THERMOPHILIC IRON REDUCTASES FROM *THERMUS SCOTODUCTUS*

BY

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Meaning of Life

The meaning of life differs from man to man, and from moment to moment. Thus it is impossible to define the meaning of life in a general way. Questions about the meaning of life can never be answered by sweeping statements. “Life” does not mean something vague, but something very real and concrete. They form man’s destiny, which is different and unique for each individual. No man and no destiny can be compared with any other man or other destiny. No situation repeats itself, and each situation calls for a different response. **Sometimes the situation in which a man finds himself may require him to shape his own fate by action**. At other times it is more advantages for him to make use of an opportunity for contemplation and to realize assets in this way. Sometimes man may be required simply to accept fate, to bear his cross. Every situation is distinguished by its uniqueness, and there is always only one right answer to the problem posed by the situation at hand.

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This dissertation is dedicated to the memory of my father.

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LIST OF ABBREVIATIONS

AQDS	Anthraquinone 2,6-disulfonate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CM	Carboxylmethyl
Da	Dalton
DEAE	Diethylaminoethyl
DMSO	Dimethyl sulfoxide
EDTA	Ethylene diaminetetraacetic acid
Fe(III)-NTA	nitrilotriacetic acid
FeR	*A. fulgidus* ferric reductase
Ferrozine	[3-(2-pyridyl)-5,6-bis-(4-phenylsulfonic acid)-1,2,4-triazine]
FMN	flavin mononucleotide
Fre	*E. coli* flavin reductase
g	Acceleration due to gravity
Hepes	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid
HIC	Hydrophobic interaction chromatography
K_m	Michaelis constant
KDO	2-keto-3-deoxyoctonate
MOPS	3-(*N*-Morpholino)-ethanesulfonic acid
Mr	Relative molecular mass
NADH	Nicotinamide adenine dinucleotide
OD	Optical density
PEG	Polyethelene glycol
pI	Isoelectric point
PMSF	Phenylmethylsulphonyl fluoride
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Tris	2-Amino-2-(hydroxymethyl)-1,3-propandiol
TMAO	Trimethylamine oxide
Tris-HCl	(Tris (hydroxymethyl)-aminomethane- HCl)
U/ml	Activity expressed in Units per milliliter
UF	Ultrafiltration

CHAPTER 1

1.1 General Introduction

Extreme environments provide opportunities for isolation and characterization of microorganisms that are physiologically adapted to “adverse” conditions. These microorganisms are able to live in environments such as high concentration of metals (Lovley, 1995a), excesses in temperature, pressure, salinity, pH (Takai *et al*., 2001) and ambient radiation (Daly and Minton, 1995; Fredrickson *et al*., 2000). Microorganisms that thrive under these extreme conditions have attracted considerable interest because of their novel metabolic properties that may be of potential value to the industry for applications in bioremediation and biotechnology. Many questions arise about their possible origin and limits of life. Explorations of extreme microbial communities, whether indigenous or altered by human activity, allow investigators to probe the potential genetic diversity of these microorganisms (Whitman *et al*., 1998).

In natural environments, the abundances of metals are relatively low and are mainly present in sediment, soils and mineral deposits. However, elevated metal levels occur in specific natural locations, for example, hot springs (Ferreira *et al*., 1997), volcanic soils, aquatic sediments (Huber *et al*., 1987; Mortimer, 1941) and a variety of subsurface terrestrial settings (Bourg, 1988; Fredrickson and Onstott, 1996; Lovley, 1993; Lovley, 1997; Luu and Ramsay, 2003), and sometimes as result of industrial activities or past mining (Prusty *et al*., 1994; Salomons, 1995). It has been observed that some industrial or mining activities are responsible for mobilization of various metals above rates of natural geochemical cycling that give rise to increased deposition in aquatic and terrestrial environments (Summers and Silver; 1978). Therefore, microorganisms encounter various kinds of metals in these environments, not surprising that they should interact with them, sometimes to their benefit, at other times to their disadvantage.

Metals are known to be a potent toxicant to most microorganisms. Although some microorganisms in their natural habitats mainly depend on the metal concentration and physico-chemical attributes of that environment (Duxbury, 1995), for this function, the metal concentration must occur in sufficient concentration locally to meet the organisms' demand. Uptake of trace metals and their subsequent incorporation into metalloenzymes or utilization in enzyme activation occurs in all microorganisms (Wackett *et al*., 1989). However, a number of microorganisms have the potential to reduce metals to conserve energy whereas others have evolved metal detoxification/resistance systems that often incorporate changes in the oxidation states of metals (Ehrlich, 1997; Silver, 1996; Silver and Phung, 1996; Silver and Walderhaug, 1992).

Recently, the microbial reduction of metals has attracted interest as these transformations can play crucial roles in the cycling of both inorganic and organic species and therefore has opened new and exciting areas of research with potential practical application (Anderson and Lovley, 1999; Anderson *et al*., 1998; Lovley and Anderson, 2000; Rooney-Varga *et al*., 1999). The environmental impact of such transformations may offer the basis for a wide range of innovative biotechnological processes, for instance the mobilization of toxic metals with potentially harmful effects on human health (Kashefi and Lovely, 2000; Lloyd, 2003; Lovley, 1993; Lovley, 1995 a & b).

1.2 Microbial interaction with metals

The biogeochemical significance of dissimilatory metal reduction by microorganisms was recognized only in the last 20 years. Non-enzymatic processes were generally considered to account for most of the redox speciation of many metals in the subsurface (Zehnder and Stumm, 1988). However, it is now clear that many metals can be enzymatically or non-enzymatically concentrated and dispersed by microorganisms in the environment (Ehrlich, 1997).

The way microorganisms interact with metals depends on whether it is needed for enzymes involved in cellular reactions (Ehrlich, 1997; Neilands, 1981; Silver and

Walderhaug, 1992; Wackett *et al*., 1989) or required in energy metabolism (Lovley, 1993; Lovley, 1995b; Lovley, 1997; Lovley, 2000; Nealson and Saffarini, 1994). Microbial detoxification of harmful metals is a third type of interaction. In this process, toxic metal species may be coverted to a less toxic or non-toxic entity (Busenlehner *et al*., 2003; Ehrlich, 1996; Rosen, 1996; Silver, 1996)

Metals in trace amounts that serve structural and/or catalytic functions are essential to virtually all organisms (Ehrlich, 1997), but pose problems of poor solubility. Microorganisms have therefore evolved various mechanisms to counter the problems imposed by their metal dependence (Andrews *et al*., 2003), but for such uses, low environmental concentration of metals are sufficient (Ehrlich, 1997; Outten and O'Halloran, 2001). Microbial interactions with small quantities of metals do not exert a major impact on the environment (Ehrlich, 1997).

However, interactions with larger quantities of metals, as are required in energy metabolism or due to detoxification/resistance systems, have a noticeable impact on the metal distribution in the environment (Ehrlich, 1997; Lovley and Coates, 1997; Lovley and Coates, 2000). It is becoming increasingly apparent that microbial metal reduction may be manipulated to aid in the remediation of environments and waste streams contaminated with metals and certain organics. Metals of particular interest include **iron**, **manganese** (Nealson and Saffarini, 1994; Troshanov, 1969), **chromium** (Lloyd, 2003; Lovley, 1995a), **cadmium** (Kersten, 1988; Trevors *et al*., 1986), **technetium** (Lloyd and Macaskie, 1996; Lloyd *et al*., 2000b), **cobalt** (Caccavo *et al*., 1994; Lloyd, 2003), **selenium** (Hallibaugh *et al*., 1998; Oremland *et al*., 1989), **molybdenum** (Lloyd, 2003; Tucker *et al*., 1997), **uranium** (Lovley and Phillips, 1992; Payne *et al*., 2002), **gold** (Lloyd, 2003; Kashefi *et al*., 2001), and **mercury** (Lloyd and Lovely, 2001; Lovley and Coates, 1997).

1.3 Fe(III) as electron acceptor

Iron is the fourth most abundant element in the earth's crust, and makes up by mass about 5.1%. It is a first-row transition metal that mainly exists in one of two interconvertible redox states: the reduced ferrous form [Fe(II)] and the oxidized ferric form [Fe(III)]. Both the ferrous and the ferric form can adopt different spin states (high or low), depending on its ligand environment (Andrews *et al*., 2003).

In order for microorganisms to use Fe(III) as an electron acceptor, it must first have a proper redox potential: one low enough to be nontoxic and high enough to be energetically useful to the cell when coupled to organic oxidation (Nealson and Saffarini, 1994). Once the acceptor has a proper redox potential, it should next be sufficiently abundant to support dissimilatory metabolism. The standard redox potential of the Fe(II) and Fe(III) couple (-770 mV) is applicable only in a strongly acidic solution (pH<2.5), in which both ions are well soluble. At neutral pH, the redox transition occurs for instance, mainly between $Fe(OH)_3$ (ferrihydrite) and the Fe(III) ion at a redox potential around + 150 mV (Widdel *et al*., 1993). The redox potential, at which neutrophilic iron reducers release their electrons, is in the range of + 30 mV. Under these conditions, the solubility of iron (III) hydroxides is extremely low. The free Fe(III) ion concentration in a $Fe(OH)_3$ saturated neutral solution is around 10^{-19} M (Stumm and Morgan, 1981). Therefore, Fe(III) reducing bacteria have to deliver their electrons to an essentially insoluble acceptor system.

One important difference between Fe(III) and other electron acceptors is that it can form different oxides and hydroxides, each with different crystalline structures, redox potentials and oxidation states of the metal. Even though an iron oxide may have the same chemical formula, its crystal structure, and hence kinetic and thermodynamic properties also may differ (Nealson and Saffarini, 1994). Today a total of 16 different ferric iron oxides, hydroxides or oxide hydroxides are known, and they are often collectively referred to as iron oxides (Schwertmann and Fitzpatrick, 1992; Schwertmann and Taylor, 1989).

1.4 Microbial interactions with Fe(III)

1.4.1 Assimilatory vs. Dissimilatory Fe(III) reduction

Studies performed during the last decade have indicated that microbial reduction of ferric iron [Fe(III)] to ferrous iron [Fe(II)] is a biologically significant process (Coates *et al*., 1998; Nealson and Saffarini, 1994). Iron plays an essential metabolic role in cellular processes as a component of metalloproteins or as co-factor for enzymatic reactions, as well as serving as an energy source in catabolic iron metabolisms of some microorganisms. The reduction of Fe(III) for the purpose of intracellular incorporation into metalloenzymes or utilization in enzyme activation, is called assimilatory iron reduction (Ehrlich, 1997; Guerinot, 1994; Schröder *et al*., 2003). In contrast, dissimilatory iron reduction is the process in which microorganisms transfer electrons to external ferric iron [Fe(III)] reducing it to ferrous iron [Fe(II)] without assimilating the iron (Lovley, 2000). Dissimilatory Fe(III) reducing microorganisms can be separated into two major groups, those that support growth by conserving energy from electron transfer to Fe(III) (Ehrlich, 1997; Lovley, 1991; Lovley, 1993; Nealson and Saffarini, 1994; Schröder *et al*., 2003) and those that do not (Lovley, 1987). The dissimilatory as well as the assimilatory iron reduction pathway is considered to be essential to the global iron cycle.

Ferric reductases do not form a single family, but appear to be distinct enzymes suggesting that several independent strategies for iron reduction may have evolved (Schröder *et al*., 2003). Dissimilatory and assimilatory iron reduction in bacteria is well studied; however knowledge about archaea is limited.

1.5 Assimilatory iron reduction

It has been deduced that assimilatory ferric reductases became physiologically important only when the amount of Fe(II) decreased significantly around 2.4 billion years ago as a result of oxygen generation by oxygenic phototrophs (Schröder *et al*., 2003). Almost all organisms require iron for enzymes involved in essential cellular reactions, but around neutral pH, Fe(III) exists mainly in a water-insoluble form. Many

environments that are rich in iron actually contain a free iron concentration of less than 1 µM, which is considered to be the threshold to sustain life (Neilands, 1981). Assimilatory ferric reductases are therefore, essential components of the iron assimilatory pathway that generate the more stable soluble ferric iron [Fe(III)], which is then incorporated into metalloenzymes or serves as a co-factor for enzymatic reactions (Andrews *et al*., 2003; Ehrlich, 1997; Neilands, 1981; Schröder *et al*., 2003). This type of enzyme activity is present in most prokaryotes that live in aerobic, neutral environments**.** Despite the importance of ferric reductases for aerobic life, it appears that these enzymes may have evolved divergently in prokaryotes and eukaryotes since the enzymes of both groups differ in their primary amino acid sequences and biochemical properties (Schröder *et al*., 2003).

Bacterial assimilatory ferric reductases are most often flavin reductases and therefore the ferric reductase gene expression is not regulated by iron (Fontecave *et al*., 1994). Considerable progress has been made in the last few years to determine the identity and biochemical properties of assimilatory ferric reductases. This includes the solution of two three-dimensional structures, those of *Archaeoglobus fulgidus* FeR (ferric reductase) and *Escherichia coli* Fre (flavin reductase) enzymes (Chiu *et al*., 2001; Ingelman *et al*., 1999).

1.5.1 Assimilatory ferric reductases of bacteria

Assimilatory ferric reductase activities have been identified and characterized in animals, plants, yeast and bacteria. It is found in all living systems except for a few lacto-homofermentative lactic acid bacteria (Archibald, 1983).

Many bacterial ferric reductases have been described over the past 30 years. All the soluble ferric reductases appear to be very similar biochemically and may be classified as flavin reductases (Fontecave *et al*., 1994). These prokaryotic ferric reductases lack cytochromes and bound flavin, but require exogenous FMN, FAD or riboflavin for optimal activity. The majority of ferric reductases use NADH or NADPH as the electron donor (Schröder *et al*., 2003).

Ferric reductases of bacterial species have been identified in the cell's cytoplasm, periplasm and cytoplasmic membrane. Certain pathogenic bacteria have been found to secrete their ferric reductase enzymes into the culture medium or to expose them on their cell surface (Hohmuth *et al*., 1998). Bacteria may also produce several ferric reductases that may have different cellular locations (Poch and Johnson, 1993).

1.5.1.1 Bacterial enzymes

Many bacterial ferric reductases that serve an assimilatory role have been described from a variety of different bacteria (Schröder *et al*., 2003).

1.5.1.1.1 Cytoplasmic and periplasmic ferric reductases

Thus far, the majority of ferric reductases that have been described are localized in the bacterial cytoplasm or in the periplasm of gram-negative bacteria. The cells of the virulent *Legionella pneumophila* produce at least two enzymes that are localized in the periplasm and cytoplasm (Poch and Johnson, 1993). This periplasmic enzyme prefers reduced glutathione as the electron donor but the cytoplasmic fraction utilizes NADPH. *Pseudomonas aeruginosa* contains a periplasmic ferripyochelin reductase activity that uses both NAD(P)H and reduced glutathione although the cytoplasmic ferric citrate reductase activity is strictly NAD(P)H-dependent (Cox, 1980). In some bacteria, it was not possible to distinguish between periplasmic and cytoplasmic ferric reductases on the basis of their electron donor and Fe(III) substrate usage. All of these ferric reductases exhibit wide substrate specificity towards complexed Fe(III) compounds and may even reduce free Fe(III) (Arceneaux and Beyers, 1980; Coves and Fontecave, 1993; Halle and Meyer, 1992a; Huyer and Page, 1989; Le Faou and Morse, 1991). Several ferric reductases appear to be loosely associated with the cytoplasmic membrane suggesting concerted function with a transporter to control iron uptake into the cell (Cox, 1980; Le Faou and Morse, 1991; Noguchi *et al*., 1999).

1.5.1.1.2 Extracellular ferric reductases

The bacterium *Listeria monocytogenes* produces a surface-bound ferric reductase that may be secreted into the culture medium (Barchini and Cowart, 1996; Deneer *et al*., 1995). The only extracellular ferric reductase isolated thus far is from the culture supernatant of *Mycobacterium paratuberculosis.* This enzyme appears to be cell surface-associated and also utilizes NADH as an electron donor. The activity of the enzyme is stimulated by the addition of Mg^{2+} and no flavin seems to be required (Hohmuth *et al*., 1998).

Extracellular ferric reductases are dependent on the environmental supply of NAD(P)H, reduced gluthathione and even flavin unless the enzyme firmly binds the latter. Neither NAD(P)H, reduced gluthathione nor flavin is membrane permeable or known to be secreted by any cell. Therefore, it is hypothesized that extracellular ferric reductases may only be physiologically functional if these cofactors are provided to the bacteria (Schröder *et al*., 2003). Extracellular ferric reductases are only known to be produced by obligate or opportunistic intracellular pathogens and may be considered as one of several virulence factors (Barchini and Cowart, 1996).

1.5.1.1.3 Membrane-bound ferric reductases

The ferric reductases of *Spirillum itersonii* (Dailey and Lascelles, 1977)*, E. coli* (Fischer, 1993)*,* and *Staphylococcus aureus* (Lacelles and Burke, 1978) were reported to be associated with the membrane. Thus far, none of these enzymes has been purified and characterized. These ferric reductases were suggested to be part of a membrane-bound electron chain that uses NADH, succinate, glycerol-3 phosphate, or L-lactate as donors to reduce Fe(III) (Schröder *et al*., 2003).

1.5.1.2 Ferric reductases or flavin reductases

In general, all the ferric reductases exhibit very broad substrate specificity towards complexed Fe(III) compounds and may even reduce free Fe(III) (Coves and Fontecave, 1993; Fontacave *et al*., 1994; Huyer and Page, 1989; Le Faou and

Morse, 1991). Significant progress has been made by research groups on unraveling the reason for the apparent lack of substrate specificity for most ferric reductases. It was demonstrated that the flavin produced by the ferricpyoverdine reductase from *P. aeruginosa*, is responsible for the reduction of Fe(III). Previously it was thought that the enzyme reduced the Fe(III), but it is now clear that $FMNH_2$ can chemically reduce Fe(III) under anaerobic conditions. *P. aeruginosa* provided the first evidence of a NADH:FMN oxidoreductase activity when the Fe(III) substrate was absent (Cox, 1980; Halle and Meyer, 1989; Halle and Meyer, 1992 a & b). The Fre enzyme of *E. coli* is the best characterized flavin reductase that also physiologically serves as a major ferric reductase (Fontecave *et* al., 1987). Fre was demonstrated to catalyze the reduction of free flavins. Once the flavins (FMN, FAD, ribloflavin) are reduced, they can transfer electrons to a variety of ferric siderophores including some that cannot be used for iron assimilation (Coves and Fontecave, 1993). Therefore, the Fre enzyme is regarded as a flavin reductase rather than a ferric reductase. It was suggested that the seemingly broad substrate specificity of *E. coli* Fre and *P. aeruginosa* ferripyoverdine reductases for ferric siderophores and the ferric iron-containing proteins such as ferritins could be explained by a reaction in which reduced flavin non-specifically reduces ferric iron (Fontecave *et al*., 1994; Halle and Meyer; 1992b).

The redox potential of the Fe(III) complexes also determines the rate of ferric iron reduction (Coves and Fontecave, 1993; Fontecave *et al*., 1994; Halle and Meyer, 1989; Halle and Meyer, 1992a). Therefore Fe(III) complexes, with redox potentials more negative than that of the flavin/dihydroflavin couple (-216 mV), can be chemically reduced only in the presence of strong Fe(II) chelators such as ferrozine, which is used to determine ferric reductase activity in vitro (Pierre *et al.*, 2002). The reduction of ferric siderophores with low redox potentials under physiological conditions still remains a problem. The reduction of these compounds has to be coupled to the utilization of the membrane potential, a very high affinity transport system or to an intracellular high affinity iron-binding protein in vivo (Schröder *et al*., 2003).

1.5.1.3 Ferric iron-specific reductases

Only a few ferric reductases have been described that are unable to reduce the broad spectrum of ferric compounds reduced by dihydroflavins. They are very specific for certain Fe(III) chelates. Therefore the question arises whether some of these enzymes are ferric iron-specific reductases. The ferric reductase of *Rhodopseudomonas sphaeroides* readily reduces ferric citrate but cannot reduce ferric siderophores (Moody and Dailey, 1985). Two ferric reductases were reported for *P. aeruginosa,* a ferric siderophores reductase that acts as a flavin reductase and a distinct ferric citrate reductase (Cox, 1980; Halle and Meyer, 1992b). The ferric citrate reductases of *R. sphaeroides* and *P. aeruginosa* have not yet been purified. Therefore, it is difficult to ascertain their nature as flavin- or ferric ion-specific reductases.

1.5.1.4 Dual functions of flavin reductases

If ferric reductases are indeed flavin reductases, several independent functions within the cell could be assumed for these enzymes. The reduced flavin then may be used both in enzymatic reactions, as a cofactor, and in non-enzymatic reactions.

The Fre of *E. coli* was found to be part of a multi-component complex formed with aerobically expressed ribonucleotide reductase, a key enzyme in DNA biosynthesis (Fontecave *et al*., 1987). The activation of ribonucleotide reductase requires the flavin reductase to reduce its non-heme diferric center directly or indirectly via a ferrous ion intermediate (Coves *et al*., 1997). It is still not clear whether the flavin reductase may play a regulatory role by regulating ribonucleotide reductase activity and therefore DNA biosynthesis (Coves *et al*., 1995).

The *Bacillus subtilis* ferric reductase was demonstrated to act as both a flavin reductase and ferrisiderophore reductase. This ferrisiderophore reductase forms a complex with chorismate synthase and dehydroquinate synthase that functions both in aromatic amino acid biosynthesis (Gaines *et al*., 1981; Hasan and Nester, 1978). The flavin reduced by the reductase is implicated to play a catalytic role as cofactor

during the synthesis of chrorismate (Bornermann *et al*., 1996). It has also been found that the siderophore, 2, 3 -dihydroxybenzoic acid produced by *B. subtilis* is a product of the aromatic acid biosynthesis pathway (Downer *et al*., 1970). Therefore, the flavin reductase may possibly have a regulatory role that somehow interfaces the need for iron with increased production of siderophores (Gaines *et al*., 1981).

1.5.1.5 Regulation of ferric reductases

Ferric reductases are usually constitutively produced; that is in contrast to the differential expression of other genes involved in iron assimilation such as siderophore biosynthesis and iron transporter genes. The Fur regulatory protein represses the expression of these genes in response to high iron availability allowing the expression only at low iron concentrations (Hantke, 2001; Panina *et al*., 2001). The bacterium, *Magnetospirillum magnetotacticum* is an exception in that its ferric reductase activity increases with increased concentration of Fe(III)-quinate in culture medium up to a 5 µM concentration (Noguchi *et al*., 1999).

1.6 Dissimilatory iron reduction

Geochemical and microbiological evidence suggest that the reduction of Fe(III) may have been an early form of respiration on early earth and dissimilatory ferric reductases are predicted to have evolved 3.5 billion years ago (Vargas *et al*., 1998). Several hyperthermophilic, deep-branching archaea and bacteria have been demonstrated to reduce Fe(III) to Fe(II), indicating that Fe(III) reduction was most likely a respiratory process of the last common ancestor (Chiu *et al*., 2001; Vargas *et al*., 1998). Many thermophilic as well as mesophilic microorganisms also have the ability to use Fe(III) as a terminal electron acceptor (Childers and Lovley, 2001; Lovley, 1993; Lovley *et al*., 1999; Lovley *et al*., 2000). The concept that Fe(III) is an early form of respiration agrees with geological scenarios that suggest that Fe(III) has been an abundant substrate on early earth, due to the possible photochemical oxidation of Fe(II) in the Archaean seas and the discharge of Fe(III) from hydrothermal vent fluids (Ehrenreich and Widdel, 1994; Vargas *et al*., 1998; Walker, 1987; Widdel *et al*., 1993). The large accumulations of magnetite in the precambrian

iron transformations indicate that the accumulation of Fe(III) on prebiotic earth was biologically reduced early in the evolution of life on earth. This and other geochemical considerations suggest that Fe(III) reduction was the first globally significant mechanism for organic matter oxidation (Lovley, 1991; Walker, 1987).

1.6.1 The mechanism for dissimilatory iron reduction

Iron respiration has received little attention while other respiratory pathways have been studied extensively (Lovley and Phillips, 1986 a & b; Nealson and Saffarini, 1994). However, several research groups have made significant progress the last few years.

In dissimilatory iron reduction, the ferric reductase acts as the terminal reductase of an electron transport chain that is somehow linked to the cytoplasmic membrane. The reduction of Fe(III) is coupled to the generation of a proton motive force across the cytoplasmic membrane (Myers and Nealson, 1990). The membrane-bound ATP synthase uses the proton motive force to generate ATP that will fuel active transport of nutrients or drive motility (Schröder *et al*., 2003). Both, inorganic Fe(III) precipitates and a variety of complexed Fe(III) species can be used as terminal electron acceptors in dissimilatory reduction (Lovley and Coates, 2000; Nealson and Saffarini, 1994). Thus far, little is known about dissimilatory ferric reductases, and the mechanism by which iron reduction is couple to energy generation (Schröder *et al*., 2003). The predominant proteins in electron transfer to Fe (III) are *c*-type cytochromes (DiChristina *et al*., 1988; Magnuson *et al*., 2000; Magnuson *et al*., 2001; Seeliger *et al*., 1998). Dependent on its coordination, heme *c* can assume a range of redox potentials suitable to bridge the redox spans from a variety of electron donors to Fe(III), and possibly also to other electron acceptors in branched electron chains (Schröder *et al*., 2003). These cytochromes are also ideal for electron transfer between proteins, to and from quinones, and to terminal insoluble Fe(III) (Lovley *et al*., 1998; Lovley *et al*., 2000; Nevin and Lovley, 2000; Scott *et al*., 1998).

Very little is known about the possible diversity of these types of enzymes in the variety of Fe(III) reducing bacteria. The molecular basis of respiratory metal reduction

processes have not been studied in such fine detail, although rapid advances are expected in this area. The mechanisms of Fe(III) reduction, and to a lesser degree Mn(IV) reduction, have been studied in most detail in *Shewanella oneidensis* MR-1 and *Geobacter sulfurreducens.* The availability of genome sequences (available at http://www.tigr.org) and suitable genetic systems for the generation of deletion mutants will greatly facilitate the future identification of components, including the terminal reductases that may play a role in metal reduction.

1.6.2 Strategies for dissimilatory iron reduction

Fe(III) reducing bacteria which use solid substrates as terminal electron acceptors for anaerobic respiration are presented with a unique problem: they must somehow established an electron transport link across the outer membrane between large particulate metal oxides and the electron transport chain in the cytoplasmic membrane (Myers and Myers, 1992; Myers and Nealson, 1990). Therefore, different strategies have evolved that allow for the usage of Fe(III) as electron acceptor. Four strategies for dissimilatory reduction of insoluble Fe(III) oxides have been identified thus far and will be discussed in the following sections.

1.6.2.1 Humic substances as electron shuttle

Humic substances were found to be naturally abundant in many environments (Lovley *et al*., 1998) and are formed from degradation of plants, animals and microorganisms (Schnitzer, 1978). Reduction of Fe(III) is actually an abiotic process when humics or other extracellular quinones serve as electron shuttles (Lovley and Blunt-Harris, 1999; Lovley *et al*., 1998). Evidence suggests that electron shuttling by humic substances is an important mechanism for reduction of Fe(III) in subsurface sediments (Nevin and Lovley, 2000). Humic-reducing bacteria have been recovered from diverse environments (Coates *et al*., 1998) and include hyperthermophilic (Lovley *et al*., 2000) and fermenting bacteria (Benz *et al*., 1998). Thus far, all microorganisms that can use humics as electron acceptors can also reduce Fe(III) (Lovley, 2000).

The mechanisms by which microorganisms oxidize humics are not yet fully understood. Humic substances cannot enter the cell because of their size and therefore, humic reduction is likely to take place outside the cell. Fe(III) reducing microorganisms were demonstrated to transfer electrons that were gained through oxidation of organic compounds or hydrogen to humic substances (Lovley *et al*., 1996; Lovley *et al*., 1998). Once reduced, the extracellular redox compound is chemically reoxidized using Fe(III) oxides as an electron sink. This indirect Fe(III) reduction by microorganisms is faster than Fe(III) reduction in the absence of humic substances because it alleviates the need for Fe(III) reducing microorganisms to have direct physical contact with the Fe(III) oxides (Lovley *et al*., 1996). Therefore, humics-mediated electron shuttling may improve the rates of organic matter oxidation couple with the reduction of Fe(III) (Scott *et al*., 1998).

The possibility that quinones serve as electron accepting moieties during the transference of electrons to humic substances was investigated. Electron spin resonance measurements showed a direct correlation between the electron-accepting capacity of humics and the number of quinone groups (Scott *et al*., 1998).

