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Metal oxidoreduction by microbial cells

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SUMMARY

For many organisms, some heavy metals in external media are essential at low concentrations but are toxic at high concentrations. Strongly toxic heavy metals are toxic even at low concentrations. Recently, it was proven that changes of valencies of Fe, Cu and Mn were necessary for these metals to be utilized by organisms, especially microorganisms. The valencies of Hg and Cr are changed by reducing systems of cells in the process of detoxifying them. Thus, the processes of oxidoreduction of these metals are important for biological systems of metal-autoregulation and metal-mediated regulation. Metal ion-specific reducing enzyme systems function in the cell surface layer of microorganisms. These enzymes require NADH or NADPH as an electron donor and FMN or FAD as an electron carrier component. Electron transport may be operated by transplamsa-membrane redox systems. Metal ion reductases are also found in the cytoplasm. The affinities of metal ions to ligand residues change with the valence of the metal elements and mutual interactions of various metal ions are important for regulation of oxidoreduction states. Microorganisms can utilize essential metal elements and detoxify excess metals by respective reducing enzyme systems and by regulating movement of heavy metal ions.

INTRODUCTION

Living organisms contain and utilize many kinds of metal elements [22]. The biological functions of monovalent metal ions and alkaline earth metal ions have been well investigated and are understood in detail. Studies on heavy metals have also been made from the aspect of their functions as cofactors of metalloenzymes and of the properties of specific metal-binding proteins, but detailed biochemical mechanisms of metabolism and regulation of these heavy metals in living cells remain unclear. For many organisms, some heavy metals in external media are essential at low concentrations but are toxic at high concentrations, while strongly toxic heavy metals such as Hg(II) and Cd(II) are toxic even at low concentrations [22]. Many organisms have adaptation mechanisms against these metals [73]. Some organisms develop resistance to the metal toxicity at high concentrations. These phenomena indicate that many organisms have regulation systems for heavy metal metabolism and suggest that these metals play an important role in regulating gene expression and the activity of functional biomolecules. However, mechanisms of biological utilization of many metal elements are still unclear. Recently, it was proven that changes of valencies of Fe, Cu, and Mn were necessary for these metals to be utilized by organisms, especially microorganisms [12,99]. This demonstrates that the processes of oxido-reduction of these metals are important for biological systems of metal-autoregulation and metal-mediated regulation. The biological importance of oxidoreduction of heavy metals by microorganisms will be described and discussed in this short review.

Oxidizing and reducing reactions in living cells include many heavy metal-related reactions. Fe, Cu, and Mn, for example, have important roles as cofactors in metalloproteins (metalloenzymes), and the functions of metalloproteins are accompanied with the oxidoreducing reactions of these metals. Valencies of Hg and Cr are changed by reducing systems of cells in the process of detoxification of these metals [34,92]. Reduction of heavy metals by microorganisms requires appropriate electron donors produced by specific energy metabolism systems. Prokaryotic microorganisms (bacteria) have electron transport systems in the plasma membrane and reduce heavy metals in the cell surface layer. In eukaryotic microorganisms (e.g. yeasts, fungi, protozoa), mitochondria mainly supply intracellular heavy metal oxidoreduction systems with electron donors produced from electron transport systems. Glycolysis and fermentation may also supply electron donors. In eukaryotic cells, transplasma-membrane redox systems may be involved in the oxidoreduction of heavy metals in the cell surface layer [12]. Transition metals change their valencies depending on the biological systems. The oxidoreduction of Fe, Cu, Mn, Cr, and Hg in microbial cells mediated by physiological reducing forces and specific enzymes will be described in the following sections.

IRON REDUCTION

Iron under aerobic conditions is predominantly in a trivalent state (Fe(III)). Ferric iron is insoluble as ferric hydroxides in aqueous solutions at neutral pH. The formation of water-soluble Fe(III)-chelates is required for iron to be incorporated into microbial cells [12]. It was suggested that iron

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was transported into the cytoplasm as a lipophilic iron-chelate or Fe(II) because Fe(III) ion was impermeable through the plasma membrane [12]. Crane et al. [12] reviewed iron uptake by plants which involves the formation of an iron-chelate and the reduction from Fe(III) to Fe(II) by a trans-plasma-membrane electron-transport system [12,67,72]. In bacteria, iron uptake processes include: 1) chelate formation between Fe(III) and citrate or various siderophores; 2) Fe(III)-chelates binding to specific receptor proteins; 3) release of iron from Fe(III)-chelates; and 4) iron transport into cells through the plasma membrane as Fe(II) [12,73]. It was proposed that iron release from Fe(III)-siderophores involved protonation of the Fe(III)-siderophore, degradation of siderophore by hydrolase and iron reduction catalyzed by enzymatic Fe(III)-reducing systems in the plasma membrane.

