BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **226,** 135–139 (1996)
ARTICLE NO. 1322

Active Oxygen Intermediates and Chlorophyllin Bleaching

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Received July 29, 1996

Bleaching of chlorophyllin, a water soluble copper containing porphyrin molecule, was investigated with regard to the potential role of active oxygen intermediate involvement. It was found that the bleaching was highly aerobic and also biphasic in nature. The aerobic photobleaching and the dark bleaching were effectively prevented by the addition of reductants such as ascorbate and cysteine. In addition, the reductant and peroxyl radical scavenger, Trolox, was highly effective in preventing bleaching. Catalase was moderately effective in preventing photobleaching whereas peroxidase and superoxide dismutase hastened the photobleaching process. It is concluded that the bleaching of chlorophyllin is a peroxidative process which does not involve singlet oxygen, superoxide, nor the $^{\cdot}OH$ radical.

Photosynthetic processes generate an oxidant capable of oxidizing water to oxygen as well as a reductant capable of reducing pyridine nucleotides such as NADP. These photoreactions carry the potential for generation of active oxygen intermediates (O_2^-, H_2O_2, $^{\cdot}OH$, and $^1O_2^*$) [1-2] that can lead to deleterious reactions among which is the photodestruction of chlorophyll. Active oxygen intermediates can be formed through autoxidations of various components of the photosynthetic electron transport chain [1-2] or by photosensitization by antenna chlorophyll molecules or the reaction center [3,4]. We have utilized a simple model system consisting of the water-soluble form of chlorophyll, chlorophyllin, to analyze the role of active oxygen intermediates in the bleaching process. The advantage of this system is that it allows us to examine the destruction of the sensitizer molecule devoid of the associated electron transport chain components found *in vivo.*

MATERIALS AND METHODS

The Na salt of chlorophyllin (Sigma Chemical Co.) was dissolved immediately prior to use in 50 mM potassium phosphate, pH 7.0. Aliquots of the solution (9.9 ml) were placed in clear glass scintillation vials (20 ml) to which 100 μl of various agents were added. To the control samples, 100 μl of 50 mM potassium phosphate, pH 7.0 was added. Five control and five sample vials were positioned 28 cm distance from two fluorescent lamps (GE F30T CW RS Cool White) in a chamber completely lined with aluminum foil. The temperature of the chamber was 24°C and was maintained by fan-driven air circulation. The light intensity (4,500 Lux) was kept low in order to yield a slow level of photobleaching. Dark controls were treated identically to the experimental samples except they were covered completely with foil. Aliquots were removed at the start of the experiment as well as at each subsequent time point and the OD_{630} determined using a Perkin Elmer 552 recording spectrophotometer. For confirmation of absorption peaks as well as routine spectral scanning, a Hewlett Packard 8452A Diode Array Spectrophotometer was used.

Assessment of bleaching in an anaerobic environment was performed in a chamber (Forma Scientific model #1031) equilibrated for 24 hours prior to the start of the experiment with 80% N_2, 10% CO_2 and 10% H_2. Solutions were equilibrated with the above gas mixture for 24 hours prior to the initiation of experiments. Illumination was obtained using the same type of fluorescent bulbs as described above with an illumination intensity equivalent to 4,500 Lux. Aliquots were withdrawn by means of a syringe and immediately placed into cuvettes to determine the OD_{630}. Dark

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0006-291X/96 $18.00

samples were treated identically except that they were maintained in aluminum foil covered vials and further insulated from light by placing the vials in a light-tight chamber.

Percent inhibition was calculated as (1 − (mean of sample values/mean of control values)) × 100%. Resampling simulation [5] was used to calculate 95% confidence intervals for percent inhibition based on 100 simulations for each compound. Simulations were performed with Resampling Stats for DOS, Version 3.16 (Resampling Stats Inc.).

All chemicals were of reagent grade and were purchased from Sigma Chemical Co. with the following exceptions: DABCO and Trolox, Aldrich Chemical Co.; dithionite, J. T. Baker; glutathione, Eastman Organic Chemicals; EDTA, Kodak International Biotechnologies; and dithiothreitol, Fisher Biotech.

RESULTS

The aerobic dependency as well as the time course of the light and dark bleaching of chlorophyllin is shown in Figure 1A and 1B. In air, chlorophyllin was bleached in a biphasic manner. There was an initial rapid photobleaching lasting 5 hours, followed by a slower bleaching process over the course of 28 hours (Figure 1A). In an atmosphere devoid of oxygen, chlorophyllin did not photobleach. Likewise under anaerobic conditions, in the dark, chlorophyllin did not bleach (Figure 1B). However, after 5 hours in the presence of air, chlorophyllin showed a gradual decrease in OD_{630} indicative of a non-photochemical, aerobic chlorophyllin bleaching. An examination of the absorption spectrum of bleached chlorophyllin revealed that the 630 nm peak was greatly diminished (data not shown).

