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The role of *c*-type cytochromes in the photosynthetic electron transport pathway of *Rhodobacter capsulatus*

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(1) Short flash excitation of membrane vesicles of a cytochrome-c_2-deficient mutant of *Rhodobacter capsulatus* (strain MT-G4/S4) led to rapid oxidation of a *c*-type cytochrome. In redox titrations, the photooxidation of *c*-type cytochrome was attenuated with a midpoint of approx. +360 mV. Vesicles from a control strain, MT1131, gave similar results. These findings are consistent with those of Prince et al. (Prince, R.C., Davidson, E., Haith, L.E. and Daldal, F. (1986) Biochemistry 25, 5208–5214). (2) In anaerobic intact cells the extent of rapid re-reduction of *c*-type cytochrome oxidised after a flash was less in MT-G/S4 than in MT1131. Cytochrome *c* reduction in both strains was inhibited by myxothiazol. The myxothiazol-sensitive component of the electrochromic absorbance change in cells indicated that rapid charge separation through the cytochrome bc_1 complex was less extensive after a flash in MT-G4/S4 than in MT 1131. (3) In anaerobic intact cells and in chromatophores of *Rb. capsulatus* strain MT-GS18, a mutant deficient in both cytochrome c_1 and cytochrome c_2, flash excitation led to the oxidation of *c*-type cytochrome. Redox titrations and spectra of chromatophores suggested that this is the same cytochrome as was photooxidised in vesicles of MT-G4/S4 and MT1131. This result is in contrast with earlier findings (Prince, R.C. and Daldal, F. (1987) Biochim. Biophys. Acta 894, 370–378) in which it was reported that no photooxidation of *c*-type cytochrome occurred in the absence of c_1 and c_2, and argues against the possibility that cytochrome c_1 can rapidly and directly donate electrons to the reaction centre. (4) It is proposed that a previously uncharacterised, membrane-bound *c*-type cytochrome ($E_{m7} \approx +360$ mV) is present in *Rb-capsulatus* MT1131, in the c_2-deficient mutant MT-G4/34 and in the c_1/c_2-deficient mutant MTGS18. This cytochrome and cytochrome c_2 are alternative electron donors to the reaction centre in strain MT1131.

Introduction

Until about 4 years ago, a clear consensus on the nature of the photosynthetic electron transport pathway in *Rhodobacter capsulatus* and *Rb. sphaeroides* (formerly *Rhodopseudomonas capsulata* and *Rps. sphaeroides*) was emerging [1,2]. The available evidence indicated that the photosynthetic reaction centre oxidised cytochrome c_2, a soluble periplasmic protein, and reduced ubiquinone. The cytochrome bc_1 complex was thought to complete the cycle by re-oxidising ubiquinol and by re-reducing cytochrome c_2. This was consistent with a general view of electron transport in other bacteria and in mitochondria and chloroplasts, in that exchange of reducing equivalents between large membrane-bound complexes was considered to be mediated by the lateral diffusion of quinones within the relatively hydrophobic domain of the membrane or by small water-soluble proteins at the membrane surface [3]. Experiments with the photosynthetic bacteria in support of this model were particularly critical, since they were performed at short time resolution and thus provided a valuable kinetic analysis.

The construction by Daldal and co-workers of a cytochrome c_2-deficient mutant of *Rb. capsulatus* which was able to grow rapidly by photosynthesis, at least at high light intensities, changed the perception of electron transport in this genus [4]. Although the fact that cyto-

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Abbreviations: P, primary electron donor in the photosynthetic reaction centre; bc_1, cytochrome bc_1 complex (ubiquinol cytochrome *c* oxidoreductase); PMS, phenazine methosulphate; PES, phenazine ethosulphate; DAD, diaminodurene; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone.

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chrome c_2 can act as a direct and rapid electron donor to the reaction centre was not disputed, it became clear that another pathway can also operate. The most radical suggestion to arise from these experiments was that cytochrome c_1 is able rapidly to transfer electrons directly to the reaction centre [4–6]. Initially, this proposal was based on a comparison of the kinetics and the extent of oxidation of *c*-type cytochromes in the cytochrome-c_2-deficient mutant and in a control strain [5]. Strong support for the proposal was provided by the observation than in membranes prepared from mutants of *Rb. capsulatus* in which the genes for both cytochrome c_1 and cytochrome c_2 were deleted, no photooxidation of *c*-type cytochrome could be detected [6]. Thus in the strain (MT1131) that was wild-type in its electron transport system, cytochrome c_1 and cytochrome c_2 were thought to be alternative direct donors to the reaction centre [4–6]. Since the rate of phototrophic growth of the cytochrome-c_2-deficient mutant was slower in dim light than that of the control strain, it was reasoned that the electron transport pathway lacking cytochrome c_2 was less efficient [4]. Not only did this hypothesis conflict with the established view of electron transport in *Rhodobacter* sp., it also raised the wider possibility that in other organisms electron exchange between large membrane-bound complexes might proceed directly without mediation by smaller, more rapidly diffusing components. There has been some dissent from the view that cytochrome c_1 can directly donate electrons to the reaction centre, based on observations with reconstituted systems [7,8], but conclusions on the experiments with physiological membranes [4–6,] are now widely recognised [9].