Assimilatory reduction by prokaryotes

Ferric iron can be reduced by electron carriers of the respiratory chain in the plasma membrane of bacteria. Dailey and Lascelles [10] detected the reduction of iron as ferric citrate by membranes from *Spirillum itersonii* in the presence of NADH or succinate using ferrozine (3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-tetrazine) as a specific ferrous iron-chelating reagent [10], and suggested that reduction of iron occurred at one or more sites on the respiratory chain [13]. Iron reduction was also observed in other bacteria including *Rhodopseudomonas spheroides*, *Rhodopseudomonas capsulata*, *Paracoccus denitrificans*, and *Escherichia coli*.

Iron-reducing activity was observed also in the cytoplasmic fraction. Arceneaux and Byers [2] reported that ferrisiderophore reductase activity (NADPH: ferrisiderophore oxidoreductase) was detected in the presence of ferrozine and was associated with the soluble rather than the membrane fraction of *Bacillus megaterium*. Iron-reducing activity was inhibited by heat, protease, and iodoacetamide treatments and varied with the ferric chelates (siderophores) used as substrates. Both NADH and NADPH were active cofactors (electron donors) for the reduction of iron, but NADPH was more effective. Iron reductase activity was also found in the periplasmic space of bacteria. This suggests that Fe(III) is reduced to the transportable form, Fe(II). Cox [11] analyzed iron reductases from *Pseudomonas aeruginosa*. The reductase for ferric citrate as a substrate was found in the cytoplasmic fraction and another for ferripyochelin as a substrate was found in the periplasmic fraction; both enzymes required NADH [11].

The determination of net enzyme activity is difficult because non-enzymatic iron reduction by crude enzyme extracts varies with ferric chelates, electron donors, and the presence of high affinity reagents for ferrous iron. It is necessary, therefore, to isolate an iron reductase for characterization of iron-reducing systems. Gaines et al. [29] isolated a cytoplasmic ferrisiderophore reductase from *Bacillus subtilis*, which required NADPH and was stimulated by FMN and some divalent cations, and suggested that the ferrisiderophore reductase activity was associated with an aromatic biosynthetic enzyme complex. Lodge et al. [57] reported that the ferriagrobactin (ferrisiderophore) reductase activity in *Agrobacterium tumefaciens* required NADH as an electron donor, FMN, and anaerobic conditions.

Moody and Daily [62,63] purified two ferric iron reductases (iron reductase A and B) from the facultative photosynthetic bacterium *Rhodopseudomonas sphaeroides*. Molecular weights of reductase A and B were 41 000 Da and 32 000 Da, respectively. These enzymes were cytoplasmic soluble proteins and required NADH and FMN. The apparent K_m values of iron reductase B for NADH, Fe(III), and FMN were 18.2, 8.3, and 3.2 μM, respectively. Huyer and Page [36] purified the ferric reductase from the cytoplasm of *Azotobacter vinelandii*. The iron reductase activity was measured in the presence of NADH, ferric citrate, $MgCl_2$, and ferrozine. The optimum pH was about 7.5 and K_m values of the enzyme for Fe(III) (ferric citrate) and NADH were 10 and 15.8 μM, respectively. The enzyme consisted of two major proteins with molecular weights of 44 600 Da and 69 000 Da and required FMN and Mg^{2+} for maximum activity. These cytosolic enzymes may be involved in regulation of the valence of intracellular iron. The reaction of iron reductase described above is summarized as follows:

$$2Fe(III)+NAD(P)H+H^+ \xrightarrow[enzyme(FMN)]{} 2Fe(II)+2H^+ + NAD(P)^+$$

Some reducing substances can non-enzymatically reduce Fe(III) to Fe(II) with or without ferrous-chelating agents and iron reduction varies with ferric chelates. Recently, Coves and Fontecave [9] reported on the reduction and mobilization of iron by a NAD(P)H : flavin oxidoreductase in *Escherichia coli*. This enzyme catalyzes the reduction of flavins such as FMN or FAD by NADPH or NADH. Reduced flavins transfer their electrons to physiological ferric complexes: ferrisiderophores (especially, ferrichrome and ferrienterobactin), ferric citrate, and ferritins. The reactions are inhibited by molecular oxygen and greatly stimulated with Fe(II)-acceptors: ferrozine as a Fe(II) specific binding reagent, and the iron-free form of ribonucleotide reductase (apoprotein) as a physiological ferrous trap. The enzyme was purified by Fontecave et al. [23] and the gene (*fre*) has been cloned, sequenced and mapped by Spyrou et al. [75]. Iron reduction by reduced flavin produced by flavin oxidoreductase is summarized as follows:

$$Flavin + NAD(P)H + H^+ \rightarrow Flavin{\cdot}H_2 + NAD(P)^+$$

$$2Fe(III) + Flavin{\cdot}H_2 \xrightarrow[(nonenzymatic)]{enzyme} 2Fe(II) + 2H^+ + Flavin$$

Assimilatory reduction by eukaryotes

Eukaryotic microorganisms also contain iron reductase related to iron uptake systems [21]. Anderson and Morel [1] found evidence for a ferrous-mediated uptake of iron by cultures of *Thalassiosira weissflogii* (phytoplankton) and for the extracellular reduction of Fe(III) to Fe(II) in the presence of bathophenanthroline disulfonate (BPDS, 4,7-diphenyl-1,10-phenanthrolinedisulfonic acid) as a strong Fe(II) complexing agent [4]. BPDS inhibited the uptake of iron by *T. weissflogii*.

Lesuisse et al. [50] assumed that iron should be reduced prior to being taken up by the yeast *Saccharomyces cerevisiae*

on the basis of the phenomena that Fe(II) was taken up faster than Fe(III) and uptake was strongly inhibited by an Fe(II)-trapping reagent such as ferrozine. It was suggested that iron was physiologically reduced by a transmembrane redox system, which was induced in iron-deficient conditions. Lesuisse and Labbe [51] suggested the possibility of two mechanisms contributing to iron uptake by *S. cerevisiae* using haem-deficient strains lacking inducible ferric-reductase activity: one with high affinity iron uptake systems mediated by siderophores and the other with low affinity iron reductive dissociation from the ligand prior to uptake. Lesuisse et al. [52] detected two cytosolic iron-reducing enzymes in *S. cerevisiae*. One of the enzymes was purified and identified to be a flavoprotein (FAD) of 40 kDa using NADPH as an electron donor. They also purified a plasma membrane-bound ferric reductase system (NADPH as an electron donor and FMN as a prosthetic group) involved in the reductive uptake of iron in vivo.

Dancis et al. [14,15] isolated a mutant of *S. cerevisiae* lacking iron reductase activity and found that deficiencies of iron reductase activity and ferric uptake were due to a single mutation (*fre1-1*); the level of the gene transcript was regulated by iron. Since a ferrous uptake system is present in both wild-type and mutant cells, they suggested that iron uptake in *S. cerevisiae* was mediated by an iron reductase and a ferrous transport system. The reaction of iron reductase described above is summarized as follows:

$$2Fe(III) + NAD(P)H + H^+ \xrightarrow[\text{enzyme(FMN/FAD)}]{} 2Fe(II) + 2H^+ + NAD(P)^+$$

S. cerevisiae excretes no siderophores. Lesuisse et al. [53] examined the production and excretion of fluorescent materials by *S. cerevisiae* in relation to the iron state in the cells, and suggested that there should be some link between the excretion of aromatics and iron metabolism including iron-reducing steps [53]. Eide and Guarente [20] selected genes that enhanced growth of wild-type cells of *S. cerevisiae* in an iron-limiting medium and suggested that a gene, *fep1*, encoded a hydrophilic 43-kDa protein, and regulated the synthesis of gene products involved in iron uptake including iron-reducing steps. Kremer and Wood [48] suggested that cellobiose oxidase functions as an Fe(III) reductase in a fungus *Phanerochaete chrysosporium*. The cellobiose oxidase resembles flavocytochrome b_2 from *S. cerevisiae*. For clarification of the physiological roles of iron reductases, it is necessary to investigate the genes involved in iron-reducing enzymes and the mechanisms of iron reduction at the active site.

