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OXIDATIVE PHOSPHORYLATION AND EFFECTS OF AEROBIC CONDITIONS ON *RHODOPSEUDOMONAS VIRIDIS*

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SUMMARY

Particles prepared from photosynthetically grown *Rhodopseudomonas viridis*, which has previously been described as an obligate photo-organotroph, catalyse oxidative phosphorylation. The P:O ratio is 0.3 with succinate as substrate and 0.45 with NADH. The reaction is inhibited by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and oligomycin. Antimycin A or 2-heptyl-4-hydroxyquinoline-*N*-oxide inhibit succinate or NADH oxidation as well as oxidative phosphorylation.

After repeated transfers of cultures from agar plates, kept aerobic in the dark, cells of *R. viridis* were capable of limited exponential aerobic growth. In such cells no bacteriochlorophyll and very little carotenoid was detected; there was also a loss of the characteristic membrane structure found in photosynthetic cells. The K_m for oxygen of these cells was lowered from 0.35 ($\pm$0.09) to 0.007 ($\pm$0.002) mM and an additional cytochrome-like pigment was observed, with an absorption maximum at 560 nm at 77 °K. There was also an increased amount of a CO-binding pigment in aerobically grown cells.

INTRODUCTION

The purple non-sulphur bacterium *Rhodopseudomonas viridis* is reputed to be an obligate photo-organotroph[1], unlike most members of the *Athiorhodaceae*. Cytochromes of the *c*-type (*c*550.5, *c*553 and *c*558) and small amounts of what is possibly cytochrome *cc'* or a *b*-type cytochrome have been detected in whole cells of this organism[2], but no *o*- or *a*-type cytochromes have been reported.

Previously we have demonstrated energy-dependent NADH formation in *R. viridis*[3] by a reversed electron flow process indicating the presence of an energy conserving site between NADH and the cytochrome chain. A NADH oxidase system was also shown in the particulate fraction, but since *R. viridis* is supposedly unable to grow aerobically it was unlikely to proceed through a coupling site.

In this paper we describe evidence for both NADH and succinate oxidase systems and for oxidative phosphorylation in *R. viridis*. Following repeated

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; HQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

incubation aerobically in the dark, we have obtained cells of *R. viridis* capable of limited aerobic growth and compared properties of such cells with those grown anaerobically in the light.

MATERIALS AND METHODS

Growth of organisms and preparation of particles

The strain of *R. viridis* used (Pfennig's original strain) was grown either anaerobically in the light and particles prepared from cultures as described previously[3], except that 20 mM *N*-tris(hydroxymethyl)methyl-χ-aminoethanesulphonic acid (TES) (pH 7.0) was used, or grown aerobically in the same medium at 30 °C. For aerobic growth cultures were incubated in erlenmeyer flasks filled to 20% of their nominal capacity and shaken vigorously in an orbital incubator. Samples were checked for purity by aerobic growth on agar plates and by subsequent illuminated anaerobic growth.

Measurement of rate of growth

Doubling times of cultures grown aerobically in the dark and anaerobically in the light were measured by two methods; both by spectrophotometric measurement of absorbance of the culture at 680 nm, and by viable counts. For viable counts, 0.1 ml aliquots were removed from the cultures at various time intervals during growth, and serial dilutions were spread on agar plates to give between 30 and 300 cells per plate. Plates were incubated under light anaerobic conditions for 5–7 days and the colonies formed were counted. Thus the doubling time was determined both from a doubling of absorbance at 680 nm and of the number of cells per ml, during exponential growth.

Enzyme assays

Succinate and NADH oxidase activity were measured polarographically using a Clark-type oxygen electrode in a 5-ml reaction vessel maintained at 30 °C. Cytochrome *c* oxidase was measured at 30 °C by following the rate of oxidation of reduced horse heart cytochrome *c* in a dual wavelength spectrophotometer at 550 *minus* 540 nm. Cytochrome *c* reductase was measured at 30 °C as the rate of reduction of horse heart cytochrome *c* at 550 *minus* 540 nm. Reoxidation of reduced cytochrome *c* was prevented by adding 8 mM KCN to the 20 mM TES (pH 7.0) used for this assay. The extinction coefficient of 19.1 mM^{-1} given by Chance[4] for reduced *minus* oxidised cytochrome *c* was used.

