Dissimilatory iron(III) reduction by *Rhodobacter capsulatus*

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The photosynthetic proteobacterium *Rhodobacter capsulatus* was shown to be capable of dissimilatory Fe(III) reduction. Activity was expressed during anaerobic phototrophic and microaerobic growth with malate as the carbon source, but not during equivalent aerobic growth. A variety of Fe(III) complexes were demonstrated to act as substrates for intact cells and membrane fractions of strain N22DNAR using a ferrozine assay for Fe(II) formation. Rates of reduction appeared to be influenced by the reduction potentials of the Fe(III) complexes. However, Fe(III) complexed by citrate, which is readily reduced by *Shewanella putrefaciens*, was a poor substrate for dissimilation by *R. capsulatus*. The Fe(III)-reducing activity of *R. capsulatus* was located solely in the membrane fraction. The reduction of Fe(III) complexes by intact cells was inhibited by 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO), suggesting the involvement of ubiquinol:cytochrome c oxidoreductases in the electron transport chain. Lack of sensitivity to myxothiazol plus data from mutant strains implies that the cytochrome bc₁ complex and cytochrome c₃ are not obligatory for dissimilation of Fe(III)(maltol). Alternative pathways of electron transfer to Fe(III) must hence operate in *R. capsulatus*. Using strain N22DNAR, the reduction rate of Fe(III) complexed by nitrilotriacetic acid (NTA) was elevated compared to that of Fe(III)(maltol), and moreover was sensitive to myxothiazol. However, these differences were not observed in the absence of the electron donor malate. The governing factor for the reduction rate of Fe(III)(maltol), thus appears to be the limited Fe(III)-reducing activity, whilst the reduction rate of Fe(III) complexed by NTA is controlled by the flux of electrons through the respiratory chain. The use of mutant strains confirmed that the role of the cytochrome bc₁ complex in Fe(III) reduction becomes apparent only with the superior substrate. The energy-conserving nature of Fe(III) reduction by *R. capsulatus* was demonstrated by electrochromic measurements, with the endogenous carotenoid pigments being employed as indicators of membrane potential generation in intact cells. Using Fe(III)EDTA as electron acceptor, periods of membrane potential generation were directly proportional to the quantity of complex added, and were extended in the presence of HQNO. Fe(III)-dependent carotenoid bandshifts were abolished by addition of the protonophoric uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone.

**Keywords:** dissimilatory Fe(III) reduction, *Rhodobacter capsulatus*, membrane-bound Fe(III) reductase activity, iron chelators, carotenoid bandshift

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**Abbreviations:** DFO, desferrioxamine-B; DMHP, 1,2-dimethyl-3-hydroxy-4-pyridinone; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; ferrozine, 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine; HQNO, 2-heptyl-4-hydroxyquinoline-N-oxide; maltol, 2-methyl-3-hydroxy-4-pyranone; NTA, nitrilotriacetic acid.
INTRODUCTION

The bacterial dissimilation of Fe(III) in anaerobic and microaerobic environments can have a significant influence on a number of biogeochemical element cycles (Lovley, 1991, 1993, 1995; Nealson & Saffarini, 1994). Despite the wealth of ecological data, there is still relatively little information available concerning Fe(III) respiration in pure cultures. The most widely studied species of Fe(III)-dissimilating bacteria are Geobacter metallireducans and Shewanella putrefaciens, which can both grow anaerobically with Fe(III) as the sole terminal electron acceptor (Lovley & Phillips, 1988; Lovley et al., 1989). Fe(III) reductase activity for both these organisms has been located in cell membranes (Gorby & Lovley, 1991; Myers & Myers, 1993a). Other bacteria found to possess a membrane-bound Fe(III) reductase include Spirillum itersonii (Dailey & Lascelles, 1977) and Staphylococcus aureus (Lascelles & Burke, 1978), the former organism having been demonstrated to translocate protons in response to Fe(III) (Short & Blakemore, 1986). Work concerning the pathways of electron transport to Fe(III) during dissimilation of the cation has thus far been focused on S. putrefaciens, with membrane-bound cytochromes, menaquinone and methylmenaquinone, and a [3Fe–4S] centre all shown to be involved in the reductive process (Myers & Myers, 1992, 1993b; Tsapin et al., 1994).

