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MEMBRANES OF *RHODOPSEUDOMONAS SPHAEROIDES*

VII. PHOTOCHEMICAL PROPERTIES OF A FRACTION ENRICHED IN NEWLY SYNTHESIZED BACTERIOCHLOROPHYLL *a*-PROTEIN COMPLEXES *

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Summary

Previous pulse-chase studies have shown that bacteriochlorophyll *a*-protein complexes destined eventually for the photosynthetic (chromatophore) membrane of *Rhodopseudomonas sphaeroides* appear first in a distinct pigmented fraction. This rapidly labeled material forms an upper band when extracts of phototrophically grown cells are subjected directly to rate-zone sedimentation. In the present investigation, flash-induced absorbance changes at 605 nm have demonstrated that the upper fraction is enriched two-fold in photochemical reaction center activity when compared to chromatophores; a similar enrichment in the reaction center-associated *B*-875 antenna bacteriochlorophyll complex was also observed. Although *b*- and *c*-type cytochromes were present in the upper pigmented band, no photoreduction of the *b*-type components could be demonstrated. The endogenous *c*-type cytochrome (E_m = +345 mV) was photooxidized slowly upon flash illumination. The extent of the reaction was increased markedly with excess exogenous ferrocytochrome *c* but only slightly in chromatophores. Only a small light-induced carotenoid band shift

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Abbreviations: *B*-875 and *B*-850, light-harvesting bacteriochlorophyll complexes absorbing maximally near 875 and 850 nm, respectively; *P*-605, reaction center absorption band which upon oxidation, exhibits a maximal absorbance decrease near 605 nm; P^+, the photooxidized form of *P*-605; *P*-870, reaction center absorption band with a maximum near 870 nm; Mops, 3-(*N*-morpholino)propanesulfonic acid; BChl, bacteriochlorophyll.

was observed. These results indicate that the rapidly labeled fraction contains photochemically competent reaction centers associated loosely with *c*-type and unconnected to *b*-type cytochrome. It is suggested that this fraction arises from new sites of cytoplasmic membrane invagination which fragment to form leaky vesicles upon cell disruption.

Introduction

The components involved in photosynthetic energy conversion in the facultatively photoheterotrophic bacterium *Rhodopseudomonas sphaeroides* are localized within an intracytoplasmic membrane system [1]. Upon cell disruption, this structure gives rise to closed membrane vesicles termed chromatophores, which exhibit an essentially uniform sidedness with respect to the asymmetric localization of photochemical reaction center, cytochrome, and ATPase components [2]; these are oriented in a manner opposite that in the intact cell [2]. The major integral proteins of the chromatophore membrane are specific to this structure [3—6] and are associated with the reaction center [7,8] and light-harvesting bacteriochlorophyll *a* complexes [5,9]. During growth at high aeration, the formation of bacteriochlorophyll and associated polypeptide components is severely repressed. Although much information is currently available on the energy transfer and primary photochemical capabilities of antenna and reaction center components [10—16], very little is known of the mechanisms by which the structural organization essential to their function becomes established within the chromatophore membrane.

Recently, a membrane fraction enriched in newly synthesized pigment-protein complexes has been isolated from *Rp. sphaeroides* [17]. This material formed an upper pigmented band which sedimented more slowly than chromatophores during rate-zone centrifugation. The upper pigmented fraction was thought to be derived from transient membrane invagination sites that are converted continuously into the intracytoplasmic photosynthetic membrane [17].

Fluorescence yield studies suggested that photosynthetic units which consist mainly of *B*-875-reaction center complexes exist at discrete sites in the newly synthesized membrane regions [18]. Although other energy transfer studies had demonstrated that photosynthetic units are interlinked in the mature intracytoplasmic membrane [10,19,20], such units are separate during initial stages of repigmentation of aerobically grown cells, and only interlink as additional light-harvesting bacteriochlorophyll complexes are added to the membrane [21]. The pulse-chase [17] and energy transfer [18] behavior of the upper pigmented fraction has suggested that such unique membrane zones with discrete developing photosynthetic units also exist in phototrophically growing cells. The photochemical properties of this fraction are described in the present communication. (A preliminary report of these studies has appeared: Hunter, C.N., Mallon, D.E., Niederman, R.A., Holmes, N.G. and Jones, O.T.G. (1979) Fed. Proc. 38, 356.)

Materials and Methods

Rp. sphaeroides NCIB 8253 was grown phototrophically in completely filled and sealed 10-l bottles on a defined medium [22] essentially as described previously [17]. Cells were harvested in late exponential phase and washed in 1.0 mM Tris buffer (pH 7.5). Crude extracts were prepared in a French press at 20 000 lb/inch^2 and centrifuged at 10 000 × g for 10 min. For preparation of the upper pigmented and chromatophore fractions, the supernatant fraction from this centrifugation was subjected directly to rate-zone sedimentation on sucrose density gradients as described previously [17]. Materials for these studies was taken directly from the respective bands and was not purified further.

