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Identification of cytochromes involved in electron transport to trimethylamine *N*-oxide/dimethylsulphoxide reductase in *Rhodobacter capsulatus*

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The role of cytochromes in the electron-transport pathway to trimethylamine *N*-oxide (TMAO)/dimethylsulphoxide (DMSO) reductase in the photosynthetic bacterium *Rhodobacter capsulatus* was investigated. Reduced-minus-oxidized difference spectra in intact cells with TMAO or DMSO as oxidant revealed cytochrome absorbance changes with a maximum at 559 nm and a shoulder between 548 nm and 556 nm. The former change indicates a role for a *b*-type cytochrome and the latter for a *c*-type cytochrome, both of which are distinct from the cytochrome bc_1 complex. Cytochrome *c*-556 was identified in a bacterial periplasmic fraction as a redox component which could be oxidised by TMAO or DMSO. Cytochrome *c*-556 was the only cytochrome species which co-fractionated with TMAO/DMSO reductase following gel filtration of a post-chromatophore supernatant produced after French press treatment of intact cells. The mid-point redox potential (pH 7.6) of cytochrome *c*-556 was +105 mV ($n = 1$). It is suggested that TMAO/DMSO reductase and cytochrome *c*-556 form a structural and functional association in the periplasm of *Rhodobacter capsulatus*.

Introduction

The pathways of photosynthetic and aerobic respiratory electron transport in *Rhodobacter capsulatus* have been characterised in detail [1,2]. *Rb. capsulatus* also catalyses respiratory electron transport under anaerobic conditions; and the known electron acceptors are nitrate, nitrous oxide, trimethylamine *N*-oxide (TMAO) and dimethylsulphoxide (DMSO) [3]. Anaerobic respiration provides a sink for electrons during phototrophic growth of *Rb. capsulatus* on highly reduced carbon sources [4] and one of its functions appears to be to maintain the cyclic electron-transport chain near to optimal redox poise for photosynthesis [5]. The recognition that phototrophic bacteria are capable of anaerobic respiration is recent and so information about the components involved in anaerobic respiration is incomplete. In earlier work we demonstrated, through the use of inhibitors of electron transport, that electron flow to TMAO or DMSO involved the NADH dehydrogenase but not the cytochrome bc_1 complex [6] and we also found that a single oxidoreductase catalysing the reduction of TMAO and DMSO was located in the periplasmic space of *Rb. capsulatus* [7–9]. Recently, the TMAO reductase has been purified from *Rhodobacter sphaeroides* f.sp. denitrificans and found to contain a molybdenum cofactor of the pterin-type [10].

Since ubiquinol is located within the hydrophobic domain of the cytoplasmic membrane and the TMAO reductase is a soluble periplasmic enzyme [9], it can be expected that other redox centres are involved in electron transfer from ubiquinol to the terminal oxidore-

Abbreviations: DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; BChl, bacteriochlorophyll; DMSO, dimethylsulphoxide; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; PES, phenazine ethosulphate; PMS, phenazine methosulphate; SDS, sodium dodecyl sulphate; TMAO, trimethylamine *N*-oxide; $\Delta\psi$, cytoplasmic membrane potential.

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ductase. *Rb. capsulatus* possesses several membrane-bound and periplasmic cytochromes whose function is unknown [11] and the levels of certain cytochromes are known to increase in cells grown in the presence of DMSO [12]. In this paper we have examined the cytochrome absorbance changes which occur during electron transport to DMSO and TMAO in whole cells of *Rb. capsulatus* and we present evidence that *b*-type and *c*-type cytochromes are involved. These cytochromes were distinguished from the cytochromes of photosynthesis and aerobic respiration.

Materials and Methods

Bacterial strains and growth conditions

Strains of *Rb. capsulatus* used in this study were a green mutant N22DNAR$^+$ [13], a wild-type strain 37b4 obtained from Professor G. Drews (University of Freiburg, F.R.G.) and H123 a Tn5-insertion mutant of 37b4 which lacks cytochrome *c'* [14]. Cells were grown phototrophically to late exponential phase on RCV medium supplemented with 30 mM TMAO or 60 mM DMSO as described in Ref. 9. Cells were harvested, washed and resuspended in RCV medium and stored as a thick suspension on ice [6]. Experiments with cells were performed within 6 h of harvesting.