Microorganisms which have been shown to reduce humic substances can also reduce the model compound, AQDS, a humic acid analogue, providing further evidence that extracellular quinones can serve as electron acceptors for microbial respiration (Cervantes *et al*., 2000; Lovley *et al*., 1998). The addition of soil humics or AQDS to cultures of *Geobacter metallireducens* resulted in significant reduction of crystalline Fe(III) forms that were not otherwise reduced (Lovley *et al*., 1998). Even in environments with low concentrations of humic substances, microbial electron transfer to humics may still be significant. As long as Fe(III) is present, humics can transfer electrons to the oxide and thus be continually recycled; AQDS as low as 10 µM can stimulate the reduction of Fe(III) oxides in aquifer sediments (Lovley, 2000).

Humic substances may also enhance Fe(III) reduction through complexation of Fe(II) (Royer *et al*., 2002 a & b). Studies with *Shewanella oneidensis* MR1 (formerly *Shewanella putrefaciens* CN32) suggest that addition of natural organic matter improved hematite reduction via electron shuttling initially and later via complexation

mechanisms (Royer *et al*., 2002a). Fe(II) complexation occurred only after sufficient Fe(II) had accumulated in the system. This complexation likely improved Fe(III) reduction by preventing Fe(II) sorption to hematite and cell surfaces (Royer *et al*., 2002b).

1.6.2.2 Microbially secreted electron shuttling compounds

Recent evidence indicates that some bacteria reduce Fe(III) oxides by producing and secreting small, diffusible redox compounds that can serve as an electron shuttle between the microorganism and the insoluble iron substrate. Therefore, the microorganism does not need to directly contact the insoluble Fe(III) substrate. The diffusible electron shuttles secreted by *S. oneidensis* MR-1 and *Geothrix fermentans*, include hydrophilic quinones of yet unknown nature (Nevin and Lovley, 2002a; Newman and Kolter, 2000), as well as melanin by *Shewanella alga* BrY (Nevin and Lovley, 2002b, Turick *et al*., 2002).

S. alga BrY was found to secrete melanin that contains quinoid compounds that could function as the sole electron acceptor for growth (Turick *et al*., 2002). Bacterially reduced melanin can also reduce Fe(III) oxides in the absence of cells suggesting that the melanin can act as an electron shuttle between the cells and Fe(III) oxides. Only a small amount of melanin was required to significantly enhance the rate of Fe(III) reduction (Turick *et al*., 2002). The ability to produce melanin may provide *S. alga* BrY with an effective strategy for reducing insoluble Fe(III) oxides. In a separate study, *S. alga* BrY and *G. fermentans* were demonstrated to reduce amorphous Fe(III) oxide entrapped within alginate beads, providing additional evidence that both can produce extracellular electron-shuttling compounds (Nevin and Lovley, 2002 a & b). Thin-layer chromatography indicated that the electron shuttling compound excreted by *G. fermentans* has characteristics similar to a water-soluble quinone (Nevin and Lovley, 2002a).

1.6.2.3 Secretion of Fe(III) chelators

It has been demonstrated that *S. alga* BrY and *G. fermentans*, in addition to the secreting quinone compounds, also reduced Fe(III) oxides by secreting iron chelating compounds, called siderophores (Nevin and Lovley, 2002 a & b). The nature of these siderophores excreted in extracellular electron transfer is not yet established.

Most microorganisms require iron for essential processes and since Fe(III) is insoluble in most natural environments, many microorganisms rely on Fe(III) chelating siderophores (Ratledge and Dover, 2000). They are of low molecular mass (smaller than 1000 Da) and are characterized by their high specificity and affinity towards Fe(III) (Byers and Arceneaux, 1998; Ratledge and Dover, 2000). It is generally assumed that enzymes used for dissimilatory Fe(III) reduction are distinct from the enzymes involved in iron-siderophore transport for assimilation (Luu and Ramsay, 2003). It has been argued that siderophores are unsuitable as electron acceptors due to their negative redox potentials (Hernandez and Newman, 2001). However, not enough is known about either type of enzyme to rule out the possible similarities. The effectiveness of siderophores in making insoluble Fe(III) bioavailable for assimilation may also be important in making Fe(III) available as a terminal electron acceptor during respiration (Luu and Ramsay, 2003).

In addition to the microbially excreted compounds, studies with *S. alga* BrY (Nevin and Lovley, 2002b) and *G. fermentans* (Nevin and Lovley, 2002a) found that these cultures solubilized significant quantities of Fe(III) during the most active phase of Fe(III) reduction. These results suggest that the cells secreted Fe(III) solubilizing compounds, possibly siderophores, as a mechanism to access and reduce insoluble Fe(III).

However, the high affinity of siderophores for Fe(III) may pose a challenge in Fe(III) respiration. When *G. metallireducens* was grown on insoluble Fe(III), the addition of hydroxamate siderophores did not stimulate cell growth (Holmen *et al*., 1999). This may be due to the high strength and redox stability of Fe(III)-hydroxamate complex, impeding reduction by *G. metallireducens*.

1.6.2.4 Requirement for direct cell-mineral contact

The anaerobic Fe(III) reducing bacterium *G. metallireducens* must directly contact the Fe(III) oxide for reduction unless a soluble electron acceptor such as Fe(III) citrate is provided (Nevin and Lovley, 2000). It has been demonstrated to produce pilli and flagella when presented with insoluble iron for chemotaxis and attachment to the metal oxide (Childers *et al*., 2002). No appendages are formed when *G. metallireducens* is cultured in the presence of Fe(III) citrate (Nevin and Lovley, 2000).

The relationship between cell-oxide adhesion and the rate of Fe(III) reduction in *S. alga* BrY was examined. For a variety of Fe(III) oxides, the initial rate and long-term extent of iron reduction by *S. alga* BrY correlated linearly with the oxide surface area (Roden and Zachara, 1996). Therefore bacterial attachment seemed to be requisite for Fe(III) reduction, and the oxide surface area correlated directly with the concentration of surface sites available for enzymatic contact. Fe(II) biosorption by dissimilatory Fe(III) reducing bacteria also affects the rate and extent of Fe(III) reduction since *S.alga* BrY cells precoated with Fe(II) reduced Fe(III) more slowly than untreated cells (Urrutia *et al*., 1998). These results suggest that the sorption of Fe(II) and the precipitation of Fe(II)/Fe(III) solids on the surface of the cell interferes with electron transport to Fe(III) oxides. Consistent with this hypothesis is the finding that Fe(II) adsorption to iron oxide lowered the extent of Fe(III) reduction, presumably blocking the surfaces sites available for bacterial attachment (Roden and Urrutia, 1999).

Das and Caccavo (2000) examined the relationship between cell-oxide adhesion and the rate of Fe(III) reduction in *S.alga* BrY. Since both *S.alga* BrY cells and Fe(III) oxides are negatively charged, adhesion of cells to the oxides is influenced by electrostatic repulsion. The authors added various concentration of KCl to allow the cells to adsorb to the oxides more readily. The ability of the cells to reduce insoluble Fe(III) was correlated with KCl concentration and with the percentage of adhered cells. These results provide direct evidence that adhesion is required for Fe(III) reduction by *S.alga* BrY.

In contrast to previous reports (Arnold *et al*., 1990; Das and Caccavo, 2000; Roden and Urrutia, 1999; Roden and Zachara, 1996), Newman and Kolter (2000) demonstrated that *Shewanella* species can respire with Fe(III) species without attaching themselves to Fe(III) oxide by excreting quinones. This has been recently disputed by Caccavo and Das (2002), who presented evidence that *S. alga* BrY may use its flagella for attaching to Fe(III) oxide. Since deflaggelated *S. alga* BrY still attached to Fe(III) oxides but with reduced efficiency, it appears that additional proteins also function in the adhesion to the metal oxide (Caccovo, 1999; Caccavo and Das, 2002). Although flagellum-mediated adhesion was found not to be a prerequisite for Fe(III) reduction, there appears to be a correlation between adhesion and Fe(III) oxide reduction (Caccavo and Das, 2002). Adherence to the Fe(III) oxide particle may present a distinct advantage for iron-respiring microorganisms by facilitating Fe(III) oxide respiration (Schröder *et al*., 2003). It is still not clear whether Fe(III) oxide adherence in *S. alga* BrY is regulated by the presence of soluble Fe(III) electron acceptors.

1.6.3 Dissimilatory ferric reductases

1.6.3.1 Ferric iron reduction in Shewanella *species*

S. oneidensis MR-1 is the best studied gram negative bacterium that can respire with a variety of electron donors including Mn(IV) and Fe(III). It has been demonstrated that *c*-type cytochromes are major components of iron and manganese reduction either in electron transfer or as a possible terminal reductase (Myers and Myers, 1997). These cytochromes are mainly located in the outer membrane (Myers and Myers, 1992; Myers and Myers, 1993; Myers and Myers, 2001) or located in the periplasmic space and are expressed anaerobically regardless of the electron donor (Myers and Myers, 1992; Tsapin *et al.*, 1996). Efforts for the identification and purification of the ferric reductases in *S. oneidensis* MR-1 have led to the elucidation of functions for the proteins that are involved in iron respiration. More than 500 of the total ferric reductase activity was localized to the outer membrane (Myers and Myers, 1993). The outer membrane is an unusual location for a respiratory enzyme although it is perfectly suitable for the utilization of an insoluble substrate (Schröder *et al*., 2003). Biochemical analysis of the outer membrane of *S. oneidensis* MR-1 revealed

four cytochrome *c*-containing proteins that could be reduced by formate and reoxidized by Fe(III) and Mn(IV), therefore demonstrating that at least one or more of these proteins could act as ferric reductases (Myers and Myers, 1997). It was demonstrated that two of the *c*-type cytochrome, OmcA and OmcB, do not participate in Fe(III) reduction but are more likely involved in Mn(IV) reduction (Myers and Myers, 2001). However, OmcA was purified and characterized from *Shewanella frigidimarina* NCIMB4000 and revealed the existence of 10 *c*-type hemes (Field *et al*., 2000). The redox potentials of the hemes enable the protein to function as a putitative ferric reductase, since Fe(III)-EDTA can reoxidize the reduced protein. It was also found that the proteins easily detach from cells and may contact insoluble substrates.

The outer membrane protein MtrB of *S. oneidensis* MR-1 has been suggested to be a component of the ferric and manganese reductases, since a transposon mutation that inactivated the *mtrB* gene resulted in the loss of both the Fe(III) and Mn(IV) reductase activities (Beliaev and Saffarini, 1998). It was also found that MtrB was required for reduction of AQDS, suggesting that the reductase(s) responsible for transferring electrons to humic acids and AQDS is also located in the outer membrane (Shyu *et al*., 2002).

The question arises what other components may participate in the electron transfer to Fe(III). Iron respiration is dependent on the presence of menaquinone since it was demonstrated that menaquinone–deficient mutants lost the ability to respire with Fe(III) as electron acceptor (Myers and Myers, 1993; Saffarini *et al*., 2002). The involvement of several genes, including *cymA, mtrA and mtrC,* were revealed after a screen for *S. oneidensis* MR-1 mutants that were greatly impaired in their ability to reduce Fe(III) (Beliaev *et al*., 2001; Myers and Myers, 1997; Myers and Myers, 2000). The latter two genes form an operon with *mtrB* described above. The genes are predicted to be located in either the periplasmic space (CymA and MtrA) or in the outer membrane (MtrC), and they all encode heme *c*-containing proteins (Beliaev *et al*., 2001). A *mtrC* deletion mutant resulted in reduced electron transfer activity to Fe(III) as well as impaired ferric reductase activity catalyzed by the membrane (Beliaev *et al*., 2001). The electron transfer activity from formate to Fe(II) and Mn(IV) by whole cells was found to be impaired with a deletion of *mtrA*, although the ferric

reductase activity assayed in the membrane fraction was still intact (Beliaev *et al*., 2001).

A c_3-type cytochrome encoded by *cctA* was identified in *S. frigidimarina* NCIMB400. This cytochrome acts as a periplasmic electron shuttle that is involved in iron respiration (Gordon *et al*., 2000). Iron respiration was almost completely impaired with a disruption of *cctA,* although it had no affect on growth with nitrate, fumarate, TMAO, DMSO and several other electron acceptors (Schröder *et al*., 2003).

A 150-kDa outer membrane protein in *S. oneidensis* MR-1 has been demonstrated to specifically interact with the surface of goethite and thus facilitated electron transfer to the insoluble iron. Biological force microscopy has been used to probe the interface between a living cell of *S. oneidensis* MR-1 and the surfaces of goethite under aerobic as well as anaerobic conditions on a nanoscale level. *S. oneidensis* MR-I responded to the surface of goethite by rapidly developing stronger adhesion energies at the surfaces. It was demonstrated that goethite as the terminal electron acceptor, actively produces and/or mobilizes proteins (the 150-kDa putitative outer membrane protein and perhaps others) that interact with the mineral surface under anaerobic conditions (Lower *et al*., 2001).

1.6.3.2 Ferric reductases in Geobacter *species*

It is likely that *c*-type cytochromes of *Geobacter* species are involved in some aspect of electron transport of Fe(III) at or near the cell surface. The role of these cytochromes in Fe(III) reduction is currently under investigation (Gaspard *et al*., 1998; Magnuson *et al*., 2000; Seeliger *et al*., 1998). Gaspard *et al*. (1998) reported that 80% of the total ferric reductase activity associated with the membrane fraction of *Geobacter sulfurreducens* was located in the outer membrane.

It was demonstrated that ferric reductase activity associated with the membrane fraction of *G. sulfurreducens* could be measured with NADH or reduced horse heart cytochrome *c* as electron donors. In addition, solubilization of ferric reductase from whole cells with 0.5M KCl without any disruption of cells indicated that the ferric

reductase is a peripheral protein on the outside of the outer membrane (Gaspard *et al*., 1998).

A membrane-associated ferric reductase was isolated from *G. sulfurreducens* (Gorby and Lovley, 1991) and the molecular mass was determined to be 300-kDa (Magnuson *et al*., 2001). Cofactor analysis of the purified reductase demonstrated that it contains a hemoprotein and flavin adenine dinucleotide. This enzyme complex consists of at least five polypeptides, one of which is an 89-kDa *c*-type cytochrome and FAD as co-factor. The redox potential of the 89-kDa *c*-type cytochrome was determined to be about -100 mV, and therefore electron transfer to Fe(III) compounds by *G. sulfurreducens* is possible (Magnuson *et al*., 2001). It is considered that ferric reductases must be membrane-bound to allow direct contact with extracellular Fe(III) oxides and also to provide mechanisms for the generation of a proton-motive force via the oxidation of NADH (Champine and Goodwin, 1991). Due to its large size and membrane distribution, it may be possible that this ferric reductase spans both the inner and outer membrane, and therefore allows electron transfer from NADH to Fe(III) oxyhydroxides. Inhibitor studies suggested that this ferric reductase enzyme complex consists of a NADH-dehydrogenase and a cytochrome *c*-terminal ferric reductase (Magnuson *et al*., 2000).

Lloyd *et al*. (2001) identified a 41-kDa outer membrane *c*-type cytochrome involved in the transfer of electrons to insoluble Fe(III) oxides by *G. sulfurreducens*. Treatment of whole cells by protease resulted in selective digestion of the 41-kDa cytochrome, localizing it to the surface of the cell.

A 9.6-kDa periplasmic *c*-type cytochrome, designated PpcA, was purified from *G. sulfurreducens* (Lloyd *et al*., 2003). It was suggested that this cytochrome was released into the environment, where it could serve as a soluble electron shuttle between the cell and insoluble Fe(III) oxides (Seeliger *et al*., 1998). This hypothesis was questioned in a subsequent study, which reported that the 9.6-kDa protein was not the dominant *c*-type cytochrome secreted by *G. sulfurreducens*, nor did it function as an electron shuttle between whole cells and Fe(III) oxides (Lloyd *et al*., 1999). The present results suggest that PpcA functions as an intermediary electron carrier in

electron transport from acetate to ferric reductases in the outer membrane (Lloyd *et al*., 2003).

A preliminary model for electron transport to insoluble Fe(III) in *G. sulfurreducens* has been proposed (Figure 1.1) (Lovley, 2000). In this model, a NADH dehydrogenase localized in the inner membrane is part of a respiratory complex that contains an 89-kDa *c*-type cytochrome. This cytochrome can transfer electrons to a 9-kDa periplasmic cytochrome, which can in turn transfer electrons to a 41-kDa membrane-bound cytochrome. The 41-kDa cytochrome is associated with the outer membrane and therefore is able to contact insoluble Fe(III) and donate electrons to Fe(III).

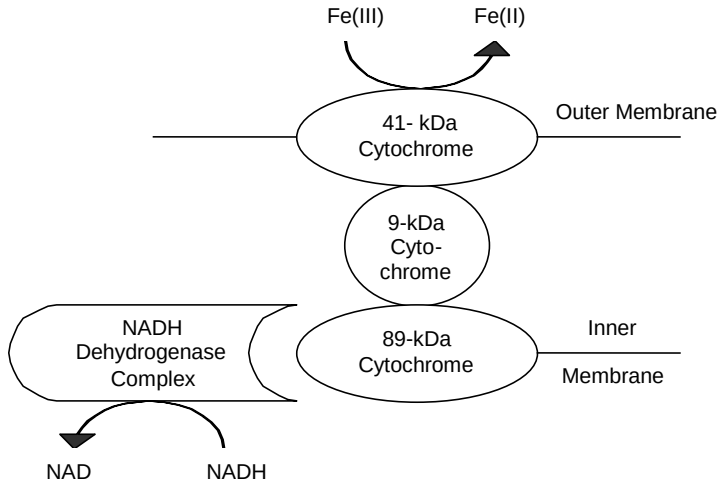


Figure 1.1 Proposed model for electron transport to extracellular Fe(III) in *G. sulfurreducens.*

A soluble ferric reductase has also been isolated from *G. sulfurreducens.* This enzyme consisted of two subunits with molecular masses of 87- and 78-kDa and had a native molecular mass of 320-kDa (Kaufmann and Lovley, 2001). The protein also contains FAD, iron and acid-labile sulfur suggesting the presence of several Fe-S centers (Schröder *et al*., 2003). It was indicated that this ferric reductase is a redox-active protein with the potential to reduce soluble forms of Fe(III). This soluble ferric reductase is unlike any previously described enzyme with the capacity for Fe(III) reduction. It still has to be established whether this enzyme acts physiologically as

ferric reductase or as a ferredoxin:NADP oxidoreductase (Kaufmann and Lovley, 2001).

Membrane-associated and soluble ferric reductase activities were also reported for *Geobacter metallireducens*, but have not yet been characterized (Gorby and Lovley, 1991).

1.7 Archaeal ferric iron reductase

Only the archaeal ferric reductase of the hyperthermophile, *Archaeoglobus fulgidus* has been isolated and characterized extensively (Vadas *et al*., 1999) and therefore we must be careful when generalizing about how assimilatory ferric reductases may have evolved. It is still unclear whether the enzyme of *A. fulgidus* serves as an assimilatory or dissimilatory ferric reductase. Recently, a novel hyperthermophilic archaeon *Geoglobus ahangari* that can grow autotrophically on hydrogen with Fe(III) serving as the sole electron acceptor has been isolated (Kashefi *et al*., 2002b). The hyperthermophilic archaea, *Pyrobaculum islandicum* and *Pyrobaculum aerophilum* have also been shown to conserve energy to support growth from Fe(III) reduction (Huber, 1987; Lovley *et al*., 2000). Therefore, the existence of at least three definite dissimilatory archaeal Fe(III) reducers is known.

1.7.1 The ferric reductase of *Archaeoglobus fulgidus*

The anaerobic hyperthermophilic sulfate reducing archaeon, *A. fulgidus* contains high Fe(III)-EDTA activity exclusively in its soluble fraction (Vadas *et al*., 1999; Vargas *et al*., 1998). Whether this enzyme is involved in the citrate-complexed Fe(III) reduction with H_2 as electron donor, is still not clear because of the absence of a genetic system for *A. fulgidus*. The *A. fulgidus* ferric reductase is very similar to bacterial flavin reductases, which act as ferric reductases, because it was demonstrated to reduce FAD as well as FMN in the absence of a Fe(III) electron acceptor (Vadas *et al*., 1999).

The fact that *A. fulgidus* utilizes flavin as a substrate suggests that it functions as a NAD(P)H:flavin oxidoreductase. Since it was possible to reconstitute the ferric reductase with FMN, this enzyme clearly classifies as a flavoprotein, and an enzyme-bound flavin is suggested to be involved in catalysis (Vadas *et al*., 1999).

The physiological role of the ferric reductase in *A. fulgidus* is still uncertain. This type of enzyme may have evolved in bacteria, and *A. fulgidus* may have acquired the enzyme via horizontal gene transfer when it came into contact with more oxygenated environments**.** Functionally the enzyme is more similar to assimilatory ferric reductases, but because of its high cellular abundance, it seems possible that this enzyme serves a dissimilatory function (Vargas *et al*., 1998). Thus far, it is difficult to recognize ferric reductase-encoding genes in the various sequenced genomes. More archaeal ferric reductases need to be identified in order to trace the evolutionary roots of this important class of enzymes.

1.7.2 *Pyrobaculum islandicum*

P. islandicum conserves energy to support growth by coupling the oxidation of hydrogen or organic compounds to the reduction of Fe(III) (Kashefi and Lovley, 2000). In contrast to the bacterial Fe(III) reducers that contain both soluble and membrane-associated ferric reductase activities (Schröder *et al*., 2003), the activity in *P. islandicum* was mainly localized in the soluble fraction (cytoplasm). The absence of *c*-type cytochromes in *P. islandicum* is the most significant difference between *P. islandicum* and other well studied dissimilatory Fe(III) reducers. Several features of this ferric reductase activity suggest that the mechanism for Fe(III) reduction in *P. islandicum* is significantly different from the models for electron transport to Fe(III) that have been proposed for *Shewanella* and *Geobacter* species (Childers and Lovley, 2001).

1.8 Diversity of Fe(III) reducing microorganisms

Iron respiration has become widely accepted as an important process mediating the biogeochemical cycling of organic matter, nutrients and contaminants under anaerobic conditions (Lovley and Phillips, 1986b). The two most intensely studied genera of Fe(III) reducers are *Geobacter* and *Shewanella* (Coates *et al*., 1996; Longergan *et al*., 1996). *S. oneidensis* and *G. metallireducens* were the first organisms demonstrated to conserve energy to support growth through metal reduction (Myers and Nealson, 1988). Since then numerous organisms have been isolated that are capable of Fe(III) reduction coupled to growth.

Early studies on the reduction of Fe(III) focused mainly on organisms that could grow predominantly via fermentation of sugars with the utilization of metals as minor electron acceptors (Lovley, 1991). Sulphate reducing bacteria were also demonstrated to reduce Fe(III) and other electron acceptors, although it has not been shown that these reactions support growth (Coleman *et al*., 1993). A wide phylogenetic diversity of microorganisms, including archaea as well as bacteria, are capable of dissimilatory Fe(III) reduction. Most microorganisms that reduce Fe(III) also can transfer electrons to Mn(IV), reducing it to Mn(II) (Lovley, 2000).

1.8.1 *Shewanella – Ferrimonas- Aeromonas*

Shewanella species and related organisms in the gamma subdivision of the *Proteobacteria* (a range of *Shewanella*, *Ferrimonas* and *Aeromonas*) have been studied as models for Fe(III) reducing microorganisms (Nealson and Saffarini, 1994). This group has several notable properties. They use a variety of electron acceptors (NO_3^-, NO_2^-, S^0) and are generally able to use only a restricted range of small organic acids and hydrogen as electron donors (Lloyd, 2003). In contrast to other Fe(III) reducers, which only grow anaerobically, several of the *Shewanella* species and related organisms, can grow both aerobically and anaerobically. Under anaerobic conditions they can use Fe(III), Mn(IV), or other electron acceptors to conserve energy (Lovley, 2000). *Shewanella* species and related organisms (Caccavo *et al*.,

1992; Coates *et al*., 1999a) can be recovered from a variety of sedimentary environments including various aquatic sediments and the subsurface (Fredrickson *et al*., 1998). In contrast to the organisms in *Geobacteraceae* which are found to be numerous in both molecular and culturing analysis of widely diverse environments where Fe(III) reduction is important, the distribution of *Shewanella* is more variable (Lovley, 1997; Lovley, 2000). Another Fe(III) reducing microorganism that may be related to this group is an unidentified microorganism referred to as a "pseudomonad," which was the first organism found to grow with hydrogen as the electron donor and Fe(III) as the electron acceptor (Balashova and Zavarzin, 1980).

1.8.2 *Geobacteraceae*

Although the environmental distribution of Fe(III) reducing prokaryotes remains poorly understood, most studies indicated that members of the *Geobacteraceae* are the key components of Fe(III) reducing communities in subsurface environments (Röling *et al*., 2001; Rooney-Varga *et al*., 1999).

G. metallireducens (formerly GS-15) was the first microorganism found to conserve energy to support growth from the oxidation of organic compounds coupled to Fe(III)-reduction. This bacterium was isolated from iron-rich sediments of the Potomac River and categorized in the delta *Proteobacteria*. Most of the known Fe(III) reducing bacteria that can oxidize organic compounds completely to carbon dioxide with Fe(III) serving as the sole acceptor are in the family *Geobacteraceae*. The family *Geobacteraceae* comprises the genera *Geobacter*, *Desulfuromusa*, *Desulfuromonas* and *Pelobacter* (Lonergan *et al*., 1996). These organisms with the exception of the *Pelobacter* species, are able to completely oxidize a wide range of organic compounds including acetate when respiring using Fe(III) and Mn(IV). This metabolism is significant because acetate is probably the primary electron donor in most sedimentary environments (Lovley, 2000). The *Pelobacter* species are also more restricted in the range of electron donors utilized. Some members of the *Geobacteraceae* are also able to use aromatic compounds including toluene, phenol and benzoate as electron donors for metal reduction (Lovley *et al*., 1993).

1.8.3 *Geothrix*

G. fermentans and closely related strains have been recovered from petroleum-contaminated aquifers (Anderson *et al*., 1998; Coates *et al*., 1999 a & b). Like *Geobacter* species, *G. fermentans* can oxidize short-chain fatty acids to carbon dioxide with Fe(III) serving as the sole electron acceptor (Anderson *et al*., 1998; Coates *et al*., 1999 a & b). It can also use long-chain fatty acids as well as hydrogen as an electron donor for Fe(III) reduction and can grow fermentatively on several organic acids (Lovely, 2000). Studies in which Fe(III) reducing bacteria were isolated in culture media suggested that organisms closely related to *G. fermentans* might be as numerous as *Geobacter* species in the Fe(III) reduction zone of a petroleum-contaminated aquifer (Anderson *et al*., 1998). However, analyses of 16S rDNA sequences have indicated that *Geothrix* species are probably several orders of magnitude less numerous than *Geobacter* species in such environments (Rooney-Varga *et al*., 1999).

1.8.4 *Geovibrio ferrireducens* and *Deferribacter thermophilus*

Other Fe(III) respiring bacteria that have been characterized recently include the mesophile *Geovibrio ferrireducens* (Caccavo *et al*., 1996) and the thermophile, *Deferribacter thermophilus* (Greene *et al*., 1997). They have been recovered from hydrocarbon-impacted soils and a petroleum reservoir. These organisms are more closely related to each other than any other known Fe(III) reducing microorganisms and grow with similar electron donors. An interesting feature of the metabolism of these organisms is the ability to use some amino acids as electron donors for Fe(III) reduction. The environmental distribution of these organisms has not been studied in detail (Lovely, 2000).

1.8.5 *Ferribacter limneticum*

Ferribacter limneticum recovered from mining-impacted lake sediments, is unusual in that it can reduce Fe(III) but not Mn(IV). It is the only organism in the beta subdivision of the *Proteobacteria* that is known to conserve energy to support growth from Fe(III) reduction (Cummings *et al*., 1999).

1.8.6 *Sulfurospirillum barnesii*

Sulfurospirillum barnesii was initially isolated because of its ability to use selenate as an electron acceptor (Oremland *et al*., 1994). Although it has commonly been found that if one organism in a close phylogenetic group has the ability to reduce Fe(III) then others in the group also will be Fe(III) reducers (Lonergan *et al*., 1996; Lovley *et al*., 1995), *Sulfurospirillum arsenophilum* does not reduce Fe(III) (Stolz *et al*., 1999). *Wolinella succinogenes*, which is also in the epsilon subdivision of the *Proteobacteria*, also can reduce Fe(III) and metalloids (Lovley, 1997), but whether *W. succinogenes* conserves energy to support growth from metal reduction has not been determined.

1.8.7 Thermophilic Fe(III) reducing microorganisms

In addition to *D. thermophilus* mentioned above, a number of other thermophilic Fe(III) reducing microorganisms can conserve energy to support growth. Several different *Bacillus* strains have been isolated and characterized as metal reducers, but only *Bacillus infernus* is capable of dissimilatory metal reduction coupled to growth. Increasing temperatures are associated with greater depth and may select for thermophilic Fe(III) reducing bacteria. The thermophile *B. infernus* that can grow at 60°C was isolated from the subsurface in the Taylorsville Triassic Basin in Virginia (Boone *et al*., 1995). This organism can conserve energy to support growth by oxidizing formate or acetate coupled to the reduction of Fe(III) and Mn(IV). *Thermus* species that have been isolated from a thermal spring and a South African gold mine were capable of Fe(III) and Mn(IV) reduction at 65°C, with lactate as the electron

donor (Kieft *et al*., 1999). *Thermoterrabacterium ferrireducens* has also been recovered from the subsurface (Slobodkin *et al*., 1997).