The situation became more complex when it was found that cytochrome-c_2-deficient mutants of *Rb. sphaeroides* were unable to grow photosynthetically [10] but that other mutants could be subsequently isolated in which the PS^- phenotype was suppressed [11]. The ability of these mutants to grow correlated with the synthesis of an 'iso-cytochrome c_2' (cytochrome *c*-552). These findings illustrate at least that there are essential differences between the electron transport pathways in *Rb. capsulatus* and *Rb. sphaeroides*. This was recently confirmed [12] by transferring the gene coding for the cytochrome bc_1 complex from *Rb. sphaeroides* into strains of *Rb. capsulatus* which were lacking either the bc_1 complex or both the bc_1 and cytochrome c_2.

During the course of experiments to monitor H^+ efflux from right-side-out vesicles of *Rb. capsulatus* [13] we became increasingly aware of inconsistencies in the view that cytochrome c_1 can directly transfer reducing equivalents to the reaction centre. This communication describes an account of these findings and presents a new model for electron transfer to the reaction centre in *Rb. capsulatus* that explains the original findings of Daldal et al. [4].

Materials and Methods

Rb. capsulatus strains MT1131 and MT-G4/S4 (both provided by Dr F. Daldal, University of Pennsylvania) were grown phototrophically as described in the preceding paper [13]. *Rb. capsulatus* strain MT-GS18 (also supplied by Dr Daldal) was grown aerobically in the dark in an orbital shaker at 120 rpm at 30 °C in 250 ml conical flasks containing 100 ml 'RCV medium' [14] (or in 2.5 litre flasks containing 1 litre medium) supplemented with 25 μg/ml kanamycin and 25 μg/ml spectinomycin. This strain was routinely checked on RCV-agar plates, to confirm its inability to grow phototrophically under strictly anaerobic conditions (see Ref. 6).

Procedures for the preparation of intact cells and osmotically-shocked vesicles were as described [13]. Chromatophores of MT-GS18 were prepared essentially as for strain N22 [15]. Other analytical methods and transient optical absorbance spectroscopy were carried out as in Ref. 13.

Results

Photooxidation of c-type cytochrome in the c_2-deficient mutant of Rb. capsulatus

Flash-induced oxidation of *c*-type cytochrome was studied in osmotically-shocked vesicles of the cytochrome-c_2-deficient mutant, MT-G4/S4, of *Rb. capsulatus*. The advantage of using vesicles is that soluble periplasmic redox components are largely removed during preparation [13] and therefore membrane-associated reactions predominate. Nevertheless, the results were entirely consistent with those described for chromatophores (inside-out vesicles) of MT-G4/S4 [5]. Thus after flash excitation there was a rapid oxidation (complete in less than 1 ms) of a *c*-type cytochrome (λ_{max} in the α-band region at approx. 552 nm, see Fig. 1b), which in redox titrations was attentuated with an $E_{m7.6}$ of about +360 mV (Fig. 1a). In earlier work this reaction was ascribed to photooxidation of cytochrome c_1 [5].

In further agreement with the earlier experiments, it was found that results with vesicles of the control strain, *Rb. capsulatus* MT1131, were very similar to those in the c_2-deficient mutant (Fig. 2). This is expected because the cytochrome c_2 present in MT1131 was largely removed during preparation of the vesicles [13]. At this juncture it is interesting to note that there were consistent differences between strains MT1131 and N22, which are both considered to be 'wild-type' strains of *Rb. capsulatus* with respect to their electron transport systems. Oxidation of *c*-type cytochrome in vesicles of N22 after a single flash was slower than in MT1131 and the reaction was attenuated with a significantly lower potential [13]. These strain differences will be discussed later.