Preparation of periplasmic fractions and chromatography of soluble fractions

Periplasmic fractions were prepared from cells treated with lysozyme as described previously [9]. Total soluble fractions were prepared from 2 l phototrophically grown cells which had been harvested, washed, and resuspended in 10 ml 20 mM Tris-HCl (pH 7.6). Cells were broken by a single passage through a French press [15]. Unbroken cells and chromatophore membranes were pelleted by two steps of centrifugation [15] and the supernatant was collected after sedimentation of the chromatophores. This supernatant is henceforth described as the postchromatophore supernatant. It was concentrated to a volume of 1 ml by ultrafiltration using an Amicon macrosolute concentrator. The sample was applied to a Sephacryl-S200 column (bed vol. 400 ml) and was eluted using 20 mM Tris-HCl (pH 7.6) at a flow rate of 6 ml $\cdot$ h^{-1}. 5-ml fractions were collected and assayed for TMAO reductase activity using dithionite-reduced methyl viologen as electron donor [9]. Peak fractions of TMAO reductase activity were pooled and concentrated by ultra filtration.

Spectroscopy and redox titration

Reduced minus oxidised spectra of intact cells were recorded at 30 °C using a Shimadzu UV-3000 spectrophotometer in double-beam mode. Cells were suspended in RCV medium in completely filled 3 ml stoppered glass cuvettes. Ferricyanide (200 μM), TMAO (2 mM) or DMSO (2 mM) were also added to the reference cuvette as indicated in figure legends. No further additions were made to the sample cuvette. To ensure anaerobiosis the RCV medium was sparged with argon (containing less than 3 p.p.m. O_2) before use and cell suspensions were left for 20 min in the stoppered cuvettes in the dark prior to collection of data.

Absorbance changes due to cytochromes and electrochromic carotenoid pigments were measured in intact cells by dual-wavelength spectrophotometry using a Shimadzu UV-3000 spectrophotometer. Procedures for maintaining anaerobiosis during such measurements have been detailed elsewhere [16]. Reduced minus oxidised spectra of periplasmic fractions and TMAO reductase-containing fractions were recorded using a Kontron Uvikon 810 spectrophotometer.

Redox titration of cytochrome *c*-556 was performed in 20 mM Tris-HCl (pH 7.6) using the Shimadzu spectrophotometer according to Dutton [17].

Protein and bacteriochlorophyll determination

Protein concentrations were measured as in Markwell et al. [18] using bovine serum albumin as a standard. Bacteriochlorophyll was measured as described by Clayton [19].

Results

Oxidation of cytochromes by TMAO and DMSO in intact cells

Fig. 1a shows a reduced minus oxidised difference spectrum of intact cells of *Rb. capsulatus* strain N22 DNAR$^+$ with malate as reductant and DMSO as oxidant. Strain N22 DNAR$^+$ is a partially carotenoid-deficient mutant [3] and this facilitated spectroscopic studies of cytochromes. The main spectral features (Fig. 1a) were an absorbance maximum at 559 nm and a broad shoulder between 548 nm and 556 nm. A malate-reduced minus TMAO-oxidised spectrum of the same cells had similar absorbance characteristics (Fig. 1b). Spectra recorded in the presence of the uncoupler FCCP at concentrations sufficient to eliminate any electrochromic absorbance changes associated with the generation of a membrane potential during electron flow to TMAO and DMSO [6] were essentially the same as those in Fig. 1. The uncoupler-insensitive absorbance changes were, therefore, the result of TMAO and DMSO-dependent oxidation of cytochromes. These cytochrome absorbance changes were not affected by the presence of myxothiazol (3 μM) or antimycin A (3 μM). These concentrations of the inhibitors were sufficient to block electron flow via the cytochrome bc_1 complex [20] (not shown). A malate-reduced minus ferricyanide-oxidised spectrum has an absorbance maximum at 550 nm which can probably be attributed to the oxidation of both cytochrome c_1 of the cytochrome bc_1 complex

