Photooxidase Activity of Heated Chromatophores of Rhodospirillum Rubrum [1]

E. S. Lindstrom [2]

Department of Botany, University of Minnesota, Minneapolis 14

Van Niel's classical distinction between bacterial and green plant photosyntheses is based on the differences in the metabolism of the photooxidant (16). But direct measurement of the oxidizing power in bacterial photosynthesis is, at best, difficult with the usual spectrophotometric equipment. Light-induced oxidations of endogenous cytochromes have been demonstrated in whole cells and extracts of *Rhodospirillum rubrum* (2, 4, 15), but special spectrophotometers were always required. The oxidizing power generated by light was easily measured by Vernon and Kamen using as the assay the photooxidation of reduced indophenol or exogenous mammalian cytochrome *c* (18, 19). However, this photooxidation of exogenous indicators was oxygen dependent and heat resistant causing Smith to doubt its physiological significance (14). The photooxidation of reduced indophenol could merely represent a manifestation of the well-known ability of bacteriochlorophyll to mediate a sensitized photooxidation (7).

Mild heat has been shown to destroy two absorption bands characteristic of the spectrum of bacteriochlorophyll in the chromatophore of *R. rubrum* (3, 8) while the photooxidase assay is relatively heat insensitive (10, 18). By comparing the effect of mild heat treatments on both the spectrum and on the photoxidase activity of the chromatophore, a validation was sought of the photooxidase assay using reduced indophenol. The rate of photooxidation of indophenol was not related to the strength of any absorption peak of bacteriochlorophyll. This participation of other heat-labile factors suggested that this in vitro assay is related to the in vivo metabolism of the oxidant.

Materials & Methods

Rhodospirillum rubrum (Esmarch) Molisch strain S1 was usually grown for 48 hours at 28 C in a lactate-yeast extract medium and was harvested and washed by centrifugation, frozen, and ground with alumina (10). For cultures grown while fixing nitrogen, inocula were grown in the light in 1 % Difco yeast extract for 48 hours at 28 C. A 1 % inoculum was added to a low nitrogen medium (DL-malic acid, 2.5×10^{-2} M; K_2HPO_4, 1×10^{-2} M; $MgSO_4$, 2×10^{-3} M; $CaCl_2$, 1×10^{-4} M; ferric ammonium citrate, 1.3×10^{-6} M Fe^{3+}; Na_2MoO_4, 4×10^{-9} M; biotin, 2×10^{-10} M; to pH 7.5 with KOH) which was incubated for 5 days in a lighted water bath at 28 C under an atmosphere of 5 % CO_2 and 95 % N_2. Chromatophores were isolated by centrifuging in 0.1 M potassium glycylglycine pH 7.6 (6). The photooxidase assay (18) measured the light dependent oxidation of sodium 2,6-dichlorobenzenoneindophenol (Fisher A.C.S. certified) that had been reduced to a faint persisting blue with solid sodium hydrosulfite. It was modified by omitting the cyanide (the dark oxidations were slight) and substituting glycylglycine buffer for phosphate. Photooxidation was measured as an increase of absorbancy at 6,000 A (A_{6000}) which was continuously monitored in a Cary model 14 spectrophotometer. The integral exciting light, mounted below the cuvette, was filtered through a Corning 2600 deep red 7-69 filter (cutoff below 7,200 A), and did not interfere with the A_{6000} measurements. With this geometry photooxidation was light saturated at 3.9 amperes to the exciting lamp (G.E. lamp No. 1615 operating at 6 v ac) while determinations were made at 4.3 amperes. To obtain the rates reported, the average of the changes of A_{6000} in the dark periods of 2 minutes each that preceded and followed the light period were subtracted from the change of A_{6000} in the 2 minute light period. The kinetics of the observed light reaction was complex with an initial rapid rate of oxidation falling off with time.

The chromatophores were heat treated in glycylglycine buffer in a water bath with a calibrated thermometer immersed in the suspension. Spectra of the unheated and heated chromatophores were determined in the Cary spectrophotometer.

