CYTOCHROME c_2 – REACTION CENTRE COUPLING IN CHROMATOPHORES OF *RHODOPSEUDOMONAS SPHAEROIDES* AND *RHODOPSEUDOMONAS CAPSULATA*

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1. Introduction

In cells and chromatophores of the photosynthetic bacteria *Rhodopseudomonas sphaeroides* and *Rhodopseudomonas capsulata* the donor to photo-oxidised reaction centres is cytochrome c_2.

For several years it has been considered that there are 2 cyt c_2 molecules attached to each reaction centre. This has been based on assays of the amount of cyt c_2 and reaction centre in chromatophores [1] and on redox titrations of the pattern of cytochrome c_2 oxidation on double flash excitation of chromatophores [2]. These findings are in contrast with the situation in *Rhodospirillum rubrum* cells, in which there is only 1 cyt c_2/2 reaction centres [3].

We show here that in our preparations of chromatophores of both *Rps. sphaeroides* Ga and *Rps. capsulata* BY 761 there is only 1 cyt c_2 haem/reaction centre and < 1/reaction centre in *Rps. capsulata* A1a pho$^+$. We also show that earlier redox titrations of the extents of cyt c_2 oxidation following 2 closely spaced flash excitations of chromatophores were misleading because the full extent of cytochrome c_2 oxidation on flash 1 was not observed due to significant antimycin A-insensitive reduction of oxidized cyt c_2.

Abbreviations: DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; MOPS, morpholinopropane sulphonate; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; UHDBT, 5-*n*-undecyl, 6-hydroxy,4,7-dixobenzothiazole; cyt c_2, cytochrome c_2; P-870, reaction centre bacteriochlorophyll dimer; $E_{m(7.0)}$, midpoint reduction oxidation potential at pH 7.0; $E_{h(7.0)}$, reduction oxidation potential at pH 7.0; ϵ^{red-ox}, difference in extinction coefficients between reduced and oxidised species

2. Methods

Cells of *Rps. sphaeroides* Ga (carotenoid containing), *Rps. capsulata* BY 761 (kindly provided by B. Marrs) (carotenoid containing) and *Rps. capsulata* A1a pho$^+$ (carotenoidless) were grown anaerobically in the light and chromatophores prepared as in [4].

Flash-induced absorbance changes were recorded using the equipment in [4]. Redox titrations were performed using a stirred anaerobic redox cuvette similar to that in [5]. Great care was taken to check that the concentrations of the mediating redox dyes were sufficient to allow reliable redox equilibration between the components of the chromatophores and the electrodes, but not so high as to significantly interfere with the flash-induced kinetics measured in the $\times$ 10-ms time range. Valinomycin (2 μM) was routinely added to prevent the buildup of a membrane potential during flash excitation.

Flash excitation was provided by a Xe flash lamp of $\sim$5 μs pulse width. The saturating effect of the flash was determined by exciting chromatophores poised at $E_{h(7.0)}$ 440 mV with a train of flashes 20 ms apart. At this potential because cyt c_2 is oxidised, the oxidation of *P*-870 on each flash was readily resolved. In this way it was found that 90–95% of the reaction centres were oxidised by the first excitation, the remainder being oxidised by the second excitation. It was assumed that a similar level of saturation occurred on the first flash at lower redox potentials.

The UHDBT was a generous gift of Professor Karl Folkers (no. IBR 17728). It was added to chromatophore suspensions as a solution in ethanol.