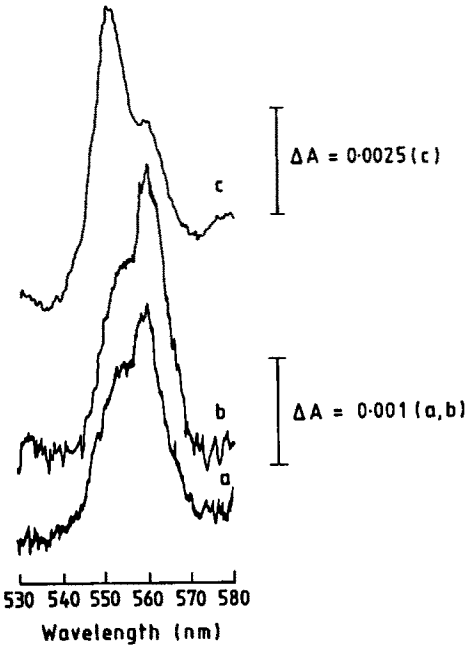

Fig. 1. Reduced minus oxidised absorption spectra of intact cells of *Rb. capsulatus* strain N22DNAR$^+$. Bacteria were suspended in argon-sparged RCV medium in completely filled 3 ml glass cuvettes. The final concentration was 20 μM BChl. DMSO (2 mM), TMAO (2 mM) or potassium ferricyanide (200 μM) were added in 10 μl volumes from concentrated stock solutions to the reference cuvette. No further additions were made to the sample cuvette. The cuvettes were sealed using plastic air-tight stoppers and 20 min allowed for the cell suspensions to become anaerobic before spectra were collected. The samples were scanned at 20 nm per min. (a) Reduced minus DMSO oxidised; (b) reduced minus TMAO oxidised; (c) reduced minus ferricyanide oxidised.

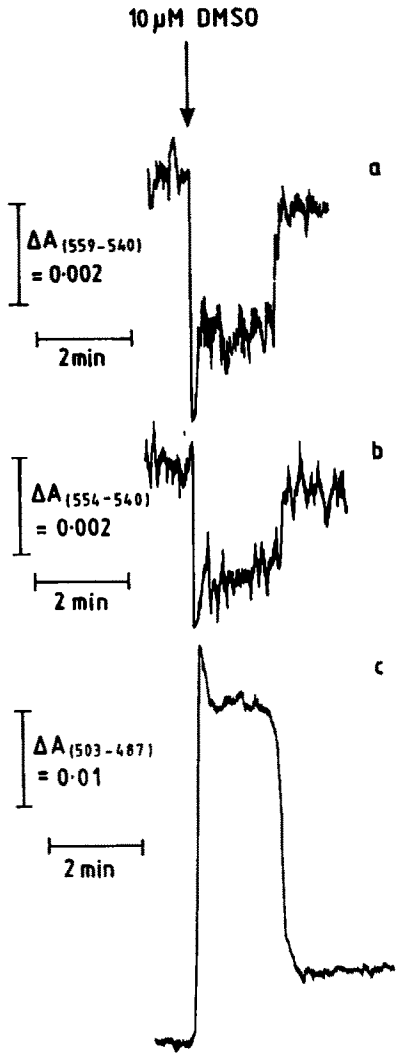

Fig. 2. DMSO-dependent cytochrome and carotenoid absorbance changes in intact cells of *Rb. capsulatus* strain N22DNAR$^+$. Cells were suspended to a final concentration of 20 μM BChl in 2.5 mls of RCV medium, in a magnetically stirred, stoppered cuvette. Anaerobiosis was maintained by gently blowing argon over the surface of the suspension via butyl rubber gas trains and stainless steel inlets and outlets through a rubber cuvette stopper. DMSO was injected in 10 μl volumes from a concentrated stock solution through the stainless steel gas outlet. (a) Cytochrome absorbance changes measured at 559–540 nm; (b) cytochrome absorbance changes measured at 554–540 nm; (c) electrochromic absorbance changes measured at 503–487 nm.

and cytochrome c_2 [11,12] (Fig. 1c). The shoulder at 560 nm in Fig. 1c is attributed to the oxidation of *b*-type cytochromes including the high-potential cytochrome *b* of the cytochrome bc_1 complex [22]. It also appears that the maximum absorbance of the cytochrome oxidized by TMAO or DMSO and giving rise to the shoulder in Fig. 1a and b lies to longer wavelengths than the combined absorbance maxima of cytochromes c_1 and c_2 (Fig. 1c).

