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RESOLVED DIFFERENCE SPECTRA OF REDOX CENTERS INVOLVED IN PHOTOSYNTHETIC ELECTRON FLOW IN *RHODOPSEUDOMONAS CAPSULATA* AND *RHODOPSEUDOMONAS SPHAEROIDES*

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Summary

1. In *Rhodopseudomonas sphaeroides* the Q_x absorption band of the reaction center bacteriochlorophyll dimer which bleaches on photo-oxidation is both blue-shifted and has an increased extinction coefficient on solubilisation of the chromatophore membrane with lauryldimethylamine-*N*-oxide. These effects may be attributable in part to the particle flattening effect.

2. The difference spectrum of photo-oxidisable *c* type cytochrome in the chromatophore was found to have a slightly variable peak position in the α-band (λ_{max} at 551—551.25 nm); this position was always red-shifted in comparison to that of isolated cytochrome c_2 (λ_{max} at 549.5 ± 0.5 nm). The shift in wavelength maximum was not due to association with the reaction center protein. A possible heterogeneity in the *c*-type cytochromes of chromatophores is discussed.

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Abbreviations: Mops, 3-(*N*-morpholinopropanesulfonate; LDAO, lauryldimethylamine-*N*-oxide; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole; $(BChl)_2$, reaction center bacteriochlorophyll dimer; $(BChl)_2^+$, oxidised dimer; Q_I, primary acceptor ubiquinone; Q_{II}, secondary acceptor uniquinone; $\Delta\epsilon^{red\text{-}ox}$, extinction coefficient difference between reduced and oxidised forms; E_h, the oxidation-reduction potential referred to the standard hydrogen electrode; $E_{m(x)}$, the E_h at which a redox couple is half reduced at pH *x*.

Supplementary data to this article are deposited with, and can be obtained from, Elsevier/North-Holland Biomedical Press B.V., BBA Data Deposition, P.O. Box 1345, 1000 BH Amsterdam, The Netherlands. Reference should be made to No. BBA/DD/166/48009/635 (1981) 167—186. The supplementary information includes lists of normalizing factors for the cytochrome c_2 and reaction center changes in *Rps. sphaeroides*.

3. Flash-induced difference spectra attributed to cytochrome *b* were resolved at several different redox potentials and in the presence and absence of antimycin. Under most conditions, one major component, cytochrome b_{50} appeared to be involved. However, in some circumstances, reduction of a component with the spectral characteristics of cytochrome b_{-90} was observed.

4. Difference spectra attributed to $(BChl)_2$, Q_{II}^-, *c* type cytochrome and cytochrome b_{50} were resolved in the Soret region for *Rhodopseudomonas capsulata*.

5. A computer-linked kinetic spectrophotometer for obtaining automatically the difference spectra of components functioning in photosynthetic electron transfer chains is described. The system incorporates a novel method for automatically adjusting and holding the photomultiplier supply voltage.

Introduction

In chromatophores from the purple non-sulfur bacteria, light-induced absorbance changes measured at single or dual wavelengths have been attributed to cytochrome c_2 ($E_{m(7.0)}$ 295 mV in *Rhodopseudomonas sphaeroides* [1,2], $E_{m(7.0)}$ 345 mV in *Rhodopseudomonas capsulata* [3]), the photochemical reaction center bacteriochlorophyll dimer ($(BChl)_2$) ($E_{m(7.0)}$ 440 mV in *Rps. sphaeroides* [1] and *Rps. capsulata* [3]), cytochrome b_{50} ($E_{m(7.0)}$ 50 mV [1,3—5]), cytochrome b_{155} ($E_{m(7.0)}$ 155 mV [1,4]), and the secondary acceptor ubisemiquinone anion radical (Q_{II}^-) of the photochemical reaction center [6,7]. A third *b*-type cytochrome (b_{-90}, $E_{m(7.0)}$ — 90 mV) has been resolved by redox titration [1], but has not previously been reported to undergo redox changes following flash activation.

The wavelengths used in single and dual wavelength kinetic measurements are chosen so as to be able to examine the kinetics of individual components. It is important to determine the in situ difference spectra of the components involved in single and defined multiple turnovers of the electron transport system (as listed above), since, without such spectra, the choice of appropriate wavelengths for kinetic measurements is somewhat arbitrary. It is also interesting to compare these spectra with the reduced-minus-oxidised difference spectra of the isolated components, or to components resolved in situ by dark equilibrium redox titration.

In this paper we present resolved light-minus-dark difference spectra of components in the 400—620 nm wavelength region, which were obtained using flash excitation and a variety of manipulations (time resolution, redox potential resolution, and the use of specific inhibitors and mutants). We also describe an automatic procedure for obtaining and processing the kinetic traces from which the spectra are obtained.

Materials and Methods

Cells were cultured and chromatophores prepared as described in [7]. The bacterial strains used were *Rhodopseudomonas sphaeroides* Ga (a green strain), *Rps. sphaeroides* R26 (carotenoidless), *Rhodopseudomonas capsulata* BW604 (carotenoidless), *Rps. capsulata* Ala pho$^+$ (also carotenoidless), *Rps. capsulata* R126 (a green strain incapable of growing photosynthetically but which posses-

ses photoactive reaction centers), and *Rps. capsulata* MT113 (a green strain lacking cytochrome c_2 but which possesses active reaction centers). *Rps. capsulata* R126 and MT113 were grown in the dark under low oxygen tension (1%). Under these conditions they synthesized reaction centers and light harvesting complexes. Both strains were isolated, and grown for us, by Dr. Barry Marrs and co-workers. All the carotenoidless strains lacked the B-800—850 light-harvesting complex [8]. The *Rps. capsulata* cytochrome c_2 was kindly provided by Dr. R.C. Prince, and the *Rps. sphaeroides* cytochrome c_2 by Dr. R.E. Overfield. Isolated reaction centers were provided by Dr. M.W. Kendall-Tobias and Dr. R.E. Overfield. They were prepared as described by Overfield et al. [9]. The reaction center concentration in chromatophores was estimated by poising the sample at an $E_{h(7.0)}$ of $\leqslant$300 mV and exciting with a train of flashes (20 ms between flashes) in the presence of antimycin to inhibit cyclic electron flow, and valinomycin to inhibit build-up of a membrane potential and eliminate the associated responses of the light harvesting pigments [2,26]. The maximal steady excitation-induced absorption change was measured at 542 nm, and reaction center concentration calculated using an extinction coefficient at 542 nm of 10.8 $mM^{-1} \cdot cm^{-1}$ (from Dutton et al. [2], after correction for the different wavelength used, based on the spectrum in Fig. 1 (▲) of this paper).

The computerised single-beam scanning spectrophotometer used to obtain absorption spectra is described elsewhere [11]. The monochromator of this instrument and that of the single beam kinetic spectrophotometer were calibrated using the emission line at 546 nm of a mercury vapour lamp. Techniques used for redox poising have been described previously [7]. All experiments were done under anaerobic conditions unless otherwise stated. Valinomycin was normally added to the reaction medium to accelerate the decay of the light-induced membrane potential to eliminate absorbance changes of the light-harvesting pigments in response to membrane potential.

Computer-linked kinetic single beam spectrophotometer

In order to facilitate the acquisition of the spectra of flash-induced absorption changes with minimum effort, we have developed computer software and peripherals to make the process automatic and flexible.

