Photosynthetic Production of Hydrogen Peroxide by Anacystis nidulans

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ABSTRACT
A sensitive assay based upon fluorescence of scopoletin allowed continuous recording of H2O2 production in illuminated intact cells of Anacystis nidulans. Onset of illumination was followed by a 5 to 10 second lag, a burst of very rapid production continuing for up to 5 minutes, and finally a slow and continuing steady rate of H2O2 production. Size of the H2O2 burst was decreased by 3-(3,4-dichlorophenyl)-1,1-dimethylurea, by low O2, and by certain Calvin cycle intermediates; it was increased by high light intensity, CO2 depletion, Calvin cycle inhibitors (as iodoacetamide), cold shock, carbonyl cyanide m-chlorophenylhydrazone, and certain organic acids as glycolate. The H2O2 burst was explained by the following hypothesis: a low potential reductant is produced more rapidly than it can be used in the normal pathway to CO2 reduction and, instead, reacts with oxygen. H2O2 production is regarded as a metabolic defect observable in Anacystis most dramatically during the transition from a very low rate of oxidative dark metabolism to a high rate of photosynthetic metabolism.

The classical experiments of Mehler and coworkers (28-30) demonstrated the production of H2O2 by isolated chloroplasts. In subcellular preparations the role of molecular oxygen as a Hill oxidant and production of H2O2 has been well established, even in the absence of autoxidizable additives (3, 7, 15, 17, 20). However, it has remained uncertain whether reduction of oxygen to H2O2 is a normal event or an artifact induced by damage to the photosynthetic apparatus during isolation. Very small amounts of H2O2 production have been reported in flash-illuminated cells of Chlorella (11) and in Anacystis nidulans (38). We now report the direct, continuous measurement of photosynthetic production of H2O2 by intact cells of Anacystis nidulans.

MATERIALS AND METHODS
Modification of the scopoletin fluorescence assay (1, 34) allowed continuous recording of production of H2O2 by illuminated algal cells. Scopoletin is oxidized by H2O2 in the presence of peroxidase so that decrease in scopoletin fluorescence is a direct measure of amount of H2O2. The assay apparatus is described in Figure 1. The routine assay reaction mixture contained 1 nmole of scopoletin and 80 units of peroxidase in a total volume of 3.3 ml. Cuvette temperature did not rise significantly above room temperature (24-26 C). We confirmed the previously reported requirement for peroxidase and the 1:1 stoichiometry for scopoletin-H2O2 in our apparatus using H2O2 solutions standardized against KMnO4. Oxygen exchange was measured by a Beckman oxygen macroelectrode in a cuvette of about 1.5 ml at a sensitivity of about 0.02 μl O2/ml (32).

Anacystis nidulans Richter (strain T6 20 of our laboratory) was grown in medium C (24) in a continuous culture apparatus at 25 C or 30 C, aerated with 1% CO2 in air. Although no detailed study of culture conditions was made, it was noted that cells grown under comparatively low light intensity (four 40-w tubular tungsten lamps at 40 cm, cell doubling time about 24 hr) consistently gave greater initial bursts and higher steady rates of H2O2 production than cells grown under higher intensity light at higher growth rates. Cells grown in this way were routinely used. For experiments, harvested cell suspensions (about 3 μl cells/ml) were centrifuged at 3300g for 10 min and resuspended in a medium C containing only the major salts (1 mM MgSO4, 5 mM NaNO3, 2 mM KNO3, 6 mM K2HPO4; pH 7.3). This stock cell suspension (about 1 μl cells/ml) was held at 26 C, illuminated with two 20-w warm white fluorescent lamps, and aerated with 1% CO2 in air until use.

Scopoletin and pCMB (sodium salt) were purchased from Mann Research Laboratories. Scopoletin solutions were prepared in 1 or 2 liter quantities (2 mg/l) and stored frozen until use. Peroxidase (type II, horseradish) and iodoacetamide were from Sigma Chemical Company. Carbonyl cyanide m-chlorophenylhydrazone was a gift of Dr. F. P. Healey. Diquat (ethylene viologen) was obtained as the dibromide salt from Ortho Division of California Chemical Company.

Cell concentrations were determined by centrifuging the cell suspension for 1 hr at 2200g in centrifuge tubes with calibrated capillary bottoms. Chlorophyll a was estimated in 80% acetone extracts (absorption coefficient = 82.04 cm2/mg at 663 nm).

