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ENZYMES OF RESPIRATORY IRON OXIDATION

PROGRESS REPORT

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DISCLAIMER

I. In situ electrolysis was exploited to achieve enhanced yields of iron-oxidizing bacteria

A principal practical goal during the current grant period was to assemble and test an electrochemical apparatus whereby large quantities of autotrophic iron oxidizing chemolithotrophic bacteria could be achieved on a reproducible basis. The hypothesis to be tested was whether we could supply bacteria that respire on iron with an endless supply of growth substrate by electrochemically reducing the soluble iron in the media in situ. The chemistry behind this in situ electrolysis is illustrated schematically in Fig. 1. The iron in the growth medium is continually reduced at the cathode; the electrons for this reduction are derived from the oxidation of water at the anode. The bacteria experience an endless supply of the growth substrate and continue to grow until something else in the medium becomes limiting.

We successfully demonstrated enhanced yields of representative members of each phylogenetically-distinct group of iron oxidizing bacteria available from commercial culture collections. Typical yields of Thiobacillus ferrooxidans, Acidianus brierleyi, Metallosphaera sedula (the latter 2 are thermoacidophilic iron oxidizers), and Sulfobacillus thermosulfidooxidans (strain BC, a Gram positive moderate thermophile) increased from 40 mg wet weight/liter to over 2.0 g wet weight/liter, while the corresponding yields of strain M-1 (a Gram negative straight rod that respires only on iron, not sulfur) and Leptospirillum ferrooxidans increased from 20 mg wet weight/liter to over 1.0 g wet weight/liter. Furthermore, the bacterial cells obtained by this electrolytic procedure appeared to be identical to corresponding cells grown by normal batch-wise culture methods with regard to cytochrome content and kinetic properties of aerobic respiration on reduced iron. We have been able to achieve enhanced yields of moderately and extremely thermophilic iron oxidizers on a 40-liter scale, while the mesophilic iron oxidizers have been cul-

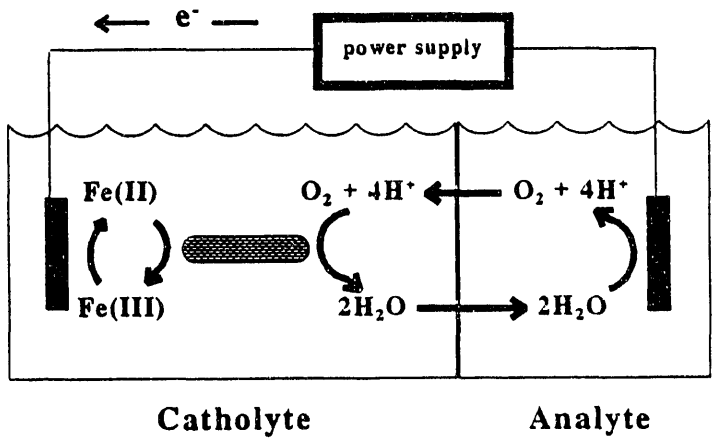


Fig. 1. Chemistry of in situ electrolysis apparatus.

tured electrolytically on a 200-liter scale; we thus have the means to routinely generate sufficient cell mass for subsequent enzyme purification and other experimental purposes. A book chapter that includes a preliminary description of this electrochemical apparatus and its performance was published in Biohydrometallurgy '89, representing the proceedings of the international biohydrometallurgy meetings held in Jackson Hole, Wyoming in August, 1989.

It is our intention to prepare an additional manuscript on this electrochemical culture method for a peer-reviewed journal. We are in the process of conducting a study of the relationship between the maximum growth yield and current passed (expressed as mg dry weight of bacteria per Coulomb) for each phylogenetically distinct iron oxidizer that shows an increased cell yield in the above apparatus. Differences in the individual values of maximum growth yields should reflect differences in the efficiency of energy conservation from the same substrate by different organisms, and thereby the probability of different substrate oxidation pathways, electron transport mechanisms, and modes of energy conservation.

