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H_2O_2 formation by Photosystem II

Thomas Wydrzynski, Jonas Ångström and Tore Vänngård

Chalmers University of Technology and University of Göteborg, Department of Biochemistry and Biophysics, Göteborg (Sweden)

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Upon addition of catalase to Photosystem II-enriched samples suspended under low sucrose conditions at pH 6.3, the observed steady-state O_2 evolution increases significantly. It compares well quantitatively with the O_2 evolution of samples suspended under high sucrose conditions or with the dichlorophenolindophenol photoreduction of samples suspended under low sucrose conditions. The extent of the catalase-induced enhancement of O_2 evolution is not affected by the presence of EDTA and is largely independent of the chloride concentration, but is eliminated by raising the pH to 7.5. The light intensity dependence of the catalase-induced enhancement is similar to the dependence of the overall observed O_2 evolution. For Tris-inhibited samples the addition of catalase does not stimulate any light-induced O_2 evolution using either Mn^{2+} or diphenylcarbazide as the electron donor and phenyl-*p*-benzoquinone as the electron acceptor. The results suggest that under conditions of low sucrose, H_2O_2 is formed by PS II and that the apparent H_2O_2 formation does not involve free Mn nor the acceptor side of PS II. Rather, the H_2O_2 formation occurs in relatively intact PS II centers close to or at the water-splitting site. The implications of H_2O_2 formation by PS II in terms of the water-splitting mechanism is discussed.

Introduction

The intermediate reaction steps in the oxidation of water to O_2 by Photosystem II (PS II) in chlorophyll *a*-containing organisms are not known. It is well-established, however, that four primary photochemical events in PS II act co-operatively in the release of O_2. This is usually expressed in terms of the S-state model proposed by Kok and co-workers [1,2] i.e.:

$$\begin{array}{c} S_0 \xrightarrow[e^-]{h\nu} S_1 \xrightarrow[e^-]{h\nu} S_2 \xrightarrow[e^-]{h\nu} S_3 \xrightarrow[e^-]{h\nu} S_4 \\ \uparrow \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad \downarrow \\ O_2 + 4H^+ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad 2H_2O \end{array}$$

S_n ($n = 1$, 2, 3 and 4) represents a state of the O_2-evolving complex which is more oxidized by one electron equivalent than the previous S_{n-1} state. Upon reaching the S_4 state, O_2 is released and the S_0 state is regenerated. In the current literature, the S_n states are often used in reference to the oxidation level of the manganese-containing component that is known to be essential for the reactions [3].

In the original Kok hypothesis, it was assumed that water oxidation occurs via a four-electron concerted reaction mechanism during the $S_3 \rightarrow S_4 \rightarrow S_0$ transition. However, water oxidation chemistry can involve a number of highly reactive intermediates [4]. Measurements of proton evolution by PS II have shown that protons are released from the O_2-evolving complex during the early S-state transitions, usually in the stoichiometry of 1, 0, 1 and 2 for the $S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$, $S_2 \rightarrow S_3$ and $S_3 \rightarrow S_4 \rightarrow S_0$ transitions, respectively [5]. Thus, it is often considered that water binds to the O_2-evolving complex in the early S-state transitions and becomes oxidized as the S-state cycle progress. Several models, for example, suggest that partially oxidized water species or specially bound peroxides are formed during the reaction sequence [6,7]. On the other hand, there also exists data to indicate that a concerted water oxidation mechanism does indeed occur [8]. It still remains to be clarified whether the protons released in connection with the early S-state transition represent protons coming directly from water or from other protonable groups of the O_2-evolving complex.

Abbreviations: Chl, chlorophyll; DCIP, 2,6-dichlorophenol indophenol; DCMU, 3-(3,4-dichloro)-1,1-dimethylurea; DMSO, dimethylsulfoxide; DPC, diphenylcarbazide; Hepes, 4-(2-hydroxyethyl)-1-piperazinesulfonic acid; Mes, 4-morpholineethanesulfonic acid; PPBQ, phenyl-*p*-benzoquinone; PS II, Photosystem II.

Correspondence (and Present address): T. Wydrzynski, Max-Volmer-Institut für Biophysikalische und Physikalische Chemie der Technischen Universität Berlin, Strasse des 17. Juni 135, D-1000 Berlin 12, F.R.G.