The photosynthetic proteobacterium Rhodobacter capsulatus is an attractive candidate in which to study dissimilatory Fe(III) reduction for a variety of reasons. Although it has a particularly complicated electron-transport system that includes components of cyclic photosynthetic electron transport and aerobic respiration, R. capsulatus has been demonstrated to utilize numerous terminal electron acceptors under anaerobic conditions (Ferguson et al., 1987; McEwan, 1994). This is a property shared with the highly versatile facultative anaerobe S. putrefaciens (Nealson & Saffarini, 1994). Furthermore, a number of respiratory mutant strains of R. capsulatus are available (Bell et al., 1992), allowing evidence to be gleaned as to components of the electron transport chain to any Fe(III) reductase. Finally, conservation of energy during anaerobic respiration can be easily investigated in R. capsulatus, with the electrochemical response of the endogenous carotenoid pigments providing a convenient non-invasive technique for monitoring membrane potential in vivo (McEwan et al., 1982, 1983, 1985; Bell et al., 1992; Richardson et al., 1994).

When elucidating rates of Fe(III) reduction by bacteria, the question of Fe(III) speciation must be addressed. In aqueous solution, the mononuclear hexa-aquo Fe(III) complex exists only under highly acidic conditions. As pH increases, protons are lost from the ligating water molecules, leading to the formation of poorly soluble oxo- and hydroxo-bridged polymeric Fe(III) species (Powell & Heath, 1994). During recent work on S. putrefaciens, it was noted that the presence of certain Fe(III) chelators can increase the rate of reduction of the cation by two orders of magnitude compared to uncomplexed Fe(III) in pH 7 buffered assays on whole cells (Dobbin et al., 1995). In the present study a number of these Fe(III) chelators (Fig. 1) have been used to demonstrate that R. capsulatus is capable of dissipimilatory Fe(III) reduction.

METHODS

Bacterial strains. R. capsulatus strains MT1131, MTG4SA, MTBC1 and MTG518 were a generous gift from Dr F. Daldal, University of Pennsylvania, USA. The mutants MTG4SA, MTBC1 and MTG518 lack cytochrome c₃, the cytochrome bc₃ complex, and cytochrome c₅₅₃/cytochrome bc₃, respectively, whilst MT1131 is the parent strain (Daldal et al., 1986, 1987). Strains H123 and 37b4 were obtained from Dr H. Hudig, University of Freiburg, Germany, and are a cytochrome c₇ deficient mutant and the wild-type, respectively (Hudig et al., 1986). N22DNAR’ is a nitrate-respiring isolate (McEwan et al.,
The anaerobic respiratory properties of all these strains are fully described by Bell et al. (1992).

**Growth conditions.** Cultures of strains MT1131, MTG4S4, N22DNAR+, 37b4 and H123 were grown to late exponential phase under anaerobic phototrophic conditions at 30 °C in RCV medium (Weaver et al., 1975), with malate as carbon source and ammonium as nitrogen source (Richardson et al., 1988). For strains MTBC1 and MTGS18, which are incapable of phototrophic growth, 100 ml cultures were grown microaerobically at 30 °C in 250 ml foam-plugged conical flasks by shaking at 160 r.p.m. until late exponential phase was reached. Microaerobic growth of the photosynthetic strains was performed in total darkness. A 20 ml culture of N22DNAR+ was grown aerobically, at 30 °C and in darkness, in a 2 l foam-bunged conical flask by shaking at 250 r.p.m. until late exponential phase was attained. Antibiotics were added to the medium where appropriate (Bell et al., 1992).

**Cell fractionation.** For the preparation of a periplasmatic fraction, a 100 ml culture of R. capsulatus strain N22DNAR+ was harvested and washed with 100 mM Tris/HCl, pH 7.4, prior to suspension in 10 ml 0.5 M sucrose, 5 mM EDTA, 100 mM Tris/HCl, pH 7.6, containing 40 mg lysozyme. Following incubation at 30 °C for 30 min, debris was removed by ultracentrifugation at 45000 r.p.m. for 1 h in a Beckman type 70Ti rotor. For membrane preparation, a 100 ml culture of strain N22DNAR+ was harvested and washed with 10 mM Tris/HCl, pH 8.1, prior to suspension in 30 ml of the same buffer. Following two passes through a chilled French pressure cell operated at 1000 p.s.i. (6.9 MPa), lysozyme and MgCl₂ were added to final concentrations of 0.5 mg ml⁻¹ and 1 mM respectively and the mixture stirred at 20 °C for 10 min. A few crystals of DNase I and RNase A were then added and stirring was continued for a further 10 min. The membranes were pelleted by ultracentrifugation at 45000 r.p.m. for 1 h in a Beckman type 70Ti rotor.

