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A THERMODYNAMIC CHARACTERISATION OF THE CYTOCHROMES OF CHROMATOPHORES FROM *RHODOPSEUDOMONAS CAPSULATA*

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SUMMARY

1. The cytochromes of chromatophores from photosynthetically grown *Rhodopseudomonas capsulata* have been characterised both spectrally, using the carotenoid free mutant Ala Pho$^+$, and thermodynamically, using the technique of redox titrations. Five cytochromes were present; two cytochromes *b*, $E'_0 = 60$ mV at pH 7.0; and three cytochromes *c*, $E'_0 = 340$ mV, $E'_0 = 120$ mV, $E'_0 = 0$ mV at pH 7.0.

2. Redox titrations at different values of pH indicated that the mid point potentials of all the cytochromes varied with pH over some parts of the range between pH 6 and 9, with the possible exception of cytochrome c_{340}.

3. The effects of succinate and NADH on the steady state reduction of the cytochromes are reported. Succinate could reduce cytochromes c_{340}, c_{120} and b_{60}; NADH could reduce cytochromes c_{340}, c_{120}, b_{60} and b_{-25}. Cytochrome c_0 could be reduced by dithionite but not by the other substrates tested.

INTRODUCTION

An investigation of energy conservation in *Rps. capsulata* necessitated spectral and thermodynamic characterisation of the electron carriers involved. Klemme [1] has demonstrated the presence of cytochrome *b* and *c* in particulate preparations of *Rps. capsulata*, and Kamen et al. [2] has isolated and characterised cytochrome *c* and *cc'* from the soluble supernatant fraction. The half time of laser-induced cytochrome *c* oxidation is 20–40 μs at 25 °C, but no light-induced cytochrome *c* oxidation at low temperatures has been demonstrated [3]. We have characterised the cytochromes of chromatophores from *Rps. capsulata*, and related the redox characteristics to their reducibility by different substrates. In later papers (Evans, E. H. and Crofts, A. R., see ref. 18) we will identify the cytochromes involved in photosynthetic electron

Abbreviations: MES, 2-(*N*-morpholino)ethanesulphonic acid; TES, *N*-(trishydroxymethyl)-methyl)-2-aminoethanesulphonic acid; PES, phenazine ethosulphate; PMS, phenazine methosulphate.

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flow, together with their involvement in reactions associated with the coupling of electron flow to ATP synthesis.

METHODS

Preparation of chromatophores

Cells of *Rps. capsulata* wild-type and Ala Pho$^+$ carotenoid-deficient mutant [4] were grown as previously described [5] in the medium of Sistrom [6], except that, following inoculation Ala Pho$^+$ cells were allowed to become anaerobic in the dark for 24 h before transfer to the light. Chromatophores were prepared and stored essentially as previously described [5], except that cells were broken by extrusion through a French press at 10 tons pressure for wild-type cells, and 15 tons for Ala Pho$^+$.

The bacteriochlorophyll content was determined by acetone/methanol extraction as described by Clayton [7].

Measurement of redox potential of cytochromes

Dark redox titrations of *Rps. capsulata* wild-type chromatophores were performed essentially as described by Dutton [8] using an anaerobic suspension of chromatophores. Cytochrome changes were monitored using a dual wavelength spectrophotometer as previously described [9].

Cytochromes of the *b*-type were measured at 561 nm with 570 nm as reference wavelength; *c*-type cytochromes were measured at 551 nm with 540 nm as reference wavelength. Chromatophores were suspended in 50 mM KCl, 50 mM buffer, where the buffers used were 2-(*N*-morpholino)ethanesulphonic acid (MES) for pH 6.0 and 6.5; *N*-(trishydroxymethyl)methyl)-2-aminoethanesulphonic acid (TES) for pH 7.0 and Tricine between pH 7.4 and 8.6. The kinetics of light-induced cytochrome changes were also measured in the dual-wavelength spectrophotometer.

