OBSERVATIONS ON LIGHT-INDUCED OXIDATION REACTIONS IN THE ELECTRON TRANSPORT SYSTEM OF *CHROMATIUM*

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ABSTRACT Light-induced cytochrome oxidations in *Chromatium* subchromatophore particles were studied in detail. These reactions were found to be dependent not only on redox potential, but also on the efficiency of coupling of the redox buffer electrons to the cytochrome system. Light-induced oxidation of the high potential cytochrome (*c*-556) was dependent on (*a*) the availability of reduced cytochrome and (*b*) the rate of light-induced oxidation (as determined by light intensity) vs. rate of cytochrome rereduction. *Chromatium* high potential iron-sulfur protein ("HiPISP") enhanced the rate of *c*-556 rereduction by mediating electron flow from artificial redox buffers to *c*-556. In these experiments, the light-induced oxidation of the low potential cytochrome (*c*-552.5) is dependent not only on the above parameters, but also on the rate of oxidation of the primary electron acceptor *X*. The interactions of purified *Chromatium* cytochromes with the light-induced cytochrome oxidation system are discussed.

INTRODUCTION

In 1960 Olson and Chance (1, 2) studied the light-induced oxidation of cytochromes in the photosynthetic bacterium *Chromatium*. They found that the environment of the cell dictated which of the available cytochrome species was observed to undergo light-induced oxidation. Further study of this bacterium has involved cell breakage and fractionation to produce membrane fragments which retain the physical attributes of whole cell, light-induced absorbance changes (3–5). Experimenters have utilized various physical methods to study these light-induced changes. Manipulation of the redox environment of the membrane fragments is necessary to produce light-induced cytochrome oxidations which mimic those of whole cell preparations. Different dyes and other redox mediators have been employed to this end.

The present work was undertaken to elucidate the pseudobiological activity of these artificial redox components and their relation to light-induced cytochrome

oxidation in membrane fragments. The effectiveness of some of the soluble redox proteins of *Chromatium* in this regard has also been investigated.

MATERIALS AND METHODS

Chromatium was cultured as described previously (6). Bacteria grown in malate medium were used for these preparations. Cells were harvested and broken immediately or stored at −20°C before use. The cells were suspended in 3 volumes of 50 mM Tris-HCl, pH 8.0, and processed with a Sorvall Ribi Cell Fractionator (Ivan Sorvall, Inc., Norwalk, Conn.) operated at 20,000 psi. Subchromatophore "fraction A" was isolated by the method of Thornber (5).

Absorption spectra were recorded using a Cary Model 14R recording spectrophotometer (Cary Instruments, Monrovia, Calif.) equipped with a combination 0–1.0, 2.0 and 0–0.1, 0.2 slide wire. The spectrophotometer was equipped with a monochromatic actinic light source for illuminating the sample cuvette at right angles to the measuring beam axis.

The anaerobic apparatus and electrode assembly used for light-induced absorbance changes were as described previously (7). The cuvettes used were those of Cusanovich et al. (4) which allowed anaerobic transfer of solution between the reference and sample cuvettes.

The suspending phosphate buffer, 0.1 M, pH 7.0, was initially deaerated in the assembly, then redox buffers and membrane fractions were added by syringe to the final desired concentrations. Gentle bubbling with argon (free of oxygen) served to mix the solution in the main cuvette, and the redox potential was adjusted with an anaerobic solution of 2.0% $Na_2S_2O_4$ in 0.1 M potassium phosphate, pH 7.0, or 100 mM $K_3Fe(CN)_6$ to the desired value. Some of the reaction mixture was then anaerobically transferred to the reference cuvette so that both the reference and sample cuvettes contained the same solution. The solution was then incubated in the dark for the desired time before the sample cuvette was subjected to actinic illumination. The light minus dark difference spectra were continuously monitored using the 0–0.1, 0–0.2 slide wire.

The starting redox buffer for the high potential range (between 400 and 200 mv) was 100 μM $K_3Fe(CN)_6$. To mediate the low potential light reactions (between 200 and −100 mv), a mixture which consisted of 10 μM indigotetrasulfonic acid, 10 μM indigodisulfonic acid, 150 μM $FeCl_3$, 5.0 mM potassium oxalate, and 20 μM phenazine methosulfate (PMS) was used.

Unless otherwise indicated, the "cross-illumination" source was a 200 w quartz-iodine lamp. The actinic light was filtered through a 5 cm water filter (to remove far infared) and through a Baird-Atomic, Inc. (Bedford, Mass.) B1NIR 890 nm interference filter with a 15 nm band pass. A maximum monochromatic intensity of 4.9×10^3 ergs/cm² sec was measured using a KSR, Inc., Model 68 Radiometer (Kettering Scientific Research, Inc., Yellow Springs, Ohio). The actinic beam was focused on the sample by a simple lens and was controlled by a photographic shutter which opened with a speed of about 5 msec.

RESULTS

Other studies have shown (1, 2, 4) that the cytochromes participating in light-induced oxidation in membrane fragments are the high potential cytochrome *c*-556 (termed *c*-422 elsewhere [1, 2]) and the low potential *c*-552.5 (termed *c*-423.5 elsewhere [1, 2]). Most of the work reported here concerns the reaction of cytochromes in the subchromatophore particle first isolated by Thornber (5), designated fraction A. The particle preparation involves treatment of the cell membrane fraction with sodium

dodecyl sulfate (SDS) and subsequent chromatography on hydroxylapatite (HAT) followed by precipitation with ammonium sulfate.

Physical Characterization of Fraction A

Subchromatophore particles from *Chromatium* were prepared by the method of Thornber (5) and this fraction A preparation was used for the remainder of the light-induced absorbance change experiments. The spectrum of the particle is shown in Fig. 1. Spectrally identical particles have been prepared from bacteria grown under various growth conditions.