To assay for cyt c_2, $\epsilon = 19\ mM^{-1}\ cm^{-1}$ measured

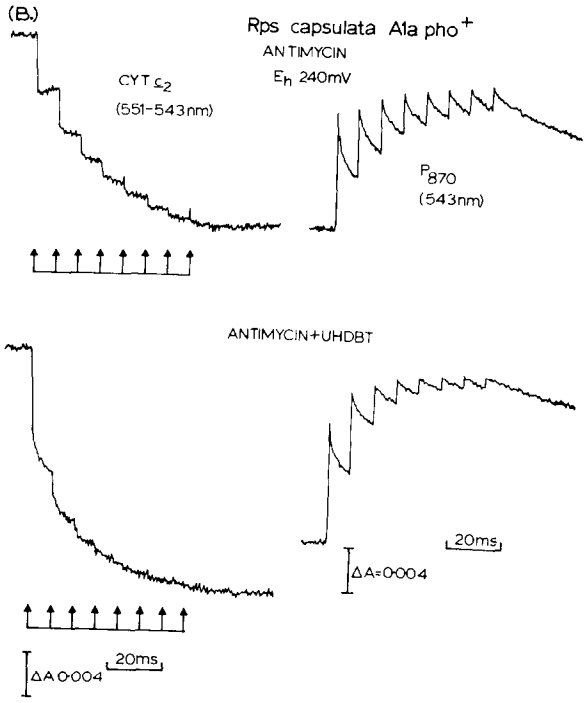
at 550–540 nm in *Rps. sphaeroides* Ga was used [1], although 551–542 nm or 551–543 nm were found to be more appropriate wavelength pairs to measure cyt c_2 in *Rps. capsulata* (our unpublished observations). To assay for *P*-870, ϵ = 10.3 mM^{-1} cm^{-1} at 540 nm (or 542 nm or 543 nm) or ϵ = 19.5 mM^{-1} cm^{-1} at 605 nm, also obtained using *Rps. sphaeroides* Ga [1] were used. Our results suggest a value for *Rps. capsulata* chromatophore *P*-870 close to that determined for *Rps. sphaeroides* Ga and the *Rps. sphaeroides* extinction coefficients were also applied to *Rps. capsulata*.

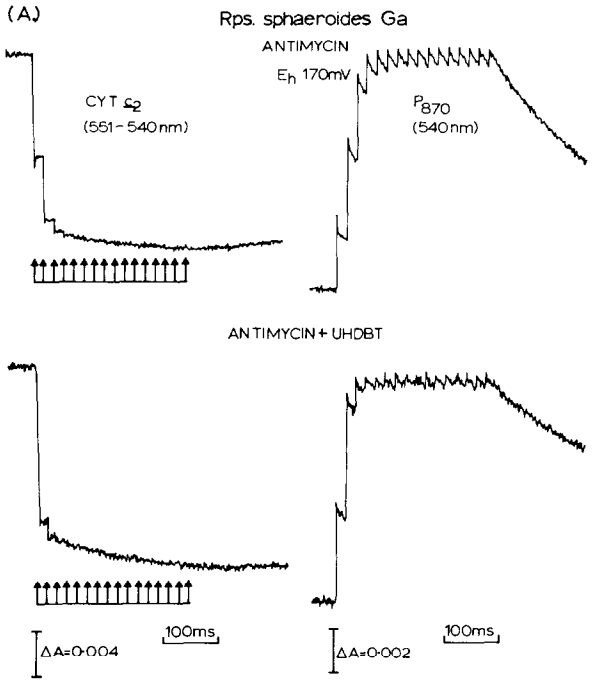
Redox titration data were analyzed using a titration fitting programme (TITFIT) adapted from the programme in [6] and modified for running on a PDP 11/34 computer (Digital Equipment Corp.).

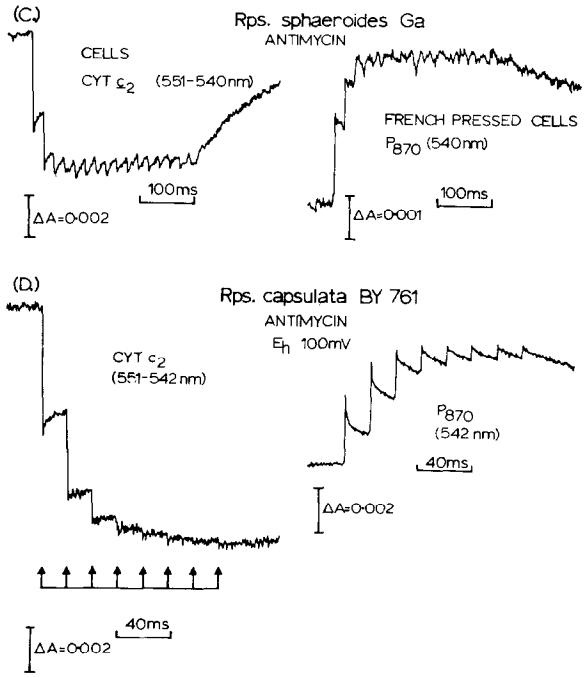
3. Results and discussion

3.1. *The amounts of photo-oxidisable cyt* c_2 *and reaction centre*

The traces in fig.1A–C show the patterns of *P*-870 and cyt c_2 photo-oxidation on multiple flash excitation in several different preparations. The traces do