Flash-induced spectral changes were measured on a rapid-response, dual-wavelength spectrophotometer [23]. Each of the photomultipliers was masked with a Corning blue glass filter (No. 9782). Signals were stored and averaged on an LSI-11 microprocessor. Photosynthetic reactions were activated with flashes from a xenon lamp of approximately 40 μs duration that were passed through a near-infrared filter (Kodak Wratten 88A). Near-infrared absorption spectra were obtained with a computer-linked spectrophotometer [24] in which the photomultiplier was protected by an orange filter. The approximations of Crounse et al. [25] were used to correct the absorbances at 850 and 875 nm. The computer-linked spectrophotometer was also employed for measurements of continuous illumination-induced changes in carotenoid absorption bands with the actinic light source and filter combination described previously [26]. A split-beam spectrophotometer [27] was used to obtain difference spectra at 77 K by procedures described previously [28]. Oxidation-reduction midpoint potentials were obtained by described equilibrium potentiometric titration procedures [29]. E_m values were assigned on the basis of computer analysis as described by Dutton and Jackson [30].

Bacteriochlorophyll was determined after acetone/methanol (7 : 2, v/v) extraction [22] using an extinction coefficient of 82 mg^{-1} · ml · cm^{-1} at 770 nm calculated from the molar extinction coefficient of Clayton [31]. Protein was determined by the method of Lowry et al. [32] with bovine serum albumin as a standard. Samples were maintained in 0.5 N NaOH for 60 min at 40°C prior to the color reaction [33].

Results

A generalized scheme for light-driven cyclic electron flow in chromatophores of *Rp. sphaeroides* is presented in Fig. 1. The reactions in the upper pigmented fraction for which evidence will be presented below are included within the solid lines. The energy transfer properties of the light-harvesting bacteriochlorophyll complexes (Reaction 1) have been described already [18]. Near-infrared absorption spectra indicate that the ratio of absorbance at 875 relative to that at 850 nm in this fraction was twice that of chromatophores (Fig. 2). The elevated *B*-875 level suggested by this observation is characteristic of early stages of chromatophore membrane development [37]. Since in chromatophores, *B*-875 is present in an essentially fixed stoichiometry with *P*-870 [38], an enrichment in reaction center bacteriochlorophyll levels within the

