Biochimica et Biophysica Acta, 396 (1975) 220–228

BBA 46958

DETECTION OF TWO FURTHER *b*-TYPE CYTOCHROMES IN *RHODOPSEUDOMONAS SPHEROIDES*

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(Received January 24th, 1975)

SUMMARY

The photosynthetically-incompetent mutant V-2 of *Rhodopseudomonas spheroides* which is incapable of synthesising bacteriochlorophyll was grown aerobically under conditions of both high and low aeration. Potentiometric titration at 560 nm minus 570 nm revealed the presence of several different components tentatively identified as *b*-type cytochromes. Two such components of oxidation-reduction midpoint potentials of $+390$ mV $\pm$ 10 mV and $+255$ mV $\pm$ 7 mV have not previously been detected in membranes of *Rps. spheroides*. These components have also been resolved by difference spectra at controlled oxidation-reduction potentials and fourth derivative spectra. Neither component appeared to react with CO. With increasing aeration of the culture medium the relative concentration of these two *b*-type cytochromes diminished, whilst that of the *a*-type oxidase increased.

INTRODUCTION

Rhodopseudomonas spheroides can grow both photosynthetically, under anaerobic conditions in the light, and aerobically in darkness. Accordingly the mechanism of adaptation of its electron transport system may be usefully studied to give information about the assembly of energy conserving membranes. In a previous communication three membrane-bound *b*-type cytochromes have been identified by potentiometric titration in both aerobically- and photosynthetically-grown cells of *Rps. spheroides* [1]. In aerobically-grown cells there is evidence for the development of a terminal oxidase of the *a*-type [2–6] with oxidation reduction midpoint potentials similar to those of cytochromes *a* and a_3 of the mitochondrial cytochrome oxidase [7]. Furthermore, the concentration of this *a*-type cytochrome appears to increase with increasingly efficient aeration of the culture.

Even photosynthetically-grown cells, which lack *a*-type cytochrome, can respire and furthermore possess some CO binding pigments. In an attempt to detect high potential components, other than the *a*-type, which may function as respiratory oxi-

Abbreviations: TES, *N*-Tris[hydroxymethyl]methyl-2-aminoethane sulphonic acid; TMPD, Tetramethyl-*p*-phenylene diamine.

dases for *Rps. spheroides* further studies on electron transport components of the *b*-type have been carried out and are described in this communication.

Since we have found that even under aerobic conditions there is sufficient synthesis of bacteriochlorophyll in *Rps. spheroides* to interfere with absorbance measurements at 560 nm minus 570 nm at high potentials, mutant V-2 which is incapable of synthesizing bacteriochlorophyll [7] has been used in these studies. This mutant was grown for the most part under conditions of relatively low aeration to suppress development of the *a*-type oxidase.

METHODS

Growth of cells and preparation of particles

Mutant V-2 was isolated as previously described [7] and grown at 30 °C in the medium of Sistrom [8] either in culture vessels filled to 65 % of their nominal capacity with medium and sparged with air via a glass sinter, or in a vigorously aerated fermenter.

Particles were prepared from cells disrupted in a French pressure cell, as described previously [6].

Spectrophotometry

The split beam spectrophotometer used in this work [9] had a reciprocal dispersion of 26Å/mm. For spectra at 77 °K the slit widths were set at 0.5 mm. Difference spectra at fixed oxidation-reduction potentials were obtained using gas tight low temperature cuvettes modified from the design of Dutton [10].

Difference spectra at controlled potentials and fourth derivative spectra were obtained at room temperature using a mini-computer interfaced with a single beam scanning spectrophotometer [11]. Syringes containing dithionite and ferricyanide were attached to a stepper motor under the control of the computer which regulates additions of dithionite or ferricyanide according to data received from the platinum-calomel electrode pair within the cuvette, so as to maintain the desired redox potential in the cuvette. The dual wavelength spectrophotometer used was essentially based on the design of Chance [12].

Determination of oxidation-reduction mid-point potentials

The anaerobic procedures were those of Dutton et al [13] using a stirred, gassed cuvette fitted with platinum and calomel electrodes. Samples were withdrawn for low temperature spectroscopy using gas-flushed syringes as described by Dutton [10].