The actinic light source (55-W quartz halogen lamp with collimating lens) was covered by a Wratten 88A filter and the photomultiplier was screened by a Corning blue glass filter (No. 9782).

Measurement of difference spectra

Difference spectra were measured by accumulating uncorrected spectra in a mini-computer (PDP 11/10, Digital Equipment Co., Reading, England) interfaced with a Hilger D330 monochromator (Rank Precision Industries) in which the wavelength drive unit was replaced by a stepper motor (G. Berger, Lahr, Germany, Type RDM 50/A). The monochromator was incorporated into a simple single beam spectrophotometer, in which the current from the measuring photomultiplier (Type 9695B, EMI, Hayes, Middlesex) was logarithmically amplified (Analog Devices, operational amplifier Type 755P) before being read by the analogue to digital conversion system (LPS 11-S, Digital Equipment Co.). Base line corrected difference spectra were derived by subtraction of the uncorrected spectra as suggested by Norris and Butler (see ref. 10), and plotted on a CRT display (Tekronix 603 storage monitor) as a 512 or 1024 point trace, with appropriate software-generated labels and scales. The spectrophotometer cuvette housing could accommodate either a conventional cuvette, or an anaerobic redox cuvette with overhead stirring, redox electrodes,

nitrogen flushing ports and a self sealing rubber septum for anaerobic additions, similar in principle to that of Dutton [8].

MATERIALS

Simple organic or inorganic reagents were of Analar grade where obtainable, but otherwise of the highest grade commercially available.

Antimycin was obtained from Sigma (London) Chemical Co.

RESULTS AND DISCUSSION

Difference spectra of Rps. capsulata Ala Pho$^+$ chromatophores

Fig. 1 shows reduced minus oxidised spectra of *Rps. capsulata* Ala Pho$^+$ chromatophores. Fig. 1a shows a cytochrome with α peak at 551 nm and β peak at 522 nm which was oxidised by ferricyanide but reduced by sodium ascorbate. Suc-