Approximately 2% of the 890 nm absorbance was reversibly bleached by light (P-883) (5). There was an accompanying blue shift of 800 nm bacteriochlorophyll (BChl) (P-800). Thornber (5) has estimated that the "890" particle contains 1 P-883 per 45 890 BChl's ("B-890").

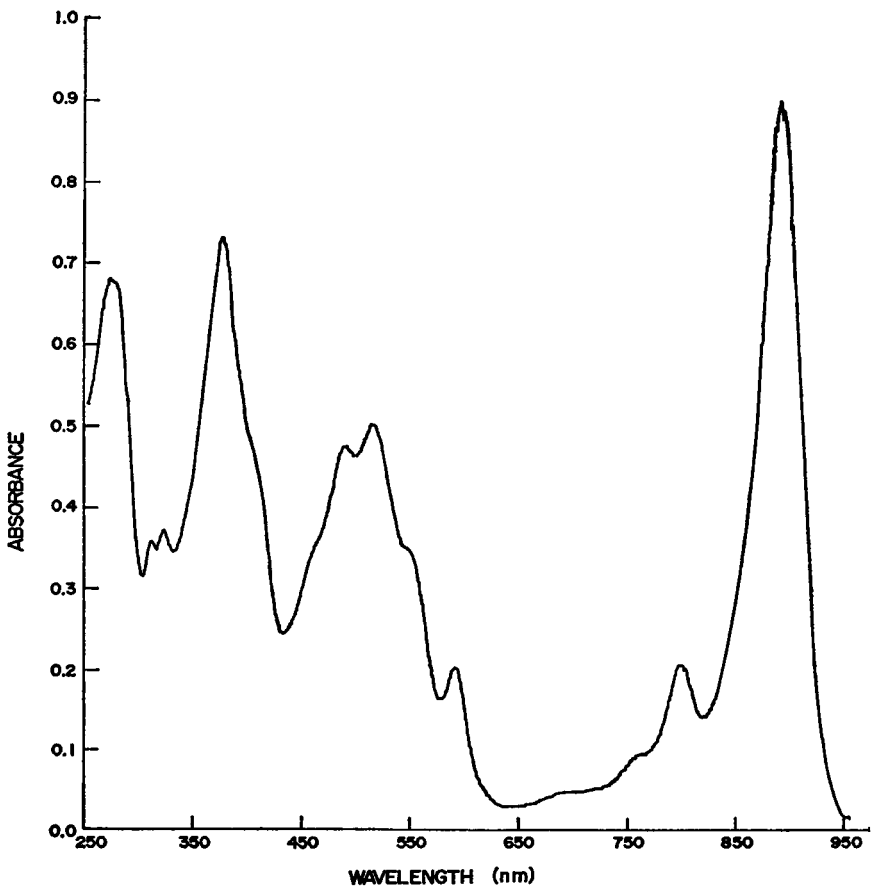

FIGURE 1 Absorption spectrum of *Chromatium* fraction A. The concentration of P-883 $\simeq$ 0.2 μM in 50 mM Tris, pH 8.0.

Cytochrome measurements on fraction A which had been extracted with acetone: methanol (7:2, v/v) to remove interfering pigments indicated four to six *c*-type hemes per active center BChl P-883. These estimates were based on the proposed extinction coefficient for P-883, $\epsilon_{1mM} = 100$ (5). Chemical difference spectra (5) indicated two *c*-556s and three to four *c*-552s per P-883. The *c*-556 was usually in the reduced state in the isolated particles, while *c*-552.5 was oxidized. Consequently *c*-556 was assumed to be a cytochrome with a redox potential significantly more positive than that of *c*-552.5. Chemical redox titrations of the cytochromes in fraction A have indicated *c*-556 $E_{m,7} \sim 345$ mv and *c*-552.5 $E_{m,7} \sim -8$ mv (7). This cytochrome complex has been extracted and purified (7). The complex contained both *c*-556 and *c*-552.5 in approximately a 1:2 ratio. The effect of this preparation, termed "cholate-soluble" cytochrome complex, on the light-induced cytochrome oxidation in fraction A is reported in later sections.

Light-Induced Absorbance Changes of the High Potential Cytochrome c-556

Since this cytochrome is usually reduced in fraction A as isolated, no external redox potential buffering is necessary to demonstrate its light-induced oxidation. The kinetics and magnitude of the changes using these ill-defined conditions are variable.

Table I shows the effect of adding PMS ($E_{m,7} \simeq 60$ mv) to the particles. This added reductant coupled efficiently with the cytochrome and caused a rapid increase in the rate of dark rereduction as well as 50% enhancement in the magnitude of the

TABLE I

EFFECTS OF SULFIDE, PMS, AND *CHROMATIUM* HiPISP ON *c*-556 LIGHT-INDUCED OXIDATION IN FRACTION A

Chromatium HiPISP	Sulfide	PMS	Maximal oxidation	$T_{1/2}$ rereduction
μM	*μM*	*μM*	%	*sec*
—	—	—	36	large
—	50	—	100	42
—	100	—	100	55
—	200	—	90	45
—	200	10	17	1
—	100	—	98	46
0.4	100	—	89	22
2.0	100	—	20	1
2.0	100	10	17	1
—	—	—	51	130
—	—	5	62	88
—	—	10	85	64
—	—	20	100	64
—	—	50	100	20
—	—	100	94	15
—	—	150	94	4
—	—	250	94	2