Protein assay

Proteins were determined by the method of Lowry et al. [14]

RESULTS

When cells of mutant V-2 are grown aerobically by sparging with air the development of *a*-type cytochrome is suppressed to some extent, when compared with cells grown with vigorous aeration in a fermenter (i.e. the concentration of *a*-type

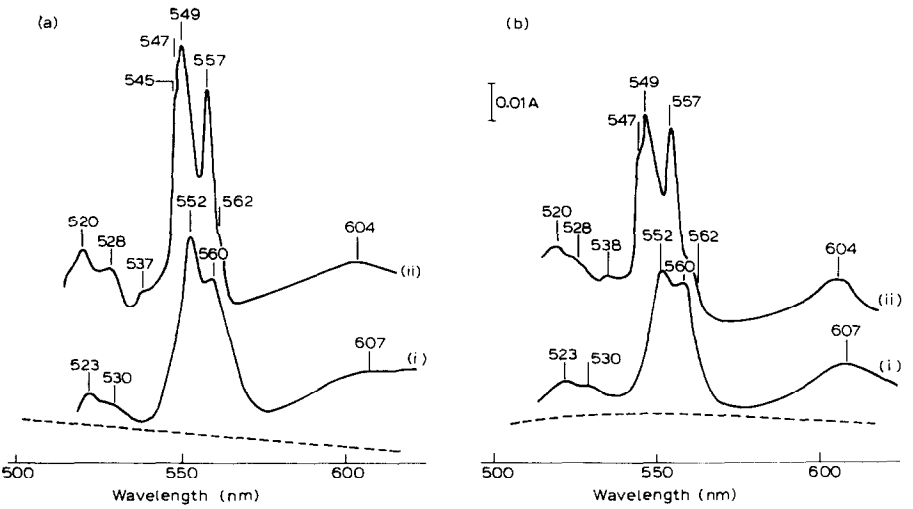

Fig. 1. Dithionite-reduced minus oxidised difference spectra of particles from aerobically-grown *Rps. spheroides* strain V-2. Particles prepared from cells grown (a) aerobically by sparging with air; (b) aerobically with vigorous aeration in a fermenter were suspended at a concentration of approximately 1.8 mg protein/ml in 50 mM TES, 50 mM KCl, 10 % (w/v) sucrose (pH 7.0). Dithionite was added to the test cuvette (i) spectrum recorded at room temperature; (ii) spectrum recorded at 77 °K. - - -, baseline.

