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A LIGHT-INITIATED, DARK OXIDATION OF BACTERIOCHLOROPHYLL FROM REACTION CENTER PARTICLES

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SUMMARY

When reaction center particles from *Rhodopseudomonas spheroides* strain R26 are illuminated and then extracted with methanol, about one-third of the extracted bacteriochlorophyll slowly becomes oxidized, The oxidation does not occur under anaerobic conditions or in the absence of the detergent lauryldimethylamine oxide. Alkaline conditions also prevent the reaction. A dark interval between illumination and extraction delays the onset of bacteriochlorophyll oxidation in a predictable way. These results are consistent with the hypothesis that illumination generates a reaction initiator which is fairly stable in methanol but decays with a half-life of about 4.5 min in reaction center particles after illumination ceases.

INTRODUCTION

The initial photochemical reaction in bacterial photosynthesis is the transfer of an electron from a specialized reaction center bacteriochlorophyll molecule or dimer to the primary electron acceptor [1, 2]. This light-driven electron transfer also occurs in reaction center particles prepared from photosynthetic bacteria using detergents [3–5]. Earlier experiments in this laboratory suggested that one of the products of this photoreaction, oxidized bacteriochlorophyll, could be extracted by rapid injection of methanol into a sample of illuminated reaction center particles [6]. However, recent experiments, in which minor refinements have speeded the extraction procedure, have shown that all of the bacteriochlorophyll is in the reduced form initially after extraction and that it then becomes oxidized on standing in the methanol extract. Conditions of illumination of the reaction center particles prior to extraction control bacteriochlorophyll oxidation in the extract. These and several other experimental variables which affect the bacteriochlorophyll oxidation reaction are the subject of this report.

MATERIALS AND METHODS

Reaction center particles were prepared from *Rhodopseudomonas spheroides* strain 2.4.1/R26, a carotenoidless mutant, as described by Clayton and Wang [4],

except that the Biogel chromatographic step was omitted, since it appears to be unnecessary. The particles were maintained in 0.01 M Tris–HCl, pH 7.5, containing 0.3 % (v/v) lauryldimethylamine oxide.

Absorption changes due to oxidation and reduction of the bacteriochlorophyl were measured at 769 nm, its absorption maximum in methanol, using a Cary 14R spectrophotometer. Light intensity was measured with a YSI radiometer (Yellow Springs Instrument Co., Yellow Springs, Ohio); 2.2 mW/cm^2 was used throughout.

A typical experiment was as follows: test tubes containing 0.1 ml of reaction center particles ($5 \cdot 10^{-5}$ M) were illuminated with infrared light ($\lambda > 800$ nm) for a period of 1.5 min, as described by Straley and Clayton [6]. Either immediately, or after a timed dark period, 3.0 ml of anhydrous methanol was rapidly added.

In experiments in which the dark period was long, the samples were kept away from room lights by placing the samples in a closed drawer and performing subsequent manipulation with the room lights extinguished.

Denatured protein was removed by sintered glass filtration, aided by a water vacuum line, directly into the Thunberg cuvette. This process took 45–60 s after addition of the methanol to the reaction center particles.

When anaerobic conditions were required, air was removed from the test tubes containing reaction centers by a standard evacuation and argon-flushing techniques. The methanol used was also anaerobic and was transferred from a sealed stock vessel via a syringe. All procedures were then done under an argon atmosphere.

For examination of the effect of lauryldimethylamine oxide on the reaction, a known amount of the lauryldimethylamine oxide was contained in the methanol solution added after illumination. For the zero lauryldimethylamine oxide experiment, an lauryldimethylamine oxide-free reaction center preparation was kindly donated by Dr Colin A. Wraight.