Results

Data in the second column of table I show the progressive loss of photooxidase activity which was observed as the intensity of the heat treatment increased. With unheated preparations a rate of 0.36 μM indophenol oxidized/minute/μM bacteriochlorophyll was obtained using the rate during the initial 20 seconds. This compared favorably with a rate of 0.25 to 1.0 μM diphosphopyridine nucleotide (DPN) reduced/minute/μM bacteriochlorophyll obtained with similar chromatophores (Frenkel, unpublished data). With unheated chromatophores the indophenol can be reduced with reduced diphosphopyridine nucleotide (DPNH) using the chromatophoral diaphorase (18). If the DPNH and indophenol were first respectively

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[2] On sabbatical leave from the Department of Bacteriology, The Pennsylvania State University, University Park.

oxidized and reduced by the unheated chromatophores in the dark, a simultaneous reduction of DPN and oxidation of reduced indophenol was observed when the exciting light was turned on. The rates of photooxidation of indophenol were comparable to those observed with chemically reduced dye, but the rate of the photoreduction of DPN was much less than could be obtained if succinate were the trap for the photooxidant (5). Heat treatments inactivated this diaphorase so all data reported were obtained with chemically reduced indophenol. The heat treatments also depressed the absorption at 8,000 and 8,800 A precluding a satisfactory bacteriochlorophyll determination. Rates with the heated extracts were reported on a volume equivalent basis with the unheated extract. A heat treatment of 80 C for 15 seconds usually resulted in complete photooxidase inactivation while 80 C for 30 seconds always resulted in complete inactivation. Chromatophores isolated from cells grown while fixing N_2 gave comparable results.

Data in the last two columns of table I express some of the spectral changes seen in figure 1. As had been reported by Duysens and Goedheer (3, 8) who used more drastic treatments, heating destroyed the peak at 8,000 A, depressed the peak at 8,800 A, and created a peak at 7,800 A characteristic of bacteriochlorophyll in organic solvents. As heating can increase the selective scattering of these extracts, the region from 7,800 to 8,200 A was checked with opal glass diffusers (13) to insure that no hidden 8,000 A peak was present. The 80 C-10 second heat treatment freed no soluble material from the chromatophore that absorbed light between 4,000 and 9,000 A. The photooxidase activity of the 80 C-10 second preparation was also determined using saturating white light to show that its low activity was not the result of exciting light of improper wavelength.

Table I

Effect of Heating on Photooxidase Activity & Bacteriochlorophyll Peaks of Chromatophores

Treatment	Photooxidase activity*	A_{8800}-A_{8200}	A_{8000}-A_{8200}
Unheated	0.21**	0.84†	0.034†
76 C for 10 sec	0.12***	0.80	0.004
78 C for 10 sec	0.082***	0.70	−0.006
80 C for 10 sec	0.038***	0.62	0.000

* The photooxidase assay contained 0.1 μM reduced indophenol and 0.1 ml chromatophoral suspension (0.026 μM bacteriochlorophyll) in 3.0 ml glycylglycine buffer and was carried out in air at 25 C. The molar absorbancies used were 2×10^4 at A_{6000} for indophenol and 2.24×10^4 at A_{8000} for bacteriochlorophyll.

** μM reduced indophenol oxidized/minute/μM bacteriochlorophyll.

*** μM reduced indophenol oxidized/minute/volume equivalent of extract.

† Height of peak as determined by the difference in absorbancies at the indicated wavelengths.

The spectra of the chromatophores from the yeast extract grown cells were qualitatively similar to the spectra from N_2 grown cells except that N_2 grown chromatophores had a distinct peak at 5,500 A in

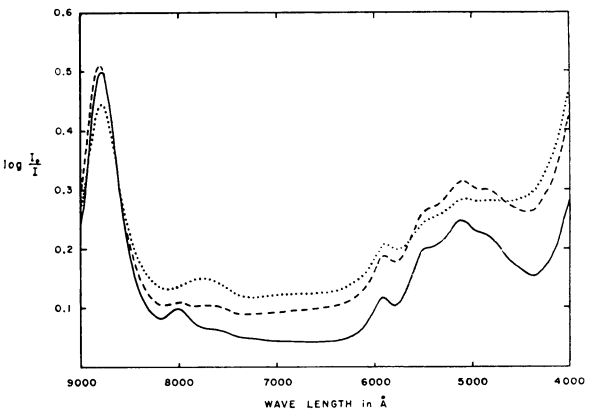

Fig. 1. Absorption spectra of chromatophores from lactate-yeast extract grown *Rhodospirillum rubrum* (0.05 ml chromatophoral suspension per 3.0 ml buffer). Before heating (——), after 76 C for 10 seconds (- - - -), and after 80 C for 10 seconds (. . . .).