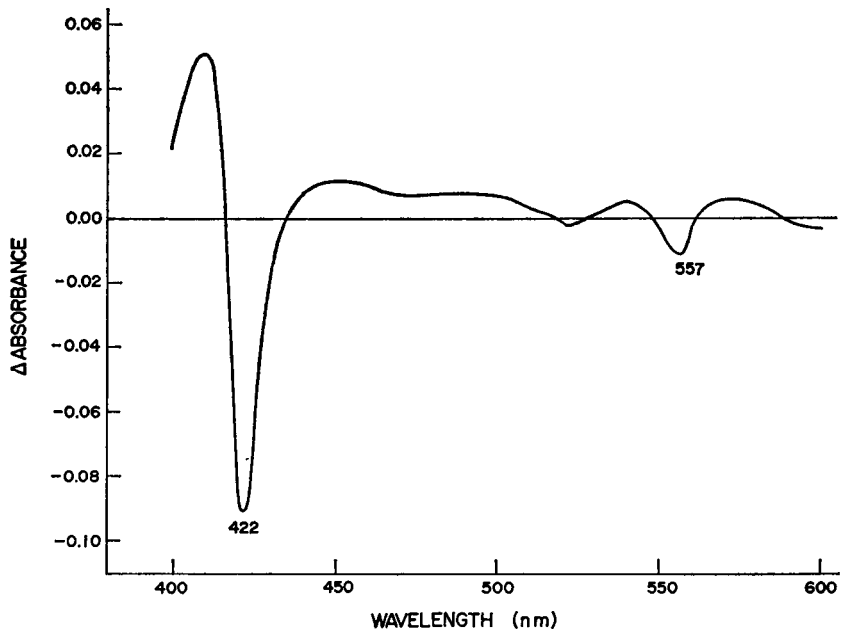

FIGURE 2 Steady-state, light-induced difference (absorption) spectrum of *Chromatium* *c*-556. The sample contained A_{890} cm^{-1} = 2.0 of fraction A (0.44 μM P-883) in 20 μM PMS and 50 mM Tris, pH 8.0. Cross-illumination was as described in Materials and Methods.

light-induced change. This indicated that some of the cytochrome in this particular particle preparation was in the oxidized state.

The steady-state, light-induced difference spectrum, replotted from a Cary 14R trace to correct for base line absorbance, is shown in Fig. 2. The α peak maximum of this cytochrome (*c*-556) is observed to vary from 555 to 557 nm, for which no explanation can be offered at present. The maximum, steady-state, light-induced cytochrome oxidation of fraction A in PMS corresponds to the total amount of *c*-556 in the particle as determined by chemical difference spectrum; therefore, in steady-state illumination, *c*-556 becomes nearly completely oxidized.

A plot of the *c*-556 light-induced absorbance change as a function of redox potential is shown in Fig. 3. For this experiment 100 μM $K_3Fe(CN)_6$ and an appropriate amount of $Na_2S_2O_4$ is added to the reaction system to buffer the redox potential at the desired value (see Materials and Methods for details). The amount of light oxidation is limited by the amount of *c*-556 in the reduced form at the given potential. As the potential is lowered, the rate of rereduction of cytochrome becomes faster than the light-induced oxidation, and so the absorbance change diminishes and finally disappears.

The high potential *c*-556 light reaction is not affected by the addition of any of the soluble *Chromatium* heme proteins: cytochrome *c'* (8–10), flavocytochrome *c*-553 (8, 10), or the high potential cytochrome *c*-553(550) (11). Addition of the membrane-

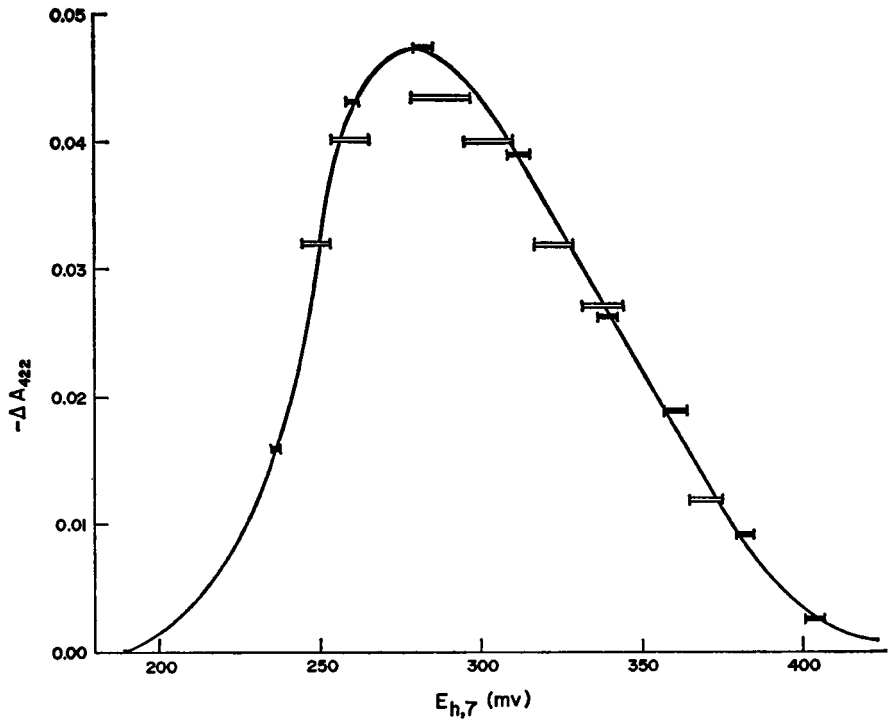

FIGURE 3 The extent of *Chromatium* c-556 light-induced oxidation as a function of redox potential. The sample contained A_{890} cm^{-1} = 1.0 of fraction A (0.22 μM P-883) in 0.1 M potassium phosphate plus 100 μM $K_3Fe(CN)_6$, pH 7.0. The redox potential was adjusted by reductive titration with 2% $Na_2S_2O_4$, closed symbols; and subsequently by oxidative titration with 100 mM $K_3Fe(CN)_6$, open symbols. The 890 nm actinic light intensity was 4.9 × 10^3 ergs/cm^2 sec.

bound cytochrome complex *c*-556 and *c*-552.5, solubilized by cholate extraction and purified as described elsewhere (7), does not affect *c*-556 light-induced oxidation in this system.

