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LIGHT-DEPENDENT REDUCTION OF HYDROGEN PEROXIDE BY INTACT SPINACH CHLOROPLASTS

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Intact spinach chloroplasts, washed four times in buffered sorbitol to decrease catalase contamination, supported O_2 evolution in the dark at very low rates (less than 2 μmol/mg Chl per h) in the presence of low concentrations of H_2O_2 (0.25 mM); H_2O_2 was not significantly metabolised under these conditions. In the light, washed chloroplasts supported H_2O_2-dependent O_2 evolution at rates of 28–46 μmol/mg Chl per h in the presence of 0.1–0.25 mM H_2O_2; the concentration of H_2O_2 supporting $0.5V_{max}$ was estimated to be 25 μM. O_2 evolution in the light was associated with H_2O_2 consumption and ceased after the production of 0.45 mol per mol H_2O_2 consumed. Both O_2 evolution and H_2O_2 consumption were abolished by 5 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea. Washed intact chloroplasts contained endogenous pools of GSH and ascorbate estimated at 10 and 33 mM, respectively. H_2O_2-dependent O_2 evolution in the light was associated with a decrease in these levels which increased as O_2 evolution gradually ceased. The results are consistent with the hypothesis that H_2O serves as eventual electron donor for the reduction of H_2O_2 in illuminated chloroplasts and that GSH/GSSG and ascorbate/dehydroascorbate serve as intermediate electron carriers. Preincubation of chloroplasts in the dark with 0.1 mM H_2O_2 abolished O_2 evolution in the light.

Introduction

Illuminated chloroplasts support the reduction of O_2 to H_2O_2 utilizing reducing equivalents emanating from Photosystem I [1–3]. H_2O_2 is a potent inhibitor of chloroplast metabolism [4] at micromolar concentrations [5]. Current evidence suggests that glutathione peroxidase, which provides an important mechanism for the reduction of low concentrations of H_2O_2 in animals [6], is not associated with plants [7,8] and it has been proposed that in chloroplasts NADPH could serve as electron donor via the sequence NADPH $\rightarrow$ GSSG/GSH $\rightarrow$ dehydroascorbate/ascorbate $\rightarrow H_2O_2/H_2O$ [9]. The enzymes catalyzing the component reactions of this sequence are associated with chloroplasts [9–11] and, in illuminated ruptured chloroplasts, H_2O can serve as eventual electron donor for the sequential reduction of exogenous GSSG, dehydroascorbate and H_2O_2 with the concomitant evolution of O_2 [10–13]. Since intact chloroplasts contain endogenous pools of ascorbate [14] and GSH [9] and the chloroplast envelope is presumably permeable to H_2O_2 then, according to theory, the metabolism of exogenous H_2O_2 by intact chloroplasts should exhibit characteristics consistent with the properties of the pathway. Here we report that intact washed chlo-

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Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

roplasts support the reduction of exogenous H_2O_2 with the concomitant evolution of O_2 in the light, but not in the dark, and that the perturbations of the endogenous pools of GSH and ascorbate are consistent with the light-dependent transfer of reducing equivalents from H_2O to H_2O_2 via these substrates.

Materials and Methods

Intact unwashed chloroplasts were prepared from spinach (*Spinacia oleracea* cv Yates Hybrid 102) ad described in Ref. 15 except that ascorbate was omitted from the extracting medium. After washing the surface of the chloroplast pellet [15] the chloroplasts were resuspended in 50 ml of 0.33 M sorbitol adjusted to pH 7 with 10 mM Hepes-KOH buffer and recentrifuged. The latter procedure was repeated a further three times to decrease catalase contamination. All reactions were conducted in medium containing 0.33 M sorbitol, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 4 mM EDTA, 100 mM Hepes-KOH buffer, pH 7.6, washed chloroplasts (100 μg Chl/ml) and 10 mM DL-glyceraldehyde to inhibit endogenous CO_2-dependent O_2 evolution [16]. Incubations were performed in red light (300 $W \cdot m^{-2}$) at 20°C in O_2 electrodes [17] supplied by Hansatech, King's Lynn, U.K. (Light plus H_2O_2)-dependent O_2 evolution was initiated with H_2O_2 (0.1–0.25 mM); the concentration of H_2O_2 supplied was determined polarographically by O_2 evolution in the presence of catalase (220 U/ml). Catalase activity associated with chloroplast preparations was also determined by O_2 evolution using 2.5 mM H_2O_2 as substrate in the dark; all other conditions were as described for (light plus H_2O_2)-dependent O_2 evolution. Catalase activity was corrected for O_2 evolution in the presence of 0.1 mM NaN_3 and is expressed as azide-sensitive activity.

