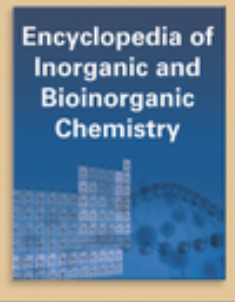
Editors
Valeria Culotta · Robert A. Scott

Metals in Cells



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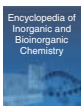

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METALS IN CELLS

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- Copper in Immune Cells

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- Manganese in Neurodegeneration

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- Nitrogenase Cofactor Assembly

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- Cyanobacterial Models that Address Cross-Talk in Metal Homeostasis

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- Selenoenzymes and Selenium Trafficking: An Emerging Target for Therapeutics

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- Resistance Pathways for Metalloids and Toxic Metals

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- [NiFe]-Hydrogenase Cofactor Assembly

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- Molecular Basis of Hemochromatosis

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- Selenoenzymes and Selenium Trafficking: An Emerging Target for Therapeutics

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Series Preface

The success of the *Encyclopedia of Inorganic Chemistry* (EIC), pioneered by Bruce King, the founding editor-in-chief, led to the 2012 integration of articles from the *Handbook of Metalloproteins* to create the newly launched *Encyclopedia of Inorganic and Bioinorganic Chemistry* (EIBC). This has been accompanied by a significant expansion of our Editorial Advisory Board with international representation in all areas of inorganic chemistry. It was under Bruce's successor, Bob Crabtree, that it was recognized that not everyone would necessarily need access to the full extent of EIBC. All EIBC articles are online and are searchable, but we still recognized value in more concise thematic volumes targeted to a specific area of interest. This idea encouraged us to produce a series of EIC (now EIBC) books, focusing on topics of current interest. These will continue to appear on an approximately annual basis and will feature the leading scholars in their fields, often being guest coedited by one of these leaders. Like the *Encyclopedia*, we hope that EIBC books continue to provide both the starting research student and the confirmed research worker a critical distillation of the leading concepts and provide a structured entry into the fields covered.

The EIBC books are referred to as ''spin-on'' books, recognizing that all the articles in these thematic volumes are destined to become part of the online content of EIBC, usually forming a new category of articles in the EIBC topical structure. We find that this provides multiple routes to find the latest summaries of current research.

I fully recognize that this latest transformation of EIBC is built upon the efforts of my predecessors, Bruce King and Bob Crabtree, my fellow editors, as well as the Wiley personnel, and, most particularly, the numerous authors of EIBC articles. It is the dedication and commitment of all these people that is responsible for the creation and production of this series and the ''parent'' EIBC.

Robert A. Scott
University of Georgia

September 2013

Volume Preface

Our understanding of metals and other trace elements in cells has witnessed an explosion over recent years. This has been prompted by a combination of new methods to probe intracellular metal locations and the dynamics of metal movement in cells, high-resolution detection of metal–biomolecule interactions, and the revolution of genomic, proteomic, metabolic, and even ''metallomic'' approaches to the study of inorganic physiology. Environmental metals and metalloids, including iron, copper, zinc, cobalt, molybdenum, selenium, and manganese, are all accumulated by cells and organisms in the micro- to millimolar range. Yet despite this abundant sea of diverse metals, only the correct metal cofactor is matched with a partner metalloprotein—mistakes in metal ion biology rarely occur. At the same time, free metal ions can be detrimental to cellular components and processes, so systems have evolved to control carefully the trace element concentrations and locations (homeostasis). The mechanisms underlying this ''perfect'' handling of metals are the goal of studies of the cell biology of metals.

Metals in Cells covers topics describing recent advances made by top researchers in the field including: regulated metal ion uptake and trafficking, sensing of metals within cells and across tissues, and identification of the vast array of cellular factors designed to orchestrate assembly of metal cofactor sites while minimizing toxic side reactions of metals. In addition, it features the aspects of metals in disease, including the role of metals in neurodegeneration, liver disease, and inflammation, as a way to highlight the detrimental effects of mishandling of metal trafficking and response to ''foreign'' metals.

While it is not possible to provide a comprehensive treatment of transport, homeostasis, sensing, and regulation of the entire ''biological periodic table,'' what *Metals in Cells* does, is give a broad sampling of the current knowledge and research frontiers in these areas. The reader will get a sense of some of the general principles of biological response to trace elements, but will also marvel at the disparate evolutionary responses of different organisms to a variable and changing inorganic environment. One of the ultimate goals in this area is to find the principles of inorganic chemistry in the biological responses.

Metals in Cells also gives an up-to-date description of many of the current tools being used to study inorganic cell biology. Genetics and biochemistry are combining with more recent genomic, proteomic, and metallomic approaches. Increasingly sophisticated microscopy and imaging technologies provide information about dynamic distribution of inorganic elements in cells and subcellular compartments. There is yet more room for improvement by collaborative approaches among physicists, chemists, and biologists.

With the breadth of our recently acquired understanding of inorganic cell biology, we believe that *Metals in Cells*, featuring key aspects of cellular handling of inorganic elements, is both timely and important. At this point in our progress, it is worthwhile to step back and take an expansive view of how far our understanding has come, while also highlighting how much we still do not know.

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Athens, GA, USA

September 2013

Periodic Table of the Elements

Group →	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Period → 1	1 H 1.0079																	2 He 4.0026
2	3 Li 6.941	4 Be 9.0122											5 B 10.811	6 C 12.0107	7 N 14.0067	8 O 15.9994	9 F 18.9984	10 Ne 20.179
3	11 Na 22.9898	12 Mg 24.305											13 Al 26.9815	14 Si 28.0855	15 P 30.9738	16 S 32.066	17 Cl 35.453	18 Ar 39.948
4	19 K 39.0983	20 Ca 40.078	21 Sc 44.9559	22 Ti 47.867	23 V 50.9415	24 Cr 51.996	25 Mn 54.9380	26 Fe 55.845	27 Co 58.933	28 Ni 58.693	29 Cu 63.546	30 Zn 65.409	31 Ga 69.723	32 Ge 72.64	33 As 74.9216	34 Se 78.96	35 Br 79.904	36 Kr 83.798
5	37 Rb 85.4678	38 Sr 87.62	39 Y 88.9059	40 Zr 91.224	41 Nb 92.9064	42 Mo 95.94	43 Tc 98.9062	44 Ru 101.07	45 Rh 102.9055	46 Pd 106.42	47 Ag 107.8682	48 Cd 112.41	49 In 114.818	50 Sn 118.710	51 Sb 121.760	52 Te 127.60	53 I 126.9045	54 Xe 131.29
6	55 Cs 132.9054	56 Ba 137.327	57 * La 138.9055	72 Hf 178.49	73 Ta 180.9479	74 W 183.84	75 Re 186.207	76 Os 190.2	77 Ir 192.22	78 Pt 195.08	79 Au 196.9665	80 Hg 200.59	81 Tl 204.3833	82 Pb 207.2	83 Bi 208.9804	84 Po (209)	85 At (210)	86 Rn (222)
7	87 Fr (223)	88 Ra (226.0254)	89 ** Ac (227.0277)	104 Rf (261.1088)	105 Db (262.1141)	106 Sg (266.1219)	107 Bh (264.12)	108 Hs (277)	109 Mt (268.1388)	110 Ds (271)	111 Rg (272)							

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* LANTHANIDES	58 Ce 140.12	59 Pr 140.9077	60 Nd 144.24	61 Pm (147)	62 Sm 150.36	63 Eu 151.96	64 Gd 157.25	65 Tb 158.9254	66 Dy 162.50	67 Ho 164.9304	68 Er 167.26	69 Tm 168.9342	70 Yb 173.04	71 Lu 174.967
** ACTINIDES	90 Th 232.0381	91 Pa 231.0359	92 U 238.0289	93 Np 237.0482	94 Pu (244)	95 Am (243)	96 Cm (247)	97 Bk (247)	98 Cf (251)	99 Es (252)	100 Fm (257)	101 Md (260)	102 No (259)	103 Lr (262)

Based on information from IUPAC, the International Union of Pure and Applied Chemistry (version dated 1st November 2004).
For updates to this table, see http://www.iupac.org/reports/periodic_table.

PART 1
Introduction

Mechanisms Controlling the Cellular Metal Economy

Benjamin A. Gilston and Thomas V. O'Halloran

Northwestern University, Evanston, IL, USA

1 INTRODUCTION

This book introduces an authoritative and extensive set of articles on the chemistry of transition metals in cells. The reader will find several in-depth overviews of progress at the confluence of several fields. In this brief introductory article, we discuss some emerging concepts and controversial ideas, which are addressed in more detail elsewhere. Biomedical research as an enterprise is undergoing a major shift in understanding the roles of transition metals in biology. Our understanding of the cellular roles of transition metals is not as well developed as, for instance, lipid biology, for a number of historical reasons, the first of which is evident in the etymology of the word bioinorganic chemistry. The term inorganic of course originates in an archaic grouping of elements; those found in living things were classified as organic and those that were not were classified as inorganic. Analytical methods applied at the cellular level are now revealing a host of inorganic elements once invisible to science. The legacy of artificial divisions is clear in other misnomers within the field. The term ''biological trace elements'' is commonly associated with transition metals, and this usage unfortunately obscures the true portrait of how cellular processes are carried out. As students of biology consider the roles of metals in cellular processes, one hurdle they must overcome involves the seemingly small number of metal ions that ''trace'' element implies. After all, if something is trace, there is hardly anything there, and if there is hardly anything there, how important can it be? From the cellular perspective, transition metals are anything but trace elements (Figure 1): intracellular metals such as zinc and iron are not present at low levels but are routinely maintained in most cells at surprisingly high levels (i.e., 0.5 mM) even when cells are grown in a medium that has metal concentrations stripped down to nanomolar levels. In fact, the minimal required metal quotas for zinc and iron are so high that they guide major cellular decisions including growth, spore formation, differentiation, or death. Furthermore, a growing body of evidence links disorders in transition metal physiology to neurological disorders and metabolic and infectious diseases. Such findings underscore the imperative to establish and test a set of fundamental principles that relate the chemistry and cellular functions of transition metal ions.

Over the past 20 years, there have been a series of breakthroughs describing the structure, properties, mechanisms, and physiology of metal-trafficking and -sensing machinery. These studies have helped the biological community to realize that the subgroup of metallic elements known as transition metals are much more complex than their distant cousins in the periodic table, namely the essential alkali and alkaline earth metal ions (K, Ca, and Mg). For instance, many well-trained biomedical researchers would find it difficult to describe the difference in bonding and reaction chemistry of the alkaline earth metal such as magnesium on the one hand

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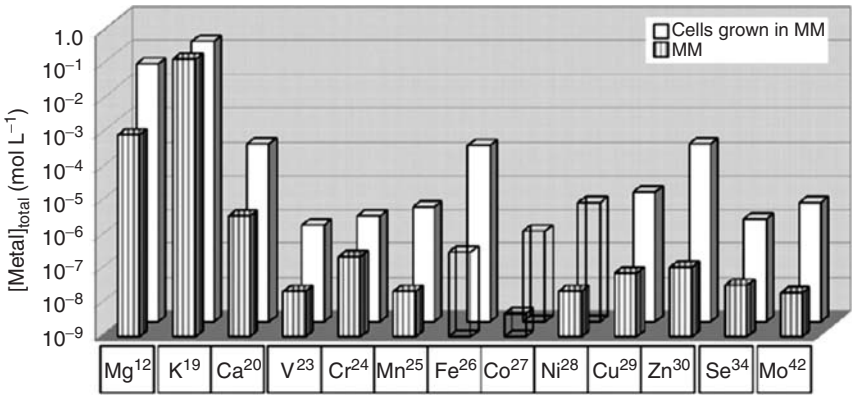

Figure 1 Depicted in this graph is the *E. coli* metallome, that is, the total metal content of the cell. The *y*-axis corresponds to the moles per cellular volume for cells grown in minimal medium and compared with the total metal concentrations in the relevant growth medium. These graphs highlight the high concentrations of transition metal ions with which *E. coli* cells retain metals from the media they are grown in. These measurements were obtained using ICP-MS (inductively coupled plasma–mass spectrometry). The unfilled columns represent detection limits for low-abundance elements under these experimental conditions. (Reproduced with permission from Ref. 1. © AAAS, 2001.)

and the transition metal manganese on the other. Their reaction chemistry is as different as night and day: the former has one available oxidation state and forms bonds that are strictly ionic in character, that is, nondirectional, whereas the latter has several accessible oxidation states and forms coordination bonds that have significant covalent character. This affords the transition metal the ability to form complex ions with a wide variety of biopolymer side chains using a variety of specific geometries. The case is becoming clear that transition metals are employed in regulatory and metabolic circuitriesof the cell; their functional roles go well beyond catalytic widgets or a type of ionic glue that helps hold together various biopolymers.