Kinetic traces for the spectra were obtained using a single beam spectrophotometer described elsewhere [7,12]. Flash excitation was provided by a xenon flash lamp filtered by Wratten 88A glass giving a far-red flash of 25 μs duration at half maximal intensity. The photomultiplier was protected by a blue glass filter (Corning glass No. 9780 or No. 9782). Rapid digitisation of the photomultiplier linear amplifier output was performed by a Datalab DL905 Transient Recorder (Data Labs, Mitcham, Surrey, CR4 4HR). The spectrophotometer was interfaced to a Digital Equipment Corporation (DEC) PDP 11/34 via a LPS-11 Laboratory Peripheral System. Bulk data storage was provided by a RX 01 dual diskette unit.

The LPS-11 digital output was used, via a homemade multiplexer, to control the measuring wavelength, the photomultiplier voltage supply, the flash excitation regime and triggering of the transient recorder. The LPS-11 programmable relays were used to turn off the stirrer of the anaerobic redox cuvette before

measurements and to open a shutter which protected the sample from exposure to the measuring beam except during measurement.

A system was developed to adjust the photomultiplier voltage supply automatically and hold it at a fixed value during measurement. The photomultiplier voltage supply was a Kepco ABC 1500, programmed in voltage mode. The control voltage was provided by a 16-bit digital to analog converter. Just before a measurement (shutter open), the control voltage was adjusted by use of a 16-bit synchronous up/down counter, which clocked in a direction and at a rate determined by comparison of the photomultiplier output with a reference voltage. When the photomultiplier output voltage had stabilized within an appropriate error margin of the reference value, the control voltage (and hence the supply voltage) was held constant while the flash-excitation induced absorption change was measured under computer control. After amplification and backing off, an output signal was available at a sensitivity equivalent to 25 V for 100% T.

Under normal operation, at each wavelength, the required number of kinetic traces for average was accumulated in the computer (stored as 512 data values), and then written into a file on diskette. To check for changes in the sample during the experiment (normally taking no more than 1—2 h), a measurement at an appropriate reference wavelength was made several times at appropriate intervals.

Input to the controlling program included the list of wavelengths at which the light-induced changes were to be measured, the required number of traces to be averaged at each wavelength, and the time between each measurement. The controlling program was developed from an earlier version written by Dr. David Crowther.

When dark-adapted chromatophores were required, an anaerobic redox-poised reservoir of material connected to a flow cuvette was used. Material was pumped using a peristaltic pump under computer control, so that a fresh dark-adapted sample could be provided before each flash or flash train. It was possible to record spectra of the millisecond kinetics with 512 point resolution for the flash-induced change on each of up to 8 flashes from the dark-adapted state, with any length of time >50 ms between each flash.

Data processing software for time-resolved spectra

The data stored on diskette (a file of wavelengths and a file of kinetic traces) were processed into time-resolved spectra by a FORTRAN program calling several Assembly Language subroutines to operate the display equipment.

For each kinetic trace, any two out of the 512 data values could be selected, one subtracted from the other, and the result appropriately scaled and plotted against wavelength, either on a Tektronix 603 storage oscilloscope or on a Tektronix 4662 Interactive Digital Plotter. The program could also read off diskette a set of normalising factors relating the light-induced absorbance change at each wavelength to the value at an appropriate reference wavelength for a previously resolved component. Thus, for example, it was possible to measure the reaction center change at 542 nm or 605 nm without significant interference from other components and then to use the set of normalising factors previously obtained for the reaction center to subtract away from the kinetic trace at each

wavelength an appropriately normalised reaction center contribution. Up to two components could be subtracted automatically in this fashion. (Details deposited in BBA Data Bank.)

Results and Discussion

Difference spectra in the spectral region 530—620 nm

Reaction center. The spectrum of the reaction center change was obtained by single flash-excitation of chromatophores poised at a high redox potential (about 440 mV) so that cytochrome c_2, the only rapid electron donor to the photo-oxidised reaction center, was oxidised before excitation. Under these conditions, reaction center re-reduction was very slow, with a $t_{1/2}$ of several seconds, and no absorption changes due to cytochrome c_2 occurred on the time scale used. In the absence of antimycin, spectral changes due to the reduction of cytochrome *b* are negligible on the first flash from the dark-adapted state [7].

The solid triangles in Fig. 1 show the spectrum obtained for *Rps. sphaeroides* Ga chromatophores. The isosbestic point is at 587 nm, and the peak of the 600 nm band is at 603 nm. The ratio of the absorption change at 605 nm to that at 540 nm is −1.96. This may be compared with a value of −1.89 calculated from

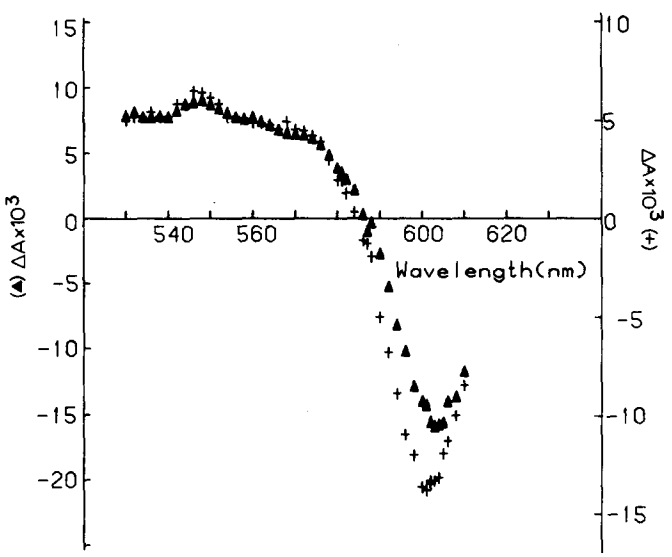

Fig. 1. ***Rps. sphaeroides*** **Ga reaction center difference spectra. ▲, In intact chromatophores. Chromatophores were suspended to about 1.54 μM reaction center in 50 mM Mops, 100 mM KCl, pH 7.0, containing 2 μM valinomycin and a total of 2 mM potassium ferrocyanide and ferricyanide. The E_h was 431 mV. Kinetic traces for the spectrum were not averaged. The time between each measurement was 60 s. The instrument response time was 5 ms. The points show the change 18 ms after a single flash. +, After addition of LDAO to 1% to chromatophores. Chromatophores were suspended in the solution described above. Conditions as above, except that the spectrum shows the change 20 ms after the second of two flashes (20 ms between flashes). The change was smaller due to reduced flash saturation, owing to dissociation of the reaction centers from the light harvesting antenna. Both spectra were normalised at 540 nm for display purposes.**

the extinction coefficients of Dutton et al. [2]. The crosses in Fig. 1 show the difference spectrum obtained after addition of the detergent lauryldimethylamine-*N*-oxide (LDAO) to chromatophores (to 1%). The two spectra have been normalised at 540 nm. Dutton et al. [2] showed that addition of 1% LDAO caused a 26% increase in $\Delta\epsilon^{red\text{-}ox}$ at 605 nm, and a 22% increase at 540 nm, so that the ratio of the change at 605 nm to 540 nm was changed to −1.95. Our own spectrum gives a value for $\Delta\epsilon^{red\text{-}ox}_{605}/\Delta\epsilon^{red\text{-}ox}_{540}$ of −2.3 in the presence of LDAO. Comparison of the extent of the steady absorbance change achieved on multiple flash excitation of chromatophores before and after addition of LDAO (and after appropriate re-adjustment of redox potential) indicated a 6% decrease when measuring at 540 nm, and a 16% increase when measuring at 602 nm. It seems that the decrease at 540 nm might reflect a slight loss of photochemical activity, so that the true increase of the extinction coefficient at 602 nm may be nearer 17%. The spectrum obtained in the presence of LDAO also differs from that in its absence in that both the isosbestic point and the peak of the bleached band have shifted to the blue, the former to 584.5 nm, and the latter to 601 nm. The spectrum obtained in the presence of LDAO was identical to that obtained using isolated *Rps. sphaeroides* R26 reaction centers in an aqueous solution containing 0.1% LDAO. This indicates that addition of LDAO to the chromatophores had effectively solubilised the reaction centers, and also that the specifically bound carotenoid molecule in the Ga reaction center had no effect on the absorption change in this spectral region (see also Ref. 16).