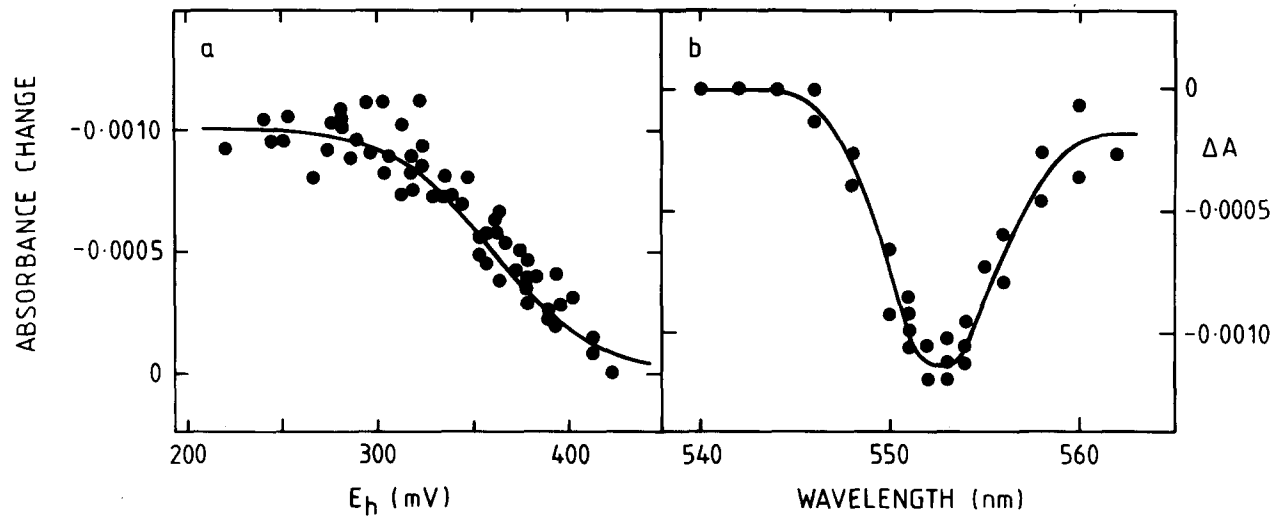

Fig. 1. Redox titration and spectrum of the extent of photooxidation of *c*-type cytochrome after a single actinic flash in vesicles of *Rb. capsulatus* strain MT-G4/S4. Vesicles were suspended to 0.2 μM P in 10 mM potassium phosphate (pH 7.6) with 2 μM PMS, 2 μM PES, 10 μM DAD, 5 μM myxothiazol and 5 μM FCCP. In (a) the absorbance changes were recorded at 552-542 nm and the E_h was adjusted with dilute solutions of potassium ferricyanide and sodium dithionite. In (b) the vesicles were poised at an E_h of approx. +250 mV and all measurements were recorded relative to the change in absorbance at 542 nm.

Photo-oxidation of the *c*-type cytochrome in vesicles of MT-G4/S4 and MT1131 was attenuated with a mid-point of approx. +360 mV (Figs. 1 and 2). The same reaction in chromatophores (at a slightly different pH) titrated with a midpoint of approx. +350 mV [5] and was proposed to represent direct oxidation of cytochrome c_1 by the reaction centre [5]. A discrepancy in this hypothesis is that these mid-point potentials differ appreciably from the $E_{m7.0}$ of cytochrome c_1 of +285 mV revealed by conventional redox titration in darkened membranes [16]. Another set of observations which is not consistent with the hypothesis of Prince et al. [5] is summarised in Figs. 3 and 4. From their model it is predicted that if the ubiquinone pool is reduced then 'cytochrome c_1', oxidised by the reaction centre after flash excitation, would subsequently become rapidly re-reduced by electron transport through the cytochrome bc_1 complex. The extent of 'cytochrome c_1' rapid re-reduction in membranes of MT-G4/S4 should be greater than, or at least similar to, the extent of (cytochrome c_1 plus cytochrome c_2) re-reduction in membranes of MT1131. A comparison of Fig. 3a with Fig. 4a shows, however, that in strictly anaerobic intact cells the re-reduction of *c*-type cytochrome after oxidation by a flash was much less extensive in MT-G4/S4 than in MT1131. Re-reduction of *c*-type cytochrome in cells of MT-G4/S4 was certainly myxothiazol-sensitive (Fig. 3b and c), showing that bc_1 was the source of electrons, but the extent of the myxothiazol-sensitive component of re-reduction (Fig. 3c) was less than in

