Changes in the cytochrome composition of *Rhodopseudomonas sphaeroides* grown aerobically, photosynthetically and on dimethyl sulphoxide

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(Received 16 November 1982/Accepted 15 March 1983)

Several strains and mutants of *Rhodopseudomonas sphaeroides* can be grown anaerobically in the dark in the presence of dimethyl sulphoxide as an electron acceptor. During adaptation to this fermentative mode of growth, two major *c*-type cytochromes are synthesized, one with M_r 45 000 and the second with M_r 20 000 and a midpoint potential of +120 mV. These cytochromes are barely detectable in membranes prepared from cells grown in aerobic or photosynthetic conditions. An electrophoretic method is presented for the detection of the *b*-type and *c*-type cytochromes of pigmented or unpigmented membranes. The method resolves three *b*-type cytochromes and four *c*-type cytochromes in membranes from aerobically and photosynthetically grown cells.

The bacterium *Rhodopseudomonas sphaeroides* is able to grow aerobically or photosynthetically in the presence of simple organic compounds as reductants. Much of the attention focused on this bacterium results from its usefulness as a simple model for the process of membrane differentiation. An extensive intracytoplasmic membrane system develops in photosynthetic conditions; its formation is inhibited by oxygen and it is absent from aerobically grown cells.

A third, dark anaerobic, mode of growth is possible in some of the photosynthetic bacteria. Uffen & Wolfe (1970) demonstrated that *Rps. sphaeroides*, *Rhodopseudomonas palustris* and *Rhodopseudomonas viridis*, as well as *Rhodospirillum rubrum*, could grow under anaerobic dark conditions with ethanol, sodium acetate or pyruvate as the energy source. In addition, Yen & Marrs (1977) reported anaerobic dark growth of *Rhodopseudomonas capsulata*, *Rps. sphaeroides* and *Rps. palustris* with dimethyl sulphoxide as terminal electron acceptor. It has been shown that *Rps. sphaeroides* can use dimethyl sulphoxide as a terminal electron acceptor in place of oxygen; a concomitant increase in the amount of a cytochrome of apparent M_r 41 000 was observed (Ward & Jones, 1980). Similar studies were conducted on the closely related bacterium *Rps. capsulata* by Zannoni & Marrs (1981).

In the present paper we examine the response of *Rps. sphaeroides* to variations in growth conditions by monitoring changes in the type and amount of cytochromes present during photoheterotrophic, chemoheterotrophic (aerobic) and fermentative growth; in each case a mixture of organic acids such as succinate and glutamate supplies reducing equivalents. We describe an analysis of the cytochromes of *Rps. sphaeroides* membranes by using potentiometric titrations, haem-staining and fluorescent gel techniques.

Experimental

Growth of cells

All cultures were grown in the medium described by Sistrom (1960) at 30°C. Strain Ga, which accumulates the carotenoids neurosporene and chloroxanthin, is green in appearance, unlike the brown wild-type, which accumulates spheroidene and spheroidenone. Photosynthetic growth took place in sealed 10-litre bottles illuminated by 40 W tungsten bulbs. Aerobic cells were grown in a 10-litre jar containing 8 litres of medium; air was bubbled through the medium by means of a sintered-glass sparge. Inocula for growth on dimethyl sulphoxide (80 mm) were taken through three sterile transfers on dimethyl sulphoxide-supplemented medium. The cells were grown in sealed 10-litre jars stored in the dark for 48 h. Membranes were prepared from French-press-disrupted cells by differential centrifugation, as described by Barrett *et al.* (1978).

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Spectrophotometry and redox titrations

Reduced-minus-oxidized difference spectra were recorded at 300 K and 77 K in a split-beam spectrophotometer built with the use of a Hilger D330 monochromator. This apparatus was also used for redox titrations to obtain a series of difference spectra at various oxidation-reduction potentials with mediators present (Cross *et al.*, 1981).

