Detection of Cytochrome b_{+50} in Membranes of *Rhodospirillum rubrum* Isolated from Aerobically and Phototrophically Grown Cells

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1. Dark equilibrium potentiometric titrations were conducted on membranes purified from *Rhodospirillum rubrum* in an effort to identify *b*-type cytochrome components reported in other Rhodospirillaceae. In preparations from aerobically grown cells virtually devoid of bacteriochlorophyll *a*, three components were observed at 560–540 nm. Their oxidation–reduction midpoint potentials assigned by computer-assisted analysis were +195, +50 and –110 mV at pH 7.0; each of these fitted closely to theoretical single-electron equivalent curves. 2. In chromatophores from phototrophically grown carotenoidless mutant G-9, three components were also observed with E_0' +190, +50 and –90 mV. 3. The α -band of the +50 mV component exhibited an absorption maximum near 560 nm in difference spectra obtained at fixed oxidation–reduction potentials. 4. This component could be demonstrated most readily in purified membrane preparations and may have been obscured in previous studies by residual cytochrome *c'*. 5. This is the first definitive report of cytochrome b_{+50} in membranes from *Rs. rubrum* and aligns this bacterium with other Rhodospirillaceae in which this component functions in light-driven cyclic electron flow.

The role of *b*-type cytochromes in the electron-transport pathways of facultatively photoheterotrophic Rhodospirillaceae has been a subject of active investigation (Jones, 1977). Membranes isolated from *Rhodopseudomonas sphaeroides* and *Rhodopseudomonas capsulata* grown under both aerobic and phototrophic conditions contain significant quantities of a *b*-type cytochrome with an oxidation–reduction potential midpoint near +50 mV (Dutton & Jackson, 1972; Connelly *et al.*, 1973; Evans & Crofts, 1974; Zannoni *et al.*, 1974, 1976b; Saunders & Jones, 1975; Crofts *et al.*, 1977). This component has been shown to function directly in the pathway of light-driven cyclic electron flow, where it is involved in both electron and proton transfer (Dutton & Jackson, 1972; Crofts *et al.*,

1977) and has also been assigned a possible respiratory role in *Rp. capsulata* (Zannoni *et al.*, 1976a). Several high-potential *b*-type cytochromes have also been described (Saunders & Jones, 1975; Zannoni *et al.*, 1974, 1976b). In aerobically grown *Rp. capsulata*, components with E_0' +413 and +270 mV were detected (Zannoni *et al.*, 1974, 1976b) and assigned roles as the respective CO-insensitive (Zannoni *et al.*, 1974) and CO-sensitive (Zannoni *et al.*, 1976b) oxidases of the branched (LaMonica & Marrs, 1976; Zannoni *et al.*, 1976a) respiratory chain. In a photosynthetically incompetent mutant of *Rp. sphaeroides*, two such components with E_0' +390 and +255 mV were reported by Saunders & Jones (1975); however, neither of these components appeared to bind CO.

In studies of the oxidase system of the related bacterium *Rhodospirillum rubrum* by Taniguchi & Kamen (1965), two *b*-type cytochromes were identified in membranes from aerobically grown cells. One was a component of apparent low oxidation–reduction potential believed to be associated with succinate oxidation, and the other was a higher-potential CO-binding component which functioned as an oxi-

Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Mops, 4-morpholine-propanesulphonic acid.

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dase. In preparations of pigmented membranes (chromatophores) obtained from the phototrophically grown carotenoidless mutant G-9, high- and low-potential *b*-type cytochromes have also been described (Kakuno *et al.*, 1971; Dutton & Jackson, 1972); these were assigned E_0' values of +170 and -105 mV respectively (Dutton & Jackson, 1972). In addition, a third component was resolved with E_0' -5 mV that was identified tentatively as cytochrome *c'* (Kakuno *et al.*, 1971; Dutton & Jackson, 1972). The latter was not detected in aerobically grown cells by either spectrophotometric or immunochemical analyses (Taniguchi & Kamen, 1965).

Since to our knowledge, neither the high-potential CO-insensitive nor the cytochrome b_{+50} components have been reported in *Rs. rubrum*, a thorough thermodynamic analysis of the cytochrome components of membranes from both aerobically and phototrophically grown cells was undertaken. Because it seemed possible that the presence of significant residual quantities of soluble cytochrome *c'* might obscure some *b*-type components, procedures developed for the purification of membranes from *Rs. rubrum* (Collins & Niederman, 1976*a,b*) were employed in the present study. A preliminary report of these findings has been presented (Niederman *et al.*, 1979).