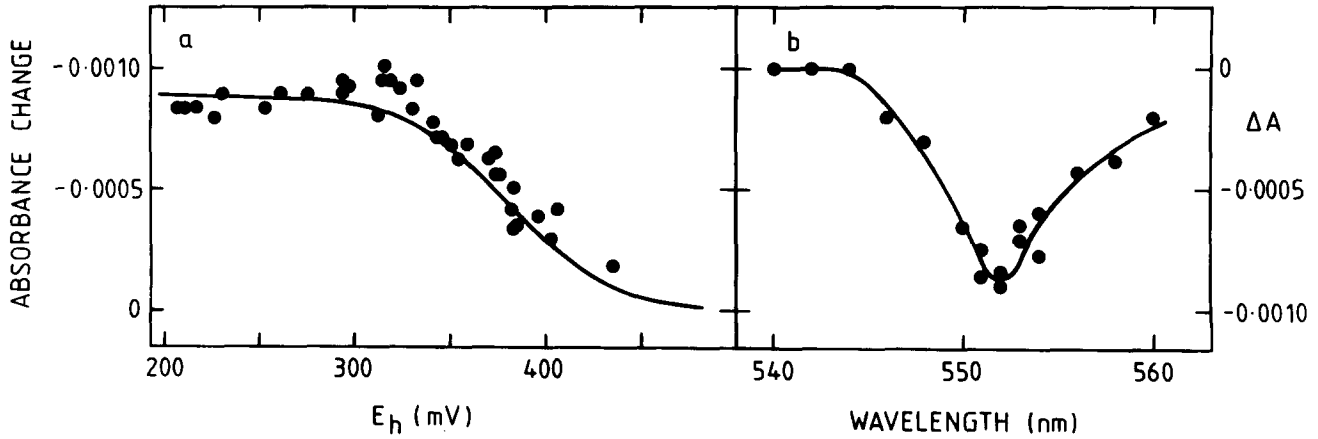

Fig. 2. Redox titration and spectrum of the extent of photooxidation of *c*-type cytochrome after a single actinic flash in vesicles of *Rb. capsulatus* strain MT1131. Experimental details as in legend to Fig. 1.

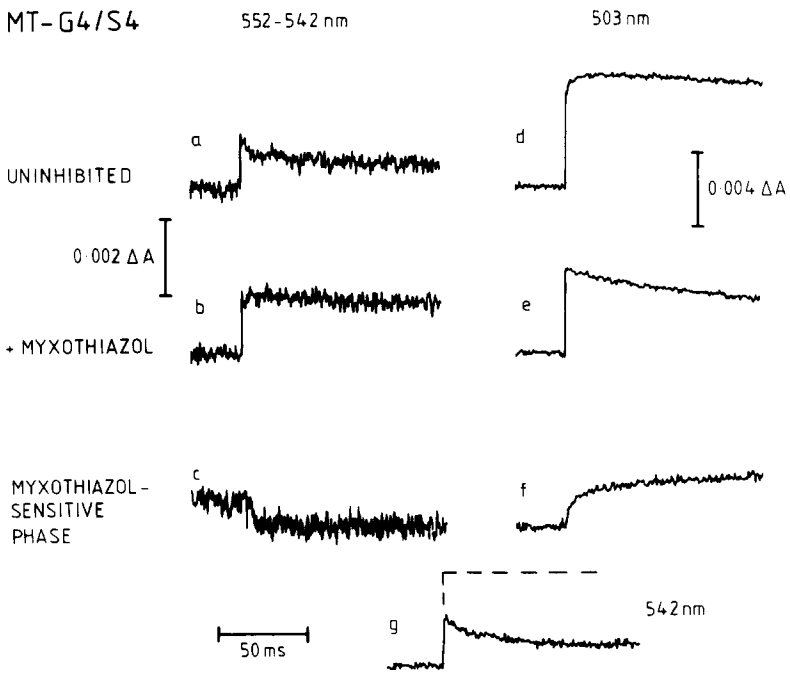

Fig. 3. Flash-induced absorbance changes associated with cytochrome *c* (a–c), the electrochromic band shift (d–f) and P (g) in intact cells of strain MT-G4/S4. Cells were suspended to 0.2 μM P in 10 mM potassium phosphate (pH 7.6) under strictly anaerobic conditions. In (b) and (e) myxothiazol was added to 5 μM. Traces (c) and (f) were obtained by subtracting data collected in the presence of myxothiazol from that collected in the absence of myxothiazol. In (g) the dotted line indicates the expected extent of P oxidation after the flash (determined separately using a train of closely spaced flashes).