II. Structure/function studies were performed on redox-active biomolecules from Thiobacillus ferrooxidans.

Rusticyanin, a type I blue copper protein, is a prominent redox-active biomolecule synthesized by T. ferrooxidans during autotrophic growth on soluble ferrous ions. The complete amino acid sequence of the purified rusticyanin from T. ferrooxidans ATCC 23270 (the type strain) was determined in collaboration with Dr. John Shively, City of Hope, Duarte, CA. **A manuscript entitled "Amino acid sequence of the blue copper protein rusticyanin from Thiobacillus ferrooxidans" was published in Biochemistry (reprint enclosed).** The results demonstrated a protein of 155 amino acids containing one cysteine and no arginine residues. The structure was established using sequences from two peptide maps (trypsin and Asp-N protease). Individual peptides were analyzed by FAB/MS to confirm molecular weights, and the intact protein was analyzed by electrospray mass spectrometry to confirm its molecular weight. In the latter case the agreement was within one amu, confirming that the structure determined was correct and that no unusual or unexpected post-translational modifications were present. Comparisons of the rusticyanin sequence to those of other blue copper proteins and the use of secondary prediction algorithms and CD studies led us to conclude that the structure was primarily β-sheet with an unusual amino-terminal region that may be α-helical or β-sheet. A novel feature of the model is the hypothesis that Asp73 (as opposed to a His residue in other blue copper proteins) may serve as a fourth copper ligand.

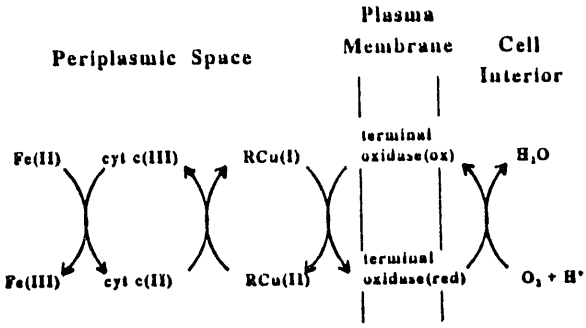
Subsequent structural experiments have focused on the determination of the tertiery structure of the rusticyanin. Dr. Menachem Shoham, Case Western Reserve University, Cleveland, OH, has succeeded in crystallizing purified rusticyanin. The current crystals are of sufficient integrity to achieve an X-ray diffraction pattern at 2.0 A resolution. **A manuscript entitled "Crys-**

tallization and preliminary X-ray crystallographic studies of rusticyanin from Thiobacillus ferrooxidans" was submitted as a Crystallization Note to the Journal of Molecular Biology (copy not included due to preprint/reprint limit). This laboratory has sent Dr. Shoham additional purified protein in the hopes that rusticyanin crystals that yield X-ray diffraction patterns at even greater resolution may be achieved. In the meantime, we have also sent purified protein to Dr. Jane Dyson, Scripps Institute, La Jolla, CA. Dr. Dyson will attempt to determine the tertiery structure of rusticyanin in solution by proton-, ^{15}N-, and ^{13}C-NMR techniques. Preliminary interpretation of the proton-NMR spectrum confirmed that the rusticyanin is largely β-pleated sheet. Autotrophic growth of T. ferrooxidans in the presence of the appropriate isotopes and purification of the isotopically-labeled rusticyanin is currently in progress. By one means or another, we will obtain the tertiery structure of purified rusticyanin.

Functional studies were also conducted on purified rusticyanin. The one-electron transfer reactions between soluble iron and purified rusticyanin were studied by stopped flow spectrophotometry in acidic solutions containing each of 14 different anions. These experiments were conducted, in part, to generate a broad picture of the electron transfer reactivity between soluble iron and rusticyanin under a variety of solution conditions. **A manuscript entitled "Effects of divers anions on the electron-transfer reaction between iron and rusticyanin from Thiobacillus ferrooxidans" was published in Biochemistry (reprint enclosed).** The results of these kinetic experiments indicated that the rusticyanin may possess at least two distinct electron transfer pathways for liganded iron. The first pathway was characterized by a protein-independent pattern of electron transfer reactivity. For liganded iron in this category, the anion-dependent reactivity pattern could be quantitatively described solely by the physicochemical properties of the individual iron-anion complexes. The second pathway, utilized by citratoiron(II) and oxalacetatoiron(II), produced second order rate constants for the liganded iron-dependent reduction of rusticyanin that were as much as 2 orders of magnitude greater than those anticipated on the basis of the physicochemical properties of the two complexes. The principal conclusion was that anion-specific interactions between the rusticyanin and selected liganded iron species do occur.