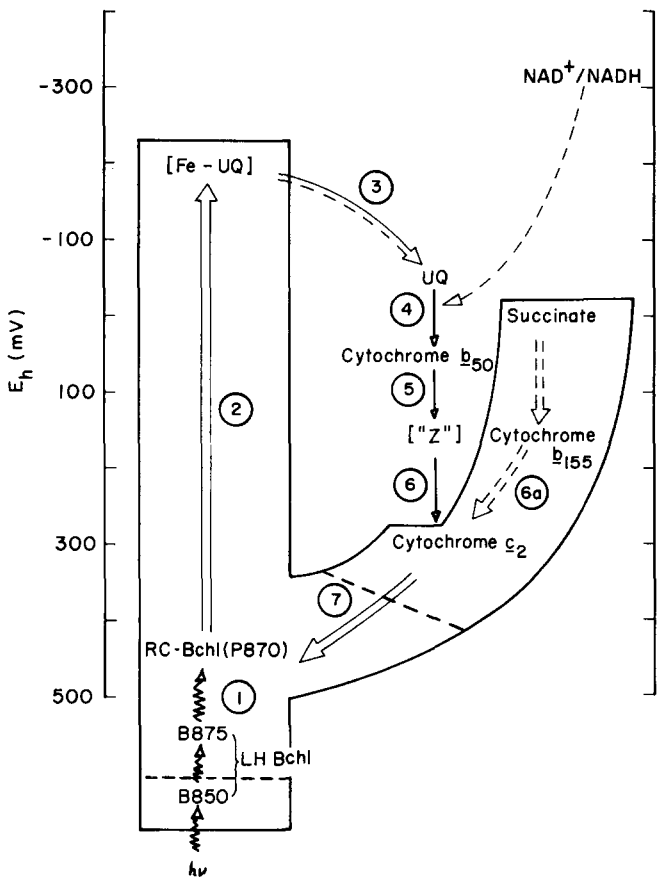

Fig. 1. Scheme for light-driven cyclic electron flow in *Rp. sphaeroides* chromatophores. It is based upon those of Jones [12] and Dutton and Prince [34]. The demarcation of various portions of the cycle denote activities detected in the upper pigmented fraction; these are included within the continuous solid lines. The double arrows indicate reactions where firm evidence has been obtained (heavy arrow) or if the evidence is tentative (dashed arrow). The single arrows denote reactions that could not be demonstrated in this fraction. The dashed line bisecting the transfer of electrons between cytochrome c_2 and *P*-870 indicates that this reaction occurs on a slow time scale probably as a result of dissociation of the cytochrome from the appropriate binding sites on the reaction center; that bisecting the reaction between *B*-850 and *B*-875 indicates that energy transfer between these complexes is incomplete in the upper fraction [18]. 'Z' is a UQ pool that is believed to form part of the UQ-cytochrome b/c_2 oxidoreductase [35,36]. Bchl, bacteriochlorophyll *a*. Other details on the portions of the cycle present within the upper fraction are provided in the text.

upper pigmented fraction would also be expected. This is borne out in the photochemical results presented below.

Dithionite-reduced minus ferricyanide-oxidized difference spectra were obtained at 77 K to determine if other components of cyclic electron flow were present in the upper pigmented band. The α-bands of both *b*- and *c*-type cytochromes were observed in the isolated fraction (Fig. 3). These were reported previously in room temperature spectra of a relatively unpigmented cytoplasmic membrane fraction from phototrophically grown cells [6]. Such fragments of cytoplasmic membrane are also present in the isolated upper pig-

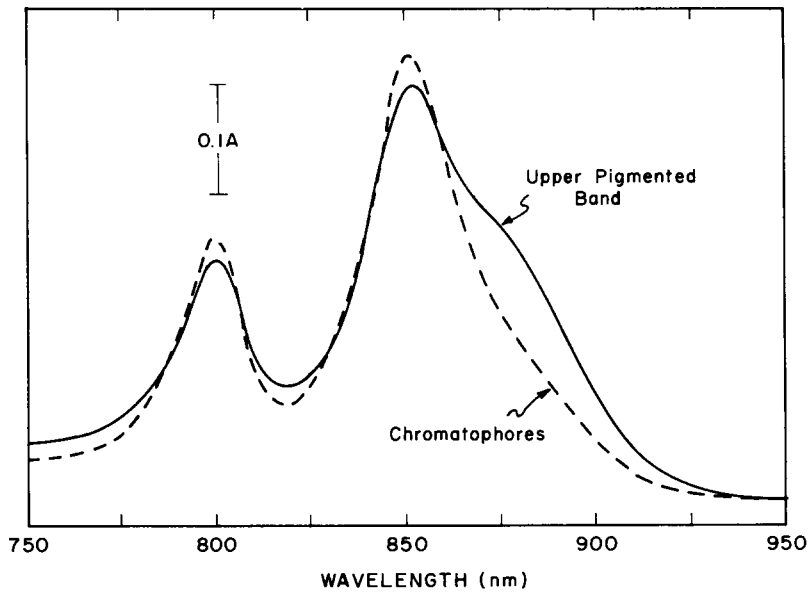

Fig. 2. Near-infrared absorption spectra of chromatophore and upper pigmented fractions obtained on a computer-linked spectrophotometer. Each sample contained 3.0 μg of bacteriochlorophyll/ml in 20 mM Mops buffer (pH 7.0) with 0.1 M KCl.

mented fraction [17]. The α-band maximum at 549 nm with a shoulder at 547 nm is characteristic of cytochrome c_2 [28]. The absorption maximum near 557 nm observed in the upper pigmented fraction indicates the presence of the α-band of *b*-type cytochromes of higher oxidation-reduction midpoint potentials, whereas the shoulder at 560 nm is consistent with the presence of *b*-type cytochrome with lower E_m values [28]. The presence of multiple *b*-type cytochrome components was also suggested by results of dark potentiometric titration of the upper pigmented fraction at 560—540 nm. Tentative E_m values near +230, +65, and —60 mV, respectively, were assigned to the three components detected. Values of +155, +50, and —90 mV were reported for the three *b*-type cytochromes observed in chromatophores of *Rp. sphaeroides* carotenoidless mutant R-26 [30]; in membranes of an aerobically grown mutant, five components with E_m values of +390, +255, +160, +40, and —90 mV were described [28].