cells of MT1131 (Fig. 4c). The observations were reinforced by measurements of electrochromic absorbance changes following flash excitation of cells of MT1131 and MT-G4/S4 (Figs. 4d–f and 3d–f). Although the extent of oxidation of *c*-type cytochrome in the two strains was similar (compare Fig. 3b and Fig. 4b), the

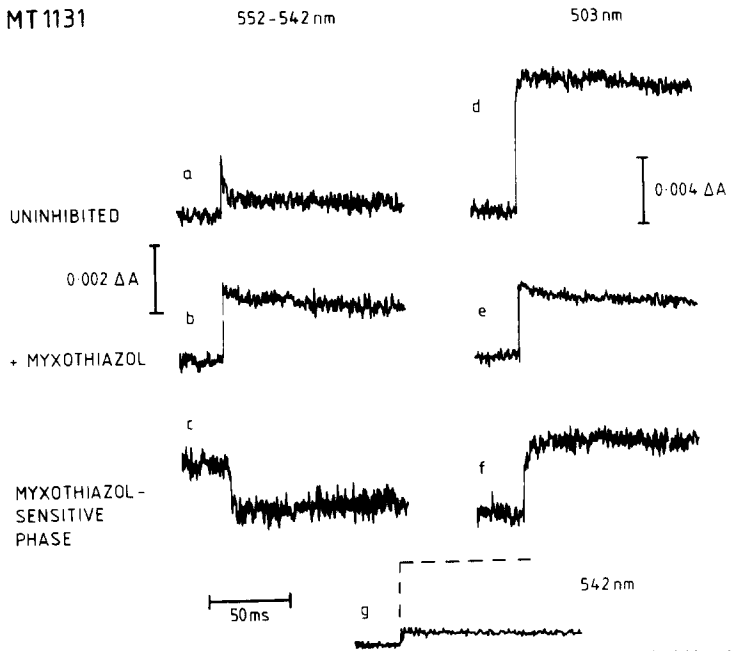

Fig. 4. Flash-induced absorbance changes associated with cytochrome *c* (a–c), the electrochromic band shift (d–f) and P (g) in anaerobic intact cells of *Rb. capsulatus* strain MT1131. Experimental details as in legend to Fig. 3.

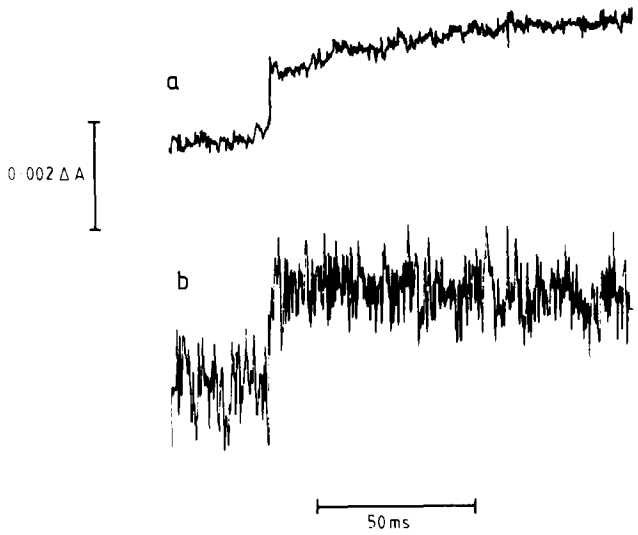

Fig. 5. The effect of a single saturating actinic flash on the redox state of cytochrome *c* in chromatophores (a) and intact cells (b) of *Rb. capsulatus* strain MT-GS18. Absorbance changes were recorded at 551–542 nm. In (a) chromatophores were suspended in 10% sucrose, 100 mM KCl, 8 mM $MgCl_2$ and 50 mM K^+-Tricine (pH 7.4) with 2 μM PMS, 2 μM PES, 10 μM DAD and 5 μM FCCP. The E_h was poised at approx. +250 mV. In (b) cells were suspended to 0.2 μM P in 10 mM potassium phosphate (pH 7.6) under strictly anaerobic conditions. The low reaction centre context of cells of strain MT-GS18 grown semi-aerobically results in a highly scattering sample and hence a poor signal-to-noise ratio.

myxothiazol-sensitive component of the electrochromic absorbance change, reflecting charge separation in the bc_1 complex, was substantially less extensive in MT-G4/S4 than in MT1131 (Figs. 3f and 4f). Re-reduction of the oxidised reaction centre (P^+) generated by the flash was also less extensive in anaerobic cells of MT-G4/S4 than in MT1131 (Figs. 3g and 4g), reflecting the loss of cytochrome c_2 as a donor in the mutant. Although the kinetics were not completely resolved, it is evident that approx. 85% of the P^+ was reduced within 1 ms of the flash in cells of MT1131 but only 50% of P^+ was reduced in this time in MT-G4/S4. Note that, although the data are shown in Figs. 3 and 4 for only one batch of cells of MT-G4/S4 and MT1131, respectively, the experiments were highly reproducible on other preparations, provided that strictly anaerobic conditions were employed. Also in vesicles derived from MT-G4/S4 and poised at an E_h of approx. +100 mV, the cytochrome *c* and electrochromic absorbance changes in the presence and absence of myxothiazol indicated only a slow rate of electron transport through the bc_1 complex compared with that in vesicles of MT1131 (not shown).