An acid-stable, membrane-associated iron:rusticyanin oxidoreductase was discovered in cell-free extracts of iron-grown T. ferrooxidans. **A manuscript entitled "Respiratory enzymes of Thiobacillus ferrooxidans: kinetic properties of a partially purified, acid-stable iron:rusticyanin oxidoreductase" was submitted to the Journal of Biological Chemistry (preprint enclosed).** Limited disaggregation and purification of the iron:rusticyanin oxidoreductase was achieved by ion exchange chromatography on SP-Sephadex in the presence of Zwittergent 3-12. An SDS-PAGE pattern of the partially purified enzyme showed a major band that stained with Coomassie Blue at 19,000 daltons, along with several minor bands with higher molecular masses. Kinetic experiments with the most highly purified preparations

revealed that the oxidoreductase-catalyzed, iron-dependent reduction of the rusticyanin exhibited 3 kinetic properties characteristic of aerobic iron oxidation by whole cells. (i) A survey of 14 different anions indicated that catalysis by the oxidoreductase occurred only in the presence of sulfate or selenate, an anion specificity identical to that of whole cells. (ii) Saturation with both sulfatoiron(II) and the catalyst produced a concentration-independent rate constant of 3 s^{-1} for the reduction of the rusticyanin, an electron transfer reaction sufficiently rapid to account for the flux of electrons through the iron respiratory chain. (iii) Values for the oxidoreductase-catalyzed pseudo-first order rate constants for the reduction of the rusticyanin showed a hyperbolic dependence on the concentration of sulfatoiron(II) with a half-maximal effect at 250 μM, a concentration similar to the apparent K_M for iron shown by whole cells. On the basis of these favorable comparisons between the behavior patterns of isolated biomolecules and those of whole cells, this iron:rusticyanin oxidoreductase was postulated to be the primary cellular oxidant of ferrous ions in the iron respiratory chain of T. ferrooxidans, according to the following scheme:



where cyt c represents the iron:rusticyanin oxidoreductase, RCu represents rusticyanin, and "terminal oxidase" represents the poorly characterized remainder of the respiratory chain.

In collaborative experiments performed with Dr. Tsuyoshi Sugio, Okayama University, Okayama, Japan, the existence of a hydrogen sulfide:ferric ion oxidoreductase (which heretofore had been investigated exclusively in one strain of T. ferrooxidans) was shown to be a general property of mesophilic and moderately thermophilic iron-oxidizing bacteria. **A manuscript entitled "Existence of a hydrogen sulfide:ferric iron oxidoreductase in iron-oxidizing bacteria" was published in Applied and Environmental Microbiology (reprint enclosed).**

III. Novel redox-active biomolecules were demonstrated in other iron autotrophs.

As part of a continuing effort to identify and investigate the biomolecules responsible for aerobic respiration on soluble ferrous ions, this laboratory conducted a comparative study of the principal electron transport components expressed during autotrophic growth by representative members of each of the 5 phylogenetically distinct groups of iron-oxidizing organisms dis-

cussed above. **A manuscript entitled "Enzymes of aerobic respiration on iron" was submitted to FEMS Microbiological Reviews to be published as part of the proceedings of Biohydrometallurgy '91, an international conference held in Troia, Portugal, in September, 1991 (copy not included due to preprint/reprint limit).** These investigations revealed that phylogenetically distinct groups of iron-oxidizing organisms, in turn, express biochemically distinct electron transport chains when grown chemolithotrophically on iron. Each type of electron transport chain is characterized by its own distinctive acid-stable, redox-active biomolecule. Absorbance spectra of the principal acid-stable biomolecules expressed by each group are presented in Fig. 2. Each spectrum in Fig. 2 represents an electrophoretically homogeneous protein that is stable and redox-active in 0.01 N sulfuric acid. Fig. 2A shows the oxidized and reduced spectra of rusticyanin, the type I blue copper protein that dominates the electron transport chains of T. ferrooxidans and strain M-1. Fig. 2B shows the oxidized and reduced spectra of the novel red cytochrome (cytochrome 579) that dominates the electron transport chains of L. ferrooxidans and similar organisms. The alkaline pyridine hemochromogen spectrum of this cytochrome revealed that it was clearly not an a-, b-, or c-type heme (data not shown), and that it represents a new, heretofore undescribed, heme with possibly a novel structure unique to respiratory iron oxidation in these organisms. Fig. 2C shows the oxidized and reduced spectra of the novel yellow chromophore (factor 458) that dominates the electron transport chains of S. thermosulfidooxidans and other Gram-positive moderate thermophiles that respire on iron. Fig. 2D shows the oxidized and reduced spectra of the novel yellow cytochrome (cytochrome 572) that dominates the electron transport chains of M. sedula and other extremely thermophilic, acidophilic archaebacteria.