Several papers have recently dealt with the interaction of H_2O_2 with PS II and the S-states. In the original paper by Velthuys and Kok [9], treatment of thylakoid membrane samples with H_2O_2 and alkaline pH was used as a means to chemically reduce the O_2-evolving complex. After removal of H_2O_2 from the sample, the O_2 flash yield pattern was found to be phase-shifted (the first peak O_2 yield occurred after the fifth rather than after the third flash). The authors interpreted this result to indicate that the O_2-evolving complex could reach a state even more reduced than S_0, which they defined as a S_{-1} state. More recent papers have shown that H_2O_2, added in relatively high concentrations (80–400 mM), can be photooxydized by PS II samples which were altered by the removal of the 33 kDa and/or the 23 and 16 kDa extrinsic polypeptides [10,11]. The PS II-catalyzed O_2-evolution from H_2O_2 by these samples was described in terms of side reactions involving non-functionally bound manganese and the PS II primary photochemistry. However, other papers have indicated that added H_2O_2 can interact directly with the S_1 and S_2 states [12,13]. In all of these experiments, H_2O_2 was added to the samples.

There are some reports that PS II can generate H_2O_2 under special conditions. Thus, it was proposed that the interaction of ADRY reagents with the oxidizing side of PS II can lead to H_2O_2 formation [14]. Under conditions in which the extrinsic 16 and 23 kDa polypeptides have been removed, H_2O_2 may also be formed [10], possibly through back reaction of the S-state cycle [15].

In this communication, we report our observations on apparent H_2O_2 formation by intact PS II preparations without the addition of ADRY reagents. This H_2O_2 formation is suggested by a catalase-induced enhancement of O_2 evolution rates under conditions of low sucrose concentrations. Evidence is presented that the H_2O_2 comes from a fraction of intact PS II centers and does not involve free manganese. The implications of H_2O_2 formation by PS II in terms of water splitting function are discussed.

Experimental

Sample preparation

Triton X-100 prepared PS II samples were made from greenhouse grown spinach as described earlier [16]. In the preparation procedure, the PS II samples were washed three times in the standard buffer medium (20 mM Mes (pH 6.3), 400 mM sucrose, 5 mM $MgCl_2$ and 15 mM NaCl) in order to remove the excess detergent. As such, the PS II samples could easily be pelleted by centrifugation at $12000 \times g$ for 10 min. The PS II samples were then stored in concentrated form (8–12 mg Chl/ml) as small beads frozen in liquid nitrogen until used in the experiments. After storage, the PS II samples gave steady-state O_2 evolution rates of 550–650 μmol O_2/(mg Chl · h), i.e. 2200–2600 μequiv/(mg Chl · h), in the standard buffer medium at 26 °C using PPBQ as the electron acceptor.

Cl^- removal and Tris inhibition

Cl^- was removed by washing the PS II samples twice (35–40-fold dilution each time) in 20 mM Mes (pH 6.3). This procedure was carried out in the dark at ice temperatures and a 10 min incubation period was used between the two washing steps. The washed samples were resuspended to 2 mg Chl/ml in 20 mM Mes (pH 6.3), before use in the measurements. For the data in Fig. 1 and Table IV samples were washed in a medium with reduced Cl^- concentration. Upon dilution of the sample into Cl^--free buffer the initial Cl^- concentration was 65 μM. The activity of the washed samples was found to be stable for at least 6 h when stored on ice in the dark.

Tris-inhibited samples were prepared by suspending the stock PS II samples to 200 μg Chl/ml in 0.8 M Tris buffer, pH 8.3. The suspension was continuously mixed in room light, on ice, for 30 min and then pelleted by centrifugation. The Tris-inhibited samples were then carried through the Cl^--removal procedure as described above.

Activity measurements

The Cl^- dependence for O_2 evolution was measured as the initial rate of the light-induced, steady-state rate of O_2 evolution upon increasing Cl^- concentrations in the assay medium. The samples were allowed to incubate for 3 min in the assay medium before the light was turned on. The assay medium consisted of 20 mM Mes (pH 6.3), and 50 μg Chl/ml. Other variations in the assay medium are described in the figure and table legends. For some data in Table III, 20 mM Hepes (pH 7.5), was substituted for the Mes buffer. The Cl^- concentration in the assay medium was adjusted by standard additions from either 10 or 100 mM NaCl stock solutions. For most measurements PPBQ prepared in DMSO was used as the electron acceptor, giving a final DMSO concentration of 5%. When catalase was used, it was added to the assay medium to a final concentration of 0.5 mg/ml at which the catalase-induced increase in the observed rates of O_2 evolution was saturated. The purified powder of catalase from bovine liver (thymol free) was obtained from Sigma Chemical Company. In Fig. 2, heat-inactivated catalase was also used, in which case a 10 mg/ml solution was heated in boiling water for 5 min and the coagulated protein was resuspended using a glass homogenizer.