**Ferrozine assay for Fe(III) reduction.** This assay was described and validated fully by Dobbin et al. (1995); it is based upon monitoring the formation of the highly stable 3:1 ferrozine:Fe(II) complex at its λₘₐₓ value of 562 nm. An Aminco DW2000 spectrophotometer set in split-beam mode was employed. Rates of formation of Fe(II)(ferrozine)₃ correlate strongly with rates of dissimilatory Fe(III) reduction if the Fe(III) complex under investigation is reduced to an Fe(II) complex of low kinetic and/or thermodynamic stability. EDTA is unsuitable for use in the assay since ferrozine does not compete effectively for Fe(II) if the cation is complexed by this hexadentate aminocarboxylate.

**Electrochromic measurements.** The underlying theory of and basic methodology for the measurement of carotenoid pigment absorption changes in intact cells of R. capsulatus as an indicator of membrane potential generation in response to light or the introduction of external electron acceptors is detailed by Clark & Jackson (1981) and McEwan et al. (1982). In this study a Shimadzu UV-3000 dual-wavelength/double-beam spectrophotometer was used with wavelength pairs of 503–487 nm for strain N22DNAR+ and 528–511 nm for strain 37b4. Fe(III)(malto)₃ is unsuitable for use in electrochromic measurements owing to the absorbance spectrum of the complex interfering with carotenoid signals.

**Chemicals.** All Fe(III) ligands used in this study are commercially available with the exception of DMHP, which was synthesized according to Kontoghiorghes & Sheppard (1987). Stock solutions of complexes were prepared using a 3:1 molar ratio of bidentate ligand to Fe(III) or a 1:1 molar ratio of tri-, tetra- or hexadentate ligand to Fe(III). Stock solutions of the respiratory inhibitors HQNO (3.85 mM) and myxothiazol (410 μM) and the protonophore FCCP (100 μM) were prepared in methanol.

**RESULTS**

**Reduction of Fe(III) monitored by the ferrozine assay using intact cells of strain N22DNAR+**

The dissimilatory reduction of Fe(III) could be readily detected in harvested intact cells of R. capsulatus strain N22DNAR+ grown under anaerobic phototrophic conditions using the ferrozine assay to monitor Fe(II) formation. For example, a time-course observed using Fe(III) complexed by NTA, in the presence and absence of malate, is shown in Fig. 2. Steady-state (linear) rates of Fe(II)(ferrozine)₃ formation were calculated with data from the first 100 s of these kinetic traces. Rates obtained in assays using a variety of Fe(III) complexes are presented in Table 1, together with data regarding the influence of malate, HQNO and myxothiazol. Malate was found not to catalyse Fe(III) reduction with no cells in the assay mixture. In the absence of electron donor or inhibitor, Fe(III) complexed by NTA was reduced slightly faster by intact cells of strain N22DNAR+ than Fe(III)(malto)₃. However, significantly greater enhancement of reduction rate was achieved upon adding malate to the assay mixture using Fe(III) complexed by NTA compared to Fe(III)(malto)₃. Addition of HQNO caused similar inhibition of Fe(II) formation from Fe(III)(malto)₃ and Fe(III) complexed by NTA. In comparison to Fe(III)(malto)₃, Fe(III) complexed by citrate was reduced much more slowly by intact cells of strain N22DNAR+, with the rate of Fe(II) formation being only 2.4 % of that obtained with the former Fe(III) species. Furthermore, the reduction rate of Fe(III) complexed by citrate was not influenced by malate or HQNO. Whilst
**Table 1.** Effects of various Fe(III) ligands on the rate of Fe(II)(ferrozine)₃ formation in the whole-cell ferrozine assay using strain N22DNAR⁺

These representative data correspond to the same cell batch. Experiments were repeated on a further two batches, and all formation rates were within 12% of those presented. NP, Experiment not performed.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Steady-state rate of Fe(II)(ferrozine), formation [nmol min⁻¹ (mg dry weight cells)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No addition</td>
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<tr>
<td>Maltol</td>
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<tr>
<td>NTA</td>
<td>5.1</td>
</tr>
<tr>
<td>Citric acid</td>
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</tr>
<tr>
<td>Acetohydroxamic acid</td>
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<td>Salicylic acid</td>
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<td>DMHP</td>
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</table>

**Table 2.** Effects of various Fe(III) ligands on the rate of Fe(II)(ferrozine), formation in the ferrozine assay using membranes of strain N22DNAR⁺, with 500 µM NADH present