The simplest explanation for the change in the reaction center difference spectrum on disruption of the chromatophore membrane by LDAO is that, in the chromatophore, the absorption is affected by a particle flattening effect. Flattening results from the fact that the pigment molecules are not homogeneously distributed in solution, but are aggregated into chromatophores [13–15]. Such an effect was shown to account for the discrepancy between the difference spectrum of P-680 (the primary donor of Photosystem II) in situ and that of an isolated complex [13].

The main absorption band in this spectral region is the Q_x band of the light-harvesting bacteriochlorophyll, peaking at 590 nm. Since the differential flattening effect (ΔA solution/ΔA chromatophore) increases as the absolute absorption increases, a greater flattening effect would be expected on the blue side of the 600 nm reaction center band than around 540 nm. Addition of LDAO to 1% to chromatophores of *Rps. sphaeroides* Ga resulted in a small increase in absolute absorption at 590 nm, giving a flattening factor (Absorption of 'solution'/Absorption of chromatophore) of 1.1 at 590 nm. Fig. 2 shows the difference spectrum corrected according to the method of Pulles [13] and the equations of Duysens [14] and Amesz [15] using flattening factors of 1.1 (○) and 1.25 (+). The observed flattening factor (1.1) cannot account for the measured difference. A flattening factor of 1.25 gives a better fit to the spectrum of the solubilised reaction centers, causing a slight shift to the blue of the peak. It may be that the analysis used [14,15], which assumed that chromatophores (diameter about 60 nm) are homogeneous spherical pigment aggregates, is inappropriate, since the pigment is actually confined to the chromatophore membrane. The treatment neglected the effect of scattering,

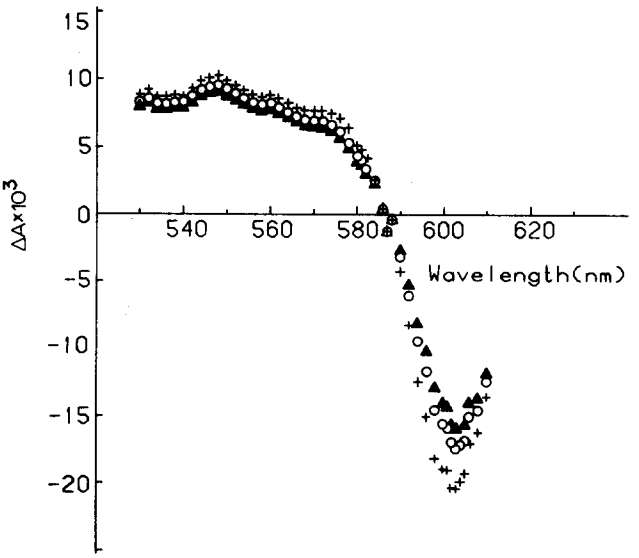

Fig. 2. Correction of the reaction center difference spectrum of *Rps. sphaeroides* Ga chromatophores for differential flattening factor. ▲, Uncorrected; ○, using absolute flattening factor at 590 nm of 1.1; +, using absolute flattening factor at 590 nm of 1.25.

but this is unlikely to be significant, owing to the small size of chromatophores. However, neither scattering nor particle flattening can cause a shift of isosbestic point, as the sign of the absorption change is not affected. It thus seems likely that other factors, in addition to flattening, must be invoked. One possibility is that removal of the reaction center protein from its membraneous environment differentially affects the absorption intensities of the two Q_x transitions of the exciton-coupled bacteriochlorophyll dimer (Ref. 17 and see also Refs. 18—21).

Comparison with in vitro spectra [22] indicates that the broad absorbance increase at wavelengths less than the isosbestic point is attributable to bacteriochlorophyll oxidation [22], but the hump at 545 nm is thought to be associated with bacteriopheophytin [23,24]. The 545 nm change may include a band shift in response to the charge on $(BChl)_2^+$, but Vermeglio and Clayton [25] recently showed that the bacteriopheophytin Q_x transition(s) undergo different spectral band shifts in response to the formation of the primary semiquinone anion (Q_I^-) and the secondary semiquinone anion (Q_{II}^-). We have yet to determine whether there are any flash-number-dependent effects on the reaction center difference spectrum in chromatophores, in this spectral region. Normalising factors calculated from spectra obtained as in Fig. 1 were used to correct spectra obtained under widely varying conditions of redox potential, flash number, etc.

The difference spectrum of the reaction center in *Rps. capsulata* BW604 chromatophores was similar to that of *Rps. sphaeroides*. The isosbestic point was at 587 nm, the peak position was at 605 nm, and the ratio of the change at 605 nm/540 nm was −1.82.

Cytochrome c_2. On flash excitation at redox potentials when cytochrome c_2 is reduced before the flash, it rapidly re-reduces the photo-oxidised reaction

center. The kinetics are biphasic with 40% of the change showing a $t_{1/2}$ of 3—5 μs, and 60% with a $t_{1/2}$ of 200—400 μs [2,9,26]. In the presence of the inhibitor 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT), the ferricytochrome c_2 is re-reduced very slowy [26—28]. UHDBT also appears to totally eliminate absorbance changes due to cytochrome *b* reduction [27], so that the principal changes are those due to cytochrome c_2 and the reaction center. The difference spectrum of cytochrome c_2 is then obtained by subtraction of the residual reaction center change at each wavelength.

Fig. 3 shows a spectrum (●) obtained in this way in *Rps. sphaeroides* Ga, using 603 nm (a wavelength at which there is negligible contribution from cytochrome c_2) as a reference wavelength for the reaction center change. It shows isosbestic points at 542.5 nm and 557.5 nm, with the peak in the bleached band at 551.0 nm. Superimposed is the oxidised-reduced difference spectrum (for reduction by ascorbate) of isolated *Rps. sphaeroides* R26 cytochrome c_2, at pH 7.0 in 20 mM NaCl. This spectrum is shifted 1.5 ± 0.5 nm to the blue of the light-minus-dark difference spectrum in situ. Cytochrome c_2 isolated from *Rps. capsulata* had an identical difference spectrum to that from *Rps. sphaeroides*. The difference spectrum of photo-oxidised cytochrome c_2 in chromatophores from *Rps. capsulata* was also red-shifted in comparison to the isolated cytochrome, with isosbestic points at 542 nm and 558 nm, and a peak at 551.25 nm.