GSH, ascorbate, chlorophyll and chloroplast intactness were determined as described in Refs. 18, 14, 19 and 20, respectively.

Results

Unwashed chloroplasts were heavily contaminated with catalase as determined by azide-sensitive O_2 evolution in the dark in the presence of 2.5 mM H_2O_2 but this was decreased approx. 20-fold by four washes with buffered sorbitol (pH 7) (Table I). Nevertheless, chloroplasts washed four times contained significant catalase activity (16 μmol/mg Chl per h for the experiment shown in Table I) when determined by the above procedure. Catalase, however, has a low affinity for H_2O_2 [21]. At 0.2 mM H_2O_2, the rate of O_2 evolution catalysed by washed chloroplasts in the dark was negligible (Fig. 1, trace B). By contrast, illuminated washed chloroplasts in the presence of 0.2 mM H_2O_2 supported O_2 evolution at rates of 28–46 μmol/mg Chl per h (Fig. 1, trace A). O_2 evolution in the light ceased after the production of 0.45 mol O_2 per mol H_2O_2 supplied. The rate of O_2 evolution was constant until approx. 50% of this value was attained, thus implying that the light-dependent reaction, unlike catalase [21], had a relatively high affinity for H_2O_2. A further addition of 0.2 mM H_2O_2 to illuminated chloroplasts reinitiated the sequence described above although the rate of O_2 evolution invariably attained only 61–71% of the rate associated with the first addition of H_2O_2 (Fig. 1, trace A). Addition of exogenous catalase after cessation of O_2 evolution did not reinitiate O_2 evolution, indicating complete removal of H_2O_2 in the light. Whereas a second application of 0.2 mM H_2O_2 slightly enhanced light-independent O_2 evolution, caused by endogenous catalase activity, the large amount of O_2 released on addition of exogenous catalase (0.36 mol O_2 per mol H_2O_2 supplied: Fig. 1, trace B) indicates that low concentrations of H_2O_2 were not rapidly metabolised by washed chloroplasts in the dark. Since both light and low concentrations of H_2O_2 are required to support O_2 evolution and H_2O_2 consumption by washed intact chloroplasts, this activity is hereafter referred to as (light plus H_2O_2)-dependent O_2 evolution.

In the dark, although the rate of O_2 evolution at H_2O_2 concentrations below 0.25 mM did not exceed 2 μmol/mg Chl per h, the rate increased with the H_2O_2 concentration up to at least 1.6 mM. In the light, however, 0.1 mM H_2O_2 supported O_2 evolution at approx. 80% of the maximum rate. When corrected for activity in the dark, the optimum concentration of H_2O_2 for the light-dependent reaction was 0.1–0.4 mM; higher concentrations were inhibitory (Fig. 2). The concentration of

TABLE I

EFFECT OF A SERIES OF WASHES UPON SOME PROPERTIES OF ISOLATED CHLOROPLASTS

Chloroplasts were isolated (referred to as unwashed chloroplasts) and washed 1, 2, 3 or 4 times with 0.33 M sorbitol adjusted to pH 7 with 10 mM Hepes-KOH as described in Materials and Methods. The uncoupled rate of ferricyanide-dependent O_2 evolution was determined after osmotic shock [20].