not resolve the full extent of *P*-870 photo-oxidation on the first few flashes because of the rapid rereduction by cyt c_2. In *Rps. sphaeroides* Ga and *Rps. capsulata* BY 761, in the presence of antimycin A to block cyclic electron transport [7] both *P*-870 and cyt c_2 reached steady state levels of photo-oxidation on multiple flash excitation. The patterns of cyt c_2 photo-oxidation with flash number were essentially similar to those in [1,8,9]. However, in contrast to these reports, our results show that the ratios of the steady state levels of cyt c_2 and *P*-870 photo-oxidised were ~1:1 in both cases (see table 1). The ubiquinone

Table 1
Amounts of cyt c_2 in chromatophores and cells of *Rps. sphaeroides* and *Rps. capsulata*

Preparation	Photo-oxidisable cyt c_2/ photo-oxidisable *P*-870	Total[c] cyt c_2/photo-oxidisable *P*-870
Rps. sphaeroides Ga chromatophores	0.88[a]	0.97
Rps. sphaeroides Ga cells	0.97[b]	–
Rps. capsulata BY761 chromatophores	1.06[b]	–
Rps. capsulata A1a pho$^+$ chromatophores	0.75[a]	0.78

[a] In presence of antimycin A and UHDBT
[b] In presence of antimycin A
[c] Determined from chemically reduced–oxidised difference spectra

Fig.1. Multipulse kinetics of cyt c_2 and *P*-870. Where shown, the arrows indicate when flashes occurred.

(A) With antimycin A: *Rps. sphaeroides* Ga chromatophores were suspended to ~1.1 μM reaction centres in 50 mM MOPS, 100 mM KCl (pH 7.0) containing 2 μM antimycin A, 2 μM valinomycin, 7 μM DAD, 7 μM 1,4-naphthoquinone, 7 μM 2-hydroxy,1,4-naphthoquinone, 7 μM 1,2-naphthoquinone and 4 μM PMS at E_h 170 mV. Traces are an average of 2. Chromatophores were subjected to 16 flashes with 20 ms between flashes. The instrument response time was 100 μs. With antimycin A and UHDBT: Conditions were as above except that no PMS was present, UHDBT was added to 40 μM, and traces were not averaged.

(B) With antimycin A: *Rps. capsulata* A1a pho$^+$ chromatophores were suspended to ~1.5 μM reaction centres in 50 mM MOPS, 100 mM KCl (pH 7.0) containing 5 μM antimycin A, 2 μM valinomycin, 1 μM DAD, 10 μM 1,4-naphthoquinone, 10 μM 2-hydroxy,1,4-naphthoquinone, 10 μM 1,2-naphthoquinone and 0.5 μM PMS at E_h 240 mV. Traces were not averaged. Chromatophores were subjected to 8 flashes with 8.3 ms between flashes. The instrument response time was 100 μs. With antimycin A and UHDBT: Conditions were as above except that UHDBT was added to 25 μM.

(C) *Rps. capsulata* BY 761 chromatophores were suspended to 0.52 μM reaction centres in 50 mM MOPS, 100 mM KCl (pH 7.0) containing 2 μM antimycin A, 2 μM valinomycin, 4 μM DAD, 4 μM 1,4-naphthoquinone, 4 μM 1,2-naphthoquinone and 4 μM 2-hydroxy,1,4-naphthoquinone at E_h 100 mV. Traces are an average of 2. Chromatophores were subjected to 8 flashes with 18.5 ms between flashes. The instrument response time was 100 μs.