RESULTS
Hydrogen peroxide production, as described herein for Anacystis nidulans, is not a universal phenomenon in algae nor in blue-green algae. By the same methods we sought but did not find evidence of H2O2 production by Anabaena cylindrica, Agmenellum quadruplicatum, and Chlorella pyrenoidosa (Emerson strain), either in freshly harvested or in CO2-starved cells. Subsequent to the main body of our work, further exploration has revealed H2O2 production in some other blue-green algae, although not always with the same pattern seen in Anacystis.

Time Course. Production of H2O2 by Anacystis after onset

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SUMMARY
The high rate of photosynthetic H2O2 production by Anacystis nidulans was shown to be an artifact of the assay procedure. Several observations indicate that H2O2 production is a low potential reductant that reacts with oxygen to form H2O2, rather than being a specific reaction of the Calvin cycle.

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1 Abbreviations: CCCP: carbonyl cyanide-m-chlorophenylhydrazone; pCMB: p-chloromercuribenzoate.
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Fig. 1. Apparatus for H$_2$O$_2$ assay. Fluorescence of scopoletin in cuvette C was excited by 365 nm provided by a 100-w mercury lamp; UV, was via a Corning 5860 plus interference filter, $F_1$. Scopoletin fluorescence at 410 to 470 nm was measured by the IP21 photomultiplier; PM was of an Aminco photometer via a compound filter, $F_2$, containing 1 cm of 1.0 m CuSO$_4$, and Wratten filters 2A, 2B(2), and 47B. Photosynthesis of algal cells in the cuvette was driven at 620 or 675 nm by a Kodak projector; $I_n$ was via an 8 cm water cell and blocked interference filters, $F_5$.

Fig. 2. Time course of H$_2$O$_2$ production in Anacystis. The reaction cuvette (Fig. 1) contained 2 ml of 50 mM phosphate buffer equilibrated with 1% CO$_2$, 100 $\mu$l of scopoletin stock solution (2 mg/l in water), 1 ml of cell suspension in medium C-m containing 1 $\mu$l cells and equilibrated with 1% CO$_2$ in air, and 50 $\mu$l of a peroxidase solution (4 mg/ml in water). In all experiments, the complete reaction mixture was equilibrated in the cuvette in the dark for 5 min before onset of illumination. The reaction mix was aerated with 1% CO$_2$ in air throughout each experiment. Illumination was at 620 nm, and the intensity shown on each curve is in $\mu$watts/cm$^2$. The immediate upward displacements at the beginnings (↓) and equal downward displacements at the ends (↑) of illumination are artifacts resulting from incomplete protection of the photomultiplier against 620 nm. Estimation of burst size is shown for one intensity.

of illumination typically followed a complex time course (Fig. 2) consisting of three stages: an initial lag of several (5–10) sec, a burst or very rapid production continuing for up to 5 min, and finally a slower and continuing steady rate of production. Return to darkness during the steady rate dropped H$_2$O$_2$ production rate to zero within the instrumental time response (about 2 sec). If illumination ended during the burst stage, H$_2$O$_2$ production was observed to continue for several (up to 20) sec in darkness. The character of the time course was not dependent on wavelength of illumination.

Illumination Effects. While the length of the lag period and the size of the steady rate varied with light intensity, the most dramatic effect of intensity was on the size of the burst (Fig. 2). Figure 3 shows burst size and rate of oxygen evolution versus light intensity as determined in parallel experiments on the same cell suspension. The burst disappeared at low intensity; illumination at 200 to 500 $\mu$w/cm$^2$, 620 nm, gave no burst but supported a low steady rate. Still lower intensities (<200 $\mu$w/cm$^2$, 620 nm) gave no measurable H$_2$O$_2$ production at all. Light saturation of the burst size required intensities

