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PROPERTIES OF THE CYTOCHROME *a*-LIKE MATERIAL DEVELOPED IN THE PHOTOSYNTHETIC BACTERIUM *RHODOPSEUDOMONAS SPHEROIDES* WHEN GROWN AEROBICALLY

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SUMMARY

A photosynthetically-incompetent mutant *Rhodopseudomonas spheroides* that lacks bacteriochlorophyll was isolated. Spectroscopic evidence from CO difference spectra and cyanide difference spectra suggested that a cytochrome oxidase was present in this mutant that contained two components, corresponding to cytochromes *a* and a_3 of mitochondria. Potentiometric titration at 607 nm also showed the presence of two components with oxidation–reduction mid-point potentials of +375 mV and +200 mV. They were present in a ratio close to unity. No cytochrome of the *c*-type corresponding to mitochondrial cytochrome *c* was detected, but a minor *c* component (near 10% of the total cytochrome *c*) with an oxidation–reduction mid-point potential of +120 mV was found

Growth of the mutant in medium with low aeration or lacking added copper diminished the concentration of the *a*-type cytochrome but not the concentrations of cytochromes of the *b* and *c*-type.

INTRODUCTION

The purple non-sulphur bacterium *Rhodopseudomonas spheroides* grows anaerobically in the light using the light energy harvested by the carotenoids and chlorophylls that are characteristic of this organism when grown photosynthetically. Aeration suppresses the formation of these pigments and leads to the development of what is apparently a new terminal oxidase [1, 2, 3, 4], a pigment with absorption maxima at 445 and 607 nm, spectroscopically similar to the cytochrome oxidase of mammalian and plant mitochondria. In our study [5] of the kinetic and thermodynamic properties of the membrane-bound cytochromes of aerobically- and photosynthetically-grown *R. spheroides* it became apparent that there was little change in the nature of the *b* and *c* cytochromes, i.e. about 90% of the cytochrome *c* from each type of cell had a redox mid-point potential around +290 mV. The same three *b*-cytochromes that had been described in photosynthetically-grown *R. spheroides* by Dutton and Jackson [6] were present in the aerobically-grown cells, although the proportion of the cytochrome *b* with a potential near +42 mV was less when the

cells were grown aerobically [5]. It thus appeared that the respiratory electron transport chain was constructed using the same haem-protein components that were present in the photosynthetic chain and that the new cytochrome oxidase interacted with these components. Since these bacteria appear to lack a cytochrome *c* with the solubility, mid-point potential, and high isoelectric point of the cytochrome *c* that couples with mitochondrial cytochrome oxidase we have studied the properties of this 607 nm absorbing material to determine whether it differs from the mitochondrial $a+a_3$ type of oxidase.

Even in aerobically-grown cells sufficient synthesis of bacteriochlorophyll occurs to interfere with the measurement of absorbance changes due to the presumed oxidase (reaction centre bacteriochlorophyll has an absorption maximum near 605 nm). We have therefore used in our work a mutant of *R. spheroides*, designated V-2, that is incapable of synthesising bacteriochlorophyll.

METHODS

Preparation of the bacteriochlorophyll-less mutant of R. spheroides

The mutant, V-2, was prepared by treatment of cells of strain 2.4.1. with *N*-methyl-*N*-nitroso-*N*-nitroguanidine [7]. Photosynthetically-incompetent (Pho^-) cells were selected using a penicillin-screening technique [8].

Growth of cells

The mutant was normally grown at 30 °C in liquid media in a vigorously aerated continuous culture apparatus [4] in the medium of Sistrom [9]. For some experiments the cells were grown in the same medium in partly-filled Erlenmeyer flasks in a shaking incubator. The rate of aerobic growth (3–3.5 h doubling time) was the same for mutant V-2 as for wild type cultures. Particles were prepared from cells disrupted in a French pressure cell, as described previously [4].

Spectrophotometry

The split beam spectrophotometer used in this work (ref. 10) had a reciprocal dispersion of 26 Å/mm. For spectra at 77 °K the slit widths were set at 0.15 mm.

Determination of oxidation–reduction mid-point potentials

The anaerobic procedures were those of Dutton et al. [11], using a stirred, gassed cuvette, fitted with platinum and calomel electrodes [5].