Chromatium HiPISP (12) does show some dramatic effects on the high potential cytochrome rereduction. Reduced HiPISP added to the 890 particles in amounts approximating the *c*-556 concentration causes little effect on the light-induced reaction; however, if PMS is also present in the mixture, the *c*-556 reduction rate is enhanced. Addition of higher concentrations of HiPISP increases the rate of *c*-556 reduction to the point where the reduction rate exceeds the light-induced oxidation rate, and an attenuation of steady-state *c*-556 light-induced oxidation results. Plastocyanin will not replace *Chromatium* HiPISP in this function, but $K_4Fe(CN)_6$ and HiPISP from *Thiocapsa pfennigii* mimic the effect, although they are less efficient.

Addition of sulfide together with PMS causes a very rapid coupling of low potential electrons to *c*-556 so that the steady-state signal is reduced some 80% and the rereduction is very fast. Addition of reduced HiPISP together with sulfide shows the

same effect. The addition of sulfide alone produces no changes in the kinetics of rereduction, but does slowly reduce *c*-556, thus enhancing photooxidation. These effects are summarized in Table I.

Light-Induced Absorbance Changes of the Low Potential Cytochrome

The low potential cytochrome *c*-552.5 is oxidized in isolated fraction A and so must be chemically reduced before the light oxidation can occur. The low potential redox buffering system used for this purpose is described in Materials and Methods. Using a constant 890 nm cross-illumination intensity of 4.9×10^3 ergs/cm^2 sec, the kinetics of oxidation and rereduction are found to vary with redox potential (Fig. 4).

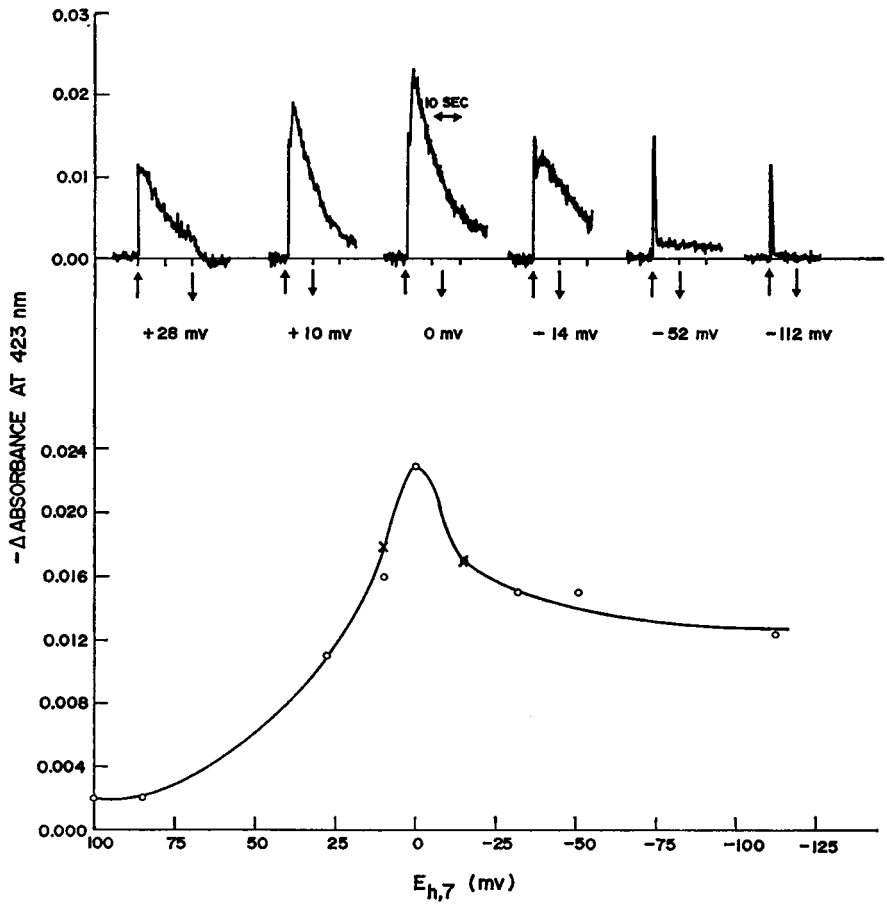

FIGURE 4 The kinetics and extent of *Chromatium* *c*-552.5 light-induced oxidation as a function of redox potential. The sample contained A_{890} cm^{-1} = 1.0 of fraction A (0.22 μM P-883) buffered and illuminated as described in Materials and Methods. A 20 min dark period separated each illumination. Circles, reductive titration; ×'s, oxidative titration. Arrows pointing upward indicate the beginning of illumination, and downward arrows indicate the end of illumination.

An interpretation of the kinetics of the *c*-552.5 light-induced reaction (Fig. 4) which first comes to mind is that the oxidation initially overshoots the equilibrium condition and then cytochrome rereduction "catches up" to establish the steady state. Further experiment has shown that even though all of the cytochrome is re-reduced in less than 1 min, full light-induced oxidation requires about a 20 min dark period. This indicates that the extent of cytochrome reduction is not the limiting factor in the extent of light-induced oxidation. A plot of magnitude of cytochrome oxidation as a function of dark recovery period at the optimum redox level, $E_{m,7} = 0$ mv, is illustrated in Fig. 5.