Dissimilatory reduction

Lovley [58] reviewed dissimilatory Fe(III) and Mn(IV) reduction in detail. Oxidation of organic matter coupled to the reduction of Fe(III) or Mn(IV) is recognized as one of the most important biogeochemical reactions in aquatic sediments, soils, and ground water. Lovley and co-workers have showed microorganisms, e.g. GS-15 and *Shewanella putrefaciens*, which can effectively couple the oxidation of organic compounds to the reduction of Fe(III) or Mn(IV). These microorganisms can oxidize fatty acids, hydrogen, or aromatic compounds with Fe(III) or Mn(IV) as the sole electron acceptor, producing energy for growth.

Recently, Coleman et al. [8] described on the basis of geochemical and microbiological studies that formation of siderite concretions in a salt-marsh sediment might have resulted from the activity of sulfate-reducing bacteria and that some of these bacteria could produce siderite ($FeCO_3$) rather than iron sulfide through an enzymatic mechanism [8]. In marine environments, sulfate-reducing bacteria produce hydrogen sulfide, which can non-enzymatically reduce iron oxyhydroxides to iron sulfide. The equations for iron reduction and siderite formation by sulfate-reducing bacteria are cited below:

$$4Fe_2O_3 + CH_3COO^- + 7H_2O \rightarrow 8Fe^{2+} + 2HCO_3^- + 15OH^-$$
$$Fe_2O_3 + H_2 + H_2O \rightarrow 2Fe^{2+} + 4OH^-$$
$$Fe^{2+} + HCO_3^- + OH^- \rightarrow FeCO_3 + H_2O$$

Thiobacillus thiooxidans, *Thiobacillus ferrooxidans*, and *Sulfolobus acidocaldarius* reduced Fe(III) to Fe(II) [7,76]. Sugio et al. [77] demonstrated that intact cells of *T. ferrooxidans* reduced Fe(III) with elemental sulfur in anaerobic conditions according to the following equations:

$$S^0 + 4FeCl_3 + 3H_2O \rightarrow H_2SO_3 + 4FeCl_2 + 4HCl$$
$$H_2SO_3 + 2FeCl_3 + H_2O \rightarrow H_2SO_4 + 2FeCl_2 + 2HCl$$
$$6FeCl_2 + 6HCl + 3/2O_2 \rightarrow 6FeCl_3 + 3H_2O$$

Under aerobic conditions, the Fe(II) produced was immediately reoxidized by the iron oxidase of the cell. Furthermore, Sugio et al. [76–83] characterized the ferric iron-reducing (FIR) system and purified sulfur : ferric ion oxidoreductase from *T. ferrooxidans* [78–80,83]. The enzyme was composed of two subunits, each of molecular weight 23 000 Da, was dependent on the presence of reduced glutathione, and was suggested to be localized in the periplasmic space. Recently, the same authors reported the isolation of a novel moderately thermophilic iron-oxidizing bacterium with an optimum pH and temperature for Fe(II) oxidation of 2.8 and 50 °C, respectively [84].

COPPER REDUCTION

In the external liquid environment of organisms, copper under aerobic conditions exists predominantly in the divalent state and binds to amino acids, proteins and nucleic acids. Cu(I) is also found in biological systems and many compounds containing copper are important in oxido-reducing reactions in the same manner as iron [40]. For many organisms, copper is essential at low concentrations but toxic at high concentrations [95,96]. Metallothionein is induced by excess copper and the copper bound to thionein is detected as Cu(I) [49,103,105]. Proteins and peptides containing thiols such as glutathione and other cysteine-rich γ-glutamyl peptides also bind with copper [25,68]. These observations suggest the involvement of thiol residues and/or sulfur metabolism in copper metabolism.

Many microorganisms produce hydrogen sulfide, which in

turn forms insoluble copper sulfide (CuS). Ashida et al. [3] and Kikuchi [43] reported on the copper resistance of *S. cerevisiae*, which involves generation of hydrogen sulfide and the formation of insoluble sulfide as a trap of excess copper in the yeast cell wall [3,43,44]. Generation of hydrogen sulfide was regulated by enzymes related to the sulfate-reducing system [60,104]. Lin and Kosman [55] demonstrated a relationship between the sulfate-reducing system and the exchangeable copper-binding pool in the cell wall of *S. cerevisiae* [55,56]. When various strains of yeasts were grown in media containing varied concentrations of copper, the formation of copper sulfide, as black precipitates, was recognized on the cell surface of many yeasts which produced hydrogen sulfide [37]. The yeast strains with higher ability of hydrogen sulfide production were more resistant to copper. However, the relationship between hydrogen sulfide production and copper resistance is a problem awaiting a full solution.