1.8.8 Hyperthermophilic archaea and bacteria

Several hyperthermophilic archaea and bacteria have also been shown to grow using Fe(III) as an electron acceptor including *Pyrobaculum islandicum*, *Pyrobaculum aerophilum*, *Thermotoga maritima* (Vargas *et al*., 1998), *Geothermobacterium ferrireducens* (Kashefi *et al*., 2002a), *Geoglobus ahangari* (Kashefi *et al*., 2002b) and *Ferroglobus placidus* (Tor and Lovley, 2001).

1.9 Bioremediation of organic and metal contaminants

Fe(III) reducing microorganisms have been shown to play a major role in removing organic contaminants from polluted aquifers. Fe(III) reducers were found to remove aromatic hydrocarbons naturally from petroleum-contaminated aquifers (Anderson *et al*., 1998; Lovley, 1995a; Lovley, 1997) and this process can be artificially enhanced with compounds that make Fe(III) more available for microbial reduction (Lovley, 1997). The Fe(II) minerals formed as a result of microbial Fe(III) reduction can be important reductants for the reduction of nitroaromatic contaminants (Hofstetter *et al*., 1999). Minerals containing Fe(II) also may serve to reductively dechlorinate some chlorinated contaminants (Fredrickson and Gorby, 1996).

Some Fe(III) reducers have the ability to substitute several high-valency metals for Fe(III) as an electron acceptor, converting them to less soluble forms, which are less mobile in groundwater or can be precipitated from waste streams or soil washings (Francis, 1994; Fredrickson *et al*., 2000; Lloyd and Lovley, 2001). Microorganisms currently used in commercial processes or with potential biotechnological applications, are ubiquitous in nature (Lloyd, 2003). Table 1.1 summarizes the reduction of metals by some Fe(III) reducers. Reduction of soluble U(IV) to insoluble U(VI) can effectively precipitate uranium from contaminated groundwater and surface water (Lloyd, 2003; Lloyd *et al*., 2002; Lloyd *et al*., 2003; Lovley, 1993). Some Fe(III) reducing microorganisms can precipitate technetium from contaminated waters by

reducing soluble Tc(VII) to insoluble Tc(IV) (Lloyd *et al*., 2000b). Soluble radioactive Co(III) complexed to EDTA can be reduced to Co(II) which is less likely to be associated with EDTA found in contaminated groundwater and more likely to adsorb to aquifer solids (Caccavo *et al*., 1994; Gorby and Bolton, 1998). Some Fe(III) reducers convert soluble, toxic Cr(VI) to less soluble toxic Cr(III) (Lloyd, 2003; Lovley and Phillips, 1992; Meyers and Meyers, 1998). Reduction of soluble selenate to elemental selenium can effectively precipitate selenium in sediments or remove selenate from contaminated water in bioreactors (Tomei *et al*., 1992; Tomei *et al*., 1995).

Table 1.1 Reduction of metals by Fe(III) reducing microorganisms.

Fe(III) reducer	**Metals**	**Reference**
G. metallireducens	Mn(VI), U(VI), Tc(VII), Au(III), Hg(II)	Lovley *et al*., 1993; Lloyd *et al*., 2000b.
S. putrefaciens	Mn(VI), U(VI), Cr(VI), Np(V)	Lloyd *et al*., 2000a; Myers and Nealson, 1988; Myers *et al*., 2000; Wade and DiChristina, 2000.
P. islandicum	Tc(VII), Co(III), Au(III), Cr(VI), Mn(IV), Au(III)	Kashefi and Lovley, 2000; Kashefi *et al*., 2001.
Thermus SA-01	Mn(IV), Co(III), Cr(VI) U(VI)	Kieft *et al*., 1999.
D. desulfuricans	Mn(VI), U(VI), Cr(VI), Tc(VII), Pd(II), Se(VI), Mo(VI), V(V)	Lloyd *et al*., 1998; Tucker *et al*., 1997; Yong *et al*., 2002.

1.10 Conclusions

The capacity of Fe(III) reduction is found in phylogenetically diverse microorganisms throughout the bacterial and archaeal domains. However, the biochemistry of microbial Fe(III) reduction is still a relatively new area of study, with most of the work focusing on pure cultures of *Shewanella* and *Geobacter* species (Luu and Ramsay, 2003).

The discovery that all hyperthermophiles that have been evaluated for Fe(III) reduction have this capacity, has raised the question whether mechanisms for dissimilatory Fe(III) reduction have been conserved throughout microbial evolution. However, this hypothesis has not been adequately evaluated because of the lack of information on the mechanisms for Fe(III) reduction in a diversity of Fe(III) reducing microorganisms. As more dissimilatory Fe(III) reducers, including thermophilic microorganisms, are identified and studied, new mechanisms for accessing and reducing Fe(III) will likely emerge.

Many studies have suggested that *c*-type cytochromes are integral components in electron transport to Fe(III) in mesophilic dissimilatory Fe(III) reducing microorganisms (Schröder *et al*., 2003). Thus, if previous models for dissimilatory Fe(III) reduction by mesophilic bacteria are correct, then it is unlikely that a singly strategy for electron transport to Fe(III) is present in all dissimilatory Fe(III) reducing microorganisms. Several features of the *P. islandicum* ferric reductase suggest that the mechanism for Fe(III) reduction differs from the models proposed for *Shewanella* and *Geobacter* species (Childers and Lovley, 2001). This suggests that phylogenetically diverse Fe(III) reducing microorganisms may have different mechanisms for Fe(III) reduction. The Fe(III) reduction mechanisms in *P. islandicum* and other Fe(III) reducing microorganisms deficient in *c*-type cytochromes, such as *Pelobacter carbinolicus* still need to be evaluated (Lovely *et al*., 1995). Thus, further investigations into the process of Fe(III) reduction in hyperthermophilic and thermophilic microorganisms are warranted to better understand the biochemical diversity of Fe(III) reduction.

Although the full environmental relevance of these Fe(III) reducing microorganisms in metal reduction processes has only recently become apparent, rapid advances in understanding these important transformations have been made (Lloyd *et al*., 2003). The microbial reduction of metals by Fe(III) reducing microorganisms can play crucial roles in the cycling of both organic and inorganic species in a range of environments and, if harnessed, may offer the basis for a wide range of innovative biotechnological processes.

CHAPTER 2

Introduction to the present study

Iron respiration is utilized by a diverse group of bacteria and archaea as a means to support growth. While other respiratory pathways have been studied extensively, iron respiration has received little attention. Many Fe(III) reducing microorganisms have the potential to reduce toxic metals, converting them to less soluble forms which are less mobile in groundwater or can be precipitated from waste streams and also play an important role in degradation of organic matter. Thus, Fe(III) reducers show promise as useful agents for the bioremediation for environments contaminated with organic and/or metal pollutants.

Dissimilatory ferric reductases catalyze the reduction of ferric iron [Fe(III)] to ferrous iron [Fe(II)]. Thus far little is known about the dissimilatory ferric reductases and the mechanism by which Fe(III) reduction is coupled to generation of energy. Only recently, significant progress has been made by several research groups on unraveling the strategies used for iron respiration. The biochemistry of dissimilatory Fe(III) reduction has only been studied intensively in mesophilic species like *Shewanella* and *Geobacter*. Indeed research on these organisms has been given added impetus through the availability of the genome sequences and suitable genetic systems for the generation of deletion mutants for both of these organisms (Coppi *et al*., 2001; Schröder *et al*., 2003). Although the terminal reductase has yet to be identified unequivocally in either organism, the involvement of *c*-type cytochromes is implicated in electron transport to Fe(III). The dissimilatory ferric reductase from the hyperthermophilic archaeon *Pyrobaculum islandicum* has been characterized and the activity was found to be mainly localized in the cytoplasm (Childers and Lovley, 2001). The absence of *c*-type cytochromes is the most significant difference. This suggest that phylogenetically diverse Fe(III) reducing microorganisms may have different mechanisms for Fe(III) reduction. Fe(III) reducing bacteria are able to reduce Fe(IIII) chelated or in insoluble inorganic minerals such as ferrihydrite, goethite and others. The question arises whether Fe(III) is taken up and reduced in the periplasm

or cytoplasm or whether electrons are transferred to Fe(III) minerals outside of the cells.

The thermophilic bacterium, *Thermus scotoductus*, was isolated and Kieft *et al*. (1999) reported on the organism's ability to reduce several metals including Fe(III). The dissimilatory reduction associated with this microorganism was investigated to shed some light into the process of Fe(III) reduction in thermophilic microorganisms that are warranted to better understand the biochemical diversity of Fe(III) reduction.

CHAPTER 3

Materials and methods

3.1 Materials and chemicals

Analytical reagent grade chemicals were obtained from commercial sources and were used without any further purification. Unless mentioned otherwise, all reagents were purchased from Sigma or Merck. Tryptone was obtained from Biolab. Yeast extract was from Difco. The following chromatography media were obtained from: (1) Tosohaas: Toyopearl Phenyl-650M, Toyopearl CM-650M and Toyopearl Super Q-650S, (2) Bio-Rad: Bio-Gel P60. The ultrafiltration stirred cell and YM – 30 (molecular cutt-off of 30 000) membranes were from Amicon.

3.2 Bacterial strain

A thermophilic bacterium was isolated from hot, alkaline groundwater sampled at a depth of 3.2 km in a South African gold mine by Tom Kieft and co-workers. The 16S rRNA gene sequence analysis indicated that it bears a close phylogenetic relationship to several well-characterized strains that belong to the genus *Thermus*. This was the first report of a *Thermus* sp. being able to couple the oxidation of organic compounds to the reduction of Fe(III), Mn(IV) and S° and other metals (Kieft *et al*., 1999). After recovery of the Thermus strain, it was designated *Thermus* SA-01 but recently it has been renamed to *Thermus scotoductus* (Balkwill *et al*., 2004).

3.3 Culturing of *Thermus scotoductus*

Themus scotoductus was cultured microaerophilically in TYG medium containing tryptone [0.5% (w/v)], yeast extract [0.3% (w/v)] and glucose [0.1% (w/v)] or anaerobically in TYG medium containing KNO_3 [0.1% (w/v)]. The strain was maintained aerobically on plates containing TYG medium with the same composition as above, with bacteriological agar [1.6% (w/v)]. The TYG plates were incubated aerobically at 65°C.

A modified TYG medium containing Fe(III) citrate (10 mM or 20 mM) and acetate [0.082% (w/v)] with glucose omitted was used to ensure the use of Fe(III) as an electron acceptor. Alternatively, KNO_3 [0.1% (w/v)] was used as an electron acceptor. The medium was adjusted to pH 6.5.

Liquid cultures were prepared by inoculating the bacterium into 5 ml TYG or modified medium and were grown microaerophilically or anaerobically overnight (pre-inoculum) at 65 °C.

Further culturing was achieved by transferring the pre-inoculum aseptically to bottles (100 ml) containing either TYG or modified medium (80 ml) (target optical density A_{660} ~ 0.01) with the headspace flushed with nitrogen to provide an anoxic environment and grown at 65 °C. Alternatively the pre-inoculum was transferred to an Erlynmeyer flask (500 ml) filled with TYG or the modified medium (250 ml) (target optical density A_{660} ~ 0.1) and grown at 65 °C under microaerophilic conditions (Figure 4.1). The microaerophilic cultures in the late exponential phase were incubated in a COY anaerobic chamber overnight at room temperature to induce the anaerobic ferric reductase activity, and the cells were subsequently harvested by centrifugation (10 000 x g for 10 min at 10 °C).

Aliquots were periodically withdrawn under sterile conditions and cells were assayed for ferric reductase activity in vitro as described in section 3.6.2.2 (Figure 4.1). The Fe(II) that accumulated in the media was determined by the assay described in section 3.6.2.1.

Biomass of cells was determined by relating the dry cell weight to absorbance at 660 nm (Figure 3.1).

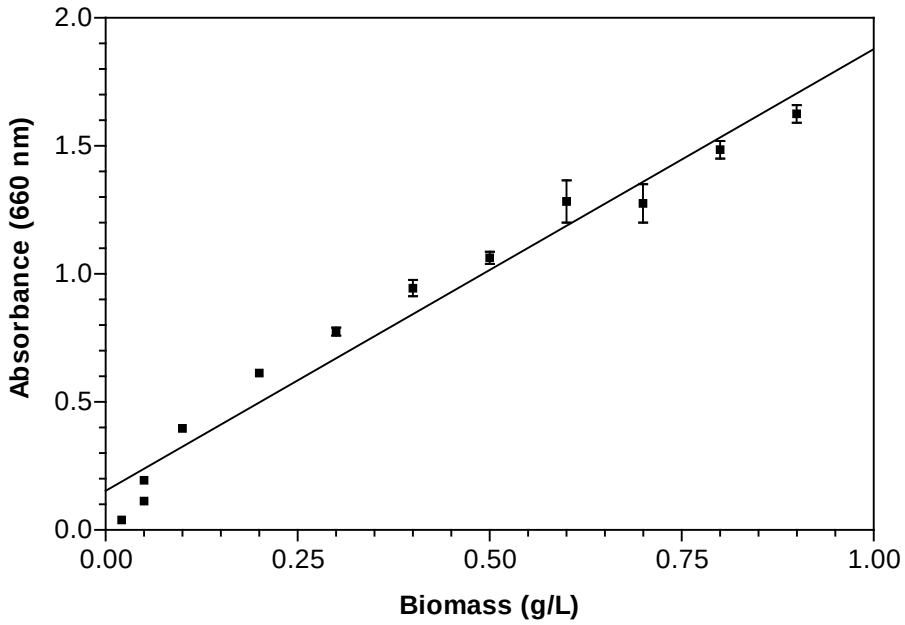


Figure 3.1. Standard curve relating biomass to OD_{660}. Standard deviations of duplicate readings are shown.

3.4 Fe(III) reduction under non-growth conditions

Fe(III) reduction by resting cells under non-growth conditions was monitored as described in section 3.2.6.1. Cells were cultured anaerobically in the modified medium with KNO_3 as the electron acceptor (section 3.3). Cells were harvested by centrifugation and washed three times with 30 mM bicarbonate (pH 7) / 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7) buffer, resuspended in 30 mM bicarbonate (pH 7) / 50 mM HEPES (pH 7) buffer containing 10 mM acetate as the electron donor and 10 mM Fe(III) citrate as electron acceptor to a final volume of 10 ml with the headspace flushed with nitrogen to provide an anoxic environment. Cell suspensions were incubated at 65 °C with shaking. The controls consisted of tubes containing the same buffer without cells. Anaerobic tubes were transferred to the anaerobic chamber, shaken vigorously to resuspend any settled solids, and then sampled with a 1 ml syringe to determine Fe(II) formation as described in section 3.6.2.1 (Figure 4.2).

3.5 Fe(III) and NO_3^- reduction experiments

Themus scotoductus was cultured microaerophilically in the modified TYG medium containing 10 mM Fe(III) citrate in the absence and presence of 10 mM KNO_3. Liquid cultures were prepared by inoculating the bacterium into 5 ml of modified medium. Cells were grown microaerophilically overnight (pre-inoculum).

Further culturing was achieved by transferring the pre-inoculum aseptically to Schott bottles (250 ml) containing the modified medium (200 ml) (target optical density A_{660} ~ 0.1). Cells were grown at 65 °C under microaerophilic conditions (Figure 4.3).

Aliquots were periodically withdrawn under anoxic conditions and Fe(II) formation in the supernatant was analyzed via the ferrozine method as described in section 3.6.2.1 (Figure 4.3). Cells were assayed for ferric reductase activity in vitro as described in section 3.6.2.2.

3.6 Assays

3.6.1 Protein determination

Protein concentrations were determined either by absorbance at 280 nm or the bicinchoninic acid (BCA) method (Smith *et al*., 1985). The Pierce BCA protein assay reagent is a highly sensitive reagent for the spectrophotometric determination of protein concentration. The commercially available kit from Pierce was used according to the manufacturer's instructions. A standard curve relating protein concentration to absorbance at 562 nm was constructed (Figure 3.2). A set of protein standards was prepared with bovine serum albumin (BSA). 50 µl of each standard / unknown sample was pipetted into a test tube (50 µl of diluent was used for blanks), 1 ml of working reagent was added to each tube and vortexed. Tubes were incubated at 37 °C for 30 min and the absorbance was read at 562 nm.

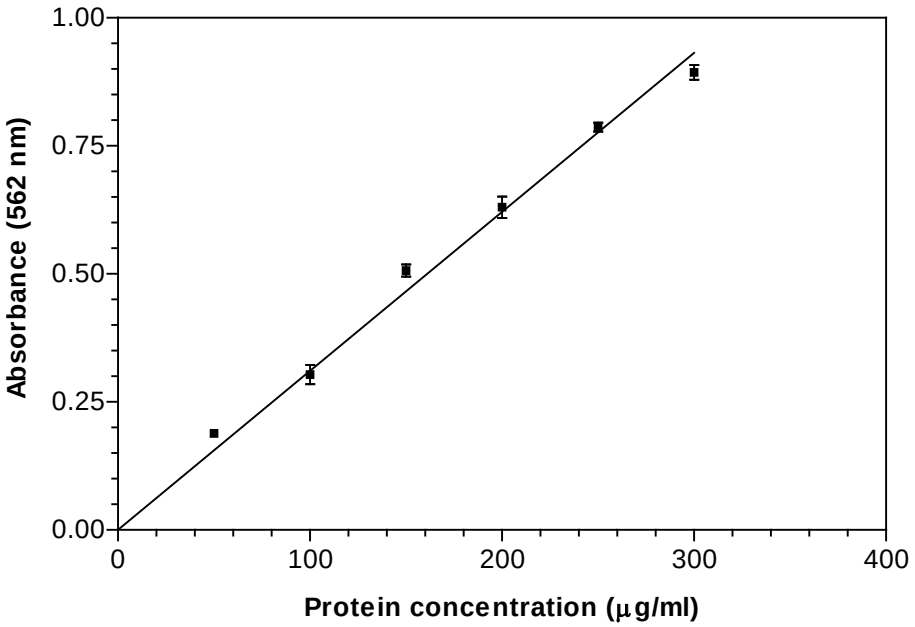


Figure 3.2. Standard curve for the BCA-protein assay with BSA as the protein standard for the test tube protocol. Error bars indicate standard deviations.

Alternatively, the standards as well as the protein samples were prepared in a 96 well flat-bottomed microtitre plate and read at a wavelength of 540 nm using a microtitre plate reader (Figure 3.3). This protocol was used for monitoring the protein concentrations of the fractions collected after application on chromatographic media (section 3.11). A set of protein standards was prepared with bovine serum albumin (BSA). 25 µl of each standard / unknown sample was pipetted into the appropriate microtitre plate wells (25 µl of diluent was used for blank wells), 200 µl of working reagent was added to each well. The microwell plate was incubated at 37°C for 30 min and the absorbance was read at 562 nm.

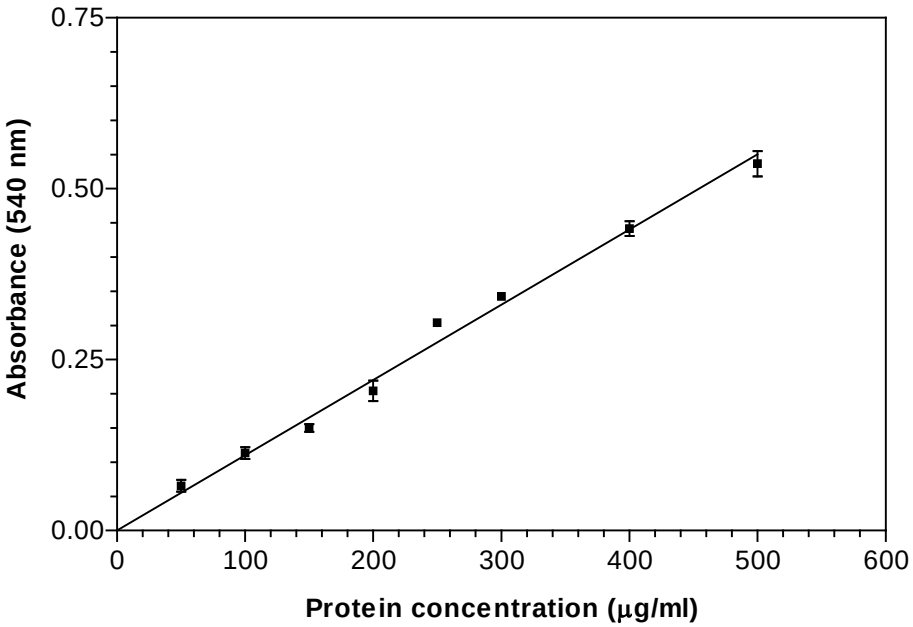


Figure 3.3. Standard curve for the BCA-protein assay with BSA as the protein standard for the microwell plate protocol. Error bars indicate standard deviations.

3.6.2. Ferrozine-based assays

3.6.2.1 Fe(III) reduction assay

Fe(III) reduction was monitored by measuring the accumulation of Fe(II) by the HCl-extractable ferrozine assay (Phillips and Lovley, 1987). The ferrozine compound [3-(2-pyridyl)-5,6-bis-(4-phenylsulfonic acid)-1,2,4-triazine] reacts with Fe(II) to form a stable complex that may be used for the direct determination of Fe(II) (Stookey, 1970).

0.1 ml extracellular samples were withdrawn and added to 5 ml of a 0.5 N HCl stock solution. After 30 minutes of extraction, 20 µl of the HCl-soluble Fe(II) was added to 1 ml of a ferrozine (1g/L) solution in 50 mM HEPES buffer (pH 7). The Fe(II) was quantified by using a calibration curve relating Fe(II) to absorbance at 562 nm (Figure 3.4).

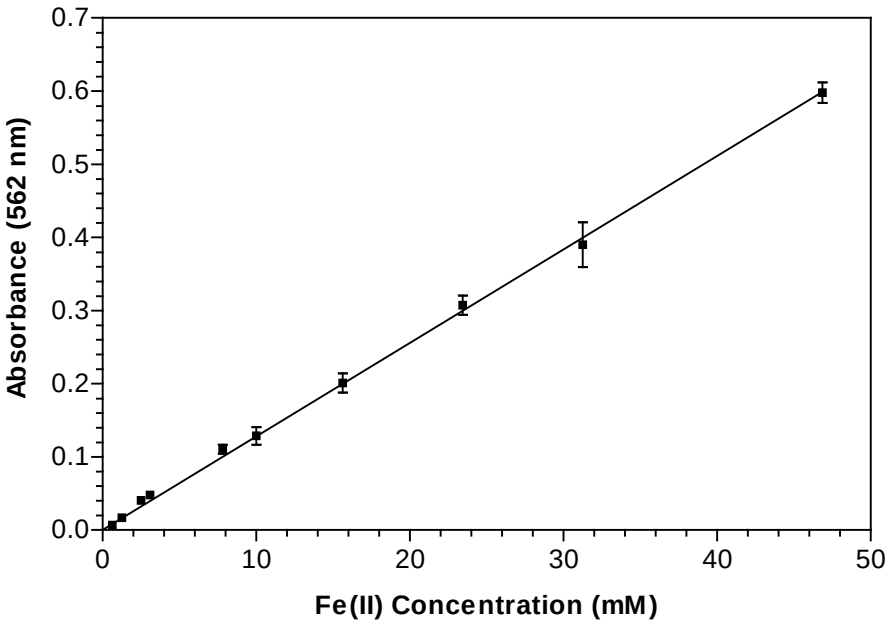


Figure 3.4. Standard curve for assay of Fe(II) with the ferrozine method using ferrous(II) chloride tetrahydrate as standard. Error bars indicate standard deviations.

3.6.2.2 Fe(III)-NTA reductase assay

During purification, the ferric reductase activity was assayed by monitoring the production of Fe(II) over time as adapted from Lascelles and Burke (1978). Ferric reductase activity was assayed anaerobically in 1 ml reaction mixture containing 1 mM NADH, 1 mM ferrozine, 2 mM Fe(III)-nitrilotriacetic acid (NTA) in 50 mM HEPES buffer (pH 7) / acetate buffer (pH 5.5). The reactions were initiated by the addition of NADH and were incubated at 65°C. Absorbance was measured after a fixed time using a Spectronic Genesys 5 spectrophotometer at 562 nm (Figure 3.5).

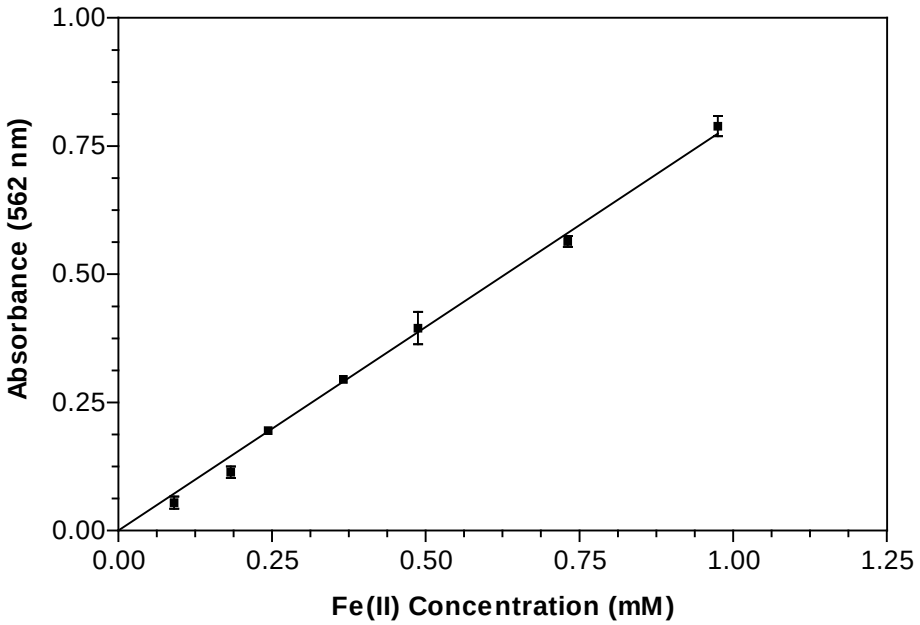


Figure 3.5. Standard curve for assay of Fe(II) during purification with the ferrozine method using ferrous(II) chloride tetrahydrate as standard. Error bars indicate standard deviations.

3.6.3 Chromate reductase assay

Chromate reductase activity was determined by measuring the decrease of hexavalent chromium during enzyme assays. Cr(VI) was analysed by the *s*-diphenylcarbazide method as described by Urone (1955). 2.5 ml samples were withdrawn and added to a 0.12 M H_2SO_4 stock solution. 0.2 ml of the *s*-diphenylcarbazide reagent (dissolved in acetone) was added to the reaction mixture to a final concentration of 0.01% (w/v). Absorbance was measured using a Spectronic Genesys 5 spectrophotometer at 540 nm and chromate quantified using a calibration curve relating chromate concentration to absorbance at 540 nm (Figure 3.6).

During purification, the enzyme was assayed in 1 ml reaction mixtures containing (to a final concentration) 20 mM MOPS buffer (3-(*N*-Morpholino)-ethanesulfonic acid) (pH 7.0), 0.1 mM CrO_3, 0.3 mM NADH and 0.05 ml of the enzyme preparation at 65°C.

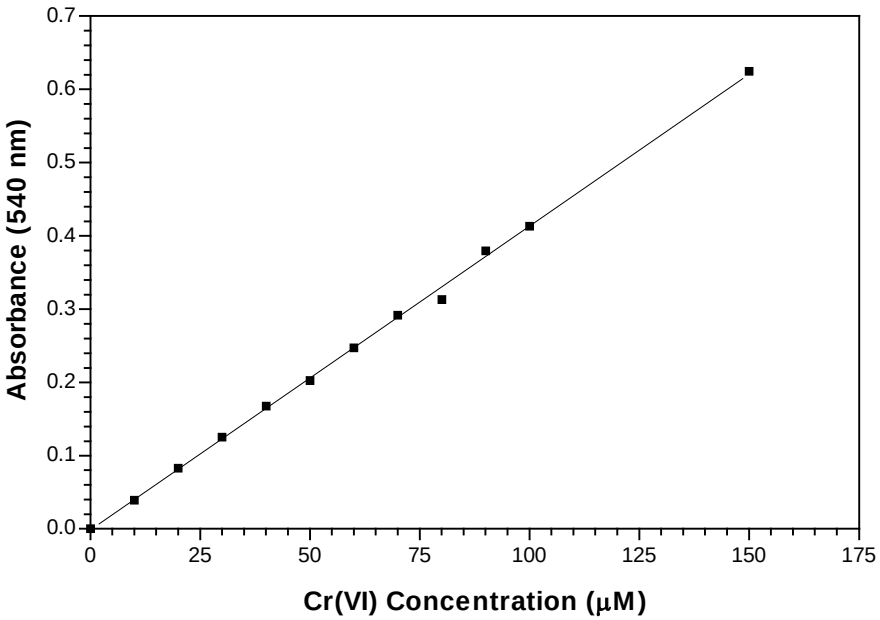


Figure 3.6. Standard curve relating chromate concentration to A_{540}. Error bars indicate standard deviations.