Chloroplasts	Intactness (%)	Ferricyanide-dependent O_2 evolution (μmol/mg Chl per h)	Azide-sensitive catalase activity (μmol O_2/mg Chl per h)
Unwashed	76	299	289
1 wash	78	331	60
2 washes	82	390	34
3 washes	78	307	33
4 washes	75	287	16

H_2O_2 supporting $0.5V_{max}$ for the light-dependent reaction was estimated to be 25 μM. DCMU (5 μM) abolished (light plus H_2O_2)-dependent O_2 evolution and H_2O_2 was not metabolised under these conditions. Experiments similar to those shown in Fig. 1 involving unwashed chloroplasts and azide (0.1 mM) to inhibit catalase were unsatisfactory due to azide inhibition of (light plus H_2O_2)-dependent O_2 evolution. However, the latter reaction was less sensitive to azide than catalase activity (Fig. 3). Osmotically shocked chloroplasts (diluted 9-fold) supported O_2 evolution at a rate of 32 μmol/mg Chl per h upon addition of 0.23 mM H_2O_2 but this ceased after the evolution of only

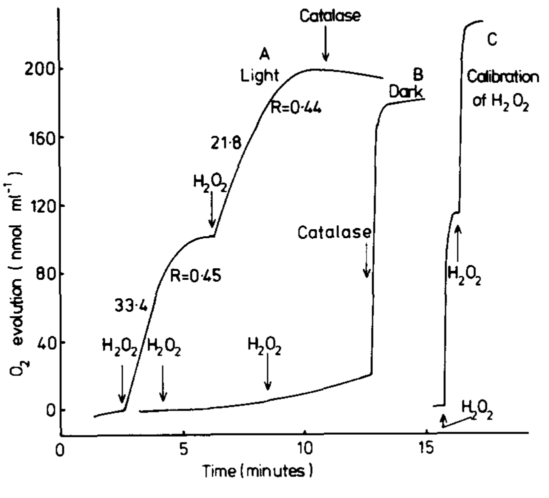

Fig. 1. Effect of H_2O_2 on O_2 evolution by washed chloroplasts (88% intact, washed four times) in (A) light and (B) dark. Values beside trace A denote the rate of O_2 evolution in μmol/mg Chl per h and R represents the ratio of O_2 evolved: H_2O_2 supplied for successive additions of 0.22 mM H_2O_2. Reaction mixtures A and B contained 100 μg Chl/ml. Catalase (220 U/ml) was added as shown. The concentration of individual additions of H_2O_2 supplied in the experiment was determined polarographically by O_2 evolution in the presence of 220 U/ml catalase (trace C).

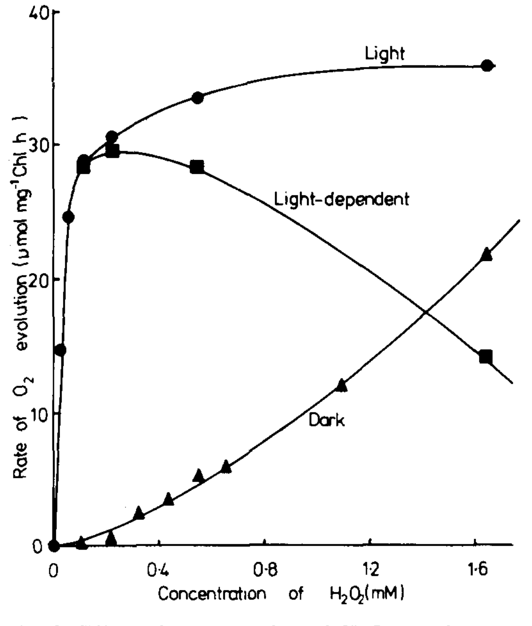

Fig. 2. Effect of concentration of H_2O_2 on the rate of O_2 evolution by washed chloroplasts (88% intact, washed four times) in the light (●) and in the dark (▲). All other conditions were as described for (light plus H_2O_2)-dependent O_2 evolution except that the concentrations of H_2O_2 were as specified. The light-dependent activity (■) is also shown.