In order to investigate further the aerobic bleaching phenomenon, we analyzed the effect of various active oxygen intermediate scavenging enzymes, singlet oxygen traps, and reductants upon the rapid photobleaching phase of chlorophyllin. The results shown in Table 1 reveal that superoxide dismutase was ineffective in preventing photobleaching as was peroxidase. In fact, they hastened the rate of bleaching (negative percent inhibition). Catalase offered a moderate degree of protection but this could not be further enhanced when used in combination with superoxide dismutase. Compounds shown to be effective $^{\bullet}OH$ trapping agents [6] were also without significant effect, although azide conferred some degree of protection.

The protective effectiveness of singlet oxygen trapping agents was minimal. Further support against the involvement of $^1O_2^*$ in bleaching comes from the experiments in deuterated solvents. When chlorophyllin was placed in D_2O, there was a 47% inhibition of photobleaching and a 25% inhibition of dark bleaching compared to water. Due to the ten fold longer half-life of $^1O_2^*$ in deuterated solvent [3], one would expect a greater singlet oxygen reactivity resulting in a faster bleach, i.e. zero inhibition.

The greatest degree of protection against bleaching was clearly seen in the presence of reductants. Reduced thiols and ascorbate were most effective whereas NAD(P)H offered a moderate degree of protection against photobleaching. Trolox, a tocopherol analogue with reductant characteristics and peroxyl radical scavenging ability [7, 8] was highly effective in preventing bleaching. Reductants also proved to be effective protecting agents in prolonged bleaching experiments carried out in the dark (Table 2). Finally, when μmolar amounts of H_2O_2 were added to a chlorophyllin solution kept in the dark, bleaching occurred (Table 3). This peroxidative bleaching of chlorophyllin could be prevented by the addition of cysteine or Trolox, thereby further demonstrating the ability of reductants to deter the oxidative destruction of chlorophyllin.

DISCUSSION

Chlorophyllin bleaching is a complex process consisting of at least two elements. Bleaching occurs rapidly in the light, is essentially complete in 5 hours under the conditions employed and is dependent upon oxygen. In the dark there is a slower oxidative bleaching. Neither the fast photobleach nor the slower dark component appeared to be affected by superoxide dismutase, implying that O_2^- was not a major contributor to the process. In fact the photobleaching was

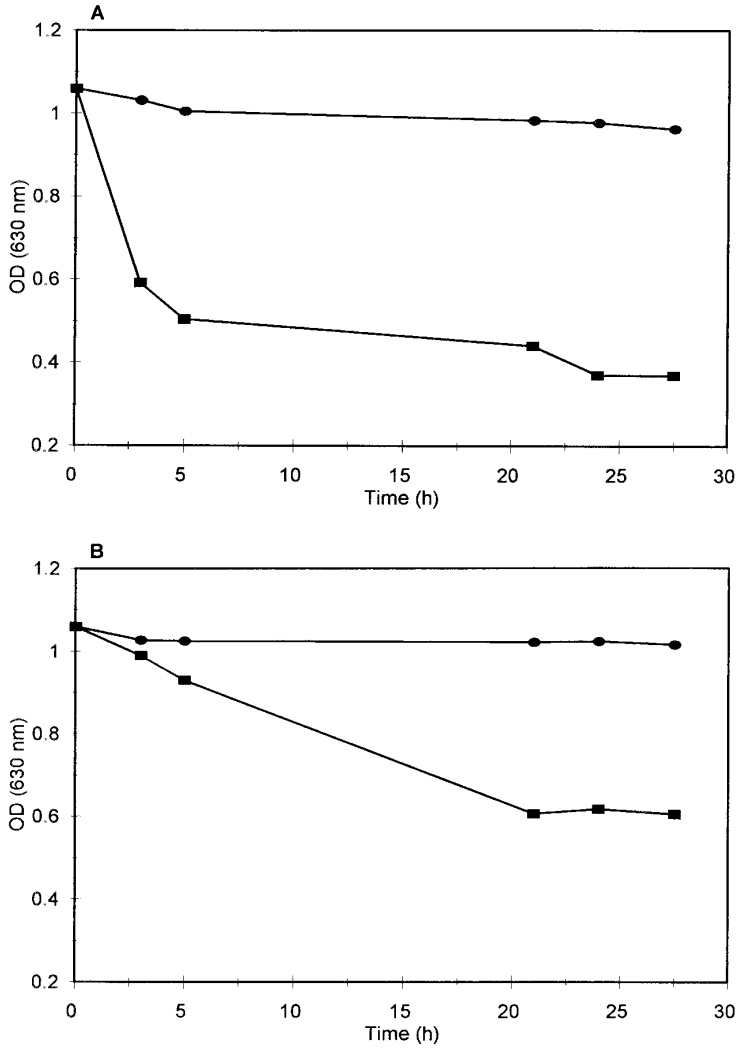

FIG. 1. Time course of chlorophyllin bleaching in light and dark under aerobic and anaerobic conditions. Experiments were performed as described under Materials and Methods. Points are representative of one typical experiment. (A) Illuminated, (B) dark; (■ — ■) aerobic; (● — ●) anaerobic.

slightly elevated in the presence of superoxide dismutase. This phenomenon might be explained by the catalytic production of H_2O_2. Catalase afforded some protection thereby demonstrating the H_2O_2 may play a role in photodestruction. Indeed, we have observed the rapid bleaching of chlorophyllin in the dark upon addition of micromolar amounts of peroxide (Table 3). Peroxidase, like superoxide dismutase also caused a slightly higher rate of photobleaching. This may be due to a peroxidation of chlorophyllin. Compounds associated with $^{\bullet}OH$ sequestration [6] were not effective in preventing bleaching thereby implying that under our conditions, hydroxyl radical played little role.