3.7 Electrophoresis

3.7.1 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to assess the homogeneity of the purified fractions and to estimate the relative molecular mass (Mr) of the enzyme by comparing its electrophoretic mobility with the molecular masses of known standard proteins, by using a 12.5% resolving gel and 4% stacking gel.

SDS-PAGE was performed using the “Mighty small” miniature slab gel electrophoresis unit, SE 200 from Hoefer Scientific Instruments. The protocol described by Laemmli (1970) was used. Electrophoresis was performed on approximately 10 µg (20 µl of prepared sample) protein. Protein bands were detected with silver staining according to the method described by Rabillot *et al*. (1989).

The determinations of the relative molecular masses of the denatured ferric reductases were based on the relative distance of migration of either of two sets of molecular standards. The protein standards used from BIO-RAD (broad range) were

myosin (200 000 Da), β-galactosidase (116 250 Da), phosphorylase b (97 400 Da), serum albumin (66 200 Da), ovalbumin (45 000 Da), carbonic anhydrase (31 000 Da), trypsin inhibitor (21 500 Da), lyzosyme (14 400 Da) and apoprotinin (6 500 Da).

The second set of protein standards was the prestained protein standards from BIO-RAD that included recombinant protein standards with the following molecular masses: 250 000 Da, 150 000 Da, 100 000 Da, 75 000 Da, 50 000 Da, 37 000 Da, 25 000 Da, 20 000 Da, 15 000 Da and 10 000 Da.

3.7.2 Native polyacrylamide gel electrophoresis (PAGE) and Zymogram

Native PAGE was performed by the same protocol described for SDS-PAGE with the exception that the anionic detergent, SDS, was omitted from gels and the samples, and the samples were not incubated prior to the loading of the gel. A sample (approximately 200 µg protein) after dialysis and concentration with a Whatman ultrafiltration cell (12 000 Da) was applied to a 10% resolving gel and 4% stacking gel.

Activity staining of the ferric reductases was performed after native gel electrophoresis. A procedure adapted from Moody and Dailey (1985) was used. After completion of the electrophoresis, the gel was washed for 1 min in 100 ml of 50 mM HEPES (pH 7) / 50 mM acetate (pH 5.5) buffer. After the washing buffer was removed, the gel was placed in 100 ml of the same buffer containing 1 mM NADH, 1 mM ferrozine and 2 mM Fe(III)-NTA at room temperature in a COY anaerobic chamber. After approximately 20 to 40 min, a pinkish-red color appeared at the position of the ferric reductase. Zymogram analysis was done at pH 5.5 for the membrane-associated ferric reductase and pH 7 for the soluble (cytoplasmic) ferric reductase.

3.7.3 Isoelectric focusing (IEF)

The pI values of the purified protein were determined by isoelectric focusing. The method used was that of Robertson *et al*. (1987). The pharmalyte carrier ampholytes

pH 3-10 was used to determine the pI of the protein. 25 mM sodium hydroxide was used as catholyte and 20 mM acetic acid for the anolyte. The gel was prefocussed at 150 V for 30 min and after loading the sample (approximately 20 µg protein), it was focused for 1½ h at 200 V and 1½ h at 400 V. Native and denaturing IEF gels containing 8 M urea were performed. The native IEF gel was stained for ferric reductase activity as described in section 3.7.2.

For the determination of the pI, a vertical gel strip was cut into 1-cm pieces. The pH of each gel piece was determined and a curve relating pH to distance, was constructed (Figure 3.7). Isoelectric focusing is the use of electrophoresis for examination of the electrophoretic mobility as a function of pH. The net charge on a protein is pH-dependent. Proteins below their isoelectric pH are positively charged and migrate in a medium of fixed pH to the negatively charged cathode. Above the isoelectric pH, a protein is deprotonated and negatively charged and migrates towards the anode.

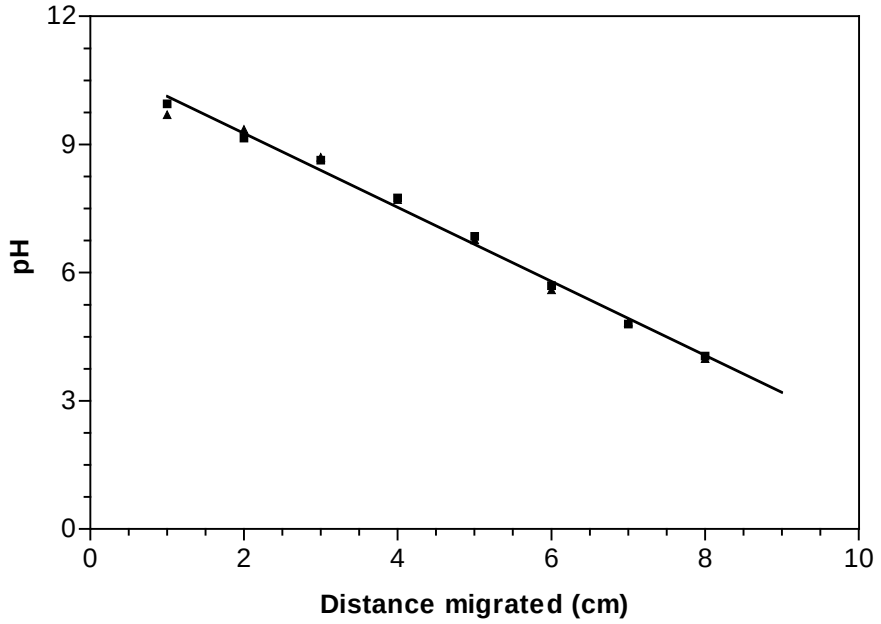


Figure 3.7. A graph relating pH to distance was constructed for determination of the pI.

3.7.4 Two-dimensional gel electrophoresis

2D PAGE was performed, where proteins are separated in each dimension on the basis of independent physiochemical properties (usually charge and size). The

method used, is that described by O'Farrel (1975) using a combination of IEF under denaturing conditions in the first dimension and SDS-PAGE in the second dimension.

The first dimension was prepared as described in section 3.7.1. After focusing, the lane containing the protein sample was removed and placed in transfer buffer (50 mM Tris-HCl (Tris (hydroxymethyl)-aminomethane- HCl), pH 6.8; Urea (6 M); glycerol [30% (v/v)]; SDS [1% (w/v)]; Bromophenol blue [0.01% (w/v)]) for 5 to 10 min. The strip was then directly placed into a 12.5% SDS resolving gel. The gel was run at a constant current (20 mA) and visualized by silver staining (Rabillot *et al*., 1989).

3.8 Preparation of subcellular fractions

Care was taken to subject samples to an anaerobic environment. The COY anaerobic chamber was flushed, activated and reactions were performed in this environment. Subcellular fractions were prepared as described by Kaufmann and Lovley (2001). Cells were grown and harvested as described in section 3.3. Cells were then resuspended in 20 mM Tris-HCl (pH 7) buffer containing 25% (w/v) sucrose. To accomplish cell wall lysis, lysozyme (20 mg) was added to cell suspension (approximately 1 g wet weight) and stirred for 20 min. Na_2-EDTA was added to a final concentration of 5 mM and stirred for another 15 min. Finally $MgCl_2$ was added to a final concentration of 13 mM and the suspension was stirred for another 15 min. Separation of the spheroplasts from the periplasmic fractions was obtained by centrifugation at 20 000 x g for 30 min. Spheroplasts were resuspended in 20 mM Tris-HCl (pH 7) / 50 mM acetate (pH 5.5) buffer.

To obtain the membrane and cytoplasmic (soluble) fraction, a protocol adapted from Gaspard *et al*. (1998) was used. A few crystals of DNase were added to the EDTA-lysozyme-treated cell suspension, and the cells were broken by ultrasonic treatment (10 times, 50 W, 30 s) with a sonifier (Branson Sonic Power Cell Disruptor B-30) in an ice-water bath (Table 4.1). The suspension was centrifuged at 100 000 x g for 1½ h at 10 °C, yielding the cytoplasmic (soluble) fraction and the membrane fraction (pellet). The pellet was resuspended in 20 mM Tris-HCl (pH 7) / 50 mM acetate (pH 5.5) buffer (Table 4.2).

3.9 Separation of outer and cytoplasmic membranes

Separation of the outer and cytoplasmic membranes was achieved according to a procedure adapted from Meyers and Meyers (1992). 1 ml of the membrane fraction (approximately 2 mg protein) was layered onto 28 ml of a 40 to 70% (w/v) sucrose gradient and centrifuged for 17 h at 90 000 x g. Two colored bands were observed. The bands were retrieved from the tube by removing fractions of 0.5 to 0.8 ml. Protein determinations (section 3.6.1) were done and the fractions were assayed for activity (section 3.6.2.2) (Figure 4.5). To identify the fractions containing the outer membrane, the content of KDO (2-keto-3-deoxyoctonate), a specific constituent of the lipopolysaccharide in the outer membrane, was determined as described by Karkhanis *et al*. (1978).

3.10 Solubilization of ferric reductase activity

Whole cells, spheroplasts or membrane fractions were stirred for 1 h at room temperature in a high-ionic salt buffer containing 0.5 M KCl (20 mM Tris-HCl [pH 7] / 50 mM acetate [pH 5.5] buffer) (Gaspard *et al*., 1998).

3.11 Isolation of ferric reductase(s) by chromatographic methods

A number of different isolation procedures were performed and assessed. Unless otherwise mentioned, purification procedures were conducted under anoxic conditions at room temperature.

3.11.1 Isolation of the cytoplasmic (soluble) ferric reductase

The cytoplasmic (soluble) fraction was prepared as described in section 3.8, and subjected to further purification on chromatographic media.

3.11.1.1 First isolation of the cytoplasmic (soluble) ferric reductase

Thermus scotoductus was cultured microaerophilically in the modified medium with KNO_3 as the electron acceptor (section 3.3). The cytoplasmic (soluble) fraction was dialyzed, using a dialysis tube with molecular weight cut-off of 14 000 Da, against 20 mM Tris-HCl (pH 7.8). The buffer was changed at least two times.

The dialysate was applied to Super-Q Toyopearl (8 cm x 2.8 cm) previously equilibrated with 20 mM Tris-HCl buffer (pH 7.8); thus the pH of the enzyme as well as that of the loading buffer was 7.8. The column was washed with 20 mM Tris-HCl until the $A_{280\ nm}$ readings were less than 0.01. A salt gradient of 0 – 0.5 M NaCl at a flow rate of 40 mlh^{-1} was used to elute proteins. The fractions collected (2.5 ml) were monitored for protein concentration by the BCA protein assay (section 3.6.1) and tested for activity (section 3.6.2.2) (Figure 4.6). The fractions containing the bulk of activity were pooled.

Polyethylenglycol (PEG) 20 000 was used to concentrate the pooled fractions in SnakeSkin pleated dialysis tubing (Pierce) with a molecular weight cut-off of 7 000 Da.

The concentrated fractions were loaded onto a Biogel P60 gel filtration column (50 cm x 1.5 cm) equilibrated with 20 mM Tris-HCl buffer (pH 7.8). Enzyme elution was performed using the same buffer. The proteins eluted at a flow rate of 10 mlh^{-1} and fractions (2 ml) were collected. The protein concentrations of the fractions were determined (section 3.6.1), and the activity was assayed (section 3.6.2.2) (Figure 4.7). The active fractions were pooled and prepared for SDS-PAGE (section 3.7.1).

3.11.1.2 Second isolation of cytoplasmic (soluble) ferric reductase

The following isolations were done to increase yields and purity of the ferric reductase.

Thermus scotoductus was cultured microaerophilically in the modified medium with KNO_3 as the electron acceptor (section 3.3).

The cytoplasmic (soluble) fraction was applied to a CM Toyopearl column (8 cm x 2.8 cm) equilibrated with 50 mM acetate buffer (pH 5.8). The pH of the enzyme as well as that from the loading buffer was 5.8. The column was washed with 50 mM acetate buffer until the $A_{280\ nm}$ reading was less than 0.01. The non-binding fraction was tested for activity (section 3.6.2.2). The unabsorbed activity found in the non-binding fraction was re-applied to the CM Toyopearl column. The proteins were eluted with salt gradient of 0 – 0.5 M NaCl over 400 ml at a flow rate of 35 mlh^{-1} and fractions were collected (3 ml). Fractions were monitored spectrophotometrically at 280 nm and tested for activity (section 3.6.2.2) (Figure 4.8). The active fractions were pooled and concentrated as described in section 3.11.1.1.

The concentrated fractions were loaded onto a Biogel P60 gel filtration column (50 cm x 1.5 cm) equilibrated with 50 mM acetate buffer (pH 5.5). Enzyme elution was performed using the same buffer. The proteins eluted at a flow rate of 9 mlh^{-1} and fractions (2 ml) were collected (Figure 4.9). The absorbance was monitored at 280 nm and the fractions were tested for activity (section 3.6.2.2). The active fractions were pooled. SDS-PAGE was performed as described by section 3.7.1.

3.11.1.3 Third isolation of cytoplasmic (soluble) ferric reductase

Thermus scotoductus was cultured microaerophilically in the modified medium with KNO_3 as the electron acceptor (section 3.3). The pH of the cytoplasmic (soluble) fraction was adjusted to 5.3 and applied to CM Toyopearl (6 cm x 2.8cm) previously equilibrated with acetate buffer (pH 5.3). The column was washed with 50 mM

acetate buffer until the $A_{280\ nm}$ reading was less than 0.01. The flow rate was adjusted to 60 mlh^{-1}. The fractions (3 ml) were collected, monitored spectrophotometrically at 280 nm and assayed for enzyme activity (section 3.6.2.2) (Figure 4.10). The activity was collected in two fractions, Fraction 1 and Fraction 2. The purity of the fractions was assessed by SDS-PAGE (section 3.7.1).

Ammonium sulphate was added to both fractions to a final concentration of 1M and loaded onto Phenyl Toyopearl (5 cm x 2.8 cm) equilibrated with 50 mM acetate buffer (pH 5.5) containing 1 M ammonium sulphate. The non-binding fraction eluted with 50 mM acetate buffer (pH 5.5) containing 1 M ammonium sulphate. The binding fraction eluted using a linear gradient of 1 – 0 M ammonium sulphate at a flow rate of 40 mlh^{-1}. The fractions were tested for activity (section 3.6.2.2) and the absorbance monitored at 280 nm (section 3.6.1). Fraction 1 (Figure 4.11) and Fraction 2 (Figure 4.12) were purified to apparent homogeneity by the above mentioned chromatography step as indicated by SDS-PAGE (section 3.7.1). The preceding purification procedure was used in all the other purification attempts.

The fractions containing activity (Fraction 1) were pooled and concentrated on an Amicon ultrafiltration (UF) unit equipped with a membrane with a molecular weight cut-off 30 000 Da.

To ensure the purity of the soluble (cytoplasmic) ferric reductase for amino acid sequencing, Fraction 1 was applied to Biogel P60 (50 cm x 1.5 cm) equilibrated with 50 mM acetate buffer (pH 5.5). The proteins eluted at a flow rate of 9 mlh^{-1} and fractions (2 ml) were collected (Figure 4.13). The protein peaks were monitored spectrophotometrically at 280 nm and the activity was tested (section 3.6.2.2). The fractions containing activity were pooled. The pooled active fraction was prepared for SDS-PAGE (section 3.7.1) (Figure 4.22), zymogram (section 3.7.2) (Figure 4.23). IEF (section 3.7.3) (Figure 4.24) and 2D -PAGE (section 3.7.4) (Figure 4.24).

3.11.1.4 Fourth isolation of cytoplasmic (soluble) ferric reductase

Thermus scotoductus was cultured microaerophilically in the modified medium containing KNO_3 as the electron acceptor (section 3.3).

The isolation of the protein was performed as described in section 3.11.1.3, except that the spheroplasts were broken by less harsh ultrasonic treatment. Instead of 10 times, the spheroplasts were sonicated 5 times at 50 W for 30 s periods to prevent detaching of the loosely membrane-associated ferric reductase activity, in an attempt to increase the yields for the soluble (cytoplasmic) fraction.

The CM Toyopearl column was run as described in the previous section. The protein peaks were monitored spectrophotometrically at 280 nm and the activity was tested (section 3.6.2.2) (Figure 4.14).

The pooled active fraction was applied to Phenyl Toyopearl as described in section 3.11.1.3. The absorbance was monitored at 280 nm and tested for activity (3.6.2.2) (Figure 4.15). The active fractions were prepared for SDS-PAGE (section 3.7.1).

3.11.2 Isolation of the extracted ferric reductase from the membrane

The membrane fraction was prepared as described in section 3.8. Extraction of the ferric reductase from spheroplasts with a high-ionic salt buffer was done as described in section 3.10, following centrifugation at 20 000 x g for 30 min at 10 °C.

3.11.2.1 First isolation of the extracted ferric reductase

Thermus scotoductus was cultured microaerophilically in the modified medium with KNO_3 as the electron acceptor (section 3.3). The KCl-extracted protein was dialyzed against 20 mM Tris-HCl buffer (pH 7.8), using a dialysis tube with molecular weight cut-off of 14 000 Da, with buffer changes several times until the pH of the dialysate was pH 7.8.

The dialysate was applied to Super-Q Toyopearl (8 cm x 2.8 cm) previously equilibrated with 20 mM Tris-HCl buffer (pH 7.8). The column was washed with Tris-

HCl buffer until the $A_{280\ nm}$ readings was less than 0.01. A salt gradient of 0 – 0.5 M NaCl at a flow rate of 40 mlh^{-1} was used to elute proteins. The protein concentrations of the fractions (3 ml) collected were determined by the BCA protein assay as described in section 3.6.1. The fractions were monitored for activity (section 3.6.2.2) (Figure 4.16). The fractions containing the bulk of activity were concentrated as described in section 3.11.1.1.

The concentrated fraction was loaded onto a Sephacryl S-100-HR gel filtration column (50 cm x 1.5 cm) equilibrated with 20 mM Tris-HCl buffer (pH 7.8). Enzyme elution was performed using the same buffer. The proteins eluted at a flow rate of 10mlh^{-1} and fractions (2 ml) were collected. The protein concentrations of the fractions were determined (section 3.6.1) and tested for activity (section 3.6.2.2) (Figure 4.17).

3.11.2.2 Second isolation of the extracted ferric reductase

Thermus scotoductus was cultured microaerophilically in the modified medium with KNO_3 as the electron acceptor (section 3.3). The KCl-extracted protein was dialyzed against a 50 mM acetate buffer (pH 5.8), using a dialysis tube with molecular weight cut-off of 14 000 Da, with buffer changes several times until the pH of the dialysate was pH 5.8.

The dialysate was applied to a CM Toyopearl column (8 cm x 2.8 cm) equilibrated with 50 mM acetate buffer (pH 5.8). The enzyme was applied to the column and the non-binding fraction tested for activity (section 3.6.2.2). The column was washed with 50 mM acetate buffer until the $A_{280\ nm}$ readings were less than 0.01. The bound proteins were eluted with salt gradients of 0 – 0.5 M NaCl at a flow rate of 35 mlh^{-1}. Fractions were collected (3 ml), monitored spectrophotometrically at 280 nm and tested for activity (section 3.6.2.2) (Figure 4.18). Fractions containing activity were pooled and concentrated as described in section 3.11.1.1.

The concentrated fractions were loaded onto a Biogel P60 gel filtration column (50 cm x 1.5 cm) equilibrated with 50 mM acetate buffer (pH 5.5). Enzyme elution was performed using the same buffer. The proteins eluted at a flow rate of 9 mlh^{-1} and

fractions (2 ml) were collected (Figure 4.19). The absorbance was monitored at 280 nm and the fractions were tested for activity (section 3.6.2.2). The active fractions were pooled and prepared for SDS-PAGE (section 3.7.1).

3.11.2.3 Third isolation of the extracted ferric reductase

Thermus scotoductus was cultured microaerophilically with KNO_3 as the electron acceptor (section 3.3).

Ammonium sulphate was added to the KCl-extracted protein to a final concentration of 1M and loaded unto Phenyl Toyopearl (5 cm x 2.8 cm) equilibrated with 50 mM acetate buffer (pH 5.5) containing 1 M ammonium sulphate. The non-binding fraction eluted with 50 mM acetate buffer (pH 5.5) containing 1 M ammonium sulphate. The binding fraction eluted using a linear gradient of 1 – 0 M ammonium sulphate at a flow rate of 40 mlh^{-1}. The fractions were tested for activity (section 3.6.2.2) and the absorbance monitored at 280 nm (section 3.6.1) (Figure 4.20). The active fractions were pooled and the ammonium sulphate salt concentration of the pooled fraction was determined by conductivity measurement and application of a standard curve. The ammonium sulphate concentration of the pooled fraction was adjusted to 1M.

The pooled fraction was applied to a second Phenyl Toyopearl column (Figure 4.21) that was run as described for the first column. The fractions containing the bulk of activity were pooled and prepared for SDS-PAGE (section 3.7.1) (Figure 4.22).

3.12 Characterization of ferric reductase

The characterization of the soluble (cytoplasmic) ferric reductase was done on the enzyme obtained after Phenyl Toyopearl (section 3.11.1.4). The characterization of the membrane extracted ferric reductase was done on the enzyme obtained after Phenyl Toyopearl (section 3.11.2.3). The activity was measured with the ferrozine method as described in section 3.6.2.2. All assays were performed on a Spectronic Genesys 5 spectrophotometer.

3.12.1 Optimum pH

A 50 mM buffer “cocktail” was prepared using equimolar amounts of sodium acetate, Bis-Tris propane and glycine. Optimum pH for activity was determined over a pH range of 3-11 by adjusting the pH with NaOH or HCl. Assays were done in triplicate together with a blank rate at each pH. The rest of the assay procedure was the same as described in section 3.6.2.2. The pH with the highest activity was taken as 100% (Figure 4.25).

3.12.2 Optimum temperature

A temperature range of 50-90°C was tested. The assay mixture was equilibrated at the specific temperature. Assays were done in triplicate as described in section 3.6.2.2 with a blank rate at each temperature. The temperature with the highest activity was taken as 100% (Figure 4.26).

3.12.3 Temperature stability

The enzyme was incubated at three temperatures namely 50°C, 70°C and 90°C for different time intervals and aliquots were periodically withdrawn and immediately placed on ice. The total incubation time was 120 min and the samples were assayed for residual ferric reductase activity as described in section 3.6.2.2 (Figure 4.27) and the half-lives of the enzyme were determined for the different temperatures (Table 4.10).

3.12.4 Effect of EDTA

The enzyme was incubated with EDTA (ethylenediaminetetraacetic acid) to final concentrations in the range of 1 - 10 mM for 30 min at 65°C. The residual activity was assayed as described in section 3.6.2.2. The activity of the control (enzyme without EDTA) was taken as 100% (Figure 4.28).

3.12.5 Effect of metals

The enzyme was incubated for 30 min at 65 °C with 1 mM, 5 mM and 10 mM of metal solutions to assess the effect of metals on the ferric reductase activity. The metals tested were the following: $BaSO_4$, $CuCl_2.2H_2O$, $CoCl_2.6H_20$, $CaCl_2$, $Cr(SO_4)_3.K_2SO_4$, $MnSO_4$, $MgCl_2$, $Hg_2(NO_3)_2$, $Pb(NO_3)_2$ and $ZnSO_2$. Enzyme assay were done as outlined in section 3.6.2.2 with a blank rate for each metal. The activity of the control (assay solution without metal) was taken as 100% activity (Table 4.11).

3.12.6 Electron donor specificity

Electron donors were tested for specificity of the enzyme, with the assay described in section 3.6.2.2, which included NADPH, benzyl viologen, bromophenol blue, and neutral red (Gaspard *et al*., 1998). The final concentration of the electron donors in the reaction mixtures was 0.5 mM. The assay solution, containing the electron donor NADH, was taken as 100% activity (Table 4.12).

3.12.7 Electron acceptor specificity

The specificity of the enzyme for Fe(III)-NTA and Fe(III)-EDTA was tested (Dobbin *et al*., 1995). The final concentration of the Fe(III) electron acceptors in the reaction mixtures was 2 mM and the activity was determined (section 3.6.2.2). The activity of the assay solution containing Fe(III)-NTA was taken as 100% (Table 4.13).

3.12.8 Addition of FMN

The effect of 1 mM flavin mononucleotide (FMN) on ferric reductase activity was assessed (Myers and Myers, 1993). The rest of the assay procedure was the same as described in section 3.6.2.2. The activity of the assay solution without FMN was taken as 100%.

3.12.9 Kinetic properties

Different concentrations of Fe-NTA, ranging from 0.4 to 45 mM, were prepared with the concentration of the NADH kept constant. The rest of the assay procedure was the same as described in section 3.6.2.2 (Figure 4.29).

3.12.10 Structural characterization

3.12.10.1 Modification of acidic residues

The enzyme was incubated with 100 mM of the water-soluble carbomiide [1-ethyl-3 (3-dimethylamino-propyl) carbodiimide] that is known for modification of acid residues. The pH of the enzyme solution was adapted to a pH of 4.5. Aliquots were withdrawn periodically and the remaining activity assayed (section 3.6.2.2). The modification is also reversible by raising the pH with the addition of a nucleophile (Lundblad, 1995). The initial activity was taken as 100%.

3.12.10.2 Modification of serine

The effect of PMSF (phenylmethylsulfonyl fluoride) on ferric reductase activity was tested by incubating the enzyme with 50 mM PMSF for 24 hours. The PMSF was prepared in 100% ethanol with the final concentration of the solvent in the reaction mixture being 10%. The control contained enzyme with 10% ethanol without PMSF. The activity of the control was taken as 100%. The remaining activity was assayed as described in section 3.6.2.2.

3.12.11 Effect of urea

The enzyme was incubated in buffer containing 9 M urea for up to 6 h. Wavelength scans were performed to monitor any unfolding of the enzyme over a period of time (Figure 4.30).

The enzyme was also incubated in buffer with urea to final concentrations in the range of 0 – 6 M for up to 5 h at 65 ºC. After incubation, samples were diluted 6 - 8 fold in the buffer and left to stand for 1 h. The assays were done before and after dilution as described in section 3.6.2.2. The initial activity was taken as 100%.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Optimization of growth conditions and ferric reductase activity

Thermus scotoductus was grown microaerophilically or anaerobically in the modified medium containing 10 mM acetate and 10 mM Fe(III) citrate / 10 mM KNO_3 (Figure 4.1) as described in section 3.3. Cell yields for growth on acetate and Fe(III) and acetate and KNO_3 were found to be nearly equal. According to Senko and Stoltz (2001), cell yield prediction based on thermodynamic calculations revealed that acetate and NO_3^- and acetate and Fe(III) are nearly identical in their numbers of moles of cell carbon per mole of substrate carbon and therefore explained the nearly identical cell yields. Growth under microaerophilic conditions was monitored until it entered the stationary phase.

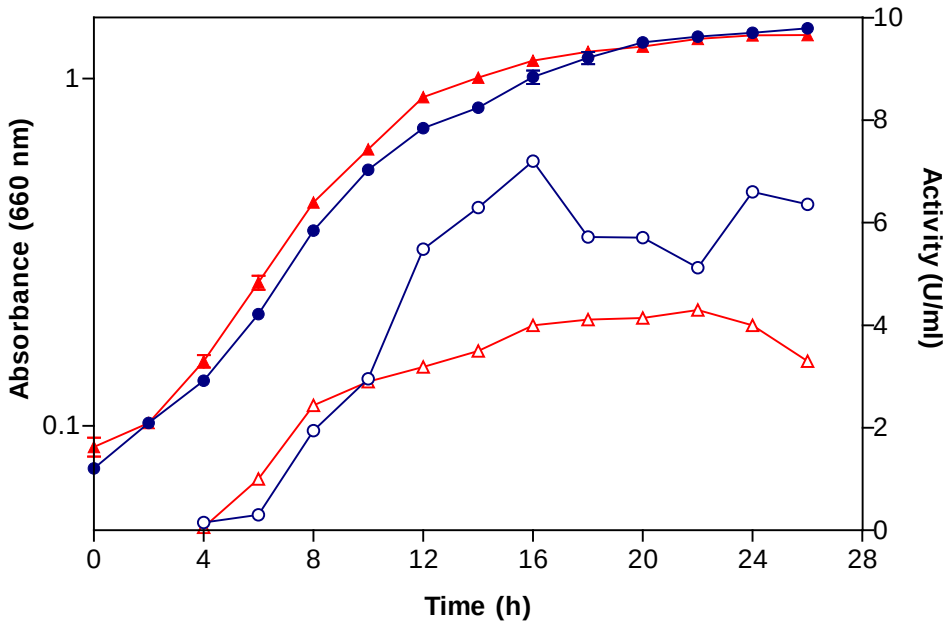


Figure 4.1. *Thermus scotoductus* grown microaerophilically in the modified medium + 10 mM Fe(III) citrate (●) and the modified medium + 10 mM KNO_3 (▲) with correlation to ferric reductase activity of the cells, with (o) representing activity of cells grown with Fe(III) and (Δ) cells grown with KNO_3. Error bars indicate standard deviations.