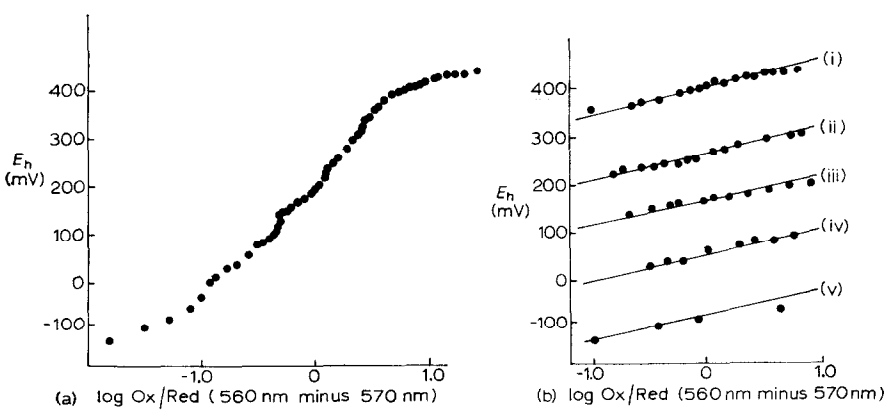

Fig. 2. Potentiometric titration at 560 nm minus 570 nm in particles from aerobically-grown *Rps. spheroides* strain V-2. Particles prepared from cells grown aerobically by sparging with air were suspended in 50 mM TES, 50 mM KCl, 10 % (w/v) sucrose (pH 7.0) at a concentration of approximately 2.0 mg protein/ml. 100 μM potassium ferricyanide, 40 μM diaminodurol, 25 μM phenazine methosulphate, 25 μM phenazine ethosulphate, 20 μM pyocyanine and 20 μM 2-hydroxy 1,4-naphthoquinone were present as mediators. Potentials were made more negative by successive additions of succinate, NADH and dithionite and more positive by adding potassium ferricyanide. Oxidative or reductive titrations, produced similar results. (b) The data from (a) have been replotted according to the method of Dutton et al. [13]. The lines drawn through the points are theoretical $n = 1$ lines derived from the Nernst equation. E'_0 (pH 7.0) from these lines: (i) +395 mV; (ii) +260 mV; (iii) +165 mV (iv) +45 mV; (v) −95 mV.

falls from approximately 0.2 nmole/mg protein to 0.07 nmole/mg protein). This is shown in the dithionite reduced minus oxidised difference spectra of particles (Fig. 1).

Potentiometric titration at 560 nm minus 570 nm (Fig. 2) of particles obtained from aerobically-grown cells low in *a*-type cytochrome indicates the presence of the same three *b*-type cytochromes that have previously been found in *Rps. spheroides* [1, 15] with oxidation-reduction midpoint potentials at pH 7.0 of +160 mV +40 mV and -90 mV, but in addition two other components were detected with midpoint potentials at pH 7.0 of +390 mV ±10 mV and +255 mV ±7 mV. Analysis of the titration curve shows that these two components contribute approximately 20 % and 15 % respectively to the total absorbance change at 560 nm minus 570 nm within the potential range of +450 mV and -150 mV. These two components were also detected in membranes obtained from cells grown with vigorous aeration and therefore richer in *a*-type cytochrome. However in such membranes under such growth conditions both components contributed less to the total absorbance change at 560 nm minus 570 nm (approximately 10 % for the +390 mV component, and for the +255 mV component).

It was not possible to detect such high potential components in particles from photosynthetically-grown cells due to interference presumably from bacteriochlorophyll bleaching at these oxidation-reduction potentials.

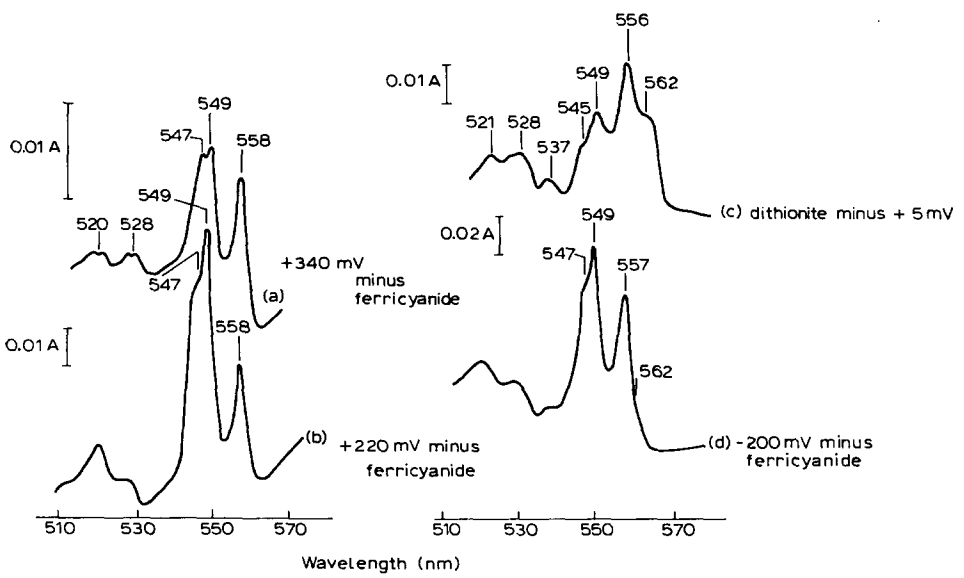

Fig. 3. Low temperature difference spectra at controlled oxidation-reduction potentials of particles from aerobically-grown *Rps. spheroides* strain V-2. Particles from cells grown aerobically by sparging with air were suspended in 50 mM TES, 50 mM KCl, 10 % (w/v) sucrose (pH 7.0) at a concentration of approximately 2.5 mg protein/ml in the redox cuvette. The mediators, as described in Fig. 2(a), were added. At the oxidation-reduction potentials shown samples were removed from the cuvette using a gas flushed syringe (see Methods) and injected into one of the gas flushed low temperature cuvettes, the other cuvette contained particles either oxidised with ferricyanide or reduced with dithionite. The low temperature cuvettes were immediately plunged into liquid N_2 and the difference spectra recorded. Note the change of scale for each figure.

