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PHOTOSYNTHETIC REACTION CENTER TRANSIENTS, P435 AND P424, IN *CHROMATIUM* D*

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SUMMARY

1. The correlation of kinetic, saturation and potentiometric evidence shows that P435 is a spectral component of the same reaction center as P883 in *Chromatium* D.

2. There is only one cytochrome oxidizing reaction center P883/P435, since the spectrum of P435 is the same independent of which cytochrome (C553 or C555) is oxidized and the P435 reduction kinetics correlate with the oxidation kinetics of either cytochrome, depending upon which is being oxidized.

3. The oxidation half-time of P883/P435 is less than 50 nsec.

4. The E_m of P883/P435 is +486 mV and that of its primary electron acceptor is −134 mV.

5. P424 is observable only when the primary electron acceptor, X, is in the reduced state prior to the flash: (A) at room temperature in chromatophores between −318 and −145 mV and (B) at 77°K over the same potential range (first laser flash) or at higher potentials (subsequent laser flashes) under conditions in which the first laser flash permanently reduces X.

6. P424, P883/P435, cytochrome C553 and cytochrome C555 are all associated with Thornber's subchromatophore Fraction A, but not Fraction B.

7. The rise half-time of P424 in chromatophores is 50 nsec at room temperature and 200 nsec at 77°K. Its recovery half-time is 2.2 to 2.4 μsec at both temperatures in chromatophores or 16 msec in whole cells at 77°K.

8. P424 might represent either an oxidation of bacteriochlorophyll spectrally modified by the presence of reduced primary electron acceptor or a light-induced reduction state of bacteriochlorophyll.

9. Thermodynamically, it is possible that this new species might be a link in the direct reduction of NAD^+.

INTRODUCTION

A previous paper[1] reports the discovery of a laser-induced transient of increased absorbance in *Chromatium* D chromatophores similar to P435 but occurring at

Abbreviations: BChl, bacteriochlorophyll; E_h the electrical potential of an oxidation–reduction system referred to the standard hydrogen electrode; E_m, the midpoint potential of an oxidation–reduction couple at a specific pH (7.4 for the experimental values reported in this work).

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oxidation–reduction potentials so low that P435 is inactivated. This work reports on observations which establish P435 as a spectral component of the same reaction center bacteriochlorophyll as P883 and describes further evidence concerning the relationship between this reaction center and the low potential transient which we shall call P424.

P435 was originally attributed to oxidized bacteriochlorophyll by DUYSENS[2] and DUYSENS *et al.*[3] and considerable kinetic[4–9] and saturation level[4,6,7,10] evidence in chromatophores of *Rhodopseudomonas spheroides*, *Rhodospirillum rubrum* and *Chromatium* D has subsequently tended to confirm this interpretation. Certain other results, however, have conflicted with this conclusion: (1) in the msec time range P435 was found to be less reversible than the infrared changes[11] in *Chromatium* D cells, (2) P435 was reported to react differently to various light intensities[12] and saturated at a lower light level than P883[13] in cells of *R. rubrum*, (3) P435 and P883 were reported to react differently to dithionite in both *Chromatium* D and *R. rubrum*[14] and (4) ratios of P435 to P883 were reported to vary in different preparations of *R. rubrum*[7,8] chromatophores.

The discrepancies found in the measurements obtained in msec and longer time scales may be explained as interference from other slower reactions, especially at low actinic light levels (see ref. 12, Fig. 5). The reaction center transient recovers so rapidly that it should be observable only at very high light intensities under steady state actinic conditions. Its kinetics can thus be properly determined only in the microsecond time range. The early observations by CHANCE AND SMITH[15] of P435 using phenylmercuric acetate can probably be explained as blockage of cytochrome reduction by the inhibition causing a pile up of both oxidized cytochrome reaction center bacteriochlorophyll (and perhaps bulk bacteriochlorophyll). The dithionite discrepancies were obtained using laser activation and can be explained as confusion of P435 with P424, especially if we can show that the phenomenon persists to 77°K (see ref. 16). A preliminary report of this work has been presented.[17]

METHODS

Sample preparation

Chromatium D was grown photoautotrophically as before[18] except that the average illuminance on the cell culture bottles from banks of 75 W bulbs placed along the sides of the growing tanks was 5400 lux. Chromatophores were prepared sonically using a previous method[18], except that the 0.1 M Tris–HCl buffer pH was adjusted to 7.4 and the particles were "washed" twice before resuspension in a minimal amount of buffer (storage was at 2°C under nitrogen). Sub-chromatophore soluble fractions of *Chromatium* D were obtained by the method of THORNBER[19].

Laser-spectrophotometer

Most of the equipment has been described elsewhere[18,20,21]. Actinic pulses were 20–30 nsec long at 694 nm, and the minimum response time of the spectrophotometer, set by the photomultiplier preamplifier circuit, was found to be 50 nsec by direct measurement. Telefunken XP-1080 and EMI-9524B photomultiplier tubes were used. The measuring light from a high pressure, 1000 W mercury arc (selected low noise AH-6 lamps from GE and PEK Labs, Inc.) passed through a 250 mm Bausch and

Lomb grating monochromator. Since this combination gives approximately 40 times as much light in the 400 to 450 nm range as the previously used boosted 45 W tungsten–iodine lamp, it became necessary to determine the actinic effect of the measuring light.

Fig. 1 shows the result of such measurements. Each point gives the change in transmission due to cytochrome oxidation (measured at 423 *minus* 460 nm using a dual wavelength spectrophotometer with a much lower measuring light intensity) after an exposure to the singlebeam measuring light of wavelength as labelled on the curves for a length of time given on the abscissa. The laser-induced absorbance changes reported in this study were obtained by exposing a sample to the measuring light for 9–13 msec prior to a flash. Thus one calculates that at most the measuring light oxidizes 10 % of the cytochrome that could be oxidized by a single laser flash knowing that the flash causes a 2.2 % transmission change. Additional evidence showing that the intense measuring light did not significantly affect the experiments is that the results in Fig. 2 and 3 were duplicated using the boosted tungsten–iodine measuring light source (although more noise was present).