As an initial approach to the assessment of the photochemical capabilities of the upper pigmented fraction, the spectrum of absorbance changes induced by single-turnover flashes was examined in the region of 540—620 nm with 540 nm as the reference wavelength (Fig. 4). After a single saturating xenon flash, the maximal absorbance decrease near 605 nm suggested that a photochemically active reaction center was present within both the upper pigmented and chromatophore fractions. The absorbance decrease maxima near 550 nm indicated significant photooxidation of a *c*-type cytochrome also within both fractions (Fig. 1, Reaction 7). The molar ratios of the single-flash oxidizable *c*-type cytochrome/*P*-605 for the upper pigmented and chromatophore fractions were 0.78 and 0.97, respectively. The *P*-605 absorbance changes have also

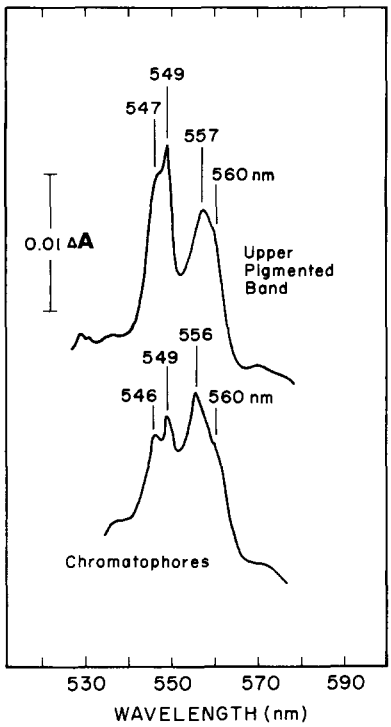

Fig. 3. Dithionite-reduced minus ferricyanide-oxidized difference spectra of upper pigmented and chromatophore fractions recorded at 77 K. The upper pigmented and chromatophore preparations contained 15 and 62 μg of bacteriochlorphyll/ml, respectively, in 20 mM Tris/0.2 M sucrose buffer (pH 7.0). Spectra were obtained by procedures described previously [28].

permitted a calculation of the relative size of the photosynthetic units (total bacteriochlorophyll:reaction centers) which in chromatophores was about twice that in the upper fraction. This two-fold enrichment in reaction center bacteriochlorophyll in the latter fraction is consistent with the elevated *B*-875 level observed in the near-infrared absorption spectra (Fig. 2).

In contrast to the flash-induced absorption changes near 550 and 605 nm in the upper pigmented band, an appreciable positive absorbance increase with a maximum near 560 nm could be demonstrated only in chromatophores (Fig. 4). This suggests that significant photoreduction of *b*-type cytochrome (Fig. 1, Reaction 4) occurred only in the latter fraction; a half-time of 11.8 ms was observed for this reaction, somewhat slower than that observed for flash-induced changes in *Rp. sphaeroides* Ga chromatophores [30]. The small change observed near 560 nm in the upper fraction can be ascribed to cytochrome c_2 photooxidation (Bowyer, J.R., personal communication). No photoreduction of *b*-type cytochrome in this fraction was observed with either flash or constant illumination under conditions in which the reaction was demonstrated in chromatophores. A slow, antimycin-sensitive photooxidation of *b*-type cytochrome in this fraction has been suggested on the basis of a preliminary examination of constant-illumination-induced absorption changes measured at