In order to determine the separate spectra of the various components, difference spectra have been constructed at controlled oxidation-reduction potentials at 77 °K (Fig. 3) using particles from cells grown aerobically by sparging with air (see Methods). These spectra confirm the presence of *b*-type components with midpoint potentials higher than those previously reported. At +340 mV (Fig. 3a) there is reduction of a component with α-band maximum at 77 °K of 558 nm tentatively identified as a *b*-type cytochrome. This is accompanied by some reduction of cytochrome c_2 (the major membrane-bound *c*-type component is of oxidation-reduction midpoint potential +295 mV with some at +340 mV), with α-band maximum at 549 nm, shoulder at 547 nm. At +220 mV (Fig. 3b) there is an increase in reduction at 558 nm and cytochrome c_2 is almost completely reduced, acting as an internal check on the potential within the membrane. The difference spectrum in Fig. 3c shows reduction of *b*-type cytochromes with maximum at 556 nm and shoulder at 562 nm presumably corresponding to cytochrome *b* +40 and cytochrome *b* −90 [1] both of which should be contributing to the difference spectrum at that potential. There is also reduction of some *c*-type (presumably not cytochrome c_2 or *c* +120 which have previously been detected in aerobically-grown V-2 [7]). Fig. 3d shows the difference spectrum over the total oxidation-reduction range covered by the titration.

Reduced minus oxidised difference spectra were obtained at various redox potentials using a computerised spectrophotometer as follows: a series of spectra were recorded at certain controlled redox potentials and stored in the computer memory store, difference spectra were then obtained by subtraction of one such

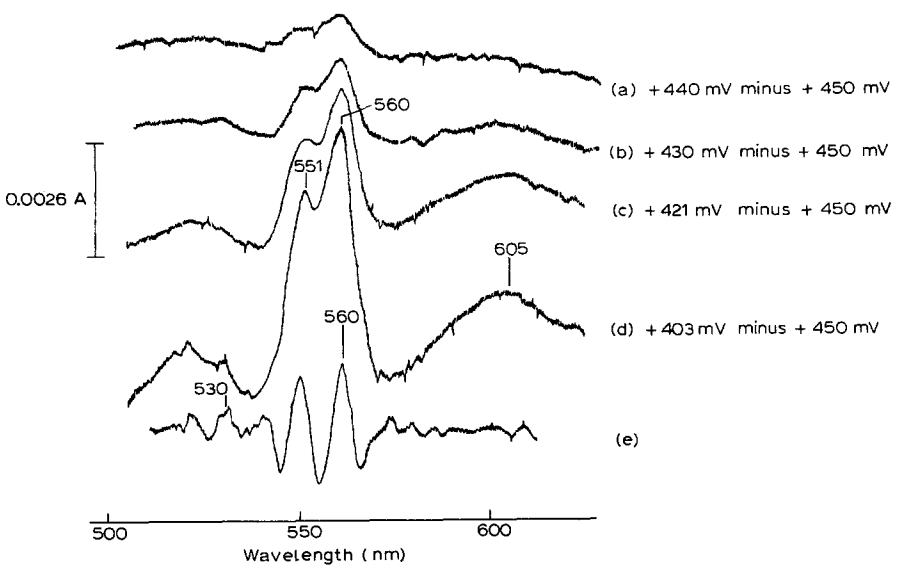

Fig. 4. Difference spectra at controlled oxidation-reduction potentials of particles from aerobically-grown *Rps. spheroides* strain V-2. Particles from cells grown aerobically by sparging with air were suspended in 50 mM TES, 50 mM KCl (pH 7.0) at a concentration of approximately 4.0 mg protein/ml in the redox cuvette. Spectra were recorded at room temperature at the redox potentials shown (see Methods for detail of the computerised spectrophotometer used) and stored in the computer memory store. The desired difference spectra ((a), (b), (c), (d)) were then obtained by subtractions. Fig. 4(e) is the fourth derivative of the difference spectrum shown in (d).