Photo-oxidation of c-type cytochrome in mutants deficient in cytochrome c_1 and c_2

The strongest support for the view that cytochrome c_1 is a direct and rapid electron donor to the reaction centre come from the finding [6] that, although in the cytochrome-c_2-deficient mutant, MT-G4/S4, there was a fast phase of cytochrome *c* oxidation, no such reaction was detected in the cytochrome-c_1/cytochrome-c_2-deficient double mutant of *Rb. capsulatus*, strain MT-GS18. In complete contrast with this report we found that both in intact cells and in chromatophores of the double mutant a fast phase of oxidation of a *c*-type cytochrome was elicited by a short flash. Fig. 5a shows

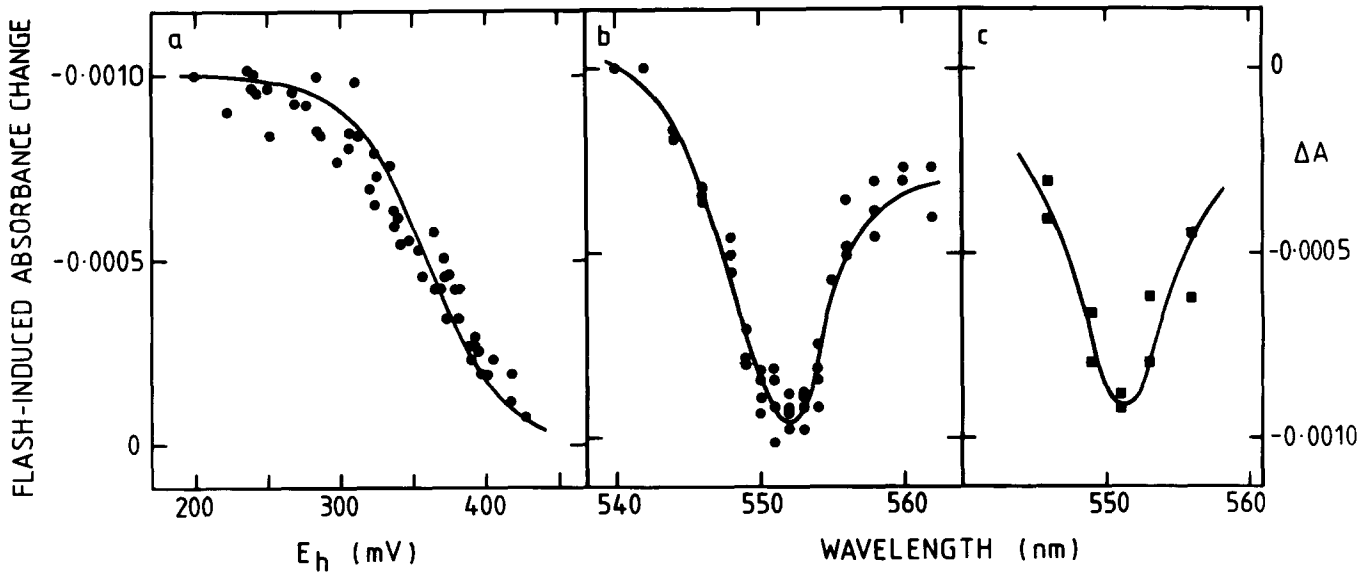

Fig. 6. Redox titration and spectra of the extent of photooxidation of *c*-type cytochrome after a single actinic flash in chromatophores (a, b) and intact cells (c) of *Rb. capsulatus* strain MT-GS18. Intact cells were suspended to 0.2 μM P in 10 mM potassium phosphate (pH 7.6) under strictly anaerobic conditions and chromatophores were suspended to 0.2 μM P in the medium described in Fig. 5a. In (a) the absorbance changes were recorded at 552–542 nm and the E_h was adjusted with potassium ferricyanide and sodium dithionite. In (b) and (c) all measurements were recorded relative to 542 nm. In (b) the chromatophores were poised at an E_h of approx. +250 mV.