Fig. 3. Light intensity curves for steady-state oxygen evolution (A) and for the H$_2$O$_2$ burst (B) in Anacystis. Harvested cells were centrifuged, resuspended in medium C-m at 0.6 $\mu$l cells/ml, and aerated with 1% CO$_2$ in air at 25 C under very low tungsten illumination until use. Chlorophyll concentration was 6.6 $\mu$g/$\mu$l cells. Illumination was provided by a Kodak projector using Baird Atomic interference filters at 620 nm (15 nm half band width) or 675 (30 nm half band width) together with infrared blocking filter 6143 and a 8 cm H$_2$O cell. Intensity was varied by screens and measured by a calibrated thermopile (A) or a silicon cell calibrated against the thermopile (B). Compensating intensity for 620 nm was estimated to be 30 $\mu$watts/cm$^2$. A: The curves for 620 and 675 nm were normalized only on the intensity scale by adjustment factor 4.04. B: The 620-nm curve was drawn for best fit by inspection; the 675-nm curve was obtained by doubling intensity values for the 620-nm curve and drawing a new curve for the points thus generated. Reasonable fit is thereby attained with experimental points for 675 nm. The curve fitting procedure was selected to make evident that for equal photosynthetic effectiveness 620 nm is about two times as effective as 675 nm in producing the peroxide burst.
ties much higher than those necessary to saturate photosynthetic oxygen evolution. Figure 3 also shows that for equal photosynthetic effectiveness, 620 nm is about twice as effective as 675 nm in producing the \( \text{H}_2\text{O}_2 \) burst.

The burst could be entirely eliminated by slow (rather than abrupt) increase from zero to high light intensity or by illumination by long flashes (\( \leq 10 \) sec, \( 1500 \, \mu\text{W/cm}^2 \), 620 nm) separated by dark periods (\( \geq 10 \) sec). However, neither of these regimens eliminated the final steady rate. The entire time course (lag, burst, steady production) could be observed repeatedly from a single cell suspension if sufficient dark time (5-10 min) was allowed between light doses of sufficient intensity and duration.

**CONDITIONS INFLUENCING THE BURST**

**Decrease of Burst Size.** DCMU cut burst size in half at 0.01 \( \mu\text{M} \) and abolished all \( \text{H}_2\text{O}_2 \) production at 0.1 \( \mu\text{M} \). Similarly, all \( \text{H}_2\text{O}_2 \) production was abolished by 0.5 \( \mu\text{M} \)-o-phenanthroline. These results are taken as evidence that the photosynthetic apparatus is the source of reductant for \( \text{H}_2\text{O}_2 \) production. \( \text{H}_2\text{O}_2 \) production was also abolished by decreased \( \text{O}_2 \) concentration obtained by aeration with 1\% \( \text{CO}_2 \) in \( \text{N}_2 \).

**Increase of Burst Size.** Production of \( \text{H}_2\text{O}_2 \) was markedly increased after 5-min incubation with \( p\text{CMB} \) or iodoacetamide, two Calvin cycle inhibitors (2, 14). At 1 \( \mu\text{M} \), \( p\text{CMB} \) doubled burst size and increased the final steady rate 3-4-fold. Iodoacetamide at 1 \( \mu\text{M} \) increased burst size 10-fold and increased the steady rate 20-fold. \( \text{H}_2\text{O}_2 \) production was also increased by \( \text{CO}_2 \) depletion. After aeration with alkali-scrubbed air, burst was increased 3-4-fold; steady rate was increased 4-5-fold.

Effects of cold shock were studied with cell suspensions held at 4\% \( \text{C} \) for 45 min in darkness and rewarmed to 25\% \( \text{C} \) before assay. This cold shock tripled the burst size and doubled the final steady rate. Similar cold treatment is known to decrease photosynthetic rates and cause pteridine excretion in *Anacystis* (12).

The uncoupler CCCP (18) gave increasing burst size with increasing concentration (3-30 \( \mu\text{M} \)) as indicated in Figure 4. At 30 \( \mu\text{M} \), burst size was doubled, but photosynthetic \( \text{O}_2 \) evolution was decreased to about half its control rate. Both \( \text{H}_2\text{O}_2 \) burst and rate of \( \text{O}_2 \) evolution declined at higher concentrations of CCCP. We note the contrast between inhibition by the uncoupler CCCP and by blocking of electron flow with DCMU.

Effects of the oxidizable electron acceptor, diquat (40), were of a different kind. Production of \( \text{H}_2\text{O}_2 \) occurred in darkness at a measurable rate which decayed slowly with time. At the onset of illumination the rate increased abruptly with no detectable lag, reached a very high maximum rate in about 1 min, and continued without decrease until return to darkness. Introduction of a catalase-ethanol trap (28), we also made conventional measurements of \( \text{O}_2 \) uptake mediated by diquat. At saturating concentration (15 \( \mu\text{M} \)) and light intensity, the rate of \( \text{O}_2 \) uptake was somewhat greater than the rate of \( \text{O}_2 \) production in unpoisoned cells.

