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CYTOCHROME PHOTOOXIDATIONS IN *CHROMATIUM* CHROMATOPHORES

EACH P870 OXIDIZES TWO CYTOCHROME C422 HEMES

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SUMMARY

A single, 20-nsec actinic flash oxidizes all of the P870 in *Chromatium* chromatophores, but only about one half of the cytochrome C422. A second flash, 1–10 msec later, oxidizes most of the remaining cytochrome. The cytochromes which undergo oxidation on the first and second flashes are indistinguishable with respect to their absorption spectra, their kinetics of oxidation and reduction, and their response to *N*-methylphenazonium methosulfate (PMS) or continuous actinic illumination. The effect of PMS is to increase the total amount of cytochrome C422 which is in the reduced form in the dark, and which is available for photooxidation. The conclusion is that each P870 reaction center is responsible for the oxidation of two C422 hemes.

INTRODUCTION

The preceding paper[1] discusses the reactions which occur on the reducing side of the primary photochemical reaction of bacterial photosynthesis. The pairs of short actinic flashes which proved valuable in this study should also help one to expose the reactions on the oxidizing side.

Because of the complication that photosynthetic bacteria may contain two different types of photochemical systems, which oxidize different cytochromes and serve different physiological functions[2,3], it is advantageous to begin such a project by investigating chromatophores, rather than whole cells. Unless one lowers the redox potential artificially[3], only one photochemical system appears to operate in chromatophores. A single actinic flash causes the oxidation of a special bacteriochlorophyll trimer, called P870, with a quantum yield near 1.0 (ref. 4). Oxidized P870 ($P870^+$) returns to the reduced state by oxidizing a *c*-type cytochrome, called C422 and C555, also with a quantum yield near 1.0 (ref. 4). If the photochemical reaction center contains only one C422 heme per P870 complex, a single saturating flash presumably could oxidize all of this cytochrome. A second actinic flash might reoxidize the P870, but this would not lead to additional cytochrome oxidation. The second flash would cause cytochrome oxidation if the reaction center contains more than

Abbreviation: PMS, *N*-methylphenazonium methosulfate.

one C422 heme, or if it contains another species of cytochrome, or if a second photochemical system becomes functional.

Ultimately, the C422 which loses an electron after the first flash regains one by cyclic electron return from the primary electron acceptor, and it then is able to undergo reoxidation. Double flash experiments are most revealing if the two flashes are spaced close enough together for cyclic electron return to be negligible. Fortunately, a second flash does have an obvious actinic effect in *Chromatium* chromatophores, and one can study it conveniently with an interflash delay of about 1 msec (ref. 1), whereas cyclic electron return requires more than 10 times as long. The cytochromes which respond to the first and second flashes turn out to be indistinguishable. Each P870 reaction center apparently contains at least two cytochrome C422 hemes.

METHODS

Earlier reports[1,4,5] describe all of the relevant materials and methods, except for the following details. All measurements were made at pH 7.4 and approx. 22°. The P870 oxidation measurements of Fig. 7 were made with a Xe flash measuring lamp, and with a photomultiplier with an S-20 spectral response (EMI 9558QA, Whittaker Corp., Plainview, N.Y.). The photomultiplier tube was specially selected for high photocathode sensitivity. The S-20 spectral response is critical for this experiment, because it is very low for wavelengths greater than 850 nm. This allows better rejection of bacteriochlorophyll fluorescence (which is maximal at approx. 920 nm) than one can achieve by using a photomultiplier with an S-1 response. In addition to a monochromator, the measuring light path between the sample cuvette and the photomultiplier included an interference filter with peak transmission at 883 nm (Type B3-NIR, Baird-Atomic Corp., Cambridge, Mass.), a broad band infrared-transmitting interference filter (IRT-211, Fish-Schurman Corp., New Rochelle, N. Y.), and a Schott RG-N9 filter.

The measuring light source for the cytochrome kinetic measurements of Fig. 2 was a 45-W tungsten–iodine lamp. The lamp was run continuously at 7 V, 6.5 A. For the measurement, the voltage was boosted by discharging 55 V from a 5300-μF capacitor through the series combination of a small inductance (approx. 300 μH), a silicon controlled rectifier, and the lamp. This procedure doubles the light output from the lamp. The light reaches a peak 10–15 msec after the silicon controlled rectifier fires.