The shift in the peak position of the α band maximum of cytochrome c_2 in the chromatophore in comparison to the isolated cytochrome cannot be due to

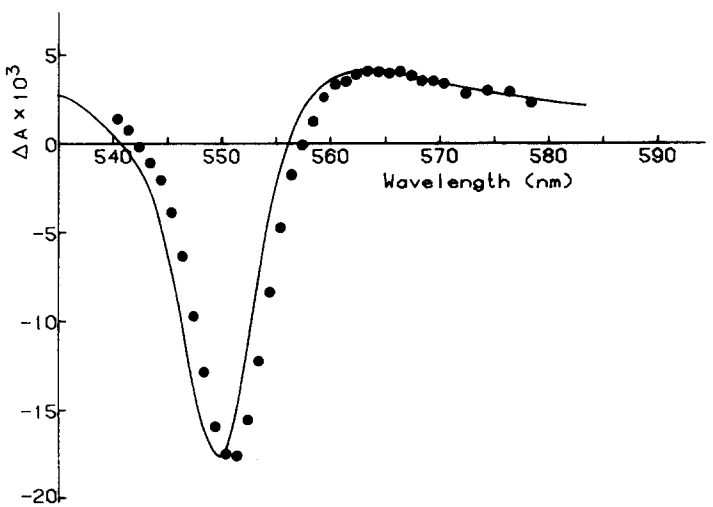

Fig. 3. Difference spectra of cytochrome c_2 in *Rps. sphaeroides*. ●, Light-minus-dark difference spectrum of cytochrome c_2 in *Rps. sphaeroides* Ga chromatophores. Chromatophores were suspended to 1.2 μM reaction center in 50 mM Mops, 100 mM KCl, pH 7.0, containing 10 μM each of 2,3,5,6-tetramethyl-*p*-phenylenediamine, 1,4-naphthoquinone and 2-hydroxy-1,4-naphthoquinone, and 1 μM valinomycin, 4 μM antimycin and 25 μM UHDBT. The redox potential was 180 ± 2 mV. Kinetic traces were not averaged, and the time between each measurement was 33 s. The spectrum shows the change 13 ms after a single xenon flash, after subtraction of the reaction center change using 603 nm as the reference wavelength. ———, Ascorbate-minus-oxidised difference spectrum of isolated *Rps. sphaeroides* R26 cytochrome c_2. The cytochrome c_2 was dissolved in 50 mM Mops, 20 mM NaCl, pH 7.0.

the flattening effect since the flattening factor is uniform over this spectral region. The shift does not appear to be specific to cyctochrome c_2 oxidised by the photochemical reaction center in the presence of UHDBT, since the chemical reduced-minus-oxidised (ferricyanide-ascorbate, or from redox titration) difference spectrum of the cytochrome c_2 in situ in the absence of UHDBT was also red shifted (up to 551.75 nm in *Rps. capsulata* Ala pho$^+$ and *Rps. sphaeroides* Ga).

The light-minus-dark difference spectrum of isolated cytochrome c_2 oxidised by purified detergent-solubilised reaction centers or reaction centers in liposomes (phosphatidylserine or equimolar phosphatidylserine and phosphatidylcholine) on flash excitation was the same as the chemical reduced-minus-oxidised spectrum of the pure cytochrome (Bowyer, J.R. and Overfield, R.E., unpublished data). This indicates that the spectral shift is not due to binding to the reaction center protein. There are several earlier reports in the literature which indicate a difference in redox properties of the major fraction of the cytochrome c_2 in *Rps. sphaeroides* Ga chromatophores in comparison to the isolated cytochrome. The isolated cytochrome c_2 has an $E_{m(7.0)}$ of 350 ± 15 mV and undergoes a fall in E_m at high pH [29,30]. In chromatophores, about 30% of the cytochrome c_2 has these properties [2], but the major component has an $E_{m(7.0)}$ of 295 mV with no fall in E_m at high pH [30,31]. Although it was suggested that the 295 mV component represents cytochrome c_2 bound to the reaction center [30], this seems unlikely since the $E_{m(7.0)}$ value for cytochrome c_2 is 295 mV in membranes lacking reaction centers owing to the growth conditions or mutation [32—34].

A link between the spectral shift and some membrane component other than the reaction center is also indicated by the fact that chromatophores prepared from spheroplasts showed an almost total loss of cytochrome c_2 oxidisable by flash excitation, but retained a significant though variable amount of *c* type cytochrome which could be chemically oxidised. The bound cytochrome *c* had a spectrum similar to that of cytochrome c_2 in chromatophores. However, it is clear that it must be bound to a site other than the reaction center. One possibility we are investigating is that the normal complement of cytochrome c_2 in cells and chromatophores consists of two populations. One of these, with $E_{m(7.0)}$ 350 ± 15 mV and λ_{max} 550 ± 0.5 nm may be associated with the reaction center, with a weak binding constant, and would be readily washed off on preparation of spheroplasts. The other, with $E_{m(7.0)}$ 295 ± 10 mV and λ_{max} 552 ± 0.5 nm would be more tightly bound to the reduction site (probably the Rieske-type FeS center [28]), and would be available for oxidation only by reaction with loosely bound cytochrome c_2. This situation would be analogous to the presence of bound and free cytochromes in mitochondria and some algae, and to the presence of bound cytochrome *f* and free plastocyanin in chloroplasts [35,36]. During the reviewing of this paper, it came to our attention that Wood [37] had shown that the bound cytochrome *c* species with $E_{m(7.0)}$ 295 mV, λ_{max} 552 nm was a different protein from cytochrome c_2.

An inhomogeneity in cytochrome *c* content might account for the strongly biphasic oxidation kinetics observed in chromatophores, the fast phase being attributed to c_2 bound to the reaction center, although similar biphasic kinetics are seen in the reaction between isolated cytochrome c_2 and detergent-solu-

bilized reaction centers [9]. Preliminary measurements of the difference spectra of the cytochrome *c* oxidised in the fast (5—10 μs) and slow (100—300 μs) phases support this conclusion (Crofts, A.R., unpublished data). In the work presented in this paper, the two components were not resolved spectrally in the chromatophore, and 'cytochrome c_2' refers to one or both of the two cytochromes.

Cytochrome b. Some controversy exists as to the mechanistic involvement of cytochrome *b* in the photosynthetic electron transport system [38—40]. There appear to be up to three different cytochrome *b* species present [1,4] which may be differently involved at different redox potentials or inhibitor regimes and which appear to have different spectra, and spectral responses to antimycin (Ref. 40 and Meinhardt, S.W., and Crofts, A.R., unpublished data). Two approaches were used to resolve the cytochrome *b* species involved in light-activated electron transfer under conditions optimised so as to eliminate other absorbance changes. At high redox potential, in the presence of antimycin, multiple turnovers of the photochemical reaction center result in cytochrome *b* reduction without concomitant redox changes due to cytochrome c_2, which remains oxidised on the time scale of the measurements. Fig. 4 shows a spectrum corrected for the reaction center change as determined above, of the change induced by a flash, given 10 s after the first, the first being given after a 2-min dark period. Chromatophores were from *Rps. capsulata* Ala pho$^+$. (Very little cytochrome *b* reduction occurred on the first flash due to the operation of a two electron gate in the reaction center acceptor complex [7].) The difference spectrum peaks at 560.5 nm, and has isosbestic points at approx. 552 nm and 569.5 nm. There is some variation in the isosbestic points which pre-

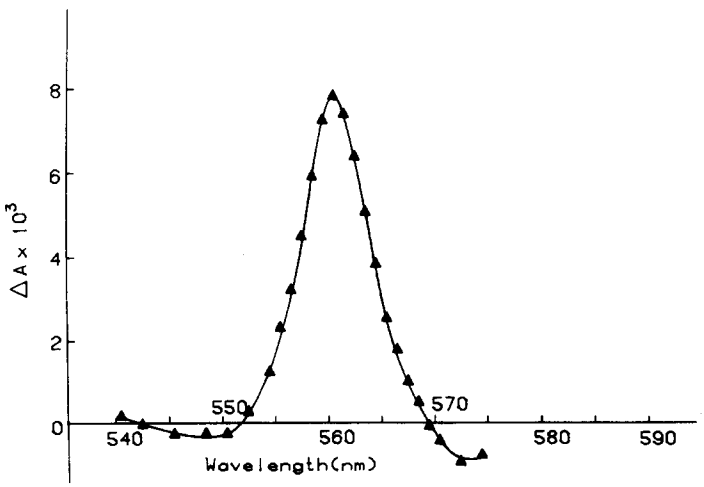

Fig. 4. Light-minus-dark difference spectrum of cytochrome *b* in *Rps. capsulata* Ala pho$^+$. Chromatophores were suspended to about 1.4 μM reaction center in 50 mM Mops, 100 mM KCl, pH 7.0, containing a total of 2.5 mM potassium ferrocyanide and ferricyanide, 2 μM valinomycin, and 2 μM antimycin. The redox potential was 400 mV. Kinetic traces were not averaged. The spectrum shows the change 100 ms after a xenon flash given 10 s after the first flash from the dark-adapted state, after correction for the reaction center contribution using 542 nm as the reference wavelength.

sumably reflects other small absorbance changes that have yet to be characterized.