The steady-state rates of O_2 evolution were measured with a Hansatech Clark O_2 electrode. Saturating continuous white light was provided by a 250 W projector lamp and focussed onto the assay vessel (1 ml total volume) by a lens through an 8 cm water filter and a

heat reflectance filter. The temperature of the assay vessel was regulated at 26 °C by a circulator water bath for all measurements. DCIP photoreduction shown in Table II was measured spectrophotometrically on a Johnson Foundation type DBS-1 dual wave-length spectrophotometer at 540 nm (λ_{ref} = 522 nm). The sample was excited by red light ($\geq$ 630 nm). The concentration of DCIP for the photoreduction measurements was 50 μM and the sample was 5 μg Chl/ml. The reproducibility of the activity measurements was $\pm$3%.

Results

Under certain experimental conditions, addition of catalase to PS II samples enhances significantly the observed rates of O_2 evolution. Initially, we anticipated that the catalase-induced enhancement in O_2 evolution was connected to the Cl^- requirement for PS II, since, for some preparations, the catalase effect was much greater at low than at high Cl^- concentrations. Fig. 1 shows a set of typical results. In this experiment, the Cl^- content of PS II was reduced by simply washing and diluting the sample in Cl^--free medium. We avoided treatments that are typically used to 'deplete' Cl^- from the sample (i.e., a high-pH shock or addition of SO_4^{2-} or chelators; see, for example, Ref. 17) because these treatments may induce other kinds of changes in PS II. As can be seen in Fig. 1, the addition of catalase significantly increases the observed rates of O_2 evolution. Readdition of 25 mM Cl^- did not substantially suppress the catalase effect in this case.

Since catalase is known to dismutate specifically H_2O_2 to O_2 and H_2O, the above results suggest that H_2O_2 is being produced by the washed PS II samples. The specificity of the catalase effect is indicated in Fig.

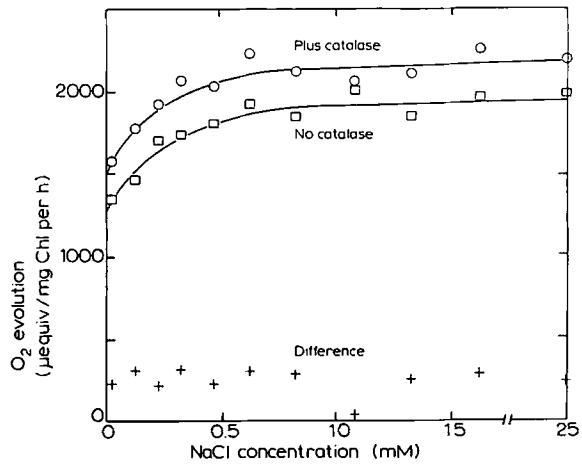

Fig. 1. The effect of catalase on the O_2 evolution of PS II-enriched membrane fragments at pH 6.3. The rate was measured as a function of Cl^- concentration in 20 mM Mes, 1 mM PPBQ, 5% DMSO and 26 °C. □, without catalase; ○, with 0.5 mg/ml catalase; +, difference between the rates with and without catalase.

