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The influence of chelating agents upon the dissimilatory reduction of Fe(III) by *Shewanella putrefaciens*. Part 2. Oxo- and hydroxo-bridged polynuclear Fe(III) complexes

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The susceptibility to dissimilatory reduction of polynuclear oxo- and hydroxo-bridged Fe(III) complexes by *Shewanella putrefaciens* intact cells and membranes has been investigated. These complexes were ligated by the potential tetradentates heidi (H_3heidi = *N*-(2-hydroxyethyl)iminodiacetic acid) or nta (H_3nta = nitrilotriacetic acid), or the potential tridentate ida (H_2ida = iminodiacetic acid). A number of defined small complexes with varied nuclearity and solubility properties were employed, as well as undefined species prepared by mixing different molar ratios of ida or heidi:Fe(III) in solution. The rates of Fe(III) reduction determined by an assay for Fe(II) formation with ferrozine were validated by monitoring *c*-type cytochrome oxidation and re-reduction associated with electron transport. For the undefined Fe(III) polymeric species, reduction rates in whole cells and membranes were considerably faster in the presence of heidi compared to ida. This is believed to result from generally smaller and more reactive clusters forming with heidi as a consequence of the alkoxo function of this ligand being able to bridge between Fe(III) nuclei, with access to an Fe(III) reductase located at the cytoplasmic membrane being of some importance. The increases in reduction rates of the undefined ida species with Fe(III) using membranes relative to whole cells reinforce such a view. Using soluble synthetic Fe(III) clusters, slow reduction was noted for an oxo-bridged dimer coordinatively saturated with ida and featuring unligated carboxylates. This suggests that sterically hindering the cation can influence enzyme action. A heidi dimer and a heidi multimer (17 or 19 Fe(III) nuclei), which are both of poor solubility, were found to be reduced by whole cells, but dissimilation rates increased markedly using membranes. These data suggest that Fe(III) reductase activity may be located at both the outer membrane and the cytoplasmic membrane of *S. putrefaciens*. Slower reduction of the heidi multimer relative to the heidi dimer reflects the presence of a central hydroxo(oxo)-bridged core containing nine Fe(III) nuclei within the former cluster. This unit is a poor substrate for dissimilation, owing to the fact that the Fe(III) is not ligated by aminocarboxylate. The faster reduction noted for the heidi dimer in membranes than for a soluble ida monomer suggests that the presence of ligating water molecules may relieve steric hindrance to enzyme attack. Furthermore, reduction of an insoluble oxo-bridged nta dimer featuring ligating water molecules in intact cells was faster than that of a soluble monomer coordinatively saturated by nta and possessing an unligated carboxylate. This suggests that steric factors may override solubility considerations with respect to the susceptibility to reduction of certain Fe(III) complexes by the bacterium.

Keywords: dissimilatory Fe(III) reduction, membrane-bound Fe(III) reductase, polynuclear Fe(III) complexes, *Shewanella putrefaciens*

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Introduction

The importance of microbial processes which couple the oxidation of organic matter to the dissimilatory reduction of Fe(III) in marine and freshwater environments has been thoroughly documented (Lovley 1991, 1993, 1995, Nealson & Saffarini 1994). Biogeochemical cycling of iron is thought to play a significant role in the aquatic distribution of other elements such as carbon, nitrogen, sulphur and phosphorus. Studies with pure cultures of Fe(III) dissimilating bacteria have largely concentrated upon *Shewanella putrefaciens*. This facultative anaerobe can express a wide variety of terminal respiratory reductases (Myers & Nealson 1988) and has been implicated in denitrification (Brettar & Hofle 1993). Induction of many of these enzymes is governed by the *etrA* gene, which is homologous to the *fnr* of *Escherichia coli* (Saffarini & Nealson 1993). Anaerobic growth of *S. putrefaciens* has been achieved with Fe(III) as the sole terminal electron acceptor present, either in the form of poorly soluble amorphic Fe(III) oxide or soluble Fe(III) citrate (Lovley *et al.* 1989), and the bacterium has also been demonstrated to translocate protons in response to Fe(III) citrate (Myers & Nealson 1990). Although the enzyme or enzymes responsible for Fe(III) reduction in *S. putrefaciens* are presently unidentified, activity has been located in the membrane fraction (Myers & Myers 1993a). Some information regarding the components of the electron transport chain to Fe(III) has been obtained by both genetic and biochemical techniques. Mutants deficient in menaquinone and methylmenaquinone lose the ability to reduce the cation (Myers & Myers 1993b), whilst sensitivity of Fe(III) reduction rates to 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) suggests involvement of a quinol:cytochrome oxidoreductase (Obuekwe *et al.* 1981, Arnold *et al.* 1986a, Myers & Myers 1993a, Dobbin *et al.* 1995). Furthermore, the elevated *b*-, *c*- and *d*-type cytochrome content resulting from anaerobic or microaerobic growth of *S. putrefaciens* is accompanied by a marked increase in Fe(III) reductase activity compared to fully aerobic cultures (Obuekwe & Westlake 1982, Myers & Myers 1992, Dobbin *et al.* 1995), whilst mutants with lowered Fe(III) reductase activity possess suppressed cytochrome levels (DiChristina *et al.* 1988). Recently, Tsapin *et al.* (1994) have used EPR spectroscopy to reveal a [3Fe–4S] centre in *S. putrefaciens* grown anaerobically on Fe(III) that is absent in aerobically grown cells.