These representative data correspond to the same membrane batch. Experiments were repeated on a further two batches, and all formation rates were within 7% of those presented. NP, Experiment not performed.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Steady-state rate of Fe(II)(ferrozine), formation [nmol min⁻¹ (mg dry weight cells)⁻¹]</th>
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</thead>
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<tr>
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<td>No addition</td>
</tr>
<tr>
<td>Maltol</td>
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<tr>
<td>NTA</td>
<td>0.17</td>
</tr>
<tr>
<td>Citrate</td>
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<tr>
<td>Acetohydroxamic acid</td>
<td>0.14</td>
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<tr>
<td>DFO</td>
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</table>

Fe(III)(acetohydroxamate)₃ was reduced at a rate comparable to Fe(III)(maltol)₃, rates of Fe(II) formation from Fe(III) complexed by salicylate and Fe(III)(DMHP)₃ were an order of magnitude lower.

Additional ferrozine assays indicated the presence of myxothiazol (1, 5 or 10 µM) to have insignificant effect upon rates of Fe(II) formation from either Fe(III)(maltol)₃ or Fe(III) complexed by NTA in the absence of malate (data not tabulated). However, with 500 µM malate in the assay mixture, the presence of 5 µM myxothiazol inhibited Fe(II)(ferrozine)₃ formation from Fe(III) complexed by NTA, but had no effect upon the malate-stimulated reduction of Fe(III)(maltol)₃. (Table 1). The presence of FCCP (1.7 µM) slowed the reduction rate of Fe(III)(maltol)₃ to 64% of that obtained in the absence of the protonophore (data not tabulated). When HQNO was used at lower concentrations (10 or 20 µM), Fe(III) reduction rates did not vary significantly from the values quoted in Table 1 using 40 µM of this inhibitor.

The rates of Fe(II) formation from Fe(III)(maltol)₃ and Fe(III) complexed by NTA using strain N22DNAR⁺ grown microaerobically were 90% and 87% respectively of those obtained using phototrophic anaerobic cells.

No formation of Fe(II)(ferrozine)₃ from Fe(III) complexed by NTA or Fe(III)(maltol)₃ was noted in assays with aerobically grown cells of strain N22DNAR⁺.

**Fe(III) reduction by membranes of strain N22DNAR⁺**

Upon fractionation of strain N22DNAR⁺, all Fe(III) reductase activity was found in the membranes. No formation of Fe(II) from either Fe(III)(maltol)₃ or Fe(III) complexed by NTA was observed using a periplasmic fraction pre-reduced with dithionite. Comparative rates of Fe(II)(ferrozine)₃ formation in the ferrozine assay using N22DNAR⁺ membranes with NADH as electron donor and various Fe(III) chelates (Table 2) showed slight variation from intact cell data. Whilst Fe(III)(maltol)₃ was
Table 3. Rates of Fe(II)(ferrozine)₃ formation in the ferrozine assay using whole cells with Fe(III) complexed by NTA or Fe(III)(maltol)₃, and 500 µM malate present

The representative data in each row correspond to the same cell batch. Experiments were repeated on a further two batches for each strain, and all formation rates were within 12% of those presented.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutant phenotype</th>
<th>Steady-state rate of Fe(II)(ferrozine)₃ formation [nmol min⁻¹ (mg dry weight cells)⁻¹]</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>From Fe(III)</td>
<td>From Fe(III)(maltol)₃</td>
</tr>
<tr>
<td></td>
<td>complexed by NTA</td>
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</tr>
<tr>
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<td>Parent strain</td>
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</tr>
<tr>
<td>MTG4S4</td>
<td>cyt c₂</td>
<td>13</td>
</tr>
<tr>
<td>MTCBCI</td>
<td>cyt bc</td>
<td>8-4</td>
</tr>
<tr>
<td>MTGS18</td>
<td>cyt c₂/cyt bc</td>
<td>3-1</td>
</tr>
<tr>
<td>769</td>
<td>Parent strain</td>
<td>9-1</td>
</tr>
<tr>
<td>H123</td>
<td>cyt c⁻</td>
<td>12</td>
</tr>
</tbody>
</table>

**Fig. 3.** Electrochromic bandshifts of the carotenoid pigments of whole cells of strain N22DNAR₆. (a) In response to illumination from a 100 W tungsten bulb, followed by additions of 25 µl 100 mM NaHEPES, pH 7-0, and Fe(III)EDTA to concentrations of 40 µM and 80 µM; (b) In response to 40 µM Fe(III)EDTA subsequent to additions of 10 µM HQNO and 1 µM FCCP. The shift upon illumination was some five times the magnitude of that upon injection of Fe(III)EDTA. The shift upon injection of buffer is probably due to a small amount of dissolved oxygen being present. Harvested and washed whole cells were suspended in 1 ml 100 mM NaHEPES, pH 7-0, per 60 ml culture, a 100 µl aliquot being used in constantly stirred 2-5 ml assays with the same buffer. Further details are given by Clark & Jackson (1981).