The data are consistent with the hypothesis set forth by Parson (13) that the hypothetical primary electron acceptor (X) is only slowly reoxidized in the particle and that as the light-induced oxidation proceeds, X becomes progressively reduced to the point that the transfer of electrons from cytochrome to X cannot continue. Under the conditions used, only about one-fourth of the *c*-552.5 present in the particles becomes light oxidized even at the optimum $E_{h,7}$ of 0 v (Fig. 4).

The light-induced oxidation of *c*-552.5 was monitored as a function of redox mediator concentrations which were varied from one-half- to fourfold the values

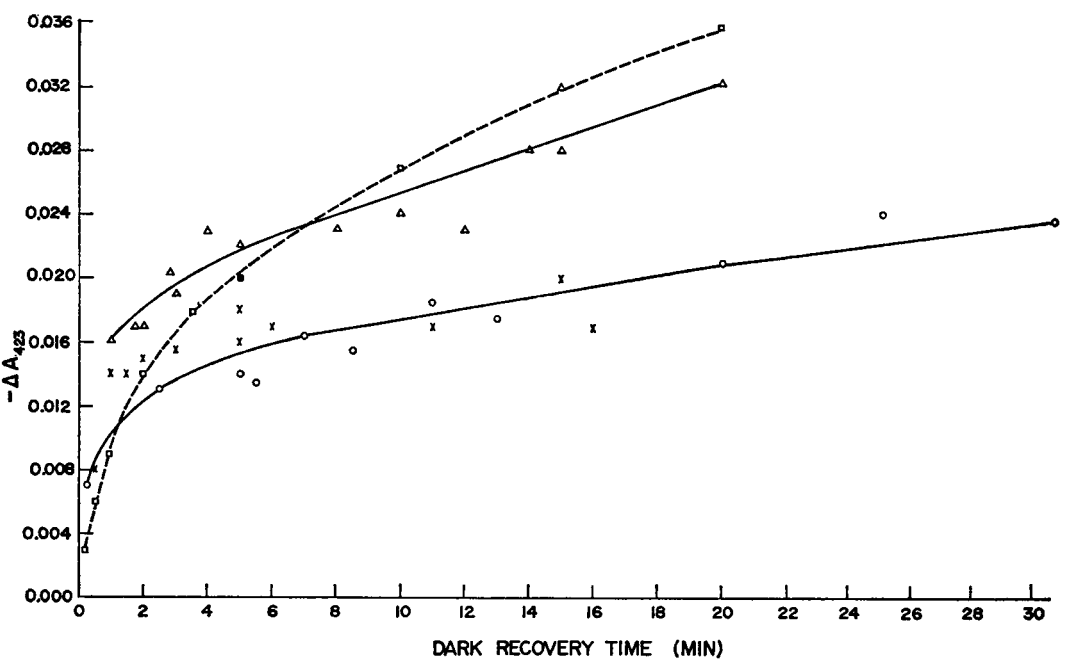

FIGURE 5 The extent of *Chromatium* *c*-552.5 light-induced oxidation as a function of dark recovery period. Conditions as for Fig. 4 with $E_{h,7} = 0$ mv. Circles and bottom solid curve, fraction A only; ×'s, fraction A plus 10 μM reduced *Chromatium* HiPISP; squares and dashed curve, fraction A plus 2 μM flavocytochrome *c*-553; triangles and upper solid curve, fraction A plus 1.9 μM in heme of purified, cholate-soluble cytochrome complex.

given in Materials and Methods. Although the rate of rereduction of cytochrome increased with concentration of mediator, the magnitude of the cytochrome oxidation remained unchanged. The oxidized redox buffer components apparently were unable to accept electrons from *X* fast enough to alleviate the postulated accumulation of reductant. Physiological redox components were not tested for this function.

The dependence of the *c*-522.5 light-induced oxidation on redox potential is shown in Fig. 4. Like the light reaction of *c*-556 (Fig. 3), the extent of light oxidation of *c*-552.5 depends on the fraction of reduced *c*-552.5 in the particle at the given potential. At lower potentials, however, a decrease in the magnitude of the oxidation is observed, presumably because of a rapid light-induced reduction of *X* as previously described. Unlike the situation with *c*-556, complete light-induced oxidation of *c*-552.5 was not achieved.

Addition of reduced HiPISP to the reaction mixture did not affect the magnitude of the light-induced absorbance change at 423 nm (associated with *c*-552.5), but it did shorten the period of dark recovery necessary to achieve maximal cytochrome oxidation (Fig. 5). Addition of flavocytochrome *c*-553 at a concentration approximating that of bound *c*-552.5 caused an increase in the extent of light-induced cytochrome oxidation, but did not alter the time dependence of dark recovery. Since full