A number of discoveries have led the biomedical research community to examine more deeply the chemical biology of transition metals. Evidence of the pressure to understand the mechanisms of metal homeostasis at the molecular level can be seen in three collective advances in the field. First is the realization that approximately 30% of the known protein-encoding genes in human and microbial genomes correspond to transition-metal-dependent proteins.[2,3] Second, the number of studies showing disruptions of metal metabolism associated with human diseases is significant and growing.[4–9] Finally, as previously mentioned, it is clear that intracellular concentrations of metals, such as zinc and iron, are not negligible but in fact are routinely maintained at much higher levels.[1] In order to accomplish this task, a host of cellular machinery is needed to sort out and allocate these reactive species to the appropriate address in the cell. These insights, as well as the linkage of metal physiology to toxicology,[10,11] neurological disorders,[12–16] and metabolic[4,6,7] and infectious diseases,[17–20] underscore the imperative to establish the fundamental principles governing cellular transition metal ion regulation. Finally, a significant number of other connections between human health and fundamental aspects of metalloregulation have emerged in the past few years.[21–40]

In this article, we highlight a few of the emerging themes in the field of inorganic physiology and as such our account is neither comprehensive nor complete. As an introduction to the field, we selected a few key unanswered questions: how do cells control the overall metal economy for a given growth condition, differentiation state, or various stages in host–pathogen conflict? What are the common principles involved in cellular metal sensing, allocation, uptake, storage, and processing? How do the normal metal-trafficking, -sensing, and management processes differ between a baseline and an activated state of any given cell? In order to tackle these challenging questions, researchers use interrogation of the physiochemical mechanisms of the metalloregulatory proteins, metallochaperones, from a diverse array of species including *Escherichia coli*, *Saccharomyces cerevisiae*, *Mus musculus*, and *Homo sapiens*.

2 UNDERSTANDING THE CELLULAR METALLOME

The total intracellular concentration of essential metal ions is referred to as the metallome, a term coined twice in 2001: once to describe the profile of transition metal concentrations in *E. coli* grown under metal replete and depleted conditions,[1] and independently by R.J.P. Williams[41] in an impressive commentary on the future of metallobiology. When the number of metal ions was considered on a cell volume basis for *E. coli* grown under a variety of growth conditions, it became clear that cells maintain tight regulation of the numbers of intracellular metal ions in terms of total metal concentration.[3,42] The idea that other cell types might

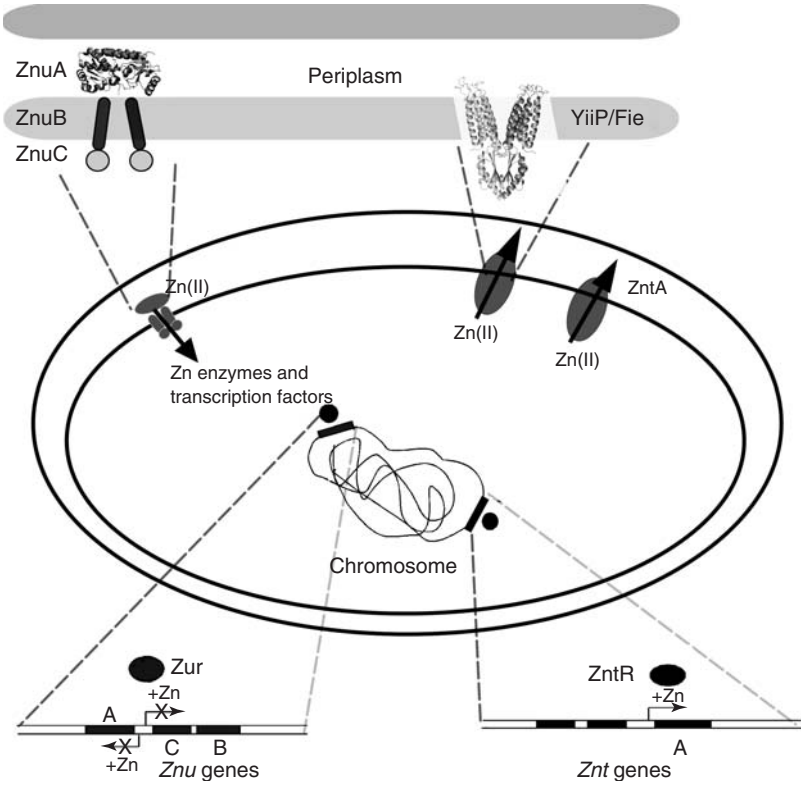

Figure 2 Here, we show a simplified version of an *E. coli* cell which uses both transport proteins (ZnuABC and ZntA) and metalloregulatory proteins (Zur and ZntR) to maintain a steady-state concentration of Zn (II) ions in the cell.[46] Metalloregulatory proteins Zur and ZntR function to repress zinc importer genes (znu genes) and activate zinc exporter genes (znt genes), respectively based on the changing environment of the cell.[47] Both ZnuA and YiiP were crystallized bound to zinc.[48,49] While the YiiP protein has been shown use a proton antiport mechanism to shuttle iron and zinc into the periplasmic space, its regulatory mechanism is unknown.[50] (Image prepared in part by Caryn E. Outten, unpublished.)

also maintain similarly high intracellular metal concentrations is being examined in fungal and mammalian systems as well.[43–45] The question then arises: how does the cell maintain such tight control over the metal economy and keep metal quotas constant in the face of metal shortages and excesses within the growth environment? Some of the factors that regulate the cellular zinc economy in *E. coli* are shown in Figure 2; however, overall regulation is perhaps best understood as a convergence of regulatory networks, structurally specific and energetically tuned metal-trafficking mechanisms, soluble metal receptors, and integral membrane transport systems. Physical characterization of gene regulatory switches has led to some general principles and mechanisms that control metal ion homeostasis in normal and disease states.

3 MOVING METALS ACROSS CELLULAR MEMBRANES

Recent structural characterization of metal transporter proteins has shed light on the movement of transition metals across cellular membranes for both prokaryotes and eukaryotes.[51] First characterized in 1995, eukaryotic zinc transporters shuttle Zn(II) ions across cell membranes and are classified into two families. ZIP (zinc IRT-like protein) and CDF (cation diffusion facilitator) work in opposition to one another, bringing zinc into and out of the cytoplasm, respectively. To date, 14 members of the ZIP family (Zip 1–14) and 10 members of the CDF family (ZnT 1–10) have been identified.[52] Interestingly, malfunctions in the transporters may play a role in diseases such as Alzheimer's disease,[14] type 2 diabetes,[53] and zinc deficiency in breast milk.[54] Owing to the importance of these proteins, researchers have set out to characterize structurally these transmembrane proteins and understand their mechanism of movement. In 2007, the first CDF member YiiP was structurally characterized from *E. coli* as a homodimer in a Y-shaped structure.[55] This protein utilized a highly conserved network of salt bridges at the dimer interface to position the transmembrane α-helices for stable movement of Zn(II) ions across the membrane.[56] Surprisingly, the crystal structure revealed that the portion of this large protein located in the cytoplasm contains a metallochaperone-like fold, which is conserved among many CDF family proteins. Previous

researchers indicated that many metallochaperones such as Atx1, Ccc2, and CopA contain a ferredoxin-like structural fold (βαβ βαβ) and were shown to aid in shuttling transition metal ions in the cytosol.[57] Taken together these findings suggest that these proteins serve two purposes: safely transferring across the membrane and stabilizing the zinc within the cytoplasm of the cell. In-depth reviews of metal transporter proteins and specifically zinc transporters can be found in ***Zinc Transporters and Trafficking in Yeast***.

4 INSIGHTS INTO IRON, COPPER, AND ZINC HOMEOSTASES

4.1 Biological Approach to Discriminate Between Transition Metals

The chemical difficulty in biological regulatory and trafficking machinery is essentially one of metal recognition and binding. Distinguishing between metal ions that often have similar properties such as size and charge is not trivial. This begs the question: how do cells regulate fluctuations in different transition metals? One successful approach has been identifying biopolymers involved in monitoring and/or selecting the correct metal ions. Several groups have focused on identification and molecular characterization of metal receptor proteins that control, regulate, or maintain the cellular metal ion economy.[58] One class of receptor proteins, characterized by the ability to switch on and off gene expression in a metal-dependent manner, are term metalloregulatory proteins (see also ***Metal Specificity of Metallosensors***). Characterization of metalloregulatory receptors is revealing new biological coordination chemistry and thermodynamics and opening new views of cell biology of essential and abundant cofactors that cannot be synthesized or destroyed by cellular machinery. The first two characterized metalloregulatory proteins in prokaryotes were mercury regulatory protein (MerR), which mediates Hg(II)-responsive transcription of mercury resistance genes, and Fur (ferric uptake regulator), which mediates the transcription of iron-responsive genes. Both of these proteins are members of large conserved families of proteins that bind to a specific DNA target and control transcription of the adjacent genes as a function of metal occupancy. MerR-related proteins sense changes in intracellular copper and zinc availability (CueR and ZntR), to activate transcription of a particular set of genes, whereas Fur-related proteins sense changes in transition metals including iron and zinc to repress transcription. A large focus of this book has been placed on the research of metalloregulatory proteins in light of recent advances in both structural and mechanistic understanding of how these proteins function. These metal sensors are utilized to control the transcriptional machinery and achieve specific types of physiological states within the cell. Our increased understanding of how metalloregulators perform these functions is outlined in this book. Additional information on another bacterial transcriptional regulator NikR can be found in ***NikR: Mechanism and Function in Nickel Homeostasis***.

4.2 Cells Maintain Robust Systems to Control Intracellular Homeostasis of Transition Metal Ions

Several teams are working to understand the mechanisms by which cells maintain metal homeostasis at the molecular, structural, and energetic levels. One of the generalizations that have emerged from researchers across the field is that the coordination chemistry of metal-trafficking and regulatory proteins is quite different from that of a major class of their client proteins, namely metalloenzymes. The metalloregultory proteins characterized to date are DNA- or RNA-binding proteins, which exert metal-responsive transcriptional control over a wide variety of genes. These proteins can be separated into two groups: proteins that maintain homeostasis of essential metals (iron, zinc, copper, etc.)[1,59,60] and proteins that detoxify the cell of highly toxic metals (e.g., mercury, lead, or arsenic).[61]

The molecular basis of metal ion specificity and recognition has been delineated in several cases that metalloregulatory proteins use mechanistic aspects of an allosteric control mechanism. Here, allosteric binding refers to a key control element in many biological switches and typically involves a series of subtle conformational changes at a distance from the primary site of interaction.[62] Progress in a variety of metalloregulatory systems reveals the intricate network of communication linked by a binding event at the control site.[63–69] A number of lessons have been learned that connect bacterial inorganic physiology to eukaryotic systems and human physiology. These events are at the heart of metal homeostasis processes in both microbes and humans. Several articles within this book describe new mechanisms for transcriptional control by metalloregulatory proteins (***Metal Specificity of Metallosensors***, ***Metal Homeostasis and Oxidative Stress in Bacillus Subtilis***, ***The Iron Starvation Response in Saccharomyces cerevisiae***, ***NikR: Mechanism and Function in Nickel Homeostasis***, and ***Regulation of Zinc Transport***). Understanding metal transfer by metallochaperone and metal-trafficking proteins is discussed elsewhere, but here we provide a brief overview of some of the emerging general concepts and controversies in this area.