The second approach was to utilize the observation that under certain conditions, following flash excitation, $(BChl)_2^+$ and cytochrome c_2 relax more rapidly to their dark redox states than cytochrome b. In the presence of antimycin, 3 μM *N*-methylphenazonium methosulfate and 3 μM *N*-ethylphenazonium ethosulfate, at an $E_{h(7.0)}$ of about 100 mV (cytochrome b_{50} ~13% reduced and cytochrome b_{155} ~90% reduced before excitation), cytochrome b is reduced following a single flash and becomes reoxidised with a $t_{1/2}$ of approx. 250 ms, whereas $(BChl)_2^+$ and ferricytochrome c_2 are re-reduced on a tens of ms time scale (somewhat variable from preparation to preparation). In an experiment using *Rps. sphaeroides* Ga chromatophores under these conditions, the spectrum of the absorption change after 43 ms was very similar to that shown in Fig. 4. After only 2 ms (Fig. 5, solid triangles), contributions from $(BChl)_2^+$ and cytochrome c_2 were still apparent. These are successively subtracted out in Fig. 5, open circles and solid circles, to leave a residual change fairly similar to that seen without correction after 43 ms. The correction for cytochrome c_2 can only be made after correction for the reaction center change, since the reference wavelength used for cytochrome c_2, 551 nm, normally includes a reaction center change.

In *Rps. sphaeroides* chromatophores, at lower redox potentials in the absence of antimycin, cytochrome b becomes oxidised on flash excitation, and remains oxidised on a seconds time scale. Ferricytochrome c_2 and $(BChl)_2^+$ are rapidly re-reduced. Fig. 6 shows a spectrum obtained in the absence of antimycin, at E_h ~ 50 mV (when cytochrome b_{50} is half reduced and cytochrome b_{155} is fully reduced before excitation). The band width is slightly greater than

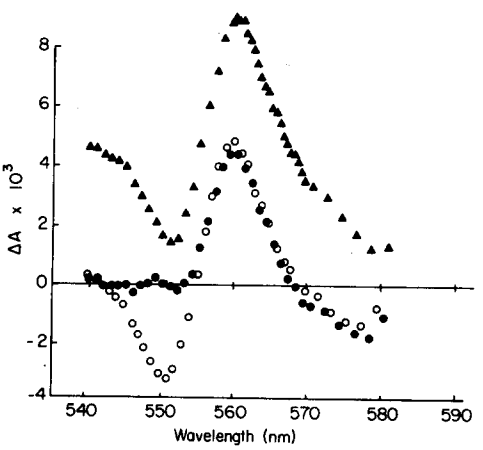

Fig. 5. Light-minus-dark difference spectra of *Rps. sphaeroides* Ga. Chromatophores were suspended to 0.94 μM reaction center in 50 mM Mops, 100 mM KCl, pH 7.0, containing 3 μM *N*-methylphenazonium methosulfate and *N*-ethylphenazonium ethosulfate, 10 μM each of 2,3,5,6-tetramethyl-*p*-phenylenediamine, 1,4-naphthoquinone, 1,2-naphthoquinone and 2-hydroxy-1,4-napthoquinone, and 1 μM nigericin, 2 μM valinomycin, and 2 μM antimycin. The redox potential was 100 mV. Kinetic traces were not averaged, and the instrument response time was 50 μs. ▲, The change 2 ms after the flash. ○, The change corrected for the reaction center change using 542 nm as the reference wavelength. ●, The change further corrected for the cytochrome c_2 change, using 551 nm as the reference wavelength.

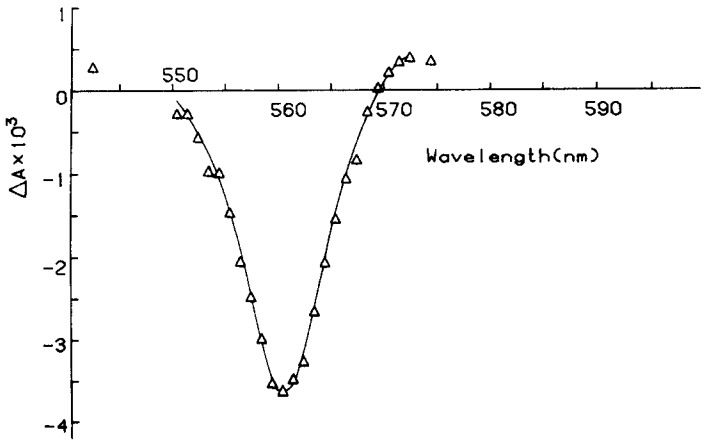

Fig. 6. Light-induced cytochrome *b* oxidation in *Rps. sphaeroides* Ga chromatophores. Chromatophores were suspended to 1.2 μM reaction center in 50 mM Mops, 100 mM KCl, pH 7.0, containing 10 μM each of 2,3,5,6-tetramethyl-*p*-phenylenediamine, 1,4-naphthoquinone, 1,2-naphthoquinone and 2-hydroxy-1,4-napthoquinone, and 3 μM *N*-methylphenazonium methosulfate and *N*-ethylphenazonium ethosulfate, 1 mM $MgCl_2$, 2 μM valinomycin, and 10 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. The redox potential was 50 mV. The kinetic traces were an average of 2, and the instrument response time was 5 ms. The spectrum shows the change 100 ms after a single xenon flash, corrected for reaction center changes (603 nm reference wavelength) but not cytochrome c_2 changes (which were very small).

that of cytochrome *b* reduced at higher E_h in the presence of antimycin, but the band maximum is in the same position. Redox titration of the extent of cytochrome *b* reduction on multiple flash excitation in the presence of antimycin indicates that cytochrome b_{50} is the main species reduced under these conditions [4,41]. Furthermore, the difference spectra of the cytochrome *b* photoreduced in the presence of antimycin resemble that of cytochrome b_{50} rather than cytochrome b_{155}, as resolved by equilibrium redox titration in the presence or absence of antimycin (Fig. 7 and Meinhardt, S.W., and Crofts, A.R., data unpublished). Van den Berg et al. [40] have suggested that antimycin causes a 1—2 nm red shift of the α band maximum of this particular population, a result we were not able to confirm. We will report in greater detail on the effects of antimycin elsewhere.

The spectra in Fig. 8A show the absorption changes induced by each of two flashes, 20 ms apart, in the presence of antimycin, after subtraction of the reaction center change. Fig. 8B shows the spectra after further correction for cytochrome c_2. The spectrum of the first flash change resembles that of cytochrome b_{50} although small contributions from other components are apparent as a broadening of the spectrum and a shoulder at 566 nm. The second flash change more closely resembles that of the low potential cytochrome b_{-90} (compare with Fig. 7). This low potential cytochrome therefore appears to be available as an electron acceptor on a millisecond time scale, but it remains to be established whether it is on a side path or on the pathway to cytochrome b_{50}. The relatively small absorption of this component at 560—540 nm or 560—570 nm would explain why it has not been detected in redox titrations of photoreducible cytochrome *b*.