The effect of pH on the reaction was obtained by adding sufficient amounts of 100 mM aqueous buffer to give a 3.3 mM final buffer concentration in the methanol. The buffers used were glycine–NaOH, pH 9.8; Tris–HCl, pH 8.8; Tris–HCl, pH 7.7; Tris–HCl, pH 6.9; 2-(*N*-morpholino)-ethanesulphonic acid–NaOH, pH 6.0; 2-(*N*-morpholino)-ethanesulphonic acid–NaOH, pH 5.5. Although the unknown methanolic solvent effects on the buffer prevent accurate pH determination, the buffers provided a useful indication of the relative acidity of the methanol.

RESULTS

A typical time course for bacteriochlorophyll oxidation is illustrated in Fig. 1. Adding a few grains of solid ascorbic acid to the extract after oxidation restored the absorption at 769 nm to very nearly the initial value. The ΔA at 769 nm was usually about 25 % of the initial value. Substracting the bacteriophaeophytin absorption at this wavelength, this means that approximately one-third of the bacteriochlorophyll present in the extract becomes oxidized, as was found previously [6].

Requirement for O_2

Under anaerobic conditions, bacteriochlorophyll oxidation does not occur, indicating that O_2 is probably the oxidant. The presence of O_2 during illumination,

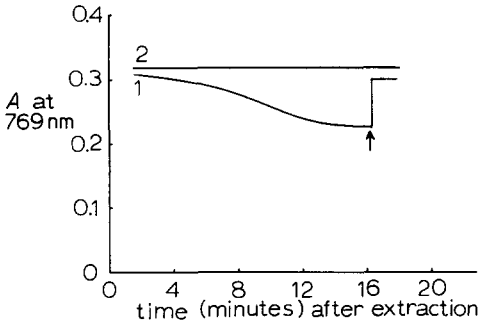

Fig. 1. Bacteriochlorophyll oxidation in a typical methanol extract from reaction center particles (1) Methanol extract from reaction center particles illumination for 1.5 min prior to extraction. Arrow, + ascorbic acid. (2) Methanol extract from a dark control.

however, is not necessary, since bacteriochlorophyll oxidation occurred when O_2 was admitted, after intervals of up to 16 min, to anaerobic methanol extracts from reaction center particles illuminated under anaerobic conditions. The reaction kinetics after aeration were always as shown in Fig. 1, with a several-minute lag period preceding the detectable onset of the oxidation.

The requirement of O_2 does not explain the fact that only one-third of the bacteriochlorophyll becomes oxidized, since calculations based on the solubility of O_2 in alcohol [7] indicate that O_2 should be present in at least a 100-fold excess over the amount of bacteriochlorophyll oxidized. Furthermore, increasing the quantity of reaction center particles extracted results in a proportional increase in the ΔA at 769 nm.

Effect of pH

Alkaline buffers (pH values of 9.8 and 8.8) prevented or at least strongly hindered the reaction. When the added buffer had a pH of 7.7 or below, the reaction occurred normally. The inhibition by high pH was not due to irreversible destruction of any component in the methanol extract, since subsequently decreasing the pH by adding 0.1 ml of 1 M Tris–HCl, pH 7.4 buffer allowed the reaction to proceed. The inhibitory effect of high pH suggests the involvement of the superoxide anion radical, since high pH likewise inhibits the superoxide-mediated oxidation of reduced flavins [8].

Effect of lauryldimethylamine oxide

As shown in Fig. 2, the maximum rate of bacteriochlorophyll oxidation measured at the midpoint of the reaction increased with increasing concentration of lauryldimethylamine oxide up to about 0.1 %. No bacteriochlorophyll oxidation occurred in the absence of lauryldimethylamine oxide. When lauryldimethylamine oxide was added to an extract prepared from illuminated, lauryldimethylamine oxide-free reaction center particles 25 min after extraction, bacteriochlorophyll oxidation ensued after a typical lag period. When lauryldimethylamine oxide was present, the total ΔA at 769 nm was independent of its concentration.