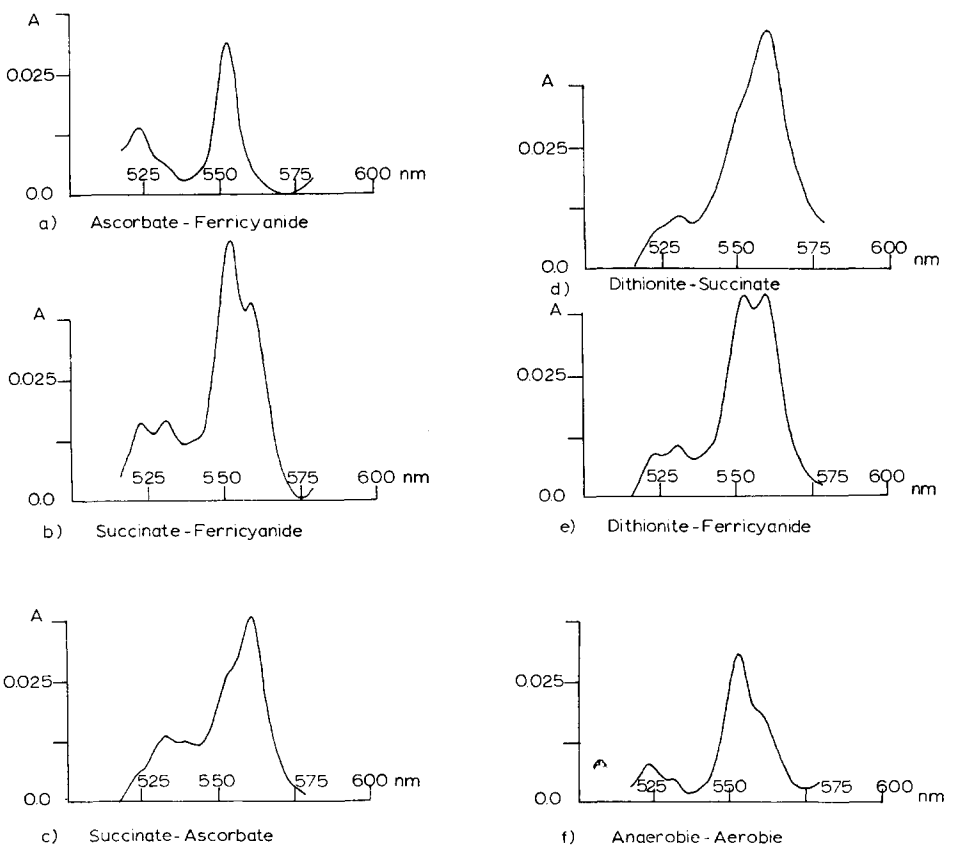


Fig. 1. Oxidised minus reduced difference spectra of *Rps. capsulata* Ala Pho$^+$ chromatophores. Chromatophores suspended to 89 $\mu g \cdot ml^{-1}$ in 200 mM glycylglycine buffer (pH 7.6). Additions were 200 μM $K_3Fe(CN)_6$ (FeCN), 400 μM sodium ascorbate (asc), 1 mM sodium succinate (succ), and sodium dithionite (a few crystals) as indicated.

cinate increased the level of the reduction with a small shift to 552 nm and 528 nm of the peaks at 551 nm and 522 nm, and caused reduction of a cytochrome with α peak at 560 nm and β peak at 532 nm (Figs 1b and 1c). Further cytochromes were reduced by addition of dithionite, increases in absorption at 552 (α), 523 (β), 559 (α) and 531 (β) being seen (Figs 1d and 1e). These changes could be ascribed to the reduction by dithionite of both *c*- and *b*-type cytochromes which were not reduced by succinate. On transition from anaerobic to aerobic conditions oxidation of cytochromes with peaks at 551 and 560 nm was observed, to give a difference spectrum similar to that observed for succinate minus ferricyanide (Fig. 1f). The full extent of the change took several minutes to develop.

Dark redox titrations of cytochromes

Dark redox titrations of *Rps. capsulata* wild-type chromatophores at 551 nm with 540 nm as reference, at pH 7.0, could be resolved into three components with mid-point redox potentials of 340 mV (cytochrome c_{340}), 120 mV (cytochrome c_{120}) and 0 mV (cytochrome c_0). In the titration shown, these components comprised 50, 34 and 16 % of the change respectively (Fig. 2a) but these values were somewhat variable. In some preparations cytochrome c_{120} was barely detectable. The potential values may be related to reduced minus oxidised spectra of Fig. 1 in that the component with a mid-point potential at 340 mV would be expected to be reduced by sodium ascorbate, but oxidised by ferricyanide, that at 120 mV would be reduced by succinate (see on) and that at 0 mV only by dithionite.