The ferric reductase activity of the whole cells was determined in vitro by the Fe(III)-NTA assay as described in section 3.6.2.2. The activity was found to be expressed during the exponential growth phase of the microaerophilically grown cells (Figure 4.1). Therefore, cells were harvested as they entered the late exponential phase. They were then placed in a COY anaerobic chamber to induce the anaerobic ferric reductase activity. Cells grown anaerobically with acetate as the electron donor and KNO_3 as the electron acceptor also contained ferric reductase activity. Therefore, it is apparent that the substrate Fe(III) is not required for the expression of ferric reductase activity. The putative dissimilatory ferric reductase of *Geobacter metallireducens* (formerly GS-15) was also expressed in nitrate-grown cells (Gorby and Lovley, 1991), although the Fe(III) grown cells had twice as much ferric reductase activity.

Reduction of Fe(III) citrate under non-growth conditions was assessed as outlined in section 3.4. Fe(III) citrate was found to be reduced with acetate as the electron donor as confirmed by Figure 4.2.

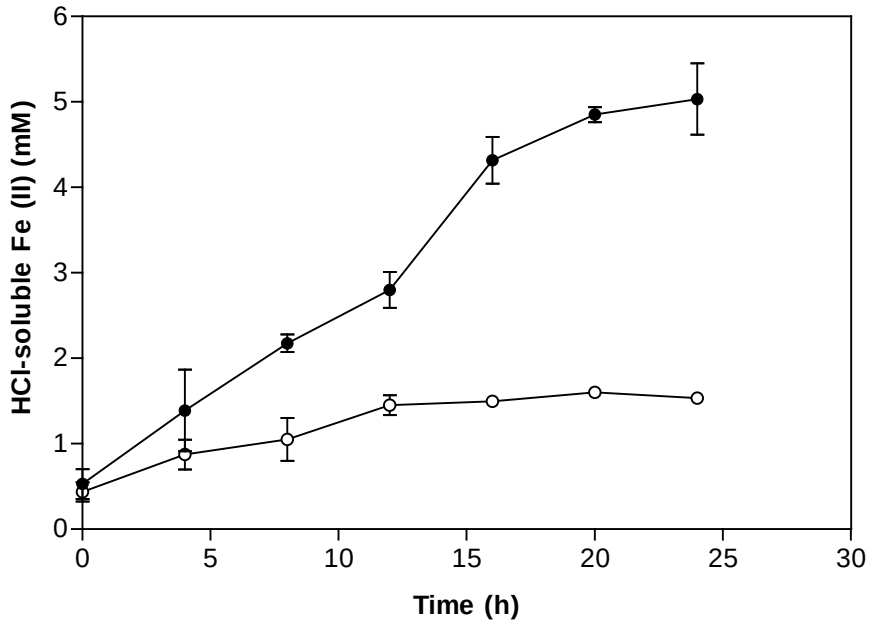


Figure 4.2. Reduction of Fe(III) citrate under non-growth conditions by suspension of cells in buffer containing acetate as the potential electron donor. Filled circles (●) show results for Fe (III) reduction by cells under non-growth conditions; open circles (○) show results for control (no electron donor). Error bars indicate standard deviations.

4.2 Fe(III) and NO_3^- reduction experiments

Fe(II) concentrations in the medium were monitored by the HCl-extractable Fe(II) ferrozine assay as described in section 3.6.2.1, until growth entered the stationary phase. No Fe(II) could be detected under either microaerophilic or anaerobic conditions with glucose serving as the electron donor. Glucose was also found to be a weak electron donor for Fe(III) reduction by Balkwill *et al*. (2004). Acetate served as a good electron donor to *Thermus scotoductus* and this finding was also confirmed by Balkwill *et al*. (2004). When grown microaerophilically with acetate and Fe(III) citrate (section 3.5), there was a rapid increase in the Fe(II) concentration after growth entered the exponential phase (Figure 4.4). When grown anaerobically, a black Fe(II) precipitate was visible at the bottom within 3 to 4 days (Figure 4.3).

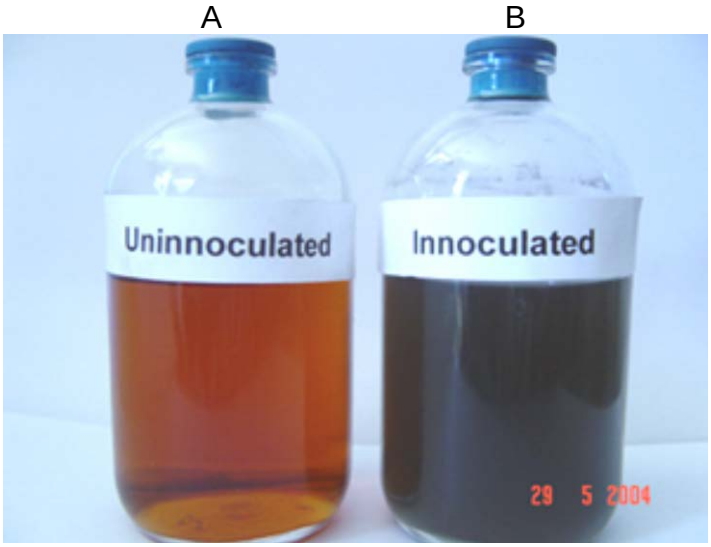


Figure 4.3. Reduction of Fe(III) citrate under anaerobic conditions by suspension of cells after 3 days of incubation at 65°C. A: Control without cells. B: Inoculated with *Thermus scotoductus* cells.

In the presence of KNO_3, *Thermus scotoductus* was unable to reduce Fe(III) under either microaerophilic (Figure 4.4) or anaerobic conditions. 10 mM KNO_3 inhibited Fe(III) citrate reduction by microaerophilically grown cells up to 96%. Previous researchers have also reported NO_3^- inhibition of Fe(III) reduction by *Shewanella putrefaciens* species. DiChristina (1992) reported that 15 mM initial NO_3^- inhibited Fe(III) chloride reduction by 46 to 92% and inhibited Fe(III) citrate reduction by 2 to 22%. Obuekwe and Westlake (1982) reported that the presence of 1 mM NO_3^- resulted in a 50% decrease in the rate of soluble Fe(III) phosphate (2% solution)

reduction by a *Pseudomonas* sp. (later identified as *Shewanella putrefaciens* 200). Recent experiments by Lee *et al*. (2000) with *Shewanella putrefaciens* DK-1 also showed significant inhibition of 10 mM Fe(III) citrate in the presence of 10 mM NO_3^-. The inhibition of Fe(III) reduction by NO_3^- could result from simple kinetic competition between two electron acceptors, a mechanism previously described to explain the inhibition of Fe(III) reduction by oxygen (Arnold *et al*., 1990). Another possibility is that the microbial NO_3^- and Fe(III) reduction produces NO_2^- and Fe(II) respectively, which then abiotically react to reduce NO_2^- to N_2O with the subsequent oxidation of Fe(II) to Fe(III) (Arnold *et al*., 1986; Cooper *et al*., 2003).

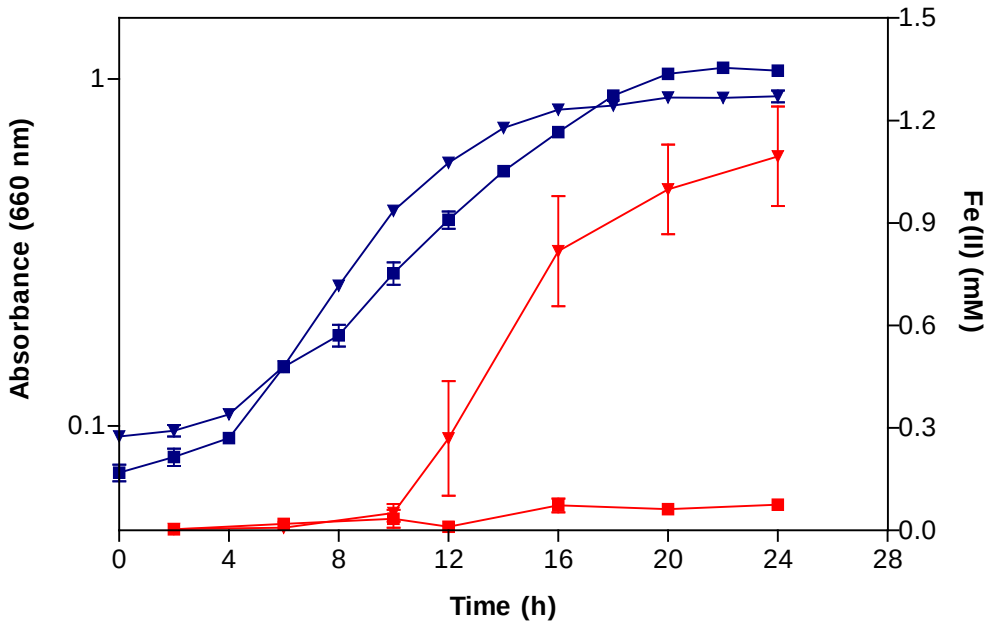


Figure 4.4. *Thermus scotoductus* grown microaerophilically in the modified medium with 10 mM Fe(III) citrate (▼) and the modified medium with 10 mM Fe(III) citrate + 10 mM KNO_3 (▼) with correlation to HCl-soluble Fe(II), with (■) representing Fe(II) formation by cells grown with Fe(III) citrate and (■) Fe(II) formation by cells grown with Fe(III) citrate + KNO_3.

The whole cells cultured in the presence of Fe(III) and KNO_3, were tested in vitro for ferric reductase activity with the Fe(III)-NTA assay as described in section 3.6.2.2. In contrast to the inhibition of Fe(III) reduction by KNO_3 during growth (Figure 4.4), no inhibition was experienced by cells when tested in vitro for ferric reductase activity .

4.3 Fractionation studies

Although most of the fractionation steps were conducted under anoxic conditions, the ferric reductase activity of *Thermus scotoductus* was not irreversibly inhibited by exposure to oxygen. This was also found for *Shewanella putrefaciens* MR-1, since no loss of activity was experienced after all the cell fractionation steps were conducted in the presence of oxygen (Myers and Myers, 1993).

Table 4.1. Localization of ferric reductase activity after preparation of subcellular fractions.

Fraction	Total [Protein] (mg)	Specific Activity (Units/mg)	Total activity (Units)	%
Spheroplasts	150.3	11.5	1729	100
Periplasm	87.6	0.2	17.5	1
After sonic	183.0	11.1	2031.3	118

*Units = mM Fe(III) reduced per minute. Ferric reductase activity with NADH as the electron donor.

Fractionation was done as described in section 3.8 and the results indicated that there was almost no ferric reductase activity in the periplasm (soluble fraction) (Table 4.1). The minor ferric reductase activity recovered in the soluble periplasmic fraction after preparation of spheroplasts is probably due to the release of loosely membrane-associated ferric reductase (Gaspard *et al*., 1998).

Breakage of the cells led to an increase of ferric reductase activity in comparison with unbroken cells, when NADH was used as the electron donor. Similarly, Gaspard *et al.* (1998) also experienced increase in activity for *Geobacter sulfurreducens* after cells were broken and suggested that the NADH gained better access to the NADH dehydrogenase.

Table 4.2. Localization of ferric reductase activity obtained after preparation of the membrane and cytoplasmic fractions.

Cytoplasmic fraction vs. membrane fraction	Total [Protein] (mg)	Specific Activity (Units/mg)	Total activity (Units)	%
Cytoplasmic (soluble)	76.6	2.7	208.6	47.2
Membrane	71.2	3.3	233.0	52.8

*Units = mM Fe(III) reduced per minute. Ferric reductase activity with NADH as the electron donor.

Approximately 47.2% of the total NADH-dependent ferric reductase activity recovered after ultracentrifugation was located in the soluble (cytoplasmic) fraction, whereas 52.8% was associated with the membrane fraction (Table 4.2). The ultrasonic treatment may account for some of the ferric reductase activity present in the soluble (cytoplasmic) fraction. Gaspard *et al*. (1998) reported that some of the membrane-associated ferric reductase activity from *Geobacter sulfurreducens* was retrieved in the soluble (cytoplasmic) fraction after cells was broken.

Distribution of the total NADH-dependent ferric reductase activity recovered in the membrane and soluble fraction of *Geobacter sulfurreducens* were 83% and 17%, respectively (Gaspard *et al*., 1998). With NADPH as the electron donor, 2% of the ferric reductase activity was recovered in the membrane fraction and 92% was associated with soluble fraction of *Geobacter sulfurreducens* (Kaufmann and Lovley, 2001). The major part of the NADPH-dependent ferric reductase activity (86%) of *Pyrobaculum islandicum* was located in the soluble fraction.

The ferric reductase activity present in both the soluble (cytoplasmic) and membrane fraction indicated the existence of two possible distinct enzymes. Recently, both membrane-associated and soluble ferric iron-reducing enzymes were purified from *Geobacter sulfurreducens* (Kaufmann and Lovley, 2001; Magnuson et al., 2000).

4.4 Separation of outer and cytoplasmic membranes

The distribution of the ferric reductase activity in the membrane fraction was evaluated by highly concentrated sucrose gradient separation (Figure 4.5) as described by the method in section 3.9.

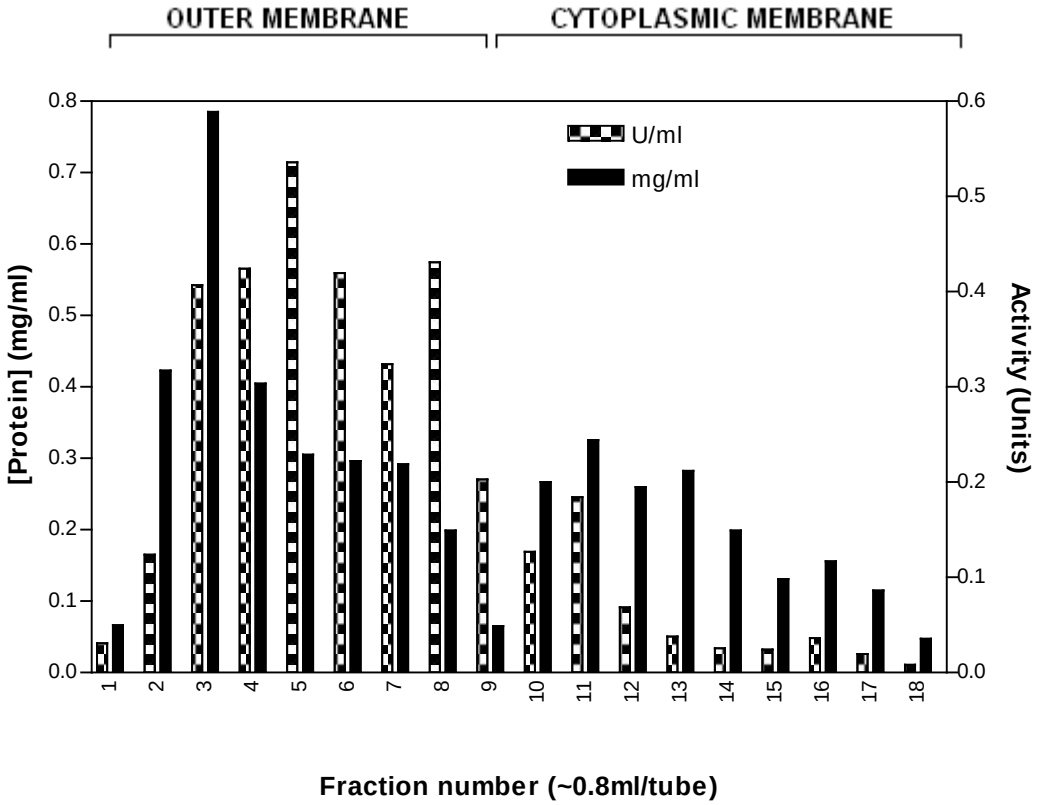


Figure 4.5. Highly concentrated sucrose gradient separation of the outer and cytoplasmic membranes for evaluation of the ferric reductase distribution in the membrane fraction.

To identify the fractions containing the outer membrane, the content of KDO (2-keto-3-deoxyoctonate), a specific constituent of the lipopolysaccharide in the outer membrane was determined as described by the method in section 3.9. Both the cytoplasmic (Fraction 9-18) and the outer membrane (Fraction 1-8) fractions of *Thermus scotoductus,* showed ferric reductase activity, with the major part of the ferric reductase activity (~ 65%) associated with the outer membrane fraction. Sucrose gradient separation of *Geobacter sulfurreducans* by Gaspard *et al*. (1998) indicated that about 80% of the ferric reductase activity was present in the outer membrane although Magnuson *et al*. (2001) reported that only 67.1% of the ferric reductase was associated with the outer membrane. In *Shewanella oneidensis* MR-1, only 54 to 56% of the total ferric reductase activity was localized to the outer

membrane (Myers and Myers, 1993). The ferric reductase activity found in the cytoplasmic membrane fraction could be an artifact due to the ultrasonic treatment since cell breakage by sonication can cause an extensive redistribution of membrane proteins between membranes (Sprott *et al.*, 1994). The finding that the ferric reductase activity is localized to the outer membrane fraction is in contrast to that of other gram-negative bacteria that have been studied, in which components of the respiratory electron transport chains and terminal reductases are mainly located in the cytoplasmic membranes or the periplasmic spaces (Saffarini *et al*., 2002). Although the location in the outer membrane is energetically unfavourable, it is perfectly suitable for utilization of insoluble substrates (Schröder *et al*., 2003).

4.5 Solubilization of the ferric reductase

It was possible to solubilize ferric reductase activity from the membrane fraction and spheroplasts with a high ionic salt buffer as described in section 3.10. These results imply that the ferric reductase may be a peripheral protein on the outside of the outer membrane, since a high salt concentration treatment is a well-known method for detaching peripheral membrane proteins (Ohlendieck, 1996). The soluble fraction obtained from the membrane fraction after treatment with KCl, contained 54.4% ferric reductase activity (Table 4.8).

4.6 Isolation of ferric reductase by chromatographic methods

The membrane-associated NADH-dependent and soluble NADPH-dependent ferric iron-reducing enzymes from *Geobacter sulfurreducens* are the only purified dissimilatory ferric reductases thus far (Kaufmann and Lovely, 2001; Magnuson *et al*., 2000; Lloyd *et al*., 2003). A few cytochromes implicated in Fe(III) reduction have also been purified (Lloyd *et al*., 2003; Magnuson *et al*., 2001; Dobbin *et al*; 1999).

A wide range of fractionation methods and chromatographic media were implemented in the purification of these ferric reductases and cytochromes (Dobbin *et al*., 1999; Childers and Lovley, 2001; Gaspard *et al*., 1998; Kaufmann and Lovley, 2001; Lloyd *et al*., 2003; Magnuson *et al*., 2001; Magnuson *et al*., 2000). After fractionation, further isolation was done by chromatographic methods. Ion exchange was the most commonly used method by the various authors (Dobbin *et al*., 1999;

Kaufmann and Lovley, 2001; Lloyd *et al*., 2003; Magnuson *et al*., 2001; Magnuson *et al*., 2000). Gel filtration and hydrophobic interaction have been used in almost two-thirds of the ferric reductase purification protocols (Dobbin *et al*., 1999; Kaufmann and Lovley, 2001; Lloyd *et al*., 2003; Magnuson *et al*., 2001; Magnuson *et al*., 2000). The use of metal affinity chromatography was also mentioned in the purification of a periplasmic cytochrome from *Geobacter sulfurreducans* (Lloyd *et al*., 2003).

Experiments were done in our laboratory with the anion exchangers, Super-Q Toyopearl and DEAE Toypopearl and the cation exchanger, CM Toyopearl to determine to which resin the enzyme would bind most effectively. The Super-Q Toyopearl and CM Toyopearl were the most promising as no activity interacted with the DEAE Toyopearl.

4.6.1 Isolation of the soluble (cytoplasmic) ferric reductase

4.6.1.1 First isolation of the soluble ferric reductase

Cultivation was carried out as described in section 3.11.1.1. Soluble (cytoplasmic) fraction prepared from cells grown with KNO_3 as the electron acceptor exhibited the same level of ferric reductase activity as did those prepared from cells grown on KNO_3. This might indicate that *Thermus scotoductus* constitutively produces this enzyme. Therefore, the soluble (cytoplasmic) enzyme was purified from cells grown on KNO_3.

Isolation of the soluble (cytoplasmic) ferric reductase was performed as described in section 3.11.1.1. Adsorption of the ferric reductase activity onto Super-Q Toyopearl was the first purification step (Figure 4.6).

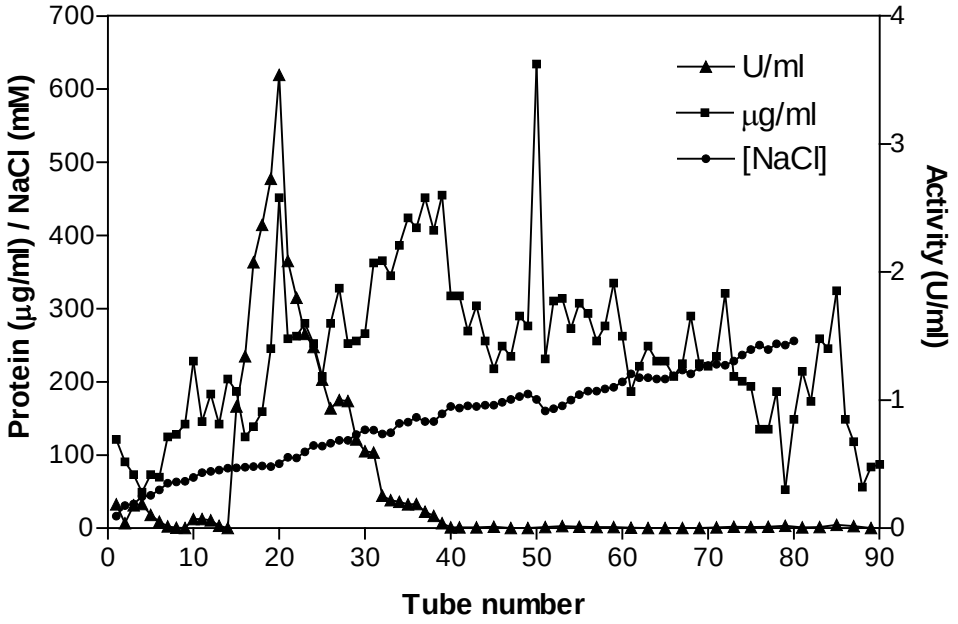


Figure 4.6. Super-Q Toyopearl elution profile of the soluble (cytoplasmic) ferric reductase. Pooled fraction = 15 - 25.

Although this chromatography step seemed to be successful, almost no protein eluted in the non-binding fraction. The active peak still contained a lot of contaminating proteins as indicated by the low specific activity and 0.8-fold purification. SDS-PAGE indicated that the final protein fractions were not homogenous.

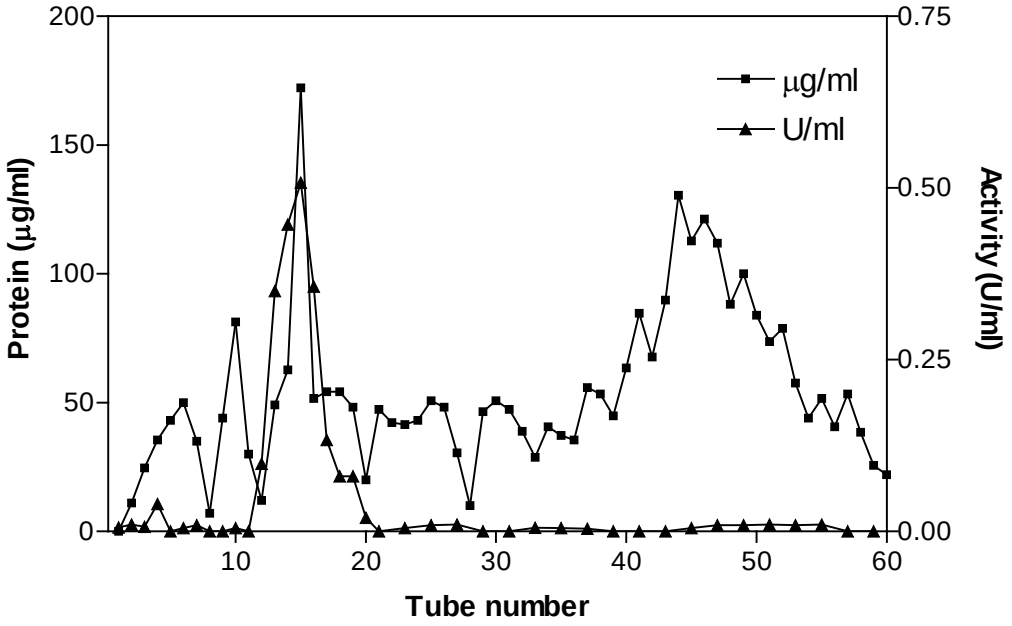


Figure 4.7. Biogel P60 elution profile of the fraction collected from Super-Q Toyopearl. Pooled fraction 1 = 13 – 15. Pooled fraction 2 = 16 – 20.

After concentration of the pooled fraction by PEG 20 000, it was loaded onto a Biogel P60 column (Figure 4.7). The ferric reductase activity was separated from most of the contaminating proteins. One active peak eluted, but was collected as fraction 1 and fraction 2. Fraction 2 (tubes 16 - 20) eluted with a second protein peak and therefore it was pooled separately. SDS-PAGE indicated that the final protein fractions were not homogenous.

Table 4.3 summarizes the purification procedure used for the first isolation of the solouble (cytoplasmic) ferric reductase.

Table 4.3. Purification Table of the soluble (cytoplasmic) ferric reductase: First isolation.

Fraction	**Total activity (Units)**	**Total [Protein] (mg)**	**Specific Activity (Units/mg)**	**Purification Fold**	**%Yield**
Cytoplasmic	81.9	13.2	6.2	1	100
Dialysis	75.5	11.6	5.7	0.9	92
Super Q	23.7	4.8	4.9	0.8	29
PEG 20 000	36.5	4.6	7.9	1.3	44.6
Biogel P60 Fraction 1	5.5	1.5	3.7	0.6	6.7

*Units = mM Fe(III) reduced per minute.

4.6.1.2 Second isolation of soluble ferric reductase

The isolation of the protein was performed as described in section 3.11.1.2. The first attempt made it clear that an ion exchanger with less general binding capacity had to be used. Therefore, it was decided to attempt the adsorption of the enzyme onto the weak cation exchanger CM Toyopearl.

Figure 4.8 shows the elution profile obtained for cytoplasmic fraction after application to CM Toyopearl.

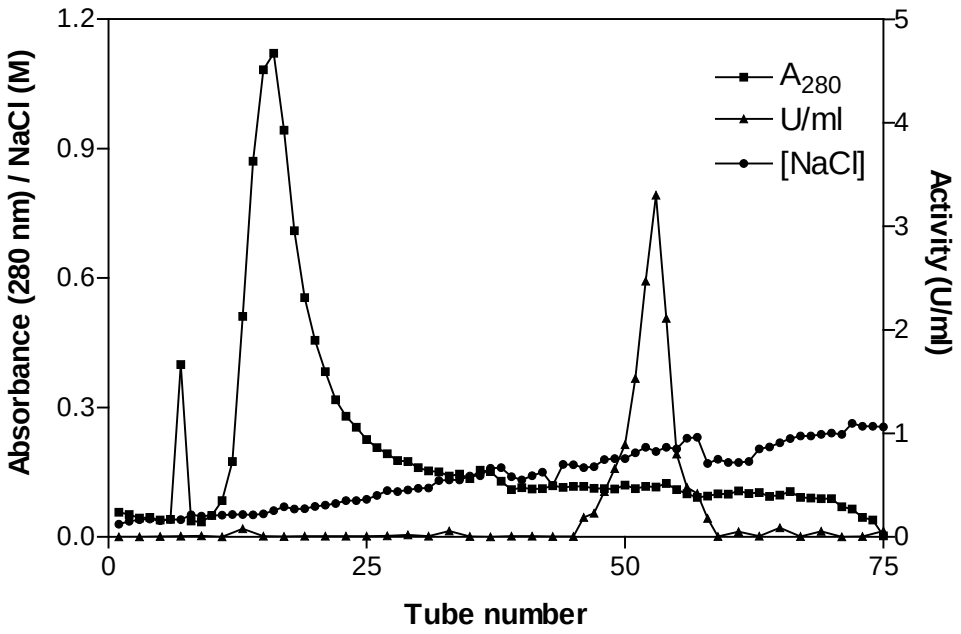


Figure 4.8. CM Toyopearl elution profile of the soluble (cytoplasmic) ferric reductase. Pooled fraction = 47 - 58.