4.3 Metalloregulatory Proteins Differ Structurally from Typical Metalloenzymes

The active sites of intracellular metal-sensing and -trafficking proteins adopt coordination environments that are unprecedented among structurally characterized metalloenzyme active sites. Several novel coordination environments have been characterized in these intracellular trafficking and sensing proteins, and none of these has precedents among

typical metalloenzymes (Figure 3). These are typically low-coordination-number environments that are poised to lower the energetic barrier for metal ion transfer between partner proteins. Conformational changes that occur on docking alter the local steric and electrostatic features of the active site in order to facilitate metal ion transfer. Some of the structurally characterized metal-binding sites are known for zinc metalloregulatory proteins,[70] the metal handling domains of copper[71] and zinc transporters,[72] periplasmic copper-trafficking proteins,[42,73,74] copper metalloregulatory proteins,[75–77] and copper homeostasis proteins.[78,79] In all cases, these proteins selectively bind a narrow subset of transition metal cations with high specificity and do so at tunable chemical potential; in other words, they bind a metal with an affinity that can vary depending on the requirements of the target physiological process. For instance, the zinc uptake regulator (Zur) protein turns off expression of zinc uptake machinery, responds to a lower concentration of free zinc than the ZntR protein, which turns on expression of zinc export proteins (Figure 2 and see the following sections). Likewise, the copper chaperone proteins are fairly selective for Cu(I) and poise the metal center on the surface of the protein, where it is accessible to Cu(I)-binding residues of a docked partner protein, but otherwise shielded from adventitious reaction. As described in the following sections, each of these coordination environments in prokaryotic and eukaryotic proteins is tuned to optimize metal binding, metal discrimination, allosteric conformational changes, and/or triggered metal release.

4.4 Metalloregulatory Proteins Respond to Vanishingly Small Changes in Free Zn(II) Ion and Free Cu(I) Ion Concentrations

The control of free zinc and copper concentrations in cells is a dynamic process that so far has been uncovered through reductionist biochemical studies on metalloregulatory proteins and the transporters they regulate. Several new

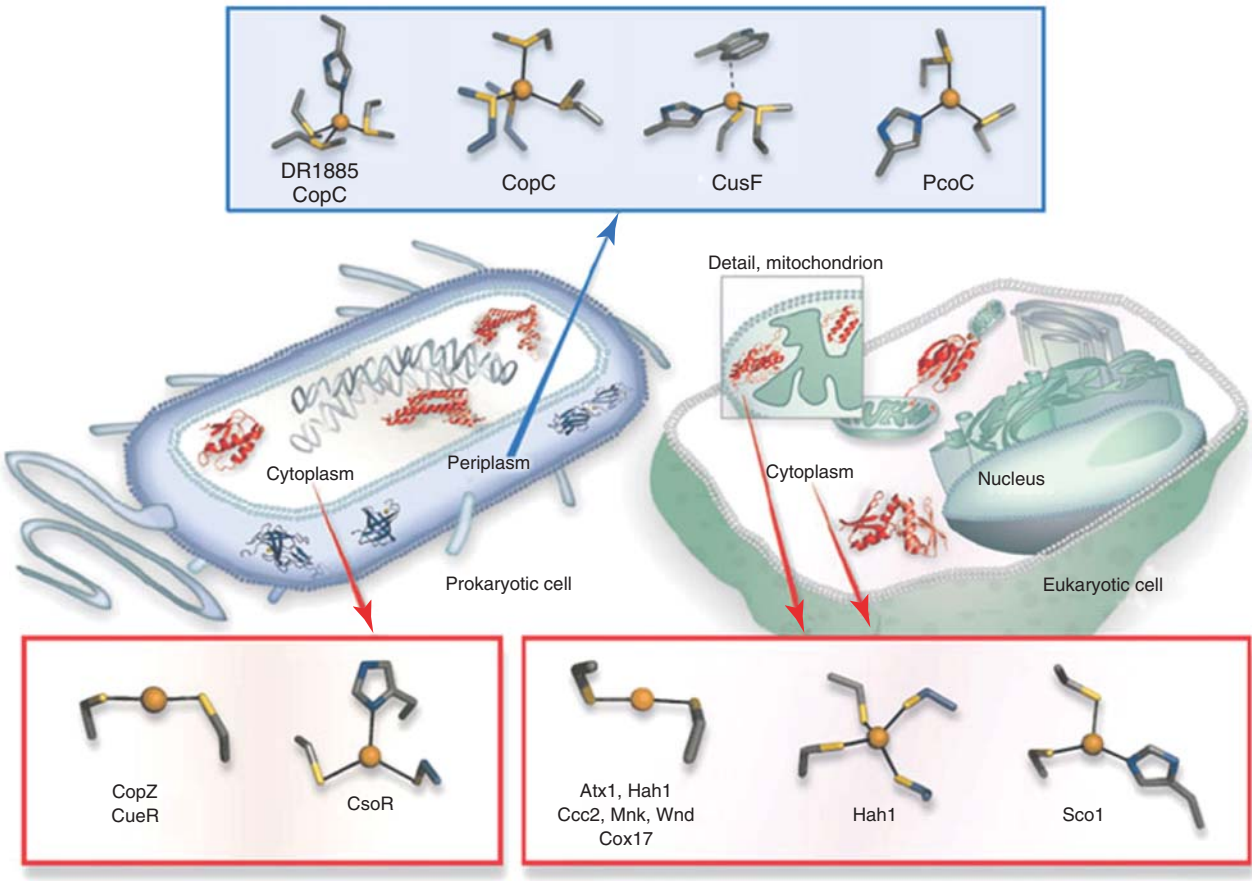

Figure 3 Summary of insights into the copper coordination environments of new metal receptor sites in prokaryotic and eukaryotic cells. Studies from our laboratory revealed structural characterization of the active sites of CueR,[75,76] CusF,[78] PcoC.[73,74] References for each of the other sites can be found in work from Davis *et al*.[77] Intriguingly, there are very few copper-binding domains known in the cytosol of bacteria, and these are all domains of exceedingly sensitive metalloregulatory proteins (CueR and CsoR) or components of export systems (CopZ). (Reproduced from Ref. 77 © Nature Publishing group, 2008.)

copper-responsive metalloregulatory systems have been identified via forward genetic screens and gene deletion studies in *E. coli*: CueR and CusRS. Several lines of evidence support the argument that steady state concentrations of free Cu(I) and Zn(II) ions are vanishingly low in bacterial cytosol.[80–83] Thermodynamic analysis of the metalloregulatory protein thermodynamics of ZntR, Zur, and CueR showed that the dissociation constants for the *E. coli* sensor proteins are in the femtomolar (10^{-15}) and zeptomolar (10^{-21}) ranges for Zn(II) and Cu(I), respectively.[1,42,70,75,76,84] Giedroc and coworkers have shown that the zinc affinities in pathogenic *Synechococcus* PCC7942 SmtB are in the 10^{-11} range, which given the small volume of the cell, formally corresponds to vanishingly few atoms of free zinc.[85] As previously mentioned, the machinery that regulates intracellular levels of Zn(II) ions are controlled at the transcriptional level by two regulatory proteins in *E. coli*: Zur and ZntR (Figure 2). While these protein families have been identified as zinc-specific metalloregulators, many of the details regarding how they regulate genes remain unanswered. Recent work from the Helmann group used site-directed mutagenesis on *Bacillus subtilis* Zur and found that the repression of DNA by Zur occurred in stepwise sequential fashion. Namely, each Zn(II)-binding event afforded a partial change in repression and allowed the protein to respond to a wider range of metal fluctuations.[86] Advances in DNA microarray technologies have led to the expansion of the number of genes regulated by these metalloregulatory proteins.[87–89] Of particular interest in these studies is the identification of a diverse subfamily of Zur-regulated GTPases called COG0523. Interestingly, these proteins may have metallochaperone properties and are conserved in other organisms as well.[90,91] These findings highlight the diversity of metal-regulated processes that are conserved in multiple species.

Extensive thermodynamic studies on Cu(I) affinities of the copper-sensing metalloregulatory proteins Ace/Cuf1 by He and coworkers suggest that the lower limit of available copper in yeast is approximately 5×10^{-21} M.[92] In addition to extremely tight binding, research from the Chen laboratory recently demonstrated that the metalloregulator CueR can switch between acting as an activator or a repressor by facilitating its own ''off switch.'' Using single-molecule FRET, it was determined that a metal-free apo-CueR molecule could quickly substitute for a metallated DNA-bound CueR to turn off transcription.[93] The structures and energetics of these atypical coordination environments (Figure 3) facilitate the extraordinary metal ion sensitivity and selectivity required for efficient management of millions of metal ions confined to exceedingly small and crowded milieu of the bacterial cytosol. Thus, many lines of evidence support the hypothesis that free copper concentrations are vanishingly low in the cytosol of both prokaryotic and eukaryotic organisms alike.[94]

4.5 Metallochaperones Facilitate Exchanges within the Cell to Get the Correct Transition Metal to the Right Site

Throughout this article we have emphasized that the total intracellular concentration of several transition metals are maintained at high levels, and next we make the case that few ''free'' ions are typically at play in the cellular economy. These observations beg the question of how the right metal ion cofactor gets into the right protein? We are far from understanding these phenomena at the molecular level for zinc and iron, but in the cases of copper and nickel, several accessory factors known as metallochaperone proteins facilitate the delivery of the correct metal to the correct protein.[95,96] Metallochaperone proteins function by binding the metal so tightly that concerns about the rate of dissociation are frequently raised. Intriguingly, both tight binding and rapid or facile metal transfer to the correct partner have been shown for the Atx1 protein and its human homolog, Atox1.[97,98] The function of the metallochaperones can be reduced to a series of bind and release events, and conformational changes induced on docking bona fide partner proteins, which provides a low-energy pathway for appropriate metal exchange. Another view is that the tight binding by metallochaperones can afford some protection to the cell. A series of recent elegant studies from the Culotta laboratory suggest that cellular control of the activity of copper, zinc, and superoxide dismutase (SOD1) may influence a number of cellular signaling pathways. In this case, the metallochaperones copper chaperone for SOD1 (CCS) participates in some of these regulatory circuits. Intriguingly, phosphorylation of SOD1 can alter its ability to be loaded with copper by CCS,[99–102] and furthermore SOD1 can alter the fundamental kinase-based pathways that regulate cellular responses to changes in oxygen and glucose availability.[103]

While there is extensive progress in the CCS field, we will briefly focus on chemistry of the Atx1 family of Cu(I) metallochaperones in light of recent reports shedding new light on this system.[57] As seen in Figure 3, protein stabilization of Cu(I) is typically achieved using a two or three coordinate system. Higher coordination numbers are thought to be blocked by steric hindrance of the protein.[96] Researchers have observed that when metallochaperone proteins utilize two cysteines to coordinate a linear Cu(I) ion, nature is capable of binding Cu(I) more tightly than any other divalent cation, except Hg(II).[96,104] These types of observations have led to interesting findings in the process of stabilizing Cu(I) ions in the cell (Scheme 1).

Beginning in the 1990s, researchers have identified a number of cytosolic proteins involved in the Cu(I) chaperone pathways.[95,105] While many of the proteins and domains involved in copper trafficking show a high degree of structural similarity, the literature estimates for the dissociation constant (K_d in Scheme 1) range over 10 orders of magnitude (10^{-5} to 10^{-18} M).[106,107] The early reports of micromolar-range Cu(I)-binding constants, determined by

$$Cu(I)_{free} + Chaperone \rightleftharpoons Cu\text{-}Chaperone \text{ with } K_d = \frac{[Cu(I)][Chaperone]}{[Cu\text{-}Chaperone]}$$

Scheme 1

titration microcalorimetry stand in contrast to a series of later papers that suggest the copper affinity of the Atx1-like domains in metallochaperones and copper ATPases, are at least 10 orders of magnitude tighter.[106,108] Recently, work from the Bertini and Banci laboratories used electrospray ionization–mass spectrometry (ESI-MS) to monitor the amount of metallated and nonmetallated chaperone protein in the presence of a competing ligand DTT (dithiothreitol) as a means of estimating the K_d of Atox1 to $\sim 10^{-14}$ M.[109] Using the known affinity DTT has for Cu(I) ions, this approach provided a uniform measurement for the protein dissociation between proteins in the Cu(I) chaperone pathway. After recalculating the dissociation constants for proteins predicted to be in the Cu(I) chaperone pathway, the group estimated that copper delivery from chaperone to intermediate to enzyme was the result of the favorable free-energy landscape.[109] Affinities measured using competition with bathocuproine disulfonate (BCS) by the Wedd laboratory reported significant differences in the apparent K_d for Cu(I) chaperones ($\sim 10^{-18}$ M).[98] Researchers questioned whether the relative concentration of species for gas-phase data (ESI) was an accurate representation of the true solution equilibrium constant. Using the probe BCS to remeasure the DTT affinity for Cu(I), Wedd suggests that the reference affinities used in the prior ESI-MS experiments were underestimated by a factor of three to four orders of magnitude.[98] It is clear that these reported protein dissociation constants depend heavily on the standards used to measure their affinities. Despite the differences in K_d, the overwhelming consensus is that the two/three cysteine metal binding sites of copper metallochaperone and copper transporter domains provide robust metal binding with Cu(I) dissociation constants in the femto- to attomolar ranges. This high affinity for Cu(I) ensures that Cu(I) is handed off from protein to protein and has a very low probability of dissociating as the free ion.[98] More information on copper transport in cells can be found in ***Structural Biology of Copper Transport***.