a recording of the flash-induced absorbance change at 552–542 nm in chromatophores of MT-GS18 poised at an E_h of +250 mV. The spectrum of the absorbance change is shown in Fig. 6b and clearly reveals the reaction was due to oxidation of a *c*-type cytochrome. The extent of the absorbance change (per mol of P) in MT-GS18 chromatophores was similar to that seen in vesicles from MT-G4/S4. The $t_{1/2}$ for the oxidation in MT-GS18 was less than 400 μs and for the re-reduction greater than 1 s. Myxothiazol at 5 μM was without any effect on the kinetics of the reaction (not shown). The absorbance change was attenuated with an $E_{m7.4}$ of approx. +360 mV (Fig. 6a) and therefore, within the precision of the measurements, resembled the absorbance change due to photooxidation of *c*-type cytochrome in the wild-type and in the cytochrome c_2 mutant (compare with Figs. 1a and 2a). Very similar results were observed with another batch of chromatophores prepared from cells of MT-GS18 grown under the same conditions of aeration as described above, a batch of chromatophores from cells grown at higher aeration and with a batch of chromatophores prepared from cells grown semi-aerobically in the presence of 60 mM dimethylsulphoxide. Interestingly, it was noted that the total amount of photo-oxidisable *c*-type cytochrome in chromatophores prepared from cells grown with high aeration was approx. 3-fold greater than that in chromatophores prepared from cells grown with lower aeration. Rapid oxidation of *c*-type cytochrome after a flash was also evident in strictly anaerobic suspensions of intact cells of *Rb. capsulatus* strain MT-GS18 (Fig. 5b). As in chromatophores the oxidation and re-reduction of this cytochrome were insensitive to myxothiazol, and the spectrum of the absorbance change gave a peak at 551 nm (Fig. 6c).

Discussion

The generation of mutants with specific cytochrome deficiencies, [4,6] is proving to be a valuable tool in the elucidation of the pathways of electron flow in the photosynthetic bacteria. The isolation of a mutant of *Rb. capsulatus* in which the gene for cytochrome c_2 is deleted and which is still capable of phototrophic growth shows unequivocally that this component is not essential for photosynthesis [4]. Observations on the absorbance changes following short flash excitation of membranes from this mutant [5], fully supported by the work described in this paper, show that another *c*-type cytochrome can serve as an electron donor to the reaction centre. It has been suggested [5] that this might be cytochrome c_1, a proposal that was later supported by the finding that cytochrome *c* photo-oxidation was not detectable in a strain in which the genes for both cytochrome c_2 and cytochrome c_1 were deleted [6]. However this proposal is questioned by the findings described above and also by the observations that in order to reconstitute cyclic electron transport between reaction centres and bc_1 complexes in liposomes, both ubiquinone and a soluble *c*-type cytochrome are required [7,8]. We have considered two reasons for the discrepancies between the observations on the cytochrome c_1/c_2-deficient double mutant MT-GS18 reported here and earlier [6]. First, although we employed several different growth conditions for strain MT-GS18 and always detected oxidation of a *c*-type cytochrome after an actinic flash in cells and in chromatophores, it is possible that under the growth conditions used in Ref. 6 this cytochrome was not expressed. Second, our experiments with cells were performed under strictly anaerobic conditions after a substantial pre-incubation under argon (see Ref. 17 for discussion), and experiments with chromatophores were also performed under strictly anaerobic conditions at a controlled redox potential in the presence of redox mediators. Thus, if the photooxidisable *c*-type cytochrome in strain MT-GS18 is only slowly re-reduced after a flash under less reducing conditions, it may escape detection during signal averaging. However the findings that the kinetics, the spectrum and the E_h-dependence of the flash-induced oxidation of *c*-type cytochrome in MT-GS18 closely resembled those in the cytochrome-c_2-deficient mutant MT-G4/S4 in our experiments (see Results) make it very unlikely that cytochrome c_1 is a direct donor to the reaction centre.