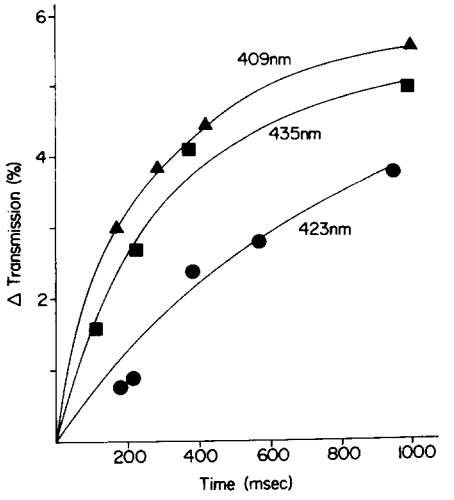

Fig. 1. The change in transmission in whole cells due to cytochrome C553 oxidation caused by the mercury arc measuring light at various monochromator wavelength settings as a function of exposure time. The measurements were made at much lower light intensity using a dual wavelength spectrophotometer (423 nm *minus* 460 nm). For comparison, a laser flash caused a ΔT of about 2.2 %. Light path, 1.6 mm; BChl concentration, 0.182 mM.

Steady illumination

A 100 W tungsten-iodine lamp focused through a 2-cm water cell and a Corning 2600 (7-69) filter provided the background illumination described in Figs. 2, 3 and 4 and Table I. The light passing through this filter combination and reaching the sample surface was measured with a silicon solar cell (Hoffman EA7-E1) calibrated against a thermopile which was in turn calibrated against a standard lamp.

Potentiometric techniques

Room temperature measurements in which the oxidation–reduction potential was controlled were made in the cuvette designed by DUTTON[22]. This cuvette allows

for continuous stirring of the sample suspension and provides for the measurement of oxidation–reduction potential by means of a platinum electrode (Radiometer No. P101) in conjunction with a saturated KCl–calomel reference electrode (Radiometer No. K401). Strictly anaerobic conditions were obtaind by flushing the chromatophore suspension with argon (< 1 ppm O_2) for a least 1.5 h before use. Various mediator dyes were added to serve as oxidation–reduction equilibration agents between the platinum electrode and the electron transport carriers in the chromatophore membrane. The electrodes were standardized[23] in a saturated quinhydrone solution (pH 7.0) at 25°C. The data in Fig. 6 for each set of redox dyes was obtained by poising the system with small additions of a buffered sodium dithionite solution when the E_m's of the dyes were less than about 150 mV or with potassium ferricyanide when the E_m's of the dyes were above 150 mV. The laser was fired at desired intervals (usually 10–20 min between flashes) as the potential slowly drifted. After this procedure more dithionite or ferricyanide was added to bring the potential back to the middle of the potential range for the dyes in use and the absorbance changes repeated as a check on the stability. Furthermore, increasing the dye concentrations by a factor of two did not significantly affect the observed absorbance changes. The overlapping of data using different dye systems provides additional assurance that equilibration of the system was achieved.

Potentiometric titrations at 77°K were made using the method of DUTTON[22]. Chromatophores were poised at room temperature and a fresh sample rapidly frozen in the dark for each potential indicated in Fig. 9. Low temperature light-induced reactions can thus be obtained as a function of the oxidation–reduction potential prior to freezing since the reduction state of the components do not change during the rapid freezing process[22].

RESULTS

Laser-induced spectra of P435 and cytochromes C555 and C553

Fig. 2 shows room temperature laser-induced P435 difference spectra in anaerobic whole cells of *Chromatium* D under conditions in which the flash is given while the cells are unilluminated (open circles) and while illuminated with steady background light (see under METHODS) (open squares). For comparison, difference spectra of the cytochromes oxidized under the same two conditions are also included. P435 and the cytochrome components were separated as indicated in the inset to Fig. 2. It is important to recognize that the laser-induced P435 difference spectrum in unilluminated cells characterized by absorbance peaks at 425 and 434 nm does not change noticeably upon illumination (except for a slight decrease in amplitude). Chromatophores poised at +415 mV exhibit a similar flash induced difference spectrum[1]. The 408 nm laser-induced peak of the cytochrome spectrum indicative of C553 oxidation[15,24], however, shifts to 410 nm (characteristic of C555 oxidation) when the cells are exposed to background illumination. As explained previously[18] cytochrome C555 is observed since these conditions serve to keep cytochrome C553 completely oxidized.

Correlation of P435 and cytochrome kinetics

From the spectra of Fig. 2 one notices that an isosbestic point for both cytochrome C553 and C555 oxidation lies around 435 nm (see also ref. 18) and that an

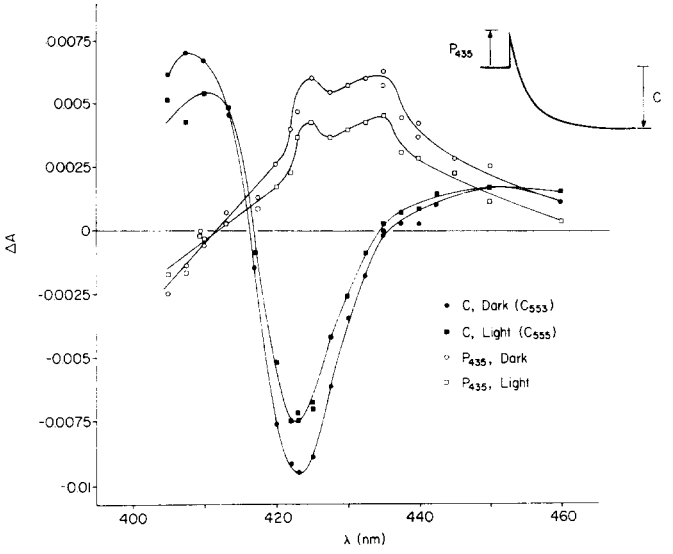

Fig. 2. Laser-induced difference spectra of P435, cytochrome C553 and cytochrome C555* reactions. Two distinct phases (within 16 μsec after a flash) were observed under conditions in which the anaerobic cells were unilluminated (○, ●) and illuminated with steady background illumination (□, ■). The inset shows how the P435 (○, □) and the cytochrome (●, ■) components were separated. Light path, 1.6 mm; bandwidth, 4.5 nm; BChl concentration, 0.182 mM; background illumination, 0.38 nEinsteins·cm^{-2}·sec^{-1}.