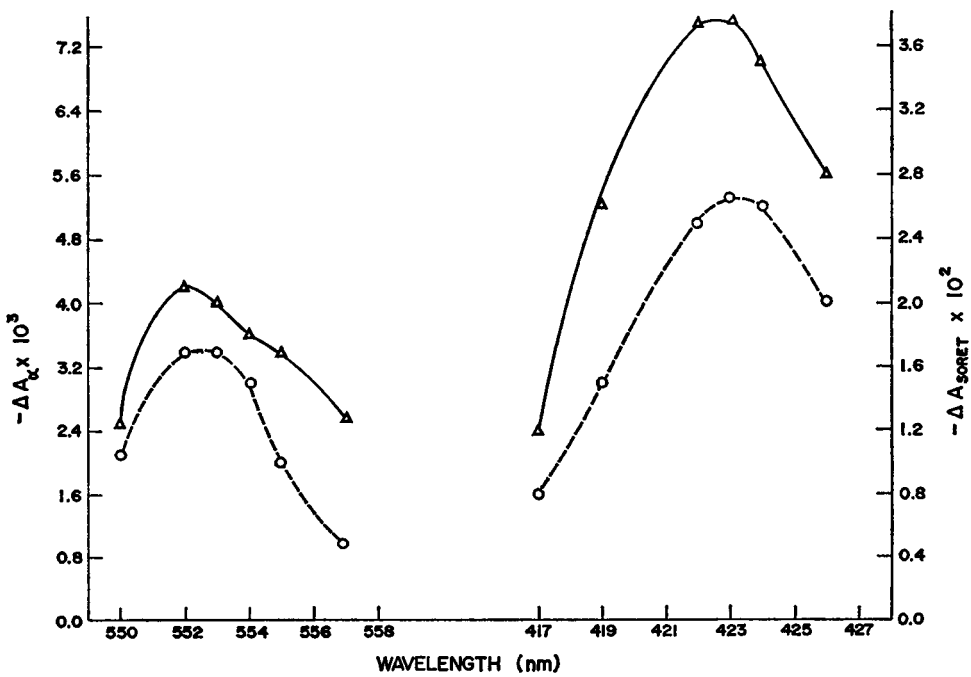

FIGURE 6 Wavelength dependence of *Chromatium* *c*-552.5 light-induced oxidation. Conditions as for Fig. 4 with $E_{h,7}$ = 0 mv and 20 min dark time between illuminations. Circles, fraction A; triangles, fraction A plus 1.9 μM (in heme) of purified, cholate-soluble cytochrome complex.

oxidation of *c*-552.5 was not achieved in the control, it is not known whether the increased light-induced absorbance change at 423 nm was due to oxidation of added flavocytochrome *c*-553 or to an enhancement of the bound *c*-552.5 light-induced oxidation.

Addition of the purified cholate-soluble cytochrome complex (7) produced an effect similar to the addition of flavocytochrome *c*-553. The wavelength dependence of this reaction in the α and Soret peak regions is presented in Fig. 6. The spectra of the light-induced cytochrome oxidation in fraction A alone, or with added cholate-soluble cytochrome complex, are the same within the limits of resolution.

DISCUSSION

General Remarks

Before illumination, provided dark incubation is long enough, the relative concentrations of all redox species in the fraction A preparation are determined only by the redox environment of the surroundings, for fraction A is devoid of endogenous reductants. At the onset of illumination, the redox equilibrium is perturbed and new steady-state redox conditions are not established for a matter of seconds in the experiments conducted as reported here (cf. Fig. 4). It has been shown (14) that the kinetics of cytochrome oxidation correlate well with the kinetics of BChl rereduction. For this reason, it is assumed that cytochrome reduces $BChl^+$ after the BChl has promoted an electron to the low potential acceptor *X* in the primary photoact. The identities of the physiological oxidant of *X*, or cytochrome reductant, have not been established.

Many kinetic parameters are involved in the interpretation of light-induced cytochrome oxidation. Primary considerations are the relative rates of cytochrome oxidation and rereduction. Both rates are influenced by the redox potential of the surroundings. The rate of rereduction is naturally faster the more reducing the environment. The rate of oxidation, however, has a twofold dependence. First, the rate and extent of light-induced oxidation depends on the concentration of reduced cytochrome. Secondly, the oxidation is also dependent on the availability of oxidized *X*. Because both the cytochrome and *X* can be reversibly oxidized and reduced, the concentrations of the involved (reduced in the case of cytochrome, and oxidized in the case of *X*) redox species for optimum light oxidation of cytochrome are dependent on the endogenous redox potential. All of the following arguments are dependent on the fact that in fraction A reduction of cytochrome by photoreduced *X* is slow in relation to the other reactions discussed.

Once cells are disrupted, the concentrations and thus reaction rates of the redox components are altered. In the preparation of chromatophores and subchromatophore particles, some components of the in vivo system are undoubtedly lost. A system of redox buffers is usually supplied as an artificial electron pool used to simulate in vivo conditions. The extent and rate of coupling of these chemicals to the

biological system components are variable and could induce artifacts in membrane structure and rates of oxidation and reduction. Thus all in vitro experiments *must be* interpreted with these restrictions in mind.

In the steady-state, dark situation for *Chromatium* with imposed high redox potential ($E_{h,7} \simeq 300$ mv), most of cytochrome *c*-556 ($E_{m,7} \simeq 345$ mv) is reduced while both cytochrome *c*-552.5 ($E_{m,7} \simeq -8$ mv) and *X* ($E_{m,7} \simeq -160$ mv) (14) are oxidized. In this case reduced *c*-556 is available for light-induced oxidation and X_{ox} is available to accept an electron from BChl in the primary photoact. At lower potentials (0 mv and below), *c*-556 is all reduced, but is not observed to undergo steady-state light oxidation. This may be ascribed to a sophisticated membrane control mechanism, or to a *c*-556 rereduction rate fast enough to mask the photooxidation at least at the actinic light intensities used here. Under this circumstance the steady-state level of oxidized cytochrome *c*-556 is very low.

The midpoint redox potentials of *c*-552.5 (−8 mv) and *X* (−160 mv) (15) are sufficiently separated to allow coexistence of ~ 90% reduced *c*-552.5 and ~ 90% oxidized *X* at E_h potentials in the −50 to −100 mv range. Therefore, nearly complete light-induced oxidation of *c*-552.5 is thermodynamically possible in this potential range; however, complete *c*-552.5 oxidation has not been accomplished. This is probably because of kinetic considerations discussed below.

Light-Induced Oxidation of c-556 in Fraction A

The *c*-556 can be completely photooxidized under the conditions described in Materials and Methods. The rate of light-induced oxidation is faster than the rereduction rate when $E_{h,7}$ is set at or above about 300 mv. If no external redox buffer is added, the rereduction step is extremely slow, but addition of reduced PMS adds reductant to the system and speeds up the rereduction phase. PMS, however, does not couple fast enough with *c*-556 to cause the reduction phase to be faster than the oxidation phase, even though it has a very low midpoint potential (~ 60 mv) relative to that of *c*-556. This is shown by the fact that even at very high PMS concentrations nearly complete *c*-556 steady-state, light-induced oxidation occurs (Table I).