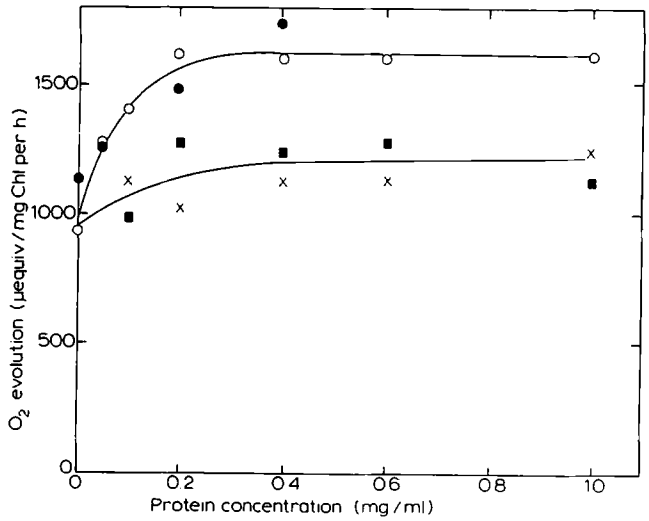

Fig. 2. The effect of added proteins on the O_2 evolution of PS II-enriched samples. The rate was measured as described in Fig. 1, in the absence of added Cl^-. ○, catalase, active enzyme; ●, catalase, active enzyme, plus 0.1 mg/ml superoxide dismutase; ■, heat-inactivated catalase (see Experimental section); ×, bovine serum albumin.

2. Here, it is shown that the catalase-induced enhancement in the observed rates of O_2 evolution is significantly larger than any osmotic effect that may be induced by the addition of protein to the sample, such as the heat-inactivated catalase or bovine serum albumin. On the other hand, Fig. 2 also shows that the inclusion of 0.1 mg/ml superoxide dismutase does not significantly alter the catalase-induced enhancement. This suggests that superoxide is probably not involved as an intermediate and that the catalase effect on O_2 evolution is specific. Furthermore, our samples do not show any inherent catalase activity, since the dismutation rate of added H_2O_2 (3.6 mM) does not increase upon sample addition, either in the dark or in the light (data not shown).

Although some of our results indicated that there may be some interaction of Cl^- on the catalase effect, it became apparent that the phenomenon was actually induced by the low sucrose concentration used in the removal of Cl^-. Table I shows the results from samples suspended in a low sucrose medium. There is a significant loss in the observed rate of O_2 evolution, which

TABLE I

Effect of sucrose and chloride on the oxygen evolution of PS II

Sucrose (mM)	Cl^- (mM)	Oxygen evolution (μequiv/(mg Chl·h))		
		no catalase	with catalase	difference
400	25	1248	1343	95
8	25	948	1204	316
8	0.5	1011	1296	285

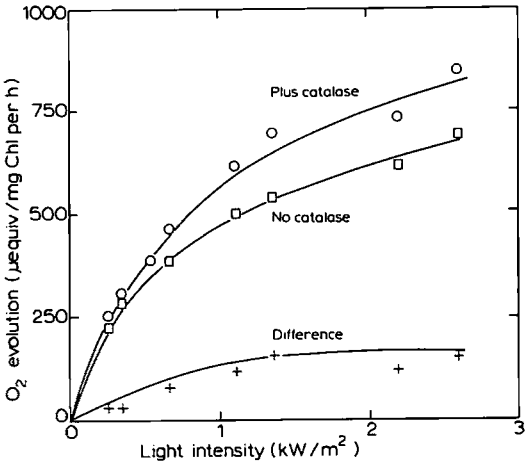

Fig. 3. The effect of catalase on the light intensity dependence of the O_2 evolution. The PS II samples were assayed in the absence of added Cl^- as described in Fig. 1. □, without catalase; ○, with 0.5 mg/ml catalase; +, difference between the rates with and without catalase.

could be overcome by the addition of catalase. In the presence of catalase, the O_2 evolution is about the same whether high or low sucrose is used. In this sample preparation, there was little effect of Cl^- on the extent of the catalase-induced enhancement of O_2 evolution. Thus, it appears that low sucrose concentrations after removal of excess Cl^- are primarily responsible for the catalase effect.

Fig. 3 shows the light intensity dependence for O_2 evolution for samples in the absence of added sucrose and Cl^- with and without catalase. The intensity dependence for the catalase-induced enhancement (i.e., the difference in Fig. 3) shows a similar behavior compared to the total O_2 evolution, indicating, therefore, that the apparent H_2O_2 formation is mediated by the PS II photochemistry.

The question arises as to whether the apparent H_2O_2 formation arises from reactions at the acceptor side, particularly when quinones are used as the electron acceptor. The catalase effect, however, does not depend on the type of acceptor used. Table II shows that significant enhancements in O_2 evolution are induced by catalase regardless of whether DCIP, PPBQ or a combination of PPBQ and ferricyanide is used as the electron acceptor. Thus, it seems unlikely that added quinones are involved in the H_2O_2 formation under these conditions.