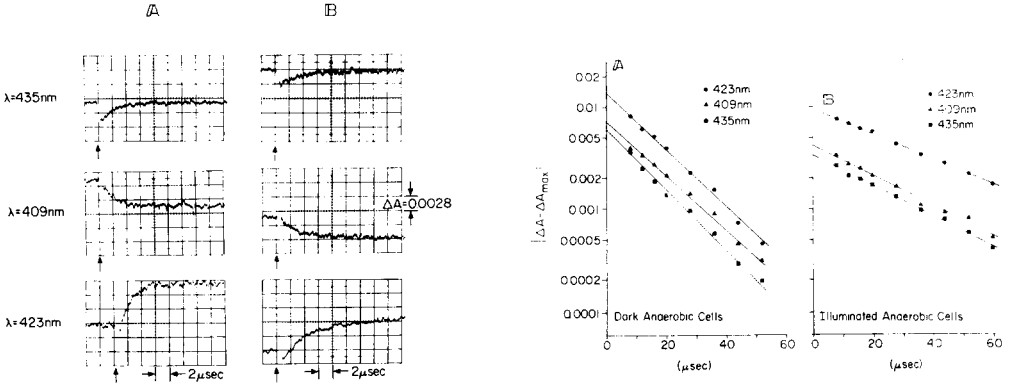

Fig. 3. Kinetic traces of the laser-induced absorbance changes of Fig. 2 at three wavelengths. Traces at 435 nm are pure P435 reactions, those at 409 nm are pure cytochrome reactions and those at 423 nm are a mixture of P435 and cytochrome reactions. In Column A the cells are unilluminated while in Column B they are illuminated (0.38 nEinsteins·cm^{-2}·sec^{-1}). Arrows indicate the point at which the laser was fired. An increase of absorbance is in the downward direction. Light path, 1.6 mm; BChl concentration, 0.182 mM.

Fig. 4. A. First-order plots of the kinetics shown in Fig. 3, Column A. B. Same as in A except that the data are from Fig. 3, Column B. Ordinates are the logarithm of the difference between the absorbance at a given time after laser flash and the absorbance eventually reached assymptotically. Abscissae are times after laser flash.

* The cytochrome C555 difference spectrum in ref. 18, Fig. 4 between 390 and 415 is incorrect. The absorbance changes in this spectral region should be multiplied by two.

isosbestic point for the P435 reaction is apparent at 410 nm (see also refs. 1 and 16). Kinetic studies at 435 nm thus afford the possibility of recording laser-induced P435 changes without cytochrome interference while those at 410 nm allow observation of the two cytochrome components without the spectral contribution of P435. Fig. 3 presents traces of laser-induced reactions at three wavelengths, 435, 409 and 423 nm, under conditions in which the cells are unilluminated (Column A) and illuminated (Column B). The kinetics at 435 nm show a rapid absorbance increase and a slow (on this time scale) absorbance decrease which recovers to the initial baseline. Traces at 409 nm show only a slow absorbance increase while those at 423 nm record a rapid absorbance increase and a slow absorbance decrease which goes substantially past the initial baseline. Thus, the first case (435 nm) shows pure P435 kinetics, the second (410 nm), pure cytochrome kinetics and the third (423 nm), a mixture of the two components.

Fig. 4 presents a plot of the logarithm of the difference between the asymptotically approached absorbance change and the absorbance change at the time of interest in Fig. 3 as a function of time after the laser was flashed. Anaerobic, unilluminated whole cells thus display in this sample cytochrome C553 oxidation half-times and P435 recovery half-times of 1.06 μsec*, while anaerobic, illuminated whole cells show cytochrome C555 oxidation half-times and P435 recovery half-times of 2.2 μsec*. Table I gives the exact figures for the individual wavelengths.

TABLE I

VALUES FOR THE HALF-TIMES OF THE TRACES IN Fig. 2

Conditions	*Wavelength (nm)*	*Half-time (μsec)*§
Anaerobic, unilluminated whole cells	435	1.0
Anaerobic, unilluminated whole cells	409	1.12
Anaerobic, unilluminated whole cells	423	1.08
Anaerobic, illuminated whole cells	435	2.05
Anaerobic, illuminated whole cells	409	2.05
Anaerobic, illuminated whole cells	423	2.3

§ The estimated error for each value is $\pm$ 0.10 μsec.

Correlation between rates of cytochrome oxidation and of P435 recovery in whole cells is thus very close for both sets of conditions. A similar correlation between cytochrome oxidation and $P883^+$ (reaction center) reduction has been observed by PARSON[14] and PARSON AND CASE[25] in *Chromatium* D chromatophores.

Correlation of the laser-induced saturation of P435 and cytochrome C553 oxidation

A comparison of absorbance changes of cytochrome C553 (410 nm) with those of P435 (435 nm) as a function of the incident laser intensity is seen in Fig. 5. The important point to note here is that both components saturate at approximately the same incident laser intensity as indicated by their respective saturation points (defined as the intersection of the initial slope at low laser intensity and the asymptote

* Average of the half-times for the three wavelengths.

at high laser intensity). A quantum efficiency of 1.06 was obtained for cytochrome C 553 oxidation using 48 % as the measured absorption[18] of the sample at 694 nm (the laser wavelength) and 42 $mM^{-1} \cdot cm^{-1}$ as the extinction coefficient change of C553 at 410 nm*.