RESULTS

Neither bacteriochlorophyll nor the carotenoids characteristic of aerobically-grown wild-type cells of *R. spheroides*, were detected in acetone–methanol extracts of the Pho^- strain, V-2. The dithionite-reduced minus oxidised difference spectra of particles prepared from strain V-2 (Fig. 1) show apparently the same *b* and *c*-type cytochromes that are found in facultatively anaerobic strains of *R. spheroides* when grown aerobically. Potentiometric titration at 560 nm shows that the same three components, that were found in particles from the green mutant of *R. spheroides* (ref. 5) were present and at 552 nm, 90% of the change was due to a component with a mid-point potential of +285 mV. The absorption bands due to a presumed *a*-type component,

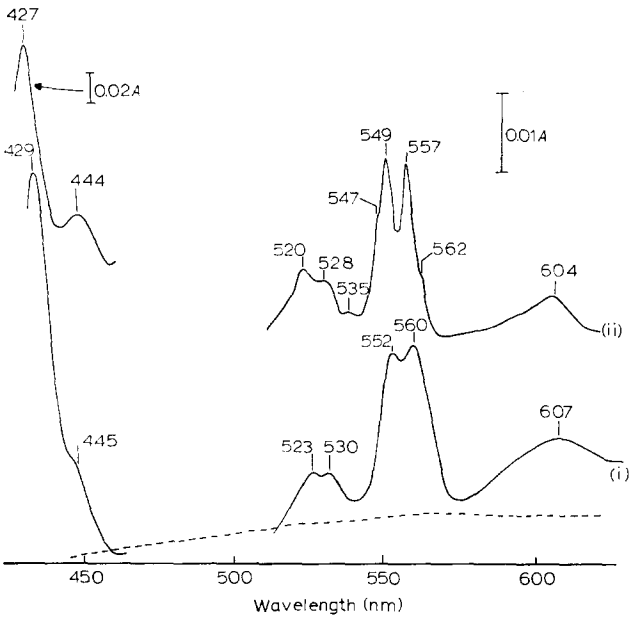

Fig. 1. Dithionite-reduced minus oxidised difference spectra of the particulate fraction of *R. spheroides* strain V-2. Particles were suspended at a concentration of approximately 2.0 mg protein/ml in 10 mM TES (pH 7.5). Dithionite was added to the test cuvette: (i) spectrum recorded at room temperature; (ii) spectrum recorded at 77 °K. - - -, baseline.

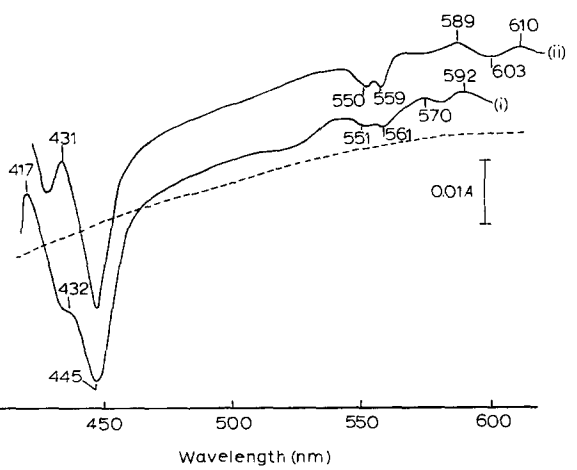

Fig. 2. CO difference spectra of the particulate fraction of *R. spheroides* strain V-2. Particles were suspended in 10 mM TES (pH 7.5) at a concentration of approximately 2.0 mg protein/ml, and reduced with dithionite. CO was bubbled into the test cuvette and spectra recorded 5 min after CO addition, (i) at room temperature; (ii) at 77 °K. - - -, baseline before CO addition.

at 607 nm and 445 nm are, however, clearer in strain V-2 than in cells of either the green mutant or wild type and it is possible to show that the proportion of *a*-type cytochrome relative to cytochromes *b* and *c* in cells of strain V-2 increases with increasingly efficient aeration of the culture.

CO-difference spectra of particles of V-2 (Fig. 2) suggest that a component forms a complex with CO, with absorption properties similar to those of cytochrome a_3 (λ_{max} at 592 nm, trough at 445 nm). Some other CO-binding pigment, with troughs at 561 nm and 551 nm, possibly an *o*-type cytochrome, was also present. For purified mitochondrial cytochrome oxidase it has been possible to obtain difference spectra of each of the haem components of the a/a_3 complex by the use of cyanide, which stabilises a_3 in its oxidised state (cf. ref. 13). When such difference spectra were constructed for particles of V-2, it appeared that two components contributed to the absorption in the 607 nm region (Fig. 3). With CN^- present in both cuvettes (keeping any a_3 in the Fe^{3+} state) the addition of dithionite to the sample caused the appearance of an absorption band at 597 nm but little absorption at 445 nm; this might correspond to the difference spectrum a^{2+} minus a^{3+}. When CN^- was present in the reference cuvette and dithionite was added to both cuvettes, a difference spectrum with maxima at 610 nm and 445 nm was obtained. By similar reasoning this might be due to the $a_3{}^{2+}$ minus $a_3{}^{3+}$ difference spectrum.