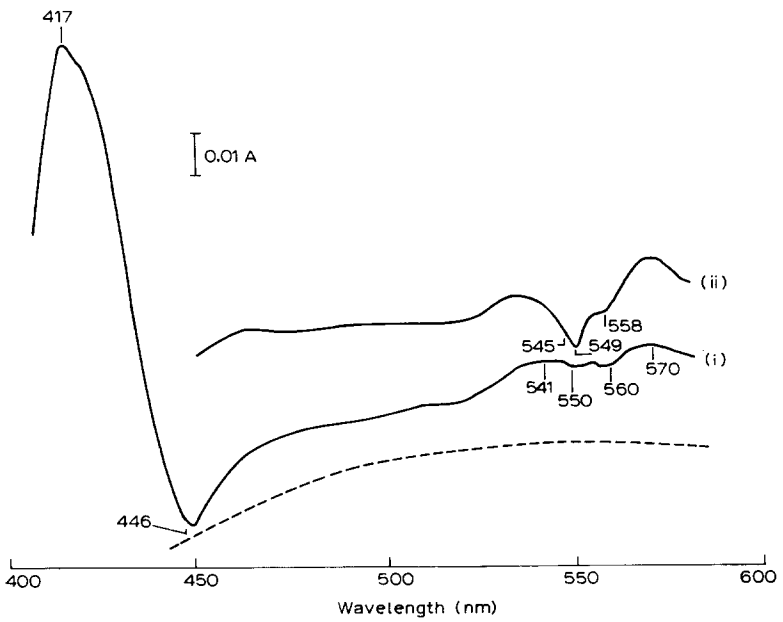

Fig. 5. CO difference spectra of particles from *Rps. spheroides* strain V-2. Particles prepared from cells grown aerobically by sparging with air were suspended in 50 mM TES, 50 mM KCl, 10 % (w/v) sucrose (pH 7.0) at a concentration of approximately 1.8 mg protein/ml. 2.0 mM ascorbate + 5.0 μM TMPD were added and particles allowed to become anaerobic by respiratory activity. CO was bubbled into the test cuvette and spectra recorded 5 min after CO addition, (i) at room temperature: (ii) at 77 °K. - - -, baseline before CO addition.

spectrum at known potential from another at a different known potential as indicated in Fig. 4. In all these spectra reduction of a component with maximum at 560 nm at room temperature is evident. In addition, there is some reduction of components with maximum at 605 nm (presumably *a*-type cytochrome of redox midpoint potential of $+375$ mV $\pm$ 10 mV [7]) and at 551 nm (presumably cytochrome c_2). The fourth derivative of the difference spectrum at $+403$ mV minus $+450$ mV (Fig. 4e) shows maxima at 560 nm and 530 nm which probably correspond to the α- and β-bands of the *b*-type cytochrome of redox midpoint potential $+390$ mV (b_{+390}).

The CO difference spectra of particles of V-2 (Fig. 5) indicate that components reduced by ascorbate /TMPD (which in the case of *b*-type cytochromes would probably reduce only the high potential components) can complex with CO. Troughs are apparent at 560 nm and at 550 nm and broad maxima at 541 nm and 570 nm suggesting the possible presence of *o*-type cytochrome in these particles. However, preliminary results taken from titrations at 560 nm minus 570 nm using a 50 % CO/ 50 % argon gas phase indicate that the redox midpoint potentials of the $+390$ mV and $+255$ mV components are not affected by CO. Such results might exclude the possible equivalence of the $+390$ mV *b*-type with the *o*-type cytochrome which is reported to be present and function as an oxidase in *Rps. spheroides* [2, 3, 6].

DISCUSSION

In a previous communication in which *b*-type cytochromes were reported for *Rps. spheroides* [1] the redox potential range over which titrations were performed was from +240 mV to −160 mV. At higher potentials measurements of absorbance changes were hindered by bacteriochlorophyll changes, thus necessitating the use of a bacteriochlorophyll-less strain in order to characterise possible high potential components.