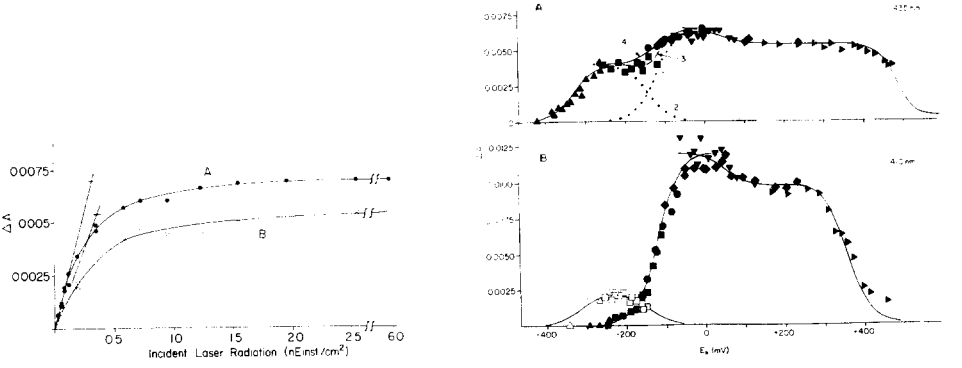

Fig. 5. A comparison of the saturation characteristics of cytochrome C553 at 410 nm (Curve A) and P435 at 435 nm (Curve B) in unilluminated anaerobic whole cells. The intersection of the initial slope with the saturation absorbance change of both curves occurs at the same incident laser intensity. Light path, 1.6 mm; BChl concentration 0.17 mM.

Fig. 6. A. Laser-induced absorbance changes as a function of oxidation–reduction potential at 435 nm for *Chromatium* D chromatophores suspended at 0.1 M Tris–HCl buffer (pH 7.4) under an atmosphere of argon gas. The absorbance changes were measured by subtracting the baseline from a fast recovering component extrapolated back to the point where the laser was fired. The solid lines drawn through the points represent $n = 1$ curves. The numbers 1–4 refer to the curves as explained in the text. B. Same as in A except that the monitoring wavelength was 410 nm. Solid symbols represent cytochrome oxidation as measured by subtracting the baseline from the change approx. 14–16 μsec after the flash (neither C553 nor C555 significantly recovers in this time range). Open symbols represent the 410 nm spectral component of the fast recovering low potential reaction measured by extrapolation back to the time of the laser flash as in A. The following mediator dyes were present in A and B: ▲, △, methyl viologen (50 μM), neutral red (50 μM) and benzyl viologen (50 μM); ■, □, sodium anthraquinone-2-sulfonate (50 μM), sodium anthraquinone-2,6-disulfonate (50 μM), and 2-hydroxy-1,4-naphthoquinone (50 μM); ●, ○, potassium indigodisulfonate (50 μM) and potassium indigotetrasulfonate (50 μM); ◆, diaminodurene (15 μM), potassium indigotrisulfonate (15 μM), methylene blue (15 μM), ferric chloride (300 μM)–EDTA (10 mM); ▼, duroquinone (33 μM), pyocyanine (10 μM), phenazine ethosulfate (40 μM), phenazine methosulfate (40 μM); ▶, potassium ferricyanide. Light path, 1 cm; BChl concentration, 38 μM; temperature, 24 ± 2°C; pH 7.4.

An oxidation–reduction potential titration of the rapid laser-induced changes at 435 and 410 nm

In order to observe the effects of externally imposed oxidation–reduction potentials on the P435 and cytochrome reactions in *Chromatium* D chromatophores, 435 nm (P435) and 410 nm (cytochrome) were chosen as monitoring wavelengths for reasons discussed earlier.

Fig. 6 is the result of such a titration at these two wavelengths. As was shown previously[1] the changes at 435 nm (Fig. 6A) are composed of two components: P435, active in the −135 to +490 mV range, and what we have tentatively termed P424 in the −320 to −135 mV range. P435 decreases slightly in amplitude between 0 and +100 mV, apparently corresponding to the switchover from cytochrome C553

* This $\Delta\varepsilon_{mM}$ was obtained from the average of data given in ref. 17, ref. 22 and Fig. 2 using 70 $mM^{-1} \cdot cm^{-1}$ as the extinction coefficient of C553 at 423 nm[18,26].

to cytochrome C555 which occurs over the same redox range[22,24,25]; the cytochromes are seen in Fig. 6B. (A similar phenomenon was noted for anaerobic whole cells in Fig. 2 where the P435 absorbance changes were slightly attenuated when background illumination was used.) The open symbols in Fig. 6B represent P424 as observed at 410 nm. (The difference spectrum of P424 in ref. 1 shows that 410 nm is not an isosbestic point as in the case for P435). It is separated from the cytochrome reaction kinetically.

Curves 1 to 4 represent an attempt to analyze the switchover between P435 and P424. Curve 1 assumes that P435 is attenuated on the low side with an E_m of -134 mV ($n = 1$) as is cytochrome C553. Curve 2 represents the possibility that P424 attenuates on the high potential side with an E_m ($n = 1$) of -145 mV as derivable from the open symbols in Fig. 6B (also Table III). Curve 3 is the sum of Curves 1 and 2. Curve 4 is a simple theoretical one-electron curve obtained using the experimental end point values at -220 and -20 mV. Here it is assumed that P424 attenuates with an E_m much higher than -145 mV. The data seem to correlate with Curve 3, indicating that P424 does in fact attenuate on the high side with an apparent E_m of around -145 mV, not significantly different from the potential at which P435 attenuates on the low side. More evidence indicating a switchover phenomenon will be presented in the section on work at 77°K.

Table II gives the half-times for the various chromatophore laser-induced components in Fig. 6 at specific redox potentials. The correlation between P435 recovery and cytochrome oxidation still holds during conditions under which either C553 (-79 mV) or C555 ($+177$ mV) are present.

TABLE II

HALF-TIMES FOR THE VARIOUS REACTIONS IN Fig. 6

Wavelength (nm)	*Description*	E_h *(mV)*	*Half-time (μsec)**
435 (P435)	Recovery	+189	2.1
410 (C555)	Oxidation	+177	2.0
435 (P435)	Rise	− 59	<0.05
435 (P435)	Recovery	− 81	0.9
410 (C553)	Oxidation	− 79	0.8
435 (P424)	Rise	−269	~0.05
435 (P424)	Recovery	−263	2.4

* The estimated error for each value is ± 5 % or 35 nsec, whichever is larger.