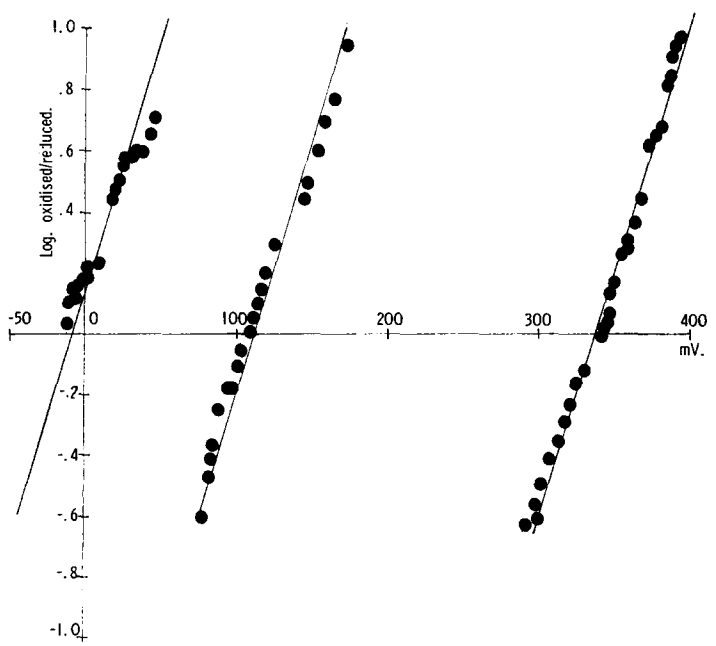
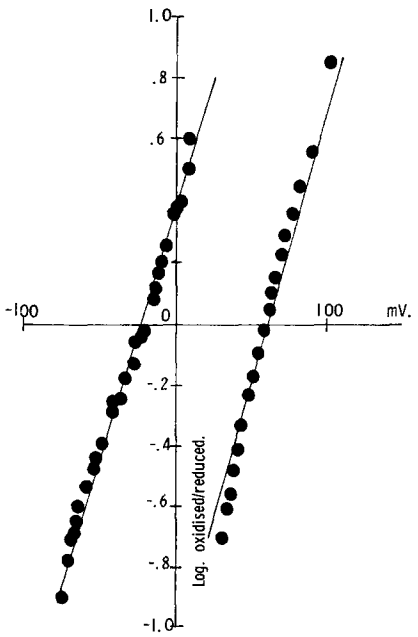
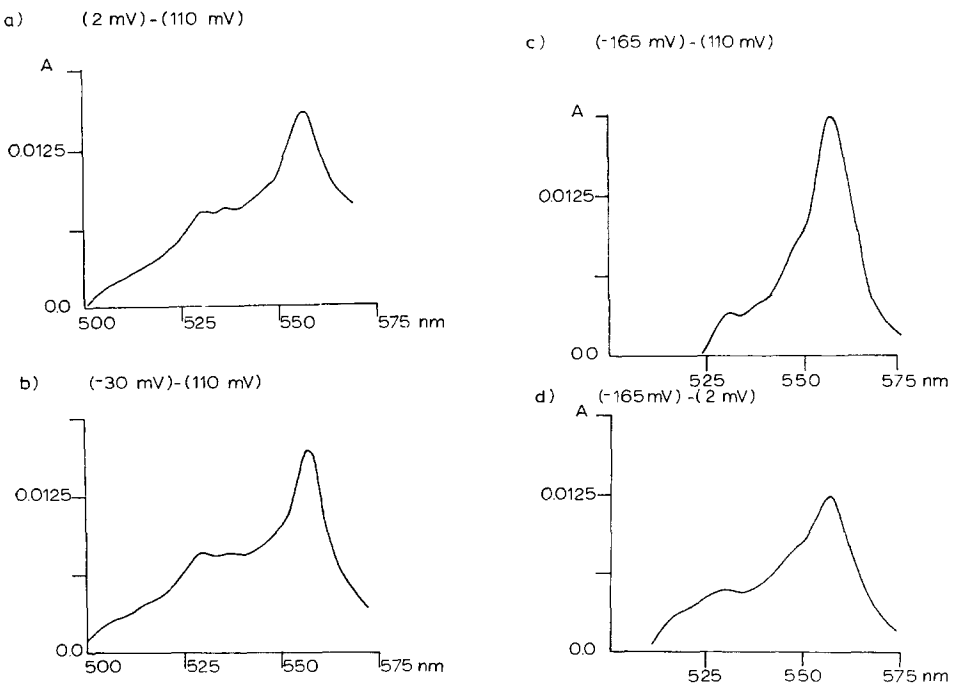


Fig. 2. Dark redox titrations of cytochromes *c* and cytochromes *b*. (a) 551 nm, with 540-nm reference wavelength. *Rps. capsulata* wild-type chromatophores suspended to 0.1 mg · ml^{-1} bacteriochlorophyll in 50 mM TES, 50 mM KCl (pH 7.0). Redox dyes present 20 μM diaminodurene, PMS, PES, 2-hydroxy 1,4-naphthoquinone, 7 μM pyocaynine and 100 μM $K_3Fe(CN)_6$. (b) 561 nm with 570 nm





reference wavelength. Conditions as for a, but with no $K_3Fe(CN)_6$. (c) Difference spectra at fixed redox potential as indicated. Chromatophores suspended to 60 $\mu g \cdot ml^{-1}$ bacteriochlorophyll in 50 mM TES, 50 mM KCl (pH 7.0). Redox dyes present 10 μM, diaminodurene, PMS, PES, 2-hydroxy-1,4-naphthoquinone, 3.5 μM pyocyanine.