Dual-wavelength spectrophotometry was used to monitor the kinetics of the TMAO- and DMSO-dependent cytochrome absorbance changes. Fig. 2a shows that addition of DMSO to anaerobic cells caused a rapid decrease in the absorbance at 559–540 nm; a steady state was then established which was sustained until all of the DMSO was consumed and the cytochromes were re-reduced. The absorbance change at 554–540 nm showed similar characteristics (Fig. 2b). In *Rb. capsulatus* generation of $\Delta\psi$ can be monitored from the electrochromic absorbance changes in endogenous carotenoid pigments and the duration of this $\Delta\psi$ can be used to monitor rates of respiration [20]. The development, duration and dissipation of $\Delta\psi$ following the addition of DMSO showed the same pattern (Fig. 2c) as the cytochrome absorbance changes (Fig. 2a and b). Similar results were obtained with TMAO as electron acceptor (data not shown). The data in Fig. 2 are consistent with the view that the cytochrome absorbance changes and the generation of $\Delta\psi$ are associated with electron flow to DMSO and TMAO. The rate of electron flow to DMSO or TMAO was reduced by addition of rotenone which inhibits NADH dehydrogenase [6] and this increased the duration of the cytochrome absorbance changes and the electrochromic absorbance changes (not shown).

Oxidation of cytochrome by TMAO or DMSO in periplasmic fractions

TMAO/DMSO reductase is located in the periplasmic space [9]. This compartment is the predominant location of *c*-type cytochromes rather than *b*-type cytochromes [23], and as a result we investigated whether any periplasmic *c*-type cytochromes could be oxidised by TMAO or DMSO. A periplasmic fraction was prepared from cells of strain H123 grown phototrophically in the presence of TMAO. Strain H123 is a mutant which lacks cytochrome *c'* [14] and this facilitated analysis of the less abundant periplasmic cytochromes. A dithionite-reduced minus air-oxidised spectrum of this preparation revealed the presence of a cytochrome with a broad α-absorption band ($\lambda_{max} = 556$ nm) (Fig. 3a). Addition of TMAO to the dithionite-reduced sample resulted in the disappearance of the peak at 556 nm (Fig. 3b). Addition of DMSO also caused the oxidation of the dithionite-reduced cytochrome (not shown).

Association of cytochrome c-556 with TMAO reductase

In view of the result in Fig. 3, a partial fractionation of soluble components released on breakage of cells was undertaken to determine whether cytochromes would co-fractionate with TMAO/DMSO reductase following gel filtration. A post-chromatophore supernatant prepared from *Rb. capsulatus* strain 374b (the wild-type parent of strain H123) was concentrated and applied to a Sephacryl S-200 gel filtration column. Fractions containing TMAO/DMSO reductase activity were identified, pooled and concentrated by ultrafiltration. Cytochromes c_2 and *c'* were eluted in later fractions (not shown) but a dithionite-reduced spectrum of the TMAO/DMSO reductase-containing sample (Fig. 4) revealed a cytochrome with absorbance maxima at 421 nm, 525 nm and 556 nm. In an air-oxidised spectrum, the Soret band was shifted to 408 nm and the peaks at 525 nm and 556 nm disappeared (Fig. 4). The spectral characteristics of this cytochrome were unlike those of cytochrome c_2 or *c'*. They were due to a *c*-type cytochrome, since the α-band of the dithionite-reduced cytochrome in the TMAO reductase sample was shifted from 556 nm to 550 nm when alkaline pyridine was added (not shown).

In the experiment of Fig. 5, sufficient dithionite was titrated into the TMAO/DMSO reductase sample just to reduce cytochrome *c*-556 (Fig. 5a). Addition of TMAO to the sample resulted in the oxidation of cytochrome *c*-556 (Fig. 5b and c). Similar experiments showed that addition of DMSO also resulted in oxidation of reduced *c*-556 (not shown). The spectra of air-oxidised and TMAO-oxidised samples containing TMAO/DMSO reductase showed an absorbance increase between 500 nm and 530 nm which was not typical of a cytochrome. These spectral changes may be