0.07 mol O_2 per mol H_2O_2 supplied; a further addition of 0.23 mM H_2O_2 did not initiate significant O_2 evolution.

Washed chloroplasts preincubated in the absence of H_2O_2 for 11 min in the light (Fig. 4, trace C) or in the dark (Fig. 4, trace B) exhibited (light plus H_2O_2)-dependent O_2 evolution when the appropriate conditions were subsequently applied; the rates were similar to those obtained by the standard procedure (Fig. 4, trace A). However, when the chloroplasts were preincubated in the dark in the presence of 0.11 mM H_2O_2, they failed to exhibit (light plus H_2O_2)-dependent O_2 evolution upon illumination even when additional H_2O_2 was supplied (Fig. 4, trace D). Subsequent addition of catalase resulted in the production of 0.26 mol O_2 per mol H_2O_2 added, implying that H_2O_2 was not extensively metabolised. The data therefore demonstrate that some component of the mechanism involved in (light plus H_2O_2)-dependent O_2 evolution is sensitive to pretreatment with low concentrations of H_2O_2 under conditions in which H_2O_2 is not metabolised (e.g., dark in the absence of significant catalase activity).

Washed spinach chloroplasts contain endogenous pools of GSH and ascorbate which we have estimated to be approx. 10 and 33 mM, respectively, assuming a chloroplast volume of 26 μl/mg Chl [22]. Thus, in incubation mixtures containing 100 μg Chl/ml the average concentrations of GSH and ascorbate were approx. 26 and 86 μM, respectively. When two additions of 0.2 mM H_2O_2 were supplied to washed chloroplasts in the dark, 100% of the GSH and approx. 80% of the ascorbate were oxidised (Fig. 5A). A single addition of H_2O_2 (0.2 mM) also decreased the GSH and ascorbate pools of illuminated chloroplasts but as the rate of O_2 evolution decreased and finally ceased, the concentrations of GSH and ascorbate increased; the GSH concentration increased to a value in excess of the initial GSH pool and ascorbate to a value somewhat less than the original. This sequence was repeated upon a further addition of 0.2 mM H_2O_2 (Fig. 5B).

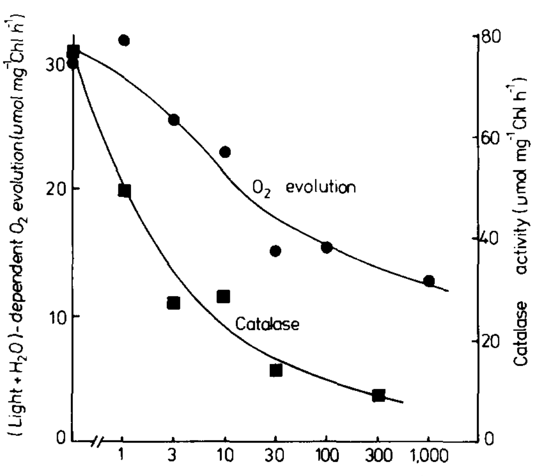

Fig. 3. Effect of concentration of azide on (light plus H_2O_2)-dependent O_2 evolution (●) and O_2 evolution in the dark in the presence of 2.2 mM H_2O_2 (■) by washed chloroplast preparations (89% intact).