RESULTS AND DISCUSSION

With *Chromatium* chromatophores in the presence of optimal amounts of PMS, a 20-nsec flash of saturating intensity causes the oxidation of about half as much cytochrome as does strong continuous actinic light (Fig. 1). The absorption spectrum and the insensitivity of this cytochrome to O_2 identify it as cytochrome C422 (ref. 4). Because one flash does not oxidize all of the available C422, it is interesting to ask whether a second short flash will oxidize any additional cytochrome. The answer is yes, if sufficient time elapses between the two flashes. The maximal effect of the second flash is about 75 % as great as that of the first.

The preceding report[1] considers the kinetics of the process which must occur

between the two flashes, before the second flash will cause cytochrome oxidation. A period on the order of 60 μsec is required for the second flash to have one half of its maximal effect. The simplest interpretation of this phenomenon is that the primary electron acceptor becomes completely reduced during the first flash, and that no

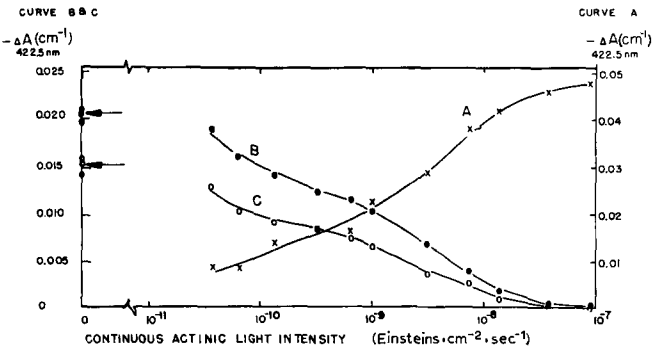

Fig. 1. Dependence of cytochrome C422 oxidation on continuous actinic light intensity. *Chromatium* chromatophores; bacteriochlorophyll, 51 μM (in a cuvette with a 1.0-cm light path); 4 μM PMS; anaerobic. Actinic light: 75-W quartz–iodine tungsten lamp with 4-mm Schott RG-N9 filter; abscissa scale is logarithmic. ×—×, Curve A, cytochrome oxidation caused by continuous light; right hand ordinate scale applies; ●—●, Curve B, cytochrome oxidation caused by 20-nsec laser flash of saturating intensity, superimposed on continuous actinic light; left hand ordinate scale applies; ○—○, curve C, cytochrome oxidation caused by a second 20-nsec laser flash, 2.0 msec after the first; left hand ordinate scale applies. (2-25-69.) See ref. 4 for a curve showing the effect of one laser flash as a function of the flash intensity.

further photochemistry can occur until it returns to the oxidized state. This interpretation rests on the demonstration that P870 oxidation, as well as C422 oxidation, is blocked during the refractory period after the first flash. If a second flash is given during this period, its energy appears to go into fluorescence and heat, rather than into an alternative photochemical reaction. 4 μsec after the first flash, the fluorescence yield from the bulk bacteriochlorophyll is roughly 4 times higher than it was before the flash. The fluorescence yield after the flash is essentially the same as that which one generates by exposing the chromatophores to saturing continuous light.

An alternative interpretation of the refractory period would be that there are two photochemical systems, that only one of them can work at a time, and that the 60-μsec process is the activation of the second system. If this were the case, one would expect to see differences between the reactions which occur on the first and second flashes. As Figs. 1–5 show, however, no significant differences were found.

Fig. 2 shows the cytochrome oxidation rates after the two flashes. The two rates are the same; both half-times are 2 μsec.

Fig. 3 shows the difference spectra of the two cytochromes. There are small, but repeatable, differences between the two spectra. These probably result from the absorption increase with a broad band centered at 430 nm which is due to an unidentified pigment[5]. This absorbance change occurs more rapidly than do the cytochrome absorbance changes, but the spectra of Fig. 3 include both, as the measurements were made approx. 50 μsec after the flashes. More rapid measurements indicated that the fast 430-nm absorbance increase was somewhat larger on the second flash than on the first. Considering the contribution which this pigment makes to the difference