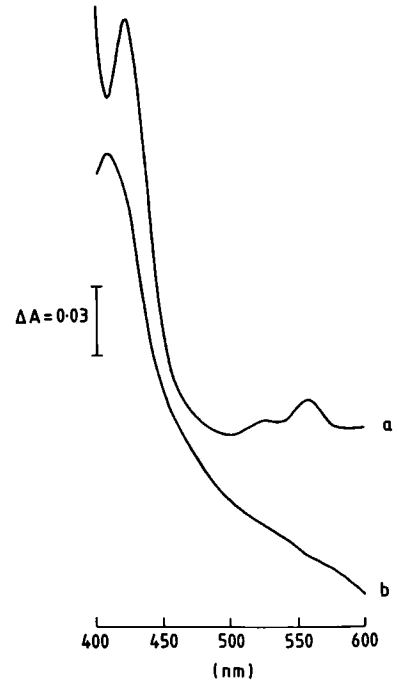

Fig. 4. Spectrum of cytochrome *c*-556 in partially fractionated post-chromatophore supernatant. The sample was prepared from fractions containing TMAO reductase activity, following chromatography, as described in the Materials and Methods. Protein concentration = 4.7 mg per ml. (a) Sample reduced by dithionite; (b) no additions.

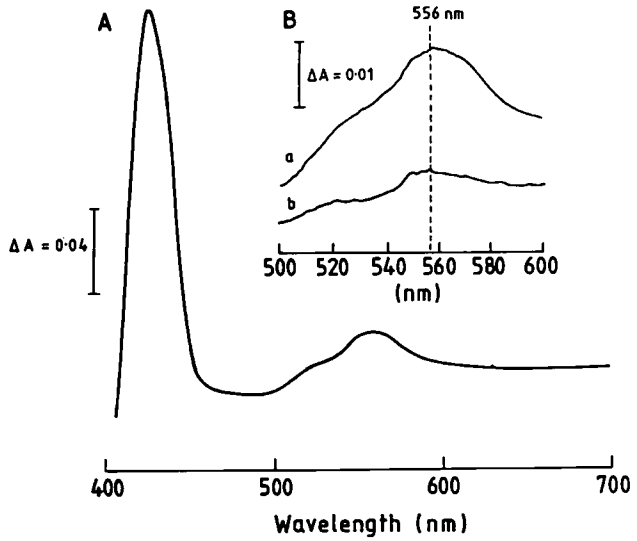

Fig. 3. Absorption spectra of a TMAO oxidisable cytochrome in periplasm from *Rb. capsulatus* strain H123. Experiments were carried out at 30 °C in 1 ml glass cuvettes, completely filled with periplasmic fractions and sealed with rubber stoppers. (A) Dithionite-reduced minus air-oxidised spectrum. The spectrum was obtained by titrating sodium dithionite into the sample cuvette until steady-state reduction was achieved. (B) Oxidation by TMAO of the reduced cytochrome: 10 µl of 100 mM TMAO was added to the sample cuvette of the experiment described in (A). 10 µl of distilled water was added to the reference cuvette. Spectra were collected at time periods over the following 5 min. (a) Spectrum prior to addition of TMAO; (b) Spectrum collected 5 min after the addition of TMAO. All additions were made via Hamilton syringes pierced through the rubber cuvette stoppers.

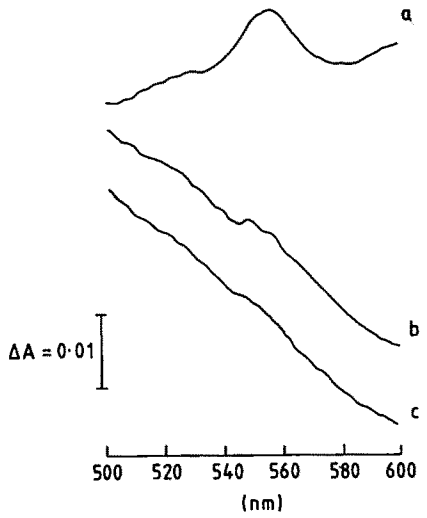

Fig. 5. Oxidation cytochrome *c*-556 by addition of TMAO. Cytochrome *c*-556 was reduced by titration with dithionite, then 3 mM TMAO was added. (a) Dithionite reduced; (b) 10 s after TMAO addition; (c) 1 min after TMAO addition. Sample prepared as described in Fig. 4, protein concentration = 1.9 mg per ml.

due to an additional uncharacterised redox centre involved in electron transfer to TMAO/DMSO reductase.