Most proteins were not bound to the column and the contaminating bound proteins eluted at dilute concentrations of NaCl. The ferric reductase activity eluted last (at high salt concentration) and at fractions of low protein content as determined by absorbance at 280 nm. Due to incomplete adsorption of the enzyme to the column, some of the activity was retrieved in the non-binding fraction. However in spite of low yields, the ferric reductase could be removed from the bulk of protein.

The pooled fraction from CM Toyopearl was concentrated using PEG 20 000 and applied to Biogel P60 (Figure 4.9).

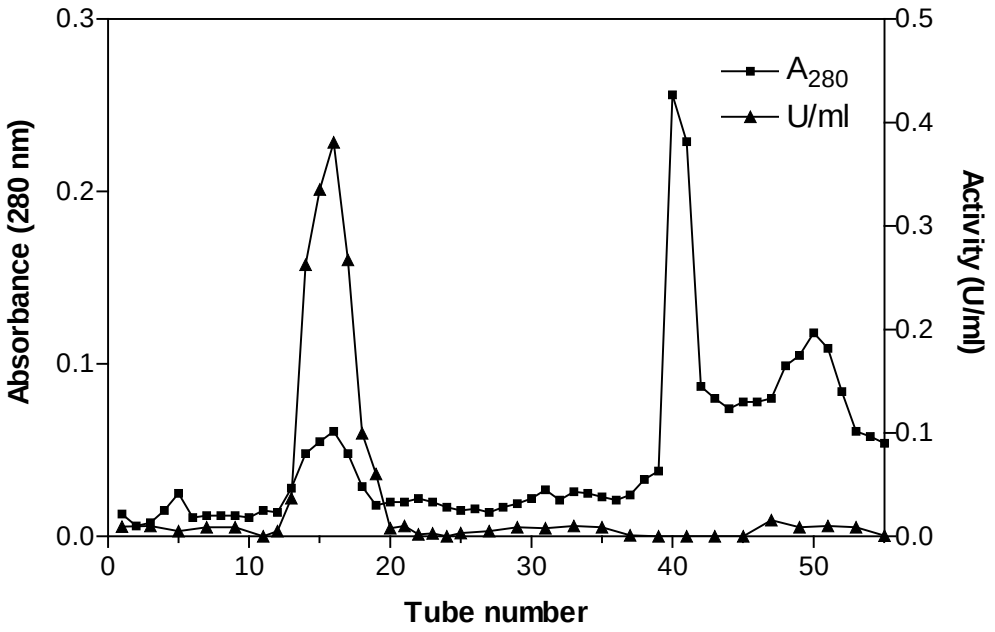


Figure 4.9. Biogel P60 elution profile of the fraction collected from CM Toyopearl. Pooled fraction = 14 – 18.

Although there was a considerable loss of activity, it was possible to separate the ferric reductase activity from the other proteins by gel filtration. SDS-PAGE was performed on the pooled fraction obtained after Biogel P60 and yielded a main protein band with relative molecular mass of 39 000 Da.

Table 4.4 summarizes the results for this is purification attempt.

Table 4.4. Purification Table of the soluble (cytoplasmic) ferric reductase: Second isolation.

Purification step	Total activity (Units)	Total [Protein] (mg)	Specific Activity (Units/mg)	Purification fold	%Yield
Cytoplasmic	341.3	35.7	9.6	1.0	100.0
Dialysis	302.8	32.5	9.3	0.9	88.7
CM	51.9	4.9	10.6	1.1	15.2
PEG 20 000	58.7	4.6	12.8	1.3	17.2
Biogel P60	14.5	1.0	14.5	1.5	4.2

*Units = mM Fe(III) reduced per minute.

4.6.1.3 Third isolation of soluble ferric reductase

The isolation of the protein was performed as described in section 3.11.1.3. The previous isolation attempt resulted in very low yields and therefore it was decided to optimize the conditions for the binding of the enzyme to CM Toyopearl. Through trial and error, it was clear that almost all the activity adsorbed to the CM Toyopearl column at pH 5.3, therefore a pH adaptation had to be made. The soluble (cytoplasmic) fraction was again applied to a CM Toyopearl column (Figure 4.10).

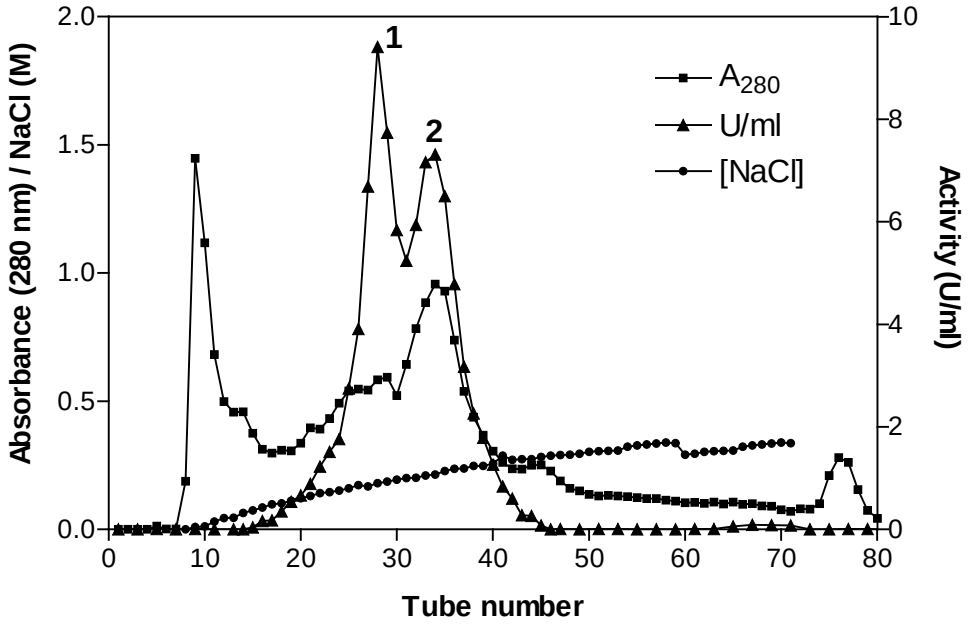


Figure 4.10. CM Toyopearl elution profile of the soluble (cytoplasmic) ferric reductase. Pooled fraction 1 = 25 - 31. Pooled fraction 2 = 32 - 38.

Most proteins were not bound to the column and the contaminating bound proteins eluted at dilute concentrations of NaCl. It is clear from Figure 4.10, that there might be two enzymes present. Therefore, the two active peaks were pooled separately and labelled: Fraction 1 (25-31) and Fraction 2 (32-38). It appeared that some of the loosely membrane associated ferric reductase activity was recovered in the soluble (cytoplasmic) fraction. Fraction 1 had a higher specific activity. Loss of activity was experienced although there was complete adsorption of the activity to the column. Fraction 1 and 2 were subjected to further separation on Phenyl-Toyopearl.

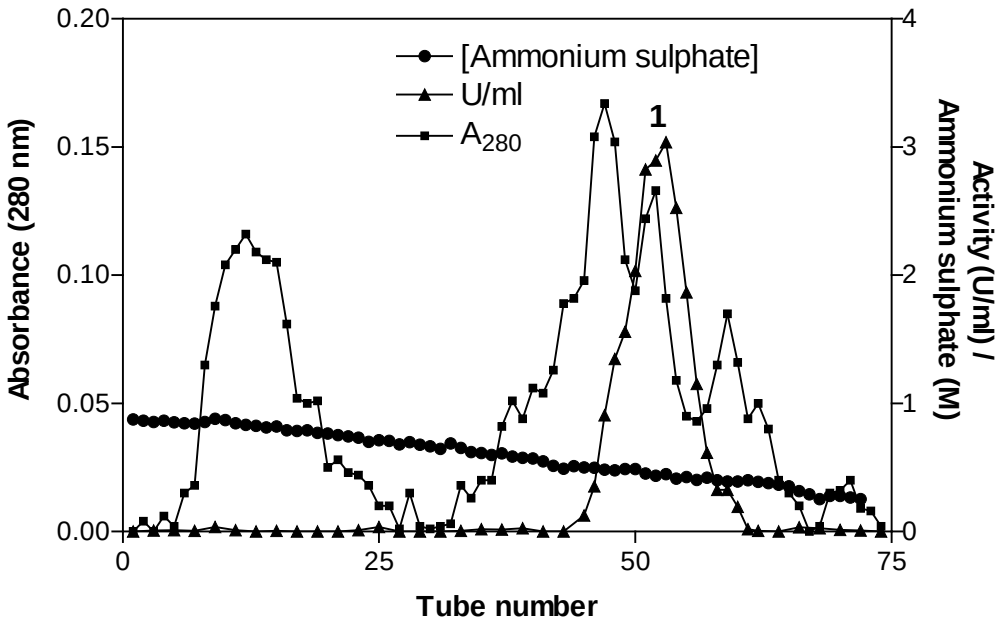


Figure 4.11. Phenyl Toyopearl elution profile of Fraction 1 collected from CM Toyopearl. Pooled fraction = 48 - 54.

From Figure 4.11 it is clear that the ferric reductase activity of Fraction 1 was separated from most of the contaminating protein. A 6.9-fold purification of the main active fraction was obtained for Fraction 1 with a specific activity of 35.1 U/mg and a yield of 14.3%.

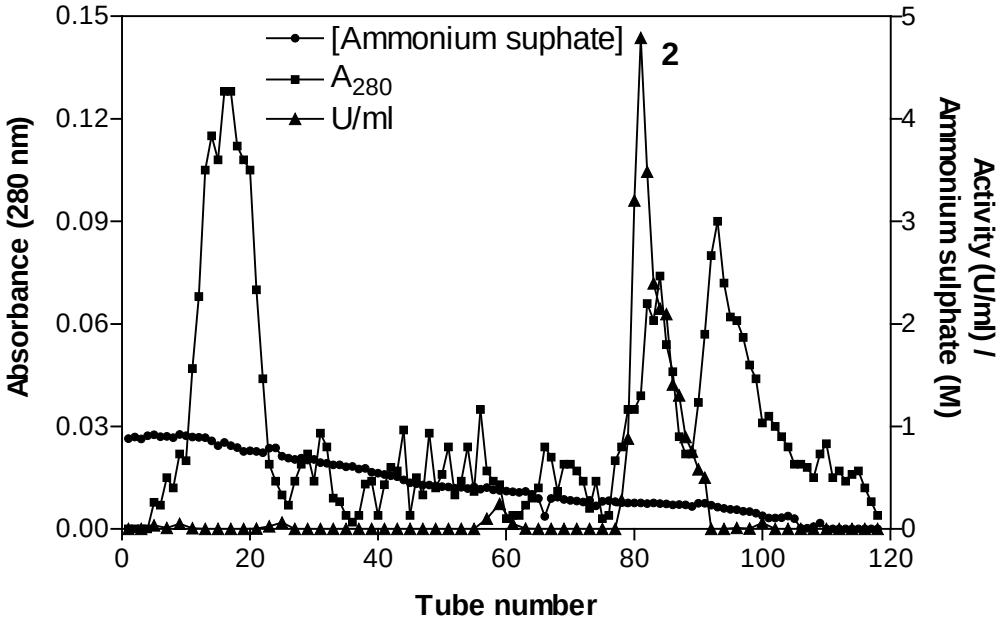


Figure 4.12. Phenyl Toyopearl elution profile of Fraction 1 collected from CM Toyopearl. Pooled fraction = 82-85.

For Fraction 2, a 10.2-fold purification of the main active fraction was obtained with a specific activity of 52 U/mg and a yield of 6.7%. This isolation was relatively successful, based on the good separation obtained for both fractions. Table 4.5 summarizes the purification procedures used for Fraction 1 and Fraction 2 during the third isolation.

Table 4.5. Purification Table of the soluble (cytoplasmic) ferric reductase: Third isolation.

Purification step	Total activity (Units)	Total [Protein] (mg)	Specific Activity (Units/mg)	Purification fold	%Yield
Cytoplasmic	541.6	106.5	5.1	1.0	100.0
CM 1	126.8	10.9	11.6	2.3	23.4
CM 2	92.1	24.1	3.8	0.7	17.0
HIC 1	77.2	2.2	35.1	6.9	14.3
HIC 2	36.4	0.7	52	10.2	6.7

*Units = mM Fe(III) reduced per minute.

SDS-PAGE performed on Fraction 1 and 2 after application on Phenyl Toyopearl. Fraction 2 was purified to apparent homogeneity whereas Fraction 1 still contained some minor contaminants that led to the chromotopgraphy on Biogel P60 in order to try to remove these small contaminants present (Figure 4.13).

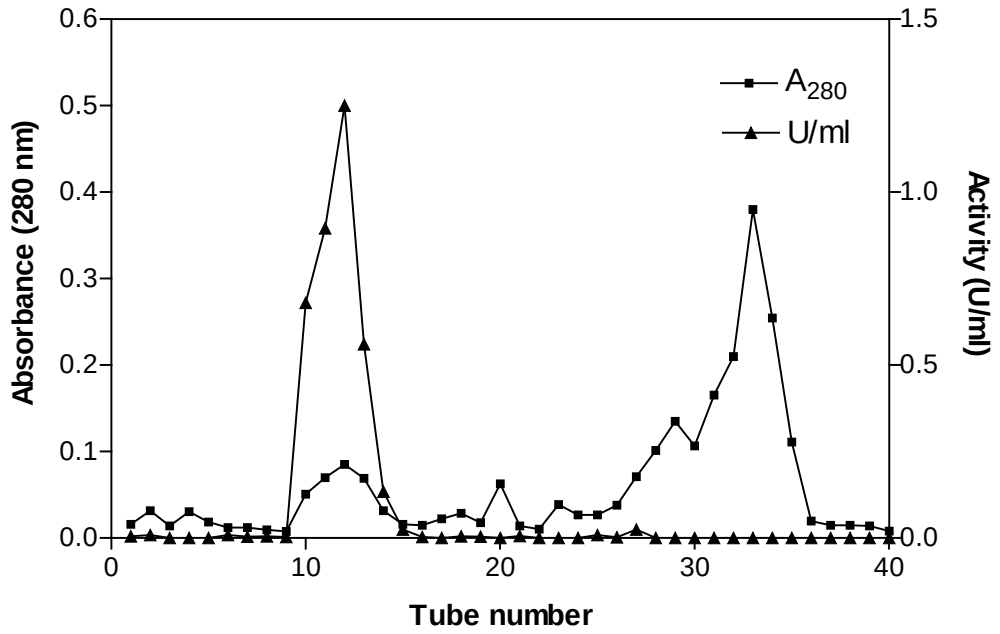


Figure 4.13. Biogel P60 elution profile of Fraction 1 collected from Phenyl Toyopearl. Pooled fraction = 10-13.

SDS-PAGE performed on this fraction after concentration with a Whatman ultrafiltration cell (12 000 Da) yielded the expected protein band with Mr 39 000 (Figure 4.22). Zymogram analysis (Figure 4.23) and Native IEF (Figure 4.24) were performed on Fraction 1 after the Phenyl-Toyopearl step and the pI was determined. 2-D SDS gel (Figure 4.24) was performed on Fraction 1 after application to Biogel P60 that confirmed homogeneity.

4.6.1.4 Fourth isolation of soluble ferric reductase

The isolation of the protein was performed as described in section 3.11.1.4. The previous isolation made it clear that some of the membrane-associated ferric reductase activity was retrieved in the soluble (cytoplasmic) fraction due to the ultrasonic treatment. In order to increase yields for the soluble (cytoplasmic) ferric reductase and to prevent detachment of loosely membrane-associated ferric reductase, milder ultrasonic treatment was used.

The first chromatography step, CM Toyopearl, resulted in a single active peak (Figure 4.14).

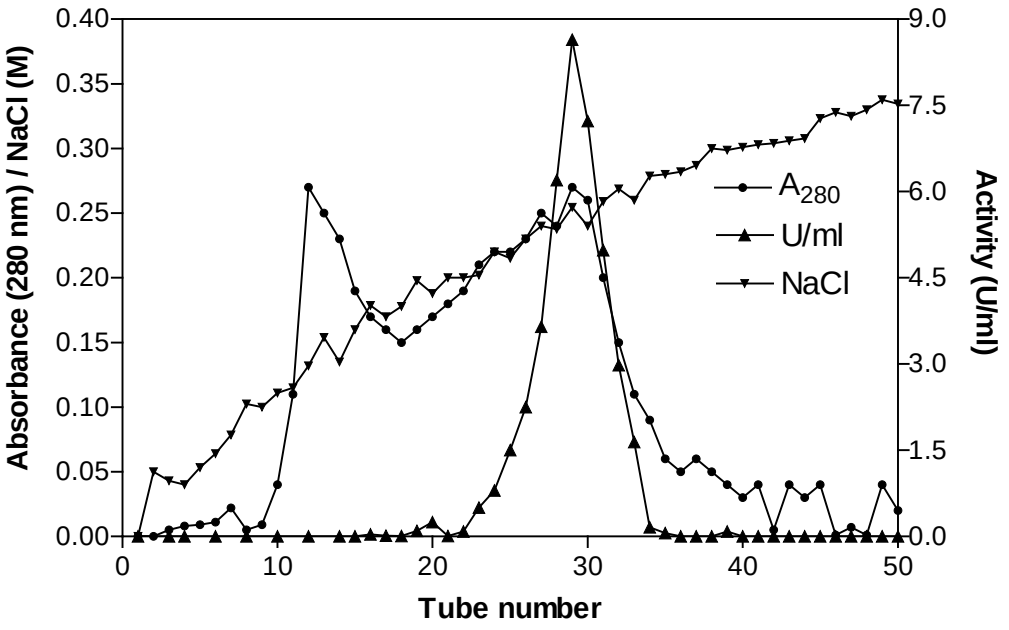


Figure 4.14. CM Toyopearl elution profile of the soluble (cytoplasmic) ferric reductase. Pooled fraction = 25 - 33.

This chromatography step seemed to be successful, as all the activity adsorbs to the column and most of the contaminating proteins were not bound to the column.

The active fractions were pooled together and applied to Phenyl Toyopearl (Figure 4.15).

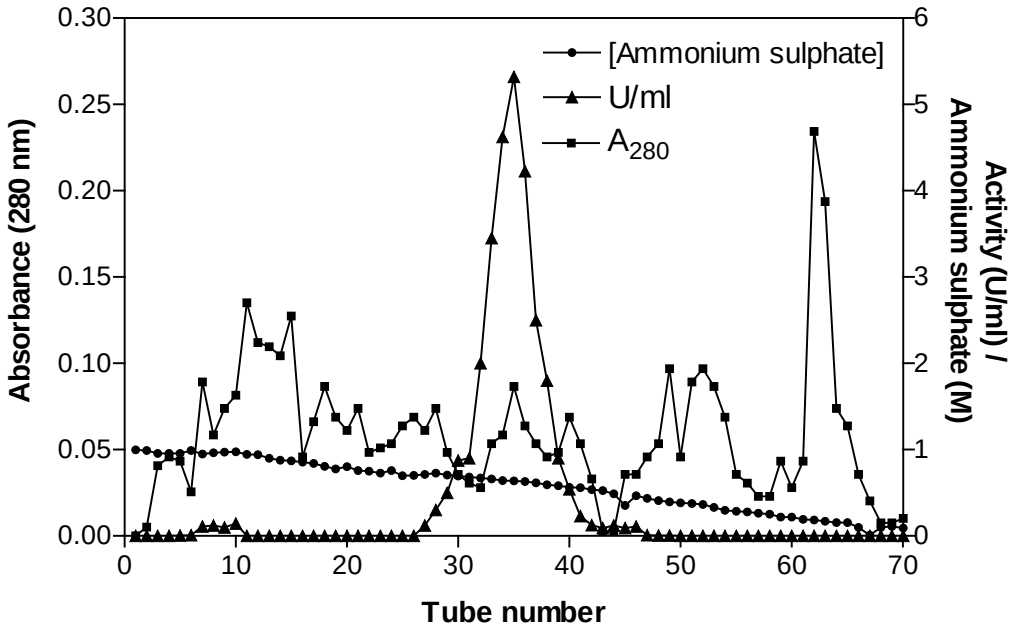


Figure 4.15. Phenyl Toyopearl elution profile of the pooled fraction collected from CM Toyopearl. Pooled fraction = 32 - 38.

The activity eluted in a single peak. SDS-PAGE was performed and yielded a protein band with the expected relative molecular mass. This isolation attempt was successful, based on good separation for both the chromatography steps.

Table 4.6. Purification Table of the soluble (cytoplasmic) ferric reductase: Fourth isolation.

Purification step	Total activity (Units)	Total [Protein] (mg)	Specific Activity (Units/mg)	Purification fold	%Yield
Cytoplasmic	245.9	38.8	6.3	1.0	100.0
CM	85.2	4.4	19.4	3.1	34.6
HIC	55.6	0.9	61.8	9.8	22.6

*Units = mM Fe(III) reduced per minute.

4.6.2 Isolation of the extracted ferric reductase from the membrane

4.6.2.1 First isolation of membrane extracted ferric reductase

Cultivation was performed as described in section 3.11.2.1. The extracted membrane-associated enzyme was purified from cells grown on KNO_3 because it was technically simpler to mass culture the organisms on KNO_3.

The isolation of the protein was performed as described in section 3.11.2.1. It was decided to use Super-Q Toyopearl as a first attempt to purify the extracted ferric reductase from the spheroplasts (Figure 4.16).

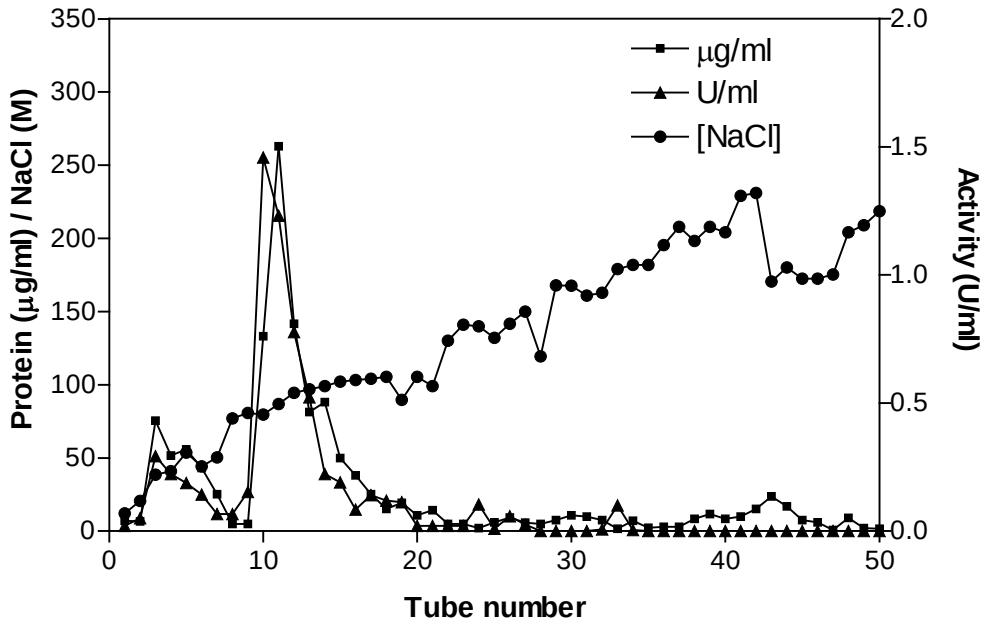


Figure 4.16. Super-Q Toyopearl elution profile of the extracted membrane-associated ferric reductase. Pooled fraction = 10 – 17.

All the activity adsorbed to the column. This chromatography step was unsuccessful since no separation of the protein occurred. A 0.4-fold purification with a 7.1% yield and specific activity of 3.8 U/mg was obtained.

The pooled fraction (tube 10-17) was concentrated and loaded onto a Sephacryl S-100-HR gel filtration column with an exclusion range of 100-100000 Da.

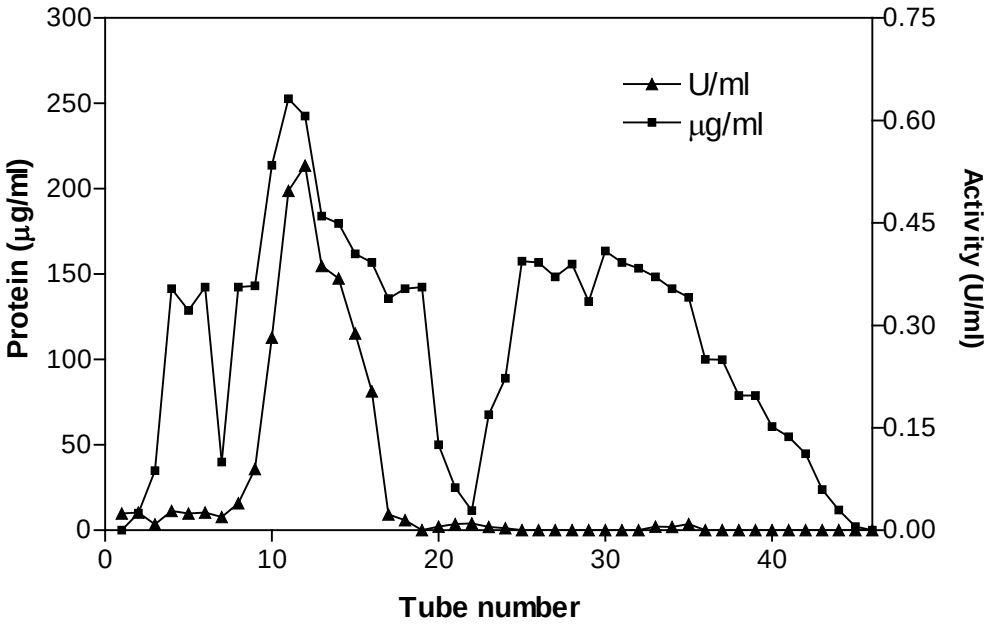


Figure 4.17. Sephacryl S-100-HR elution profile for the fraction collected form Super-Q Toyopearl. Pooled fraction 1 = 10 – 12. Pooled fraction 2 = 13 – 15.

Although this chromatography step seemed to be successful in separating the ferric reductase activity from the bulk of protein, a considerable loss of activity was experienced. A 0.6-fold purification with a 1.4% yield and specific activity of 5.9 U/mg was obtained. Due to low yields and the SDS-PAGE results which indicated the protein was not homogeneous, this isolation attempt was abandoned.

Table 4.7 summarizes the procedure followed for the purification of the extracted ferric reductase from spheroplasts.

Table 4.7. Purification Table of the ferric reductase extracted from spheroplasts: First isolation.

Purification step	Total activity (Units)	Total [Protein] (mg)	Specific Activity (Units/mg)	Purification fold	%Yield
Spheroplast	670.6	66.8	10	1	100
Solubilized	169.2	24.1	7.0	0.7	25.2
Dialysis	144.6	19.7	7.3	0.7	21.6
Super-Q	47.9	12.6	3.8	0.4	7.1
PEG 20000	55.8	10.5	5.3	0.5	8.3
Sephacryl	9.5	1.6	5.9	0.6	1.4

*Units = mM Fe(III) reduced per minute.

4.6.2.2 Second isolation of membrane extracted ferric reductase

The isolation of the protein was performed as described in section 3.11.2.2. The preparation of the subcellular fractions was carried out under the same conditions as described for the first isolation, except that the spheroplasts were fractionated into the membrane and the soluble (cytoplasmic) fraction and the ferric reductase activity was solubilized from the membrane fraction.

The solubilized ferric CM Toyopearl cation-exchange column redcutase activity was applied to (Figure 4.18). A single active peak eluted in the non-binding fraction. No activity eluted in the binding fraction. The CM Toyopearl column appeared to be quite successful despite the fact that the protein eluted in the non-binding fraction, as the vast majority of protein adsorbed to the column.

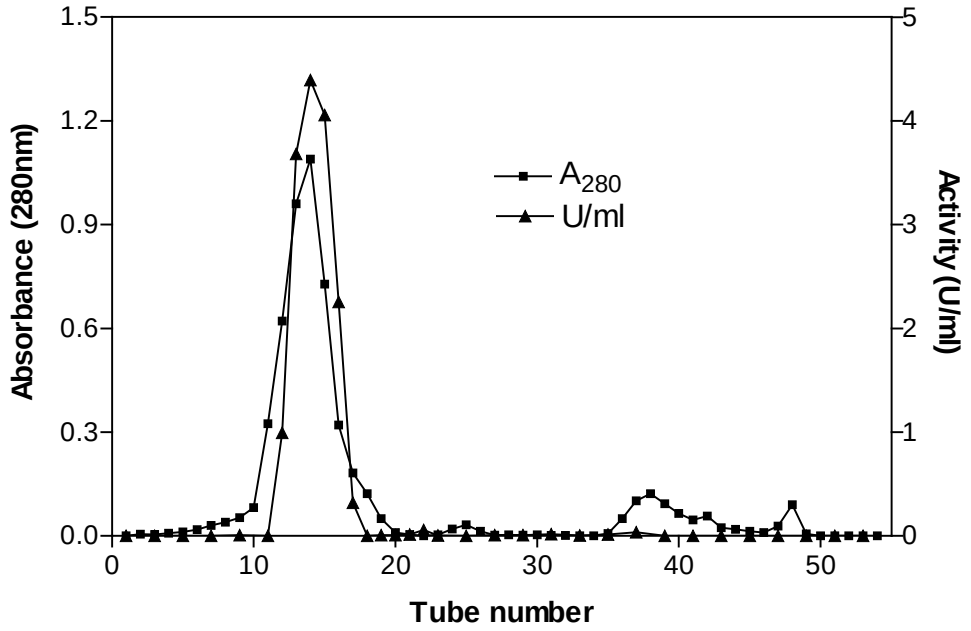


Figure 4.18. CM Toyopearl elution profile of the extracted membrane-associated ferric reductase. Pooled fraction = 12-16.