Table III reviews the apparent oxidation–reduction midpoint potentials of the various components in experiments such as that in Fig. 6.

The association of the low potential reaction with the P883/cytochrome complex

In order to further clarify the relationship between P435 and P424 experiments were performed with sodium dodecyl sulfate solubilized subchromatophore particles of *Chromatium* D (Fractions A and B) prepared according to the method of THORNBER[19]. Fig. 7A clearly shows that Fraction A poised at -257 mV displays a rapid absorbance increase and recovery after a laser flash. P424 is not, however, apparent in Fraction B (Fig. 7B) which THORNBER[19] found could reduce methyl viologen.

TABLE III

MIDPOINT POTENTIALS OF THE VARIOUS ELECTRON TRANSPORT COMPONENTS APPARENT IN Figs. 5 AND 6

Component	E_m (*Expt. 1*)*	E_m (*Expt. 2*)**	E_m (*Expt. 3*)§	*Average*	*Literature values for E_m's (pH 7.0 to 8.0)*
P435 (435 nm)	+490 mV	—	+483 mV	+486 mV	+489 mV (P883) (ref. 24); +470 mV (P435 and P600) (ref. 22)
Electron acceptor for P435 (435 nm)	−131 mV	—	−123 mV	−127 mV	−160 mV (P883) (ref. 27); −135 mV (P600) (ref. 22)
Cytochrome C555 (410 nm)	+340 mV	+362 mV	+357 mV	+353 mV	+330 mV (ref. 24)
Electron acceptor for P883 (410 nm-cytochrome C553)	−143 mV	−135 mV	−123 mV	−134 mV	−135 mV (ref. 24)
P424 (410 nm)-high potential side	—§§	—	—§§	−145 mV	—
P424 (435 nm)-low potential side	−312 mV	—	−323 mV	−318 mV	—

* ΔA measurements taken within 16 μsec after a laser flash.
** ΔA measurements taken approx. 1 msec after a laser flash.
§ These E_m's correspond to the data in Figs. 5 and 6.
§§ Single experiment values not obtained.

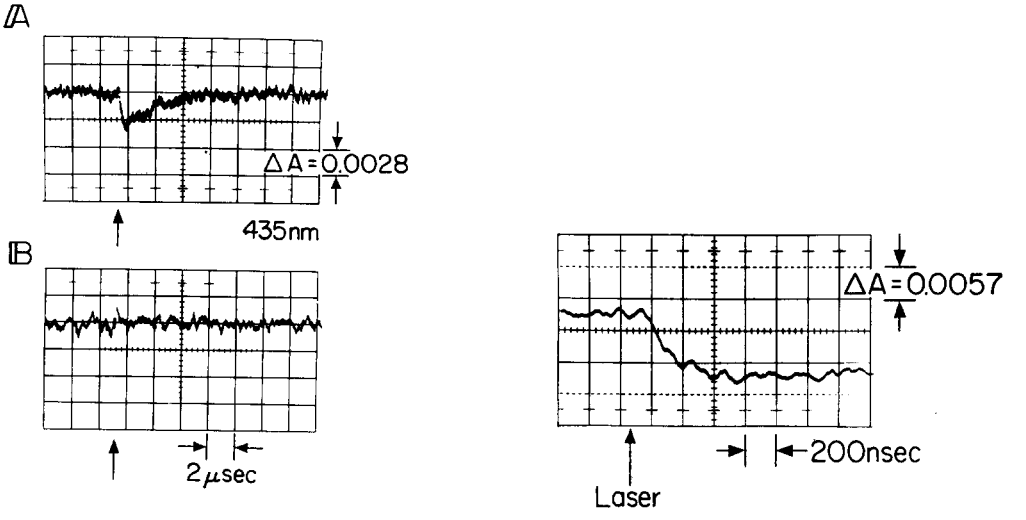

Fig. 7. A. Laser-induced absorbance changes in 435 nm in Fraction A sodium dodecyl sulfate solubilized sub-chromatophore particles of *Chromatium* D prepared by the method of THORNBER[19]. An increase in absorbance is in the downward direction. Light path, 1 cm; mediator dye benzyl viologen (15 μM); E_h −257 mV; BChl concentration 22 μM; temperature 24 ± 2°C; pH 7.4. B. Same as A except that the sample material was Fraction B. E_h −262 mV; BChl concentration 11 μM.

Fig. 8. Laser-induced rise kinetics of P424 ($t_{1/2}$ = 200 nsec) in chromatophores at 77°K. The arrow indicates the time at which the laser was fired. An increase in absorbance is in the downward direction. Light path, 1.0 mm, wavelength, 435 nm; bacteriochlorophyll concentration, 0.265 mM; E_h = −206 mV (see Fig. 9 for method); pH 7.4 before freezing.

Cytochrome oxidation and P435 reactions were observed at higher potentials in Fraction A but not in Fraction B. Fraction C was not tested because we were unable to isolate any of this fraction from cells grown under our conditions. Dr. J. P. Thornber (personal communication) has found that under some growing conditions *Chromatium* D cells do not contain any Fraction C.

Low potential laser-induced reactions at 77°K

As presented in Fig. 8 laser-induced P424 is still observable in chromatophores at 77°K. Its rise half-time of about 200 nsec is slightly longer than the 50 nsec half-time measured at room temperature. (The estimate of 75 nsec given in ref. 1 is the apparent rise time and should be corrected by taking the 50 nsec response time of the amplifier into account.) The recovery half-times at the two temperatures, however, are indistinguishable, being on the order of 2.2 to 2.4 μsec.