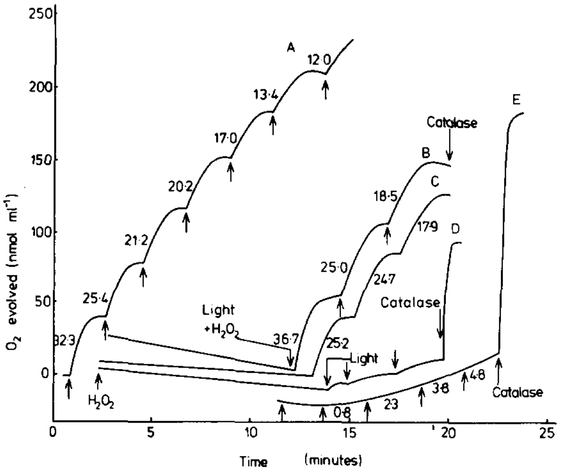

Fig. 4. Effect of pretreating chloroplasts (88% intact) in the light and dark, with and without 0.11 mM H_2O_2, on (light plus H_2O_2)-dependent O_2 evolution. Reaction mixtures A–E contained 100 μg Chl/ml and 0.11 mM H_2O_2 was added at the times shown by the unlabelled arrows. A and E constitute illuminated and dark controls, respectively, in which H_2O_2 was added immediately. B was preincubated in the dark for 11 min prior to illumination and addition of H_2O_2. C is analogous to B but was preincubated in the light. D shows the effect of preincubating chloroplasts in the dark for 11 min in the presence of 0.11 mM H_2O_2 prior to illumination. The O_2 evolution associated with the addition of catalase (220 U/ml) to D (three additions of 0.11 mM H_2O_2) and E (five additions of 0.11 mM H_2O_2) demonstrate that H_2O_2 was not extensively metabolised in these treatments. Values beside the traces denote the rate of O_2 evolution in μmol/mg Chl per h.

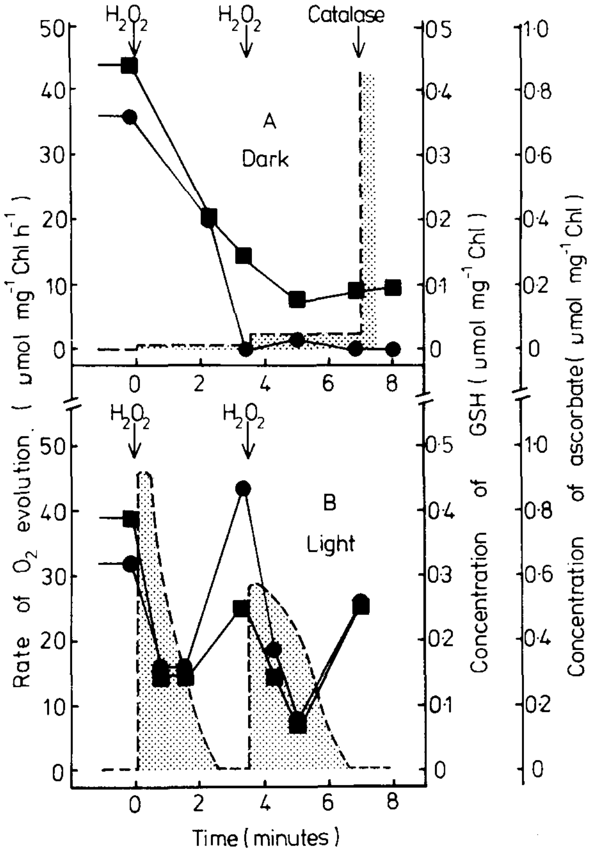

Fig. 5. Correlation between rate of O_2 evolution by intact washed chloroplasts (dotted curve) and the endogenous concentrations of GSH (●) and ascorbate (■) in (A) dark and (B) light in response to the addition of exogenous H_2O_2. Reaction mixtures contained washed chloroplasts (87% intact, 100 µg Chl/ml); H_2O_2 (0.23 mM) and catalase (220 U/ml) were added as shown. The maximum standard errors for determinations of GSH and ascorbate were 0.085 and 0.11 µmol/mg Chl, respectively.