The protein was pooled (tube 12-16), concentrated using PEG 20 000 and loaded onto Biogel P60 (Figure 4.19).

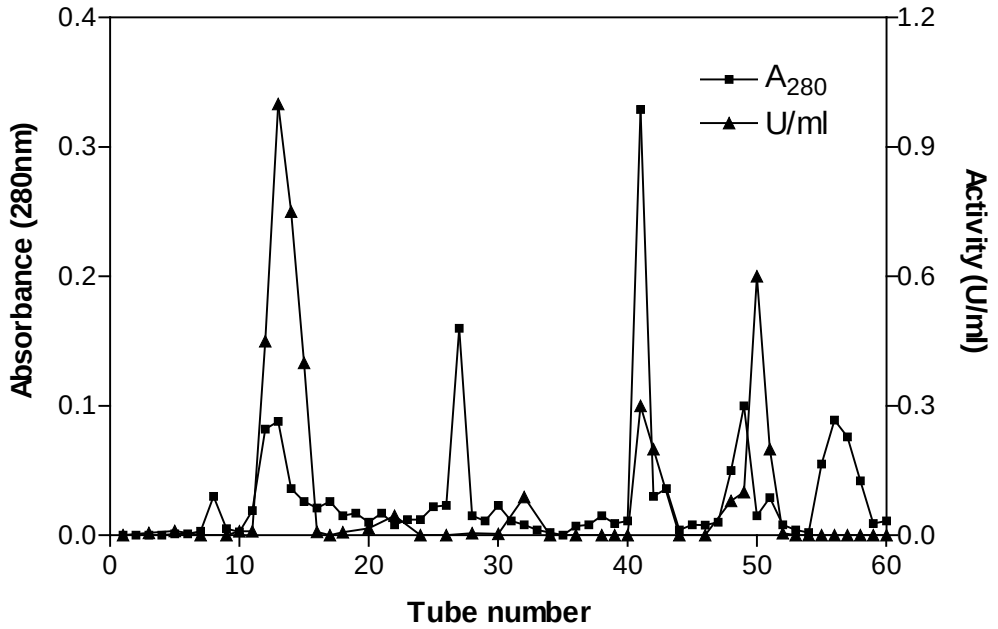


Figure 4.19. Biogel P60 elution profile of the fraction collected from CM Toyopearl. Pooled fraction = 12 – 15.

The Biogel P60 was successful in separating the ferric reductase activity from most contaminating protein although some activity eluted with the void volume. A considerable loss of activity was experienced for this chromatography step. The pooled fraction was analyzed by SDS-PAGE. Unfortunately, there was a second and a third protein present.

Table 4.8 summarizes the procedure followed for the purification of the extracted ferric reductase.

Table 4.8. Purification Table of the membrane-extracted ferric reductase: Second isolation.

Purification step	Total activity (Units)	Total [Protein] (mg)	Specific Activity (Units/mg)	Purification fold	%Yield
Membrane	161.1	33.5	4.8	1.0	100.0
Solubilized	54.4	14.1	3.9	0.8	33.8
Dialysis	41.4	11.7	3.5	0.7	25.7
CM	30.8	8.3	3.7	0.8	19.0
PEG 20 000	35.9	9.2	3.9	0.8	22.3
Biogel P60	7.5	1.6	4.7	1	4.7

*Units = mM Fe(III) reduced per minute.

4.6.2.3 Third isolation of membrane extracted ferric reductase

The isolation of the protein was performed as described in section 3.11.2.3. The solubilized protein of the membrane fraction was applied to Phenyl Toyopearl (Figure 4.20).

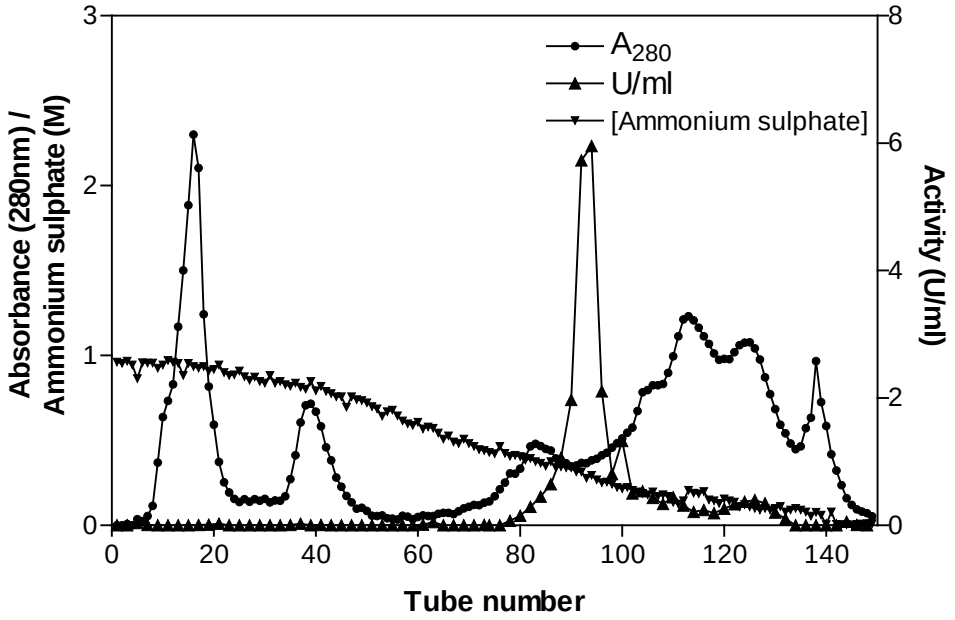


Figure 4.20. First Phenyl Toyopearl elution profile of the extracted membrane-associated ferric reductase from the membrane. Pooled fraction = 86-100.

The Phenyl Toyopearl appeared to be very successful in separating the ferric reductase from the vast majority of proteins. The SDS-PAGE gel performed on the pooled fraction indicated the presence of some contaminants, which led to chromatography on a second Phenyl Toyopearl (Figure 4.21).

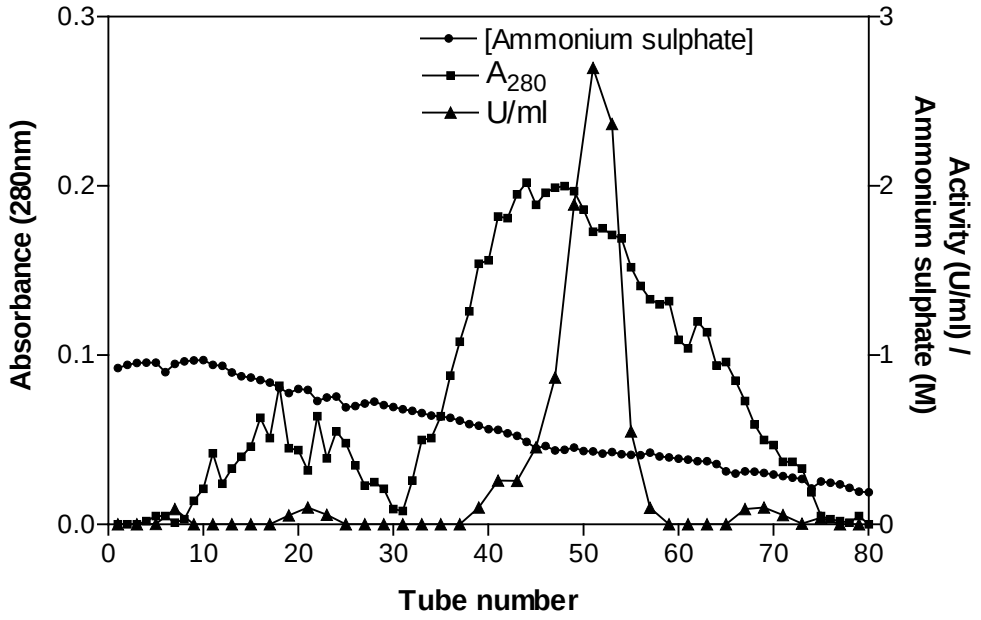


Figure 4.21. Elution profile of fraction collected from first Phenyl Toyopearl. Pooled fraction = 48 - 55.

The second Phenyl-Toyopearl chromatography step also separated the ferric reductase activity from the bulk of protein. SDS-PAGE yielded a main protein band of the expected size (Mr 49 000) (Figure 4.22).

Table 4.9 summarizes the purification procedure for the second isolation of the extracted ferric reductase.

Table 4.9. Purification Table of the membrane-extracted ferric reductase: Third isolation.

Purification step	Total activity (Units)	Total [Protein] (mg)	Specific Activity (Units/mg)	Purification fold	%Yield
Membrane	116.5	28.9	4.0	1.0	100.0
Solubilized	54.4	14.1	3.9	0.9	46.8
HIC 1	38.4	2.9	13.2	3.3	33.0
HIC 2	13.7	0.8	17.1	4.3	11.8

*Units = mM Fe(III) reduced per minute.

4.7 Electrophoretic analysis

4.7.1 SDS-PAGE

SDS-PAGE was performed as described in section 3.7.1. SDS-PAGE was used to monitor the development of the purification process, to determine homogeneity and to determine the relative molecular mass of the ferric reductases. The purified ferric reductase from the cytoplasmic fraction had a relative molecular mass of 39 000 Da (Figure 4.22 A) whereas the membrane-associated ferric reductase had a relative molecular mass of 49 000 Da (Figure 4.22 B) relative to the molecular markers.

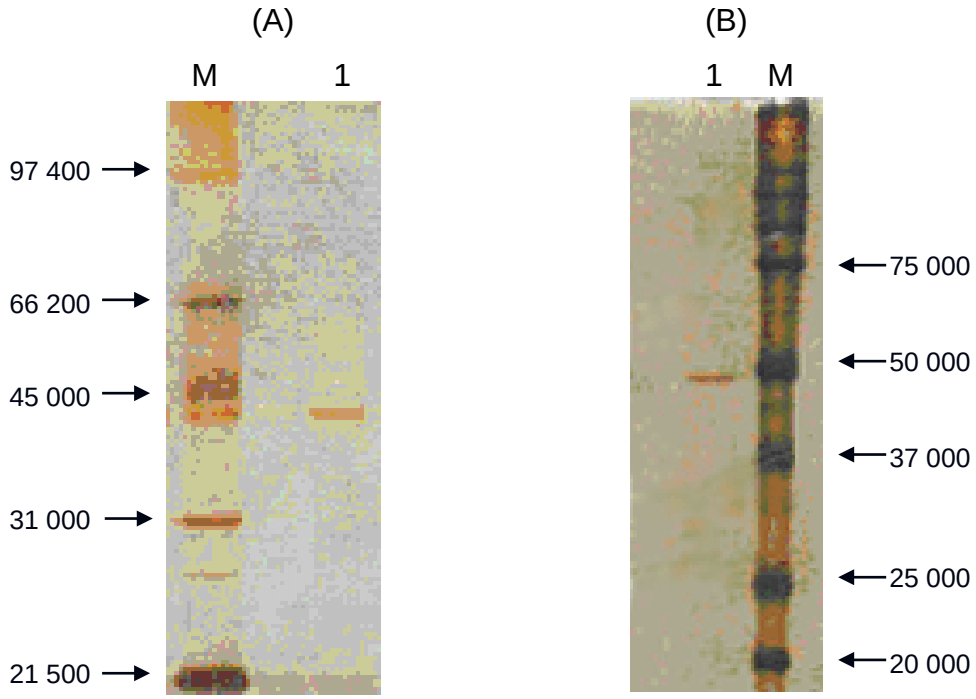


Figure 4.22. SDS-PAGE of (A) Soluble (cytoplasmic) ferric reductase and (B) Membrane-associated ferric reductase. Both gels were silver stained (section 3.7.1). M: Molecular mass marker proteins (section 3.7.1); Lane 1 (A): Biogel P60 eluate (section 4.6.1.3); Lane 1 (B): Phenyl Toyopearl eluate (section 4.6.2.3).

The membrane-associated and the soluble (cytoplasmic) ferric reductases isolated from *Geobcater sulfurreducens* have typical molecular masses in the range of 300 000 Da (Magnuson *et al*., 2000; Kaufmann and Lovley, 2001). Purified *c*-type cytochromes implicated in Fe(III) reduction were reported to have molecular sizes in the range between 9 000 and 150 000 Da. Such *c*-type cytochromes are produced by *Geobacter sulfurreducens*, 9 600 Da (Seeliger *et al*., 1998), *Geobacter sulfurreducens*, 41 000 Da (Luu and Ramsay, 2003), *Shewanella frigidimarina* NCIM

B400, 20 000 Da (Field *et al*., 2000), *Geobacter sulfurreducens*, 90 000 Da (Magnuson *et al*., 2001), and *Shewanella oneidensis* MR-1, 150 000 Da (Lower *et al*., 2001).

4.7.2 Zymogram analysis

Zymogram analysis was done for ferric reductase activity on the native electrophoresis gel as described in section 3.7.2. Both ferric reductases migrated as single bands on the native PAGE and stained for ferric reductase activity (Figure 4.23).

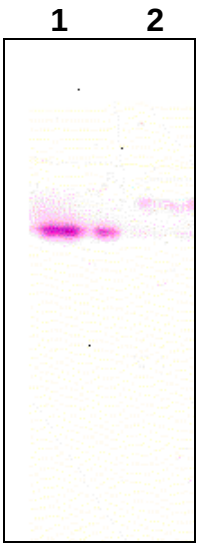


Figure 4.23. Results of zymogram analysis for both ferric reductases: Lane 1: Membrane-associated (section 4.6.2.3); Lane 2: Soluble (cytoplasmic) (section 4.6.1.3).

4.7.3 Isoelectric focusing and 2D PAGE

Isoelectric focusing and 2D PAGE were performed on the purified soluble (cytoplasmic) ferric reductase as described in section 3.7.3 and 3.7.4, respectively. The native IEF gel was stained for ferric reductase activity as described in section 3.7.2. The enzyme fraction showed a single active band on the gel with an acidic Isoelectric point of approximately 5.8 (Figure 4.24 A).

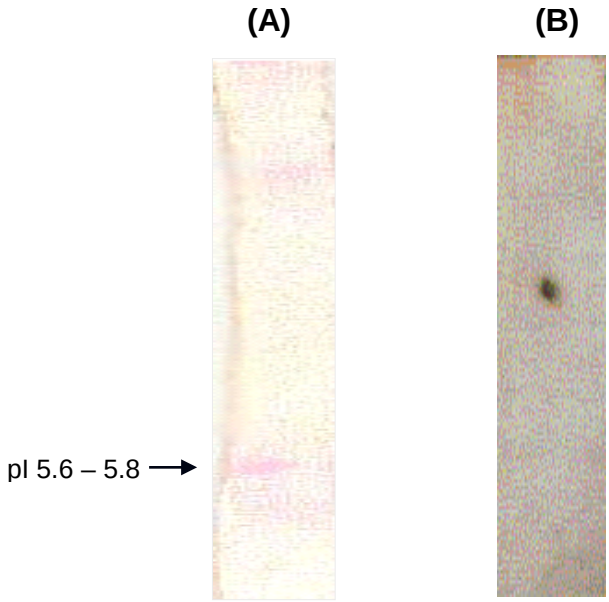


Figure 4.24. Results for purified soluble (cytoplasmic) ferric reductase (A) Native IEF (section 4.6.1.3) and (B) 2D-PAGE (section 4.6.1.3).

The results indicated that the ferric reductase from *Thermus scotoductus* has an acidic pI of approximately 5.8. An acidic pI of 5.2 was reported for the 90 000 Da *c*-type cytochrome (Magnuson *et al*., 2001) and a basic pI of 9.5 for the 9 600 Da *c*-type cytochrome (Lloyd *et al*., 2003) isolated from *Geobacter sulfurreducens*.

A denaturing IEF gel was used to perform a 2-D SDS gel on the purified soluble (cytoplasmic) fraction after application to Biogel P60 (section 4.6.1.3), and from Figure 4.24B it is clear that ferric reductase was purified to homogeneity.

4.8 Characterization of the ferric reductases

The enzyme preparations used for characterization were stored at 4°C throughout all the experiments without significant loss of activity.

4.8.1 Optimum pH

The optimum pH was determined over a pH range of 3-11 as described in section 3.12.1. The pH that showed maximum activity was taken as a 100%. The effect of the pH on both enzymes is depicted in Figure 4.25. The soluble (cytoplasmic) ferric

reductase was found to be optimally active at a pH of 6. The membrane-associated ferric reductase showed preference for acidic conditions, with maximal activity at pH 5. The soluble (cytoplasmic) ferric reductase isolated from *Geobacter sulfurreducens* showed a wide pH range, with maximal activity at 5 (Kaufmann and Lovley, 2001). The membrane-associated ferric reductase from *Geobacter sulfurreducens* showed maximum activity at neutral pH (Magnuson *et al*., 2000).

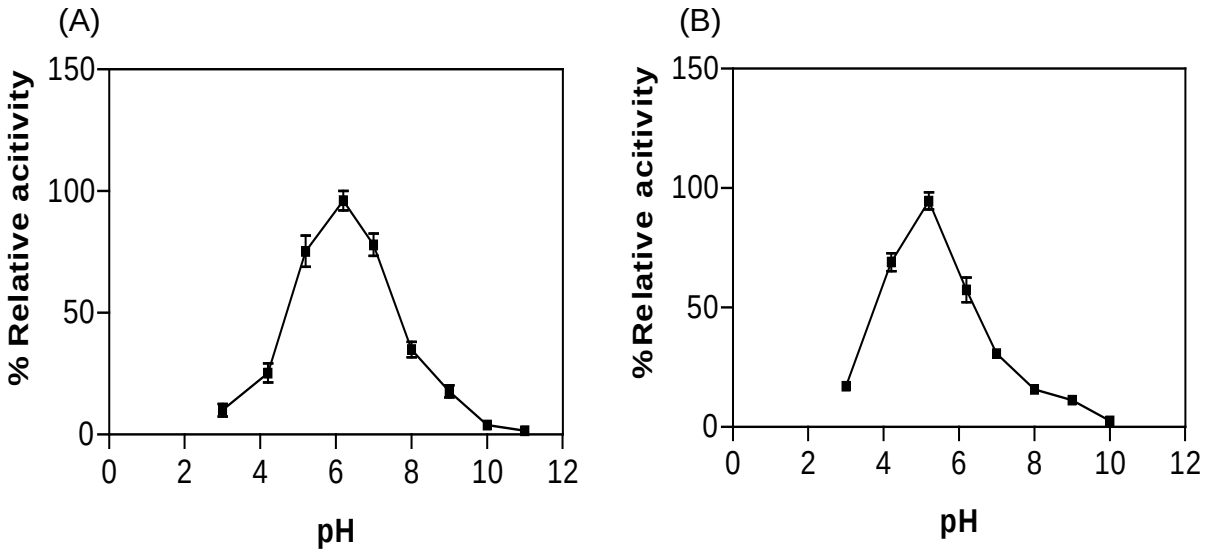


Figure 4.25. Optimum pH of (A) Soluble (cytoplasmic) ferric reductase and (B) Membrane-associated ferric reductase.

4.8.2 Optimum temperature

The optimum temperature was determined as described in section 3.12.2. The effect of temperature on the soluble (cytoplasmic) and membrane-associated ferric reductases is shown in Figure 4.26. The temperature that showed maximum activity was taken as 100%. The soluble (cytoplasmic) ferric reductase activity increased up to 60°C and then declined as the temperature was increased further. The optimum temperature for the membrane-associated ferric reductase was found to be approximately 75°C. Optimum temperatures are significantly affected by the assay used due to chemical reduction at temperatures above 65°C that made the detection and quantification of enzymatic catalysis difficult. Both the membrane-associated and the soluble (cytoplasmic) ferric reductases of *Geobacter sulfurreducens* were found to have temperature optimums at 45°C (Magnuson *et al*., 2000; Kaufmann and Lovley, 2001).

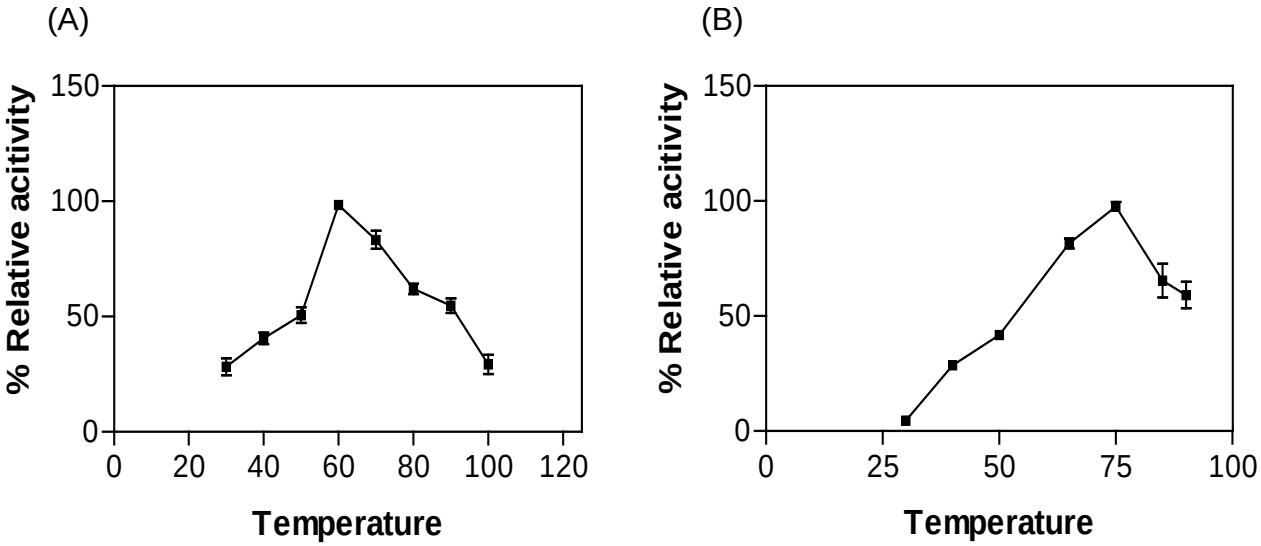
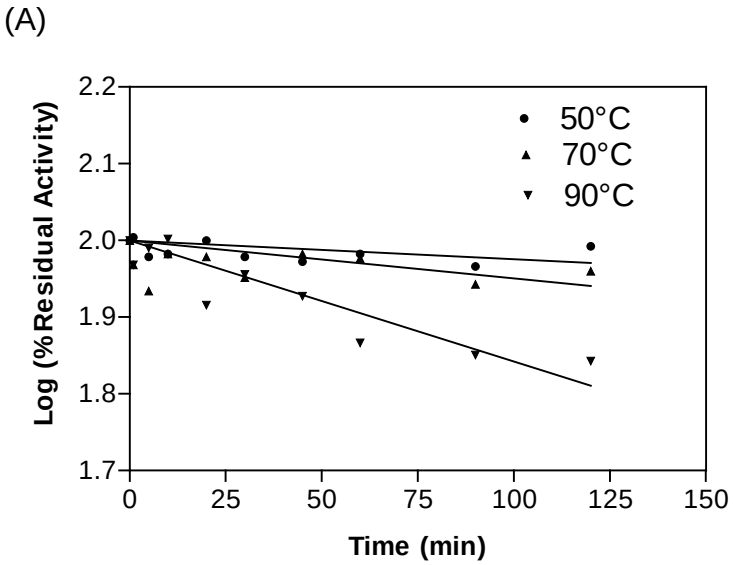


Figure 4.26. Optimum temperature of (A) Soluble (cytoplasmic) ferric reductase and (B) Membrane-associated ferric reductase.

4.8.3 Temperature stability

The thermostability of the ferric reductases was determined at various temperatures as described in section 3.12.3. The residual activity was measured after different periods (Figure 4.27).



(B)

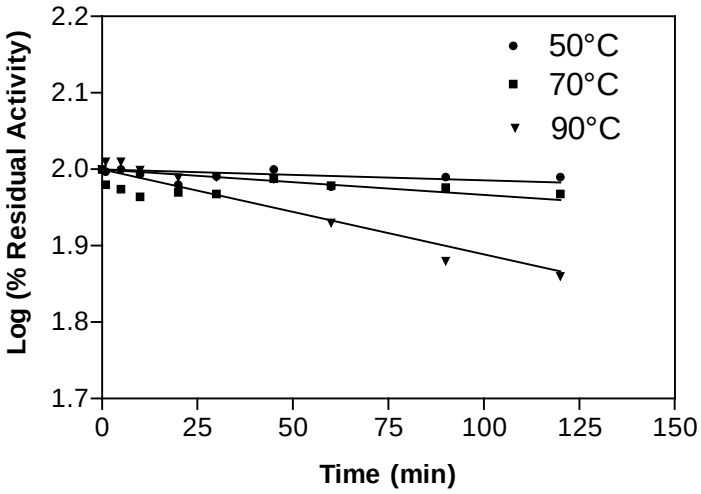


Figure 4.27. Temperature stability of (A) Soluble (cytoplasmic) ferric reductase and (B) Membrane-associated ferric reductase**.**

The half-lives (Table 4.10) calculated at the three temperatures indicate that both ferric reductases are extraordinary stable in comparison with other characterized heat stable enzymes. The ability to function at 90 °C, with an astonishing half-life of approximately 81 h at 70 °C for the membrane-associated ferric reductase, makes this enzyme novel.

Table 4.10. Half-lives of (A) Soluble (cytoplasmic) ferric reductase and (B) Membrane-associated ferric reductase.

(A)

Temperature (°C)	**Half-life (h)**
50	16
70	14
90	4

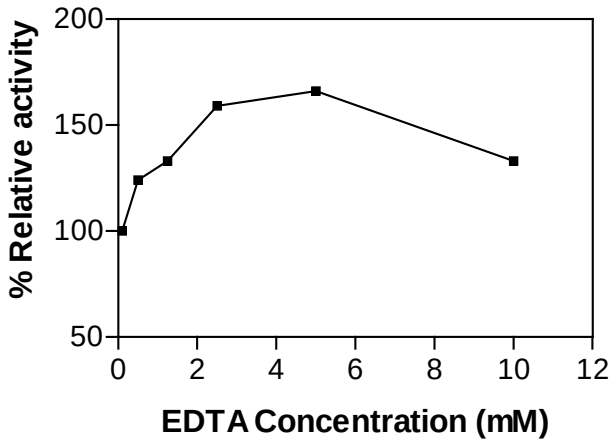
(B)

Temperature (°C)	**Half-life (h)**
50	62
70	81
90	4

4.8.4 Effect of EDTA

Some enzymes are dependent on metals for their activity, hence they are referred to as metalloenzymes. The dependence of enzymes on metals is often investigated by addition of metal chelating agents to the enzyme assay mixture as described in section 3.12.4. The effect of EDTA on the ferric reductases is depicted in Figure (4.28). Incubation of the soluble (cytoplasmic) ferric reductase with the chelating agent EDTA showed a slight inhibitory effect on the activity at low concentrations. The membrane–associated and soluble (cytoplasmic) ferric reductases were stimulated upon incubation with 0.5 mM and 3 mM EDTA, respectively. This might be the result of the inhibitory metals chelated by EDTA. Higher concentrations of EDTA slightly inactivated both ferric reductases. The inhibition of activity may be due to the EDTA that compete with the chelating agent ferrozine to form a Fe(II) complex with the reduced Fe(II)-NTA.

(A)



(B)

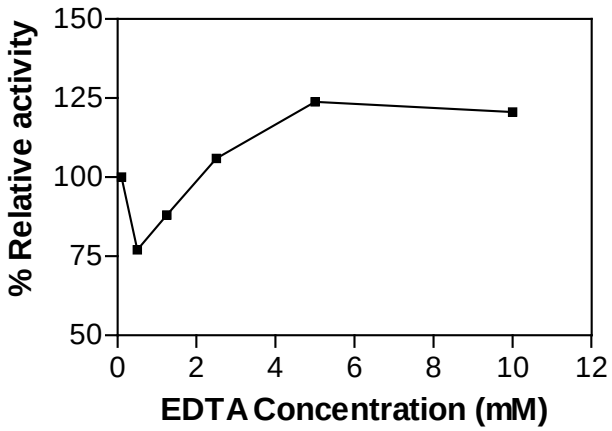


Figure 4.28. The effect of EDTA on the (A) Membrane-associated ferric reductase and (B) Soluble (cytoplasmic) ferric reductase.