The burst was sensitive to pH and to the buffer system (Fig. 5). The \( \text{H}_2\text{O}_2 \) burst was absent or very small below \( \text{pH} \) 7.0 where growth of *Anacystis* is inhibited (24). The \( \text{H}_2\text{O}_2 \) burst is increased by higher \( \text{pH} \) values during the brief (5 min) dark incubation preceding illumination. Our curve for burst size versus \( \text{pH} \) in 50 \( \mu\text{M} \) phosphate buffer is remarkably similar to the curve of Honeycutt and Krogmann (20), describing \( \text{O}_2 \) reduction by a subcellular preparation of *Anabaena variabilis*.

**Effects of Cell History.** Effects of cell history were examined for cells grown under illumination varied from our standard conditions. Although effects of light intensity during growth were not explored in detail, it was noted that cells grown at higher light intensities and higher specific growth rate gave lowered burst size. A particular condition examined was that of red light illumination which gives a high phycocyanin-chlorophyll ratio and an increased ratio of system 2-system 1 activity as viewed by enhancement (22, 23). Such cells grown under BCJ red (>660 nm) lamps gave greatly increased burst.
size observed at lower intensity of 620 nm actinic illumination (Fig. 6). Unfortunately, the culturing illumination was effectively low and gave only slow growth with doubling time of about 96 hr. Hence, it is not clear whether the increased burst size is attributable to the preceding very low growth rate or to the increased system 2-system 1 activity.

Effects of Metabolic Intermediates. Recalling effects of various intermediates in decreasing induction lags in O₂ production or CO₂ uptake by chloroplasts (4, 5, 8, 39), we examined effects of intermediates added at the beginning of the 5-min dark incubation period before assay. Four of the five Calvin cycle intermediates tested depressed H₂O₂ production (Table IA). We also examined effects of five carboxylic acids (Table 1B). Here the effects were more complex and more dependent on concentration. The most dramatic effects were seen in the 3- to 4-fold increase in burst size with added succinate and malate. None of the additives examined gave measurable H₂O₂ production in darkness.

DISCUSSION

In Anacystis the transient high rate of H₂O₂ formation (the burst) can be a significant fraction of the total electron flow. At 7 mw of 620 nm illumination a typically measured rate of H₂O₂ production was 0.4 μmole H₂O₂/mg chl-min during the 1st min of the burst. This represents about 5% of the steady light-saturated rate of electron transport (3.7 μmoles O₂/mg chl-min) or about 15% of the average rate during the same portion of the induction period (15-75 sec). Such calculations, and indeed all of our measurements based on extracellular H₂O₂, necessarily underestimate rates of intracellular H₂O₂ production. Anacystis has a strong catalase activity evident equally in whole cells and broken cell preparations (27, 33).

It is instructive to consider the metabolic peculiarity of Anacystis. As with several other blue-green algae, it will assimilate a number of exogenous substrates into cellular material in light but not in darkness (19, 36). A proposed explanation of its obligate autotrophy hinges partly on lack of ability to generate ATP oxidatively (6, 36). As compared to other blue-green algae, Anacystis is unusual in its very low rate of dark oxidative metabolism, especially when grown at low light intensities (25). From these considerations alone it might be expected that Anacystis must have a special metabolic problem in adjustment of ATP level and pool sizes of intermediates following transition from darkness to high light intensity.

Almost all of our data are consistent with the following simple hypothesis as an explanation of the H₂O₂ burst in Anacystis (Fig. 2). A low potential reductant is produced more rapidly than it can be used in the normal pathway to CO₂ reduction and instead, reacts with O₂. The preceding initial lag represents time required to deplete pool sizes or otherwise establish limitations on rate of electron acceptance by the Calvin cycle. The burst ends when such limitations are removed. In support of this hypothesis, we note especially the following. (a) The burst requires sudden transition from darkness to high light intensity; it can be abolished by gradual transition or by repeated flashes if each is shorter than the lag. (b) Partial inhibition of electron transport (DCMU) decreases the burst, while partial inhibition of phosphorylation (CCCP) or of the Calvin cycle (low CO₂, pCMB, iodoacetamide) increases the burst. (c) Cold shock, which increases the burst, has been shown to inhibit phosphorylation far more than electron transport (21). (d) Exogenous Calvin cycle intermediates

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1 Control experiments indicated the compound did not interfere with the scopoletin assay.
which decrease induction effects in chloroplasts also decrease the burst. (e) More complete diversion of electron flow to oxygen by diquat abolishes the lag, showing that there is no lag in electron transport.