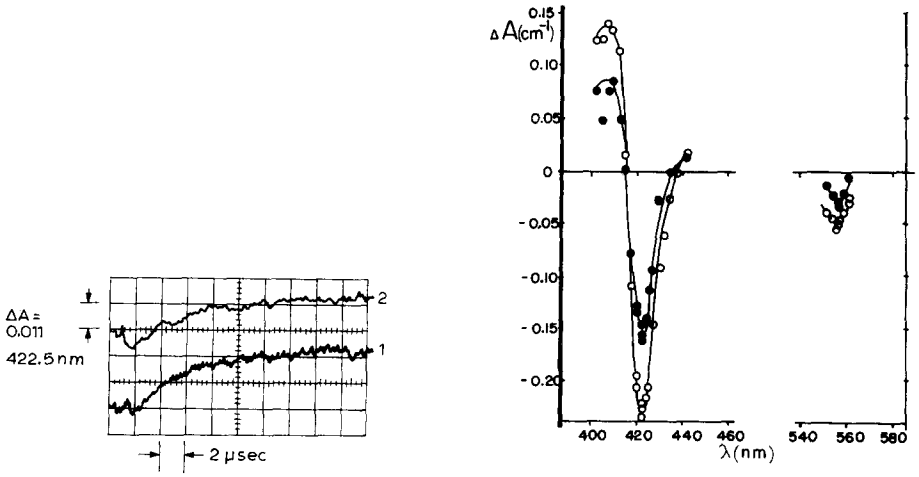

Fig. 2. Kinetics of cytochrome C422 oxidation on first flash (Traces 1) and second flash (Traces 2). Conditions as in Fig. 1. Delay between first and second flashes: 1.4 msec. (2-26-69.)

Fig. 3. Difference spectra of cytochromes oxidized by first laser flash (○—○), and by a second flash, 1.0 msec after the first (●—●). Both flashes were of saturating intensity. Bacteriochlorophyll, 505 μM (in a cuvette with a 0.1-cm light path); 10 μM PMS; 1 mM ascorbate. Monochromator bandpass, 3.2 nm. (7-30-68.)

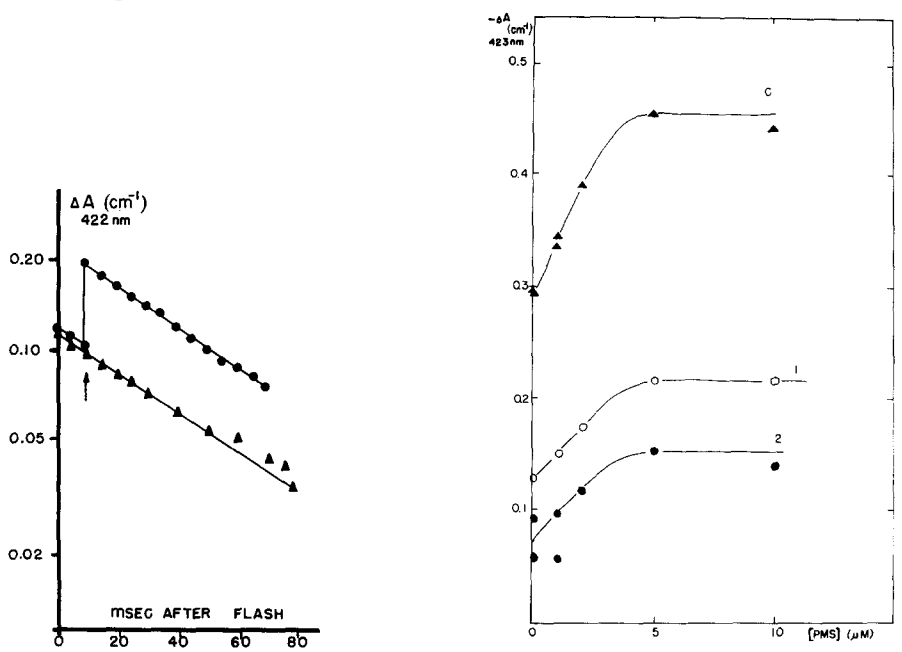

Fig. 4. Kinetics of cytochrome reduction after flash-induced oxidation. ▲—▲, one saturating flash only; ●—●, two saturating flashes, the second (at arrow) 10 msec after the first. The ordinate gives, on a logarithmic scale, deflection remaining at various times after the first flash. Bacteriochlorophyll, 489 μM (0.1-cm light path); 10 μM PMS, 1 mM ascorbate. (7-24-68.)

Fig. 5. Effect of PMS on cytochrome C422 photooxidation by continuous actinic light (▲—▲); by a single 20-nsec flash (○—○); or by a second 20-nsec flash, 1.0 msec after the first (●—●). Conditions as in Fig. 3. (8-1-68.)

spectra, the cytochrome difference spectra on the two flashes appear to be essentially identical.