Dark redox titrations at 561 nm with 570 nm as reference at pH 7.0, could be resolved into two components, one with a mid-point redox potential of 60 mV (cytochrome b_{60}) and the other of −25 mV (cytochrome b_{-25}) at pH 7.0 (Fig. 2b). They comprised 60 and 40 % of the change, respectively. From these values, the oxidised minus reduced spectra of Fig. 1 would be expected to show reduction of cytochromes b_{60} and c_{120} by ascorbate and succinate while cytochrome b_{-25} should only be reduced by dithionite. In fact, cytochrome b_{60} and c_{120} were reduced by ascorbate only very slowly and appreciable reduction was not observed under aerobic conditions. In the presence of PMS and the absence of oxygen, ascorbate was able to reduce cytochrome b_{60}, but the cytochrome would not have been reduced under the conditions of Fig. 1. Fig. 2c, shows difference spectra of the chromatophores in a suspension where the redox potential was poised at the values indicated. These show more clearly the low potential components separated according to their mid-point

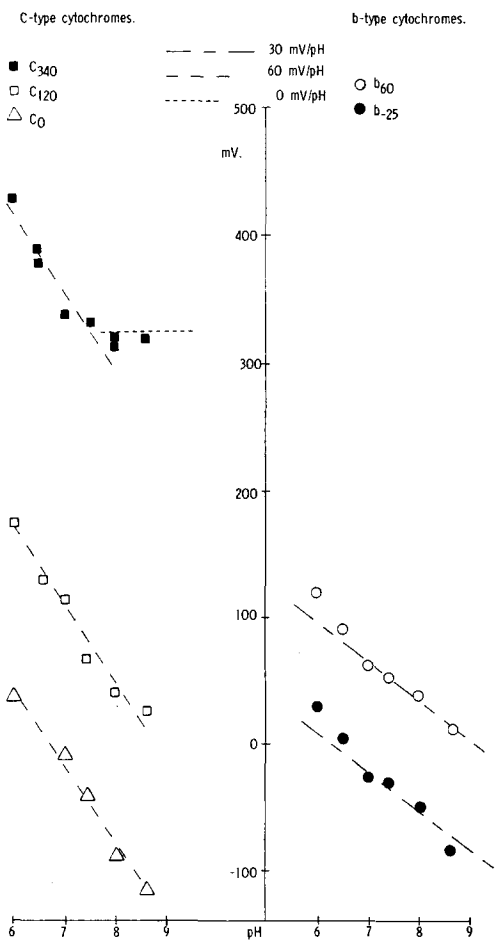


Fig. 3. The dependence on pH of the redox potentials of cytochromes of *Rps. capsulata* wild-type chromatophores. Conditions as for Fig. 2. pH 6.0, 6.5 in 50 mM MES, 50 mM KCl; pH 7.0 in 50 mM TES, 50 mM KCl; pH 7.4, pH 8.0, pH 8.6 in 50 mM tricine, 50 mM KCl.

TABLE I

CHARACTERISTICS OF *RPS. CAPSULATA* CYTOCHROMES

Cytochrome	α peak* (nm)	β peak* (nm)	E'_0 (pH 7.0)* (mV)	E'_0/pH*** (mV)	Quantity† (moles/mole bacterio-chlorophyll)	Laser-induced†† turnover (moles/mole bacterio-chlorophyll)
c_{340}	551	522	340	60†††	1:150	1:110
c_{120}	552	523	120	60	1:250	—
c_0	552	523	0	60	1:375	1:220
b_{60}	560	532	60	30	1:115	1:310 1:130
b_{-25}	559	531	−25	30	1:125	—

* At 20 °C, values are ±1 nm.