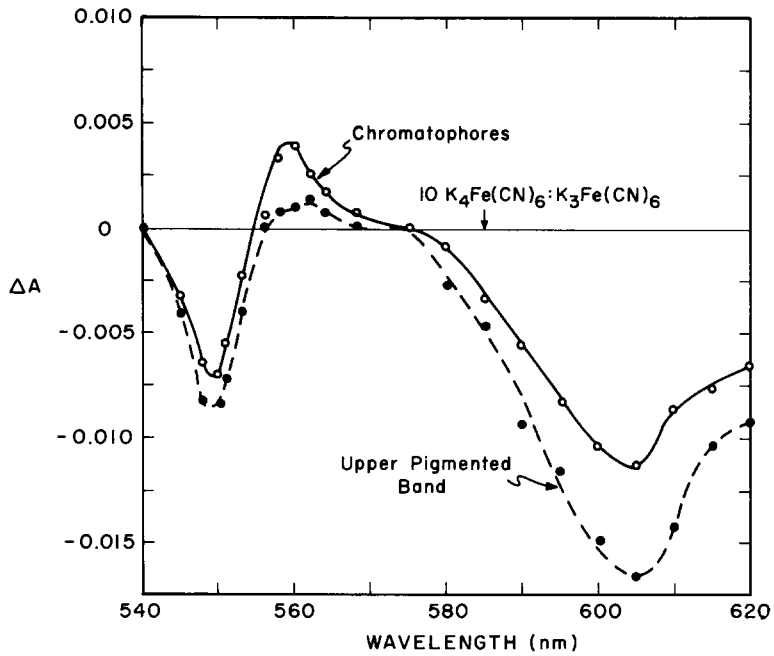

Fig. 4. Xenon flash-induced spectra of upper pigmented and chromatophore fractions measured on a rapid-response, dual-wavelength spectrophotometer. The instrument was similar to that described in Materials and Methods and is located in the laboratory of Dr. J.B. Jackson, University of Birmingham, U.K. The reference wavelength was 540 nm. Each point represents the average absorbance change for at least three measurements. The oxidation-reduction potential was established in 20 mM Mops/100 mM KCl (pH 7.0) at approximately +80 mV with 10 mM fumarate, 1 mM succinate in measurements between 540 and 580 nm; thereafter it was established at +380 mV with 5 mM ferrocyanide, 0.5 mM ferricyanide. Antimycin A (10 μg/ml) was included to minimize any cyclic electron flow between cytochromes b_{+50} and c_2 [30]. The bacteriochlorophyll concentrations for each sample were 30 μg/ml.

560—570 nm; this has been attributed tentatively to a linear substrate-linked pathway [29] (Fig. 1, Reaction 6a).

An examination of the kinetics of flash-induced changes measured from the difference in absorption at 605—540 nm is shown in Fig. 5. The bottom panel shows that the half-time for the observed re-reduction of *P*-605 in the upper pigmented fraction is 14 ms. This is in accord with the half-time of *c*-type cytochrome photooxidation (see below) which suggests that electron flow from the reduced *c*-type cytochrome to P^+ occurs within this fraction. Approximately 10% of the P^+ persists 200—400 ms after single (bottom panel) or multiple (middle panel) flash excitation indicating that this proportion of reaction centers is not re-reduced by the *c*-type cytochrome present (cf. Fig. 4 where absorbance difference spectral data indicate that as much as 20% of the reaction centers may not be connected to oxidizable *c*-type cytochrome in a different preparation of the upper pigmented fraction). The *P*-605 of chromatophores is photooxidized by a single flash of exciting light, but subsequent flashes produce further decreases in the absorption difference measured at 605—540 nm (Fig. 5, top panel). In contrast, the upper pigmented band achieves the maximum absorbance decrease at this wavelength pair following

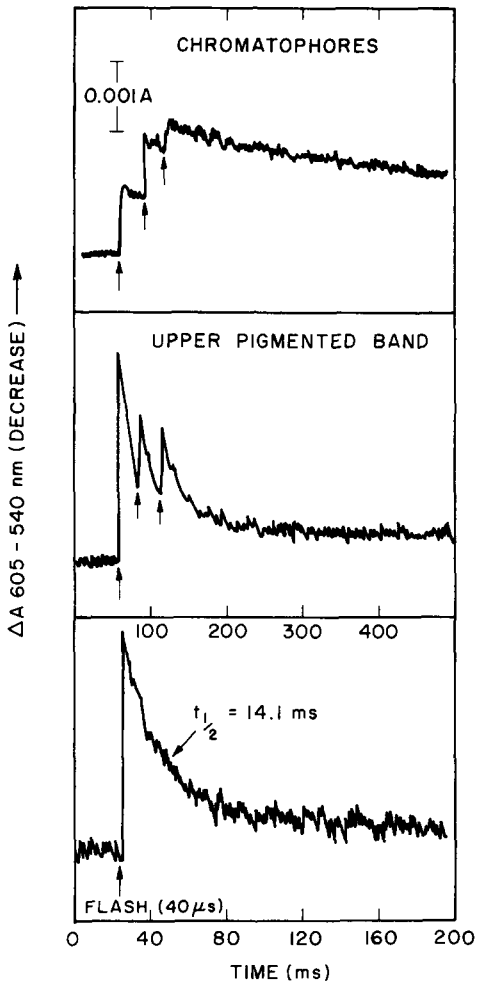

Fig. 5. Kinetics of flash-induced *P*-605 changes in the isolated fractions. Measurements were made on a fast-response, dual-wavelength spectrophotometer [23] at 605—540 nm. The oxidation-reduction potential was established at approximately +80 mV as described in Fig. 4. The bacteriochlorophyll concentrations for each sample were 17.3 μg/ml. The bottom panel shows the effect of a single flash on *P*-605 in the upper pigmented band. Only the slow phase of P^+ re-reduction [39] is shown in the trace with chromatophores (top panel); this phase was demonstrated more readily in constant-illumination-induced absorption changes.

the first flash. The second and third flashes result in a decreased photooxidation which is most easily interpreted as a difference in the size of the electron-accepting 'pools' in the two preparations. Preliminary extraction data suggests that the UQ/bacteriochlorophyll ratios are similar in the upper pigmented and chromatophore fractions. This provides support for the presence of a secondary acceptor pool in the upper pigmented band but further studies will be necessary to establish this possibility. It is also possible that the putative primary and secondary UQ acceptors might be coupled poorly to some of the reaction centers. Furthermore, the incomplete photooxidation of the reaction centers may also reflect back reactions that re-reduce *P*-605 in this fraction.