Redox potentiometry of the TMAO/DMSO reductase-containing fraction at 556–540 nm demonstrated a single cytochrome component $E_{m7.6} = +105$ mV ($n = 1$) (Fig. 6). Collection of spectra at redox potentials between 0 mV and +200 mV confirmed that the absorbance changes measured in Fig. 6 were due to the oxidation and reduction of cytochrome *c*-556. Spectra obtained with redox potentials between +200 mV and +400 mV revealed no additional high-potential cytochromes.

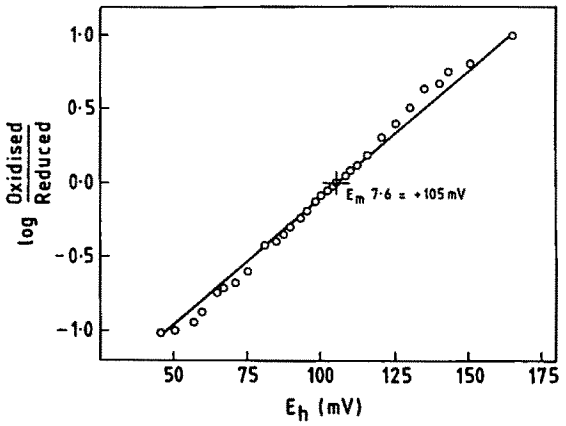

Fig. 6. Potentiometric titration of cytochrome *c*-556 in partially fractionated post-chromatophore supernatant. Experiments were carried out in a glass cuvette with a side arm attachment. The cytochrome sample was diluted to a volume of 7.5 ml with Tris-HCl buffer (pH 7.6). This entire volume was sparged with argon and placed into the redox cuvette. Anaerobiosis was maintained by keeping the sample under an atmosphere of argon during the entire experiment. Argon was passed into the cuvette via butyl rubber gas trains and stainless steel inlet and outlets. The sample was continuously stirred during the experiment by a magnetic stirrer placed at one side of the cuvette above the measuring beam. E_h changes were recorded using a combination of platinum/calomel electrode. Absorption changes were measured at 556–540 nm. The sample was oxidatively titrated with potassium ferricyanide and reductively titrated with sodium dithionite. Added redox mediators were PMS (10 μM), PES (10 μM) DAD (10 μM) and duroquinone (50 μM). The sample was allowed to equilibrate for 5–10 min at each E_h value before the absorbance was recorded.

The polypeptides in the TMAO/DMSO reductase-containing fraction were separated by polyacrylamide gel electrophoresis in SDS at room temperature as described previously [7] and stained for haem-dependent peroxidase activity [24]. Since only *c*-type haem is retained under these conditions, this procedure can identify *c*-type cytochromes [24]. A single haem-staining polypeptide (M_r = 13 500) was observed in the TMAO/DMSO reductase containing fraction (data not shown), and this supports the view that a single *c*-type cytochrome species associates with TMAO-DMSO reductase.

Discussion

This paper reports the identification of cytochromes involved in the electron-transport pathway to TMAO and DMSO in *Rb. capsulatus*. The reduced minus TMAO-oxidised difference spectra of intact cells (Fig. 1) revealed an absorbance peak at 559 nm consistent with the role of a *b*-type cytochrome and a shoulder between 548 nm and 556 nm which suggested a *c*-type cytochrome. Experiments with unfractionated periplasm and fractionated post chromatophore supernatant (Figs. 3 and 4) suggest that the absorbance shoulder in Fig. 1a and b is due to cytochrome *c*-556. The observation that the oxidation of a *b*-type cytochrome and *c*-type cytochrome by TMAO is sustained for the same period as the TMAO-dependent $\Delta\psi$ (Fig. 2) supports the view that the two cytochromes are components of the TMAO/DMSO electron-transport pathway.