Our studies on *S. putrefaciens* are concerned with the influence of Fe(III) speciation upon dissimilatory reduction of the cation. We have previously shown rates of Fe(III) reduction by whole cells and membrane fractions to be chelate-dependent, utilizing assays based upon Fe(II) formation, cytochrome oxidation and re-reduction, and Fe(III) complex disappearance (Dobbin *et al.* 1995). Data obtained with ligands that give predominantly mononuclear Fe(III) complexes of high solubility suggested that the reduction potential of a complex is a major factor in determining its rate of reduction by the bacterium. All of the Fe(III) complexes we used were more susceptible to dissimilation than the unligated cation and this was proposed to result from increased solubility. With no organic ligands present, Fe(III) will distribute as poorly soluble oxo- and hydroxo-bridged polymers in water (Powell & Heath 1994). We also postulated that the low dissimilation rate of Fe(III) chelated by desferrioxamine B might be a consequence of the role of hydroxamate-type siderophores in iron assimilation (Gram 1994). In this paper we explore the susceptibility to reduction by *S. putrefaciens* of polynuclear Fe(III) complexes, including several defined

Table 1. Characteristics of the defined Fe(III) clusters with ligands ida, heidi and nta used in this study

Compound	Formula	Appearance (solubility in H_2O)	Molecular weight (MW of Fe(III) species)
ida monomer	$K[Fe(ida)_2]\cdot 3H_2O$	yellow crystals (soluble)	411.2 (318.0)
ida dimer	$K_4\{[Fe(ida)_2]_2O\}\cdot 4.5H_2O$	dark red crystals (soluble)	889.6 (652.1)
ida hexamer	$Na_4[Fe_6(\mu_3\text{-}O)_2(\mu_2\text{-}OH)_6(ida)_6]14.5H_2O$	orange–brown powder (soluble)	1609 (1256)
heidi dimer	$[Fe(heidi)(H_2O)]_2$	yellow–green powder (insoluble)	496.0 (496.0)
heidi multimer[a]	$[Fe_{19}(\mu_3\text{-}O)_6(\mu_3\text{-}OH)_6(\mu_2\text{-}OH)_8(heidi)_{10}(H_2O)_{12}]$ $[Fe_{17}(\mu_3\text{-}O)_4(\mu_3\text{-}OH)_6(\mu_2\text{-}OH)_{10}(heidi)_8(H_2O)_{12}](NO_3)_4\cdot 60H_2O$	orange–brown powder (insoluble)	7567 (3353 and 2895)
nta monomer	$Na_3[Fe(nta)_2]\cdot 5H_2O$	yellow powder (soluble)	591.2 (432.1)
nta dimer	$Ba\{[Fe(nta)(H_2O)]_2O\}\cdot 4H_2O$	orange powder (insoluble)	749.4 (540.0)

[a]The Fe_{19} and Fe_{17} oxyhydroxide clusters of the heidi multimer are the largest synthesized to date, and crystallize in the same unit cell. Both clusters feature the inorganic core $\{Fe_7(\mu_3\text{-}OH)_6(\mu_2\text{-}OH)_4[(\mu_3\text{-}O)Fe]_2\}^{13+}$. This is linked, by various modes of bridging, to the outer shells $[Fe_{10}(heidi)_{10}(H_2O)_{12}(\mu_3\text{-}O)_4(\mu_2\text{-}OH)_4]^{12-}$ and $[Fe_8(heidi)_8(H_2O)_{12}(\mu_3\text{-}O)_2(\mu_2\text{-}OH)_6]^{10-}$. Thus only 10 Fe(III) nuclei in the Fe_{19} species and only eight Fe(III) nuclei in the Fe_{17} species are ligated by heidi. Full structural details for these multimers are given in Powell *et al.* (1995).

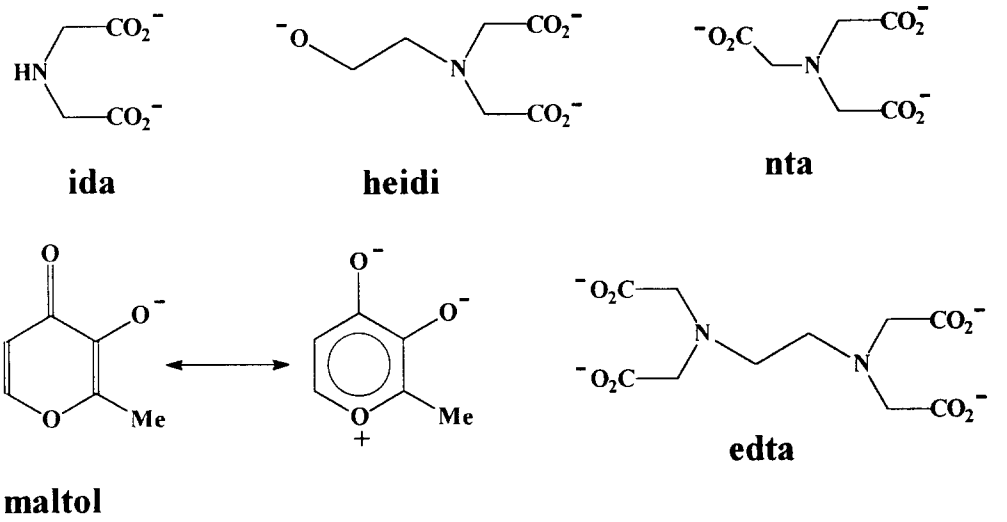

Figure 1. Structures of Fe(III) ligands (experimental and standard) used in this study.

small oxo- and hydroxo-bridged clusters (Table 1). These species are ligated by the anions generated from iminodiacetic acid (H_2ida), *N*-(2-hydroxyethyl)iminodiacetic acid (H_3heidi) or nitrilotriacetic acid (H_3nta). The ligand ida is potentially tridentate, whilst heidi and nta are potentially tetradentate (Figure 1). Bridging between Fe(III) nuclei by the alkoxo function of heidi is possible. Differences in a number of properties of both ligands and complexes featured in this study indicate the governing factors for susceptibility to reduction by *S. putrefaciens*. Furthermore, the varying nuclearity and solubilities of the clusters used enables further information to be gleaned regarding the location and nature of the Fe(III) reductase(s) of the bacterium.

Materials and methods

Bacterial growth and cell fractionation

The type strain of *S. putrefaciens* (NCIMB10471; ATCC8071) was purchased from The National Collections of Industrial and Marine Bacteria, Aberdeen, UK. Microaerobic growth was achieved in minimal media at 28–30°C as described previously (Dobbin *et al.* 1995). Cells were harvested by centrifugation at 4°C and washed twice with 100 mM NaHEPES, pH 7.0, the resulting pellet being homogenized with 1 ml of the same buffer per 20 ml culture prior to assays for Fe(III) reduction. Membranes for similar assays were prepared via sphaeroplast formation using lysozyme as previously described (Dobbin *et al.* 1995), then suspended in 1 ml of 100 mM NaHEPES, pH 7.0, per 25 ml original culture.