Discussion

Our results demonstrate that intact washed chloroplasts have the capacity to metabolise low concentrations of H_2O_2 (< 0.4 mM) at rapid rates in the light but not in the dark with the concomitant evolution of O_2. Since washed chloroplasts exhibit negligible catalase activity at the low concentrations of H_2O_2 (0.25 mM) employed in our studies but support H_2O_2-dependent O_2 evolution in the light (Fig. 1), this implies that H_2O serves as the ultimate electron donor for light-dependent reduction of H_2O_2 to H_2O:

$$H_2O_2 + H_2O \xrightarrow{h\nu} 2H_2O + \tfrac{1}{2}O_2$$

The sensitivity of both H_2O_2 consumption and H_2O_2-dependent O_2 evolution to DCMU is consistent with this proposal and implies that the requirement for light involves Photosystems I and II.

The transitory decrease and subsequent increase in the endogenous GSH and ascorbate concentrations during (light plus H_2O_2)-dependent O_2 evolution is similar to that reported for ruptured pea chloroplasts in the presence of exogenous GSH and ascorbate [13]. In the light, the levels of the reduced forms of glutathione and ascorbate approach the initial values upon complete consumption of the added H_2O_2 but remain in their oxidised forms upon addition of H_2O_2 in the dark (Fig. 5). These perturbations are consistent with the proposed role of GSH/ GSSG and ascorbate/ dehydroascorbate as intermediate electron carriers in the transfer of reducing equivalents from Photosystem I and NADPH to H_2O_2 [9,11,13]. These results in turn imply that the enzymes glutathione (oxidised) reductase, glutathione dehydrogenase and ascorbate peroxidase are associated with chloroplasts as reported previously [9–11] and, under these conditions, exhibit rates commensurate with the rate of (light plus H_2O_2)-dependent O_2 evolution. The high affinity of the reaction for H_2O_2 (Fig. 2) is in broad agreement with the K_m value of 45 µM for H_2O_2 reported for spinach chloroplast ascorbate peroxidase [11].

The inhibition of (light plus H_2O_2)-dependent O_2 evolution by azide is consistent with the properties of ascorbate peroxidase [11,13] and previous studies of H_2O_2 metabolism in spinach chloroplasts in the presence of 1 mM azide [23]. However, (light plus H_2O_2)-dependent O_2 evolution was relatively less sensitive to azide than catalase activity, exhibiting only 50% inhibition at 0.1 mM azide. Impermeability of the chloroplast envelope to azide seems an unlikely explanation, since 0.1 mM azide was without significant effect upon (light plus H_2O_2)-dependent O_2 evolution by ruptured pea chloroplasts [13]. The abolition of (light plus H_2O_2)-dependent O_2 evolution upon osmotic shock was assumed to be due to dilution of the endogenous pools of NADP(H), GSH/GSSG and ascorbate/dehydroascorbate associated with intact washed chloroplasts; shocked chloroplasts must be supplemented with these reagents to support (light plus H_2O_2)-dependent O_2 evolution [11,13].

The abolition of light-dependent H_2O_2 metabolism by pretreatment of chloroplasts in the dark in the presence of low concentrations of H_2O_2 (Fig. 5, trace B) is not understood. This raises the question as to whether light-dependent H_2O_2 metabolism is any more sensitive to inhibition by H_2O_2 than other forms of metabolism in chloroplasts and, if so, whether inhibition of the H_2O_2 reduction pathway could be causal in inhibiting other forms of metabolism.

The mechanism we describe, with its high absolute activity and high affinity for H_2O_2, would afford a mechanism for maintaining H_2O_2 in the chloroplasts of illuminated leaves at extremely low concentrations under steady-state conditions as has been suggested previously [23]. Since isolated chloroplasts readily metabolise exogenous H_2O_2 in the light, it is not impossible that illuminated chloroplasts might (also) function in the reduction of H_2O_2 produced at other sites in photosynthetic cells. The ability of chloroplasts to reduce H_2O_2 in the light further demonstrates the capacity of chloroplasts to utilise light-generated reducing equivalents in processes other than CO_2 assimilation [24]. The data presented here do not support the suggestion that the primary function of ascorbate peroxidase in chloroplasts is involved with the oxidation of excess NADPH in the dark [25].

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