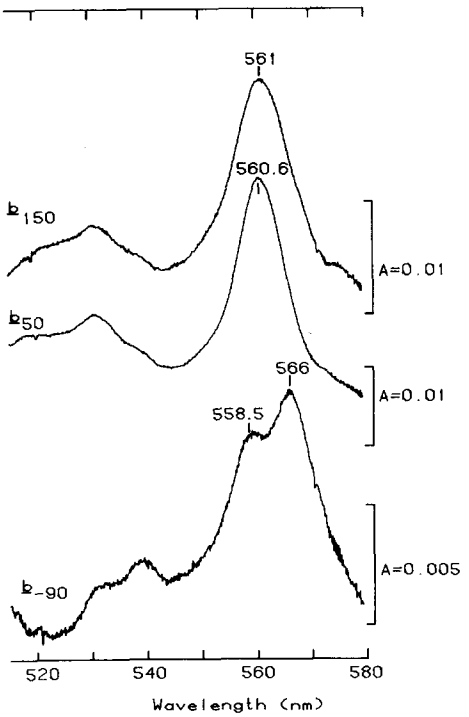

Fig. 7. Reduced-oxidised difference spectra of cytochrome *b* components in *Rps. sphaeroides* Ga chromatophores resolved by equilibrium redox titration. Chromatophores were suspended to 2—3 μM reaction center in 50 mM Mops, 100 mM KCl, pH 7.0, containing 20 μM each of *N*-methylphenazonium methosulfate, *N*-ethylphenazonium ethosulfate, pyocyanin, 1,4-naphthoquinone, 1,2-naphthoquinone, 2-hydroxy-1,4-naphthoquinone, 40 μM 2,3,5,6-tetramethyl-1,4-benzoquinone, 50 μM 2,3,5,6-tetramethyl-*p*-phenylenediamine, 2 μM valinomycin and 1 μM nigericin. The spectrum of cytochrome b_{155} ($E_{m(7.0)}$ 165 mV in this experiment) (top) was resolved by subtraction of the spectrum recorded at 200 mV from that at 110 mV; the spectrum of cytochrome b_{50} ($E_{m(7.0)}$ 50 mV) (middle) by subtraction of the spectrum recorded at 80 mV from that at 10 mV; and the spectrum of cytochrome b_{-90} ($E_{m(7.0)}$ −106 mV) (bottom) by subtraction of the spectrum at −50 mV from that at −110 mV. The spectra were normalised at λ_{max} 543 nm for display purposes.

Resolved spectra in the γ band region (420—480 nm)

Absorption changes in the γ band region are of interest because it is in this region that absorption changes due to formation of the anionic semiquinone of the primary and secondary acceptors of the reaction center are observed [6,7, 42]. Analysis is difficult, however, because the absorption change due to formation of the stable semiquinone is rather similar to that of $(BChl)_2^+$, and the extent to which electrostatic interactions between the charged species present affect the absorption spectra is not known.

$(BChl)_2$ and Q_{II} spectra. We have previously determined that at high redox potential (>390 mV) the difference spectrum obtained on the first flash following dark adaptation is different from that on the second [7]. This is partly due to different cytochrome *b* changes. We earlier attempted to time-resolve out the cytochrome *b* change, since cytochrome *b* reduction is relatively slow and also the extent of the absorbance increase due to cytochrome *b*

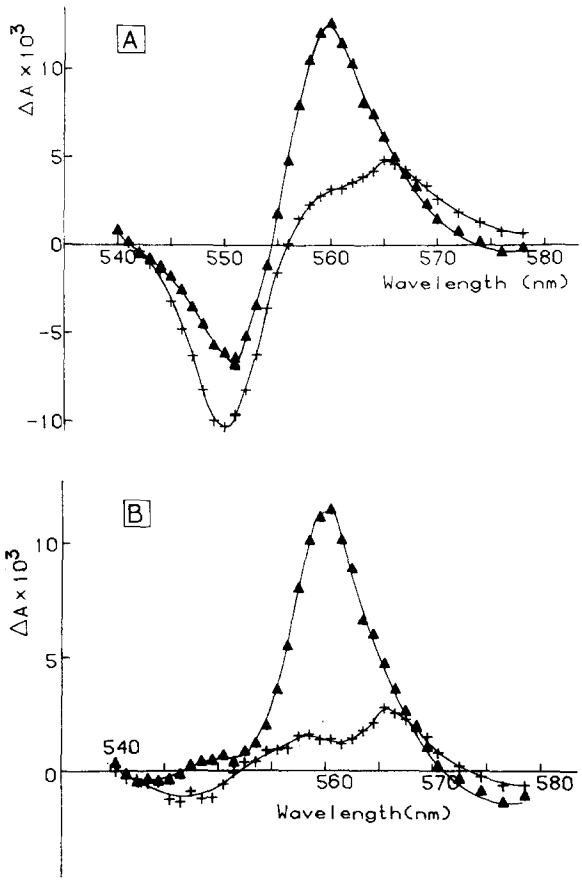

Fig. 8. Light-minus-dark difference spectra of cytochrome changes in *Rps. sphaeroides* Ga chromatophores. (A) Chromatophores were suspended to 1.2 μM reaction center in 50 mM Mops, 100 mM KCl, pH 7.0, containing 10 μM each of 2,3,5,6-tetramethyl-*p*-phenylenediamine, 1,4-naphthoquinone, 1,2-naphthoquinone and 2-hydroxy-1,4-naphthoquinone, 1 μM valinomycin and 4 μM antimycin at E_h 190 ± 5 mV. At each wavelength, chromatophores were subjected to two flashes with 20 ms between each flash. Signals were not averaged. 33 s elapsed between each measurement. ▲, The change 13 ms after the first flash. +, The change 5 ms after the second flash, using the point 17.5 ms after the first flash as the baseline. The contribution of the reaction center change was removed by subtraction of the appropriately normalised change recorded at 603 nm. (B) The spectra in Fig. 8A after additional correction for the cytochrome c_2 change by subtraction of the appropriately normalised change recorded at 551 nm (after reaction center correction).

reduction is small in the absence of antimycin. However, because this meant that we had to use a fast instrument response time, rather noisy kinetic traces were obtained. We have utilised a mutant of *Rps. capsulata* (strain R126) which, for a reason not yet understood, shows little or no cytochrome *b* photoreduction (Marrs, B., Prince, R.C., and Dutton, P.L., unpublished data; [44]). This enabled us to use a slower response time and obtain considerably less noisy difference spectra. Fig. 9 shows the spectra obtained on flash 1 and 2 (10 s between flashes) from the dark-adapted state, at E_h 395 ± 5 mV. The extent of the reaction center change, as measured at 605 nm, was the same on