Fe(III) reduction by mutant strains

Using Fe(III) complexed by NTA and Fe(III)(maltol)₃, and in the presence of malate, rates of Fe(II)(ferrozine)₃ formation for *R. capsulatus* strains MT1131, MTG4S4, reduced more slowly than Fe(III) complexed with NTA and Fe(III)(acetohydroxamate)₃ in whole cells, the converse was found using membranes. Furthermore, HQNO caused insignificant inhibition of Fe(II) formation from Fe(III)(maltol)₃ and Fe(III) complexed by NTA using membranes. However, similarly to intact cells, reduction of Fe(III) complexed with citrate by membranes was slow in comparison to Fe(III)(maltol)₃ (i.e. 7-4 %). The rate of reduction of Fe(III)DFO by membranes was also some order of magnitude lower than that of Fe(III)(maltol)₃. Succinate (500 µM) served as an electron donor comparable in efficiency to NADH for the reduction of Fe(III)(maltol)₃ and the cation complexed by NTA (data not presented). Neither NADH nor succinate catalysed Fe(III) reduction in the absence of membranes. Performing ferrozine assays on membranes with the detergent Triton X-100 (0-01-0-25 %) present gave no variation in Fe(II) formation rates from either Fe(III)(maltol)₃ or Fe(III) complexed by NTA (data not presented).
NTA, Fe(II) was formed fastest from the parent strain and in the absence of cytochrome c, variations in Fe(II) reduction rates were negligible. The Fe(II) formation rate from Fe(III)(maltol) was considerably slower for the double mutant MTGS18 than MT1131. However, the absence of cytochrome c or the cytochrome bc complex alone caused insignificant variations in Fe(III)(maltol) reduction rate relative to the parent strain. In comparison to the phototrophic anaerobic cultures, cells of strains MT1131 and MTG4S4 grown under microaerobic conditions formed Fe(II) from Fe(III) complexed by NTA at rates of 89% and 80%, respectively, and from Fe(III)(maltol) at rates of 105% and 92%, respectively (data not tabulated). For the cytochrome c-deficient mutant H123, reduction rates of Fe(III) complexed by NTA and Fe(III)(maltol) were comparable to those obtained with the parent strain 37b4 (Table 3).

Electrochromic carotenoid bandshifts induced during Fe(III) reduction

The generation of Fe(III)-dependent carotenoid bandshifts, indicative of a cytoplasmic membrane potential being created, was observed for R. capsulatus strain N22DNAR* using EDTA to chelate the cation. Fig. 3(a) presents bandshifts obtained following illumination, addition of NaHEPES or addition of Fe(III)EDTA. The bandshift observed with buffer was insignificant compared to those with Fe(III)EDTA. Doubling the concentration of Fe(III) added caused an increase in bandshift duration. However, the long periods of decay for the Fe(III)-induced bandshifts disallow any confident calculation of reduction rates. Fig. 3(b) shows bandshifts induced with 40 μM Fe(III)EDTA being extended by HQNO and abolished by the uncoupling agent FCCP. Lowering of the baseline was observed on addition of Fe(III)EDTA (data not presented). Fe(III)-dependent electrochromic carotenoid bandshifts were also obtained using R. capsulatus strain 37b4 (data not presented).

DISCUSSION

The results presented in this work clearly show that R. capsulatus can reduce Fe(III) chelates in reactions which are coupled to the generation of a membrane potential. Fe(III) can thus be added to the extensive list of anaerobic electron acceptors for the bacterium, which includes S-oxides, N-oxides and N-oxyanions (Ferguson et al., 1987; McEwan, 1994).