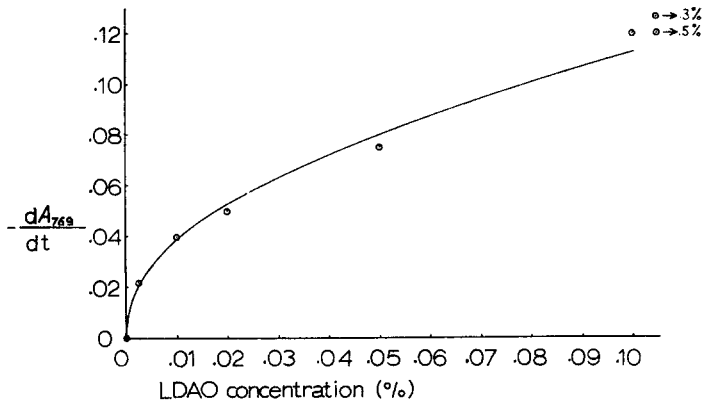

Fig. 2. Effect of lauryldimethylamine oxide concentration on the maximum rate $-dA_{769\ nm}/dt$ of bacteriochlorophyll oxidation. $-dA_{769\ nm}/dt$ was measured at the reaction midpoint and is in absorbance units per min.

Effect of a post-illumination dark interval

When dark intervals of several minutes intervened between illumination and extraction, bacteriochlorophyll oxidation still occurred. As the length of the dark

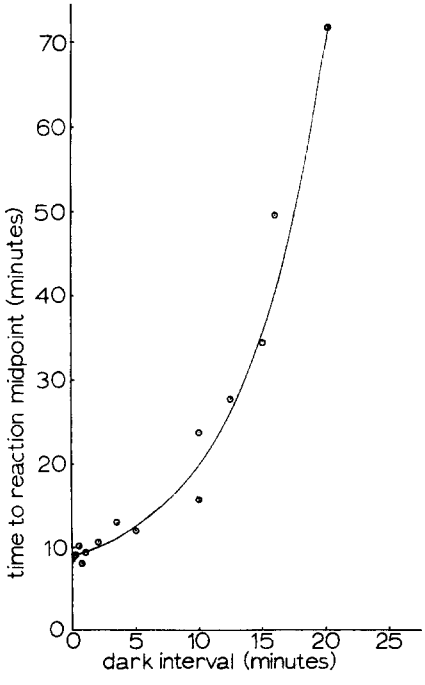

Fig. 3. Time to the reaction midpoint as a function of the dark interval intervening between illumination and extraction of the reaction center particles. Circles, experimental points; ——, calculated values from the equation $t_m = 2^{t_d/4.5} \times 3 + 6$, where t_m represents time from extraction to the reaction midpoint in minutes and t_d the length of the dark interval in minutes, respectively.

interval increased, the lag time before the onset of bacteriochlorophyll oxidation also increased as indicated in Fig. 3. The kinetics of the actual oxidation were always as shown in Fig. 1, but various intervals during which the absorption at 769 nm remained constant elapsed prior to the visible onset of the reaction. Since the division between the end of this lag period and the onset of bacteriochlorophyll oxidation was somewhat arbitrary, the time from extraction to the reaction midpoint was graphed. The lag time was always about 6 min (± 1 min) less than the time until the reaction midpoint. The extent of the reaction was the same in all cases, within the limits of experimental variability, i.e. $\Delta A_{769\ \mathrm{nm}} = -0.08 \pm 0.01$, where $A_{769\ \mathrm{nm}}$ initial ≈ 0.32.