Fig. 4 shows the recovery (reduction) rates after the flashes. The two recovery processes appear to be identical. These rates vary considerably with the chromatophore preparation. They frequently exhibit several steps, rather than the simple exponential step of Fig. 4.

Fig. 1 shows that superimposing a continuous background illumination on the chromatophores does not expose any differences between the two cytochromes. The effects of both flashes decrease smoothly as the strength of the continuous light increases. As one would expect, the effect of the second flash falls off somewhat more rapidly than does that of the first. The results of Fig. 1 differ qualitatively from the results of a similar experiment in whole cells. With whole cells, one can oxidize approximately 1/2 of the total cytochrome by weak continuous illumination, with essentially no effect on the amount of cytochrome which a flash will oxidize[6]. Apparently, whole cells contain cytochromes which respond to continuous illumination, but which do not respond directly to a short flash. As the discussion below will demonstrate, continuous illumination of chromatophores does not reveal these cytochromes because they are already in the oxidized state.

Fig. 5 shows the effect of the PMS concentration. This dye affects the two cytochromes identically. The effect of PMS appears to be to increase the total amount of cytochrome C422 which is in the reduced state, in the dark before the flash. Fig. 6 verifies this supposition, by showing that the addition of PMS to the chromatophores causes cytochrome C422 reduction. The amount of cytochrome reduction which follows PMS addition varies with the chromatophore preparation. It is quantitatively equal to

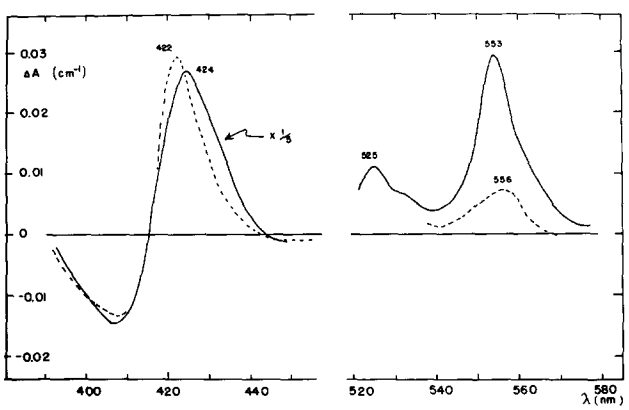

Fig. 6. Difference spectra caused by addition of PMS and $Na_2S_2O_4$ to anaerobic *Chromatium* chromatophores. – – – –, both sample and reference cuvettes contained 42 μM bacteriochlorophyll, 0.1 M glycylglycine buffer (pH 7.4), 1.0-cm light path. Sample cuvette also contained 4 μM PMS. Absorbance due to PMS was subtracted by placing a second cuvette containing 4 μM PMS in the reference beam and a second cuvette containing only buffer in the sample beam. ———, both sample and reference cuvettes contained 42 μM bacteriochlorophyll, 0.1 M glycylglycine buffer (pH 7.4). Sample cuvette was treated with an excess of solid $Na_2S_2O_4$. Reference cuvette also contained 4 μM PMS. Absorbance due to PMS was subtracted by placing a second cuvette containing 4 μM PMS in the sample beam and a second cuvette containing only buffer in the reference beam. This was a different chromatophore preparation from the one which was used for Fig. 5. The curves are redrawn from traces that were obtained with a Cary Model 15 spectrophotometer. Numbers above the curves indicate wavelengths of the maxima. (5-1-69).

the resulting increase in cytochrome photooxidation under saturating continuous light. The reservoir of endogenous reductant which supplies electrons for this process is unknown (see ref. 3). GELLER[7] recently has demonstrated that dyes similar to PMS undergo sequential photo-reduction and oxidation in *Rhodospirillum rubrum* chromatophores, and that this is the mechanism by which they catalyze cyclic electron transport. The mechanism of action of PMS in the present case is still rather mysterious, however, because at the low concentrations which are shown in Fig. 5, PMS does not accelerate the reduction of cytochrome C422 after an actinic flash. If anything, the cytochrome recovery rates are somewhat slower in the presence of small amounts of PMS.

Even in the presence of PMS, about one half of the total cytochrome in the chromatophores remains in the oxidized form. Fig. 6 shows the effect of further addition of a strong reductant, $Na_2S_2O_4$. The cytochromes which become reduced at this point are clearly different from C422. From the spectrum, they must include cytochromes C423.5 and cytochrome *cc'*. Isolated forms of these cytochromes have E_0' values of +10 mV and −5 mV (ref. 8) which probably are far below the redox potential which prevails in anaerobic chromatophores[3].