Formation of ATP by oxidative phosphorylation

[^{32}P]ATP was estimated by the method adapted from Avron[5] and Nielsen and Lehninger[6]. The reaction mixture contained in 5 ml: 20 mM TES (pH 7.0), 2.5 mM ADP, 8.0 mM $Na_2H^{32}PO_4$, 2.0 mM NaH_2PO_4 1.7 mM $MgCl_2$, particles (15 mg protein) and either 8 mM succinate or 3.3 mM NADH. Incubations were at 30 °C, in the dark and stirred continuously. The formation of ATP was confirmed using the luciferase assay according to the method of Stanley and Williams[7]. The determinations of ATP formed agreed within 5%.

Spectrophotometry

Split-beam spectra were recorded as described in ref. 3.

Protein

Protein was determined by the method of Lowry *et al.*[8], with bovine serum albumin as standard.

RESULTS

Particles prepared from cells of *R. viridis* grown anaerobically in the light catalysed the oxidation of NADH and succinate. Rates of oxidation were significantly lower than those for similar particulate preparations of other purple non-sulphur bacteria capable of aerobic growth[9,10], but like these were sensitive to cyanide, antimycin A and 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) (Table I). Illumination of *R. viridis* particles had no effect on these rates of oxidation. The respiratory activity of cell free extracts of *Rhodospirillum rubrum* is reported to be either unaffected[11] or stimulated by light[12], for *Rhodopseudomonas spheroides* we have observed light stimulation of oxygen uptake in particulate fractions.

ATP synthesis took place aerobically in the dark with either succinate or NADH as substrate in particles of photosynthetically grown *R. viridis*. The incorporation of $^{32}P_i$ under aerobic conditions was linear with respect to time. Measured P:O ratios were higher for the oxidation of NADH than for succinate (Table II). The reaction was inhibited by antimycin A and cyanide and was dependent upon the presence of ADP and oxygen. The uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and also oligomycin were inhibitory.

The effect of various inhibitors upon particulate cytochrome *c* reductase activity is summarised in Table III. The inhibition of NADH cytochrome *c* reductase by antimycin A was partially overcome by addition of *N,N,N',N'*-tetramethyl-*p*-

TABLE I

EFFECT OF INHIBITORS ON OXIDASE ACTIVITY OF PARTICLES PREPARED FROM PHOTOSYNTHETICALLY GROWN *R. VIRIDIS*

O_2 uptake was measured at 30 °C using an oxygen electrode. The incubation mixture contained in 5 ml:20 mM TES (pH 7.0) and particles (25 mg protein). 8 mM succinate or 3.3 mM NADH was added to start the reaction. Additions were made to the incubation mixture as indicated. Control rates: (μgatom O per min per mg protein) succinate oxidase 0.004; NADH oxidase 0.0054.

Addition	*Concn*	*Inhibition (%)*	
		Succinate oxidase	*NADH oxidase*
Antimycin A	2 μM	0	0
	4 μM	17	0
	6 μM	20	30
HQNO	5 μM	9	14
	10 μM	25	28
	15 μM	40	42
Rotenone	5 μM	0	66
KCN	1 mM	65	45
	2 mM	87	63
	8 mM	—	77

TABLE II

OXIDATION OF NADH AND SUCCINATE AND ASSOCIATED ATP SYNTHESIS BY PARTICLES PREPARED FROM PHOTOSYNTHETICALLY GROWN *R. VIRIDIS*

Rates of oxidation of NADH and succinate and [^{32}P]ATP formation were measured as described under Materials and Methods. The incubation mixture in 5 ml:20 mM TES (pH 7.0), 2.5 mM ADP, 8.0 mM $Na_2H^{32}PO_4$, 2.0 mM NaH_2PO_4, 1.7 mM $MgCl_2$ and either 8.0 mM succinate or 3.3 mM NADH. Particles (15 mg protein) were added to start the reaction. Final specific activity of P_i was 0.05 Ci/mole; final pH 7.2. Additions and omissions were made to the incubation mixture as indicated.