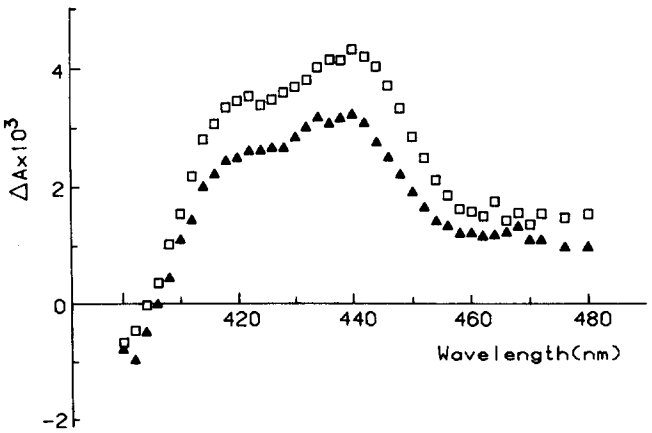

Fig. 9. Light-minus-dark difference spectra in *Rps. capsulata* R126. Chromatophores were suspended to about 0.24 μM reaction center in 50 mM Mops, 100 mM KCl, pH 7.0, containing a total of 1 mM potassium ferrocyanide and ferricyanide and 2 μM valinomycin. The redox potential was 395 ± 5 mV. Kinetic traces were not averaged, and the instrument response time was 20 ms. □, Spectrum of the change 82 ms after a xenon flash given after 120 s dark adaptation (first flash). ▲, Spectrum of the change 82 ms after a xenon flash given 10 s after the first flash (second flash).

both flashes. Fig. 10 shows the difference between the spectra of the changes elicited by flash 1 and flash 2. Superimposed is the difference spectrum for ubisemiquinone-10 anion minus ubiquinone-10 in methanol (from Ref. 43) which, as noted before [7], shows a close resemblance to the observed data. However, Fig. 10 emphasizes the point that the spectrum ascribed to formation of Q_{II}^- [6,7,42] is rather similar to that due to $(BChl)_2^+$. It is quite likely that both the spectra in Fig. 9 include contributions from $(BChl)_2^+$ and Q_{II}^-, but that the first-flash spectrum includes more Q_{II}^- than the second. Using a $\Delta\epsilon_{605nm}^{red\text{-}ox}$ of 19.5 $mM^{-1} \cdot cm^{-1}$ for $(BChl)_2^+$ [2] and $\Delta\epsilon_{445nm}^{red\text{-}ox}$ of 8.5 $mM^{-1} \cdot cm^{-1}$ [45] for Q_{II}^-, it appears that 0.95 Q_{II}^- per reaction center oxidised disappeared on flash 2. How-

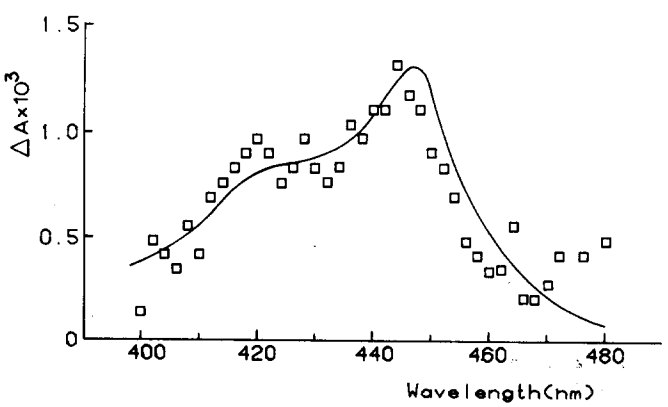

Fig. 10. The open squares show the difference between the spectra of the flash 1- and flash 2-induced changes from Fig. 9. Superimposed is the difference spectrum for ubisemiquinone-10 anion minus ubiquinone-10 in methanol.

ever, there is some uncertainty in the extinction coefficient of Q_{II}^-. Both spectra of Fig. 9 appear to involve two bands, Q_{II}^- making a greater contribution to the longer wavelength band.

Another approach was to use a mutant which lacked cytochrome c_2 (*Rps. capsulata* strain MT113 [46]). This mutant also shows negligible cytochrome *b* reduction induced by flash-excitation (Marrs, B.L., Prince, R.C., and Dutton P.L. unpublished observations; [44]). However, unlike R126, it did not show oscillations in the formation of Q_{II}^- detectable optically at any redox potential. Following a single flash excitation of chromatophores poised at $E_{h(7.0)}$ 75 mV, the absorption change relaxed slowly due to the absence of cytochrome c_2. Spectra of the change 40 ms and 80 ms after the flash (Fig. 11) were similar to those shown in Fig. 9, but with a large absorption change at 420 nm. The absence of this peak in the spectra of Fig. 9 can be attributed to oxidation of a small amount of cytochrome c_2 in this experiment; cytochrome c_2 would be approx. 10% reduced at $E_{h(7.0)}$ 395 mV in *Rps. capsulata*.

In isolated reaction centers of *Rps. sphaeroides*, a very broad positive absorption band is apparent in the light-minus-dark difference spectrum in this spectral region, which is independent of the presence of bound carotenoid [10,16]. However, there is only a very slight distinction into two peaks. The difference spectrum for monomeric bacteriochlorophyll-minus-bacteriochlorophyll$^+$ in methanol shows a corresponding absorbance increase [22], but the bandwidth at half maximum height is considerably smaller than that of the reaction center band.

Cytochrome c_2. The crosses in Fig. 12 show the change induced by a single flash in *Rps. capsulata* R126, at E_h 130 mV. The solid triangles show the change after correction for the 'reaction center' contribution (from the flash 2 spectrum of Fig. 9), using 605 nm as reference wavelength.

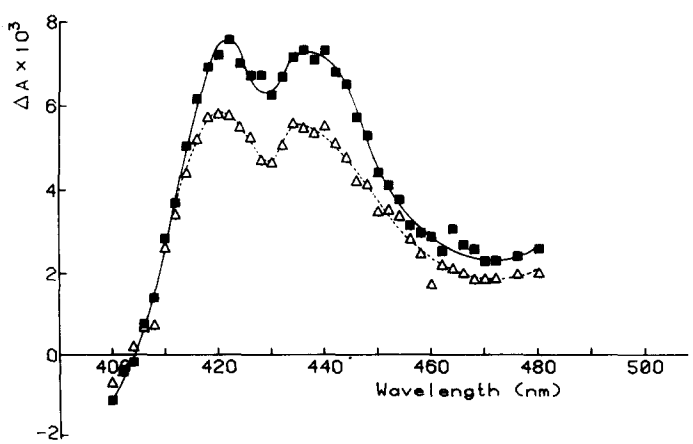

Fig. 11. Light-minus-dark difference spectra of *Rps. capsulata* MT113 chromatophores. Chromatophores were suspended to 0.5 μM reaction center in 50 mM Mops, 100 mM KCl, pH 7.0, containing 5 μM each of 2,3,5,6-tetramethyl-*p*-phenylenediamine, 1,4-naphthoquinone, 1,2-naphthoquinone and 2-hydroxy-1,4-naphthoquinone, and 2 μM valinomycin. The redox potential was 75 mV. Kinetic traces for the spectra were not averaged, and the instrument response time was 10 ms. The spectra show the change induced by a single xenon flash, 40 ms (■) and 80 ms (△) after the flash.