Low temperature oxidation–reduction potential titrations presented in Fig. 9 show that P424 (Curve A) has an apparent E_m (on the high potential side) of about —135 mV which is close to the apparent E_m of —145 mV observed at room temperature (Table III). These results were obtained using only the information from the first laser flash for a particular sample poised at the indicated potential, then quickly

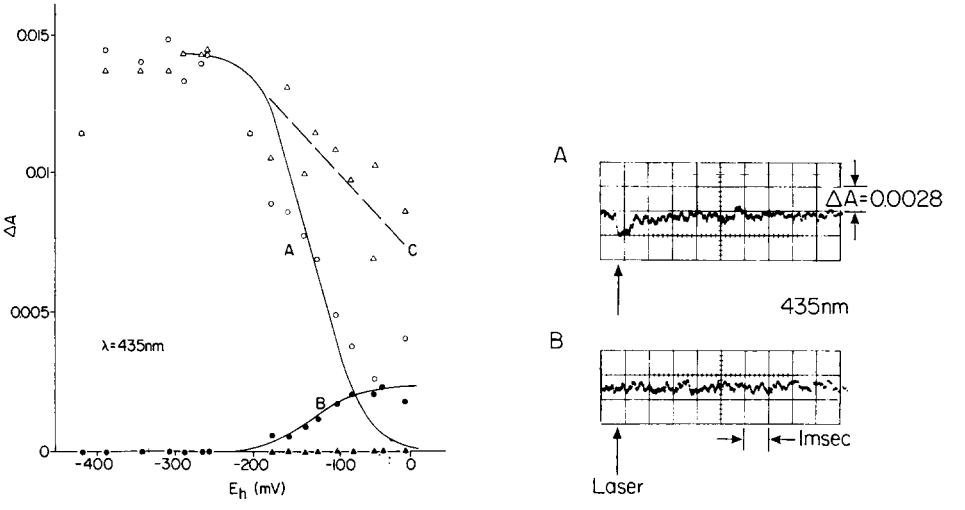

Fig. 9. An oxidation–reduction potential titration of the laser-induced absorbance changes in chromatophores at 435 nm. For each potential a sample was poised at room temperature and then rapidly frozen to 77°K in the dark. Curve A ($n = 1$) represents the attenuation of P424 (apparent E_m of —135 mV), and Curve B ($n = 1$) the appearance of P435 (E_m of —135 mV) as the potential is raised. These results were obtained with the first laser flash. With subsequent laser flashes P424 appeared at higher potentials (Curve C) while P435 (▲) was not apparent. The inactivation of P435 after the first flash is explained in the text. Mediator dyes: methyl viologen (50 μM), neutral red (50 μM), benzyl viologen (50 μM), sodium anthraquinone-2-sulfonate (50 μM), sodium anthraquinone-2,6-disulfonate (50 μM), potassium indigodisulfonate (50 μM) and potassium indigotetrasulfonate (50 μM). Light path, 1.0 mm; BChl concentration, 0.265 mM; pH 7.4 before freezing.

Fig. 10. The laser-induced oxidation and 2 msec reduction half-time of P435 in chromatophores at 77°K as a result of the first flash (A). A second flash (B) does not oxidize any more P435. Arrows indicate the time of laser flash, and an absorbance increase is in the downward direction. Light path, 1.0 mm; wavelength, 435 nm; BChl concentration, 0.265 mM; E_h = —38 mV; pH 7.4 before freezing.

frozen to 77°K in the dark. As the potential is raised P435 (Curve B) becomes apparent with the same —135 mV E_m (low potential side). Since P435 recovers with a 2-msec half-time (Fig. 10) it is easily separable from the low potential light reaction. Note that this shows still another correlation of the P435 recovery half-time with that of cytochrome C553 oxidation which is also 2 msec at 77°K[21].

Titration of the events caused by subsequent laser flashes at 77°K also presented in Fig. 9, show that P424 can be observed at higher potentials (Curve C) when P435 is inactivated. This inactivation is presumably due to the P435 acceptor's being permanently reduced by the first laser flash. The laser-induced difference spectra in Fig. 11 taken on frozen chromatophores at 77°K further illustrate this point. Curve A was obtained on a sample poised at —266 mV before freezing where no P435 would be observable. Curve B, however, was obtained from subsequent flashes on a frozen sample poised at —46 mV before freezing and shows the same characteristic single peak at around 432 nm (which is shifted about 8 nm to the longer wavelength compared with the room temperature single peak). The spectrum in Fig. 12 using anaerobic whole cells frozen to 77°K (subsequent laser flashes) also shows a single peak (at 427 nm). The recovery half-time of this component, however, was about 16 msec, or almost 4 orders of magnitude slower than P424 observed in chromatophores. Thus it can be stated that P424 is only observable when the primary electron acceptor for the P883/P435 system is initially reduced.

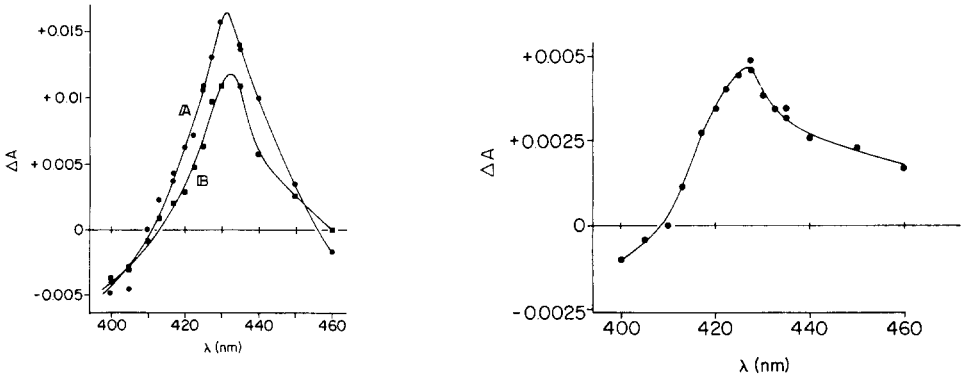

Fig. 11. Spectra of laser induced P424 in chromatophores at 77°K. The data in Curve A were taken at an E_h of —266 mV while that of Curve B were obtained (subsequent laser flashes) at an E_h of —46 mV. Light path, 1.0 mm; bandwidth of monochromator, 6.6 nm (A) and 4.5 nm (B); BChl concentration, 0.265 mM; pH 7.4 before freezing.