Materials and Methods

Rs. rubrum S1 and a carotenoidless mutant G-9 derived from it (kindly supplied by Dr. Judy Wall, Department of Biochemistry, University of Missouri, Columbia, MO, U.S.A.) were grown on a defined medium (Cohen-Bazire *et al.*, 1957). For growth in the light, completely filled 10-litre bottles were employed, and for aerobic growth in darkness similar quantities were sparged vigorously with air. Inoculum for the aerobic cultures was transferred through several passages on a gyratory shaker in the dark. For the preparation of crude extracts, deoxyribonuclease and ribonuclease were added to washed cells that were passed through a pressure cell at 138 MPa (20 000 lb/in²). After removal of unbroken cells by centrifugation at 10 000 *g* for 10 min, the extracts were centrifuged at 100 000 *g* for 90 min. For isolation of chromatophores from phototrophically grown strain G-9, the pellet was resuspended by homogenization and purified by rate-zonal (velocity) sedimentation essentially as described by Collins & Niederman (1976*b*). The cell envelope was purified from pellets obtained from aerobically grown cells by an isopycnic ultracentrifugation procedure (Collins & Niederman, 1976*a*). The sucrose gradients for these centrifugations were prepared in 10 mM-Hepes/10 mM-EDTA buffer, pH 7.5. The purified membranes were sedimented and resuspended in 20 mM-Mops/100 mM-KCl buffer, pH 7.0.

For determination of the bacteriochlorophyll *a* and protein contents of membrane preparations, the procedures described by Collins & Niederman (1976*a*) were employed.

Difference spectra at room temperature and 77 K were obtained on a split-beam spectrophotometer (Jones & Saunders, 1972) as described by Saunders & Jones (1975). Dark equilibrium potentiometric titrations were performed on a chopped dual-wavelength spectrophotometer with procedures described by Connelly *et al.* (1973). The following redox mediators were used to facilitate equilibration between the platinum electrode and the cytochrome components within the membrane: diaminodurol (Fluorochem, Glossop, Derbyshire, U.K.), 50 μ M; phenazine methosulphate and phenazine ethosulphate (both from Sigma), both 25 μ M; pyocyanine (K & K Laboratories, Plainview, NY, U.S.A.), 50 μ M; 2-hydroxy-1,4-naphthoquinone (Koch-Light, Colnbrook, Bucks., U.K.), 50 μ M. All titrations were performed in 20 mM-Mops/100 mM-KCl buffer, pH 7.0. E_0' values were assigned on the basis of a computer-assisted analysis, as described by Dutton & Jackson (1972). Both oxidative and reductive titrations produced similar results.

Results

Membrane preparations purified from vigorously aerated *Rs. rubrum* contained only 0.5–1.3% of the bacteriochlorophyll content reported for purified chromatophores by Collins & Niederman (1976*b*). This minimized the possibility that alterations in the bacteriochlorophyll spectrum at high potentials might obscure any cytochrome components. As shown in Fig. 1, dark potentiometric titrations of such membrane preparations at 560–540 nm confirmed the presence of both high- and low-potential *b*-type cytochrome components (Taniguchi & Kamen, 1965). In addition, a major component of intermediate oxidation–reduction potential is also observed. In Fig. 2, these data have been fully resolved into three components by a computer program. Each component fitted closely to theoretical single-electron equivalent ($n = 1$) curves. Mean E_0' values for these apparent *b*-type cytochrome components were calculated from the results of several titrations and are presented in Table 1. Values near +195, +50 and -110 mV were assigned; their relative contributions to the total $A_{560-540}$ change were approx. 19, 21 and 60% respectively. Although the highest potential shown in the titration depicted in Fig. 1 is +300 mV, several reductive titrations were begun at E_h values higher than +400 mV. No significant absorbance differences could be reproducibly demonstrated at these higher redox values, which suggests that *b*-type cytochromes of very high potential similar to those observed in the other

related species (Saunders & Jones, 1975; Zannoni *et al.*, 1974, 1976a) were absent from the *Rs. rubrum* membranes prepared here.

Since a cytochrome b_{+50} had not been described previously in membranes of *Rs. rubrum*, difference spectra were obtained at fixed oxidation-reduction potentials on a computer-linked spectrophotometer

Fig. 1. Dark potentiometric titration at 560–540 nm of cell envelope purified from aerobically grown cells. The protein concentration was 3.9 mg/ml. (a) Plot of data obtained from dual-wavelength spectrophotometer. The observed points are plotted about a theoretical curve for three components each accepting a single electron ($n = 1$); the curve was drawn from data generated by a computer program. (b) The data are plotted as the ratio of oxidized to reduced cytochrome, so that the multiplicity of components is visualized more easily.