Polyacrylamide-gel electrophoresis

The sample to be examined for fluorescence of demetallated *c*-type cytochrome (0.5 mg of protein in 0.5 ml of 1 mM-Tris/HCl buffer, pH 7.5) was treated with 100% acetic acid (0.2 ml), and Na₂S₂O₄ was added to 60 mM; 30 μ l of 12 M-HCl was added, and the mixture was sedimented in an Eppendorf Microfuge. The pellet was washed once with acetone and then with diethyl ether to remove lipid, pigment and protohaem (Castelfranco & Jones, 1975). Remaining material containing demetallated *c*-type cytochrome was dispersed by gentle sonication in 200 μ l of 62.5 mM-Tris/HCl buffer, pH 6.8, containing 50 mM-dithiothreitol, 2% (w/v) lithium dodecyl sulphate and 15% (w/v) sucrose, and solubilized by heating at 100°C for 60 s. After cooling, the samples were applied to the wells of a 5% polyacrylamide stacking gel containing 0.125 mM-Tris/HCl buffer, pH 6.8, and an acrylamide/bisacrylamide ratio of 30:0.8. This stacking gel of 6 mm thickness was cast on a running gel of 13.5% polyacrylamide (acrylamide/bisacrylamide ratio 30:0.8) containing 0.375 M-Tris/HCl buffer, pH 8.8. The gel was run at room temperature for approx. 16 h. Demetallated *c*-type cytochrome had a red fluorescence in u.v. light, and red-fluorescent bands were photographed with a Polaroid camera mounted over the gel placed on a u.v. light-box (Ultraviolet Products, San Gabriel, CA, U.S.A.).

Samples to be analysed for haem-containing proteins were solubilized in 62.5 mM-Tris/HCl buffer, pH 6.8, containing 50 mM-dithiothreitol, 0.4%

lithium dodecyl sulphate and 15% sucrose at 4°C and electrophoresed on 1 mm-thick polyacrylamide (5% stack, 10–15% gradient running gel) with pH values and running times as indicated above. At the end of the run the gel was stained for haem-dependent peroxidase activity as described by Thomas *et al.* (1976).

Results

Potentiometric titrations of cytochrome components

Cells of the carotenoid-less mutant R-26 synthesized relatively low amounts of bacteriochlorophyll when grown in the presence of dimethyl sulphoxide (see Table 1). A similar decrease in pigmentation was recorded by Uffen and co-workers (reviewed by Uffen, 1978), who studied anaerobic dark growth of *Rps. rubrum*. The ratio of *c*-type to *b*-type cytochromes increased whether or not the cells were inherently capable of synthesizing bacteriochlorophyll. Membranes from the mutant O1, which is incapable of synthesizing bacteriochlorophyll, were studied in some detail to avoid spectral interference from this pigment. It was shown (Fig. 1) that *b*-type cytochrome (λ_{max} , 558 nm) is produced in low amounts relative to *c*-type cytochrome (λ_{max} , 547 and 551 nm). *Rps. sphaeroides* contains at least three *c*-type cytochromes (Bartsch, 1978), and in order to determine which *c*-type cytochrome increases in amount during growth on dimethyl sulphoxide the cytochrome content of membranes from aerobically and dimethyl sulphoxide-grown cells was investigated by performing potentiometric titrations. The results of such titrations are displayed in Figs. 2 and 3. A *c*-type cytochrome with midpoint potential that was found to vary between +90 mV and +120 mV was a major component of membranes from dimethyl sulphoxide-grown cells. A cytochrome *b* with $E_{m,7.0} = -130$ mV was found as a minor component in such cells (results not shown). No attempt was made to resolve the *c*-type component of high

Table 1. Quantification of bacteriochlorophyll and cytochrome content of *Rps. sphaeroides* membranes

b- and *c*-type cytochromes were quantified from dithionite-reduced-minus-ferricyanide-oxidized difference spectra recorded on a split-beam spectrophotometer as described by Cross *et al.* (1981). The millimolar absorption coefficients used were: cytochrome *b*, $A_{560}-A_{570}$, $\Delta\epsilon = 20 \text{ mm}^{-1} \cdot \text{cm}^{-1}$; cytochrome *c*, $A_{551}-A_{540}$, $\Delta\epsilon = 20 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ (Hendry *et al.*, 1981). Bacteriochlorophyll was extracted in acetone/methanol (7:2, v/v) and quantified by using a millimolar absorption coefficient at 772 nm of $75 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ (Clayton, 1963).