(D) Cytochrome c_2 measurements: *Rps. sphaeroides* Ga cells in growth medium, taken directly from the growth chamber were placed in a stirred redox cuvette and allowed to become anaerobic. Additions were 40 μM antimycin A, 20 μM FCCP and 10 μM valinomycin. Small amounts of oxygen were stirred in before measurements were made, as described in the text. The signal was not averaged. Cells were subjected to 16 flashes with 20 ms between each flash. The instrument response time was 500 μs. The reaction centre was 0.34 μM, as determined below. Reaction centre measurement: The cells in growth medium used for the cyt c_2 measurements above were French pressed twice, and measurements made directly on the French Press effluent. Additions were 10 μM antimycin A, 5 μM valinomycin, 20 μM FCCP, 4 μM DAD, 5 μM 1,4-naphthoquinone, 5 μM 1,2-naphthoquinone and 5 μM 2-hydroxy,1,4-naphthoquinone. The E_h was 280 mV. The signal was not averaged. The suspension was subjected to 16 flashes with 20 ms between flashes. The instrument response time was 500 μs.

analogue UHDBT, dramatically affected the pattern of cyt c_2 and *P*-870 photo-oxidation with flash number, but only had a very slight effect on the final steady state levels of photo-oxidation. It seems likely that these steady state levels of cyt c_2 and *P*-870 photo-oxidation reflected the actual amounts of these components present in chromatophores. In *Rps. sphaeroides* Ga, at E_h 440 mV, the steady state level of *P*-870 photo-oxidised by multiple flash excitation, gave, after correction for the amount of *P*-870 chemically oxidised before the flash (assuming $E_{m(7.0)}$ was 440 mV) the same value for the amount of *P*-870 as that measured at E_h 170 mV. The amount of chemically reducible cyt c_2, as determined from a ferricyanide oxidised minus ascorbate reduced, difference spectrum with 10 μM TMPD as mediator, was also found to be ~1 cyt c_2/*P*-870.

Figure 1D shows the pattern of cyt c_2 photo-oxidation in *Rps. sphaeroides* Ga cells. Antimycin A is an inefficient inhibitor of electron transport in cells, probably because it does not readily penetrate the cell envelope. To inhibit cyt c_2 rereduction between flashes, small amounts of O_2 were stirred in to oxidise the donor pool to cyt c_2, so that the steady state level of cyt c_2 photo-oxidation rose to a constant level. The extinction coefficient of *P*-870 at 605 nm varies with the environment of the reaction centre and is known for *P*-870 in chromatophores, but not in cells. For this reason, cells were French pressed twice to convert them into a crude chromatophore preparation before assaying for *P*-870. The results indicated a value of 1 cyt c_2/*P*-870 in *Rps. sphaeroides* Ga cells although this may be an underestimate due to ongoing cyt c_2 rereduction during the flash train.

In *Rps. capsulata* A1a pho$^+$ in the presence of antimycin A and UHDBT, a considerably smaller fraction of the steady state level of photo-oxidisable cyt c_2 was oxidised on flash 1 (55% as compared to 73% in *Rps. sphaeroides* Ga, fig.1A,B). This implies that, since 90–95% of the reaction centres were oxidised on the first flash, a much smaller fraction were active in directly oxidising cyt c_2. Thus, for more cyt c_2 to be oxidised after subsequent excitations, cyt c_2 must be mobile between the 'active' oxidised reaction centres. This observation is consistent with observations made in *Rhs. rubrum* cells [3,10] and with second order effects observed in the interaction between cyt c_2 and reaction centres in liposomes (R. E. Overfield, personal communication), but is inconsistent with the results in [11] (fig.7) which indicated that in *Rps. sphaeroides* Ga, cyt c_2 was not mobile between reaction centres on a millisecond time scale. However, our results indicate considerable differences in the cyt c_2 reaction centre interaction in chromatophores from strains with and without the B800/850 bacteriochlorophyll and carotenoid light harvesting complex II and this may in part be a cause of discrepancies. Table 1 summarises the data for the preparations examined.