We note from the light intensity curves (Fig. 3) several characteristics which support or are consistent with the above hypothesis. Below a threshold light intensity the burst disappears. Here photochemical generation of reductant is slow, and the necessary adjustment in pool sizes is small enough that there is no accumulation of excess reductant. At high light intensities the burst size is still increasing when the steady rate of photosynthesis is saturated. We suggest that in these cells the rate-limiting step in photosynthesis is located somewhere in the Calvin cycle, at least during the first several minutes of illumination. The lower effectiveness of 675 nm (versus 620 nm) is attributed to its relatively higher contribution to cyclic photophosphorylation and a more rapid increase in available ATP as one of the pools requiring adjustment.

We have no evidence on the site of H₂O₂ production. However, none of the electron transfer intermediates between the photo reactions has been reported capable of reducing O₂ to H₂O₂. Hence, we tentatively assign H₂O₂ production to a reductant near the terminus of system 1. Production of H₂O₂ by autoxidation of ferredoxin has been reported (37), but H₂O₂ production also has been reported in membrane preparations of Anabaena, presumably washed free of ferredoxin (20). The cytochrome reducing substance has been shown to be readily oxidized by O₂ but without evidence for concurrent H₂O₂ production (13).

The final steady production of H₂O₂, though possibly related to the burst in terms of site of H₂O₂ formation, appears to be an independent phenomenon partially overlapping in time. A succession of flashes or gradual transition from darkness to high light, which eliminate the burst, have no effect on the final steady production. The final steady rate, as measured, never represented more than 0.5% of the light-saturated electron throughput rate as estimated from rate of oxygen evolution. Hence, we regard it as a small defect or leak. We have considered the notion that H₂O₂ might play some metabolic role, e.g., biosynthesis of glycolate (35), but find no supporting evidence pertinent to blue-green algae.

Effects of some added intermediates were explored but without intent of providing detailed analysis of metabolic pathways. With whole cells interpretation of such effects is clouded by questions of intracellular availability. In fact we were surprised to find effects at all and by such an array of compounds. Depression of the burst by ribose-5-P, ribulose-5-P, fructose-1,6-diP, and dihydroxyacetone-P is consistent with observations on shortening of induction effects in chloroplasts (4, 5, 8, 39). We suppose that these additives are effective by maintaining pool sizes of Calvin cycle intermediates closer to their light levels.

Activity of glycolate, succinate, and malate in increasing the H₂O₂ burst suggests that these compounds may be supplying reductant (e.g., reduced pyridine nucleotide) to the cells via succinate dehydrogenase, malate dehydrogenase (36), and glycolate oxidase (16). This would cause still greater excess of reductant at onset of illumination. Such activity suggests the operation of the tricarboxylic acid cycle or the glyoxylate shunt. However, on the basis of labeling experiments in which Anacystis cells were fed ¹⁴C-acetate, previous investigators (19, 36) have concluded that the tricarboxylic acid cycle in this organism is blocked at α-ketoglutarate and that no glyoxylate shunt is present to bypass the block. Our experiments indicate that the light metabolism in Anacystis is somehow disturbed by sub-strate levels of organic acids: our observations may be related to those of Miller et al. (31) who observed light stimulation of glycolate uptake and oxidation in two blue-green species.

Our findings contribute to but do not resolve a three-part anomaly surrounding Anacystis and H₂O₂: (a) a high sensitivity of single-cell isolates to exogenous H₂O₂ (27, 38); (b) a strong catalase-like activity observable in whole cells and not increased by cell disruption (33); and (c) a photochemical production of H₂O₂ by whole cells observable outside the cells.

We have treated a relatively small production of peroxide observed under special conditions in Anacystis. This observation and our explanation for it are not novel (9, 10), save in the sense that we have considered events occurring in intact, unpoisoned cells. We have been led to the conclusion that the peroxide production results from a metabolic defect understandable from other known metabolic characteristics of Anacystis. In a broader context we are led to great respect for the elegance of metabolic machinery of other algae. It is common practice to grow algae in the laboratory at a relatively low effective light intensity and then to study the cells at light saturation and a metabolic rate perhaps 10-fold higher than they had ever previously experienced. Detailed attention has been given relatively minor aberrant events observable during the induction period of photosynthesis. We wonder if there occurs anywhere other than in photosynthetic cells the up to 100-fold increase in over-all metabolic rate which attends transition from darkness to high light intensity.

LITERATURE CITED

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