Type of cell	Bacteriochlorophyll (nmol/mg of protein)	<i>b</i> -type cytochrome (nmol/mg of protein)	<i>c</i> -type cytochrome (nmol/mg of protein)	Ratio cytochromes <i>c/b</i>
R-26, photosynthetically grown	26.00	0.35	0.19	0.54
R-26, Me ₂ SO-grown, dark	5.00	0.68	0.93	1.37
O1, aerobically grown	—	0.76	0.64	0.84
O1, Me ₂ SO-grown, dark	—	0.61	1.23	2.02

Fig. 1. Reduced-minus-oxidized difference spectra of membranes from aerobically and dimethyl sulfoxide-grown cells of *Rhodopseudomonas sphaeroides* O1 at (a) room temperature and (b) 77 K

The membranes were suspended in 100 mM-KCl/20 mM-Mops (4-morpholinepropanesulphonic acid)/KOH buffer, pH 7.0, at a concentration of 0.88 mg/ml. Spectra were recorded in apparatus described in the Experimental section. Top, membranes from aerobically grown cells; bottom, membranes from dimethyl sulfoxide-grown cells.

midpoint potential ($E_m = +290$ mV; see Fig. 3) into two components corresponding to cytochromes c_1 ($E_m = +280$ mV) and c_2 ($E_m = +350$ mV) (Wood, 1980). Both preparations contained low-potential cytochromes in significant amounts (Figs. 2 and 3).

Electrophoretic behaviour of cytochrome components: detection of demetallated cytochrome fluorescence

It is possible to investigate the c -type cytochrome of membranes from *Rps. sphaeroides* by using a technique based on that of Wood (1980), in which the haem iron is displaced by protons to yield a

strongly fluorescent demetallated cytochrome c . When the electrophoretically separated porphyrin-proteins are excited by u.v. light (Fig. 4), red-fluorescent bands can be detected.

Respiratory and photosynthetic membranes (Fig. 4, lanes a and c) appear to be similar and to contain c -type cytochromes numbered in Table 2 as bands 3, 4 and 8/9, with M_r 34 000, 28 000 and 12 600–14 000. It is not clear whether the double fluorescent band of high electrophoretic mobility is due to two different cytochromes (Fig. 4, lane b). It is possible that proteolysis has removed a small fragment of the protein or that two isocytochromes c_2 (Prince *et al.*,

Fig. 2. Potentiometric titration of membranes from photosynthetically grown *Rps. sphaeroides* R-26. The membranes were suspended in 100 mM-KCl/20 mM-Mops buffer, pH 7.0, at a concentration of 2.06 mg/ml. Mediator concentrations were: phenazine methosulphate, phenazine ethosulphate, 2-hydroxy-1,4-naphthoquinone, anthroquinone-2,6-disulphonate, 3,6-diaminodurene and duroquinone, all at 12.5 μ M; pyocyanine (6 μ M). The titration was performed in an anaerobic cuvette as described by Cross *et al.* (1981). (a) Reductive titration; (b) replot of (a) resolved into two $n = 1$ components by repeatedly recalculating the data until the best fit was obtained.

Fig. 3. Potentiometric titration of membranes from dimethyl sulfoxide-grown *Rps. sphaeroides* R-26. Membranes were suspended at 1.53 mg/ml. Buffers, mediators and conditions were as indicated in Fig. 2 legend.