Preparation of Fe(III) complexes

All Fe(III) ligands used in this study are commercially available. The defined ida complexes were prepared using methods described by Harding *et al.* (1993) and Henderson (1994), the heidi dimer and the Fe_{19}/Fe_{17} multimer were synthesized according to Heath & Powell (1992), and the nta monomer and dimer were obtained as in Clegg *et al.* (1984) and Heath *et al.* (1992). Stock solutions of the ida clusters and nta monomer containing 10 mM Fe(III) were prepared immediately prior to an assay. Owing to the poor solubility of the heidi dimer, the heidi multimer and the nta dimer, these compounds were crushed to a fine powder and suspended in water at an identical Fe(III) concentration. Stock solutions of undefined polynuclear complexes with ida and heidi were prepared with 10 mM Fe(III) and varying molar ratios of ligand. Standard Fe(III) solutions were employed in all sets of assays, i.e. 10 mM Fe(III)(maltol)$_3$ or 10 mM Fe(III) complexed by edta (Figure 1; Hmaltol = 3-hydroxy-2-methyl-4-pyrone, H_4edta = ethylenediaminetetra-acetic acid), which were prepared with 3:1 and 1:1 ratios of bidentate and hexadentate ligand to cation, respectively.

Assay for Fe(II) formation using ferrozine

The underlying principles of and detailed methodology for assessing Fe(III) dissimilation rates by monitoring formation of Fe(II) with the bidentate ligand ferrozine (3-(2-pyridyl)-5,6-bis(4-phenylsulphonic acid)-1,2,4-triazine) have been presented in Dobbin *et al.* (1995). Briefly, an Aminco DW2000 spectrophotometer set in split-beam mode at 562 nm was employed. Anaerobic assays were performed in 50 mM NaHEPES, pH 7.0, containing cells or membranes plus 350 μM ferrozine and initiated by the injection of 100 μM Fe(III). Additions of an electron donor (500 μM) (essential for membranes) or HQNO (40 μM) were made 5 min before an assay. Fe(III)(maltol)$_3$, a model complex for high rates of reduction by *S. putrefaciens* (Dobbin *et al.* 1995), was employed as a standard in ferrozine assays on all whole cell and membrane preparations.

Assay for cytochrome oxidation

The oxidation of *b*- and *c*-type cytochromes by Fe(III) complexed with edta in *S. putrefaciens* whole cells and

membranes forms the basis of this assay (Dobbin *et al.* 1995). The reversibility of these cytochrome oxidations combined with durations proportional to the amount of Fe(III) added allow transient spectral bleaching times to be used as an indicator of Fe(III) reduction rates. In this study assays (on whole cells only) were performed by monitoring the oxidation and re-reduction of *c*-type cytochromes at 552 nm with respect to time after addition of 500 μM Fe(III). Fe(III) complexed by edta was utilized as a standard in cytochrome oxidation aasays on all cell batches. The absorbance possessed by Fe(III)(maltol)$_3$ at 552 nm negates its usefulness in this assay, whilst the kinetic stability of Fe(II) chelates with edta causes Fe(III) complexed by edta to give erroneous data in the ferrozine assay. However, comparison with other Fe(III) species has demonstrated Fe(III)(maltol)$_3$ to be reduced by *S. putrefaciens* whole cells or membranes at comparable rates to Fe(III) complexed by edta (Dobbin *et al.* 1995).

Results

Ferrozine assays

Results pertaining to the reduction of undefined polynuclear Fe(III) complexes by intact cells of *S. putrefaciens* are presented in Tables 2 and 3. It can be seen that in the presence of lactate formation rates of Fe(II)(ferrozine)$_3$ from the ida species were considerably slower than from Fe(III)(maltol)$_3$ (all below 6%), whilst rates from the heidi species were comparable to the standard chelate (100–130%). Furthermore, it is apparent that the absence of lactate or presence of HQNO slowed Fe(II)(ferrozine)$_3$ formation rates from the heidi species more markedly than from the ida species. Rates of Fe(II)(ferrozine)$_3$ formation increased upon raising the ida:Fe(III) ratio, although a significant similar trend was not apparent using heidi. Table 4 presents data obtained for the reduction of the undefined polynuclear Fe(III) complexes by membranes. Rates of Fe(II)(ferrozine)$_3$ formation from the heidi species were again comparable to Fe(III)(maltol)$_3$ (80–120%), but rates corresponding to the ida species were slightly elevated in comparison to those using whole cells (i.e. 10–15% of the standard chelate). No significant trends are apparent from altering the ratio of ligand:Fe(III) for either ida or heidi.

Rates of Fe(II)(ferrozine)$_3$ formation from the defined Fe(III) complexes of varying nuclearity catalysed by both whole cells and membranes are detailed in Table 5. The data for whole cells indicated Fe(II) to form from the ida monomer considerably faster than from the ida hexamer, which was in turn more reactive than the ida dimer. Results using membranes were in general agreement with the above except for an increment in Fe(II) formation rate from the ida dimer (16% of Fe(III)(maltol)$_3$ rate compared to 1.3% in whole

Table 2. The effects of varying ida:Fe(III) ratios on the rate of Fe(II)(ferrozine)$_3$ formation in the whole cell ferrozine assay

Fe(III) species	Steady-state rate of Fe(II)(ferrozine)$_3$ formation		
	Lactate present [nmol (min mg dry weight cells)$^{-1}$]	Lactate absent (% of when lactate present)	Lactate absent/HQNO present (% of when lactate absent)
Fe(III)(maltol)$_3$	53	24	17
0.25:1 ida:Fe(III)	1.9	NP	NP
0.5:1 ida:Fe(III)	2.0	79	51
0.75:1 ida:Fe(III)	2.4	NP	NP
1:1 ida:Fe(III)	2.9	86	46
3:1 ida:Fe(III)	2.9	NP	NP
5:1 ida:Fe(III)	3.0	69	68

NP, experiment not performed.

Table 3. The effects of varying heidi:Fe(III) ratios on the rate of Fe(II)(ferrozine)$_3$ formation in the whole cell ferrozine assay

Fe(III) species	Steady-state of Fe(II)(ferrozine)$_3$ formation		
	Lactate present [nmol (min mg dry weight cells)$^{-1}$]	Lactate absent (% of when lactate present)	Lactate absent/HQNO present (% of when lactate absent)
Fe(III)(maltol)$_3$	47	24	8.1
0.5:1 heidi:Fe(III)	46	30	11
0.75:1 heidi:Fe(III)	49	NP	NP
1:1 heidi:Fe(III)	60	17	5.4
3:1 heidi:Fe(III)	56	NP	NP
5:1 heidi:Fe(III)	58	19	18

NP, experiment not performed.