3.2. *Matching of cyt* c_2 *photo-oxidised and* P-870 *rereduced*

In contrast to the results in [1], our results show clearly that the extent and kinetics of cyt c_2 photo-oxidised and *P*-870 rereduced following a single flash in the presence of antimycin A did not match (fig.1A–C, fig.2) but matched fairly well in the presence of UHDBT. In *Rps. sphaeroides* Ga, with antimycin A but no UHDBT (fig.1A), 0.48 μM cyt c_2 remained oxidised on flash 1 in the train but 0.86 μM *P*-870 was rereduced. In the presence of antimycin A and UHDBT, 0.73 μM cyt c_2 was oxidised on the first flash and 0.71 μM *P*-870 was rereduced. In addition, it is evident from fig.2 that in the presence of antimycin A alone, there was a small apparent reductive phase in the cyt c_2 kinetics following the first flash in

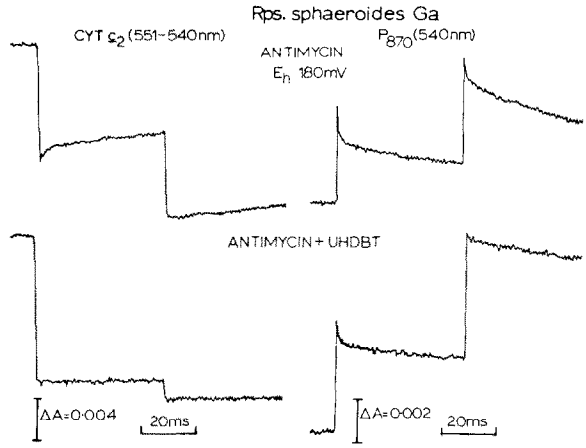


Fig.2. Double flash kinetics of cyt c_2 and *P*-870. With antimycin A: Same conditions as for fig.1A (with antimycin A). Traces are an average of 2. Time between flashes was 46 ms. The instrument response time was 100 μs. With antimycin A and UHDBT: As above but UHDBT was added to 40 μM.

Rps. sphaeroides Ga. This is also clearly apparent in [9] (fig.1). This change occurred in the absence of redox mediators, and in the presence of $\leqslant 10$ μM antimycin A but not in the presence of UHDBT. This phase means that the *P*-870 rereduction and cyt c_2 oxidation kinetics cannot match at $\leqslant 10$ ms after the flash in the presence of antimycin A alone. The results with *Rps. capsulata* also showed that the cyt c_2 and *P*-870 reactions did not match in the presence of antimycin A but matched more closely in the presence of UHDBT and enabled us to obtain an approximate value for ϵ^{red-ox} for *P*-870 at 605 nm of $18.5 \pm 3.6\ mM^{-1}\ cm^{-1}$.

We interpret the effects of UHDBT as indicating that part of the photo-oxidised cyt c_2 is rereduced in an antimycin-insensitive UHDBT-sensitive reaction, by a component J which in turn results in increased *P*-870 rereduction. Preliminary reports of our findings on J and the effects of UHDBT have been published in [12,13].

Figure 3 shows the rapid kinetics of cyt c_2 photo-oxidation in the presence of antimycin A and with antimycin A plus UHDBT. They show that UHDBT was affecting the slow phase but not the fast phase of cyt c_2 oxidation. The fast phase was complete within 20 μs. The slow phase had $t_{1/2}$ 185 μs in the presence of UHDBT. It thus appears that J rereduced cyt c_2 at < 20 μs but faster than the slow phase of oxidation.

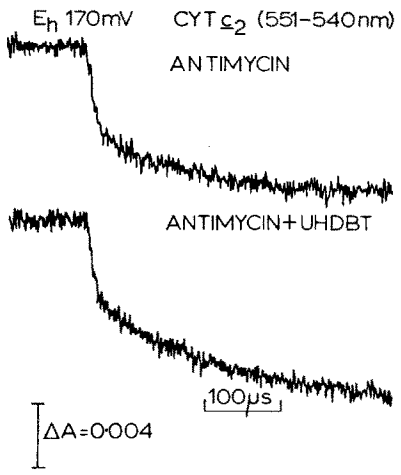


Fig.3. Rapid kinetics of cyt c_2 photo-oxidation. With antimycin A: Same conditions as for fig.1A (with antimycin A). The trace is an average of 32. The instrument response time was < 1 μs. With antimycin A and UHDBT: Same conditions as for fig.1A (with antimycin A and UHDBT). The trace is an average of 32. The instrument response time was < 1 μs.