1974) have different mobilities in sodium dodecyl sulphate/polyacrylamide-gel electrophoresis or that band 8 is monomeric cytochrome c_3 (Bartsch, 1978). Band 3 is probably cytochrome c_1 ; the M_r of this cytochrome has been variously reported as 30000 (Wood, 1980) and 33000 (Gabellini *et al.*, 1982). We observed that the relative molecular mass is dependent on solubilization temperature. Guikema & Sherman (1981), too, noted that a cyanobacterial

33 000- M_r polypeptide band had different mobilities when treated with lithium dodecyl sulphate at different temperatures. Such discrepancies may arise as a result of changes in the degree of unfolding of the polypeptides at different temperatures. Bands 1 and 7 are minor components in respiratory and photosynthetic membranes respectively. Band 4

Fig. 4. Lithium dodecyl sulphate/polyacrylamide-gel electrophoretic separation of the *c*-type cytochromes of *Rps. sphaeroides*

Sample preparation is described in the Experimental section. Approx. 500 μ g of protein was loaded into each sample well. The lanes are: (a) cytoplasmic membranes from aerobically grown mutant O1; (b) cytochrome c_2 ; (c) intracytoplasmic membranes from the photosynthetically grown wild-type strain 8253; (d) crude membranes from dimethyl sulphoxide-grown strain Ga.

appears as a minor component under all growth conditions. In comparison with the electrophoretic profiles of *c*-type cytochromes from aerobically and photosynthetically grown cells, there is a striking increase in the intensities of bands 1 and 7 of membranes from dimethyl sulphoxide-grown cells; in addition, much less low- M_r cytochrome *c* is seen. It is probable that one of the cytochromes of apparent M_r 45 000 and 20 000 is that cytochrome with a midpoint potential of approx +120 mV detected potentiometrically.

Electrophoretic behaviour: detection of haem-staining activity

Various types of *Rps. sphaeroides* membranes were solubilized in lithium dodecyl sulphate and then subjected to polyacrylamide-gel electrophoresis at 4°C. A series of haem-binding polypeptides were detected after the mild solubilization process. This procedure preserves plant chlorophyll-protein complexes (Delepelaire & Chua, 1979), *Rps. sphaeroides* bacteriochlorophyll-protein complexes (Broglie *et al.*, 1980) and cyanobacterial haemoprotein complexes (Guikema & Sherman, 1981). Membranes from aerobically, photosynthetically and dimethyl sulphoxide-grown cells were analysed by using this technique. Protohaem extraction (Castelfranco & Jones, 1975; Jones & Jones, 1970) was used to destroy *b*-type cytochrome without abolishing cytochrome *c* staining, and careful comparison with fluorescent gel profiles as in Fig. 4 allows the cytochromes to be tentatively identified as *b*-type or *c*-type (see Table 2). The spectra of bands 1 and 7 excised from non-stained preparative slab gels

Table 2. Electrophoretically separated polypeptides of dimethyl sulphoxide-grown *Rps. sphaeroides* that exhibit haem-dependent peroxidase activity

The results are of a summary of the electrophoretic profiles from several gels of dimethyl sulphoxide-grown *Rps. sphaeroides* Ga. The apparent M_r is measured in the lithium dodecyl sulphate/polyacrylamide-gel electrophoretic system run at 4°C (see the Experimental section). Treatment of the membranes with lithium dodecyl sulphate at 100°C after Fe^{2+} removal (see the Experimental section) slightly altered the electrophoretic mobility with respect to the standard protein markers. Bands 8 and 9 were occasionally resolved, but often appeared as one large diffuse staining area.

