), (2) and (6)) [15]. The rate constant of reaction (3) is four orders higher in the presence of SOD than that of the spontaneous reaction, and the addition of SOD at the proper concentration prevents superoxide reacting with ascorbate outside the thylakoid. Furthermore, SOD prevents production of monodehydroascorbate, a very effective electron acceptor in thylakoids [29]; the oxygen evolution accompanying its reduction might affect the total oxygen balance. Thus, only the reaction of superoxide with ascorbate in the lumen should increase the rate of oxygen consumption when the electron transfer rate is unchanged; in our case, the electron transport is limited by the reduction of dioxygen [10]. The proportion of electrons that produce superoxide reacting with ascorbate, in a total electron flow (P_{lu}) can be calculated as $[½(B-A)\times A^{-1}]\times 100\%$, where A and B are the rates of oxygen uptake in the absence and in the presence of ascorbate, respectively (see Appendix A). The value of P_{lu} averaged 9% based on five experiments at 1 mM ascorbate. This value could be overestimated because it was impossible to exclude the possibility of electron donation from ascorbate to PS I [30,31], and this donation, which would be greater at higher ascorbate concentration, could result in an increase (the level of which has not been estimated) in the rate of oxygen uptake in the presence of ascorbate. The typical experiments at ascorbate concentrations of 0.5 mM and 1 mM are shown in Table 3. The value of P_{lu} at 0.5 mM ascorbate is less than

Table 3
The flow of electrons participating in H_2O_2 formation in the lumen (P_{lu})

Exp.	Ascorbate	$V_{O2.}$, μmol (mg Chl)$^{-1}$ h^{-1}	P_{lu},%
I	–	−7.25±0.12	5.8
	0.5 mM	−8.10±0.17	
II	–	−7.60±0.09	11.8
	1 mM	−9.40±0.20	

Light intensity, 450 μmol quanta m^{-2} s^{-1}.
P_{lu} is expressed as a percentage of the total electron transport from water and calculated according to the Appendix. The reaction mixture contained SOD at 100 ū per ml. In the experiments with ascorbate, the thylakoids were incubated with the indicated amount for 5 min to establish an equilibrium between the medium and the lumen.

at 1 mM, however, 0.5 mM was calculated to be the lowest concentration able to trap the superoxide in lumen.

The contribution of the lumen H_2O_2 formation to the intrathylakoid H_2O_2 production was checked also in the absence of ascorbate. Since the rate constant for superoxide disproportionation sharply increases with as the pH decreases [32], it would be expected that at lower lumen pH the total H_2O_2 production would increase if superoxide diffuses into lumen. The lumen pH was controlled by the presence of Gramicidin D in the reaction mixture: at a medium pH value of 7.8, the lumen pH in the light is lower when Gramicidin D is absent [33]. It might also be assumed that an increase in the lumen volume could facilitate the location of superoxide inside the lumen. Thus the lumen volume was changed by varying the osmolarity of the medium by changing the sucrose concentration. The results of these experiments showed that neither a change of osmolarity, nor the presence/absence of Gramicidin D appreciably affected the amount of electrons participating in H_2O_2 formation (Table 2, experiments III and IV).

4. Discussion

This study suggests that the location of hydrogen peroxide production is the thylakoid membrane in chloroplasts. This seems reasonable, taking into account the evidence for superoxide generation inside the membrane [8]. However, in an earlier study [6], light-induced hydrogen peroxide formation in thylakoids in the presence of cyt *c* was not detected. Possibly, this result was due to use of weak actinic light; our data (Table 1) showed that "intrathylakoid" hydrogen peroxide formation increased with increasing light intensity. In later studies, data about hydrogen peroxide formation in the presence of cyt *c* appeared [7,8]. Takahashi and Asada [8] detected hydrogen peroxide fluorometrically at pH 7.3, but only in the presence of the uncouplers, ammonium chloride, methylamine, and Gramicidin D. The authors assumed that these substances provided the protons needed for disproportionation of superoxide radicals, which are stable in an aprotic thylakoid membrane interior. In the present study, we observed hydrogen peroxide formation in the presence of cyt *c* at physiological pH (pH 7.8) (Fig. 3), and uncouplers such as Gramicidin D (Table 1) and nigericin (data not shown) did not influence H_2O_2 formation appreciably.

At pH 7.8 the disproportionation of superoxide (pK 4.8) should be low owing to a strong electrostatic repulsion in the membrane interior with low permittivity. We propose that the reaction leading to formation of H_2O_2 inside the membrane is the reaction of $O_2^{\bullet-}$ with plastohydroquinone, PQH_2 ($pK_1 = 10.8$). Due to the large difference between the E'_0 values of the redox pairs $PQ^{\bullet-}/PQH_2$ (0.37 V) [34] and $O_2^{\bullet-}/H_2O_2$ (0.94 V) [2] (the difference does not change at pH 7.8), the reaction is thermodynamically favorable. As stated above, this means of superoxide reduction does not change the stoichiometry between electron transport and oxygen uptake. We did not find an inhibitory effect of propylgallate on oxygen uptake (data not shown), and we have no indication of a specific enzyme involved in catalysis of this reaction, however we cannot rule it out.