Other challenging questions address whether there are differences between cytosolic and compartmentalized pools of free Cu(I) and Zn(II) ions, and whether specialized eukaryotic cells might maintain different degrees of regulation as a function of developmental stage or cell cycle? Research indicates that in resting, or unstimulated cells, the free zinc concentrations in the cytosol may be maintained at quite a low steady-state concentration. This was first suggested based on the extreme thermodynamic sensitivity of the Zur and ZntR proteins described earlier.[1] The issue is not settled and continues to be tested in a variety of metalloregulatory and cellular systems using calibrated fluorescent probes[110] and green fluorescent protein (GFP)-based expression sensors. The latter have been developed in independent studies from both the Eide[52] and Palmer[111,112] laboratories and lead to estimates of cytosolic free [Zn(II)] concentrations that are substantially $<10^{-10}$ M in eukaryotic cells. The free zinc concentrations in subcellular compartments such as the vacuole in yeast or the synaptic vesicles in mammals are a different issue and can be quite high. Fierke, Thompson, and coworkers estimate that the free zinc concentration in mammalian cells is in the picomolar range, which formally corresponds to a few free ions in the mammalian cell, which have volumes as large as 3×10^{-7} mL.[113,114] Taken together, the consensus is that cytosolic free zinc levels in resting cells are quite low, perhaps no more than a few to a few hundred ions at any given time when one considers a dynamic and rapidly established steady-state equilibrium. The work of Lippard, Nakamura, Fierke, Fahrni, and Chang laboratories has developed new types of calibrated fluorescent reporter proteins and vital probes to examine cellular zinc and copper pools.[115–127] As can be seen in ***Fluorescent Probes for Monovalent Copper*** and ***Fluorescent Zinc Sensors***, the advances in probe development have helped elucidate the importance that cells place on maintaining tight regulation of transition metal ions in a wide range of organisms.

4.6 Cellular Management of Iron Pools is Enigmatic

Although free zinc and copper pools seem to be quite small, if present at all, the question of free iron pools in the cell is more controversial. For insights into these issues, we turn to iron-responsive metalloregulatory proteins. Several structures of the bacterial Fur protein are now known; however, a number of puzzling facets about microbial iron physiology remain unanswered. Fur proteins in most organisms are predicted by sequence homology to have at least two metal-binding sites: a structural zinc site rich in cysteine residues and a sensor site containing five or six nitrogen/oxygen amino acid ligands.[128] Interestingly, all of the structurally characterized Fur proteins were isolated bound to zinc, not iron.[129–133] Research from our laboratory and others identified that there is a tightly bound structural Zn(II) ion that is essential for protein folding and repressor activity for many Fur proteins.[128,134] DNA-binding experiments have demonstrated that Fur has several orders of magnitude higher affinity for zinc compared to iron and that zinc-loaded Fur binds DNA with high affinity.[134] On the basis of the DNA-binding assays, researchers suggest that the Fur affinity for Fe(II) ions is in the low micromolar range.[135] This affinity coupled with the prediction that free iron concentrations in the cytosol are in the micromolar range suggests that Fur operates as a micromolar sensor

of iron fluctuations within the cell.[135,136] Unfortunately, a specific Fe(II)–Fur site is yet to be directly characterized by crystallography and mutation of predicted amino acids involved in iron binding in many cases has little effect on Fur repression.[59] While Fur is a global iron regulator in *E. coli* based on the genes it regulates, it is tempting to characterize Fur as an Fe(II) receptor that directly senses changes in iron concentration; however, there are several issues that need to be resolved. We cannot rule out the possibility that Fur responds to fluctuations in more than one transition metal ion, including zinc, in order to control iron uptake systems. Interestingly, *Helicobacter pylori* Fur has been implicated in both on/off switch and rheostat responses within the cell.[137,138] In these studies, *H. pylori* has been shown to respond rapidly to repress the iron uptake gene *frpB*, while simultaneously autoregulating the expression of the *fur* gene itself. Research on *E. coli* Fur has shown that the number of Fur molecules within the cell is maintained at high level (~5000 per cell) and doubles on oxidative stress.[139] The increasing number of *E. coli* Fur molecules suggests that Fur likely responds as a rheostat; *E. coli* uses changes in Fur expression to combat environmental changes. Taken together, these observations suggest that there are multiple types of Fur-regulated responses across bacterial species. Further studies are required to elucidate mechanisms of specific Fur protein family members.

Bacterial iron has been studied in iron–sulfur clusters, which participate in electron transfer, iron/sulfur storage, gene regulation, and enzyme activity.[140] In addition to Fur, iron–sulfur clusters provide an additional cellular response to iron starvation and oxidative stress. The regulatory mechanism for an iron–sulfur cluster assembly is quite complex; however, recent work from the Outten laboratory outlined a novel sulfur transfer pathway (the *suf* operon) for the Fe–S cluster assembly under iron starvation and oxidative stress.[141–143] Future research into the *suf* and other iron–sulfur assembly pathways will lead to a better understanding of the many processes that iron contributes to in the cell. Further details on bacterial and eukaryotic Fe–S assembly can be found in ***Fe–S Cluster Biogenesis in Archaea and Bacteria***.

Significant new structural and biochemical insights into eukaryotic iron physiology have emerged in studies of iron-dependent regulation of translation by iron regulatory proteins (IRPs).[144–147] Furthermore, a number of additional breakthroughs in eukaryotic iron trafficking come from studies providing evidence for two types of iron chaperones.[148–150] The low affinity, protein partnerships, and client proteins remain open issues and are examined in more detail in ***Iron and Heme Transport and Trafficking***.

5 ROLE OF TRANSITION METALS IN DIFFERENTIATION AND DEVELOPMENT

Temporal fluctuations in the total metal content and availability of metals within cells can play a significant regulatory role in differentiation and development. Significant changes in metal content in some differentiated cells are directly relevant in human disease states such as diabetes[43] as well as key steps in developmental biology.[44,45] By analyzing changes in metal content during human egg oocyte maturation and fertilization, we discovered an unexpected role for zinc fluxes in controlling embryonic development. We determined the metallome of individual mouse oocytes[45] and subsequently showed that intracellular zinc levels increased by ca. 20 billion zinc ions in the last 12 hours of oocyte maturation, representing a 57% increase in total zinc. On fertilization, the egg initiates a systematic exocytosis of zinc, which we have termed the ''zinc spark,'' a phenomenon referring to the coordinated cellular exocytosis of zinc.[44] Both the zinc uptake step and the fertilization-induced rapid zinc exocytosis step are essential for normal embryo development. These findings further support the importance of maintaining the transition metal quota of a single cell to higher order eukaryotic species.

6 HIGH METAL QUOTAS IN SPECIALIZED CELLS: PATHOGENS THAT STAND OUT

A variety of infectious organisms must create and maintain unusually high metal quotas to achieve pathogenesis. Control of the metallome is a key battleground in the fight between human host and pathogen. While the metallomes of only a few pathogens have been described to date, in several cases, extraordinary changes in the metallomes of *Candida ablicans*,[151] *Cryptococcus neoformans*,[152] and *Plasmodium falciparum*[153] have been shown to be essential to the infectious process. In each case, the pathogens have been shown to sequester high levels of a specific essential metal, achieving levels that are far higher than their nonpathogenic relatives. Future work will be geared toward understanding the physiological advantage that such a distortion provides to the pathogen and perhaps the discovery of agents that selectively inhibit pathogen-specific metal acquisition processes. Other areas of future interest include analysis of metal regulation pathways as potential targets for potent anticancer and anti-angiogenesis drugs.[79] More information highlighting advances in understanding of pathogenicity and immunology can be found in ***Metals in Bacterial Pathogenicity and Immunity***.

7 CONCLUDING REMARKS

The importance of inorganic chemistry is becoming better understood across the board within the biological

community. As general principles of metal ion physiology, metal receptor structure and energetics, and the cell biology of metals are brought into focus so is an appreciation for the role of transition metal ions. Recent results, summarized in the subsequent articles of this book, argue that a series of important and disruptive biomedical concepts are emerging as the inorganic chemistry of the cell is being rethought and thoroughly uncovered. It is clear that an extensive amount of the genome and cellular machinery is dedicated to manage a complex array of essential metal ions. What is not yet clear is how all of these pieces work, individually or as an ensemble. This book provides an overview of key recent insights that will guide the next stage of inquiry.

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9 ABBREVIATIONS AND ACRONYMS

BCS = bathocuproine disulfonate; CCS = copper chaperone for superoxide dismutase 1; CDF = cation diffusion facilitators; DTT = dithiothreitol; ESI-MS = electrospray ionization–mass spectrometry; Fur = ferric uptake regulator; GFP = green fluorescent protein; ICP-MS = inductively coupled plasma–mass spectrometry; IRP = iron regulatory protein; MerR = mercury regulatory protein; SOD1 = superoxide dismutase; ZIP = zinc IRT-like proteins; Zur = zinc uptake regulator protein.

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PART 2
Probing Metals and Cross Talk in the Metallome

The Metallome

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1 INTRODUCTION

The term ''metallome'' has been introduced by analogy with the genome and proteome and refers to the complete set of metal ions in a cell.[1] An alternative definition of the metallome encompasses metalloproteins and any other metal-containing biomolecules that organisms utilize.[2] Studies of the metallome, often referred to as metallomics, is a growing area of research focused on understanding the utilization and roles of biometals and metal-containing molecules in different organisms. Except for sodium, potassium, calcium and magnesium that are required in large quantities, these metals belong to the group of trace elements, which include zinc (Zn), iron (Fe), copper (Cu), manganese (Mn), molybdenum (Mo), tungsten (W), nickel (Ni), cobalt (Co), chromium (Cr), vanadium (V), and several other metals. Although these trace metals are needed in small quantities, they are involved in critical enzymatic activities and physiological mechanisms.[2,3] Their deficiency, overload, or variations in genes involved in metal homeostasis and utilization may result in metabolic abnormalities or even death.[4,5]

Among all known trace metals, Zn and Fe are considered to be essential for all organisms.[6–8] Other metals (such as Cu, Mn and Mo) are used by some, but not all, organisms. The molecular mechanisms by which cells regulate bioavailability and utilization of metals (metal homeostasis) represent a rapidly developing research area which includes the mechanisms and control of the uptake, transport, storage and utilization of metals.[9] Specific transport systems have been characterized for many trace metals and can be divided into ATP-binding cassette (ABC) transport systems and non-ABC transporters.[10–15] In addition, nonspecific cation channels may also serve as an alternative path for cellular entry of trace metals.[16,17] On the other hand, accumulation of inappropriate amounts of certain metals in the cell may result in metal overload and toxicity. Thus, cellular processes that are involved in the detoxification of metals and thus tolerance to metal stress are needed (e.g., metallothioneins for heavy metal binding and CopA for Cu export).[18–20] In addition, crosslinks between different metal utilization have been observed.[21] Therefore, metal homeostasis should be carefully maintained to offer sufficient but not toxic levels of metal ions for biological processes. Most metals are directly inserted into metalloproteins, whereas some initially form metal-containing cofactors or complexes (e.g., molybdopterin for Mo and vitamin B_{12} for Co) before their incorporation into target proteins.[22,23]

The past decade has witnessed dramatic advances in genomics and high throughput analyses in various

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experimental systems, which have provided an opportunity to investigate the metabolism of metals. Previous studies of metalloproteins and other proteins involved in metal homeostasis have revealed the complexity of metal utilization. It is obvious that the identification of all metalloproteins in different genomes may help scientists better understand the utilization and function of metals. However, owing to the lack of reliable approaches and difficulty in analyzing metals in a high throughput manner, it is currently not possible to identify the complete set of metalloproteins (metalloproteome) in most organisms. Recently, initial attempts have been made at a genome-wide level by applying bioinformatics and ionomics [such as inductively coupled plasma mass spectrometry (ICP-MS)] analyses.[24–33] These studies have improved our understanding of the metabolism of trace metals in currently living organisms in the three domains of life. Here, we focus on several essential metals and discuss recent progress on computational and comparative genomics analyses to achieve a more integrated and system-level picture of how organisms manage these metals. We also discuss the recent contributions to ionomics studies that examined trace metals in several model organisms.