3.3. *Redox titrations of flash-induced cytochrome* c_2 *oxidation in* Rps. sphaeroides *Ga chromatophores*

Figure 4 shows the extent of cyt c_2 photo-oxidation induced by 2 flashes 50 ms apart, in the presence of antimycin A, and with antimycin A plus UHDBT. In the presence of antimycin A alone, the extent of cyt c_2 photo-oxidation on flash 1 was taken to be the amount remaining oxidised after the small apparent reductive phase. The titrations in the presence of antimycin A correspond to those in [2,14]. They give

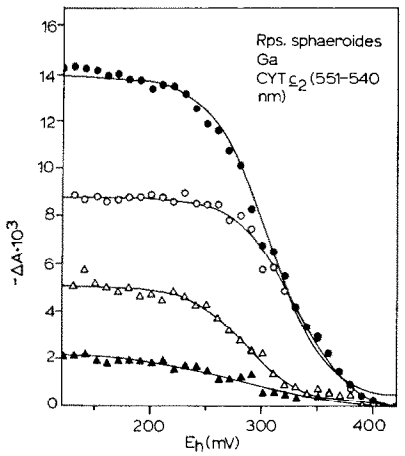


Fig.4. Redox titrations of extent of cytochrome c_2 photo-oxidation caused by 2 flashes 50 ms apart. With antimycin A: *Rps. sphaeroides* Ga chromatophores were suspended to ~1.1 μM reaction centres in 50 mM MOPS, 100 mM KCl (pH 7.0) containing 2 μM antimycin A and 2 μM valinomycin. For traces E_h 300 mV, redox mediators were $\geqslant 200$ μM potassium ferricyanide and ferrocyanide and 7 μM TMPD. Below E_h 300 mV, redox mediators were 7 μM DAD, 7 μM 1,4-naphthoquinone, 7 μM 1,2-naphthoquinone and 7 μM 2-hydroxy, 1,4-naphthoquinone. Signals were not averaged. The instrument response time was 100 μs. (○) Extent of oxidation induced by flash 1, recorded ~40 ms after the flash. (△) Extent of oxidation induced by flash 2, recorded ~40 ms after the flash. With antimycin A and UHDBT: As above except that 40 μM UHDBT was also present, and 5 μM TMPD was used above E_h 300 mV instead of 7 μM. (●) Flash 1-induced cyt c_2 oxidation. (▲) Flash 2-induced cyt c_2 oxidation.

$E_{m(7.0)}$, $n = 1$, 330 mV for the cyt c_2 oxidised on flash 1 and 285 mV for cyt c_2 oxidised on flash 2, in close agreement with the values in [14]. These results were interpreted [2,14] to indicate that 2 equivalent haems shared each reaction centre as had been proposed on the basis of similar observations in *Chromatium vinosum* [5,15]. However, our results indicate that there is only 1 cyt c_2 haem/reaction centre and that the flash 1 and 2 pattern of cyt c_2 oxidation in the presence of antimycin A does not reflect the true pattern of cyt c_2 oxidation but is due to the effect of J [13]. Thus the fit of the data in [2,14] to the theoretical model proposed would appear to be fortuitous.

In the presence of UHDBT the E_m for flash 1 oxidised cyt c_2 was 305 mV; close to the equilibrium value of 300 mV [14]. Because of the small extent of cyt c_2 oxidation on the second flash in the presence of UHDBT we cannot give a reliable E_m value for this fraction of cyt c_2.

The difference between the titrations of the extent of the cyt c_2 oxidised on flash 1 in the presence of antimycin A with and without UHDBT should be a titration of J, the rapid donor to cyt c_2. This indicated an $E_{m(7.0)}$ value for J of ~270 mV, $n = 1$, close to that of the Rieske-type Fe–S protein [16]. A more detailed description of the identity, stoichiometry, kinetics and thermodynamic properties of J will be reported later.

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