Table 4. The effects of varying ida:Fe(III) and heidi:Fe(III) ratios on the rate of Fe(II)(ferrozine)$_3$ formation in the membrane ferrozine assay using formate as electron donor

Fe(III) species	Steady-state rate of Fe(II)(ferrozine)$_3$ formation [nmol (min mg dry weight cells)$^{-1}$]	
	Ligand = ida	Ligand = heidi
Fe(III)(maltol)$_3$	2.3	2.1
0.5:1 ligand:Fe(III)	0.27	1.7
0.75:1 ligand:Fe(III)	0.34	1.9
1:1 ligand:Fe(III)	0.33	2.4
3:1 ligand:Fe(III)	0.23	2.2
5:1 ligand:Fe(III)	0.22	2.5

Table 5. Rates of Fe(II)(ferrozine)$_3$ formation from defined Fe(III) complexes in the whole cell and membrane ferrozine assays, using lactate and formate, respectively, as electron donors

Fe(III) species	Steady-state rate of Fe(III)(ferrozine)$_3$ formation [nmol (min mg dry weight cells)$^{-1}$]	
	Whole cells	Membranes
Fe(III)(maltol)$_3$	73	5.3
ida monomer	60	3.4
ida dimer	0.93	0.87
ida hexamer	13	1.0
heidi dimer	43	5.5
heidi multimer	16	2.5
nta monomer	37	3.2
nta dimer	57	5.8

cells). However, for the poorly soluble heidi clusters and nta dimer, and equating with the standard Fe(III)(maltol)$_3$, rates were considerably faster in membranes as opposed to whole cells, i.e. 104% compared to 59% for the heidi dimer, 47% compared to 22% for the heidi multimer and 109% compared to 78% for the nta dimer. Whilst formation rate of Fe(II)(ferrozine)$_3$ from the soluble ida monomer was approximately 1.5 times greater than from the insoluble heidi dimer using whole cells (Figure 2), the converse was true for the membrane fraction (Figure 3). Furthermore, Fe(II) formation from the soluble nta monomer was at a rate markedly slower than from the insoluble nta dimer using both whole cells and membranes.

Cytochrome assays

Traces obtained from the cytochrome assay for Fe(III) reduction using the cation complexed by edta, the ida monomer and the ida dimer are shown in Figure 4. Although magnitudes of ΔA_{552} were similar for all three, no cytochrome re-reduction was apparent over the 10 min assay period in the reaction involving the ida dimer. This implies a much slower rate of reduction for Fe(III) in this complex, and complements results from the ferrozine assay. Traces

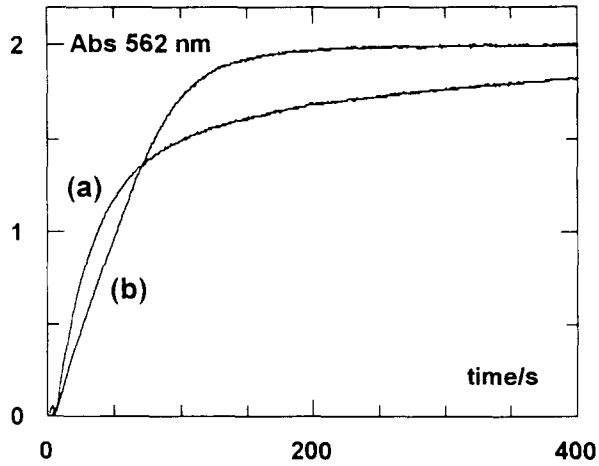

Figure 2. Whole cell ferrozine assay in the presence of lactate using (**a**) ida monomer and (**b**) heidi dimer.

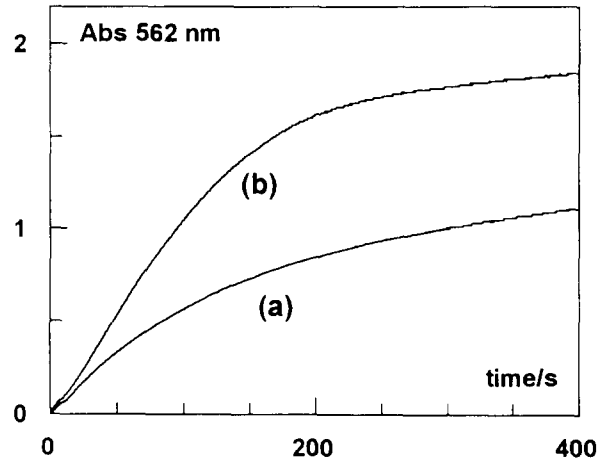

Figure 3. Membrane ferrozine assay with formate as electron donor using (**a**) ida monomer and (**b**) heidi dimer.

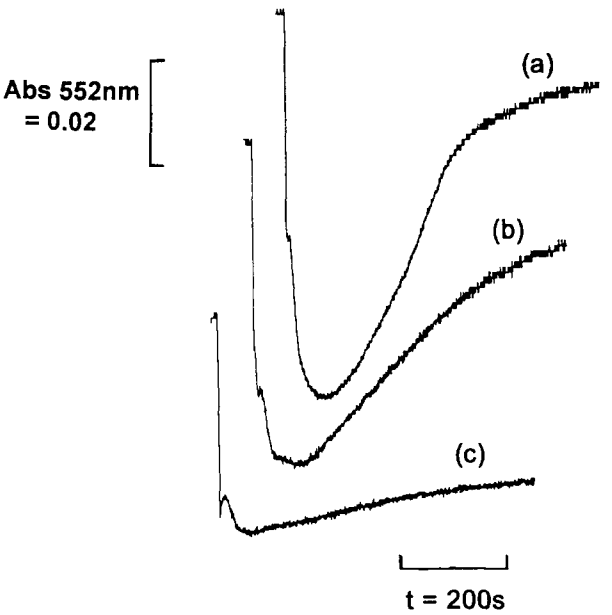

Figure 4. Whole cell cytochrome assay using (**a**) Fe(III) complexed by edta, (**b**) ida monomer and (**c**) ida dimer.

for the other defined Fe(III) species were also in agreement with the ferrozine data in that transient cytochrome oxidation periods of less than 10 min were obtained. The assays using the undefined species with heidi indicated full cytochrome re-reduction within 10 min, whilst bleaching of the spectral bands obtained with the undefined ida species continued beyond such a time.