As a strategy to clarify copper metabolism, including copper transport through the cytoplasmic membrane and copper transfer to apoproteins, copper uptake was investigated using the yeast *Debaryomyces hansenii* IFO 0023, which was selected for no detectable production of hydrogen sulfide and no copper resistance [37,95,97,99]. Copper uptake by this strain was biphasic; copper rapidly bound to the cell surface after the addition of Cu(II) to the cells suspension and was then slowly transported into cells [95]. The first phase indicated copper binding to the cell wall. The second phase indicated copper transport through the plasma membrane, corresponding to the increase of EDTA-unwashable copper taken up by the cells. Copper transport was stimulated with copper-reducible agents such as ascorbic acid and hydroxylamine, and inhibited with oxidizing agents such as hydrogen peroxide [99]. Biphasic uptake of metal ions was shown also by other researchers [6]. The same phenomena were also observed in copper uptake by protoplasts prepared from yeast cells by removing the cell wall with a cell wall lytic enzyme, Zymolyase [99]. Uptake by protoplasts was stimulated with cell wall materials prepared from intact cells. Copper was reduced from Cu(II) to Cu(I) by the cell wall materials, which contained copper-reducing mannan proteins. Cu(I) was determined using bathocuproine (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline), a Cu(I) specific colorimetric reagent [5,74]. The cell wall and periplasmic materials solubilized with Zymolyase from intact yeast cells contained more copper-reducing activity than the cell wall materials prepared by a glass beads method. These results suggested that copper bound to the cell surface was reduced to Cu(I) and then transported through the plasma membrane into the cells.

Jones et al. [42] also detected the reduction of external Cu(II) complexes in the presence of bathocuproinedisulfonate (BCDS) and redox dyes in the phytoplankton cell surface and suggested that the reductive activity was mediated by one or more plasmalemma redox enzymes. In mitochondria from the protozoan *Tetrahymena pyriformis*, Cu(II) was reduced to Cu(I) accompanied with the oxidation of electron carriers, i.e. flavoproteins and cytochromes, in the electron transport system [98]. This shows that Cu(II) can be directly bound to components of the electron transport system and then reduced to Cu(I). The copper-specific enzymatic reducing system, however, has not yet been detected.

Copper reductases were found in solubilized cell wall materials (SCWM) from *D. hansenii* [100,101]. The SCWM contained a non-enzymatic copper-reducing system mediated by SH residues and a NAD(P)H-dependent Cu(II)-reducing enzyme system. The enzyme was stimulated by FMN and the optimum pH was about 5. The molecular weight of the purified enzyme was about 100 000 Da by gel chromatography. The affinity of NADPH as an electron donor for the purified enzyme was larger than NADH. Physiological electron donors may be supplied through the plasma membrane [12,72]. Equations describing copper-reducing reactions are:

$$2Cu(II) + NAD(P)H + H^+ \xrightarrow[\text{enzyme}]{} 2Cu(I) + 2H^+ + NAD(P)^+$$

$$2Cu(II) + 2RSH \xrightarrow[\text{(nonenzymatic)}]{} 2Cu(I) + 2H^+ + RS\text{–}SR$$

Jones et al. [41] reported that the thiol-dependent reduction of Cu(II) and associated superoxide radical production in Cu(II)-treated cultures of the unicellular alga *Dunaliella tertiolecta* were induced by light (>425 nm). Thiol compounds and SH residues of proteins such as glutathione and SCWM can also reduce copper. Copper-reducing systems mediated by SH groups may be controlled by the related enzymes. As the Cu(II)-reducing enzyme systems are localized in the cell surface layer and involved in copper uptake [99], it is suggested that the copper reductase situated in the cell wall and/or periplasmic space of *D. hansenii* can regulate copper metabolism. Many metal-reducing reactions are known to exist in biological systems and it is important for an understanding of metal metabolism to clarify metal-specific enzymatic reducing systems.