Fig. 12. Laser-induced spectrum (after the first flash) of anaerobic whole cells frozen to 77°K in the dark. Light path, 1.6 mm; bandwidth of monochromator, 7.7 nm; BChl concentration 0.125 mM.

Steady light-induced changes at low potential

Although we have observed small steady light-induced changes in chromatophores (room temperature) at around —250 mV in the 390 to 470 nm range[28], an absorbance peak at 452 nm and a trough at 402 nm indicated that these are due to reactions other than the P424. Calculations indicate, however, that since the recovery rate is so rapid, it would take a steady intensity of about $7 \cdot 10^5$ nEinsteins $\cdot cm^{-2} \cdot sec^{-1}$ to saturate P424 (assuming one P424 molecule per reaction center, the

concentration of which was 0.26 nmoles/cm^2 as obtained from Fig. 6). This required intensity is about 10^4 times that of our light source so that one would not expect to be able to observe P424.

This technique, however, might prove useful in observing P424 in steady illuminated cells at 77°K since the recovery time of this component is apparently much slower in cells than in chromatophores.

DISCUSSION

The identification of P435 as a spectral component of the same reaction center as P883

In whole cells and chromatophores of *Chromatium* D, several lines of evidence have been presented which identify P435 as another spectral component of the same reaction center as P883. These are the correlation of (1) the recovery or reduction kinetics of P435 with the oxidation kinetics of both cytochromes C553 and C555 (Figs. 3 and 4, Table II) as has been done with P883[25], (2) the reduction of P435 and the oxidation of cytochrome C553 at 77°K, (3) the laser intensity required for the saturation of P435 and cytochrome C553 oxidation (Fig. 5), (4) the midpoint potentials of P435 and P883 (Table III), and (5) the midpoint potentials of the primary electron acceptor for P435 and P883 (Table III). It is noteworthy to add that the kinetic correlation between P435 and cytochrome C553 is also observed in glutaraldehyde fixed chromatophores[29]. Finally, the chemically generated BChl$^+$-*minus*-BChl difference spectrum shows an absorbance increase in the 430 nm region for the isolated compound in organic solvents[30–32] and *in vivo* BChl[5].

It would appear that previous kinetic and saturation data to the contrary must have suffered from the admixture of other components into the steady states observed. This is quite likely since the results were obtained under conditions which allow the reaction center to turnover many times.

Evidence indicating one cytochrome-oxidizing reaction center

The kinetic correlation of the transients at 435 nm with the oxidation of both cytochromes identifies them as the oxidant for both. The similarity of the difference spectra of P435 when concomitantly either C555 or C553 is oxidized is evidence that under the different conditions P435 represents the same substance (BChl$^+$). We conclude that the same reaction center oxidizes both C555 and C553 in agreement with the recent suggestions of others[18,19,22,25,33]. An explanation of evidence to the contrary in *Chromatium* D has been put forward elsewhere[18,25]. While this was being written, a paper of SCHMIDT AND KAMEN[34] appeared showing that "P836", a photo-induced bleaching at 836 nm noted by THORNBER in his fraction B subchromatophore particles, has an E_m of +320 mV on the high side. They suggest that it may represent a separate reaction center able to reduce NAD$^+$. If so, it is not a reaction center that can oxidize either cytochrome C553 or C555, both of which are found in Fraction A, nor is it correlated with P424, discussed below, which is also found in Fraction A.

The nature of P424

The identification and function of P424 is more difficult to assay than that of P435. Its continued occurrence at 77°K, however, as shown in Fig. 8 confirms the supposition mentioned in the introduction that the assumed observation of P435

in the presence of sodium dithionite[14] was actually of P424. A summary of the facts concerning P424 follows. At 77°K in whole cells after C553 has been oxidized by earlier flashes or at room temperature in chromatophores poised at oxidation–reduction potentials of around —250 mV (for maximal effect), a flash of light produces a transient absorbance increase at wavelengths from 400 to 450 nm peaking at 424 nm (room temperature). The rise half-time is about 50 nsec at room temperature and 200 nsec at 77°K. It recovers in about 2.2 to 2.4 μsec at both temperatures in chromatophores and in about 16 msec at 77°K in whole cells. The phenomenon is observed only when the P883/P435 acceptor is reduced. One might consider this to be (a) a triplet state of BChl, (b) a photochemical product of BChl, (c) reduced BChl or (d) oxidized BChl. Cases (b), (c) and (d) may be further subdivided according to whether they are considered to be formed directly from the singlet state or from the triplet state of excited BChl. It could also be possible that some other molecule might be substituted for "BChl" in cases (a) to (d). We discuss these possibilities in the following points:

(1) The quantum efficiency of P435 as measured by cytochrome C553 oxidation approaches 1 (ref. 18). The maximal peak, saturation absorbance changes of P435 and P424 are quite similar (Figs. 2 and 3 in ref. 1), with the latter usually being a little larger in chromatophores at room temperature. If P424 is excited by the same light-gathering system as P435, then the available excitation should be the same for both and hence also the product of the extinction coefficient and the quantum efficiency. This means that the quantum efficiency of P424 cannot be much smaller than 1 without implying an inordinately large extinction coefficient.

The absorption of laser 694 nm light by the photosynthetic system is around 50 % under our experimental conditions. If excitation is by a pathway different from BChl, the absorption of the alternative light-gatherer could not be much less at that wavelength than the absorption of BChl without again implying an excessively large absorbance change for P424. This would be difficult with molecules present in much smaller quantities than BChl even if their absorbance were greater than BChl at 694 nm. The fact that P424 is associated with sub-chromatophore Fraction A as is P883/P435 and that its spectrum is quite similar to that of P435 lends weight to its probable identification with the reaction center. Accordingly, we feel that we are dealing with states of BChl, particularly those of the reaction center.