Figs. 1–6 thus lead one to the conclusion that only one *c*-type cytochrome, C422, is functional in anaerobic chromatophores in the presence of PMS, and that the mechanism of its oxidation after the second flash is the same as that after the first. This leaves open the question of whether two different P870 complexes participate on the two flashes, or whether one P870 complex can oxidize two C422 hemes. Previous observations support the latter alternative. A single 30-nsec laser flash oxidizes as much P870 as does a 500-μsec burst of flashes[4]. Fig. 7 confirms this observation, using the absorbance increase at 785 nm to measure P870 oxidation. Apparently, a single short flash is adequate to oxidize all of the P870 in the chromatophores.

Parenthetically, it is worth noting that the earlier P870 measurements[4] were made from the absorbance decrease at 882 nm. It has not yet been possible to obtain a complete difference spectrum for the P870 oxidation after a flash, because of the

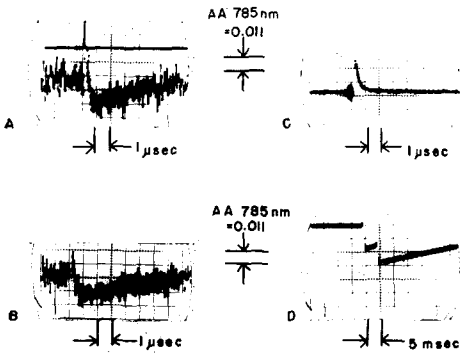

Fig. 7. P870 photooxidation by a single 20-nsec flash, or by two 400-μsec bursts of flashes. A and B show two measurements of P870 oxidation by single flashes, measured by the absorbance increase (downward deflection of trace) at 785 nm. The upper trace in A is a photodiode record of the time of the laser flash. C shows the fluorescence artifact which interferes with the first μsec of the absorbance measurements. For the measurement in C, the Xe flash measuring lamp was not used, and no offset current was applied to the preamplifier. See ref. 5 for a discussion of this way of measuring the fluorescence artifact. D shows, on a much slower time scale, the long-lived P870 oxidation by two 400-μsec bursts of flashes from the normal mode ruby laser, with 5 msec between the bursts. A single 400-μsec burst is not quite long enough to oxidize all of the cytochrome C422 and P870 in the chromatophores[1], but two such bursts are sufficient to do so. All samples had 40.6 μM bacteriochlorophyll, 4 μM PMS. (4-21-69; 4-18-69.)

speed with which the P870 returns to the reduced state, and because fluorescence from bacteriochlorophyll interferes with the absorption measurements. The measurements at 882 nm depend on a strong emission line in the Xe measuring lamp. At wavelengths immediately below or above 882 nm, the lamp provides considerably less light, and the chromatophores either absorb more of the measuring light or fluoresce more strongly. The measurements at 785 nm provide a valuable adjunct to those at 882 nm, because they show an absorbance change of opposite sign. This is important in distinguishing true absorbance changes from artifacts. Within experimental error, the fast absorbance changes between 704 and 800 nm follow the same spectrum as do the P870 absorbance changes which result from strong continuous illumination.

Additional evidence that each P870 complex is associated with two or more C422 hemes is available in the literature. CUSANOVICH *et al.*[3] have presented difference spectra for cytochrome and P870 oxidation by continuous illumination in a *Chromatium* chromatophore preparation. Under conditions which gave oxidation of predominantly C422 (Fig. 3 in ref. 3), the cytochrome absorbance change at 422 nm was about 3 times higher than the absorbance change at 883 nm due to P 870. KUNTZ *et al.*[9] published a similar spectrum with the cytochrome peak about twice as high as the P870 peak. But single flash experiments indicated that the extinction coefficient changes for cytochrome C422 and P870 oxidation are approximately the same[4]. This must mean that cytochrome C422 exceeds P870.

The two cytochromes which have been extracted from *Chromatium* (cytochrome *cc'* and cytochrome C552) both have two or more hemes per protein molecule[8], and cytochrome C422 may be similar. CUSANOVICH AND BARTSCH[10] have reported on the extraction of a cytochrome which may be C422. The extracted protein polymerizes readily. Perhaps it exists as a dimer or a higher aggregate in the chromatophores. If on P870 molecule can oxidize two or more C422 hemes, the question remains whether it does so by direct interaction with each of the hemes, or whether the hemes react in series.

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