The difference between the rate of total superoxide generation and the rate of superoxide-dependent cyt *c* reduction (Fig. 2) showed that in higher light more superoxide was trapped within the membrane, and the data in Table 1 demonstrates that the fraction of electrons participating in "intrathylakoid" H_2O_2 formation is also increased in higher light. That is to say, an increase in the light intensity leads to production of superoxide that is mainly converted into H_2O_2 *within* the thylakoid membrane. This could be the result of the majority of additional superoxide, emerging in higher light, being reduced by PQH_2. Since the oxygen reduction in the PQ-pool is limited [9], this additional superoxide formed under high light is presumably produced by PSI. The PSI cofactor that is the direct reductant of O_2 in PSI in washed thylakoids is not clearly established. The supposed reduction by PQH_2 of the majority of the additional superoxide implies that PQ-pool plays an increased role in the co-operative (that is, when superoxide produced by PSI is reduced by PQH_2) oxygen reduction to H_2O_2. This corresponds to the earlier report of an increase (up to 70%) of participation of the PQ-pool in oxygen reduction at high light intensities [10].

The model proposed implies that the reduction of dioxygen by PSI can regulate the electron pressure in PSII by removing electrons from the PQ-pool by means of PQH_2 oxidation by the produced superoxides. Scavenging of superoxide by PQH_2 could also be significant in preventing the interaction of this destructive agent with lipids and membrane proteins inside the membrane. It is consistent with the role of plastohydroquinone as an antioxidant in thylakoid membranes, as was shown earlier [35]. H_2O_2 rapidly leaves the membrane and can be scavenged in the stroma by the powerful ascorbate-peroxidase system [2].

The assumption that some superoxide can move into the lumen and are disproportionated there was put forward by Asada et al. [7] to explain their observation of hydrogen peroxide formation in the presence of cyt *c*. Our measurements showed that this flow is not large (Table 3). We speculate that most superoxide is trapped by plastohydroquinone and that the main reactive oxygen species entering the lumen is H_2O_2. The data showing the presence of ascorbate peroxidase in the lumen [36] imply that hydrogen peroxide is present there and this fits the above picture.

There is much evidence for the dependence of the initiation of adaptation reactions in plant cells on the redox-state of the plastoquinone pool [37,38]. The redox state of the PQ-pool controls the distribution of excitation energy in photosynthesis, regulating expression of chloroplastic and nuclear genes encoding some of the components of the PETC [38,39]. However, the mechanism of this control remains obscure. Hydrogen peroxide is known to be the most universal signaling molecule [1], and the generation of hydrogen peroxide with a direct role relative to the PQ-pool might be the means by which the redox state of the PQ-pool initiates the signalling chain. It seems clear that in the presence of natural electron acceptors, such as ferredoxin plus $NADPH^+$, the electron flow resulting in intramembrane H_2O_2 production is less (when expressed as a percentage of total electron flow) than that found in the present

work. Indeed the level of total H_2O_2 production should be low enough to allow for small variations in the amount of H_2O_2 to fulfil its signalling role.

Finally, it is possible to speculate that production of H_2O_2 in mitochondria and bacteria also occurs within the membranes that contain their electron-transport chains.

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Appendix A

In the absence of ascorbate, when superoxide is disproportioned or reduced to H_2O_2 with electrons from the PETC, the stoichiometry between electron flow from water, and oxygen uptake, is $4e:1O_2\downarrow$. If the measured rate of oxygen uptake, in μmol O_2 (mg Chl)$^{-1}$ h^{-1}, in the absence of ascorbate is denoted as A, then the rate of electron transport, in μeqv (mg Chl)$^{-1}$ h^{-1}, along the PETC is $4\times A$. At an unaltered rate of electron transport, the rate of oxygen uptake after ascorbate addition increases because the stoichiometry is $4e^-:3O_2\downarrow$ for the electrons producing the superoxides reacting with ascorbate [15]. If X denotes the rate of transport of these electrons, then the rate of transport of electrons producing superoxide, which is disproportioned or reduced in the PETC, is $4\times A-X$. The electrons producing the superoxide that reacts with ascorbate provide (according to the reaction $O_2^{\bullet-}$ + ascorbate = H_2O_2 + monodehydroascorbate) a rate of oxygen uptake equal numerically to X. The electrons producing the superoxide that disproportionates provide (according to reaction $O_2^{\bullet-}+O_2^{\bullet-}+2H^+=H_2O_2+O_2\uparrow$) a rate of oxygen uptake equal to ½ $(4\times A-X)$. Since, for transfer of $(4\times A)$ electrons along the PETC, dioxygen is evolved from water in PSII with the rate equal numerically to A, then the oxygen balance rate in the presence of ascorbate, B, is:

$$B = [1/2(4A - X) + X] - A.$$

After transformation we have:

$$X = 2(B - A).$$

Then the proportion of electrons producing the superoxide that reacts with ascorbate in the total electron flow is:

$$X \times (4 \times A)^{-1} = 2(B - A) \times (4 \times A)^{-1} = 1/2(B - A) \times A^{-1}.$$

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