(Fig. 3) to characterize this component further. After subtraction of the spectrum recorded at +40 mV from that obtained at +100 mV, a significant α -band with an absorption maximum near 560 nm is evident. On the assumption that the components of low and intermediate potential each accept a single electron, it was possible to calculate from the Nernst equation that approx. 90% of the absorbance difference observed between +40 mV and +100 mV would be expected from a b -type cytochrome of E_0' near +50 mV and that only 10% would be contributed by the low-potential component in this region of the titration (Fig. 3a). Even in the case where the spectrum at -40 mV was subtracted from that at +100 mV, it was calculated that about half the absorbance difference would still result from the

Fig. 2. Resolution of titration curve into individual components by computer-assisted analysis. The points calculated from the data in Fig. 1 are plotted about theoretical $n = 1$ lines derived from the Nernst equation by the method of Dutton & Jackson (1972).

Table 1. Oxidation-reduction midpoint potentials of b -type cytochromes in membranes purified from aerobically and phototrophically grown cells

Results are means $\pm$ S.E.M. for three determinations.

Growth conditions	E_0' (pH 7.0) (mV)	Approximate proportion of total absorbance change (%)
Aerobic	$+194 \pm 19$	19
	$+49 \pm 17$	21
	-111	60
Phototrophic	$+192 \pm 17$	13
	$+49 \pm 15$	29
	-91	58

Fig. 3. Absorption spectra of membranes from aerobically grown cells at fixed oxidation-reduction potentials. The potentials were established automatically at the preset values by alternative additions of dithionite or ferricyanide from syringes until stable values were achieved. The spectra were obtained with a computer-linked spectrophotometer (Evans & Crofts, 1974). The computer was programmed to record spectra at 20 mV intervals. The respective E_h values are shown at the right-side of each panel. The computer has subtracted each spectrum from that recorded at +100 mV. The fractions shown at the left side of the panels give the approximate relative contributions of the components of low (denominator) and intermediate (numerator) potential to absorbance differences observed in each spectrum, assuming that each component accepts a single electron. The E_0' values for these components determined from the titration shown in this Figure were -45 and +60 mV respectively. Their approximate relative contributions to the total absorbance at 560–540 nm observed at -100 mV were 30 and 55% respectively. The protein concentration was 4.0 mg/ml.

Fig. 4. Dark potentiometric titration at 560–540 nm of a chromatophore preparation from *Rs. rubrum* G-9. The bacteriochlorophyll concentration was 57 μ M. The Figure was generated on a Tektronix data plotter by a computer-assisted analysis coupled to a display routine. The observed points are plotted about a theoretical curve for three $n = 1$ components. Some scatter in the data is seen in the vicinity of 0 mV.

cytochrome of intermediate potential (Fig. 3b). These results support the possibility that the +50 mV component is a *b*-type cytochrome. In difference spectra obtained at 77 K in this range of potentials, the splitting of the α -band into two components with absorption maxima at 560 and 556 nm first observed by Taniguchi & Kamen (1965) was confirmed in the present study.

The detection of cytochrome b_{+50} in membranes from aerobically grown *Rs. rubrum* was of special interest because this is an integral component of light-driven cyclic electron flow in other related Rhodospirillaceae (Dutton & Jackson, 1972; Crofts *et al.*, 1977). Potentiometric titrations at 560–540 nm were therefore conducted on chromatophore preparations obtained from phototrophically grown *Rs. rubrum* G-9 (Fig. 4). It was necessary to use this carotenoidless mutant because the broad absorption bands of spirilloxanthin present in the phototrophically grown wild-type interfere with measurements of the cytochrome α -bands. Multiple components were also observed in these titrations, which resolved fully into three components after computer analysis (Fig. 5). Their mean E_0' values

Fig. 5. Resolution of titration curve of chromatophore preparation into individual components

The Figure was obtained with a Tektronix data plotter by computer-assisted analysis of the data from Fig. 4 coupled to a display routine. Other details are provided in Fig. 2.

were near +190, +50 and -90 mV and their relative absorbance contributions were approx. 13, 29 and 58% respectively (Table 1). Similar high- and low-potential components were reported for mutant G-9 chromatophores by Dutton & Jackson (1972), but a +50 mV component had not been observed previously in pigmented membranes from *Rs. rubrum*. This component was demonstrated most readily in chromatophore preparations that were purified by rate-zonal sedimentation on sucrose density gradients, a procedure that apparently removes residual cytochrome c' ($E_0' -5$ mV; Dutton & Jackson, 1972) that might obscure a cytochrome b_{+50} component in potentiometric titrations. Although the results were somewhat variable, three components also appeared in potentiometric titrations at 560–540 nm of membranes purified from mutant G-9 grown aerobically in the dark; tentative values near +250, +55 and -105 mV were observed in several trials.