Fig. 3 shows SDS-PAGE patterns for the latter 3 of the 4 purified respiratory components presented in Fig. 2. Fig. 3A shows various amounts of cytochrome 579 isolated from L. ferrooxidans, while Fig. 3B shows the purified factor 458 and cytochrome 572 from S. thermosulfidooxidans and M. sedula, respectively. Purification procedures for all 3 proteins were developed during the current grant period.

During the course of these investigations several collections of novel iron chemolithotrophs were acquired from private industrial sources. Many of the individual microorganisms in these collections exhibited distinctive morphological features, unexpected nutritional requirements, and heretofore uncharacterized respiratory cytochromes. Relevant properties of selected members of these collections are briefly summarized in Table 1. The sequence of the 16S ribosomal RNA from strain Funis was obtained in collaboration with Dr. Arthur Harrison, Jr., University of Missouri, Columbia, MO (unpublished data). The sequence indicated that, while strain Funis is unquestionably a eubacterium, it does not fit into any of the 10 recognized phyla in that kingdom. Likewise, other individual members of this collection almost certainly constitute new genera and species. It is anticipated that the information gleaned from the study of these novel, as yet poorly characterized, iron-oxidizing organisms will make a contribution to the current body of knowledge in this area.

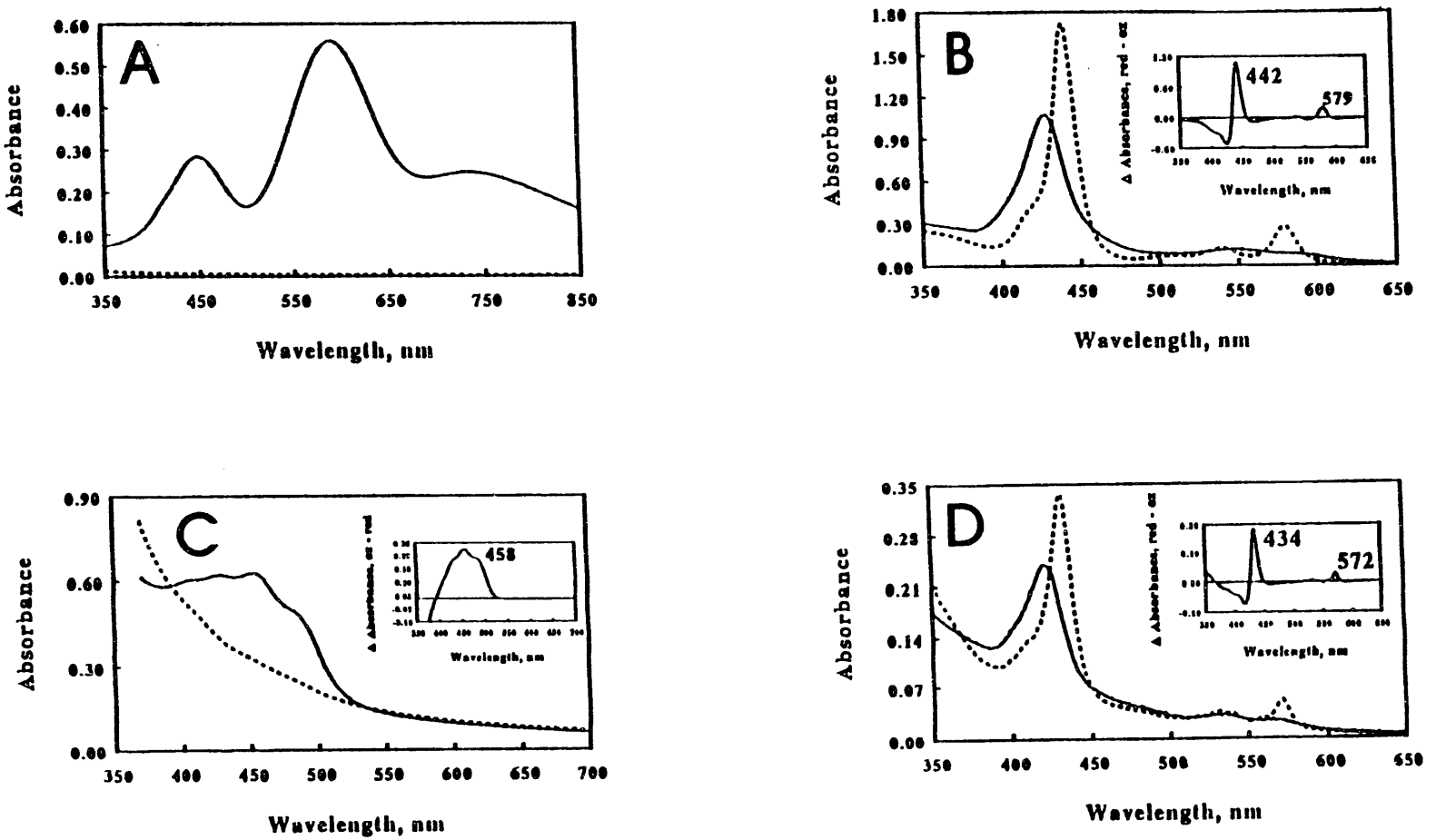


Fig. 2. Absorbance spectra of oxidized (solid line) and reduced (dashed line) acid-stable proteins from acidophilic iron-oxidizing bacteria. All spectra were determined on purified proteins in 0.01 N sulfuric acid at 25° C. The absorbance spectrum of each reduced protein was determined 10 min after mixing the sample of oxidized protein with an excess of ferrous sulfate. A, rusticyanin from strain M-1. B, cytochrome 579 from L. ferrooxidans. Inset, a difference spectrum representing the absolute spectrum of the Fe(II)-reduced protein minus that of the oxidized protein. C, factor 438 from S. thermosulfidooxidans. Inset, a difference spectrum representing the absolute spectrum of the oxidized protein minus that of the Fe(II)-reduced protein. D, cytochrome 572 from M. sedula. Inset, a difference spectrum representing the absolute spectrum of the Fe(II)-reduced protein minus that of the oxidized protein.