The interpretation of CN^- difference spectra has been made from experiments with purified cytochrome oxidase (cf. ref. 13) and may not be applicable to bacterial membrane preparations. Therefore a potentiometric titration was carried out (at 607 nm minus 630 nm) to provide additional evidence for the presence of two com-

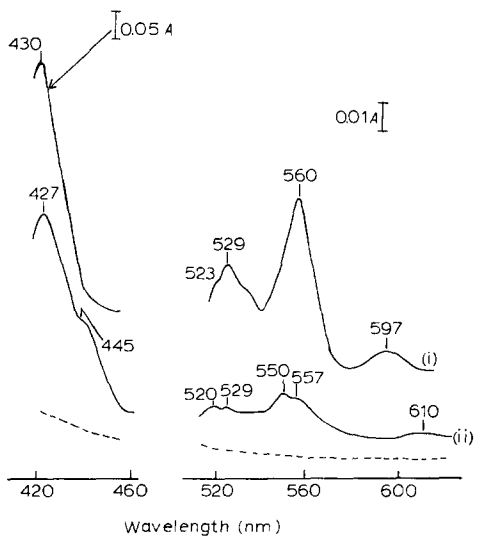

Fig. 3. Cyanide difference spectra of the particulate fraction of *R. spheroides* strain V-2. Particles were suspended in 10 mM TES (pH 7.5) at a concentration of approximately 5.0 mg protein/ml. (i) 150 μM KCN was added to both test and reference cuvettes, followed by addition of dithionite to test cuvette, spectrum recorded at room temperature. (ii) 150 μM KCN was added to reference cuvette, followed by addition of dithionite to both cuvettes, spectrum recorded at room temperature. - - -, baseline.

ponents corresponding to cytochrome *a* and a_3. Such a titration has been shown to distinguish between *a* and a_3 in mitochondria [11, 14] and in the bacterium *Nitrobacter agilis* [15]. The results of such a titration (Fig. 4) show that there are indeed two components present and a replot of these components shows that each has an *n* value of 1.0 (Fig. 5). At pH 7.0 the high potential component has a redox mid-point poten-

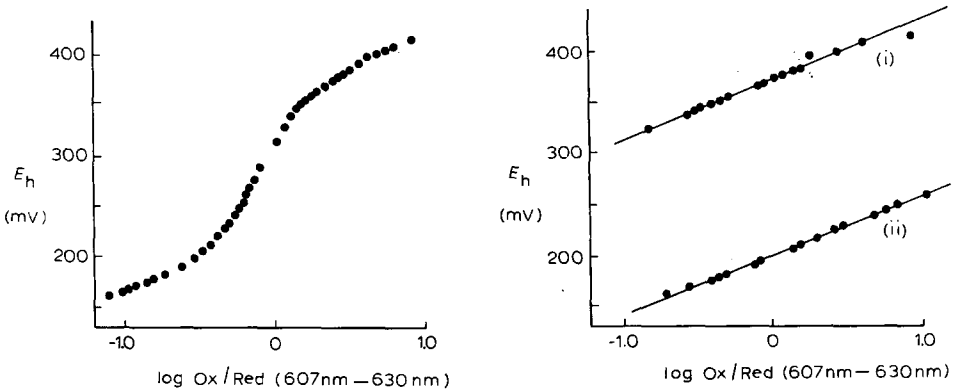

Fig. 4. Potentiometric titration at 607 nm minus 630 nm in particles prepared from *R. spheroides* strain V-2. Particles were suspended in 50 mM TES, 50 mM KCl (pH 7.0) at a concentration of approximately 6.0 mg protein/ml. 100 μM potassium ferricyanide, 40 μM diaminodurol, 20 μM phenazine methosulphate and 20 μM phenazine ethosulphate were present as mediators. Oxidation–reduction potentials were made more positive by adding potassium ferricyanide. Potentials were made more negative by successive additions of succinate, NADH and dithionite. Oxidative or reductive titrations produced similar results.

Fig. 5. Replot of the data from Fig. 4, showing the resolution of the curve into two $n = 1$ components. The lines drawn through the points are theoretical $n = 1$ lines derived from the Nernst equation. E'_0 (pH 7.0) from these lines was: (i) +375 mV; (ii) +200 mV.