2 COPPER

Cu is required by a wide range of species, from bacteria to yeast, plants, and mammals. It plays an important role as an essential cofactor in several fundamental metabolic processes, including photosynthetic and mitochondrial electron transport, oxidative stress responses, and hormone perception.[34] This metal can exist in either Cu(I) or Cu(II) state and is highly toxic because of its ability to produce radicals by shifting between the two redox forms.[35] Thus, Cu-dependent organisms have developed a conserved and complex network of proteins to handle Cu in order to prevent its deficiency and to avoid its toxic effects.

2.1 Copper Homeostasis

The strategies that organisms use to maintain Cu homeostasis include the regulation of Cu uptake in cells, Cu trafficking via P-type ATPases and Cu chaperones, and regulation of the levels of Cu proteins in response to changes in metal availability. In prokaryotes, the mechanisms governing Cu homeostasis are only partially understood. So far, no high affinity Cu uptake system has been identified in the majority of bacteria.[36] Very recently, a Cu chelator, methanobactin, has been found to mediate Cu acquisition from the environment in the methanotroph *Methylosinus trichosporium*.[37] However, such a machinery is specific for methanotrophic bacteria. To date, several Cu-related transport and resistant proteins have been characterized in various organisms, including CopA/PacS/CtaA, CusCFBA, and PcoABCDRSE.[36,38,39] Figure 1a shows the most relevant Cu homeostatic systems in *Escherichia coli*, which is among the best-studied organisms with regard to Cu trafficking.

In *E. coli*, the major component of Cu homeostasis is a Cu(I)-translocating P-type ATPase named CopA, which serves as an exporter for removing Cu(I) from the cytoplasm.[36,38] CopA belongs to the superfamily of heavy metal pumping CPx-type ATPases.[40] These proteins contain a conserved intramembranous cysteine-proline-cysteine (Cys-Pro-Cys) motif, which is involved in high-affinity Cu binding.[41] In cyanobacteria, two additional CopA homologs, named PacS and CtaA, are also required for Cu resistance and transport into the thylakoid, where the Cu proteins plastocyanin and cytochrome oxidase reside.[42,43] Studies have found that PacS is mainly located in the thylakoid membrane and likely plays an important role in Cu transport into thylakoid (thylakoid import), whereas CtaA is found to be involved in Cu import from the periplasm (cell import).[44] Very recently, the CopRS two-component system was also found to be essential for Cu resistance and thylakoid transport in cyanobacteria.[45] In *Enterococcus hirae*, two CopA homologs, named CopA (Cu importer) and CopB (Cu exporter), were reported.[39] Besides, many organisms contain a small protein, CopZ, which acts as a Cu chaperone.[46] CopZ can interact with and deliver Cu to cytoplasmic metal-binding domains (MBDs) of CopA. CopZ may also transfer Cu ions to the transmembrane metal-binding sites of CopA lacking MBDs.[47] In *E. hirae*, CopZ specifically donates Cu(I) to the cytoplasmic Cu repressor CopY, thereby releasing its bound Zn and abolishing repressor–DNA interaction, which in turn induces the expression of the *copY-ZAB* operon.[48] Recently, a stress response protein Gls24 was reported to interact with CopZ and may be also involved in Cu homeostasis in *E. hirae*.[49] A CopZ homolog, named Atx1, was found to be widely present in eukaryotes.[50] In *Saccharomyces cerevisiae*, Atx1 is required for the transport of Cu(I) into the trans-Golgi network, which delivers Cu to the Cu(I)-transporting P-type ATPase Ccc2.[51] Besides, a new Cu-binding metallothionein MymT was found to serve as a chaperone involved in CopA-related Cu(I) detoxification in several pathogenic mycobacteria.[52]

Another four-component Cu efflux pump, the CusCFBA system, was exclusively found in Gram-negative bacteria. This system includes CusA (the inner membrane substrate-binding pump), CusB (the periplasmic protein), CusC (the outer membrane channel), and CusF (a small periplasmic protein that binds Cu(I) may subsequently transport to CusB).[53] In *E. coli*, the *cus* genes are present in one operon, which is required only under conditions of extremely high Cu amount and is important under anaerobic conditions. These genes are regulated by the CusRS two-component signal transduction system, which encodes the histidine kinase CusS and the response regulator CusR.[54] The Cus system is also responsible for silver resistance in *E. coli*.[55] The recently solved cocrystal structure of the CusBA complex

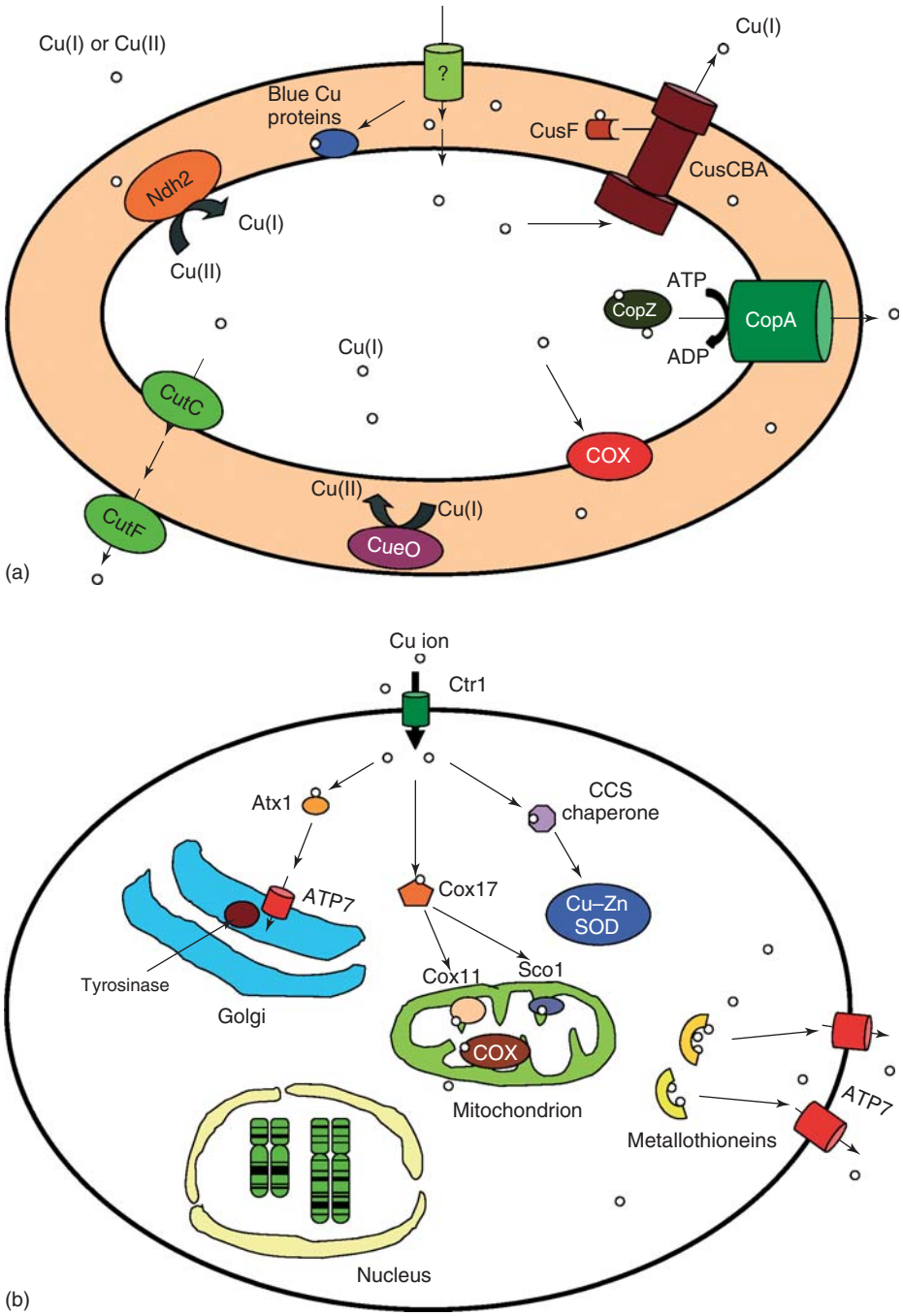

Figure 1 Schematic view of Cu homeostasis. (a) Cu homeostasis in *E. coli*. CopA, the Cu(I)-translocating P-type ATPase; CusCFBA, the four-component Cu efflux system; Ndh 2, a cupric reductase; CueO, a multicopper oxidase; CutC and CutF, two proteins involved in Cu efflux and/or homeostasis; CopZ, a Cu chaperone involved in Cu export; COX, cytochrome *c* oxidase. (b) Cu homeostasis in *D. melanogaster*. Atx1, CCS, and Cox17, Cu chaperones involved in various pathways; Ctr1, eukaryotic Cu importer; ATP7, eukaryotic Cu exporter (also involved in Cu transport to Golgi); COX11 and Sco1, two proteins involved in cytochrome *c* oxidase assembly; and Cu–Zn SOD, Cu–Zn superoxide dismutase

has revealed a $CusC_3$-$CusB_6$-$CusA_3$ model in which CusA, present as a trimer, interacts with six CusB protomers and that the periplasmic domain of CusA is involved in these interactions.[56]

Some strains of *E. coli* possess additional plasmid-encoded genes that confer Cu resistance, which include *pcoABCDRSE*.[38] The multicopper oxidase PcoA and PcoC are exported to the periplasm; PcoB is a putative outer membrane protein; PcoD might transport Cu across the cytoplasmic membrane; PcoE is a Cu-binding periplasmic protein; and PcoRS forms a two-component regulator that senses excess Cu and is required for the expression of *pco* genes.[38] Some other proteins have also been associated with Cu resistance in *E. coli*, such as the products of *cutABCDEF* genes.[57] CutC was shown to be induced late during Cu exposure and can modify intracellular Cu content.[58] The recent crystal structure of human CutC homolog suggests that this protein may function as a Cu-binding enzyme.[59]

Cu homeostasis in eukaryotes is shown in Figure 1b. Cellular acquisition of Cu in these organisms is primarily accomplished through a high affinity copper transport (Ctr) family.[60] Organisms may have multiple Ctr proteins located in different biological membranes. In *S. cerevisiae*, three Ctr proteins (yCtr1–yCtr3) have been identified.[61] yCtr1 and yCtr3 are localized to the plasma membrane, while yCtr2 protein is present in the vacuole membrane and may mobilize stored Cu to the cytoplasm when extracellular Cu is limited.[62] Humans possess two Ctr proteins, hCtr1 and hCtr2. hCtr1 is the main Cu importer, which is located predominantly at the plasma membrane and may also be present in vesicular compartments, whereas hCtr2 is localized in late endosomes and lysosomes and facilitates cellular Cu uptake.[63,64] The extracellular N-terminal regions of both human and yeast Ctr1 contain multiple methionine residues (Met-rich motifs) that are important for the acquisition of Cu(I) ions.[65,66] These motifs comprise combinations of Met residues arranged in clusters of MXM and MXXM.

Cu export in eukaryotes is mediated by a highly conserved PIB-type ATPase family, ATP7, which is homologous to bacterial CopA proteins.[67] Mammals possess two ATP7 proteins: ATP7A and ATP7B.[68] Cu-induced trafficking of ATP7A and ATP7B from the trans-Golgi network toward the plasma membrane is critical for their role in Cu homeostasis.[69] *Drosophila melanogaster* has a single DmATP7 protein, which is essential for efflux of excess Cu.[70] It was found that DmATP7 is able to functionally compensate for the absence of ATP7A, with important trafficking motifs conserved in these distantly related orthologs.[71] Yeasts possess an ATP7 ortholog Ccc2, which pumps Cu from the cytosol to the Golgi lumen. A recent study has shown that two serine residues (Ser258 and Ser971) may control sequential steps during catalysis of Ccc2 through different mechanisms.[72]

2.2 Cuproproteins

A number of cuproproteins have been discovered and characterized, which contain one or more Cu ions as prosthetic groups (Table 1). The metal sites in these proteins can be classified into several types based on their structural and spectroscopic properties. Type-1 Cu (or blue Cu) sites are mainly found in small electron transfer proteins (cupredoxins) that ferry electrons between larger enzymes such as components of the denitrification pathway and photosynthesis. It shows strong visible absorption at near 600 nm and hyperfine splittings in the electron paramagnetic resonance (EPR) spectroscopy, and the Cu atom is coordinated by a Cys and two histidine (His) residues in a trigonal planar structure. Type-2 Cu does not give evident absorption and shows a weaker signal with larger A values in the EPR spectrum, which are clearly distinct from type-1 site spectra. Most type-2 sites exhibit a square planar coordination by N or N/O ligands. Type-3 Cu could be detected by neither strong absorption nor EPR spectroscopy. These sites consist of a pair of Cu centers, each coordinated by three His residues. Some cuproproteins, such as multicopper oxidases (MCOs), contain multiple types of Cu sites.