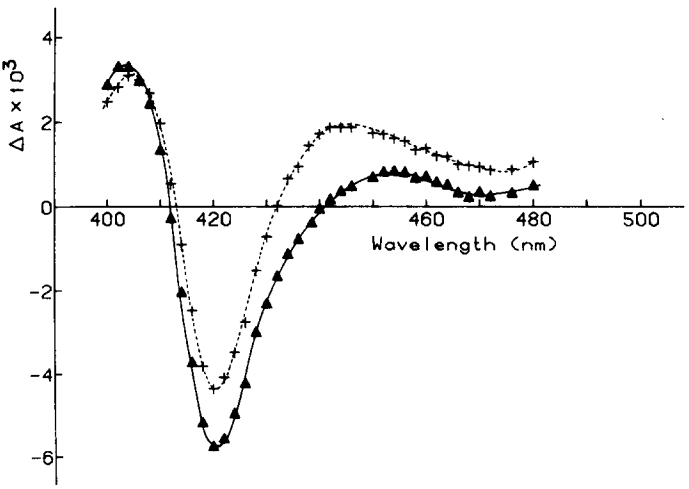

Fig. 12. Light-minus-dark difference spectra of *Rps. capsulata* R126 chromatophores. Chromatophores were suspended to 0.23 μM reaction center in 50 mM Mops, 100 mM KCl, pH 7.0, containing 10 μM 2,3,5,6-tetramethyl-*p*-phenylenediamine, 5 μM each of 1,4-naphthoquinone, 1,2-naphthoquinone and 2-hydroxy-1,4-naphthoquinone, and 1 μM antimycin and 40 μM UHDBT. The redox potential was 150 mV. Kinetic traces were not averaged, and the instrument response time was 20 ms. +, Spectrum of the change 250 ms after a single xenon flash. ▲, Spectrum after correction for the reaction center change, using the flash 2 spectrum of Fig. 9, with 605 nm as the normalising wavelength.

The spectrum recorded in the α band region under the same conditions, after correction for the reaction center change, was very similar to that shown in Fig. 3 (solid circles), and the spectrum in Fig. 12 (solid triangles) is attributed largely to cytochrome *c* components. The ratio of the change at 551 nm to that at 420 nm, after correction for reaction center changes, was 0.29. This compares with α/γ difference spectrum peak ratios of approx. 0.41 for isolated *Rhodospirillum rubrum* cytochrome c_2 [47] and approx. 0.42 for horse heart cytochrome *c* [48], but is closer to the value for isolated bovine heart mitochondrial cytochrome c_1 (approx. 0.22, [49]), a protein which is normally tightly bound into the cytochrome b-c_1 complex.

Cytochrome b. The spectrum of the change induced by a flash given 10 s after the first, in the presence of antimycin at E_h 390 mV, corrected for the reaction center, is largely attributable to cytochrome b_{50} reduction. Such a spectrum is shown in Fig. 3F of Ref. 7. The ratio of the change at 560 nm to that at 430 nm, after correction for the reaction center change, was 0.22.

Other components. In collaboration with R.C. Prince and P.L. Dutton, we have recently demonstrated that the Rieske type iron sulfur cluster [50] is oxidised by cytochrome c_2 in an antimycin-insensitive, UHDBT-sensitive reaction [28]. It seems likely that antimycin inhibits re-reduction of the Rieske cluster, so that absorbance changes due to Rieske cluster oxidation might be observed in the presence of antimycin and the absence of UHDBT. Oxidation of isolated mitochondrial Rieske cluster results in a broad absorbance increase over the range 400—600 nm with peaks at about 463 nm and 570 nm [50], having $\Delta\epsilon^{red\text{-}ox}$ values of 2.45 $mM^{-1} \cdot cm^{-1}$ and 1.6 $mM^{-1} \cdot cm^{-1}$ respectively (assuming the Rieske cluster to be a two-iron cluster). The isolated Rieske protein was

apparently contaminated with a cytochrome [51], but $\Delta\epsilon^{red\text{-}ox}$ values at the same wavelengths for isolated spinach ferredoxin, which is also a two-iron cluster, were close to those determined for the isolated Rieske protein [52]. These values are considerably smaller than those for cytochrome c_2 at 550—540 nm (19 $mM^{-1} \cdot cm^{-1}$) and for $(BChl)_2^+$ at 540 nm (10.3 $mM^{-1} \cdot cm^{-1}$) [2]. That of cytochrome *b* is unknown but is in the range 10—25 $mM^{-1} \cdot cm^{-1}$. Considering the relative amount of Rieske cluster oxidised in the presence of antimycin [28], the absorption change due to Rieske cluster oxidation would be less than one tenth of that due to cytochrome c_2.

Another component we have ignored is the special ubiquinone molecule Q_zH_2 (or ZH_2) which is thought to re-reduce ferricytochrome c_2 via the Rieske cluster [28,53]. Oxidation of Q_zH_2 is thought to proceed without the formation of a significant level of semiquinone, either because of its rapid re-reduction or oxidation to quinone, so that absorption changes in the spectral region 400—620 nm due to this component should be negligible.

In all spectra shown, measurements were made in the presence of sufficient valinomycin to collapse the membrane potential before the measurement was made. However, in the absence of valinomycin or gramicidin, the electrochromic responses of the light harvesting pigments can make very large contributions to absorption changes in the spectral regions of interest. This is described in more detail elsewhere [54].

Appropriate wavelengths at which to measure the resolved kinetics of individual components

The difference spectra shown indicate appropriate wavelengths at which to measure the resolved kinetics of redox centers in uncoupled chromatophores. The contribution of absorption changes due to spectral bandshifts of the light-harvesting pigments in response to membrane potential formation in coupled chromatophores introduces further problems, discussed elsewhere [54,55].

The isosbestic point of cytochrome c_2 at about 542.5 nm (Fig. 3) and the small contribution due to cytochrome *b* at this wavelength (Figs. 4 and 6) indicate that 542 nm is an appropriate wavelength at which to measure changes due to $(BChl)_2^+/(BChl)_2$. The kinetics and pattern of absorption changes on multi-pulse excitation recorded at 542 nm and 605 nm when concomitant cytochrome changes were occurring, were identical. However, 542 nm is a more convenient wavelength at which to measure the reaction center change since there is considerably more background absorption at 605 nm (due to the light harvesting bacteriochlorophyll and the low wavelength tail-off in the absorption band of the pale blue filter used to protect the photomultiplier from the actinic flash).

Figs. 4 and 6 indicate that there are negligible contributions from cytochrome *b* at either 551 nm or 542 nm, and Fig. 1 shows that the absorption change due to $(BChl)_2$ oxidation is almost identical at 542 nm and 551 nm. The change recorded at 542 nm may then be subtracted from the change recorded at 551 nm to obtain the kinetics of cytochrome c_2.

The cytochrome *b* kinetics are the most difficult to resolve by conventional dual wavelength techniques. In *Rps. sphaeroides* Ga and in *Rps. capsulata*, attempts to measure cytochrome *b* at 560—542 nm or at 560—540 nm, espe-

cially at high E_h, would lead to a small absorption decrease due to the contribution of $(BChl)_2^+$. Measurement at 560—570 nm would more adequately eliminate changes due to $(BChl)_2^+$ in *Rps. capsulata*, but would leave an absorption increase in *Rps. sphaeroides*. In both species, measurement at 560—540 nm would lead to a small absorption increase due to ferrocytochrome c_2 oxidation, which is eliminated by measurement at 560—570 nm. The problem can be solved by subtraction of appropriately normalised kinetic components attributed to $(BChl)_2^+$ and cytochrome c_2 from the change recorded at 560 nm. However, in routine measurements on uncoupled chromatophores in which the principal overlapping absorption change is due to cytochrome c_2, 560—570 nm is an appropriate wavelength pair with which to measure cytochrome b kinetics and is the best choice for *Rps. capsulata*.

Measurement of the kinetics of cytochrome b_{-90} cannot be made simply by dual wavelength techniques, because of overlap with the other components. The contribution of cytochrome b_{50} can be eliminated by measurements using the wavelength pair 565.5—555 nm which also compensate reasonably well for the reaction center change. The contribution of cytochrome c_2 at this wavelength pair is —0.49 of the change measured at 551—542 nm, and these values can be used to subtract out the cytochrome c_2 kinetics. Cytochrome b_{-90} does not contribute any large error to the kinetics measured at the other wavelength pairs recommended above.

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