Condition	*Rate of O_2 uptake (ngatoms O/min/mg protein)*	*Rate of incorporation $^{32}P_i$ (nmoles/min/mg protein)*	*P:O*
+Succinate	4.2	1.25	0.297
+NADH	5.7	2.5	0.438
+Succinate+FCCP (1.6 μM)	5.5	0.125	0.023
+NADH+FCCP (1.6 μM)	6.8	0.225	0.033
+Succinate+oligomycin (4 μg/ml)	4.0	0.69	0.17
+NADH+oligomycin (4 μg/ml)	5.5	1.2	0.22
+Succinate+antimycin A (6 μM)	3.36	1.0	0.29
+NADH+antimycin A (6 μM)	3.99	1.75	0.43
Omit succinate	0.17	0.05	0.29
+Succinate+KCN (1 mM)	1.47	0.45	0.3
+NADH+KCN (1 mM)	3.13	1.37	0.43
+Succinate, omit ADP	4.0	0.025	0.006
+NADH, omit ADP	5.4	0.125	0.023
+Succinate, anaerobic (under N_2)	0	0.13	—
+NADH, anaerobic (under N_2)	0	0.125	—

TABLE III

EFFECT OF INHIBITORS ON CYTOCHROME *c* REDUCTASE ACTIVITY OF PARTICLES FROM PHOTOSYNTHETICALLY GROWN *R. VIRIDIS*

Reduction of cytochrome *c* was measured at 30 °C at 550 *minus* 540 nm in 20 mM TES (pH 7.0) containing 8 mM KCN, 30 μM horse heart cytochrome *c*, particles (0.1 mg protein) and either 6 mM succinate or 1 mM NADH was added to start the reaction. Control rates: (μmoles cytochrome *c* reduced per min per mg protein) succinate cytochrome *c* reductase: 0.065; NADH cytochrome *c* reductase: 0.086. Additions were made as indicated.

Addition	*Concn (μM)*	*Rate of cytochrome c reduction as % of control*	
		Succinate–cytochrome c reductase	*NADH–cytochrome c reductase*
Antimycin A	3	0	5
HQNO	16	60	40
Rotenone	10	100	12
Antimycin A +TMPD	3 30	0	60

phenylenediamine (TMPD). However, TMPD did not abolish the inhibition by antimycin A of succinate cytochrome *c* reductase. It would seem that TMPD creates a bypass of the antimycin A inhibition site between NADH and added cytochrome *c*. No significant incorporation of $^{32}P_i$ was detected in particles of photosynthetically grown *R. viridis* in the presence of either NADH or succinate, if respiration was blocked by cyanide and added cytochrome *c* was the electron acceptor.

A soluble NADH cytochrome *c* reductase activity was found in *R. viridis*. It was unaffected by the inhibitors of particulate cytochrome *c* reductase. The specific activity of the soluble NADH cytochrome *c* reductase was 10% of the particulate NADH cytochrome *c* reductase.

An oxidation of reduced cytochrome *c*, sensitive to cyanide and CO was catalysed by particles from photosynthetically grown *R. viridis* (Table IV). Antimycin A and HQNO were without effect.

TABLE IV

EFFECT OF INHBITORS ON CYTOCHROME *c* OXIDASE ACTIVITY BY PARTICLES PREPARED FROM PHOTOSYNTHETICALLY GROWN *R. VIRIDIS*

Oxidation of cytochrome *c* was measured at 30 °C at 550 *minus* 540 nm in 20 mM TES (pH 7.0) containing 5 μM reduced horse heart cytochrome *c*. Particles (0.1 mg protein) were added to start the reaction. Additions were made as indicated.

Addition	*Concn*	*Rate of cytochrome c oxidase (μmoles cytochrome c oxidised/min/mg protein)*
None	—	0.031
Antimycin A	6 μM	0.031
	9 μM	0.031
HQNO	30 μM	0.031
KCN	3 mM	0
CO	—	0.012

After long term subjection to dark aerobic conditions on agar plates, cells of *R. viridis* were obtained that were capable of limited exponential aerobic growth in liquid medium. The generation time was approximately 15 h. The doubling time for anaerobic light-grown cells, in the same medium was 10 h.