MERCURY REDUCTION AS DETOXIFICATION

Organic and inorganic mercury compounds are taken up into organisms and strongly inhibit various metabolic processes, especially SH-related reactions. In early investigations of mercury reduction and resistance, Tonomura et al. [92,93] found the decomposition of mercurials and the vaporization as a way to remove mercury from the cells by mercury-resistant bacteria. Furukawa and Tonomura [26,28] proposed the existence of the enzyme system involved in decomposition of mercurials by mercury-resistant *Pseudomonas* and purified a metallic mercury-releasing enzyme, which reductively decomposed organic and inorganic mercurials to form metallic mercury in the presence of a NAD(P)H generating system and cytochrome *c*-I. The enzyme, a flavoprotein (FAD) composed of enzyme I and II, was induced by mercurials such as phenylmercuric acetate, *p*-chloromercury benzoate, ethyl mercuric thiosalicylate, mercuric chloride, and metallic mercury but not by HgS or other metals [26–28]. Kōmura et al. [45,47] and Kōmura and Izaki [46] also studied vaporization of mercury compounds in *Escherichia coli* and Izaki et al. [39] purified a mercuric ion-reducing enzyme from *E. coli* bearing a plasmid. The enzyme, also a flavoprotein, rapidly oxidized NADPH in

the presence of mercuric chloride but did not in the presence of organic mercurials. Tezuka and Tonomura [89] found an enzyme which catalyzed the splitting of carbon-mercury linkages of organo-mercury compounds and was involved in vaporization, i.e. reduction.

Summers and Silver [85] reported that a strain of *E. coli*, which carried genes determining mercury resistance on a naturally-occurring resistance transfer factor, converted mercuric chloride to metallic mercury. Summers and Sugarman [86] reported Hg(II)-reducing activity determined by a plasmid-borne gene in *E. coli*. Schottel [71] purified a mercury reductase which reduced Hg(II) to volatile Hg(0) and organomercurial hydrolase from the plasmid-bearing *E. coli*. The reductase contained bound FAD, and required NADPH as an electron donor together with a sulfhydryl compound for activity. The molecular weight of the enzyme was about 180 000 Da (the molecular weight of a subunit, 63 000 Da), the optimum pH was 7.5 and K_m for mercuric chloride was 13 μM. Fox and Walsh [24] and Rinderle et al. [69] also purified the mercury reductases from *Pseudomonas aeruginosa* carrying the plasmid pVS1 and from *E. coli* containing mercury resistance genes from plasmid NR1, respectively. They suggested the presence of oxidation-reduction active cysteine residues at the active site on the basis of spectroscopic similarities to lipoamide dehydrogenase and glutathione reductase. Both enzymes bound FAD, required NADPH as an electron donor and were composed of two identical subunits. Fox and Walsh proposed the equation of the reductase reaction to be:

$$NADPH + RS\text{–}Hg\text{–}SR + H^+ \rightarrow NADP^+ + Hg^0 + 2RSH$$

Distefano et al. [19], Moore and Walsh [64], and Miller et al. [61] investigated the redox-active disulfide of mercuric ion reductase, the *merA* gene product, using mutagenesis of disulfide in MerA. Recently, Silver and Walderhaug [73] reviewed plasmid ion resistance systems containing mercury reductase (MerA) and organomercurial lyase (MerB).

CHROMIUM REDUCTION AS DETOXIFICATION

Cr(VI) compounds are powerful oxidizing agents and toxic to many organisms. It is suggested that the reduction of Cr(VI) to Cr(III) is important for the detoxification of chromium on the basis of studies on the mechanisms of toxic action. Horitsu et al. [34,35] isolated a Cr(VI)-tolerant bacterium, *Pseudomonas ambigua* G-1, from activated sludge and detected enzymatic Cr(VI)-reducing activity in a cell-free extract of this bacterium. This reducing activity required NADH, not NADPH as a hydrogen donor. They demonstrated the relations between the chromium-reducing activity and the Cr(VI)-tolerance of bacteria using several Cr(VI)-sensitive mutants. Ishibashi et al. [38] detected a NAD(P)H-dependent Cr(VI)-reducing activity in the cell-free supernatant phase of *Pseudomonas putida*. Das and Chandra [16] reported reduction of chromium from Cr(VI) to Cr(III) in the presence of NAD(P)H in the cell extract and the membrane of *Streptomyces* species. Ohtake et al. [66] and Wang et al. [102] found that Cr(VI) was reduced to Cr(III) under anaerobic conditions involving the respiratory chain of the cell membrane in *Enterobacter cloacae*. Recently, Suzuki et al. [88] purified a NAD(P)H-dependent chromium(VI) reductase (molecular weight 65 000 Da) from *P. ambigua* G-1 and suggested that a Cr(V)-intermediate formed during the reduction of chromium from Cr(VI) to Cr(III) might act as a toxic and mutagenic agent [88].