With regard to the c -type cytochrome associated with the membranes purified from aerobically grown wild-type cells, spectral evidence had suggested that this component was cytochrome c_2 (Taniguchi & Kamen, 1965). The results of titrations at 551–540 nm with aerobic membrane preparations obtained here could be fitted most readily to a single component $n = 1$ curve, which was assigned an E_0'

value near +300 mV. This is in close agreement with the E_0' of +293 mV reported for cytochrome c_2 in chromatophore preparations (Dutton & Jackson, 1972); a value of 293 ± 13 mV was observed in the present study. Although a lower-potential species was sometimes present as a minor apparent c -type cytochrome component in the aerobic preparations, no reliable E_0' value could be assigned. In chromatophores from *Rp. sphaeroides* Ga, an E_0' of +295 mV has been reported for cytochrome c_2 when in close association with the membrane (Dutton *et al.*, 1975); a soluble pool with an E_0' of +345 mV is also present in such preparations, but this is apparently absent from membranes purified from *Rs. rubrum*. Only the +295 mV component was thought to be involved in re-reduction of the photo-oxidized reaction centre, but this remains to be established in *Rs. rubrum*.

Discussion

In membranes purified from both aerobically and phototrophically grown *Rs. rubrum*, a component accepting one electron with an E_0' (pH 7.0) near +50 mV has been detected here. It was identified tentatively as a b -type cytochrome on the basis of potentiometric titrations and difference spectra at controlled oxidation–reduction potentials. This component comprised approx. 20–30% of the total apparent b -type cytochrome in the potentiometric titrations of these membrane preparations. Although a cytochrome b_{+50} component has been identified in membranes from other Rhodospirillaceae (Dutton & Jackson, 1972; Connelly *et al.*, 1973; Crofts *et al.*, 1977), it had not been reported previously in *Rs. rubrum*. The present study confirms the presence of two additional b -type cytochromes, one of higher and one of lower oxidation–reduction potential in *Rs. rubrum* (Dutton & Jackson, 1972). These were observed in the membrane from cells grown under both conditions and E_0' values near +195 and -100 mV were assigned to them in close agreement with those reported previously for mutant G9 chromatophore preparations (Dutton & Jackson, 1972). In the membranes from the aerobically grown cells they represent the first precise E_0' assignments for these cytochromes. In previous studies (Connelly *et al.*, 1973; Saunders & Jones, 1975) it was shown that three b -type cytochromes with redox potentials similar to those observed here were present in membranes of both aerobically and phototrophically grown *Rp. sphaeroides*; the present results extend the observation of such shared components to *Rs. rubrum*. In further support of this possibility are the findings that immunologically identical coupling factor (Melandri *et al.*, 1971) and cytochrome c_2 (Baccarini-Melandri

et al., 1978) components function in both aerobically and phototrophically grown *Rp. capsulata*.

It has not yet proved possible to assign a functional role to the cytochrome b_{+50} component of *Rs. rubrum* observed here. In both *Rp. sphaeroides* (Dutton & Jackson, 1972) and *Rp. capsulata* (Crofts *et al.*, 1977) this cytochrome functions directly in light-driven cyclic electron flow where it is thought to oxidize one ubiquinone pool (the secondary acceptor) and reduce another, resulting in the re-reduction of photo-oxidized cytochrome c_2 (Crofts *et al.*, 1977; Dutton & Prince, 1978). Although flash-illumination studies (Parson, 1967; van Grondelle *et al.*, 1976) have suggested that both *b*- and *c*-type cytochromes also function in the cyclic pathway (Frankel, 1970) in *Rs. rubrum*, it was not possible to study light-dependent absorption changes or their dependence on oxidation-reduction potential in the chromatophore preparations isolated here.

With regard to the possible functions of the other *b*-type cytochromes observed here, studies by Dutton & Baltscheffsky (1972) on the oxidation-reduction potential dependence of pyrophosphate-induced reduction of *b*-type cytochromes in mutant G-9 chromatophore preparations have suggested a role for cytochrome b_{-100} in reversed electron flow. In addition, a low-potential *b*-type component is thought to be reduced by NADH via a NADH-haem protein oxidoreductase (Horio *et al.*, 1969). The cytochrome b_{+195} component may constitute the CO-sensitive cytochrome 'o' that forms the oxidase in the respiratory chain of aerobically grown *Rs. rubrum* (Taniguchi & Kamen, 1965; Horio & Taylor, 1965; Chance *et al.*, 1966; Smith & Pinder, 1978). On the basis of studies on oxygen-linked photoreduction (del Valle-Tascón *et al.*, 1975, 1977), these authors suggested that an alternative oxidase system might exist in *Rs. rubrum* analogous to that which forms the branch of the *Rp. capsulata* respiratory chain linked to the light-driven cyclic system (LaMonica & Marrs, 1976). In addition to involving cytochrome c_2 , this branch has as a terminal oxidase the CO-insensitive cytochrome b_{+413} component (Zannoni *et al.*, 1976a,b); however, neither the latter component nor any other cytochrome with an oxidation-reduction potential midpoint sufficiently high to oxidize ferrocyclochrome c_2 has been observed in the potentiometric titrations reported here.

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