If reducing power from artificial electron donors is efficiently coupled to *c*-556, the reduction rate exceeds the light oxidation rate, and the extent of steady-state oxidation is less than maximal. Fig. 3 shows that this does occur if fraction A, in the presence of 100 μM $K_3Fe(CN)_6$, is titrated to potentials below $E_{h,7} \sim 250$ mv with $Na_2S_2O_4$. The most efficient coupler between artificial electron donors and *c*-556 found to date is *Chromatium* HiPISP, for if reduced HiPISP is added to the reaction mixture with a source of low potential electrons (i.e., reduced PMS), the rate of rereduction of *c*-556 is greatly enhanced. If approximately equimolar ratios of HiPISP and fraction A membrane-bound *c*-556 are mixed with 50 μM PMS, the rate of *c*-556 rereduction is so fast that the extent of steady-state light oxidation is attenuated by about 20%. It takes about 10 times the concentration of $K_4Fe(CN)_6$ and 50 times the

concentration of *T. pfennigii* HiPISP to produce the same effect as is obtained with *Chromatium* HiPISP. Because whole *Chromatium* cells contain similar concentrations of HiPSIP under all growth conditions (6), this reaction could be of physiological significance.

Sulfide ion does not couple electrochemically well with *c*-556 even though it is a very strong reductant. Concomitant addition of HiPISP allows efficient rereduction and results in attenuation of the steady-state *c*-556 light-induced oxidation. This is not a result of *X* reduction by $S^=$ because steady-state spectra are observed. Thus *X* is still rapidly oxidized under these conditions. The availability of oxidized *X* does not seem to be a limiting factor in the amount of oxidation of *c*-556 at the light intensities used in these experiments because the extent of light-induced *c*-556 oxidation is found to be dependent only on the amount of reduced *c*-556 in the sample, unless, as discussed, cytochrome reduction is extremely fast.

In contrast, Parson (14) has shown that the availability of oxidized *X* limits *c*-556 oxidation in very fast light-induced reactions. He has shown that a 20 nsec pulse from a *Q*-switched ruby laser oxidizes all of the P-883, but only about half of the *c*-556 (there are two *c*-556s per P-883). After the first laser pulse, the system goes into a "refractory period" of about 60 μsec during which another flash can produce no further *c*-556 oxidation. If intervals of greater than 60 μsec separate the laser flashes, the second flash oxidizes the remaining *c*-556 in the system. This refractory period is thought to be the time necessary for X_{red} to become reoxidized. The refractory period is dependent on temperature and pH, but does not vary upon addition of PMS. If PMS is active in accelerating the oxidation of *X*, it must do so on a time scale longer than 60 μsec. This proposed slow reaction might be important in physiological situations when the light is of low intensity, or where the redox potential of the surroundings is low enough to slow the oxidation of *X*. The oxidation of *X* is inhibited by *o*-phenanthroline (14), suggesting the involvement of a transition metal ion in the reaction.

Parson interprets the refractory period as the time necessary for *X* to be oxidized by another hypothetical electron acceptor *Y*. His data may also be explained by the presence of a pool of *X*, for if only one *X* molecule can occupy a favorable position for accepting electrons from P-883, the 60 μsec refractory period may then be the time necessary for an oxidized *X* from the pool to replace the photoreduced *X* in the active site in a diffusion-limited process. For this hypothesis to be correct, *o*-phenanthroline must prevent the oxidized *X* pool from interacting with the "active site" for *X* photoreduction. This diffusion hypothesis also rationalizes the temperature, pH, and redox potential dependence of the reaction.

The amount of reduced *c*-556 in fraction A is the only factor limiting the extent of light-induced *c*-556 oxidation under the conditions used here. Supplementing the reduced *c*-556 concentration by adding purified, cholate-solubilized cytochrome in the appropriate redox form should cause an increase in the extent of the *c*-566 light-induced oxidation. As described in Results, the addition of cholate cytochrome

complex to the reaction mixture does not affect the light-induced reaction (6). Furthermore, the *c*-556 light-induced oxidation is not affected by the addition of any soluble *Chromatium* heme protein. The failure to couple added detergent-solubilized heme proteins to fraction A reactions may be due to denaturation of the added cytochrome *c*-556 by cholate, although all known physical properties of the cytochrome are retained in the extracted form. It is more likely that extracted cytochrome *c*-556 cannot couple well enough to fraction A to react with P-883. This would happen if (*a*) there were only one site for cytochrome binding which remained saturated in the isolated subchromatophore particles, or (*b*) if the detergents used to prepare either fraction A or cytochrome prevent association. The size of the cholate-soluble cytochrome complex in buffer is estimated to be ~200,000 daltons (as determined by gel filtration). Such a particle may be too large to fit in the hypothetical binding site. It should be noted that the cytochrome appears smaller (45,000 daltons) under the denaturing conditions of SDS-acrylamide gel electrophoresis (6). A possible alternative suggestion is that the added cytochrome is oxidized by $P\text{-}883^+$ but because of its "peripheral" association with fraction A it is more susceptible to rereduction and so remains essentially reduced in steady-state illumination.

Light-Induced Oxidation of c-552.5 in Fraction A

As previously discussed, *c*-552.5 has not been shown to undergo complete light-induced oxidation. The maximum extent of reaction obtained in this work corresponds to oxidation of one-fourth of the total *c*-552.5 present in the particle preparation (Fig. 4). As shown in Fig. 4, this initial light-induced *c*-552.5 oxidation decays even under continuous illumination at the maximum available intensity, and steady-state, light-induced oxidation is not observed.