Type-1 Cu proteins are a group of small proteins that contain single type-1 Cu center and show similar three-dimensional structures.[73] They play important roles in the photosynthetic and the respiratory electron transport chains in cyanobacteria, green algae and higher plants. Plastocyanin is

Table 1 Cuproproteins

Prokaryotes	Eukaryotes
Plastocyanin family	Plastocyanin family
Plastocyanin	Plantacyanin family
Amicyanin	Plantacyanin
Pseudoazurin	Mavicyanin
Halocyanin, etc.	Umecyanin
Azurin family	Stellacyanin, etc.
Azurin	Cytochrome *c* oxidase subunit I
Auracyanin	Cytochrome *c* oxidase subunit II
Rusticyanin	CuZn superoxide dismutase
Nitrosocyanin	Cu amine oxidase
Cytochrome *c* oxidase subunit I	Dopamine β-monooxygenase
Cytochrome *c* oxidase subunit II	Peptidylglycine R-hydroxylating monooxygenase
Nitrous oxide reductase	Multicopper oxidase family
NADH dehydrogenase 2	Laccase
Cu–Zn superoxide dismutase	Hephaestin
Cu amine oxidase	Ceruloplasmin
Particulate methane monooxygenase	Fet3p
Multicopper oxidase family	Ascorbate oxidase, etc.
Nitrite reductase	Hemocyanin
CueO	Tyrosinase
CotA	Cnx1G
PcoA	Galactose oxidase
Laccase	
Bilirubin oxidase	
Phenoxazinone synthase, etc.	
Tyrosinase	

the best-studied type-1 Cu protein. In photosynthesis, plastocyanin acts as a soluble carrier transferring electrons between the two membrane-embedded complexes cytochrome b_6f and photosystem I.[74] Moreover, plastocyanin was the first blue Cu proteins to be characterized by X-ray crystallography. Crystal and NMR solution structures of several plastocyanins from different organisms such as plants and algae have revealed that this protein has an eight-stranded Greek-key beta-barrel fold and that the Cu-binding site is generally conserved and contains two His, one Cys, and one Met.[75] Other blue Cu proteins include azurin, pseudoazurin, amicyanin, rusticyanin, plantacyanin, auracyanin, nitrosocyanin (the red Cu protein), and several other proteins. In addition, type-1 Cu center is found in some larger cuproproteins, such as nitrite reductase (NiR), which catalyzes the reduction of nitrite to nitric oxide, and a variety of MCOs (such as laccase, ascorbate oxidase, CueO, PcoA, hephaestin, ceruloplasmin, Fet3p, etc.) that function in intramolecular electron transfer.[73,76] These MCO proteins are enzymes which oxidize their substrate by accepting electrons at a mononuclear Cu center and transferring them to a trinuclear Cu center. Some MCOs (such as mammalian ceruloplasmin and yeast Fet3p) are ferroxidases, whereas laccases derive electrons from the oxidation of phenolic compounds (e.g., lignin). The majority of MCOs have four Cu centers: a type-1 Cu and a mixed Cu center containing a type-2 and two type-3 Cu atoms.

Cytochrome *c* oxidase (COX) and nitrous oxide reductase (N_2OR) have a binuclear Cu center (Cu_A), which is a variant of type-1 Cu. COX is a member of the heme Cu oxidase (HCO) superfamily. HCOs function as the terminal enzymes in the respiratory chain of mitochondria and aerobic prokaryotes, coupling molecular oxygen reduction to transmembrane proton pumping.[77] The two major subgroups of HCOs include COX and quinol oxidase.[78] Both groups have several catalytic subunits, and subunit I contains two heme centers: the first of these, low-spin heme a, is thought to act as an electron input device to the second heme a_3, and the high-spin heme a_3 is part of the binuclear center, with a Cu atom (CuB), and is the site of dioxygen reduction. However, COX subunit II contains the Cu_A center with two Cu atoms, whereas quinol oxidase subunit II loses the Cu_A center.[79] The HCO superfamily comprises the aa_3-, ba_3-, and cbb_3-type oxidases along with other possible subfamilies. N_2OR is an important enzyme owing to the environmental significance of the reaction it catalyzes: reduction of nitrous oxide. It carries six Cu atoms. Two of them are arranged in the COX-like binuclear Cu_A site, and four make up the catalytic center, named Cu_Z. The structure of the Cu_Z center, which is a unique tetranuclear Cu center, has opened a novel area of research in metallobiochemistry.[80]

Type-2 Cu-containing proteins include CuZn superoxide dismutase (CuZn SOD), Cu amine oxidase (CuAO), peptidylglycine R-hydroxylating monooxygenase (PHM), and dopamine β-monooxygenase (DBM):[81] (i) CuZn SOD is widespread in all three domains of life and has been especially well studied in eukaryotes, including yeast and humans. Cu is coordinated by four His residues in its oxidized form. (ii) CuAOs are ubiquitous enzymes, which belong to the quinoprotein family and play a vital role in the physiology and pathology of mammals by controlling the metabolism of various primary monoamines, diamines, and polyamines of endogenous or xenobiotic origin. CuAOs possess two cofactors: a tightly bound Cu(II) and a quinone residue, which catalyzes the oxidative deamination of primary amines with concomitant production of aldehyde, ammonia, and hydrogen peroxide. Crystal structures of CuAO from different organisms revealed that the Cu atom is coordinated by three His residues and two water molecules.[82] (iii) PHM catalyzes the stereospecific hydroxylation of the glycine α-carbon of all peptidylglycine substrates, which is essential for the activation of a variety of hormones by R-amidation, thereby improving hormone–receptor affinity. (iv) DBM converts dopamine to norepinephrine during catecholamine biosynthesis and the hydroxylation of dopamine is at the β-carbon. Since PHM is homologous to DBM, they likely evolved from a common ancestor.[83]

Other cuproproteins include tyrosinase, hemocyanin, galactose oxidase (GAO), particulate methane monooxygenase (pMMO), Cnx1G, and NADH dehydrogenase 2 (Ndh2): (i) Tyrosinases are widely distributed in nature. They are essential enzymes in melanin biosynthesis and, therefore, are responsible for pigmentation of skin and hair in mammals, where two more enzymes, the tyrosinase-related proteins (Tyrp1 and Tyrp2), participate in the pathway.[84] This family also includes catechol oxidase and polyphenol oxidase whose activities are similar to that of tyrosinase. The active site of tyrosinase is a type-3 Cu center consisting of two Cu atoms, each coordinated by three His residues. (ii) Similar to tyrosinase, hemocyanin also belongs to the type-3 Cu protein family. These proteins are found in the hemolymph of some molluscs (e.g., octopus) and arthropods (e.g., crab) and are extracellular oxygen carriers responsible for the oxygen transport from the respiratory organs to tissues.[85] (iii) GAO has been extensively studied; it is a Cu metalloenzyme containing a novel protein-derived redox cofactor in its active site, formed by cross-linking two residues, Cys and Tyr.[86] (iv) pMMO is an integral membrane cuproprotein found in methanotrophic bacteria, which catalyzes the conversion of methane to methanol. Recent advances, especially the first crystal structure of pMMO, have revealed the presence of three metal centers (one mononuclear Cu, one dinuclear Cu, and one Zn), providing new insights into the molecular details of biological oxidation of methane.[87] (v) Cnx1G is an enzyme involved in the biosynthesis of the Mo cofactor in plants. The crystal structure of Cnx1G revealed the remarkable feature of a Cu ion bound to the dithiolene unit of a molybdopterin intermediate, which provides a molecular link between Mo and Cu metabolism.[88] (vi) The Cu(II)-reductase Ndh2 contributes to bacterial oxidative protection and Cu homeostasis.

In *E. coli*, it is a membrane-bound flavoprotein linked to the respiratory chain and is involved in hydroperoxide-induced oxidative stress.

2.3 Bioinformatics Studies on Copper Utilization and Cuproproteomes

In recent years, bioinformatics studies, especially comparative genomics analysis, have been conducted to characterize important features of Cu utilization and cuproproteomes (the whole set of cuproproteins) in a variety of organisms.[28,89–93] On the basis of metal-binding patterns of metalloproteins derived from the PDB database, a computational method was developed that searched for cuproproteins in several organisms via a set of Cu-binding patterns.[89] To decrease the false positive rate in this approach, additional searches integrated with domain recognition methods were used to examine the cuproproteomes in almost 60 sequenced genomes in prokaryotes and eukaryotes.[91,93] It was found that the size of the cuproproteome is generally less than 1% of the total proteome. The majority of cuproproteins are likely to be involved in a network that is crucial for Cu utilization and homeostasis. The speciation of prokaryotic organisms appeared to only slightly affect the ancestral cuproproteome, whereas eukaryotes may have expanded their ancestral cuproproteome by either evolving new Cu domains or reusing old domains for new functions.

Recently, several comprehensive comparative analyses revealed a more clear view of Cu utilization in the three domains of life.[28,94] By identifying all known Cu transporters and solely Cu-dependent proteins, occurrence of the Cu utilization trait was examined in hundreds of organisms. The distribution of Cu-dependent and Cu-independent organisms is shown in Figure 2. In bacteria, approximately 80% of sequenced organisms were found to be Cu-utilizing. However, all or almost all organisms in some bacterial phyla (such as *Mollicutes*, *Chlamydiae*, and *Spirochaetes*) lacked known cuproproteins. In contrast, only half of the organisms appeared to utilize Cu in archaea. The major Cu exporter, CopA, was found to be widespread in bacteria and was the only Cu transporter detected in archaea. The occurrence of other Cu transporters was limited, such as the Cus system exclusively detected in Gram-negative bacteria.[94] Some organisms were found to possess multiple copies of certain Cu transporters, such as *Acidovorax sp*. JS42 (10 Cu exporters), which was isolated from nitrobenzene-contaminated sediment. Besides, many organisms, including those that lack known cuproproteins, appeared to have Cu exporters, implying that the processes of Cu utilization and detoxification are independent and that many organisms may protect themselves against Cu ions that inadvertently enter the cell. Among known cuproproteins, COX I, COX II, and MCOs were the most frequently used cuproproteins in bacteria and archaea. In contrast, the utilization of pMMO, nitrosocyanin, CuAO, and tyrosinase appeared to be quite limited. Some bacterial cuproproteins, such as azurin, nitrosocyanin, Ndh2, pMMO, and tyrosinase, were not detected in archaea. Further investigation of the cuproproteomes revealed that large cuproproteomes were mainly found in proteobacteria. *Sinorhizobium medicae* and *Sinorhizobium meliloti* contained the largest bacterial cuproproteomes (22 cuproproteins). In archaea, *Haloarcula marismortui* (Euryarchaeota/Halobacteriale) had the largest prokaryotic cuproproteome (25 cuproproteins, half were plastocyanin homologs).[94]

In eukaryotes, almost all sequenced organisms utilized Cu. Distribution of the Cu importer Ctr1 and exporter ATP7 was consistent with that of the Cu utilization trait. Although the majority of organisms possessed one to three *ctr1* genes, nematodes had many; for example, *Caenorhabditis elegans* had 11 *ctr1* genes.[94] It is possible that these Ctr1 proteins are located in different membranes and/or expressed in cell types for Cu trafficking. On the other hand, *Phytophthora* species (belonging to Oomycetes), which are crop plant pathogens, had the highest numbers of ATP7 (i.e., six ATP7 genes in *Phytophthora infestans*). With regard to the cuproproteins, almost half of prokaryotic cuproproteins could not be detected in eukaryotes; however, additional cuproproteins have evolved in eukaryotes, such as plantacyanin, PHM, hemocyanin, and GAO. Among