The length of the lag time can be predicted reasonably well from the empirical equation

$$t_l = 2^{t_d/4.5} \times 3 \text{ min}$$

where t_l represents the lag time and t_d the length of the dark interval in minutes, respectively. This can be interpreted as meaning that the lag time is inversely proportional to the amount of a light-generated reaction initiator which is inactivated in the dark after illumination but prior to extraction with a half-time of approximately 4.5 min under the conditions of these experiments. The quantity $2^{t_d/4.5}$ would be the reciprocal of the fraction of the initiator remaining in the reaction center particles after a dark interval equal to t_d. The 3-min quantity in the equation corresponds to the minimum lag time in these experiments. The time required to reach the reaction midpoint can be calculated by adding 6 min to the lag time. The solid curve in Fig. 3 is a theoretical plot of the time until the reaction midpoint.

In a dark control, in which the test tube containing the sample of reaction center particles was kept completely dark for 24 h prior to extraction with methanol, the reaction reached its midpoint 112.5 min after extraction. This corresponds to a lag phase of 106.5 min. The occurrence of a reaction in the dark control may indicate that the amount of the initiator does not continually decrease by a factor of 0.5 every 4.5 minutes but eventually reaches a steady-state level after about 25–30 min. Alternatively, the reaction may eventually occur spontaneously, even in the absence of any initiator.

The hypothetical inverse proportionality between the lag time and the amount of a light-generated reaction initiator present was tested further in a second series of experiments. Samples of reaction center particles were divided into two portions, one of which was illuminated and the other of which was not. The methanol used for extraction was also divided proportionally. After extraction, the two methanol solutions were mixed and filtered into the sample cuvette. Fig. 4 shows the time until the reaction midpoint as a function of the reciprocal of the fraction of the reaction center particles illuminated. The solid line was calculated from the equation

$$t_l = (1/f_i) \times 3 \text{ min},$$

where f_i represents the fraction of the reaction center particles illuminated and where 6 min were added to t_l to give the reaction midpoint time as before. The theoretical plots in both Figs 3 and 4 are in satisfactory agreement with the data, and the two equations are mutually consistent.

The chemical identity of the initiator of bacteriochlorophyll oxidation and the mechanism through which it controls the lag time are unknown. It is unlikely to

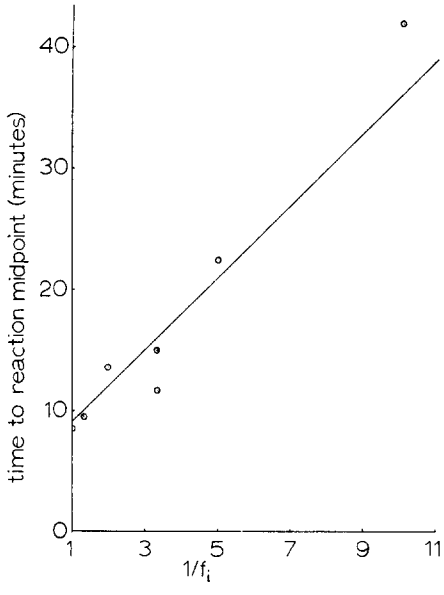

Fig. 4. Time to the reaction midpoint in extracts from various mixtures of illuminated and unilluminated reaction center particles. f_i, fraction of reaction center particles illuminated prior to extraction; circles, experimental points; ——, calculated values from the equation $t_m = (1/f_i) \times 3 + 6$, where t_m represents time from extraction to the reaction midpoint in minutes.

be either oxidized bacteriochlorophyll or the reduced primary electron acceptor since neither has a half-life greater than about 0.1 s in the dark [9]. A dark period of a few seconds, long enough to allow nearly full recovery of P870, has almost no effect on the lag. Perhaps it is a reduced secondary electron acceptor or an oxidized secondary donor. Preliminary results indicate that incubation of reaction center particles with *o*-phenanthroline, which is known to inhibit electron transfer to secondary acceptors [10], increases the lag time. The function of the initiator in reaction center particles and in vivo as well as its identity are subjects for future research.

APPENDIX

A bacteriochlorophyll cation radical EPR signal has now been detected in methanol extracts from illuminated reaction center particles. We plan to also look for EPR signals of possible free radical precursors to the bacteriochlorophyll cation radical.

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