Our results are consistent with the view that cytochrome c_2 and a novel *c*-type cytochrome (henceforth described as cytochrome c_x are alternative donors to the reaction centre in the wild-type strain MT1131. However, some structural heterogeneity in the membranes of *Rb. capsulatus* MT1131 (some reaction centres with and some without tightly-bound cytochrome c_x) is also a possibility. Such heterogeneity has been described in *Rhodospirillum rubrum* [18], with a major population of reaction centres having a small number of antenna bacteriochlorophylls and being re-reduced by a soluble cytochrome *c*-420, and a minor population having a large number of antenna bacteriochlorophylls and being re-reduced by a soluble cytochrome *c*-428. It also can not be completely ruled out that cytochrome c_x is the only donor to the reaction centre and that cytochrome c_2 and the bc_1 complex are alternative donors to cytochrome c_x. From the data presented it is not possible to determine with certainty the relative amounts of cytochrome c_2 in strain MT1131. Titration data (Fig. 2) indicate that the amount of cytochrome c_x may be substantial, but it is also possible that there was some contamination by cytochrome c_2.

Following photooxidation, cytochrome c_x is re-reduced, at least in part, by electron transfer from the bc_1 complex, as illustrated by the myxothiazol sensitivity of cytochrome *c* re-reduction and of the electrochromic

absorbance change following flash excitation of intact cells of the cytochrome c_2 deficient mutant (Fig. 3a–c). This explains why the double mutant is unable to grow phototrophically [6]. However, rapid electron transport from bc_1 to cytochrome c_x is less extensive after a flash than that from bc_1 to cytochrome c_2; compare the re-reduction of *c*-type cytochrome and the extents of the myxothiazol-sensitive electrochromic absorbance changes in intact cells of strains MT1131 and MT-G4/S4 (Figs. 3f and 4f). This probably means that during continuous illumination the rate of electron transport from $bc_1 \rightarrow c_x$ is slower than from $bc_1 \rightarrow c_2$ and may explain why MT-G4/S4 grows as fast as MT1131 at high light intensities but at a lower rate at reduced light levels [4]: only at reduced light does the relatively slow rate of electron transfer from the cytochrome bc_1 complex to cytochrome c_x limit growth; at higher intensities other factors common to both strains become limiting.

The biochemical nature of cytochrome c_x is unknown but one possibility is that it is the *c*-type cytochrome of $E_{m7} = -312$ mV observed in dark redox titrations of membranes from MT-GS18 [6]. Cytochrome c_x cannot be a soluble periplasmic component, since, in agreement with Prince et al. [6], we have found that the photooxidisable *c*-type cytochrome in the cytochrome-c_2-deficient mutant is not lost after release of periplasmic components. Cytochrome c_x must be sufficiently close to the reaction centre that electron transport can proceed in less than 100 μs (cf. *Chloroflexus aurantiacus* [19] and see Ref. 5). However, there is no evidence for the presence of a cytochrome in reaction centre preparations of *Rb. capsulatus* [20]. The situation we have described may resemble the model for electron transfer between cytochrome bc_1 and cytochrome oxidase in *Paracoccus denitrificans* [21]. In the latter case a small (M_r 22000) periferal, membrane-bound *c*-type cytochrome has been shown to mediate electron flow between the two transmembrane complexes. A similar cytochrome has been identified in *Rb. capsulatus* [22], but this component is unlikely to be cytochrome c_x, since its redox potential ($E_{m7} = +234$ mV) is too low. Electron flow between the cytochrome bc_1 complex and the reaction centre in MT-G4/S4 could, however, be catalysed by a similar cytochrome of higher redox potential. Our model predicts that one of the membrane-bound *c*-type cytochromes in MT-G4/S4 (other than cytochrome c_1) is an electron donor to P^+. If this model is correct then some PS^- mutants of the cytochrome-c_2-deficient mutant will map in a new locus, outside the *puf* and the *fbc* operons. This new locus should encode cytochrome c_x.

The model presented here does not help to rationalise differences between the electron transport pathways of *Rb. capsulatus* strain MT1131 and of *Rb. sphaeroides* strain 2.4.1. Although cytochrome c_x and iso-cytochrome c_2 seem to serve the same function in supporting photosynthetic growth in the absence of cytochrome c_2 (this work and Refs. 10,11), the evidence suggests that they are structurally quite different proteins. Finally, it is clear that not all 'wild-type' strains of *Rb. capsulatus* have the same complement of electron donors to the reaction centre. In contrast to strain MT1131, there was no evidence for the existence of cytochrome c_x in strain N22 [13]: the kinetics and redox titrations of cytochrome *c* oxidation in right-side-out vesicles were distinctly different in the two organisms. Thus, in strain N22, the data were satisfactorily and completely explained by the conventional view of high-potential electron flow from bc_1 to the photosynthetic reaction centre via cytochrome c_2. In this context it is notable that Hudig et al. [23] reported several years ago that a cytochrome-c_2-deficient mutant of *Rb. capsulatus* strain 37b4 was unable to grow photosynthetically.

Acknowledgments

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