Although a role for $^1O_2^*$ in the photodestruction of chlorophyll through oxygenation of ring constituents has been postulated [4,9], our data do not confirm its active participation. Rather, peroxidation appears to play a major role. The protective effect of reduced thiols and other

TABLE 1
Effect of Various Agents on Chlorophyllin Photobleaching

Class	Compound	% Inhibition	LCL	UCL
H_2O_2, O_2^-, and $^{\bullet}OH$	Catalase (250 U/ml)	20	14	26
Scavenging enzymes	Catalase/SOD (100 U/100 U)	. 4	0	9
	Peroxidase (100 U/ml)	−15	−18	−11
	SOD[a] (500 U/ml)	−15	−18	−12
$^{\bullet}OH$ Scavenging agents	Azide (10 mM)	22	19	24
	Benzoate (10 mM)	4	1	7
	EDTA (10 mM)	11	7	15
	Formate (0.1%)	1	−2	4
	Thiourate (10 mM)	8	7	10
	Urate (10 mM)	−8	−13	−4
$^1O_2^*$ Scavenging agents	DABCO[b] (10 mM)	6	3	9
	Histidine (10 mM)	18	17	18
Reductants	Ascorbate (10 mM)	96	94	99
	Cysteine (10 mM)	105	103	108
	Dithionite (10 mM)	80	77	f84
	DTT[c] (10 mM)	92	90	94
	Glutathione (10 mM)	84	80	86
	NADH (1 mM)	47	45	49
	NADPH (1 mM)	31	27	34
	Trolox (10 mM)	104	102	106

Note. Chlorophyllin photobleaching was determined as described under Materials and Methods. Illumination was carried out for 1 hour and the percentage inhibition of bleaching determined by comparing the rate of photobleach in the presence of the agent to the rate of photobleach in its absence. Statistical analysis was performed as described under Materials and Methods with LCL and UCL standing for the 95% lower confidence level and 95% upper confidence level, respectively.

[a] Copper-Zinc bovine superoxide dismutase.

[b] 1,4-Diazabicyclo [2.2.2] octane.

[c] Dithiothreitol.

TABLE 2
Effect of Various Reductants on Chlorophyllin Dark Bleach

Compound	% Inhibition	LCL	UCL
Ascorbate (10 mM)	67	66	69
Cysteine (10 mM)	60	58	61
Dithionite (10 mM)	52	50	55
DTT (10 mM)	58	57	59
Glutathione (10 mM)	64	62	67
NADH (1 mM)	33	31	35
NADPH (1 mM)	39	37	41

Note. Measurement of chlorophyllin bleaching was performed after 24 hour dark incubation. Analysis of data was as described in Table 1 and under Materials and Methods.

TABLE 3
Effect of Reductants on Peroxidative Bleaching of Chlorophyllin

Compound	% Inhibition	LCL	UCL
H_2O_2 (0.1 mM)	−170	−242	−105
Cysteine (10 mM) + H_2O_2 (0.1 mM)	55	25	80
Trolox (10 mM) + H_2O_2 (0.1 mM)	49	15	76

Note. Measurements of chlorophyllin bleaching were performed after one hour dark incubation. Values are compared to that of a control in which no H_2O_2 was added. A negative number for inhibition would imply a rate of bleach faster than that of the control. Analysis of the data was as described under Materials and Methods.

reductants may have implications for *in vivo* photosynthesizing systems i.e. plant and algal chloroplasts. It is conceivable that the photooxidations catalyzed by antenna chlorophyll molecules might either directly or indirectly deplete the chloroplast of reductants such as glutathione and ascorbate. This process could occur slowly in the dark but is greatly exacerbated in the light. If the rates of oxidation exceed replenishment rates, the chlorophyll molecule would be vulnerable to oxidative destruction. Our results point to the delicate balance that exists in the cell between photooxygenations which are beneficial in a synthetic manner and those which are photodestructive.

ACKNOWLEDGMENTS

The technical assistance of Mrs. Monika Oli is gratefully acknowledged. This work was supported by funds made available from the Mississippi Agricultural and Forestry Experiment Station on Project MIS-6727 and has been approved as journal article J-8965. In addition the financial support of the University of Arkansas Experiment Station is gratefully acknowledged.

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