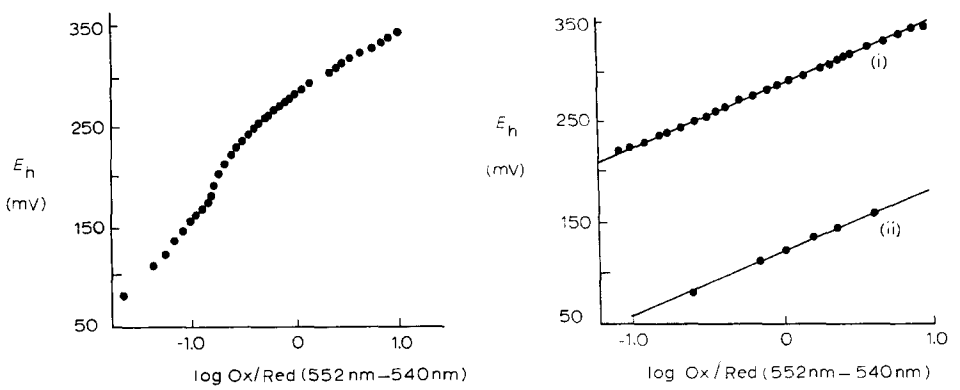

Fig. 6. Potentiometric titration at 552 nm minus 540 nm in particles prepared from *R. spheroides* strain V-2. Particles were suspended in 50 mM TES, 50 mM KCl (pH 7.0) at a concentration of approximately 4.0 mg protein/ml. Other experimental conditions were as described for Fig. 4.

Fig. 7. Replot of the data from Fig. 6, showing the resolution of the curve into two components. The lines drawn through the points are theoretical $n = 1$ lines derived from the Nernst equation. E'_0 (pH 7.0) from these lines was: (i) +285 mV; (ii) +120 mV.

tial of +375 mV (±10 mV) and the second component has a redox mid-point potential of +200 mV (±10 mV). Analysis of the curve shows that approximately 56% of the total oxidised minus reduced absorbance change at 607 minus 630 nm when the potential fell from +450 mV to +120 mV, is contributed by the high potential component and 44% by the low potential component.

Mitochondrial cytochrome oxidase is a copper-containing complex and it has been shown that when yeast cells are grown under conditions of copper limitation the cytochrome oxidase level of the cells decreases [16, 17]. Preliminary studies with the mutant V-2 show that omission of copper from the growth medium in batch culture leads to a fall in the cytochrome oxidase content of the cells of about 50–60%.

In a previous communication [2] it was claimed that an oxidase complex could be extracted from aerobically-grown *R. spheroides* with 2% Triton X-100 and that associated with this complex was a *c*-type cytochrome with a mid-point potential of +246 mV. We have carefully titrated the intact membrane preparations from V-2 in the cytochrome *c* region of the spectrum and have not detected such a component. It is possible to show that a minor cytochrome *c* component is present in the membranes to an extent of approximately 10% of the total cytochrome *c*. Replotting this minor component shows that it has a redox mid-point potential of +120±10 mV with an *n* value of 1.0. A very similar cytochrome has also been found in the soluble fraction of the cell.

DISCUSSION

In a recent short publication [18] it was shown that purified reduced cytochrome c_2 of *Rhodospirillum rubrum* was oxidised very poorly by purified cytochrome *c* oxidase of bovine heart mitochondria. This low activity may be due to the considerable difference between the net charge at pH 7.0 on cytochrome c_2 and on mammalian cytochrome *c*. *R. rubrum* cytochrome c_2 has a pI of 6.2 (ref. 19) and mammalian cytochrome *c* a pI of 10.7 (ref. 20) and their electrostatic interactions with the oxidase would be very different. Cytochrome c_2 of *R. spheroides* has a pI of 5.5 (ref. 19) and would not be expected to bind well with a mammalian cytochrome oxidase. Our results show, however, that the *a*-type cytochrome that develops in *R. spheroides* in response to aeration has some of the properties described for a mitochondrial type of oxidase. It has two haem components that can be resolved by cyanide difference spectra, CO difference spectra, and by potentiometric titration. The mid-point potentials of these two haem components correspond well with those of cytochromes *a* and a_3 of mitochondria [11, 14] and they are present in a ratio reasonably close to unity. No cytochrome *c* corresponding to mitochondrial cytochrome *c* (E_0' around +230 mV [11]) was found in *R. spheroides* membranes, nor in the soluble fraction, although a minor cytochrome *c* component, $E_0' = +120$ mV, was present.

The function of the cytochrome *o* in aerobic metabolism is far from clear. By using the V-2 mutant that lacks bacteriochlorophyll it has been possible to show clearly that the amount of *a*-type cytochrome (per mg protein) in *R. spheroides* membranes increases with increased aeration but the cytochrome *o*-type persists and the amount of *b* and *c*-type cytochrome (per mg membrane protein) does not alter significantly.

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