A spectrum of aerobically grown and photosynthetically grown cells (Fig. 1) shows that a suppression of photo pigments accompanies changes in growth conditions. No bacteriochlorophyll could be detected in acetone–methanol extracts of whole cells of *R. viridis* grown aerobically, and unlike light-grown cells, no light-induced cytochrome reactions could be demonstrated in aerobically grown cells.

The dithionite-reduced *minus* oxidised difference spectra of whole cells of *R. viridis* (Fig. 2) indicate that both aerobically and anaerobically light-grown cells contain *c*-type cytochromes. Spectra at 77 °K suggest that in addition to *c*-type cytochromes with α-peaks at 550 and 555 nm, a pigment with a α-band maximum at 560 nm, possibly a *b*-type cytochrome, was present in aerobic cells. Previously we have given evidence for the absence of *b*-type cytochromes in photosynthetically grown *R. viridis*[3].

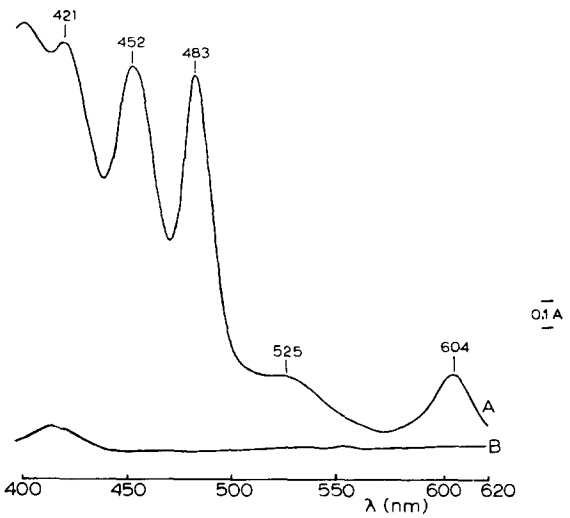

Fig. 1. Spectra of cells of (A), photosynthetically grown and (B), aerobically grown *R. viridis* Cells ((A), approx. 1.0 mg protein; (B), approx. 1.0 mg protein) were suspended in 3 ml 20 mM TES (pH 7.0) in test cuvette; 20 mM TES (pH 7.0) was in the reference cuvette. Spectra were recorded at room temperature on preparations of both types of cells adjusted to give the same absorbance at 680 nm.

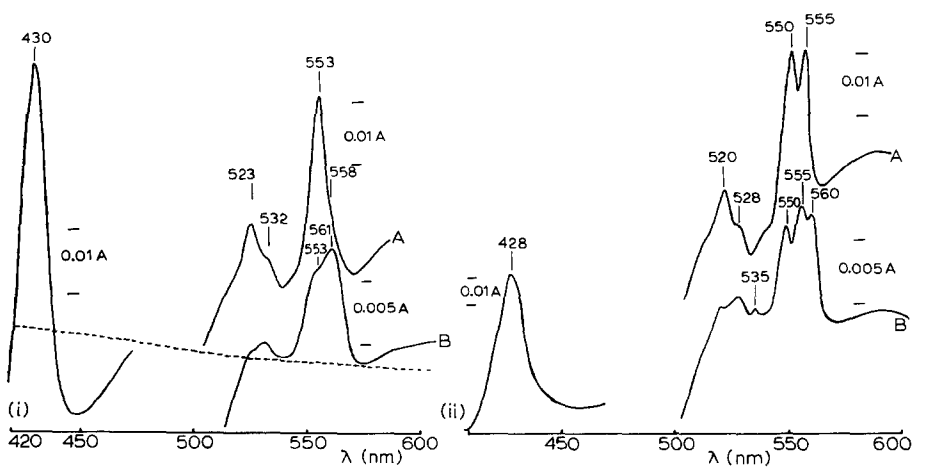

Fig. 2. Reduced *minus* oxidised difference spectra of whole cells of (A), photosynthetically grown and (B), aerobically grown *R. viridis*. Cells ((A), 20 mg protein; (B), 20 mg protein) were suspended in 5 ml 20 mM TES (pH 7.0) and divided between two cuvettes. Dithionite added to test cuvette, 0.02% (w/v) H_2O_2 and catalase to reference. (i) Recorded at room temperature, (ii) recorded at 77 °K. ------, baseline.