A kinetic analysis of the flash-induced absorbance decrease measured at 551—540 nm in the presence of antimycin A revealed a monophasic reaction with a half-time of 13.2 ms in the upper pigmented fraction. This was about 200-fold slower than the initial phase of the reaction in chromatophores ($t_{1/2}$ = 0.061 ms first phase, 0.49 ms second phase) which suggests that in the upper pigmented band, the *c*-type cytochrome is casually associated with reaction centers. Further evidence that the *c*-type cytochrome within this fraction is dissociated from reaction centers is provided from an equilibrium potentiometric titration measured at 551—540 nm (Fig. 6); only a single component accepting one electron with an E_m value of +345 mV was observed. This is consistent with results reported for cytochrome c_2 in solution [39,40]. In the chromatophores of strain Ga where cytochrome c_2 appears to be associated more closely with reaction centers, the E_m value is shifted partially to +295 mV [39].

The possibility that the reaction center polypeptides within the upper pigmented fraction existed in an orientated form within closed chromatophore-like vesicles was examined by multiple flash-induced absorbance changes measured at 551—540 nm, following the addition of a 20-fold excess of equine ferrocytochrome *c* (Fig. 7). If the reaction centers in the upper pigmented band have the same asymmetric orientation as in isolated chromatophore membranes, it would be expected that their site of interaction with *c*-type cytochrome would be localized on the inner surface of vesicle membranes (peri-

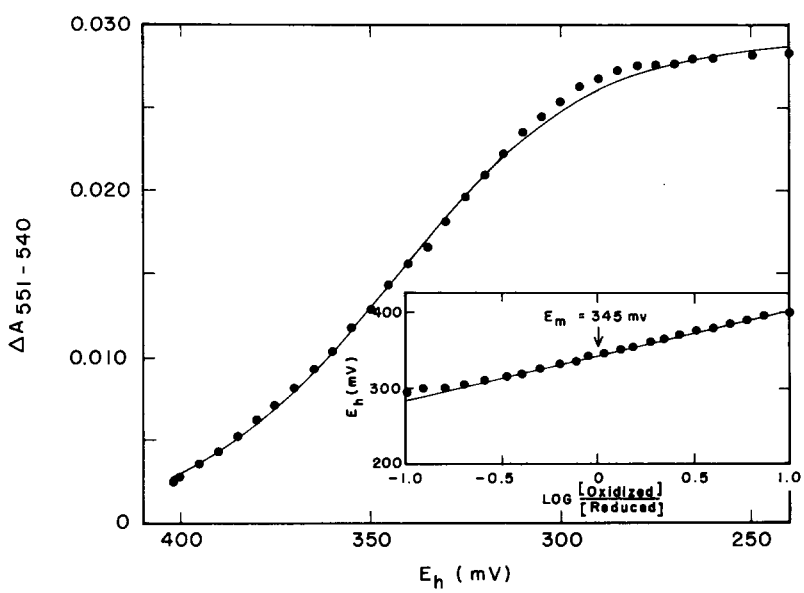

Fig. 6. Dark potentiometric titration of upper pigmented fraction at 551—540 nm. The titration was conducted in 20 mM Mops/100 mM KCl buffer (pH 7.0) by procedures described previously [29]. The bacteriochlorophyll concentration was 32 μM and 50 μM diaminodurol was used as a redox mediator. The observed points are plotted about a theoretical single electron equivalent (n = 1) curve generated by a computer program [30]. In the inset, the curve has been fully resolved into a single n = 1 component; the computer was unable to generate a two component fit with the available data. A second itration with the same preparation gave a single E_m value of +360 mV.