Nevertheless, Table II presents another interesting phenomenon. When photoreduction of the acceptor DCIP is measured instead of O_2 evolution in the absence of added sucrose and Cl^-, the rate of electron transport is significantly greater than what is obtained from the observed O_2 evolution rate. A similar observation has been reported for salt-washed PS II samples where the extrinsic 26 and 16 kDa polypeptides have been removed [18]. In our experiments, upon addition of catalase the O_2 evolution increases such that the

TABLE II

Effect of catalase on acceptor photoreduction and oxygen evolution of PS II using various electron acceptors in the absence of chloride and sucrose

Electron acceptor	Acceptor photoreduction (μequiv/(mg Chl·h))	Oxygen evolution (μequiv/(mg Chl·h))		
	no catalase	no catalase	with catalase	difference
0.5 mM DCIP	504 [a]	315	525	210
1 mM PPBQ	–	940	1527	587
0.5 mM PPBQ + 2 mM $K_3Fe(CN)_6$	–	1586	2085	499

[a] DCIP photoreduction was measured at 5 μg Chl/ml and 0.05 mM DCIP.

differences in the electron transport rates largely disappears. Thus, in the absence of catalase a significant fraction of the electron transport through PS II is not expressed as O_2 evolution but as H_2O_2 formation. All activities, with or without catalase, are abolished by DCMU (data not shown).

Since it is known that added H_2O_2 to PS II samples having the extrinsic polypeptides removed can react with non-functional manganese in a PS II-mediated reaction [11], there remains a possibility that free Mn coming from damaged centers in our samples could be oxidized by PS II and be involved in the apparent H_2O_2 formation. Table III shows the catalase effect in the presence of EDTA. Although chelators may have complex effects on PS II [19], EDTA certainly chelates any free Mn that may be present in the samples. As indicated in Table III, the presence of EDTA does not affect the extent of the catalase-induced enhancement in the O_2 evolution, thus indicating that free Mn in the sample is not involved. However, it should be pointed out that addition of EDTA under these conditions does suppress the overall O_2 evolution with and without catalase as compared in the absence of EDTA (Fig. 1).

Table III also shows that the catalase effect can be prevented by raising the pH of the assay medium to 7.5. Although changes in pH probably cause many alterations in PS II, pH 7.5 is usually considered not to

TABLE III

Effect of pH, EDTA and catalase on the oxygen evolution of PS II in 1.0 mM chloride and in the absence of sucrose

pH	EDTA (mM)	Oxygen evolution (μequiv/(mg Chl·h))		
		no catalase	with catalase	difference
6.3	–	1856	2124	268
6.3	0.5	1660	1910	250
7.5	–	794	791	3

TABLE IV

Effect of catalase on the residual oxygen evolution of Tris-washed PS II with additions of Mn^{2+}, EDTA and DPC

When added, the catalase concentration was 0.5 mg/ml. The Cl^- concentration was 0.65 mM in all experiments except those with DPC where it was 25 mM. All measurements were made in the presence of 1 mM PPBQ.

Catalase	Mn(II) (300 μM)	EDTA (0.5 mM)	DPC (1 mM)	Rate (μequiv/(mg·h))
−	−	−	−	120
+	−	−	−	100
−	+	−	+	70
+	+	−	−	110
−	−	+	−	50
+	−	+	−	70
−	+	+	−	80
+	+	+	−	90
−	−	−	+	130
+	−	−	+	140

cause irreversible damage. The loss of the catalase effect at pH 7.5 may be a consequence of a direct pH effect on the reactions giving rise to the apparent H_2O_2 formation.

Finally, in another approach to determine if nonfunctional centers are responsible for the apparent H_2O_2 formation, we measured the effect of catalase in Tris-inhibited samples. After Tris treatment, the PS II samples did not show any sustained O_2 evolution, with or without catalase, in the presence of added electron donors and acceptors. However, a small O_2 burst (which is not a heating artefact) was observed in the first 10–20 s of illumination. Table IV gives the estimated initial rates of this residual O_2 evolution activity. As can be seen, the addition of catalase has no significant effect on detectable O_2 evolution using Mn^{2+} or DPC as the electron donor and PPBQ as the acceptor. Thus, these results suggest that the apparent H_2O_2 formation is not mediated by the acceptor side of PS II but requires an intact water-splitting site.