CO-binding pigments have been found in photosynthetically grown *R. viridis*[2]. Both light-grown and aerobically grown cells appear to contain components reacting with CO (Fig. 3) and the spectra suggest that more than one CO-binding pigment is present. An increased concentration of a CO complex absorbing beyond 560 nm was apparent in the aerobically grown form.

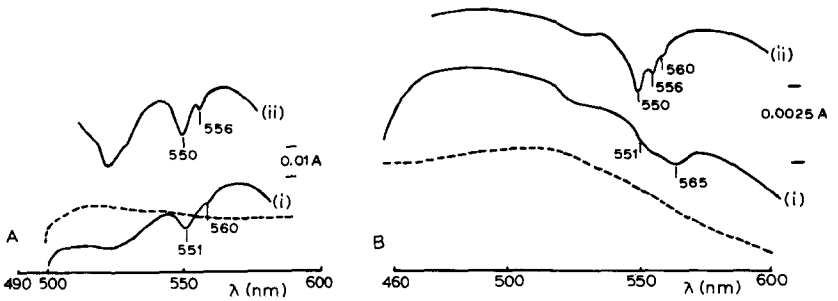

Fig. 3. CO difference spectra of whole cells of (A), photosynthetically grown and (B), aerobically grown *R. viridis*. Cells ((A), 50 mg protein; (B), 20 mg protein) were suspended in 5 ml 20 mM TES (pH 7.0) and reduced with dithionite. CO bubbled into test cuvette. Trace (i) recorded 10 min after CO addition, at room temperature. Trace (ii) recorded at 77 °K. -------, baseline before CO addition.

Oxygen electrode traces indicate that the terminal oxidase of light-grown whole cells has a higher K_m (0.35 $\pm$0.09 mM) than aerobically grown cells (K_m: 0.007 $\pm$0.002 mM) (Fig. 4). Rates of oxygen consumption in particles from photosynthetically grown cells of *R. viridis* with either NADH or succinate as substrate, were lower than rates measured in light-grown whole cells.

Electron micrographs of freeze-etched cells of aerobically grown *R. viridis* showed almost complete absence of the complex membrane system characteristic of the photosynthetically grown cells.