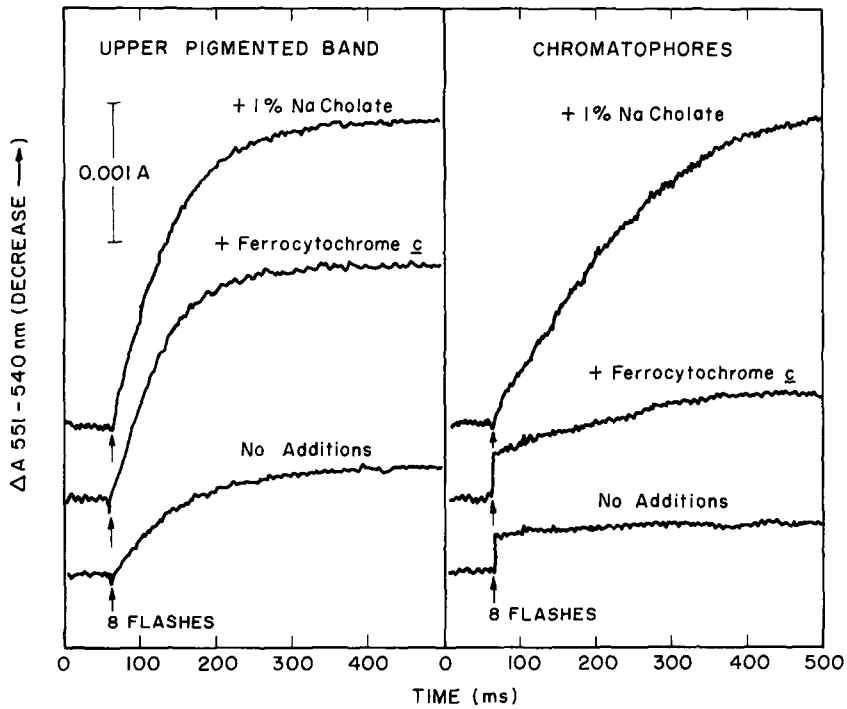

Fig. 7. Kinetics of multiple flash-induced *c*-type cytochrome changes. Ascorbate-reduced equine cytochrome *c* (50 μM) was present in the upper traces in 20-fold molar excess over the amount of *P*-605 present. Excess ascorbate was removed from the ferrocytochrome preparation by gel filtration. The ambient oxidation-reduction potential was established as described in Fig. 5. Sodium cholate (1%) was added after the change with the exogenous ferrocytochrome had been recorded.

plasmic aspect) unavailable for reaction with added ferrocytochrome *c* [2]. In the absence of the added cytochrome, photooxidation approached a maximum rapidly in chromatophores (right panel). With the added ferrocytochrome *c*, only a relatively small increase was observed which indicates that most of the sites for photooxidation of *c*-type cytochrome on the reaction center are exposed internally within intact chromatophore vesicles. In contrast, the marked increase in photooxidation upon exogenous ferrocytochrome addition in the upper pigmented band indicated that appropriate sites are exposed to the added cytochrome (left panel); the addition of sodium cholate to disrupt any barrier to exogenously added macromolecules produced little increase in ferrocytochrome *c* photooxidation in this fraction but a large increase in chromatophores. Thus, the reaction centers in the upper pigmented band may be oriented either in inside-out vesicles or at random in leaky vesicles.

An examination of the extent of the constant-illumination-induced carotenoid band shift strongly favors the latter possibility (Fig. 8). This parameter has been reported to be a sensitive indicator of chromatophore vesicle integrity [41]. Although the upper pigmented band contained significant quantities of carotenoids (the wt./wt. ratio of carotenoids/bacteriochlorophyll was 0.41 in this fraction vs. 0.34 in chromatophores), the extent of the shifts was markedly

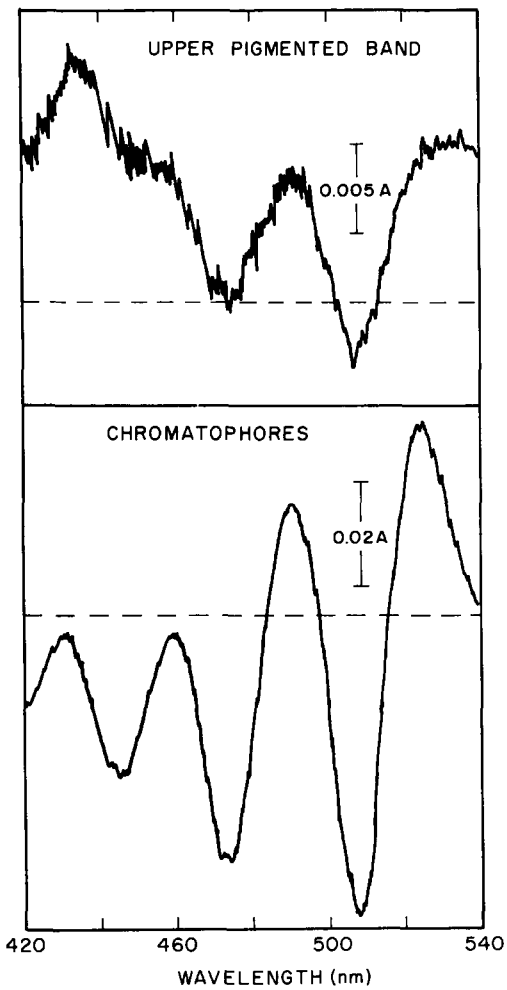

Fig. 8. Constant-illumination-induced carotenoid band shift in the isolated fractions. Recorded on a computer-linked spectrophotometer as described previously [26]. Cuvettes for the upper pigmented and chromatophore preparations contained 44 and 21 μg of bacteriochlorophyll/ml, respectively, in 50 mM Mops/100 mM KCl buffer (pH 7.0).