Earlier studies identified cytochromes in *Rb. capsulatus* and *Rb. sphaeroides* whose levels are elevated in cells grown in the presence of DMSO [12,125]. Zannoni and Marrs [12] identified a redox component attributable to a *b*-type cytochrome ($E_{m7} = 0$ mV) in chromatophores of *Rb. capsulatus* grown with DMSO as electron acceptor. This may well be cytochrome *b*-559 which is oxidised by TMAO and DMSO (Fig. 1a and b). We have established that the cytochrome bc_1 complex is not involved in electron transport to TMAO or DMSO [20]. It follows therefore that an uncharacterised oxidoreductase must catalyse the oxidation of ubiquinol during TMAO/DMSO respiration. We suggest that a *b*-type cytochrome might be a component of this uncharacterised ubiquinol oxidase.

Cytochrome *c*-556 was identified as a component of periplasmic fractions which could be oxidised by TMAO

(Fig. 3). This cytochrome also co-fractionated with TMAO/DMSO reductase following gel filtration of the chromatophore supernatant. The data in Figs. 3 and 5 do not give information about the kinetics of cytochrome *c*-556 oxidation by TMAO reductase. This is because an excess of dithionite was required to reduce cytochrome *c*-556 and, as a result the initial oxidation of the cytochrome was accompanied by an indeterminate rate of re-reduction. The mid-point potential of cytochrome *c*-556 was $E_{m7.6} = +105$ mV and so at a reasonable poise of donor and acceptor couples it could in principle participate in electron flow to TMAO (E_{m7} TMA/TMAO = +130 mV) [26] and DMSO (E_{m7} DMS/DMSO = +160 mV) [27]. *c*-type cytochromes whose levels are increased in cells grown with DMSO have been shown to have mid-point redox potentials of +134 mV and 120 mV in chromatophores of *Rb. capsulatus* [12] and *Rb. sphaeroides* [25], respectively. These may represent in situ measurements of the cytochrome *c*-556 which we have identified.

Cytochrome *c*-556 from *Rb. capsulatus* may be an example from a group of cytochromes with α-band absorption maxima in the reduced form, at 556 or 554 nm [27]. These cytochromes are present in a variety of purple non-sulphur bacteria but their function has eluded definition. *Rb. sphaeroides* and *Rhodopseudomonas palustris* are capable of respiration with TMAO and DMSO as electron acceptors [25,29] (McEwan, unpublished) and so it is possible that the cytochrome *c*-554 of *Rb. sphaeroides* and the cytochrome *c*-556 of *Rps. palustris* [28] have a functional role similar to *Rb. capsulatus* *c*-556. The cytochrome *c*-556 class does appear to share common physicochemical properties [28]. A feature of the *Rps. palustris* cytochrome *c*-556 is that its absorption maximum (α-band) shifts from 556 to 550 nm during prolonged frozen storage. A similar observation was made for the cytochrome *c*-556 of *Rb. capsulatus* during the course of this study (data not shown).

The co-fractionation of cytochrome *c*-556 and TMAO reductase following gel filtration requires comment. The subunit molecular weight of the cytochrome *c*-556 from *Rb. capsulatus* was estimated to be 13000 which is similar to that reported for the native form of *Rp. palustris* cytochrome *c*-556 [28]. Cytochrome *c'* (M_r = 26000) and cytochrome c_2 (M_r = 12000), which do not appear to function in electron transport to TMAO/DMSO, did not co-fractionate with TMAO/DMSO reductase but were eluted in later fractions. Cytochrome *c*-556 could not be expected to cofractionate with TMAO reductase during gel filtration, since the apparent molecular weight of a polypeptide staining for TMAO reductase activity after SDS PAGE is 46000 [7], which is clearly distinct from the cytochrome *c*-556 polypeptide. To explain these observations we suggest that cytochrome *c*-556 forms a structural association with TMAO/DMSO reductase: in some cases, electron transport in the periplasm may proceed via multi-redox centre complexes and not via random collision of individual redox proteins.

In *Chlorobium limicola* F *thiosulphatophilum* flavocytochrome *c*-553 has been shown to form a complex with another soluble *c*-type cytochrome, cytochrome *c*-555 [30]. This redox complex appears to be stabilised via electrostatic interactions [30]. TMAO reductase and cytochrome *c*-556 were not dissociated following gel filtration at high ionic strength (data not shown) and this may indicate that hydrophobic forces are additionally involved in the assembly of a TMAO reductase-cytochrome *c*-556 complex.

Acknowledgements

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