4.8.5 Effect of metals

To date, this is the first study concerning the effect of metal salts on dissimilatory ferric reductase activity (section 3.12.5). From literature it was apparent that gentle breakage of thioether linkages present in *c*-type cytochromes could be achieved by using silver or mercury salts (Barret and Kamen, 1961). The effects of selected metals on enzyme activity were determined for the soluble (cytoplasmic) and the membrane-associated ferric reductases (Table 4.11) at metal concentrations of 1 mM, 5 mM and 10 mM. Activities are relative to the activity of the enzyme assayed without a metal ion. Strong inhibitors for the soluble (cytoplasmic) ferric reductase were the heavy metals AgCl, $BaSO_4$ and $CuCl_2.2H_2O$. For the soluble (cytoplasmic) ferric reductase, a maximum concentration of 5 mM AgCl could be used, due to the fact that higher concentrations led to the precipitation of protein. AgCl, $Pb(NO_3)_2$ and $Hg_2(NO_3)_2$ had significant inhibitory effects on the membrane-associated ferric reductase. Results obtained indicated that $MgCl_2$, $MnSO_4$ and $CaCl_2$ had the least effect on the soluble (cytoplasmic) ferric reductase. $MgCl_2$ and $CaCl_2$ had a slight activation effect on the membrane-associated ferric reductase. The results indicated that neither enzyme is significantly affected by metal ions. Therefore it can generally be concluded that these enzymes are not dependent on metals for ferric reductase activity. The negative effect of metals could be due to metal ions that compete with the Fe(II)-NTA to form reduced complexes with the chelating agent ferrozine.

Table 4.11. The effect of selected metals on the ferric reductase activity of (A) Soluble ferric reductase and (B) membrane-associated ferric reductase.

(A)

Metal	% Relative activity		
	1 mM	5 mM	10 mM
AgCl	33.5	15.6	-
$BaSO_4$	79.4	54.0	48.1
$CuCl_2.2H_2O$	91.8	64.3	59.8
$CoCl_2.6H_20$	87.8	79.7	74.5
$CaCl_2$	101.3	100.0	92.5
$Cr(SO_4)_3.K_2SO_4$	82.0	87.9	79.2
$MnSO_4$	99.9	95.3	89.2
$MgCl_2$	99.3	98.3	89.0
$Hg_2(NO_3)_2$	78.6	71.5	66.9

(B)

Metal	% Relative activity		
	1 mM	5 mM	10 mM
AgCl	34.3	30.3	25.5
$BaSO_4$	89.7	85.0	70.1
$CuCl_2.2H_2O$	91.8	78.7	69.3
$CoCl_2.6H_20$	87.8	74.7	62.5
$CaCl_2$	116.1	105.0	88.3
$Cr(SO_4)_3.K_2SO_4$	87.0	81.3	82.2
$MnSO_4$	104.8	101.0	79.1
$MgCl_2$	98.3	94.3	89.5
$Hg_2(NO_3)_2$	78.6	68.6	44.9
$Pb(NO_3)_2$	65.7	51.7	39.4
$ZnSO_4$	86.9	70.1	61.8
$FeCl_3$	111.0	101.0	95.5

4.8.6 Electron donors

In initial attempts to develop an in vitro assay for ferric reductase activity, the use of NADH and NADPH was tested as described in section 3.12.6. The reduction potentials of the NAD^+ / NADH or $NADP^+$ / NADPH couple is -0.32 V, which place it fairly high on the electron tower; that is, NADH and NADPH are good electron donors (Madigan *et al*., 2003).

Bromophenol blue was found to support nitrate reduction at a rate 5 times greater than NADH with freshly prepared enzyme (Cambell, 1986). However, most nitrate reductases will also accept electrons from other artificial potential donors such as flavins and viologen dyes (Cambell and Smarrelli, 1986; Hewitt and Notton, 1980). It was decided to test these artificial electrons with relative low reduction potentials in the vicinity of NADH / NADPH (Table 4.12) to see whether they could act as potential donors for ferric reductase activity.

From the results obtained it was clear that the artificial electron donors could not be used as they all rapidly reduced Fe(III)-NTA in the absence of cell fractions making the detection and quantification of enzymatic catalysis very difficult. In spite of high chemical reduction, slight activity was observed for the viologen dyes. These results may imply that the electrons bypassed the NADH site of the enzyme and entered the system downstream from FAD, which is involved in accepting electrons from pyridine nucleotides (Cambell and Smarrelli, 1986; Hewitt and Notton, 1980). Gaspard *et al*. (1998) also tested artificial electron donors to see whether they could act as possible electron donors for *Geobacter sulfurreducens* but due to the high chemical reduction, they abandoned it.

Both NADH and NADPH served as electron donors. NADH was preferred over NADPH as an electron donor for the membrane-associated ferric reductase, whereas NADPH was the preferred substrate for the soluble (cytoplasmic) ferric reductase, of *Thermus scotoductus* (Table 4.12). A ferric reductase associated with the membranes of anaerobically grown *Shewanella putrefaciens* MR-1 was found to be NADH-dependent (Myers and Myers, 1993). A preference for NADH as the electron donor was found for the membrane-associated ferric reductase of *Geobacter sulfurreducens* (Gaspard *et al*., 1998), whereas the soluble ferric reductase had a strict specificity for NADPH (Kaufmann and Lovley, 2001). The soluble ferric

reductase of *Pyrobaculum islandicum* also preferred NADPH as the electron donor for the reduction of Fe(III) (Childers and Lovley, 2001). *Archaeoglobus fulgidus* was shown to use both NADH and NADPH (Vadas *et al*., 1999).

Table 4.12. Electron donors tested for the (A) Membrane-associated ferric reductase and (B) Soluble (cytoplasmic) ferric reductase.

(A)

Electron donor	Concentration (mM)	Chemical reduction	% Relative ferric reductase activity *
NADH	0.5	Yes	100.0
NADPH	0.5	Yes	64.6
Benzyl viologen	0.5	Yes	12.5
Neutral red	0.5	Yes	ND**
Bromophenol blue	0.5	Yes	ND**
Methyl viologen	0.5	Yes	5.4

* Ferric reductase activity with NADH as electron donor taken 100%

* *Not determined

(B)

Electron donor	Concentration (mM)	Chemical reduction	% Relative ferric reductase activity *
NADPH	0.5	Yes	128.5
NADH	0.5	Yes	100.0
Benzyl viologen	0.5	Yes	6.0
Neutral red	0.5	Yes	ND**
Bromophenol blue	0.5	Yes	ND**
Methyl viologen	0.5	Yes	11.5

* Ferric reductase activity with NADH as electron donor taken 100%

* *Not determined

4.8.7 Electron acceptors

The specificity of the enzymes for the substrates, Fe-NTA and Fe-EDTA was tested as described in section 3.12.7 (Table 4.13). The Fe(III) complexed with NTA and EDTA, respectively, showed that the reduction of Fe(III)-NTA occurred faster than the reduction of Fe(III)-EDTA for both enzymes. The Fe(II) species formed with EDTA may be kinetically less labile than those formed with NTA due to steric effects (Dobbin *et al*., 1995). No ferric reductase activity could be measured with Fe(III)-

EDTA for *Geobacter sulfurreducens*, and Gaspard *et al*. (1998) suggested that it could be attributed to the inability of the ferrozine to remove Fe(II) from the Fe(II)-EDTA complex. A similar finding had been described for *Shewanella putrefaciens* MR-1 by Dobbin *et al*. (1995). *Archaeoglobus fulgidus* contained high Fe(III)-EDTA activity in its soluble fraction (Vadas *et al*. 1999) when the activity was measured in the absence of ferrozine monitoring the oxidation of NADH at 340 nm. These results gave even more impetus to the assumption made by Gaspard *et al*. (1998).

Table 4.13. Electron acceptors tested for the (A) Membrane-associated ferric reductase and (B) Soluble (cytoplasmic) ferric reductase.

(A)

Electron acceptor	Concentration (mM)	% Relative ferric reductase activity*
Fe-NTA	2	100.0
Fe-EDTA	2	35.6

* Ferric reductase activity with Fe-NTA as electron donor taken 100%

(B)

Electron acceptor	Concentration (mM)	% Relative ferric reductase activity*
Fe-NTA	2	100.0
Fe-EDTA	2	49.5

* Ferric reductase activity with Fe-NTA as electron donor taken 100%

4.8.8 FMN

The effect of FMN on ferric reductase activity was evaluated as described in section 3.12.8. In contrast to previously described soluble ferric reductases, the addition of FMN did not stimulate the *Thermus scotoductus* ferric reductase activity of the soluble (cytoplasmic) ferric reductase. Addition of FMN also had no stimulatory effect on the membrane-associated ferric reductase from *Thermus scotoductus*. The soluble ferric reductase activity of *Geobacter sulfurreducans* was also not enhanced by the addition of FMN (Kaufmann and Lovley, 2001) although the ferric reductase activity in the soluble fractions of *Archaeoglobus fulgidus* (Vadas *et al*. 1999) and *Pyrobaculum islandicum* (Childers and Lovley, 2001) were markedly stimulated. The NADH-dependent ferric reductase activity associated with the membrane fractions of *Shewanella putrefaciens* MR-1 (Myers and Myers, 1993) and *Geobacter*

metallireducens (formerly GS-15) (Gorby and Lovley, 1991) was also stimulated by FMN.

4.8.9 Kinetic properties

Kinetic parameters were investigated in *Thermus scotoductus.* The double reciprocal plots showed linearity in both cases, consistent with an enzymatic reaction (section 3.12.9) (Figure 4.29). The Km (affinity/specificity) values for Fe(III)-NTA was 2.6 mM (V_{max} 7.6 mM/min) and 6.1 mM (V_{max} 7 mM/min), for the membrane-associated and soluble (cytoplasmic) ferric reductase activity, respectively. The Km of the soluble (cytoplasmic) ferric reductase is relatively high as compared to other ferric reductases (Table 4.14).

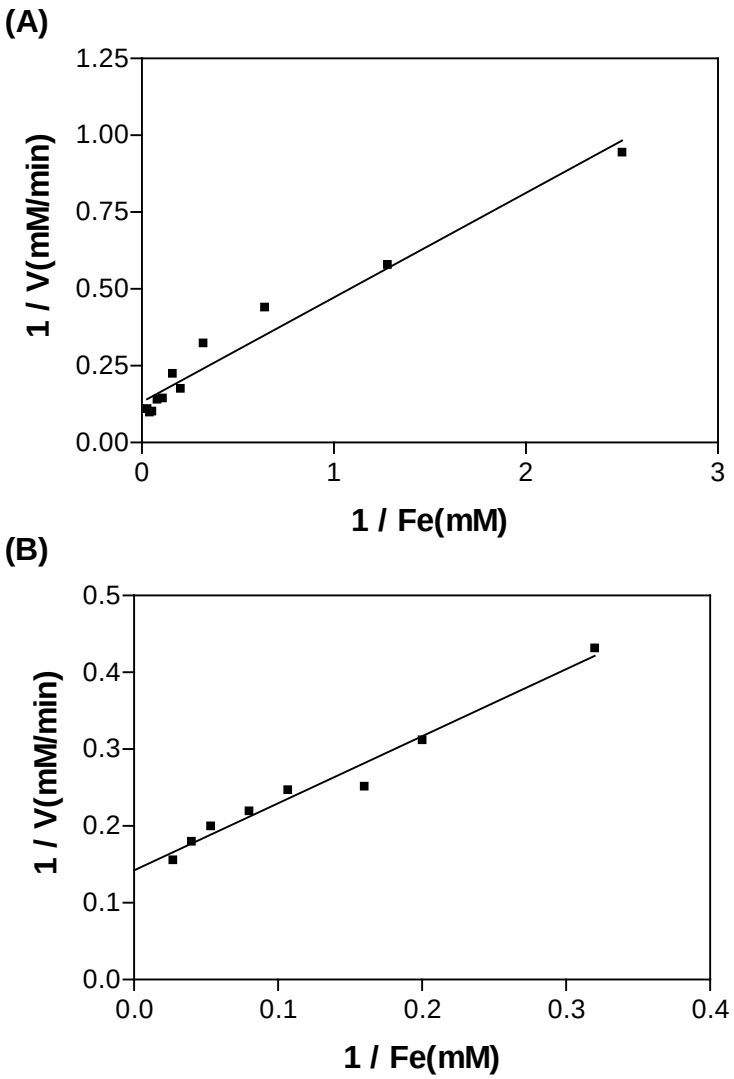


Figure 4.29. Double reciprocal plots for both ferric reductases with a constant NADH of 0.2 mM: (A) Membrane-associated ferric reductase and (B) Soluble (cytoplasmic) ferric reductase.

Table 4.14 summarizes the Km values obtained for various Fe(III) reducing microorganisms.

Table 4.14. Km v alues for ferric reductases for different Fe(III) reducing microorganisms.

Microorganism	K_m
Geobacter sulfurreducens (soluble)	1 mM
Geobacter sulfurreducens (membrane)	0.590 mM
Shewanella putrefaciens CN32 (membrane)	29 mM
Pyrobaculum islandicum (soluble)	0.37 mM

4.8.10 Structural characterization

4.8.10.1 PMSF

PMSF is a classical serine hydrolase inhibitor, and its effect on ferric reductase activity was evaluated as described in section 3.12.10.1. 50 mM PMSF resulted in a 20% and 10% decrease in soluble (cytoplasmic) and membrane-associated ferric reductase activities, respectively after incubation for 24 h. These results imply that Ser is not part of the catalytic centre for ferric reductase activity, although resistance to PMSF has been observed for enzymes known to contain Ser in their active catalytic centre (Brady *et al*., 1990).

4.8.10.2 Effect of carbodiimide

Water-soluble carbodiimide (1-ethyl-3 (3-dimethylamino-propyl) carbodiimide) are known for modification of acid residues in protein. Both enzymes were incubated with water-soluble carbodiimide as describe in section 3.12.10.2 to evaluate the involvement of a carboxylic residue in the catalytic action of the ferric reductases.

The initial activity was taken as 100%, and after addition of carbodiimide the activity was measured.

The reagent successfully modified both enzymes until no activity could be detected. After 10 min only 48% activity of the soluble (cytoplasmic)

ferric reductase remained and after 140 min no activity could be detected. The pH was restored but only 25% activity was detected after 30 min.

After 20 min only 36% of the membrane-associated ferric reductase activity remained, and no activity could be detected after 120 min. After the pH was restored, 18% activity was detected after 30 min.

Although both enzymes were inactivated by the addition of the carbodiimide, the inhibition did not occur as rapidly as experienced for enzymes that are known to contain acid residues.

4.8.11 Effect of Urea

For this study urea was used as the denaturing agent that destroys the normal water hydrogen bond structure that drives hydrophobic interaction. In folded native proteins, some of the aromatic residues are buried within the hydrophobic core of the molecule. They become exposed to the aqueous solvent during unfolding (Encinas *et al*., 1998). Both ferric reductases from *Thermus scotoductus* were incubated with different urea concentrations and activity measurements were done after 5 h (section 3.12.11). The soluble (cytoplasmic) protein lost 80% of its activity whereas the residual activity of the membrane-associated ferric reductase was less than 10% at a concentration of 6M.

The enzyme activity could be completely recovered upon 5-fold dilution of samples denatured in urea concentrations up to almost 3 M for the membrane-associated ferric reductase and 4 M for the soluble (cytoplasmic) ferric reductase.

Both enzymes were also incubated in 9 M urea and wavelength scans were performed to monitor any unfolding of the enzyme over period of time as described in section 3.12.11 (Figure 4.30). A decrease in absorbance is observed, which is significantly illustrated by the difference in absorbance at 280 nm due to the exposure of the aromatic side chains. The aromatic side chains become exposed to the aqueous solvent giving rise to absorbance in the 285 – 295 nm regions. The peak observed at 250 nm for the soluble (cytoplasmic) ferric reductase indicates the

presence of disulphide bonds (Creighton, 1995). From the results it is clear that the membrane-associated ferric reductase unfolds more readily than the soluble (cytoplasmic) ferric reductase.

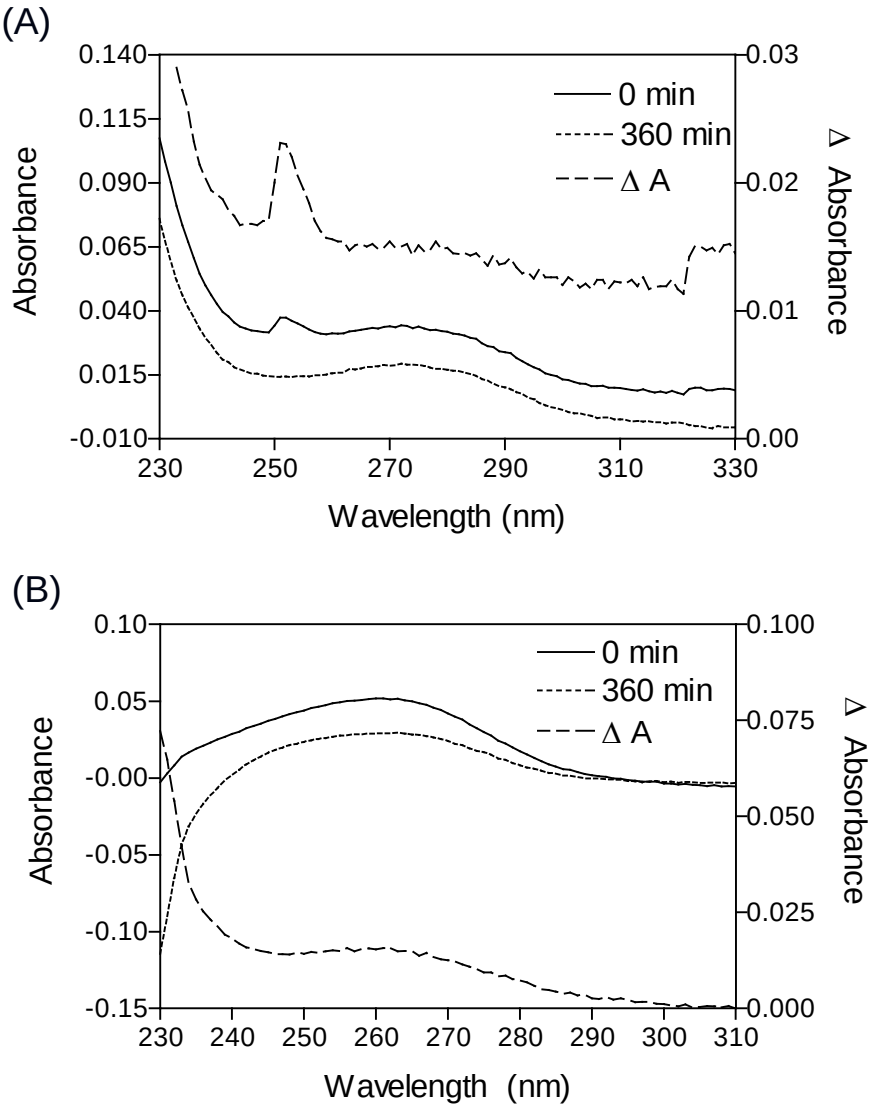


Figure 4.30. Wavelengths scan of *Thermus scotoductus* ferric reductases with 9 M urea to monitor time-dependent unfolding: (A) Soluble (cytoplasmic) ferric reductase and (B) Membrane-associated ferric reductase.

CHAPTER 5

General discussion and conclusion

The thermophilic bacterium, Thermus scotoductus, was isolated by Tom Kieft and co-workers from groundwater sampled at a 3.2 km depth in a South African gold mine. This was the first report of a Thermus sp. being able to couple the oxidation of organic compounds to the reduction of Fe(III), Mn(IV), S° and other metals (Kieft et al., 1999).

This study expands the known diversity of enzymes that may be involved in dissimilatory Fe(III) reduction The soluble and membrane-associated ferric reductases from *Thermus scotoductus* represent the first description of ferric reductases isolated from a thermophilic microorganism that conserves energy to support growth from Fe(III) reduction. In this study the ferric reductases displayed some characteristics consistent with these of other ferric reductases.

Both the soluble and membrane-associated ferric reductases were purified from *Thermus scotoductus* by chromatographic methods. Ion exchange and hydrophobic interaction chromatography were used to purify the soluble ferric reductase to homogeneity with a yield of 22.6%. Some of the characteristics differ significantly from its mesophilic counterparts, for instance its stability at high temperatures. The soluble ferric reductase showed maximal activity at pH 6.

The membrane-associated ferric reductase was purified by hydrophobic interaction chromatography to apparent homogeneity with a yield of about 11.8%. Activity over a wide range of temperatures and the ability to function at 90ºC, with an astonishing half-life of approximately 81 h at 70ºC makes this enzyme novel. The membrane-associated ferric reductase showed maximum activity at pH 5.

The characterization of both thermophilic ferric reductases isolated from *Thermus scotoductus* is an important contribution to our existing knowledge concerning the dissimilatory ferric reductases. However, further investigations into the process of Fe(III) reduction in *Thermus scotoductus* and other Fe(III) reducing microorganisms are warranted to better understand the biochemical diversity of Fe(III) reduction. Both ferric reductases displayed unique characteristics making these enzymes potentially suitable for application in bioremediation.

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SUMMARY

A thermophilic bacterium, *Thermus scotoductus*, was previously isolated form hot, alkaline groundwater sampled in a South African gold mine. *Thermus scotoductus,* was grown microaerophically / anaerobically with Fe(III) citrate or KNO_3 serving as the final electron acceptor and acetate as the potential electron donor.

Subcellular fractionation indicated that the ferric reductase activity was located in both the soluble (cytoplasmic) and the membrane fraction. The major part of the activity present in the membrane fraction was found to be associated with the outer membrane. Both the soluble and membrane-associated ferric reductases were purified.

Purification of the soluble ferric reductase included ion exchange and hydrophobic interaction chromatography resulting in a 9.8-fold purification of the main active fraction and a specific activity of 61.8 U/mg with a final yield of 22.6%. The protein was purified to homogeneity as indicated by SDS-PAGE and 2 D-PAGE with a relative molecular mass of 39 000. Isoelectric focusing was performed on the purified protein that displayed a pI of 5.6 - 5.8. The purified enzyme was analyzed for ferric reductase activity by a zymogram.

The KCl-extracted ferric reductase activity from the membrane fraction was isolated by using hydrophobic interaction chromatography. The final purification protocol resulted in a 4.3-fold purification of the main active fraction and a specific activity of 17.1 U/mg with a final yield of 11.8%. A main protein band with relative molecular mass of 49 000 was detected on SDS-PAGE. The purified enzyme was analyzed for ferric reductase activity by a zymogram.

The soluble ferric reductase exhibited an optimum temperature of 60 – 65 ºC and an optimum pH of 6. The enzyme was extremely stable yielding half-lives of 16 h at 50 ºC, 14 h at 70 ºC and 4 h at 90 ºC. The heavy metal AgCl significantly inhibited the ferric

reductase activity. The Km value for Fe(III)-NTA was estimated to be approximately 6.1 mM.

The membrane-associated ferric reductase appeared to by extremely thermostable, yielding an astonishing half-live of 81 h at 70 ºC. The half-lives for 50 ºC and 90 ºC were estimated to be 62 h and 4 h, respectively. The enzyme exhibited an optimum temperature of 70 – 75 ºC and an optimum pH of 5. With NADH as the electron donor, the Km value for Fe(III)-NTA was estimated to be approximately 2.6 mM. Of the 13 metals tested, only AgCl, $Hg_2(NO_3)_2$ and $Pb(NO_3)_2$ had a significant inhibitory effect on the activity.

Both ferric reductases were stimulated upon incubation with EDTA although higher concentrations of EDTA slightly inactivated the membrane-associated ferric reductase. PMSF had only slight effect on both ferric reductases. Water-soluble carbodiimide resulted in the inactivation of the soluble and membrane-associated ferric reductase after 125 min and 100 min, respectively. Both ferric reductases were affected by high concentrations of urea up to 6 M; minor enzyme activity was restored upon 6-fold dilution.

The membrane-associated and soluble ferric reductases differ favourably from their mesophilic counterparts in some characteristics.

The aim of this study, namely the isolation and characterization of the thermophilic ferric reductases from *Thermus scotoductus* and to compare it with that of hyperthermophilic and mesophilic species, was successfully completed.

OPSOMMING

Die termofiliese bakterie, *Thermus scotoductus*, is geïsoleer vanuit warm, alkaliese grondwater afkomstig vanaf ‘n Suid-Afrikaanse goudmyn. *Thermus scotoductus,* is onder anaerobiese / mikroaerofiliese kondisies gekultiveer met Fe(III) sitraat of KNO_3 as ‘n finale elektron akseptor en asetaat as die potensiële elektron donor.

Subsellulêre fraksionering het getoon dat die Fe(III) reduktase aktiwiteit teenwoordig was in die sitoplasmiese sowel as die membraan fraksie. Die grootste gedeelte van die Fe(III) reduktase aktiwiteit geassosieer met die membraan fraksie, is geleë in die buitenste membraan. Beide ensieme is gesuiwer en gekarakteriseer.

Suiwering van die Fe(III) reduktase teenwoordig in die sitoplasmiese fraksie is uitgevoer deur middel van ioonuitruiling en hidrofobiese interaksie chromatografie. Die suiwerings protokol het ‘n 9.8-voudige suiwering van die hoof aktiewe fraksie met ‘n spesifieke aktiwiteit van 61.8 U/mg en ‘n opbrengs van 22.6% getoon. SDS-PAGE en 2-D PAGE het aangedui dat die proteïen homogeen was met ‘n relatiewe molekulêre massa van 39 000. Iso-elektriese fokussering is uitgevoer en die gesuiwerde ensiem het ‘n pI van 5.6 - 5.8 getoon. Fe(III) reduktase aktiwiteit van die gesuiwerde ensiem is bevestig met ‘n zimogram.

Die membraan-gebonde Fe(III) reduktase is gesuiwer deur die toepassing van hidrofobiese interaksie chromatografie. Dit het gelei tot ‘n 4.3-voudige suiwering van die hoof aktiewe fraksie met spesifieke aktiwiteit van 17.1 U/mg en ‘n opbrengs van 11.8%. Fe(III) reduktase aktiwiteit van die gesuiwerde ensiem is bevestig met ‘n zimogram. ‘n Prominente proteïenband is waargeneem op SDS-PAGE met ‘n relatiewe molekulêre massa van 49 000.

Die sitoplasmiese Fe(III) reduktase het ‘n optimum temperatuur van 60 – 65 ºC getoon met ‘n optimum pH van 6. Die ensiem het ekstreme stabiliteit getoon met halfleeftye van 16 h by 50 ºC, 14 h by 70 ºC en 4 h by 90 ºC. Van die 9 metale wat getoets is, het slegs

AgCl ‘n noemenswaardige inhiberende invloed op die ensiemaktiwiteit gehad. ‘n Km waarde van 6.1 mM is bereken met Fe(III)-NTA as die substraat.

Die membraan-gebonde Fe(III) reduktase het uitermatig termostabiel vertoon en die volgende halfleeftye getuig daarvan: 62 h by 50 ºC, 81 h by 70 ºC en 4 h by 90 ºC. Die ensiem het maksimale aktiwiteit by temperatuur van 70 - 75 ºC en pH van 5. Die metaalione AgCl, $Hg_2(NO_3)_2$ en $Pb(NO_3)_2$ toon ‘n sterk inhiberende invloed op die ensiemaktiwiteit. ‘n Km waarde van 2.6 mM is bereken met Fe(III)-NTA as die substraat.

EDTA het ‘n aktiverende invloed op die ensiemaktiwiteit van beide Fe(III) reduktases getoon alhoewel dit ‘n effense inhiberende invloed gehad het op die membraan-gebonde Fe(III) reduktase by hoër konsentrasies. Dit dui moontlik daarop dat die ensieme nie afhanklik is van metale vir aktiwiteit nie. Die aktiwiteit van beide Fe(III) reduktases is slegs effens geïnhibeer deur die serien hidrolase inhibeerder, PMSF. Water-oplosbare karbodiimied, wat aanleiding gee tot die modifikasie van funksionele karboksiel groepe, het die sitoplasmiese sowel as die membraan-gebonde Fe(III) reduktase aktiwiteit geïnhibeer, met geen oorblywende aktiwiteit, na onderskeidelik 125 min en 100 min, nie. Met Urea konsentrasies so hoog as 6 M, kon slegs geringe aktiwiteit van beide ensieme herwin word na 6-voudige verdunning.

Die membraan-gebonde sowel as die sitoplasmiese Fe(III) reduktase veskil gunstiglik van die mesofiliese Fe(III) reduktases wat reeds gekarakteriseer is.

Die doel van die studie, naamlik die suiwering en karakterisering van die termofiliese Fe(III) reduktases van *Thermus scotoductus* en die vergelyking met Fe(III) reduktases van hipertermofiliese en mesofiliese spesies, is suksesvol afgehandel.