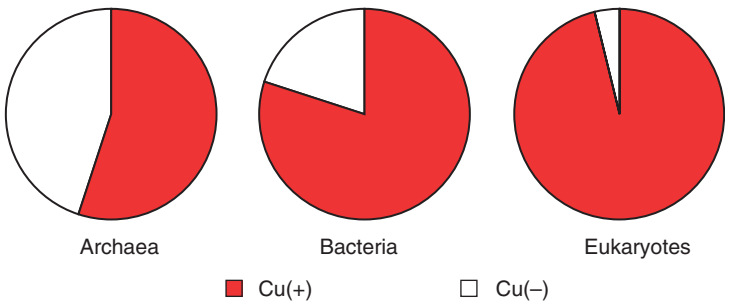

Figure 2 Occurrence of Cu utilization in the three domains of life. Proportion of Cu-dependent organisms among all organisms with sequenced genomes. Organisms were classified into two groups: Cu(+): Cu-dependent; Cu(−): Cu-independent

them, MCOs, COX I, COX II, and CuZn SOD were the most abundant cuproproteins. The largest cuproproteomes in eukaryotes were found in land plants (62 and 78 cuproproteins in *Arabidopsis thaliana* and *Oryza sativa*, respectively).[94] The majority of these cuproproteins are plantacyanin, CuAO, and MCO proteins, suggesting important roles of these families in plant metabolism. Interestingly, aerobic organisms use utilized Cu and have larger cuproproteomes, whereas the majority of anaerobic organisms do not.[28,30] This may suggest that proteins evolved to utilize Cu following the oxidizing environmental conditions.[92]

3 MOLYBDENUM

The transition-metal Mo is of essential importance for many living organisms as it is required by Mo-dependent enzymes (molybdoenzymes) catalyzing several important reactions in the metabolism of carbon, nitrogen, and sulfur compounds.[22,95–97] With the exception of bacterial nitrogenase where Mo is a constituent of the FeMo cofactor, Mo is bound to a pterin, forming the Mo cofactor (Moco), which is the active center of all other molybdoenzymes. A few thermophilic archaea utilize W that is also coordinated by pterin (Wco).[95,98] Moreover, W can be specifically transported into some prokaryotic cells by selective ABC-type of transporters and even becomes an essential element for nearly all enzymes of the aldehyde:ferredoxin oxidoreductase (AOR) family.[95,99,100]

3.1 Molybdenum Uptake and Molybdenum Cofactor Biosynthesis

Organisms take up Mo in the form of the molybdate anion (see ***Mo Cofactor Biosynthesis and Crosstalk with FeS***). This process requires specific uptake systems to scavenge molybdate in the presence of competing anions. In both bacteria and archaea, several high affinity ABC-type transporters have been described. ModABC was the first identified Mo transport system, comprising ModA (periplasmic molybdate-binding protein), ModB (membrane integral channel), and ModC (cytoplasmic ATPase).[101] In many bacteria such as *E. coli*, the *modABC* operon expression is controlled by ModE regulator.[102] *E. coli* ModE functions as a homodimer with two distinct domains, an N-terminal DNA-binding domain (ModE_N) and a C-terminal molybdate-binding helix-turn-helix domain, which contains a tandem repeat of the Mo-binding protein (Mop) domain (Di-Mop domain). Two additional Mo/W ABC transport systems, WtpABC (Mo and W) and TupABC (W-specific), were also found.[29,99,103] The regulation of these two transporters is unclear. In the absence of the high affinity molybdate transporter, molybdate may also be transported by another ABC transporter, which transports sulfate/thiosulfate as well as by a nonspecific anion transporter.[104] Recently, a member of a universal permease family, PerO, was found to be involved in Mo accumulation in *Rhodobacter capsulatus*, which is the first reported bacterial molybdate non-lABC-type transport systems.[105]

In contrast, eukaryotic molybdate transport is less well understood. Algae (*Chlamydomonas reinhardtii*) and higher plants (*A. thaliana*) have a ultrahigh-affinity molybdate transport system, MOT1, belonging to the large sulfate carrier family.[106,107] Surprisingly, MOT1 is localized to the endomembrane system or to the mitochondrial envelope and does not reside in the plasma membrane.[106–108] Quite recently, a second molybdate transporter, MOT2, was found in both *C. reinhardtii* and animals (including humans).[109] In *A. thaliana*, MOT2 is important for vacuolar molybdate export and is a novel element in interorgan translocation of Mo.[110]

In all organisms studied so far, Moco is synthesized by an evolutionarily conserved biosynthetic pathway that can be divided into four steps (Figure 3).[95–97,111] This process includes (i) conversion of GTP into the sulfur-free pterin compound cyclic pyranopterin monophosphate (cPMP); (ii) transformation of cPMP into an intermediate molybdopterin; (iii) Mo atom incorporation; and (iv) maturation to an active Moco, for example, formation of a dinucleotide form [molybdopterin guanine dinucleotide (MGD)] or substitution of a terminal oxygen ligand of Moco with a sulfur ligand. In *E. coli*, proteins required for Moco biosynthesis and regulation are encoded in the moa, mob, mod, moe or mog operons (Figure 3a).[22,97] In eukaryotes, at least six gene products (Cnx1–3 and Cnx5–7 in plants) are involved in Moco biosynthesis (Figure 3b), which are homologous to their counterparts in bacteria.[97,111–113] Some of the eukaryotic Moco biosynthesis genes are able to functionally complement the matching bacterial mutants.[96] Many organisms including plants and mammals also contain a Moco sulfurase catalyzing the generation of the sulfurylated form of Moco, which is essential for the final activation of the xanthine oxidase family of proteins such as xanthine dehydrogenase and aldehyde oxidase.[114,115] In bacteria, several enzyme-specific chaperones have been found to play a central role in the biogenesis of multisubunit molybdoenzymes by coordinating subunit assembly and Moco insertion, for example, NarJ for nitrate reductase and DmsD for dimethylsulfoxide reductase.[116,117] In eukaryotes, the general mechanism of Moco protection, storage, and transfer in mammals is still unclear. A Moco-storage/carrier protein has been described in *C. reinhardtii* (a homotetramer that can hold four Moco molecules) donating Moco to nitrate reductase.[118] Several homologous Moco-binding proteins (MoBPs) were recently identified in land plants that might be involved in the cellular distribution of Moco.[97,119] A recent study has revealed that, in *A. thaliana*, the first step of Moco biosynthesis is localized in the mitochondrial

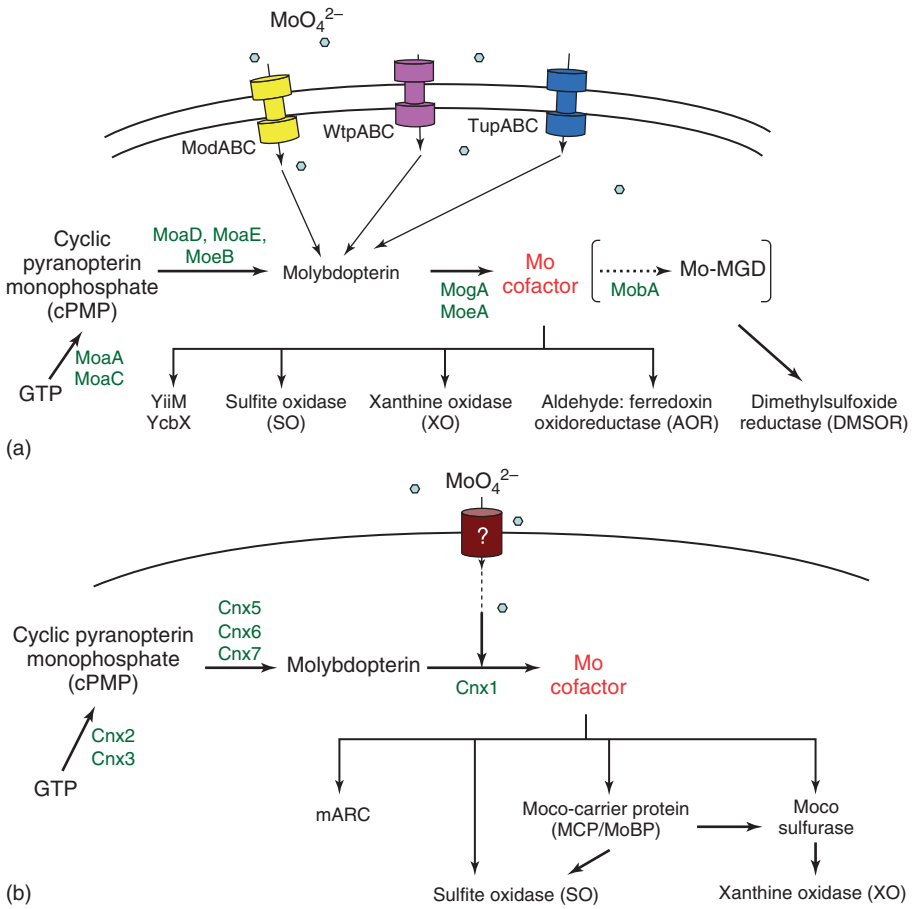

Figure 3 Biosynthesis of molybdenum cofactor. The pathway of Moco synthesis can be divided into four steps. (a) Biosynthesis of the Mo cofactor in prokaryotes. (b) Biosynthesis of the Mo cofactor in eukaryotes. The proteins from *E. coli* and *A. thaliana* catalyzing the respective steps are depicted and their names are given. MGD, molybdopterin guanine dinucleotide

matrix, and the mitochondrial ABC transporter ATM3 has a crucial role in Moco biosynthesis by transporting cPMP.[120]

3.2 Molybdoenzymes

All molybdoenzymes could be divided into two groups: (i) Fe-Mo-containing nitrogenase and (ii) Moco-containing molybdoenzymes. Table 2 shows all known molybdoenzyme families and their major members.

Nitrogenase catalyzes a key step in the global nitrogen cycle, the nucleotide-dependent reduction of N_2 to bioavailable ammonia. So far, three homologous nitrogenase families have been identified, which are distinguished by the different types of heterometals in their active sites. The most abundant and best-characterized is the Fe–Mo-dependent nitrogenase, which contains $MoFe_3S_3$ and Fe_4S_3 groups triply joined by bridging sulfurs.[121]

All other molybdoprotein families utilize Moco as a cofactor, including xanthine oxidase (XO), sulfite oxidase (SO), dimethylsulfoxide reductase (DMSOR), AOR (mostly W-containing) families, and the newly identified mitochondrial amidoxime-reducing component (mARC). Prokaryotes contain a variety of proteins belonging to these families; however, eukaryotes have only several molybdoenzymes, such as xanthine dehydrogenase (XDH) and aldehyde oxidase (AO) (members of the XO family), as well as nitrate reductase (NR) and SO (members of the SO family).[97]

The XO family has the most diverse molybdoenzymes. In general, proteins of this family catalyze the oxidative hydroxylation of a wide range of aldehydes and aromatic heterocycles.[111] The major enzymes of this family include AO (catalyzes the oxidation of aromatic and nonaromatic heterocycles and aldehydes), XDH (oxidizes hypoxanthine to xanthine and xanthine to uric acid), and a variety of bacterial enzymes such as aldehyde oxidoreductase, 4-hydroxybenzoyl-CoA reductase, and quinoline 2-oxidoreductase.