Band	$10^{-3} \times$ Apparent M_r	Haem-dependent peroxidase activity	Haem stain after protohaem extraction	Red fluorescence after Fe^{2+} displacement from haem	Assignment
1	45	+++	+++	+++	<i>c</i> (Me_2SO)
2	43	+			<i>b</i>
3	34	++	++	++	<i>c</i> (c_1) $E_m = 290$ mV
4	28	+	+	++	<i>c</i>
5	23	++			<i>b</i> (b_{+50} , b_{-90})
6	21	+			<i>b</i>
7	20	+++	+	+++	<i>c</i> (Me_2SO) $E_m = 120$ mV
8	14	++			<i>b</i>
9	13	++	+	+	<i>c</i> (c_2) $E_m = 350$ mV

exhibited a reduced-minus-oxidized absorbance peak centred around 550nm (results not shown), indicative of *c*-type cytochrome. Bands 1 and 7 are detected in large amounts in dimethyl sulphoxide-grown cells (Fig. 5, lanes *b*, *d* and *f*), in agreement with the data in Fig. 4 (lane *d*). The ‘dimethyl

sulphoxide effect’ is much more pronounced with the 20 000-*M_r* cytochrome (band 7), which is observed only in cells grown on dimethyl sulphoxide. A comparison of respiratory and photosynthetic membranes (Fig. 5, lanes *a* and *e*, and Table 3) shows that they possess *c*-type cytochromes (bands 1, 3, 4

Fig. 5. *Lithium dodecyl sulphate/polyacrylamide-gel-electrophoretic separation of b- and c-type cytochromes of Rps. sphaeroides*

Sample preparation is described in the Experimental section. Solubilized membranes in each well (approx. 80 µg) are: (a) strain O1, aerobically grown; (b) strain O1, dimethyl sulphoxide-grown; (c) strain R-26, photosynthetically grown; (d) strain R-26, dimethyl sulphoxide-grown; (e) strain Ga, photosynthetically grown; (f) strain Ga, dimethyl sulphoxide-grown.

Table 3. *Haem-staining bands in subcellular fractions of Rps. sphaeroides subjected to lithium dodecyl sulphate/polyacrylamide-gel electrophoresis*

‘Crude’ and ‘pure’ intracytoplasmic membranes were prepared by high-speed differential centrifugation and sucrose-density-gradient centrifugation respectively. For details see the Experimental section.

Band	<i>Rps. sphaeroides</i> mutant O1		<i>Rps. sphaeroides</i> Ga			
	Aerobically grown, cytoplasmic membranes	Me ₂ SO-grown, cytoplasmic membranes	Photosynthetically grown, crude intracytoplasmic membranes	Me ₂ SO-grown, crude intracytoplasmic membranes	Me ₂ SO-grown, pure intracytoplasmic membranes	Me ₂ SO-grown, small membranes
1	++	+++	+	+++	+++	
2		+		+	+	
3	++	++	++	++	++	
4	+	+	+	+	+	
5	++	++	++	++	+	
6	+	+	+	+		+
7		+++		+++		++
8	++	+	+++	++	+	+++
9	++	+	+++	++	+	+++

and 9) and *b*-type cytochromes (bands 5, 6 and 8) in common. These data are in agreement with the titration results obtained by Saunders & Jones (1975) and J. R. Bowyer, C. N. Hunter, T. Ohnishi & R. A. Niederman (unpublished work), who identified a number of cytochrome components in *Rps. sphaeroides* membranes common to photosynthesis and respiration. We have not identified the high- M_r haem-staining bands in lane (f) in Fig. 5; they are labile to heating and protohaem extract, and we suggest that they could be loosely associated cytochrome complexes such as the cytochrome *b*- c_1 complex of *Rps. sphaeroides* (M_r approx. 100 000) isolated by Gabellini *et al.* (1982). The haem-staining pattern was not affected by the presence of a variety of proteinase inhibitors, demonstrating that the bands observed could be attributed to intact cytochromes present in the membranes and not to the breakdown of a few large molecules.