(2) A spectrum of the triplet state of BChl in pyridine is given by PEKKARINEN AND LINSCHITZ[35]. This spectrum shows an absorbance increase (compared with the spectrum of the normal state) in the same region as our observations. Details such as isosbestic points do not agree entirely but allowance must be made for the differences produced by the pyridine environment. This is not a strong argument, however, for case (a) because other states of BChl such as $BChl^+$ give similar difference spectra. If our phenomenon is a triplet state, then its rise time must be the lifetime of the singlet. A 50–200 nsec singlet lifetime with high efficiency of conversion to triplet postulated above presents problems since the mean fluorescence lifetime of BChl in solution is approx. 5 nsec while that in *Chromatium vinosum* cells is about 1 nsec[36]. Until more data are gathered concerning fluorescence and phosphorescence characteristics under the conditions of our observations, we tentatively rule out a long-lived singlet and, therefore, case (a). We cannot, however, rule out the possibility that a triplet state precedes the observed transient. In this case, the intersystem crossing

occurs presumably in a few nanoseconds and the rise time observed is the lifetime of the triplet state. The observed transient thus could either be (b), (c) or (d).

(3) If a case of photochemical reaction (beyond electron transfer) occurs, case (b), it must be one that can occur reversibly at 77°K in the frozen solid. Perhaps a simple bond-breaking without too much displacement of products is conceivable. However, one would wonder how the electronic excitation energy, which is probably spread out over the whole porphyrin ring, can come to concentrate itself on a particular bond. It is a more complicated hypothesis than the following, and we are not yet driven to it by failure of the others.

(4) KRASNOVSKY[32] presents a spectrum of BChl reduced with sodium sulfide. It shows a decrease in absorbance in the region 420 to 480 nm, compared to the normal spectrum. This would tend to rule out case (c), above since we observe an absorbance increase. However, one cannot be sure that KRASNOVSKY's isolated, reduced BChl species[32] is identical with the species that one would have in the chromatophore. The fact that P424 occurs at low potential and that it is not followed by cytochrome oxidation tempts one to continue to entertain case (c) as a strong possibility. One could also postulate that the reduced "acceptor" is now acting as a donor and that P424 is followed (at least in whole cells) by reduction of a low potential chemical such as NAD^+. GOVINDJEE AND SYBESMA[37] have evidence of direct NAD^+ reduction in older *R. rubrum* cells under conditions in which the infrared bacteriochlorophyll changes are quite different from what has so far been accepted as normal. Low temperature laser work also in *R. rubrum*[16] can be interpreted as indicating that a component similar to P424 is present in this organism.

There is also the question of what turns off the reaction at —320 mV. If this were the redox midpoint potential for the BChl/$BChl^-$ couple an answer is provided since below this potential the BChl would be chemically reduced before the flash.

(5) The best available *in vivo* $BChl^+$-*minus*-BChl difference spectrum in the 430 nm region is that of P435 itself (Fig. 2; ref. 1, Fig. 2). It is possible to entertain hypothesis (d) with the reasonable assumption that the differences shown by P424 could be produced by the proximity of the pre-reduced acceptor which has settled into some form of relationship with the BChl different from the case of P435 which is observed within a few microseconds after donating an electron to the acceptor. A major difficulty with this hypothesis is the fact that cytochrome oxidation is not observed to follow. It would be necessary to conclude that when the normal acceptor is pre-reduced the reaction center cytochrome relationship is modified so that cytochrome oxidation is then inhibited. If one entertains this hypothesis then the question of an alternative acceptor (which might also contribute spectral differences) arises. Since it must remain in an oxidized state down to —320 mV, there is the possibility that it could in turn reduce NAD^+.

Case (d) fails to suggest a donor for P424. It is possible that under our conditions the electron simply returns from the alternative acceptor so that the same electron can be re-used (NAD^+ reduction would not be coupled under our conditions). Under physiological conditions in the whole cell, electrons might reach P424 through the cytochrome C553 pathway (*via* a slow leak) everytime the normal acceptor is in the reduced state.

As indicated in Fig. 13, we continue to entertain cases (c) and (d) as alternate hypotheses involving BChl with smaller probability for (a) and (b). This discussion

points out the direction which further work should take in order to distinguish between the possibilities and clarify the role of the P424 in photosynthesis.

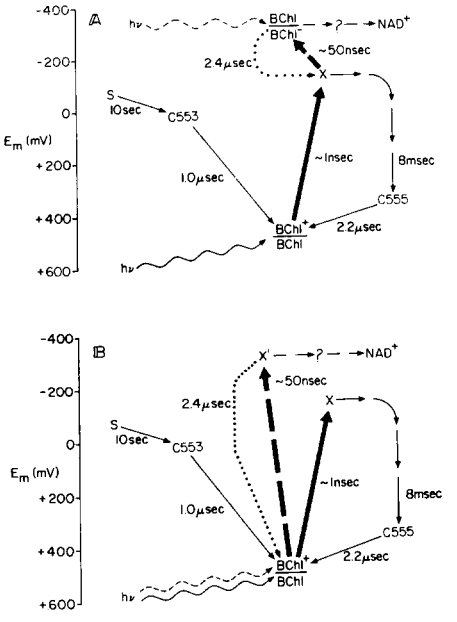

Fig. 13. Possible models incorporating P424 into the present electron transport scheme in *Chromatium* D. Thick arrows indicate light-reactions. Electron transport in the ordinary case with the reaction center operative as P883/P435 and cytochrome C553 or C555, depending on conditions, being oxidized is shown by ——→. The electron pathway for P424, operative when X is in the reduced form before a flash is indicated by – – –→. Case A suggests that P424 is a light-induced reduction of the reaction center BChl with X^- acting as a donor. Case B describes P424 as a light-induced oxidation of the reaction center BChl spectrally modified by the presence of X in its reduced form. X′ is the required alternate acceptor. In this case we would have to say that conditions have somehow inactivated cytochrome oxidation. Our data can not distinguish between these two models. We present here the recovery of P424 under our experimental conditions as a simple collapse of charge separation (· · ·→). Under physiological conditions we suppose that the electrons go to NAD^+.

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