The kinetic data of Fig. 4 show that light initially induces *c*-552.5 oxidation, but the cytochrome becomes rereduced faster than it is oxidized. The rate of rereduction increases with decreasing redox potential and seems to be independent of illumination. Furthermore, a second illumination of the sample within less than 10 min after the first gives an attenuated *c*-552.5 oxidation even though the cytochrome has been completely rereduced. The extent of light-induced *c*-552.5 oxidation as a function of dark recovery period is shown in Fig. 5. This phenomenon is like that observed by Parson (14) for *c*-556 oxidation, but on a much slower time scale, and can be interpreted similarly as the consequence of complete reduction of *X*.

Because the estimated redox potential of *X* (15) is sufficiently low to ensure that the substance is thermodynamically capable of being completely oxidized at the ambient redox potentials tested, kinetic factors must be decisive. That is, although *X* oxidation is thermodynamically favorable, it occurs at a very slow rate. This is probably because of inefficient interaction between X_{red} and the oxidized component which *X* reduces. Case et al. (16) have shown that *X* oxidation is about 1000 times slower in fraction A than in their chromatophore preparations. They postulate that

this is because of the absence of the physiological *X* oxidant in fraction A since in their experiments with chromatophores, *X* oxidation is speeded up by addition of PMS and is insensitive to *o*-phenanthroline, in contrast to the effect of PMS and *o*-phenanthroline on *X* oxidation in fraction A.

Again, the existence of the postulated secondary electron acceptor (*Y*) cannot be distinguished from the *X* pool hypothesis proposed in a previous section, because the passage of electrons from *X* to *Y* would give results identical with those of the proposed *X* diffusion process. Extraction of most of the *X* pool in the fraction A preparation could produce the results observed by Case et al. (16) if only one *X* remained in the active site for photoreduction.

The difference between the *X* oxidation rates observed by Case et al. (16) and those reported in this work may be entirely due to differences in the imposed redox potentials used. Alternatively, it may arise from the efficiency of *X* interaction with the different oxidized redox buffers used. Case et al. (16) used PMS to speed up the *X* oxidation, while the only oxidized species available for *X* oxidation in this work were the indigosulfonic acids.

In all studies to date, the availability of oxidized *X* has been the limiting factor in the extent of *c*-552.5 oxidation. Since *c*-552.5 is not completely oxidized by illumination, increasing the concentration of this cytochrome by adding purified, cholate-soluble cytochrome should have little effect on the extent of light-induced oxidation. Data noted in Results (Fig. 6), however, show that the amount of *c*-552.5 oxidized by illumination of fraction A increases upon addition of the cholate-solubilized cytochrome complex. It is possible that the *c*-552.5 component of the cholate-solubilized cytochrome efficiently mediates the oxidation of *X* by the available oxidized redox components.

The addition of soluble flavocytochrome *c*-553 also produced enhancement of the *c*-552.5 oxidation. This is probably because of enhancement of *X* oxidation in the manner suggested for the effect of cholate-soluble cytochrome. In fact, it is possible that flavocytochrome *c*-553 is the physiological *X* oxidant. This is consistent with the presence of flavocytochrome *c*-553 in chromatophores, and the absence of the cytochrome from fraction A, as is also true for the species *Y* proposed by Parson (14). Cytochrome *c'* is not active in this reaction, and so some specificity for flavocytochrome *c*-553 is indicated. It is, of course, possible that added cholate cytochrome and flavocytochrome *c*-553 directly couple with BChl P-883 and are oxidized by illumination, despite the considerations presented above.

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REFERENCES

1. OLSON, J. M., and B. CHANCE. 1960. *Arch. Biochem. Biophys.* **88**:26.
2. OLSON, J. M., and B. CHANCE. 1960. *Arch. Biochem. Biophys.* **88**:40.
3. CUSANOVICH, M. A., and M. D. KAMEN. 1968. *Biochim. Biophys. Acta.* **153**:376.
4. CUSANOVICH, M. A., R. G. BARTSCH, and M. D. KAMEN. 1968. *Biochim. Biophys. Acta.* **153**:397.
5. THORNBER, J. P. 1970. *Biochemistry.* **9**:2688.
6. KENNEL, S. J., R. G. BARTSCH, and M. D. KAMEN. 1971. *Biochim. Biophys. Acta.* **234**:458.
7. KENNEL, S. J., and M. D. KAMEN. 1971. *Biochim. Biophys. Acta.* **253**:153.
8. BARTSCH, R. G. 1963. *In* Bacterial Photosynthesis. H. Gest, A. San Pietro, and L. P. Vernon, editors. Antioch Press, Yellow Springs, Ohio. 475.
9. DUS, K., and M. D. KAMEN. 1963. *Biochem. Z.* **338**:364.
10. BARTSCH, R. G., M. L. COVAL, and M. D. KAMEN. 1961. *Biochim. Biophys. Acta.* **51**:241.
11. CUSANOVICH, M. A., and R. G. BARTSCH. 1969. *Biochim. Biophys. Acta.* **189**:245.
12. BARTSCH, R. G. 1963. *In* Bacterial Photosynthesis. H. Gest, A. San Pietro, and L. P. Vernon, editors. Antioch Press, Yellow Springs, Ohio. 315.
13. PARSON, W. W. 1969. *Biochim. Biophys. Acta.* **189**:384.
14. PARSON, W. W. 1968. *Biochim. Biophys. Acta.* **153**:248.
15. CRAMER, W. A. 1969. *Biochim. Biophys. Acta.* **189**:54.
16. CASE, G. D., W. W. PARSON, and J. P. THORNBER. 1970. *Biochim. Biophys. Acta.* **223**:122.