Influence of chelating agents on reduction of Fe(III) by intact cells

With the exception of Fe(III) complexed by citrate, comparative rates of Fe(II)(ferrozine) formation from the various Fe(III) complexes using whole cells of R. capsulatus strain N22DNAR* were in broad agreement with data obtained from S. putrefaciens (Dobbin et al., 1995), although specific activities were an order of magnitude lower in this study. These data are consistent with the view that the kinetics of Fe(III) reduction are controlled to some extent by the reduction potential of the Fe(III) chelate. The rate of reduction of high-potential Fe(III)(maltol) was an order of magnitude faster than that of low-potential Fe(III)(DMHP), in spite of the fact that these closely related Fe(III) species are both uncharged and of similar size. Using S. putrefaciens, whole-cell ferrozine assays indicated that Fe(II)(ferrozine) formed from Fe(III) complexed by citrate at a rate approximately one-third of that from Fe(III)(maltol), (Dobbin et al., 1995), and this was also found to be the case when bacterial cells were replaced with the chemical reducing agent ascorbic acid (P. S. Dobbin & D. J. Richardson, unpublished results). The comparable figure from the present study was 24% for strain N22DNAR, which demonstrates that Fe(III) complexed by citrate is a poor substrate for dissimilation by R. capsulatus. Slower flux of electrons to Fe(III) complexed by citrate is also illustrated by the lack of influence exerted by the presence of malate or HQNO upon reduction rates compared to Fe(III)(maltol), and the cation complexed by NTA. That Fe(III) complexed by citrate is a poor electron acceptor in R. capsulatus but not S. putrefaciens may indicate differences to exist between any specific Fe(III)-dissimilating enzymes in the two bacteria. A biological (as opposed to physicochemical) reason for the slow reduction of Fe(III) complexed by citrate could be the role of the ligand as a siderophore in R. capsulatus. Indeed, citrate has been implicated in iron assimilation for the close phylogenetic relation Rhodobacter sphaeroides (Moody & Dailey, 1984, 1985). By analogy with Escherichia coli (Waggeg & Braun, 1981), an outer-membrane receptor which transports Fe(III) complexed by citrate into the periplasmic compartment may well exist for R. capsulatus. In view of the high initial concentration of Fe(III) in the ferrozine assays, it seems unlikely that this receptor will utilize all the citrate-chelated cation at the expense of dissimilatory reduction.

Influence of chelating agents on reduction of Fe(III) by membranes

Again with the exception of citrate, relative Fe(II) formation rates from different Fe(III) chelates in assays using membranes of R. capsulatus strain N22DNAR generally concurred with the S. putrefaciens membrane data (Dobbin et al., 1995). Moody & Dailey (1984, 1985) have found DFO not to act as a siderophore for R. sphaeroides. If this is also the case for R. capsulatus, then the extremely low rate of formation of Fe(II)(ferrozine), observed from Fe(III) chelated by DFO with N22DNAR+...
membranes is probably a consequence of low reduction potential. The ability to utilize NADH and succinate as electron donors indicates localization of Fe(III) reduction at the cytoplasmic membrane, whilst the range of Fe(III) chelates reduced, which vary in overall size, charge and hydrophobicity, suggests that any specific Fe(III) reductase might possess a periplasmic active site. For S. putrefaciens, the reduction rates of Fe(III)(malto)₃ and the cation complexed with NTA by membranes compared to intact cells were 14% and 16%, respectively (Dobbin et al., 1995). However, from the present study on strain N22DNAR⁺, the corresponding figures were 3·5% and 2·0% (using the intact-cell rates obtained with malate present). The most probable explanation for this finding is that more of the membranes will form as inverted vesicles from cells of R. capsulatus, and hence less of the active site is available for reaction with substrate in fractions prepared from this bacterium. Such a phenomenon is related to the specialized structure of the intracytoplasmic membrane of R. capsulatus, which is formed by a process of invagination to incorporate photosynthetic pigments and pigment-binding proteins (McEwan, 1994). A possible reason for the faster reduction of Fe(III)(malto)₃ compared to Fe(III)(acetoxyhydroxamate)₃ and the cation complexed by NTA is that the former species is both uncharged and reasonably lipophilic, and thus might penetrate the membrane vesicle to access any active site inside. However, attempts to validate this hypothesis using Triton X-100 to puncture the membrane vesicles were unsuccessful.