contrast to the slight shoulder seen at 5,500 A in figure 1. Heat treatments always flattened the peak or shoulder at 5,500 A. Growth in the presence of increased Fe^{3+} levels apparently did not enhance this peak. It was not enhanced by addition of Na hydrosulfite to the suspended chromatophores but was flattened about 50 % by the addition of 1 μM ferricyanide/3 ml. Attempts to demonstrate photooxidation of this 5,500 A peak of the isolated chromatophores in the Cary spectrophotometer gave equivocal results. It is not now known if this peak is related to electron transport in the presumed photoreduction of N_2 (1, 11, 12, 16).

Discussion

Photooxidation of reduced indophenol is apparently not a simple sensitized photooxidation mediated by bacteriochlorophyll. No photooxidation could be demonstrated by the bacteriochlorophyll absorbing at 7,800 A after the 80 C-10 second heat treatment. The absorption peak at 8,000 A was essentially destroyed by the heat treatment of 76 C for 10 seconds without losing all of the photooxidase activity. There was a poor correlation between the decline in the so-called working peak at 8,800 A and in photooxidation. Participation of the 5,900 A band of bacteriochlorophyll or of the carotenoids in the primary photochemical events resulting in the photooxidation of the indophenol was ruled out here as the exciting light was cut off by the filter at 7,200 A. These data do suggest that other heat sensitive components or structures of the chromatophore are necessary for the photooxidation of the indophenol probably with the assistance

of bacteriochlorophyll in the form represented by the absorption peak at 8,800 A. This photooxidation probably requires the participation of enzymatic reactions.

If the photooxidation of reduced indophenol does not represent a sensitized photooxidation by bacteriochlorophyll, does this assay represent a valid measurement of the bacterial photooxidant? The model for chromatophoral photosynthetic phosphorylation first postulated by Frenkel (5) outlines a more or less closed system for the recombination of the photochemically generated reduced and oxidized moieties for trapping biologically useful energy. To assay for either the reductant or oxidant individually, two things are necessary: A: an acceptor for either moiety must be found which will react with a weak spot in the electron transport system. B: A trap must be introduced to prevent the other photochemical moiety from neutralizing the change to be measured. To demonstrate the photoreduction of exogenous DPN, succinate is added to trap the photooxidant. To demonstrate the photooxidation of reduced indophenol, oxygen is used to trap the photoreductant. On a bacteriochlorophyll basis, the specific activity of both the photoreduction of exogenous DPN and the photooxidation of reduced indophenol were similar. This suggests that both processes are related to the same electron flow.

That this photooxidase assay is usually carried out in air does not mean that oxygen is necessary within the cell for photooxidation. In vitro photooxidations can be shown under anaerobic conditions with sulfate (9), fumarate (17), and probably DPN substituting for oxygen. But these are only convenient methods for trapping the reductant so that the oxidant can be measured. The aerobic assay for photooxidase is a valid measurement for part of the photochemical process and is not necessarily a process that takes place within the cell.

Summary

Mild heat treatments of the chromatophore of *Rhodospirillum rubrum* (Esmarch) caused a more rapid decline in the rate of photooxidation of reduced indophenol than a destruction of the far-red absorption band of bacteriochlorophyll. This indicated that such photooxidations were not simply a passage of electrons from the reduced indophenol to the nascent photooxidized bacteriochlorophyll as other heat labile factors were also necessary for the photooxidation of the reduced indophenol. The rates of these photooxidations on a bacteriochlorophyll basis were comparable to the rates of the photoreduction of exogenous DPN. It is suggested that such photooxidations are valid measurements for the oxidant in bacterial photosynthesis.

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