** The best approximation of $n = 1$ curve to all titrations; in no case is an $n = 2$ curve a better fit to any point. All values are ±10 mV.

*** Simplest approximation to experimental values.

† Assuming extinction coefficients of 15 mM^{-1} for cytochrome *b* and *c*.

†† Laser induced turnover of P605 per bacteriochlorophyll is 1:50, using an extinction of 52 mM^{-1} determined by Prince, R. C. and Cogdell, R. J. (personal communication).

††† Only 60 mV/pH up to pH 7.0. Above pH 7.0, no apparent change, but see text.

§ With antimycin.

potentials. Fig. 3 shows the variation of the apparent mid-point potentials of the cytochromes of *Rps. capsulata* chromatophores on titration at different values of pH. With the possible exception of cytochrome c_{340} (see below) the cytochromes showed some variation with pH. In the case of cytochrome c_{340}, at values above 7 there was no dependence on pH and the dependence at lower values of pH was variable. In aged preparations of chromatophores no marked variation of *Em* with pH was observed, suggesting that the effect seen with fresh preparations may be due to sluggish equilibration at lower values of pH. We are investigating the variability of pH dependence using alternative mediating systems in the redox titrations. The significance of these effects will be discussed in a later paper [18].

Table I summarises the properties of *Rps. capsulata* cytochromes identified and characterised in this work. The variation of mid-point potential with pH shown is only approximate (Fig. 3, and above). The amount of cytochrome, and the amount of cytochrome undergoing a redox change following flash excitation [18] are in the last two columns. In view of the variability from preparation to preparation, comparison of these values may be taken to indicate that most of cytochromes b_{60} and c_{340} are involved in photosynthetic electron flow. The role of cytochrome c_0 is more ambiguous and will be discussed in greater detail elsewhere [18]. Cytochrome b_{-25} does not appear to take part in rapid light induced reactions.

The effect of substrates on the redox state of Rps. capsulata cytochromes

Table II summarises the effectiveness of succinate and NADH in reducing the total pools of cytochromes *c* and *b*. The steady-state levels of reduction of cytochromes *c* by succinate and NADH were virtually the same, and were unaffected by

TABLE II

Wavelength (nm)	Substrate	Aerobicity	% of total pool reduced
551–540	Succinate	aerobic	64
		anaerobic	
551–540	Succinate + KCN	aerobic	77
		anaerobic	
551–540	NADH	aerobic	67
		anaerobic	
551–540	NADH + KCN	aerobic	77
		anaerobic	
561–570	Succinate	aerobic	45
		anaerobic	
561–570	Succinate + KCN	aerobic	45
		anaerobic	
561–570	NADH	aerobic	22
561–570	NADH + KCN	aerobic	29
561–570	NADH	anaerobic	99

aerobic or anaerobic conditions. Cyanide caused a slight increase in the level of reduction. Under controlled redox conditions, i.e. anaerobic in the presence of redox dyes, cytochrome c_{340} and c_{120} could be reduced by ascorbate, cytochromes c_{340} and c_{120} by succinate and NADH, but cytochrome c_0 was only accessible to dithionite. In the absence of redox dyes, the proportion of the total cytochrome *c* reduced on adding succinate or NADH was similar to the proportion of the total cytochrome contributed by cytochromes c_{340} and c_{120}, suggesting that these cytochromes are readily reduced by the substrates under physiological conditions. The extent of the light-induced oxidation of *c*-type cytochrome was unaffected by the presence of succinate or NADH, although the kinetics were modified (Fig. 4). In the absence of