Evidence for a specific Fe(III) reductase

The carotenoid bandshift studies undertaken here demonstrate that reduction of Fe(III)EDTA by R. capsulatus strains N22DNAR⁺ or 37b4 is coupled to the generation of a transmembrane proton electrochemical gradient. Increasing quantities of Fe(III)EDTA extended duration of carotenoid bandshifts, and this was correlated with a longer period of Fe(III) reduction. The presence of HQNO slowed electron flow to Fe(III), resulting in protons being extruded over a longer period, whilst FCCP uncoupled Fe(III) reduction from energy conservation, leading to bandshifts being abolished. The lowering of the baseline observed upon addition of HQNO and FCCP is indicative of uncoupling, although in the presence of HQNO the proton electrochemical gradient clearly persisted with reduction of Fe(III)EDTA. The inhibition of Fe(II) formation by FCCP observed in the ferrozine assay is not consistent with a role of dissipating the protonmotive force, as a thermodynamic backpressure should be removed in uncoupled cells leading to a stimulatory effect upon Fe(III) reduction. However, inhibition of Fe(III) reduction by FCCP using S. putrefaciens has been noted (Dobbin et al., 1995), as well as inhibition of the deamination reactions of other bacteria by a range of protonophores (Walter et al., 1978).

The carotenoid bandshifts obtained in this present work with Fe(III)EDTA cannot be quantified easily and H⁺/e⁻ ratios were not calculated. We are thus not in a position to say whether any specific Fe(III) reductase of R. capsulatus functions as a proton pump. Indeed, we have not obtained any irrefutable evidence for the presence of a physiological Fe(III) reductase in the bacterium. However, some of our data do suggest that a specific enzyme donates electrons to Fe(III) as opposed to non-specific reduction of the cation by one or more electron-transport components. Primarily, the fact that activity was not present in aerobically grown cells but was inducible under phototrophic anaerobic and microaerobic conditions strongly suggests that a specific Fe(III) reductase is expressed under oxygen-limited conditions.

Amongst the electron-transport components of R. capsulatus which may non-specifically reduce Fe(III) are cytochromes. Evidence for this is provided by the finding that horse-heart cytochrome c reduces certain Fe(III) species at appreciable rates, with the cation complexed by NTA being reduced faster than Fe(III)(malto)₃ (P. S. Dobbin & D. J. Richardson, unpublished results). However, data obtained here using a periplasmic fraction of strain N22DNAR⁺ suggest that the non-specific oxidation of water-soluble cytochromes by Fe(III) is of no importance for R. capsulatus, and moreover lends support to the view that a specific Fe(III) reductase is operating. The periplasmic cytochrome c₉ of Desulfovibrio vulgaris has recently been implicated in both Fe(III) reduction and U(VI) reduction by this bacterium (Lovley, 1993; Lovley et al., 1993), although whether or not the protein is a physiological Fe(III) reductase is yet to be established (Lovley, 1995). It seems unlikely that such a system could be coupled to vectorial proton translocation since the primary electron donor is thought to be periplasmic hydrogenase.

Components of the electron transport chain to Fe(III)

The inhibition of electron transport to Fe(III) in R. capsulatus strain N22DNAR⁺ by HQNO suggests the involvement of a cytochrome b dependent quinol-oxidizing complex. However, the insensitivity to myxothiazol of Fe(II) formation from Fe(III)(malto)₃ appears to indicate that the cytochrome bc₁ complex is not involved in electron transport to this substrate, whilst the comparable reduction rates of Fe(III)(malto)₃ obtained using MT1131 and its cytochrome bc₁ deficient mutant MTCB11 support such a theory. The operation of an alternative ubiquinol:cytochrome c oxidoreductase activity in R. capsulatus for the reduction of this substrate is moreover suggested. However, it has been shown with strains of R. capsulatus possessing a poor capacity for
nitrous oxide reduction that the flux of electrons from an alternative ubiquinol:cytochrome $c$ oxidoreductase is sufficient to render respiration of this substrate nearly insensitive to myxothiazol (Richardson et al., 1989). The dependence of the reaction on the cytochrome $bc_1$ complex was only revealed when the terminal nitrous oxide reductase activity was high, and parallel pathways of ubiquinol oxidation were subsequently proposed to exist for electron transport to the substrate. Therefore, if Fe(III)-reducing activity is sufficiently low to govern the rate of reaction, then the flow of electrons to Fe(III)(maltol)$_3$ in $R.$ capsulatus may not be independent of the cytochrome $bc_1$ complex.