MANGANESE OXIDOREDUCTION

Manganese is an essential element for many organisms. The oxidation and reduction of manganese are coupled with cycling of other essential elements, and microorganisms play an important role in the natural cycling of manganese [65]. Soluble Mn(II) is oxidized to Mn(III) or Mn(IV) by microorganisms and oxidized manganese precipitates around the cells [30,65]. Spores of microorganisms are known to catalyze the oxidation of manganese [18,33,70]. Rosson and Nealson [70] suggested that a protein catalyzed the oxidation of manganese. Recently, Waasbergen et al. [94] genetically analyzed the marine manganese-oxidizing *Bacillus* sp. strain SG-1 using transposon mutagenesis and identified chromosomal loci of genes involved in the oxidation of manganese [36,94].

Manganese must be reduced to Mn(II), soluble manganese, to be utilized by microorganisms. De Vrind et al. [17] reported that vegetative cells of *Bacillus* sp. strain SG-1 were able to reduce manganese oxide, presumably during anaerobic respiration. Lovley [58] has reviewed dissimilatory Mn(IV) reduction in detail. Fe(II) can reduce Mn(IV) non-enzymatically. Sugio et al. [81,82] reported on the reduction of Mn(IV) and Mo(VI) by Fe(II) produced by sulfur : ferric ion oxidoreductase in *Thiobacillus ferrooxidans*. Lovley et al. [58] reported on the relation between Mn(IV) reduction and the growth of GS-15 and *S. putrefaciens*. Ghiorse and Ehrlich [31,32] and Trimble and Ehrlich [90,91] investigated the electron transport of Mn(IV) in the marine microorganism *Bacillus* strain 29, and demonstrated that the electron transport system consisted of a dicumarol-sensitive quinone, a Mn(II)-inducible, atebrine-sensitive flavoprotein and a cyanide- and azide-sensitive MnO_2 reductase. Recently, Sunda and Kieber [87] observed that manganese oxides produced by Mn-oxidizing bacteria oxidized humic substances to low-molecular-weight organic compounds and manganese was reduced to Mn(II). These authors suggested that Mn-oxidizing bacteria were able to use the low-molecular-weight organic compounds as a carbon source for growth [87].

SPECULATION

Organisms need to solubilize insoluble metal compounds occurring in the natural environment prior to utilization. Microorganisms can solubilize insoluble minerals by producing chelate-ligands and by oxidoreduction systems of the cell surface. Bacteria produce siderophores to form soluble iron chelates. In eukaryotic cells, metabolites such as amino acids, carboxylic acids may also act as ligands to solubilize metal ions. In blood or inside cells, metal ion-binding proteins and peptides (ferritin, ceruloplasmin, albumin, glutathione, etc) may function as solubilizers and mobilizers of metal ions.

In the process of metal ion metabolism, metal ion-specific reducing enzyme systems are known to operate in the cell surface layer of microbial cells as described above. These enzymes require NADH or NADPH as an electron donor and FMN or FAD as an electron carrier component. Electron transport may be operated by transplasma-membrane redox systems [12,72]. Physiological mechanisms of electron donor systems for metal reduction are, however, still unclear. Electron carrier proteins, e.g. cytochromes, may mediate electron transport from the cytoplasm to the cell surface. Copper reductase of yeast may require some functional substances which mediate electron transfer from the plasma membrane to the reductase, because copper reductase is localized in the cell surface layer and/or on the surface of plasma membrane [100,101]. Mercury reductase contains a disulfide as a binding and reducing site [24,69]. Further investigations of metal binding sites of each metal reductase and electron donor systems will be able to clarify the physiological mechanisms of metal reduction.

Iron and copper are transported as Fe(II) and Cu(I), respectively. Copper uptake by the yeast is apparently inhibited by amino acids, ligands to copper, added to the medium. The inhibition is caused by the decrease of copper adsorption to the cell surface layer [97]. The copper adsorption to the cell wall depends mainly on the degree of dissociation of copper-amino acid complexes to free ions [59,97]. Copper reduction by SH residues of cell wall proteins and copper reductase is also inhibited by amino acids but copper transport through the plasma membrane is not affected by amino acids [97,99,100]. Copper-reducing enzyme systems may regulate copper uptake through the plasma membrane by yeast cells. The process of copper uptake and copper reduction by yeast cells is proposed to be as shown in Fig. 1 and the following equations:

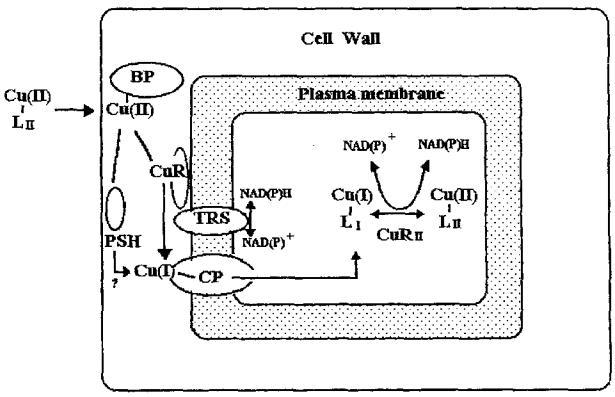

Fig. 1. The process of copper uptake and copper reduction by yeast cells. L_I and L_{II} are ligands to Cu(I) and Cu(II), respectively. Step 1: ligand exchange in Cu(II) binding to BP (copper-binding protein) in the cell surface layer. Step 2: copper reduction by CuR (copper reductase) system and/or PSH (SH residues-bearing protein or peptide), then Cu(I) is transferred to CP (carrier protein). Electrons are transported by TRS (transplasma-membrane redox systems). Step 3: copper transport in plasma membrane. Step 4: oxidation-reduction of copper by CuR_{II} (copper reductase system in cytosol) and ligand exchange. Copper is incorporated to valence-specific metal binding proteins or peptides in this step.

$$2Cu(II)\cdot L_{II} + 2L_I^- + NAD(P)H + H^+ \xrightarrow[CuR(FMN \longleftrightarrow FMNH_2)]{} 2Cu(I)\cdot L_I + 2HL_{II}^- + NAD(P)^+$$

$$2Cu(II)\cdot L_{II} + 2PSH + 2L_I^- \rightarrow 2Cu(I)\cdot L_I + PS\text{–}SP + 2HL_{II}^-$$

Copper transport through the yeast plasma membrane is strongly inhibited by SH reagents such as *N*-ethylmaleimide, and iodoacetate [97]. The uptake of external copper by yeast cells is stimulated by copper-reducing agents such as ascorbic acid or hydroxylamine [99]. Furthermore, copper uptake by yeast protoplasts is stimulated with NAD(P)H added in the extracellular fluid (Wakatsuki et al., unpublished data). The same stimulation of copper uptake is observed in the protozoan *Tetrahymena pyriformis* GL. The copper reductase of the protozoan is found in the pellicle and has been purified to homogeneity (Wakatsuki et al., unpublished data). The enzyme also requires NAD(P)H as an electron donor. These data support the proposed assumption of the copper uptake process. Metallothionein (MT) is involved in intracellular metabolism of heavy metals, but no evidence of involvement of MT is observed in copper transport through the plasma membrane of microorganisms. Lin et al. [54] represented copper transport kinetically using copper-MT-deficient *S. cerevisiae*. In *E. coli*, *cutA*, *cutB*, and *cutE* genes are known to relate to copper uptake and in *S. cerevisiae*, the *cup1* gene encodes copper-MT [73]. Bacterial genes encoding other copper-binding proteins are also reported. However, the genes involved in copper reduction are still unknown.

Metal ion reductases are found in the cytoplasm as described above. The yeast *D. hansenii* and the protozoan *T. pyriformis* also have intracellular copper reductases (Wakatsuki et al., unpublished data). These enzymes may regulate the oxidoreduction states of metal ions. For an understanding of the physiological functions of these enzymes, it is therefore necessary to clarify the relation between metal-specific oxidoreducing systems and the various oxidoreducing enzymes in the cytoplasm.

Microorganisms detoxify mercury and chromium by mercury- and chromium-specific reductases, respectively, and, on the other hand, utilize copper and iron by respective reducing enzyme systems. Fe(II) reduces Mn(IV) and Cu(II) non-enzymatically. These findings indicate that the affinities of metal ions to ligand residues change with the valence of the metal elements and that mutual interactions of various metal ions are important for the regulation of oxidoreduction states. Metal-specific oxidoreducing enzyme systems can regulate movement of metal ions, and may thus enable organisms to utilize essential elements. The same mechanisms may work for the detoxification of excess metals. To clarify the physiological role of metal reduction in microbial cells, it is important to characterize the mechanisms of metal-specific oxidoreducing systems involving valence-specific metal binding proteins or peptides.

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