We examined the effect of membrane purification on the haem-staining pattern of membranes from dimethyl sulphoxide-grown cells. Sucrose-density-gradient centrifugation (Niederman *et al.*, 1976) yielded a pigmented membrane band with the sedimentation behaviour of authentic intracytoplasmic membrane from photosynthetically grown cells. Electron microscopy (results not shown) confirms the presence of intracytoplasmic membranes in dimethyl sulphoxide-grown cells. After lithium dodecyl sulphate/polyacrylamide-gel electrophoresis of the pure membranes and haem-staining, the 45 000- M_r *c*-type cytochrome (band 1) was detected, where the 20 000- M_r *c*-type cytochrome (band 7) was found in appreciable quantities only in the 'small-membrane' fraction (Barrett *et al.*, 1978) prepared from the supernatant of the high-speed sedimentation used to prepare crude membranes. No *c*-type cytochrome of midpoint potential +120 mV was detected in potentiometric titrations of the pure membranes (results not shown); this observation is consistent with the electrophoretic data, and demonstrates that the 20 000- M_r *c* cytochrome possesses a midpoint potential of +120 mV. This cytochrome is loosely bound to the cell membrane, in contrast with the 45 000- M_r cytochrome, which appears to be firmly bound to the intracytoplasmic membrane system.

Discussion

Dimethyl sulphoxide acts as a sink to accept electrons that must have originated from succinate and glutamate supplied in the medium. The midpoint oxidation-reduction potential for the reduction of dimethyl sulphoxide to dimethyl sulphide has been calculated by Wood (1981) to be +160 mV. We suggest that the two *c*-type cytochromes identified as bands 1 and 7 in Fig. 5 are needed to mediate this

electron flow, although we have found that neither is reduced when isolated membranes are treated with substrate *in vitro* under anaerobic conditions (J. A. Ward & O. T. G. Jones, unpublished work).

Zannoni & Marrs (1981) have presented a thorough bioenergetic analysis of the growth of *Rps. capsulata* on dimethyl sulphoxide; they found that the membrane-bound electron-transport system within this bacterium differs from that established for photoheterotrophic growth. Normal electron flow through the cytochrome *b*-*c* region was restricted, and it was concluded that a non-energy-conserving NADH dehydrogenase was present in dimethyl sulphoxide-grown cells. In contrast, *Rps. sphaeroides* is able to grow in the dark in dimethyl sulphoxide medium with non-fermentable substrates (J. A. Ward & O. T. G. Jones, unpublished work). Analysis of the cytochromes present in *Rps. capsulata* demonstrated that the presence of dimethyl sulphoxide induced relatively high amounts of a cytochrome *c* ($E_{m,7.0} = +134$ mV) and a cytochrome *b* ($E_m = 0$ mV). This *c*-type component is likely to have a similar function to the component with $E_{m,7.0} = +120$ mV in our titrations.

Although growth on dimethyl sulphoxide induces the appearance of two major haem-staining components, potentiometric titrations revealed the presence of only one component, with a midpoint potential found to vary between +90 and +120 mV. It is possible that two cytochromes *c* are present with midpoint potentials too close to resolve by potentiometric titration. The 'soluble' *c*-type cytochrome in dimethyl sulphoxide-grown cells ($E_m = +120$ mV) is probably identical with cytochrome *c*-553 identified by Orlando (1962) as having M_r 25 000 and a midpoint potential of +120 mV.

The appearance of a minor haem-staining component, band 2, designated as a *b*-type cytochrome, is consistently associated with growth on dimethyl sulphoxide and may be the component of low midpoint potential identified in our potentiometric titrations.

The cytochrome *b*- c_1 complex isolated from *Rps. sphaeroides* by Gabellini *et al.* (1982) is composed of three polypeptides of M_r 40 000, 34 000 and 25 000; the 34 000- M_r component is cytochrome c_1 , in agreement with the estimated relative molecular mass of our band-3 polypeptide. We suggest that the *b*-type component with M_r 23 000 (band 5) corresponds to the 25 000- M_r component in this cytochrome *b*- c_1 complex.

J. A. W. was supported by a studentship from the Science and Engineering Research Council, and C. N. H. by a postdoctoral fellowship award from the Science and Engineering Research Council. C. N. H. and O. T. G. J. acknowledge the receipt of a Science and Engineering Research Council research grant. The

authors are grateful for the technical assistance of Mrs. Janet Fielding and Mrs. Elaine Burd.

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