Discussion

Fe(III) speciation in aqueous solution is essentially governed by the hydrolytic chemistry which arises from the ability of the cation to activate O–H bonds. Under highly acidic conditions the mononuclear hexa-aquo Fe(III) complex predominates, but as pH increases protons readily become dissociated from the ligating water molecules. This leads to formation of the poorly soluble polynuclear oxo- and hydroxo-bridged Fe(III) species which inhabit the natural environment (Figure 5a) (Powell 1993, Powell & Heath 1994). However, mononuclear Fe(III) complexes can exist at neutral pH values if suitable concentrations of certain multidentate ligands possessing high affinity for the cation are present. These ligands include siderophores such as the tris-hydroxamate desferrioxamine B and the tris-catecholate enterobactin, which are excreted by bacteria to scavenge iron required for assimilation (Hider 1984). When present in at least a 3 molar excess, bidentate hydroxamates and maltol give predominantly 3:1 ligand:Fe(III) species at pH 7 (Hider & Hall 1991). Hexadentate aminocarboxylate ligands can trap the cation as a monomer, although the formation of a chelate with octahedral stereochemistry is normally disfavoured. A water molecule may hence ligate at a seventh coordination site of Fe(III)edta, and this can participate in bridging to another Fe(III) nucleus and consequent dimer formation (Henderson 1994). In the presence of tri- and tetradentate ligands such as ida, heidi or nta, speciation of Fe(III) in aqueous solution is much more complicated. Despite possessing reasonably high affinites for Fe(III) (Table 6), these chelators do not effectively displace hydroxide ions ligated to the cation unless present in an extremely large molar excess. Indeed, a tri- or tetradentate ligand:Fe(III) molar ratio in the region of 5000:1 in solution is required for Fe(III)(ligand)$_2$ species to predominate (Hider & Hall 1991). The tendency is thus for oxo- and hydroxo-bridged polynuclear Fe(III) clusters to form (Figure 5b), with sizes

$$|Fe(H_2O)_6|^{3+} \xrightarrow{(a)} |Fe_x(O)_y(OH)_z(H_2O)_p|^m$$

$$(FeL_n)^m \xrightarrow{(b)} |Fe_xL_n(O)_y(OH)_z(H_2O)_p|^m$$

Figure 5. Schemes for the hydrolysis of Fe(III) (**a**) with no ligands present and (**b**) in the presence of ligand L, which may be a tridentate or tetradentate aminocarboxylate. The overall charges will be influenced by the charge of the ligand L.

Table 6. Thermodynamic stability constants of ligands used in this study for Fe(III) and Fe(II)

Ligand	log K_1 Fe(III)	log β_3 Fe(III)	log K_1 Fe(II)
ida	10.7	—	5.54
Hheidi[a]	11.6	—	6.78
nta	15.9	—	8.33
edta	25.1	—	14.3
maltol	11.1	28.5	NA

Data from Martell & Smith (1974) and Hider & Hall (1991). NA, data unavailable.

[a]Hheidi has the alcoholic proton associated.

theoretically linked to the tri- or tetradentate ligand:Fe(III) ratios in that increasing the molar excess of ligand should decrease the average cluster nuclearity. Varying ligand concentrations and other determining factors such as pH and solvent has recently led to a degree of control being exerted over Fe(III) hydrolytic processes, resulting in the isolation of the defined complexes used in this study (Powell 1993, Powell & Heath 1994). Some of these Fe(III) species feature small hydroxo(oxo) cores trapped by amino-carboxylate chelators, which is a situation of environmental and moreover physiological relevance if the ligating properties of citrate, phosphate, humic acids and certain protein side-chains are considered.

Previous studies regarding the influence of tri- and tetradentate ligands upon Fe(III) dissimilation by *S. putrefaciens* have been limited to citrate and nta. Arnold *et al.* (1986b, 1988) and DiChristina (1992) demonstrated these chelators to cause a large increment in reduction rate with respect to uncomplexed Fe(III) and Fe(III) oxides, and we have recently shown that solutions containing 1:1 citrate or nta:Fe(III) were reduced at rates similar to Fe(III)(maltol)$_3$ and the cation complexed by edta (Dobbin *et al.* 1995). That 1:1 heidi:Fe(III) solutions were here shown to be reduced by whole cells at comparable rates to the standards employed is in agreement with the citrate and nta data, whilst the extremely slow Fe(II)(ferrozine)$_3$ formation noted from 1:1 ida:Fe(III) is in stark contrast to the previous findings. The absence of the electron donor lactate or presence of the respiratory inhibitor HQNO slowing Fe(II)(ferrozine)$_3$ formation rates less markedly in undefined ida species compared to heidi species might be expected, since restriction of the flow of electrons to the terminal reductase or reductases should not be such an influential factor in the dissimilation of poor substrates. Validity of Fe(III) reduction rates indicated by Fe(II)(ferrozine)$_3$ formation rates in the ferrozine assay using ida was upheld by the cytochrome assays, indicating kinetic labilities of the resultant Fe(II) complexes to be high. As to the reasons for the low rates of Fe(III) reduction obtained using the undefined ida species, thermodynamic stability constants of this ligand for Fe(III) and Fe(II) are similar to the values corresponding to heidi (Table 6), and thus appear to be of no consequence. The major structural difference between ida and heidi is the alkoxo function of the latter ligand, which may participate in bridging between Fe(III) nuclei. It is conceivable that such

ligand bridging may result in the average polynuclear Fe(III) cluster size being diminished in the heidi solutions and that these smaller species may be more prone to reduction by the bacterium. In support of this view, the potential tetradentate citrate features an alkoxo function which may participate in ligand bridging, and this is illustrated in recently characterized dimeric Fe(III) complexes (Shweky *et al.* 1994). Furthermore, the average cluster size could be depressed in Fe(III) solutions with the tetradentate nta compared to the tridentate ida since one more bridging site is available in the monomeric Fe(III)(ida)$(H_2O)_3$ chelate compared to Fe(III)(nta)$(H_2O)_2$.