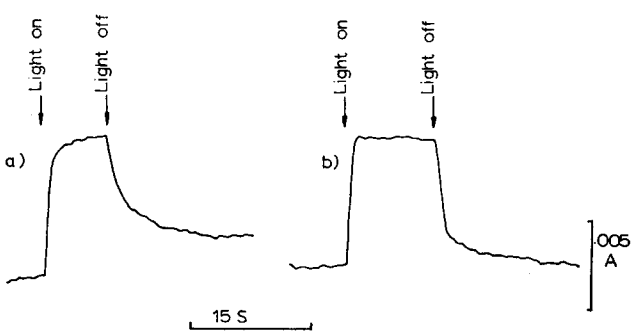


Fig. 4. The effect of succinate on light induced oxidation of cytochrome *c*. *Rps. capsulata* Ala Pho$^+$ chromatophores suspended to 50 mg · ml^{-1} bacteriochlorophyll, in 50 mM TES, 50 mM KCl (pH 7.0). (a) No additions, (b) With 10 mM succinate.

substrate, re-reduction in the dark was slow, while in the presence of succinate or NADH, cytochrome *c* oxidation was rapidly reversed. It seems likely that in the presence or absence of substrate the photooxidation observed was that of cytochrome c_{340}, and that the constant extent of oxidation indicates that the other cytochrome (c_{120}) reduced by the substrate is not readily photooxidized.

Table II also shows the extent of reduction of the total cytochromes *b* pool by succinate and NADH. In contrast to reduction of the cytochromes *c*, the same extent of reduction was not produced by both substrates. Cyanide had no effect on the extent of cytochrome *b* reduction induced by succinate, but slightly increased the level of reduction produced by NADH. Under anaerobic condition, nearly all the cytochrome *b* pool could be reduced by NADH, but the extent of reduction by succinate remained the same. When the redox potential of the suspending medium was held constant at a potential where cytochrome b_{-25} was oxidised but cytochrome b_{60} reduced, a transient reduction of cytochrome b_{-25} could be demonstrated on addition of small amounts of NADH. This suggests that NADH was able to reduce cytochrome b_{-25} more rapidly than the cytochrome was able to interact with the mediating dyes. In the absence of mediating dyes, the proportions of the total cytochromes *b* reduced by succinate and NADH were similar respectively to the proportions contributed by cytochrome b_{60}, and b_{60} plus b_{-25}. It seems likely therefore that NADH is able to react readily with cytochrome b_{-25} under physiological conditions, and through this cytochrome, with cytochrome b_{60}. Antimycin A had no effect on the level of reduction of the cytochromes *b* by substrates, but had a marked effect on the light induced cytochrome *b* reduction (Figs 5 and 6). In the absence of antimycin there was only a small light induced reduction. When a proportion of the total cytochrome *b* pool had been reduced by succinate, the light induced reduction was no longer seen but could occasionally be replaced by a small oxidation. These results are consistent with the suggestion that cytochrome b_{60} is concerned in photosynthetic electron flow, but may also be reduced by succinate. Antimycin caused an increase in the extent of light induced reduction of cytochrome *b*, as would be expected from the proposed site of inhibition of photosynthetic electron flow [11]. However, the cytochrome was readily reoxidised in the dark in the presence of antimycin. Cyanide had no effect on this reoxidation, but NADH appeared to slow it down. There may therefore be a variety of pathways for oxidation of cytochrome b_{60}.

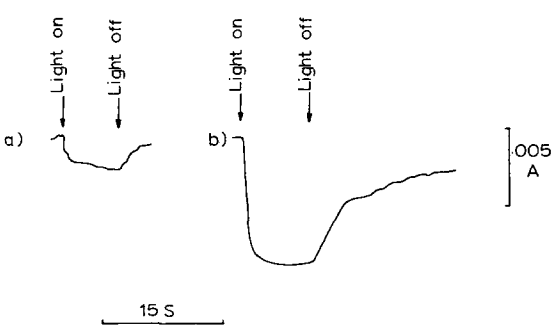


Fig. 5. The effect of antimycin on light-induced cytochrome *b* reduction. Chromatophores suspended as for Fig. 4. (a) No additions. (b) With 2 μM antimycin.