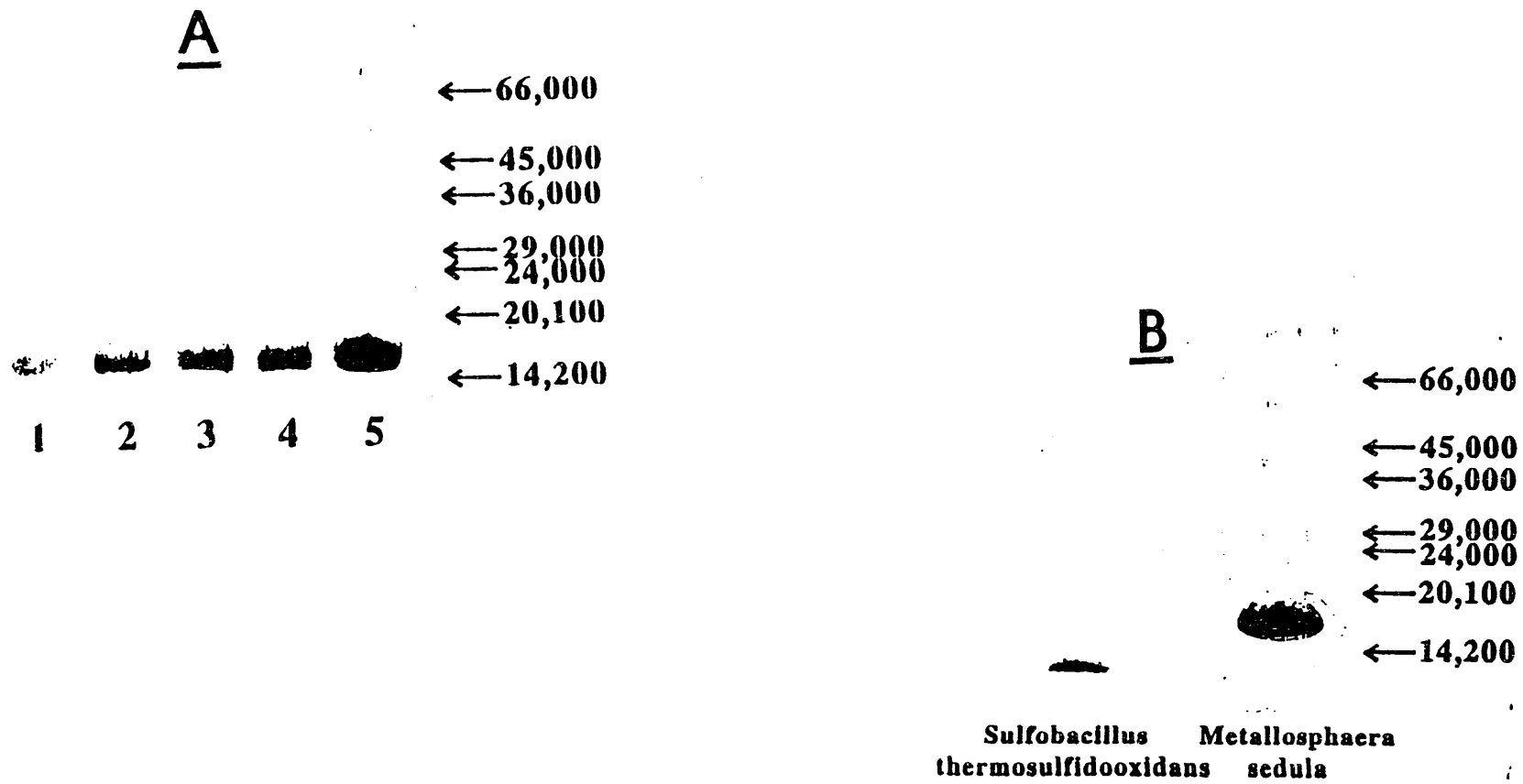


Fig. 3. Sodium dodecyl sulfate polyacrylamine gel electrophoresis patterns of purified acid-stable proteins isolated from acidophilic iron-oxidizing bacteria. SDS-PAGE under reducing conditions was performed on a Pharmacia Phastsystem using PhastGel Gradient 10-15 polyacrylamide gels and Phastgel SDS buffer strips. A, cytochrome 579 from L. ferrooxidans. The amount of cytochrome 579 was 85, 175, 255, 350, and 700 ng in lanes 1 through 5, respectively. B, factor 458 from S. thermosulfidooxidans and cytochrome 572 from M. sedula. The amounts of factor 458 and cytochrome 572 were 150 and 1000 ng, respectively.

Table 1. Properties of novel iron-oxidizing organisms.

Laboratory designation	Gram stain	Temperature °C	Growth on S°	Nature of principal respiratory molecule
Funis	negative	30-40	no	Soluble yellow cytochrome (novel)
Ch68	negative	25-35	yes	Membrane c-type
Ch7	negative	25-35	yes	Membrane novel cytochrome (386 Soret)
Carla	positive	25-40	no	Membrane c-type
Menjapur	negative	25-35	yes	Novel chromophore with peak at 305 nm
KU	negative	45-55	yes	Novel slate-grey chromophore

IV. New probes of metalloprotein electron-transfer reactivity were synthesized and characterized.

One barrier to further investigations on the electron transfer reactivity of these novel acid-stable proteins is that relatively few well characterized inorganic or small organometallic complexes are available with the appropriate electrochemical, solubility, and structural properties that are required to probe redox centers of such high reduction potential under strongly acidic conditions. Accordingly, a series of mixed ligand complexes of Fe(II) and Fe(III) with cyanide and 1,10-phenanthroline were synthesized and their electron transfer properties were investigated. By introducing different substituents on the aromatic diimine and varying the stoichiometry of the substituted phenanthroline in the final organoiron complex, a series of substitution-inert electron transfer reagents were constructed with reduction potentials from 500 to 900 mV. Kinetic and thermodynamic experiments were performed on the electron transfer reactions of each of these substitution-inert iron complexes with both sulfatoiron and rusticyanin from T. ferrooxidans. The utility of these reagents in both probing the electron transfer reactivity of the rusticyanin and in performing and mediating reduction potential measurements was illustrated. **A manuscript entitled "Mixed ligand complexes of iron with cyanide and phenanthroline as new probes of metalloprotein electron transfer reactivity: analysis of reactions involving rusticyanin from Thiobacillus ferrooxidans" was published in the Journal of Biological Chemistry (reprint enclosed).** This family of substitution-inert electron transfer reagents could prove useful in characterizing the reactivity of each of the very high potential respiratory chain components that are beginning to emerge in this research.

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