Discussion

There are now several reports in the literature showing that under certain inhibitory conditions [20,21] and in certain sample preparations [18,22], electron flow through PS II after multiple turnover can be greater than the measured O_2 yield. Under these conditions the question remains open as to what is the source of electrons. If it is indeed water and O_2 is not produced, then the question is what are the oxidized water species that are formed.

In this communication we report a similar phenomenon in PS II-enriched samples which were simply depleted of Cl^- in the absence of sucrose. Under this condition, the extent of electron flow through PS II as measured by DCIP photoreduction is significantly greater than what is measured as O_2 evolution (Table II). Upon the addition of catalase to the sample, the discrepancy in the measured electron transport rates for the most part disappears. Thus, under this condition H_2O may be oxidized to H_2O_2 instead of to O_2. This would account for the excess electrons that are measured at the acceptor side of PS II.

The phenomenon is also expressed using PPBQ or a combination of PPBQ and ferricyanide as the electron acceptor. In these cases, where the overall electron transport rates are higher, the addition of catalase also significantly increases O_2 evolution (Fig. 1; Table II). Thus, the phenomenon is not a consequence of rate limitations induced by the added electron acceptor system nor of possible side reactions of added quinones.

We had originally thought that low Cl^- conditions were necessary to induce the apparent H_2O_2 formation. However, results on the readdition of high Cl^- (25 mM) to Cl^- depleted samples were variable in suppressing the catalase effect. Instead, it became apparent that the low sucrose condition, which was used in the procedure to remove Cl^- from the samples, is primarily responsible for inducing the catalase effect. Thus, for example, samples suspended in low sucrose (8 mM), with either high (25 mM) or low (0.5 mM) Cl^-, show a reduced level of O_2 evolution which is restored to the same level as measured for samples suspended in high sucrose (400 mM) and high Cl^- (25 mM) upon the addition of catalase (Table I). In this context, it is interesting to point out that after removal of Cl^- by a simple washing and dilution procedure, there is very little Cl^- dependence on O_2 evolution, with or without catalase (Fig. 1). It is possible that this procedure does not remove the functional Cl^-, which can be achieved by the standard Cl^--depletion procedures involving high pH and counter anions. However, it is also possible that the standard Cl^- depletion procedures induce other changes in PS II which leads to an apparent Cl^- dependence. In any event, our results indicate that Cl^- in PS II is tightly bound ($K_m < 25$ μM).

The low sucrose/low Cl^- conditions used in these experiments to induce the catalase effect may also lead to a destruction of part of the PS II centers. It is, therefore, possible that the apparent H_2O_2 formation involves the free manganese from destroyed centers. However, the catalase effect occurs to the same extent when EDTA is present (Table III). EDTA should complex with free manganese and prevent any possible side reactions. Likewise, in Tris-inhibited samples, in the presence of added Mn^{2+} or DPC as electron donors, the addition of catalase did not stimulate any significant O_2 evolution (Table IV). Thus, these results together suggest that the apparent H_2O_2 formation does not involve free manganese nor the acceptor side of PS II, but

occurs near to the water-splitting site in relatively intact PS II centers.

If H_2O_2 is being formed by relatively intact PS II centers, then these observations have important mechanistic implications for the native water oxidation process. In terms of the step-wise water oxidation models, the apparent H_2O_2 formation could be arising from the proposed peroxide intermediates of the O_2-evolving complex [6].

Alternatively, for concerted water oxidation models, H_2O_2 could arise as a consequence of a short circuit in the S-state cycle, e.g., perturbed O_2-evolving complexes may advance only to the S_2 or S_3 state, release H_2O_2 and recycle back to the S_0 or S_1 state. Since H_2O_2 formation is induced by low sucrose conditions, it is likely that conformational changes are involved. Such conformational changes may influence the accessibility of water and the S-state cycling and may involve the extrinsic polypeptides [10,15]. It will become important in the future to measure H_2O_2 formation as a function of light flashes in order to discern these possibilities.

Another interesting feature in the data is that in order to observe the catalase-induced enhancement in O_2 evolution, relatively high concentrations of catalase are needed. This could imply that the H_2O_2 which is formed may react with other parts of the sample, such as lipids, in competition with the catalase reaction. Such reactions of H_2O_2 could certainly be important in the photoinhibition of PS II.

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