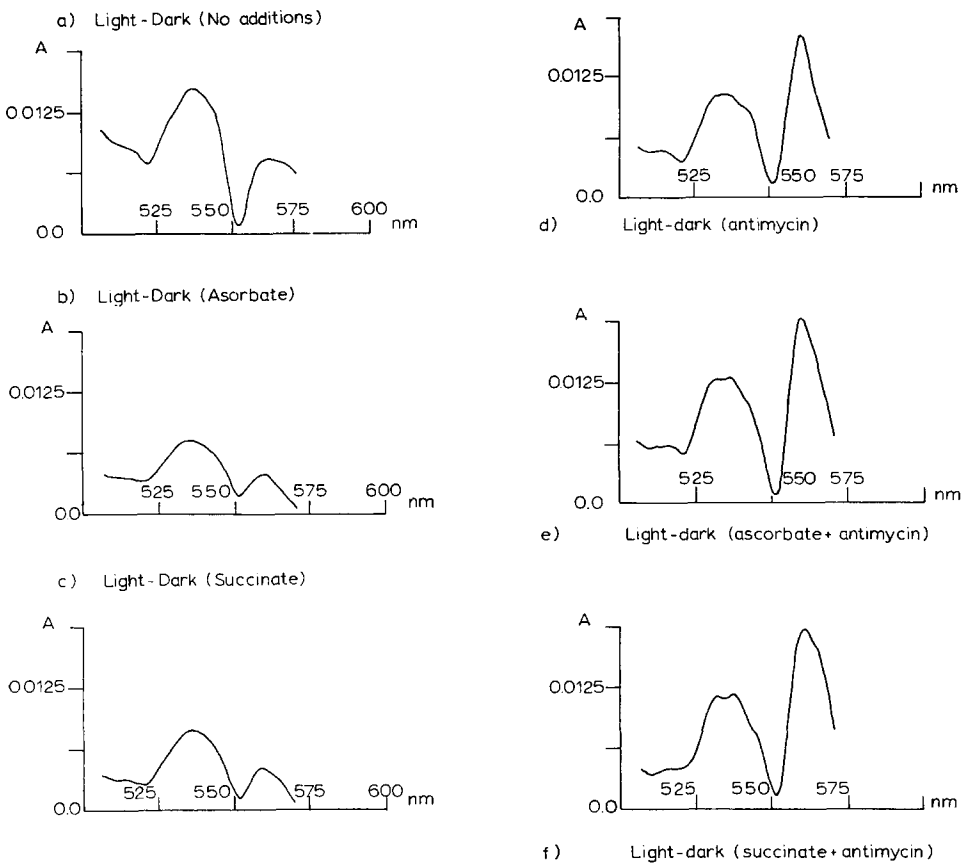


Fig. 6. Spectra of the light-induced change in the presence and absence of antimycin. *Rps. capsulata* Ala Pho$^+$ chromatophores (45 $\mu g \cdot ml^{-1}$) were suspended in 200 mM glycylglycine (pH 7.6). Antimycin (2 μM) was added where shown, with sodium succinate (4 μM), or sodium ascorbate (3.2 mM) as indicated.

CONCLUSION

Our results suggest that five cytochromes are present in chromatophores of photosynthetically grown *Rps. capsulata*. Two of these, cytochrome c_{340} and cytochrome b_{60}, are concerned with photosynthetic electron transport. Both could be reduced by succinate, after which the kinetics of the light induced changes of both cytochromes were modified. It has been suggested [12] that succinate and other reducing agents may poise the electron transport system at a redox potential optimal for coupled electron flow, and it seems likely that our observations are related to this effect. Cytochrome c_{120} could also be reduced by succinate, but cytochrome b_{-25} was reduced only by NADH. The relationship of cytochrome c_0 to electron transport is not clear from these experiments. However, such low potential cytochromes c (c_3) have been isolated from other bacteria [13, 15] and it has been proposed that they are concerned with hydrogen lyase activity [15]. In view of the rapid growth of *Rps. capsulata* in the presence of H_2 [16, 17], cytochrome c_0 may function in this capacity.

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