Potentiometric titration, difference spectra at controlled oxidation-reduction potentials and fourth derivative spectra all support the presence of high potential components absorbing at 560 nm minus 570 nm, tentatively identified as *b*-type cytochromes. CO appeared to have no effect on the redox midpoint potentials of either of these components and it has therefore been assumed that neither component reacts with CO, since for the *a*-type cytochrome oxidase of mitochondria CO induced shifts in the redox midpoints of both cytochrome *a* and a_3 (from +210 mV and +385 mV to +255 mV and +570 mV respectively). These shifts supposedly resulted from the direct liganding of the CO to cytochrome a_3 haem [see ref. 16]. On the other hand, the CO could be binding to these *b*-types without significantly affecting their redox potentials.

By virtue of its high potential the b_{+390} cytochrome could possibly function as an oxidase in respiration. However its apparent inability to complex with CO argues against it being an *o*-type cytochrome. As aeration of the culture is reduced so the relative concentration of cytochrome b_{+390} appears to increase whilst that of the *a*-type oxidase decreases. This suggests that synthesis of both b_{+390} and the *a*-type oxidase of *Rps. spheroides* are controlled by oxygen tension such that when synthesis of the *a*-type is suppressed, that of b_{+390} is increased and vice versa.

TABLE I

SUMMARY OF OXIDATION-REDUCTION MIDPOINT POTENTIALS OF *b*-TYPE CYTOCHROMES IN PARTICLES PREPARED FROM AEROBICALLY- AND PHOTOSYNTHETICALLY-GROWN *RPS. SPHEROIDES*

Oxidation-reduction midpoint potentials were measured at 560 nm minus 570 nm as described in Methods. For particles from photosynthetically-grown cells, the cytochrome change at 560 nm minus 570 nm has been taken over the potential range of +240 mV and −160 mV.

Growth conditions	E'_0 (pH 7.0) mV	Proportion of total cytochrome change (%) approx.
Aerobic*	+390±10	20
	+255± 7	15
	+160±10	20
	+ 40±10	35
	− 90±10	8
Photosynthetic	+160±10	28
	+ 45±10	55
	−100±10	15

* Cells were grown aerobically by sparging with air (see Methods).

Recently a high potential *b*-type cytochrome of redox midpoint potential at pH 7.0 of +410 mV (b_{410}) has been detected in a closely related organism *Rhodopseudomonas capsulata* [17]. This bacterium lacks *a*-type cytochrome even when grown with vigorous aeration [18]. There is substantial evidence from the use of mutants of *Rps. capsulata* that b_{410} functions as an oxidase in respiration, even though this *b*-type cytochrome does not react with CO [17]. Cytochrome b_{410} has been proposed as an atypical *o*-type or new oxidase of the *b*-type [17] and the results reported in this communication indicate some similarity between b_{+390} of *Rps. spheroides* and b_{410} found in *Rps. capsulata*. Since there are both respiratory and photosynthetic electron transport systems in *Rps. spheroides* it is perhaps not surprising that multiple *b*-type cytochromes have been found (Table I). Indeed studies on plant mitochondria [19, 20] have shown that four *b*-cytochromes are associated with the respiratory chain and that other *b*-types that are dithionite-reducible are also present.

In our study of the kinetic and thermodynamic properties of the membrane-bound cytochromes of *Rps. spheroides* [1] the same three *b*-type cytochromes were identified in both aerobically- and photosynthetically-grown cells. This was so despite the gross differences in membrane complexity between the two types of cell. However the presence or absence of these high potential *b*-type cytochromes (redox midpoint potentials +390 mV and +255 mV) in photosynthetically-grown cells cannot be established. Therefore, it is not possible to determine whether such cytochromes develop and function solely in aerobically-grown *Rps. spheroides*.

ACKNOWLEDGEMENTS

This work has been generously supported by grants from the Science Research Council. We would like to thank Dr A. R. Crofts for the use of his computerised spectrophotometer and D. Crowther for assistance with the difference spectra obtained from it.

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