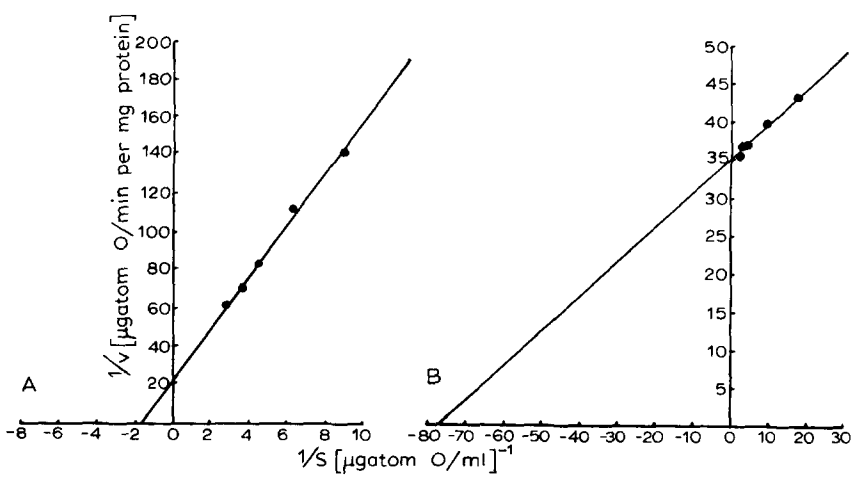

Fig. 4. Double-reciprocal plots of reaction velocity against O_2 concentration for the oxidase systems of whole cells of (A), photosynthetically grown; (B) aerobically grown *R. viridis*. Cells were washed and depleted of endogenous substrates by preincubation in 20 mM TES (pH 7.0). O_2 uptake was measured at 30 °C using an oxygen electrode. The incubation mixture contained in 5 ml: 20 mM TES (pH 7.0) and cells ((A), 10 mg protein; (B), 8 mg protein). 8 mM succinate was added to start the reaction. Michaelis constants were calculated using a computer programme of least squares regression modified by Dr P. J. England of this department. (A), K_m: 0.35 $\pm$ 0.09 mM; (B), K_m: 0.007 $\pm$ 0.002 mM.

DISCUSSION

In our previous investigation[3] of energy-linked electron transport reactions in *R. viridis* we found that ATP could be used to drive the reduction of NAD^+ by succinate. The addition of ATP to anaerobic particles induced the oxidation of both high and low potential cytochromes *c*, suggesting that each cytochrome is associated with the oxidising side of an energy conserving site. Since these cytochromes become partly oxidised upon stirring oxygen into an anaerobic suspension of *R. viridis* particles it is apparent that there is some form of autoxidisable pigment even in anaerobically grown cells and that preparations from these cells should be capable of oxidative phosphorylation as well as photophosphorylation. The results in Table II show that this speculation is well-founded and that the P:O ratios obtained using membrane fractions from *R. viridis* are comparable with those found for known facultative photosynthetic bacteria, *R. rubrum*[13] and *R. capsulata* (Melandri, B. A., personal communication), and that NADH yields a higher ratio than succinate. The dark phosphorylation system in *R. viridis* responds to uncouplers, to oligomycin and to other inhibitors in a way that is very comparable with mitochondrial oxidative phosphorylation.

Since *R. viridis* apparently carries out normal oxidative phosphorylation it might be expected to grow aerobically, in the dark. We have found that aerobic growth can be achieved if cells are repeatedly cultured, in the dark, under aerobic conditions. Growth is slow, but the effects of aerobic growth closely resemble those so well described in *R. spheroides*[14]: chlorophyll and carotenoid synthesis is almost completely inhibited. In addition, no photo-oxidation of cytochrome *c* can be induced. A new pigment is found in aerobic cells with absorption maxima at 561 nm at room temperature and 560 nm at 77 °K. This may be a *b*-type cytochrome since pyridine haemochrome spectra of aerobic cells show a shoulder on the long wavelength side of the haem *c* peak that may be due to protohaem (Saunders, V. and Jones, O. T. G., unpublished). At the same time a new or increased amount of a CO-binding pigment is observed and it appears that a new terminal oxidase is formed with a lower K_m for oxygen. The K_m for oxygen of the oxidase found in the photosynthetic cell is so high that it is above the normal dissolved oxygen concentration found in salt solutions and may account for the very slow rate of aerobic growth found initially. The relatively high K_m for oxygen of the terminal oxidase that develops on adaptation suggests that it is neither an a/a_3 type nor the *o*-type found in *R. rubrum* (compare ref. 15).

The changes in fine structure and inhibition of pigment synthesis in *R. viridis* on oxygenation indicate that some regulatory enzymes of chlorophyll and carotenoid synthesis are controlled by an oxygen-sensitive compound or compounds as has been suggested in *R. spheroides*[14]. Alternatively some substrates required for membrane and pigment production may be present only in low concentrations in aerobic conditions if they are linked to an autoxidisable component. It is remarkable that the response of *R. viridis* to changes in growth conditions is similar to that of *R. spheroides* and *R. rubrum* although its membrane structure, carotenoids and even its chlorohpyll are different. This adaptation of *R. viridis* to aerobic growth follows a long lag phase, and the exponential phase is equivalent only to a few doubling times. Such aerobically grown cells revert to the characteristic photosynthetic form

when transferred to anaerobic light conditions, with the same complement of cytochromes, carotenoids and bacteriochlorophyll that is found in photosynthetically grown cells.

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