reduced in this fraction (upper panel) and the shift in the shortest wavelength band (maximum near 460 nm) is obscured by an apparent continuous-illumination-induced reaction center bleaching [14]. The relative band shift measured at 523—509 nm varied in different upper pigmented preparations, but a mean value of 7% that observed in chromatophores indicates that more than 90% of the reaction centers within this fraction do not share the properties of uniform transmembrane orientation manifested as a carotenoid shift in the chromatophore membrane. The variability in the extent of the band shift in several preparations of the upper pigmented band suggests that it reflects the upper limit of chromatophore contamination in the isolated material and, accordingly it has provided a sensitive assay for this purpose.

Discussion

The bacteriochlorophyll-associated proteins of the upper pigmented fraction whose photochemical properties are described here were labeled rapidly when phototrophically growing cell were pulsed with radioactive amino acids [17]. In contrast, radioactivity accumulated more slowly in chromatophores due to the larger pool of pigment-protein complexes present in the mature intracytoplasmic membrane. The apparent movement of radioactivity from the upper membrane fraction to chromatophores during a chase with unlabeled amino acids was thought to represent maturation of the former to intracytoplasmic membrane with the isolation characteristics of chromatophores. Thus it was suggested that the observed pulse-chase relation was a reflection of pool sizes and the mode of membrane growth and that the upper fraction was derived from transient sites of new cytoplasmic membrane invagination [17].

The photochemical activities demonstrated here for the upper pigmented band are summarized in Fig. 1. Energy transfer in the sequences *B*-850 → *B*-875 → *P*-870 has been demonstrated but it is incomplete between *B*-850 and *B*-875 [18]. Significant photochemical reaction center activity (Reaction 2) is demonstrated here in flash illumination studies of the *P*-605 component. The size of the photosynthetic unit in the isolated fraction was half that of chromatophores and this was shown to reflect a decreased level of *B*-850 relative to that of *B*-875 characteristic of early stages in the development of the chromatophore membrane [37]. The presence of primary and secondary acceptors (Reactions 2 and 3) remains to be established. The inability to demonstate photoreduction of a *b*-type cytochrome suggests that Reaction 4—6 are absent. In contrast, electron transfer from reduced *c*-type cytochrome to P^+ as depicted in Reaction 7 was demonstrated but on a rather slow time scale. Reaction 6a has been included in the scheme because of the slow *b*-type cytochrome photooxidation observed under continuous illumination. Overall, the present results taken together with those reported previously [17,18] suggest that structural organization essential to primary photochemistry and energy transfer is established in early stages of membrane development. This had been suggested in repigmentation studies under conditions of reduced oxygen tension [42,43]; the present study extends these observations to steady-state, phototrophically growing cells.

Thus far we do not have an adequate explanation for the incomplete cycle of electron flow or the lack of appropriate transmembrane orientation within the upper pigmented fraction. It is possible that the membrane invagination sites thought to give rise to this material have fragmented in a manner that produces small, leaky vesicles. Indeed, the pigmented material cosediments with small, relatively unpigmented membrane fragments [17]. Furthermore, the absence of a carotenoid band shift suggests a lack of vesicle integrity and the photooxidation of exogenously added ferrocytochrome *c* is consistent with a significant exposure of reaction center sites ordinarily unavailable in isolated chromatophore vesicle. A consequence of the lack of vesicle integrity may also be a dissociation of *c*-type cytochrome from the appropriate binding site on the reaction center; this is consistent with the slow rate of photooxidation of the endogenous *c*-type cytochrome observed here. An additional possibility is that

new pigment-protein complexes are added to invagination zones where the lipid/protein ratio has increased thereby providing sites for insertion of the new material into a membrane otherwise saturated with protein. This is supported by the enrichment in pulse-labeled polypeptides in the upper pigmented relative to the chromatophore fractions [17] and evidence that saturation of the membrane with protein by inhibition of phospholipid synthesis prevents further insertion of new polypeptides [44]. The recent demonstration of discontinuities in lipid [45] and protein insertion [46] into membrane of synchronously dividing cell populations is also in accord with this possibility. If such special zones of membrane growth do exist, spatial separation may not permit newly inserted reaction centers to merge completely with the cytochrome components already within the cytoplasmic membrane. Inadequacies in the secondary acceptor ubiquinone pool may also be related to the absence of *b*-type cytochrome photoreduction in the postulated membrane regions. Experiments to test these possibilities further are currently in progress.

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