In contrast to results with Fe(III)(maltol)$_3$, reduction rates of Fe(III) complexed by NTA were approximately halved in strain MTCBC1 compared to the parent MT1131. Moreover, whilst Fe(III) complexed by NTA was reduced at rates twice the rate of Fe(III)(maltol)$_3$ in MT1131, reduction rates for the two species are similar in MTCBC1. These data, from internally controlled experiments, strongly suggest that the cytochrome $bc_1$ complex is involved in electron transport to Fe(III) complexed by NTA, and are corroborated by the finding with strain N22DNAR$^+$ that myxothiazol significantly inhibited reduction of the species in the presence of malate. Fe(III) complexed by NTA thus appears to be a superior substrate for reduction by $R.$ capsulatus, with Fe(III) reducing activity being sufficiently high for the inhibition of the cytochrome $bc_1$ complex to depress reaction rate. That the reduction rate of Fe(III) complexed with NTA by strain N22DNAR$^+$ in the absence of malate was unaffected by myxothiazol demonstrates availability of electrons from the ubiquinol pool to also be a controlling factor. It is conceivable that some component of the cytochrome $bc_1$ complex is responsible for reduction of Fe(III) complexed by NTA, and that similar non-specific reduction of Fe(III)(maltol)$_3$ does not occur owing to differences in reduction potentials between the two Fe(III) species.

For the cytochrome $c_2$ deficient mutant MTGS4S4, only slight lowering of Fe(II) formation rates compared to MT1131 were noted from both Fe(III)(maltol)$_3$ and the cation complexed by NTA. This suggests that the electron flow to Fe(III) in $R.$ capsulatus can operate independently of cytochrome $c_2$. Previous work has shown cytochrome $c_2$ to be obligatory for electron transport to the nitrous oxide reductase of the bacterium (Richardson et al., 1991), but reduction of nitric oxide by MTGS4S4 has been found to occur at a rate comparable with that using MT1131 (Bell et al., 1992). Hence another mediator of electron transport from the cytochrome $bc_1$ complex to the nitric oxide reductase was proposed by Bell et al. (1992). McEwan (1994) has recently suggested that the redox protein in question could be cytochrome $c_{55}$, which is thought to operate in parallel with cytochrome $c_2$ during cyclic electron transport (Jones et al., 1990).

The formation of Fe(II) from Fe(III)(maltol)$_3$ using the doubly deficient mutant MTGS18 was at a rate less than half that using the parent MT1131, whilst a similar drop in reduction rate of Fe(III) complexed by NTA using MTGS18 as opposed to cytochrome $bc_1$ deficient MTCBC1 was noted. These data show that cytochrome $c_2$ is not obligatory for electron transport from the alternative ubiquinol:cytochrome $c$ oxidoreductase to Fe(III). However, the differences in reduction rates between strains are not sufficient for firm conclusions to be drawn regarding the relative abilities of cytochrome $c_2$ and the alternative redox protein to mediate electron transport in the absence of the cytochrome $bc_1$ complex. The similar rates of Fe(II) formation from Fe(III) complexed by NTA and Fe(III)(maltol)$_3$ in MTGS18 reinforce the hypothesis that the cytochrome $bc_1$ complex contributes to reduction of the former species in wild-type cells.

Data collected with the parent strain 37b4 and its mutant H123 suggest that the pentacoordinate cytochrome $c$ plays no role in Fe(III) respiration. Similar experiments have previously shown this protein not to be involved in the reduction of nitric oxide by $R.$ capsulatus (Bell et al., 1992).

**Physiological role of Fe(III) reduction by $R.$ capsulatus**

It is now widely regarded that the physiological importance of redox poising by auxiliary oxidants is a primary reason for the expression of a variety of terminal dissimilatory reductases operative under anaerobic conditions in $R.$ capsulatus (McEwan, 1994). During anaerobic phototrophic growth on the highly reduced carbon substrates butyrate and propionate terminal electron acceptors act as electron sinks, whilst growth on the more oxidized substrates succinate and malate is enhanced at low intensities of light by auxiliary oxidants (Richardson et al., 1988; Jones et al., 1990). Unfortunately, we have thus far been unable to reproducibly demonstrate an increase in biomass when growing batch cultures of $R.$ capsulatus on propionate or butyrate in the presence of Fe(III). We believe this may be a consequence of the toxicity of the ligands at the concentrations required (60 mM maltol, 20 mM NTA or EDTA). Indeed, we have failed to grow $S.$ putrefaciens on any Fe(III) species apart from the cation complexed by citrate. Detailed studies in continuous cultures of $R.$ capsulatus, with the concentration of Fe(III) ligands being maintained at sub-toxic levels, are thus required to firmly establish whether the cation can support phototrophic growth on highly reduced carbon substrates.

$R.$ capsulatus cells obtained from both phototrophic anaerobic and microaerobic cultures were found to be capable of dissimilatory Fe(III) reduction, suggesting that respiration of the cation might occur under oxygen-limited conditions. Hence Fe(III) dissimilation could be of major importance for the survival of the bacterium in natural light-limited and/or micro-oxic environments.

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