As stated earlier, increasing tri- or tetradentate:Fe(III) ratios should theoretically reduce polynuclear cluster size. The data obtained using whole cells and Fe(III) chelated by ida shows an increase in reduction rates on raising ligand concentrations and could be indicative of greater susceptibility to dissimilation of the generally smaller clusters being formed. With heidi complexes, a reason for no such trend being observed might be that cluster sizes are not so dependent upon ligand concentration, which may arise from the bridging alkoxo function exerting fundamental limits regarding nuclearity.

The increased susceptibility to reduction of undefined Fe(III) species with ida by membranes compared to whole cells could be a consequence of access to a dissimilating enzyme bound to the cytoplasmic membrane, since large polynuclear clusters may not be able to easily enter the periplasmic compartment. The clusters of relatively reduced size formed with heidi and Fe(III) would appear to pass through the outer membrane, leading to reduction rates using both whole cells and membranes not varying relative to the standard Fe(III)$(maltol)_3$. That no apparent upward trend in Fe(III) reduction rates by membranes upon raising the ida:Fe(III) molar ratio was observed seems reasonable if susceptibility to reduction is governed significantly by access to the Fe(III) reductase.

Moving to the defined Fe(III) complexes studied, the most prone to reduction by whole cells was the ida monomer. This species features two ida ligands on an Fe(III) nucleus and as stated earlier is unlikely to form in significant quantities in solution unless a considerable molar excess of ida is employed. Accelerated reduction of the ida monomer might be predicted since the species resembles the standard Fe(III)$(maltol)_3$ in respect of octahedral stereochemistry. It is also apparent that the ida monomer is not distributed among a number of species as a consequence of dissolution when preparing the 10 mM stock solution or indeed upon further dilution to 100 μM in the ferrozine assay. Extensive displacement of ida with water and the consequente hydrolytic processes would presumably lead to a variety of polynuclear complexes being formed, resulting in similarly slow Fe(II)$(ferrozine)_3$ formation rates to those noted with the undefined species from 2:1 ida:Fe(III) solutions. In contrast to the ida monomer, the ida dimer was reduced very slowly by *S. putrefaciens* whole cells, giving a rate comparable to those obtained using undefined species. However, this complex is akin to the monomer in that it features two ida ligands per Fe(III) nucleus and it is thus unlikely to form in solution unless a large excess of ida is present. Two explanations might be given for the slow dissimilation of the ida dimer, the first being that the complex is simply distributed amongst a range of species in the stock solution or assay mixture. This implies that the Fe(II)-$(ferrozine)_3$ formation rates observed originate from reduction of undefined complexes similar to those present in the previously utilized 2:1 ida:Fe(III) solutions. However, since the ida monomer is stable in solution, and the dimer is essentially two monomers linked by an oxo-bridge, extensive displacement of the ida ligands on the dimer with water/hydroxide seems unlikely. A second and perhaps more credible proposal regarding slow bacterial reduction of the ida dimer relies upon steric considerations. It might be reasoned that since each Fe(III) in this species is hexacoordinate and thus one carboxylate is unattached per Fe(III) (i.e. one of the ida ligands on each Fe(III) nucleus is only acting as a bidentate; Figure 6), then enzyme access to the cation could be restricted. Indeed, the unligated carboxylates should also make the Fe(III) nuclei of the ida dimer less accessible to water and/or hydroxide than the cation in the ida monomer. Although in the monomer Fe(III) is fully coordinated by two ida ligands, with no water molecules ligated to the cation, it might be supposed that the Fe(III) reducing enzyme may easily enter the coordination sphere. This has previously been demonstrated using Fe(III)$(maltol)_3$ and other coordinatively saturated Fe(III) species (Dobbin *et al.* 1995). It is apparent that when water approaches the Fe(III) nucleus of the monomer, a carboxylate ligand may become uncoordinated, as suggested by the structure of the ida dimer. Thus the lability of a carboxylate ligand might also facilitate access of a reducing enzyme to the cation.

The rate of reduction of the ida hexamer by *S. putrefaciens* whole cells was found to lie between the values obtained for the monomer and dimer. Since the hexamer features one

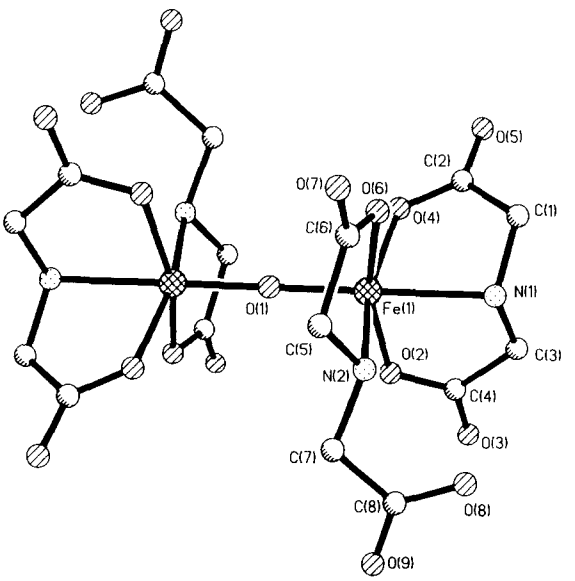

Figure 6. The molecular structure of $\{[Fe(ida)_2]_2O\}^{4-}$. Full details in Henderson (1994).

ligand per Fe(III), its formation in the undefined solutions is conceivable, although the significantly greater susceptibility to reduction of the defined species suggests it is not present at high levels. Previous work in one of our laboratories has demonstrated the ida hexamer to distribute significantly in pH 7 aqueous solution, giving as yet unidentified species over a time span dependent upon concentration (unpublished data). The apparent Fe(III) reduction rates from the dissolved ida hexamer probably do not correspond exclusively to this defined complex. However, it might be supposed that the oxo- and hydroxo-bridged clusters formed in the solutions of varying ida:Fe(III) molar ratios are less prone to dissimilation than the hexamer, perhaps by virtue of restricted access to an Fe(III) reductase located at the cytoplasmic membrane. That the hexamer (and the species it may distribute to give) can enter the periplasm is apparent from rates of reduction being similar by whole cells and membranes in comparison with the standard Fe(III)(maltol)$_3$. However, no satisfactory explanation can be given regarding the increment in reduction rate observed with the ida dimer using membranes as opposed to whole cells.

Using the insoluble heidi dimer and multimer, decreased Fe(III) dissimilation rates by intact cells were observed relative to the undefined soluble species from the various solutions of heidi with the cation. This appears to be a reasonable finding in that any reduction of Fe(III) in an undissolved cluster would have to involve an enzyme situated at the outer membrane. However, it might be supposed that the solubilities of the heidi dimer and multimer may be such that at the Fe(III) concentrations of 100 μM in the ferrozine assay considerable portions will be dissolved. Such soluble species would be able to enter the periplasm and subsequently be reduced by the cytoplasmic membrane-bound enzyme. This implies the operation of two processes, dissolution followed by reduction, instead of the one reductive dissolution process. However, the approximate doubling of reduction rates for both the dimer and multimer relative to the standard Fe(III)(maltol)$_3$ using membranes instead of whole cells demonstrates that the Fe(III) reductase located on the cytoplasmic membrane in the intact cell is now accessible to the undissolved species. Furthermore, if near totally dissolved, the rate of dissimilation of the heidi dimer by whole cells might be expected to lie in the region of figures obtained with the undefined species of heidi and Fe(III) in solution, where clusters featuring two or more Fe(III) nuclei are likely to be present. Unlike the ida dimer, the heidi dimer is ligand-bridged and has one water molecule ligated to each Fe(III), so steric hindrance of enzymatic reduction would seem unlikely. The argument for substantial dissolution preceding reduction by whole cells thus appears flawed and that some Fe(III) reductase activity is associated with the outer membrane appears likely. The apparent reduction rates of the heidi dimer and multimer could perhaps be determined by rate of dissolution of these Fe(III) species and thus confer evidence for a cytoplasmic membrane-bound enzyme only. However, this seems unlikely if it is considered that the ratios of reduction rates for the heidi dimer to the heidi multimer are similar for both whole cells and membranes.

With the question of access to an Fe(III) reductase irrelevant, and redistribution to other species not occurring, data obtained with the insoluble heidi dimer and multimer indicates the cluster of smaller nuclearity to be reduced at a faster rate by the *S. putrefaciens* enzyme systems. A possible reason for this finding is that in the dimer both Fe(III) nuclei are ligated with heidi and feature coordinated water molecules, permitting facile enzyme access to both cations. However, both the Fe_{19} and Fe_{17} clusters of the heidi multimer feature a central hydoxo(oxo)-bridged core unit with nine Fe(III) nuclei, surrounded by a shell featuring 10 or eight Fe(III) nuclei ligated with heidi (Table 1) (Figure 7) (Powell *et al.* 1995). The shell Fe(III) nuclei of the heidi multimer are structurally comparable to the Fe(III) nuclei of the heidi dimer, including the presence of ligated water molecules. The cations within the shell of the multimer should be reduced at a rate comparable to the Fe(III) dimer, whilst Fe(III) within the central unligated core is expected to be a poor substrate for enzymatic reduction in the light of previous work (Dobbin *et al.* 1995). Therefore, the finding that the heidi multimer was reduced at a rate less than half that noted using the heidi dimer appears reasonable.

Regarding the data obtained using undefined Fe(III) species prepared by mixing varying ratios of ida or heidi:Fe(III), it was postulated earlier that decreased cluster sizes are formed with the latter ligand, and that the smaller clusters are more prone to enzymatic reduction. If the results obtained using the heidi multimer and dimer are considered, then this view gains support in that formation of larger hydoxo(oxo)-bridged core units with more Fe(III) nuclei, which will be poor substrates for dissimilation, should accompany any increase in cluster size.

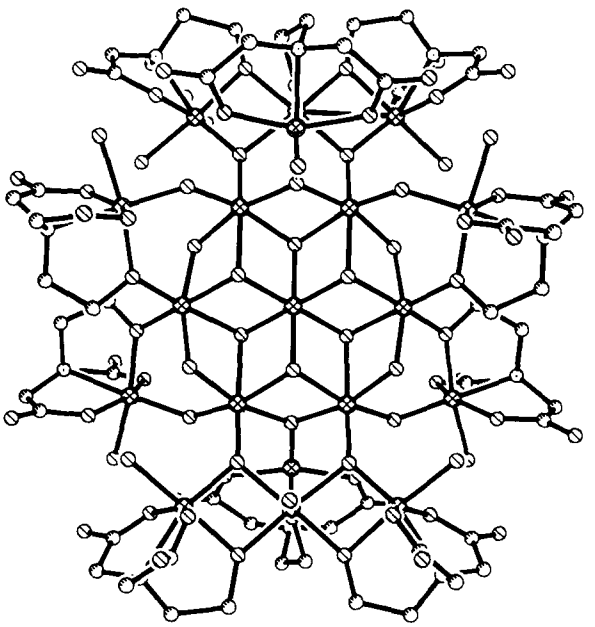

Figure 7. The molecular structure of $[Fe_{19}(\mu_3\text{-}O)_6(\mu_3\text{-}OH)_6(\mu_2\text{-}OH)_8(heidi)_{10}(H_2O)_{12}]^+$, one of the two oxyhydroxide clusters present in the heidi multimeter. Full details in Powell *et al.* (1995).

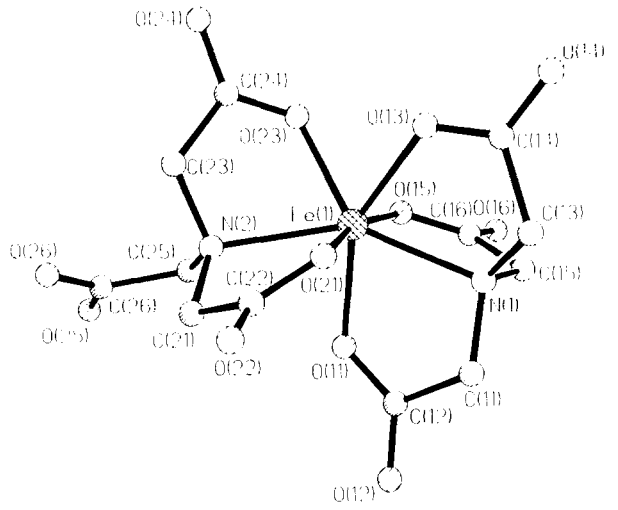

Figure 8. The molecular structure of $[Fe(nta)_2]^{3-}$. Full details in Clegg *et al.* (1984).

Comparing apparent reduction rates from the ferrozine assay, it may initially seem surprising that the insoluble nta dimer was reduced faster by whole cells than the soluble nta monomer, since only the latter complex may access a cytoplasmic membrane-bound Fe(III) reductase. However, the influence of steric hindrance upon electron transfer may well override solubility considerations for these species. The nta monomer features one unligated carboxylate, resulting from one nta acting as a tridentate rather than a tetradentate (Figure 8), and this may restrict enzyme access to the cation by steric hindrance. Furthermore, access of the reducing enzyme to the heptacoordinate Fe(III) nucleus of the nta monomer may be more difficult than access to the hexacoordinate Fe(III) nucleus of species such as the ida monomer and Fe(III)(maltol)$_3$. In contrast to the nta monomer, the nta dimer has only one tetradentate nta, and moreover a ligating water molecule, on each Fe(III). Structural similarities between the nta monomer and ida dimer, and also between the ida monomer and nta dimer, exist with respect to unligated carboxylates. However, structural similarities between the nta and heidi dimers, and to a certain extent the heidi multimer, exist with respect to the non-coordinative saturation of Fe(III) nuclei and the presence of ligating water molecules. The rates of Fe(II)(ferrozine)$_3$ formation observed using the nta monomer might be slower in comparison to the nta dimer by virtue of kinetic stability of the resultant Fe(II) monomer (i.e. steric hindrance to ferrozine ligation), but this seems unlikely since the transient cytochrome oxidation period was judged to be slightly shorter for the nta dimer. The increment in reduction rate for the insoluble nta dimer relative to the standard Fe(III)(maltol)$_3$ using membranes as opposed to intact cells is in agreement with the data from the insoluble heidi complexes, and supports the view that Fe(III) reductase activities are located at both the cytoplasmic and outer membranes of *S. putrefaciens*.

Although both the nta monomer and ida dimer are soluble, and both feature unligated carboxylates which may sterically hinder enzymatic action, the former species was reduced much faster by both *S. putrefaciens* whole cells and membranes. A possible reason for this finding could simply be a lower reduction potential for the ida dimer, arising from properties of the ligand such as thermodynamic stability constants for Fe(III) and Fe(II) (Table 6), and/or the presence of an oxo-bridge between Fe(III) nuclei. That the ida hexamer features fully ligated aminocarboxylate disallows any comparison with the ida dimer being made with respect to the influence of hydroxo- or oxo-bridging, as does the suspected distribution of the hexamer upon dissolution. The instability in solution of the hexamer also prevents confident comparison of reduction rate with those of the heidi dimer and the nta dimer, so conclusions regarding the influence of ligating water molecules upon the susceptibility of polynuclear Fe(III) complexes to dissimilation cannot be made. However, the finding that the heidi dimer was reduced faster by membranes than the ida monomer (and moreover Fe(III)(maltol)$_3$) might suggest that the presence of ligating water molecules makes for easier enzymatic attack at a labile site. Indeed, water may become readily uncoordinated from the Fe(III) nuclei of the heidi dimer and thus facilitate rapid reduction. The phenomenon was not evident using whole cells owing to the insolubility of the heidi dimer, which causes this complex to only access the outer membrane Fe(III) reductase. Although the nta dimer is also an insoluble species featuring one coordinated water molecule per Fe(III) nucleus, its reduction by either intact cells or membranes was significantly accelerated in comparison to the heidi dimer. This is most probably a consequence of the reduction potential being higher for the nta dimer than the heidi dimer, which in turn might be influenced by properties of the ligands such as thermodynamic stability constants for Fe(III) and Fe(II) (Table 6). Furthermore, the different modes of bridging between Fe(III) nuclei (one oxo-bridge for the nta dimer compared to two alkoxo-bridges for the heidi dimer) may exert some influence on reduction potentials.

In summary, results from this study suggest that a number of factors influence the susceptibility of an Fe(III) complex to dissimilatory reduction by *S. putrefaciens*. Data obtained using undefined species of the cation ligated by ida or heidi indicate cluster size to be of importance, with the smaller Fe(III) clusters formed with heidi (a potential tetradentate possessing an alkoxo function capable of bridging between Fe(III) nuclei) being reduced faster. The increase in cluster size is probably accompanied by the formation of larger hydoxo(oxo)-bridged core units, which are poor substrates for dissimilation. This argument is supported by the slower reduction of the heidi multimer compared to the heidi dimer. Also, larger undefined polymeric Fe(III) species may not easily access a reductase located at the cytoplasmic membrane. The reduction of insoluble Fe(III) species by intact cells suggests that enzyme activity is associated with both the outer membrane and the cytoplasmic membrane of the bacterium. Relatively decelerated reduction rates obtained using soluble Fe(III) species which are coordinatively saturated and feature unligated carboxylates (i.e. the

ida dimer and nta monomer) implies access of the Fe(III) reductase to the cation may be subject to steric hindrance. Indeed, steric considerations may override solubility considerations with regard to rates of Fe(III) dissimilation. The presence of ligating water molecules on an Fe(III) species is not obligatory for elevated reduction rates, since the enzyme may easily access the coordination sphere of the ida monomer and perhaps displace a carboxylate ligand. However, the faster rate of reduction in membranes for the insoluble heidi dimer compared to the ida monomer may indicate that the presence of a ligating water molecule can result in easier access of the enzyme to the cation.

Our localization of Fe(III) reductase activity to both the cytoplasmic and outer membranes of *S. putrefaciens* is in some agreement with the work of Myers & Myers (1993a). These authors employed membrane fractionation procedures followed by ferrozine assays with Fe(III) complexed by citrate to deduce that cation dissimilation occurs in the main at the outer membrane, albeit with a different strain of the bacterium grown anaerobically. Our efforts are currently directed towards the design and syntheses of more oligomeric hydroxo(oxo) Fe(III) species which may shed further light upon the location and nature of the Fe(III) reductases of *S. putrefaciens*, with particular attention being paid to nuclearity, solubility, ligand-bridging, number of ligand donor atoms and hindering enzyme access to the cation.

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