Enzymes of the SO family catalyze net oxygen atom transfer to or from a heteroatom lone electron pair rather

Table 2 Mo-dependent proteins

Group	Family	Protein
Moco-dependent proteins	Sulfite oxidase	Sulfite oxidase
		Nitrate reductase (assimilatory)
	Xanthine oxidase	Xanthine oxidase
		Xanthine dehydrogenase
		Aldehyde oxidase
		Aldehyde oxidoreductase
		Quinoline 2-oxidoreductase
		4-hydroxybenzoyl-CoA reductase
		CO dehydrogenase
		Isoquinoline 1-oxidoreductase
		Quinoline 4-carboxylate-2-oxidoreductase
		Quinaldine 4-oxidoreductase
		Quinaldic acid 4-oxidoreductase
		Nicotinic acid hydroxylase
		6-hydroxynicotinate hydroxylase
		Nicotine dehydrogenase
		Picolinate hydroxylase
		Pyridoxal oxidase
		Nicotinate hydroxylase
	Dimethylsulfoxide reductase	Dimethylsulfoxide reductase
		Biotin sulfoxide reductase
		Trimethylamine-*N*-oxide reductase
		Nitrate reductase (dissimilatory)
		Formate dehydrogenase
		Formylmethanofuran dehydrogenase
		Polysulfide/thiosulfate/arsenate reductase
		Arsenite oxidase
		Acetylene hydratase
		Pyrogallol-phloroglucinol transhydroxylase
		Ethylbenzene dehydrogenase
	Aldehyde: ferredoxin oxidoreductase (W-specific)	Aldehyde:ferredoxin oxidoreductase
		Formaldehyde ferredoxin oxidoreductase
		Glyceraldehyde-3-phosphate ferredoxin oxidoreductase
		Carboxylic acid reductase
		Hydroxycarboxylate viologen oxidoreductase
		Aldehyde dehydrogenase
	mARC	mARC/YcbX
		YiiM
Fe-Mo-binding protein	Nitrogenase	Nitrogenase

than hydroxylation of a carbon center.[122] SO, the name-giving enzyme for this family is widespread and highly conserved in eukaryotes; it is located in the mitochondrial intermembrane space and catalyzes the oxidation of sulfite to sulfate.[123] The assimilatory NR, another important member of this family, is found in autotrophic organisms to catalyze the reduction of nitrate to nitrite.[111]

The DMSOR family is detected only in bacteria and archaea and consists of a number of enzymes. It has been divided into subfamilies I, II, and III,[124] whose members are very diverse in reaction, function, and structure.[125] Most of these enzymes function as terminal reductases under anaerobic conditions. The majority of enzymes in this family include dissimilatory NR and formate dehydrogenases (subfamily I), ethylbenzene dehydrogenase (subfamily II), DMSOR and trimethylamine-*N*-oxide reductase (subfamily III), and several additional enzymes.[124,125]

AOR catalyzes the interconversion of aldehydes and carboxylates and was the first member of the AOR family to be structurally characterized as a protein containing a Wco cofactor, which was similar to that found in molybdoenzymes.[98] This family also includes formaldehyde ferredoxin oxidoreductase, glyceraldehyde-3-phosphate ferredoxin oxidoreductase, and hydroxycarboxylate viologen oxidoreductase.

Very recently, novel MoBPs have been identified in both mammals and *E. coli*.[126,127] In mammals, the mARC protein is found to bind Moco: this protein appears to catalyze the reduction of a variety of *N*-hydroxylated compounds. mARC binds a Moco that carries neither a terminal sulfur ligand (such as XO) nor a covalently bound Cys residue (such as SO), implying that these proteins represent a new family of molybdoenzymes.[128] Human mARC proteins may catalyze the *N*-reduction of a variety of *N*-hydroxylated substrates such

as *N*-hydroxy-cytosine and N_{ω}-hydroxy-L-arginine albeit with different specificities, suggesting that mARC may play an important role in drug metabolism.[128–130]

3.3 Advances in Comparative Genomics of Molybdenum Utilization and Molybdoproteomes

In the recent decade, several comparative studies have been carried out to investigate the evolutionary dynamics of Mo utilization at the level of Moco biosynthesis, and molybdoenzymes, which provided a first glance at Mo utilization and showed its widespread occurrence, yet restricted use of this metal in individual organisms.[29,30,94,131]

Generally, in archaea and bacteria, Mo was found to be used by almost all phyla (except *Mollicutes* and *Chlamydiae*), implying that its utilization is an ancient trait in prokaryotes. In eukaryotes, Mo utilization was mainly observed in animals, land plants, algae, certain fungi, and stramenopiles, whereas parasites, yeasts (saccharomycotina and schizosaccharomycetes), and free-living ciliates have lost the ability to use Mo.

ModABC is the most widespread Mo transport system in bacteria, present in approximately 90% of sequenced organisms.[94] In contrast, the occurrence of WtpABC and TupABC transport systems is much more limited. In archaea, WtpABC is the most frequently used transporter. With regard to the regulation of these transporters, the *E. coli*-type ModE was only present in a small number of Mo-utilizing organisms (<30%), implying that novel or unspecific regulatory mechanisms for molybdate uptake are present in many other organisms.[29,130] However, separate ModE_N and/or Mop/Di-Mop proteins were observed in a variety of organisms that lack the *E. coli*-type ModE, some of which contain novel domain fusions. Most of these genes are close to or are even in the same operon with *modABC*, suggesting that they are involved in regulation of ModABC.[29,130] In eukaryotes, MOT1 and MOT2 proteins may play key roles in Mo transport. MOT1 was found in some Mo-utilizing organisms, such as land plants and green algae, whereas MOT2 has a wider distribution in algae, higher plants, and animals.[109,110]

Compared to Mo transport systems, all Moco biosynthesis proteins could be detected in essentially all Mo-utilizing organisms, although a very small number of organisms, which contain homologs of molybdoenzymes, lack genes for either Moco biosynthesis proteins or Mo/W transporters.[130] It is unclear whether these molybdoprotein homologs can use Mo in these organisms.

Analysis on molybdoenzymes revealed complexity in their distribution and evolution. Except for the AOR family, which was detected only in approximately 15% of Mo/W-utilizing organisms, molybdoenzymes showed widespread occurrence in Mo-utilizing organisms (93, 65, and 64% for DMSOR, SO, and XO families, respectively). Many organisms possessed several molybdoenzyme families and several subfamilies within these families. In archaea, as in bacteria, DMSOR was also the most frequently used molybdoenzyme family (>95% of Mo-utilizing organisms). The FeMo-containing nitrogenase was detected in approximately 20% of Mo-utilizing bacteria and methanogenic archaea.

Further analysis of molybdoproteomes of individual organisms showed that proteobacteria have larger molybdoproteomes than other organisms.[130] To date, the largest molybdoproteome was observed in a dehalorespiring bacterium, *Desulfitobacterium hafniense* (Firmicutes/Clostridia), which contains at least 63 predicted molybdoenzymes, almost twice as many as other molybdoprotein-rich organisms. Almost all molybdoproteins (95%) in *D. hafniense* belong to the DMSOR family. In archaea, relatively large molybdoproteomes were observed in Crenarchaeota/Sulfolobales, and the majority of these proteins were members of the XO family.

In eukaryotes, Moco is the only known form of Mo utilization, and only two known molybdoenzyme families, SO and XO, have been found. Almost all organisms that possessed the Moco biosynthesis pathway had both families. Plants appeared to have the largest molybdoproteomes among eukaryotes (10–11 molybdoproteins). In contrast, all sequenced saccharomycotina and schizosaccharomycetes did not have known molybdoenzymes. Although a small number of unsequenced yeast species, such as *Candida nitratophila* and *Pichia anomala*, were reported to utilize Moco-containing assimilatory NR,[30,132] the absence of both this protein and the Moco biosynthesis pathway in sequenced yeast genomes strongly suggested the loss of Mo utilization in these organisms.

Recent bioinformatics studies also provided insights into the newly identified mammalian molybdoprotein mARC. In eukaryotes, mARC proteins were detected in more than 95% Mo-utilizing organisms, suggesting a wide distribution of this novel molybdoenzyme family. It is composed of two domains: N-terminal MOSC_N (pfam03476) and C-terminal MOSC (pfam03473) domains, both of which are also found in Moco sulfurases.[131] In addition, all eukaryotic mARC proteins, including the plant counterparts, are characterized by the presence of N-terminal extensions that predict a mitochondrial localization of these proteins. The MOSC domain may be involved in Moco binding with high affinity, whereas the function of the MOSC_N domain is unclear. With an average molecular mass of approximately 35 kDa and owing to the fact that they bind Moco as the only prosthetic group, mARC proteins are the smallest molybdoenzymes identified to date. It is assumed that mARC may play a detoxifying role in metabolism. Compared to other molybdoenzymes, mARC proteins do not exhibit enzymatic activity on their own but require other proteins such as cytochrome b_5 and NADH/cytochrome b_5 reductase as electron transmitters and electron donors, respectively.[133] Two additional bacterial Moco-containing proteins, YcbX and YiiM, were characterized in *E. coli*.[127] Phylogenetic analysis revealed that YcbX and mammalian mARC proteins could be

considered as orthologs (the mARC/YcbX family), whereas YiiM belonged to a different family within the MOSC superfamily, which was mainly detected in bacteria.[131] Additional research is needed to better understand the functions of these Moco-binding proteins.

Some environmental and other factors have been suggested to affect Mo utilization and molybdoenzyme families. The majority of intracellular parasites and symbionts lost the ability to utilize Mo.[94] In addition, organisms possessing AOR proteins appeared to favour anaerobic conditions, whereas organisms possessing SO or XO proteins live under aerobic conditions. Organisms containing nitrogenase mainly live under anaerobic, and warm conditions.[94,131] Moreover, a crosslink between Mo and selenium metabolism was observed, as the formate dehydrogenase alpha subunit, a major member of the DMSOR family, is also a selenocysteine-containing protein (see ***Selenoenzymes and Selenium Trafficking: an Emerging Target for Therapeutics***), which is responsible for maintaining the selenocysteine utilization trait in sequenced prokaryotes.[94] Thus, selenium-utilizing organisms are essentially a subset of Mo-utilizing organisms in prokaryotes, most likely because of formate dehydrogenase, which is not only a widespread molybdoenzyme but is also the major user of selenium in these organisms.

Table 3 Ni- and Co(B_{12})-dependent proteins

Ni-dependent proteins	Co(B_{12})-dependent proteins
Urease	Adenosylcobalamin-dependent isomerases:
Ni–Fe hydrogenase	Methylmalonyl-CoA mutase
Carbon monoxide dehydrogenase	Isobutyryl-CoA mutase
Acetyl-coenzyme A decarbonylase/synthase	Ethylmalonyl-CoA mutase
Superoxide dismutase SodN	Glutamate mutase
Methyl-coenzyme M reductase	Methyleneglutarate mutase
	Diol dehydratase
	D-lysine 5,6-aminomutase
	B_{12}-dependent ribonucleotide reductase
	Glycerol dehydratase
	Ethanolamine ammonia lyase
	Methylcobalamin-dependent methyltransferase:
	Methionine synthase (MetH)
	Other methyltransferases: Mta, Mtm, Mtb, Mtt, Mts, and Mtv
	B_{12}-dependent reductive dehalogenase CprA

4 NICKEL AND COBALT

The transition-metal Ni is an essential cofactor for a number of prokaryotic enzymes involved in energy and nitrogen metabolism. Co is a constituent of vitamin B_{12} (or cobalamin), a compound involved in methyl group transfer and rearrangement reactions, but also occurs in a few non-corrin Co-containing enzymes.[134] The list of known Ni- and B_{12}-containing proteins is shown in Table 3.

4.1 Nickel and Cobalt Uptake

As bioavailable Ni and Co ions in natural environments are usually present in trace amounts, high affinity uptake systems are required to assure intracellular metalloenzyme activities. In prokaryotes, Ni and Co use similar transport systems for uptake into cells. Both ABC and several secondary transporters have been identified in bacteria and archaea.[12] Figure 4 shows all known Ni-/Co-transport systems.

In bacteria, the best-investigated ABC-type Ni transporter is the NikABCDE system, which belongs to a large family of ABC transporters (nickel/peptide/opine transporter family). Typically, a Nik system is composed of a substrate-binding protein NikA, two integral membrane components (NikB and NikC), and two ATPases (NikD and NikE), which are encoded by an operon *nikABCDE*.[135] *E. coli* NikA may also bind heme whose binding site is remote from the Ni-binding site.[136] Several residues of NikA have been suggested to be involved in Ni binding. For example, it was reported that *E. coli* NikA binds a natural metallophore containing three carboxylate groups that coordinate a Ni ion, and that one ligand (His416) is essential for Ni transport.[137] Distant homologs of Ni ABC transporters were also identified in *Yersinia* (YntABCDE).[138] A second family of the ABC-like transport system, Cbi/NikMNQO, has also been shown to be involved in Ni or Co uptake in bacteria.[139] Analyses of the *cbi/nik* operon structures revealed that the M, Q, and O components are essential for this system, whereas CbiN (Co uptake) and NikN (Ni uptake) only have structural similarity (two transmembrane domains).[27] When NikN is absent, two additional proteins, NikK and NikL, were thought to be involved in Ni uptake, which form the NikKMLQO system.[27]

Several secondary Ni/Co transporters have been identified, which include NiCoT, UreH, and HupE/UreJ.[140] NiCoT proteins, also designated HoxN, HupN, NixA, or NhlF in different organisms, possess eight transmembrane domains. They are widely found in bacteria, archaea and eukaryotes. Various subtypes of NiCoT family have different ion preferences.[15,141] UreH and HupE/UreJ are secondary transporters, which have recently been found to mediate Ni transport.[140–142] In addition, several new of Co transporters were predicted by comparative genomics approaches, such as CbtAB, CbtC-CbtG, and CbtX.[141,143]

No high affinity Ni- or Co-uptake system has been reported in eukaryotes. In plants, soluble Ni compounds are

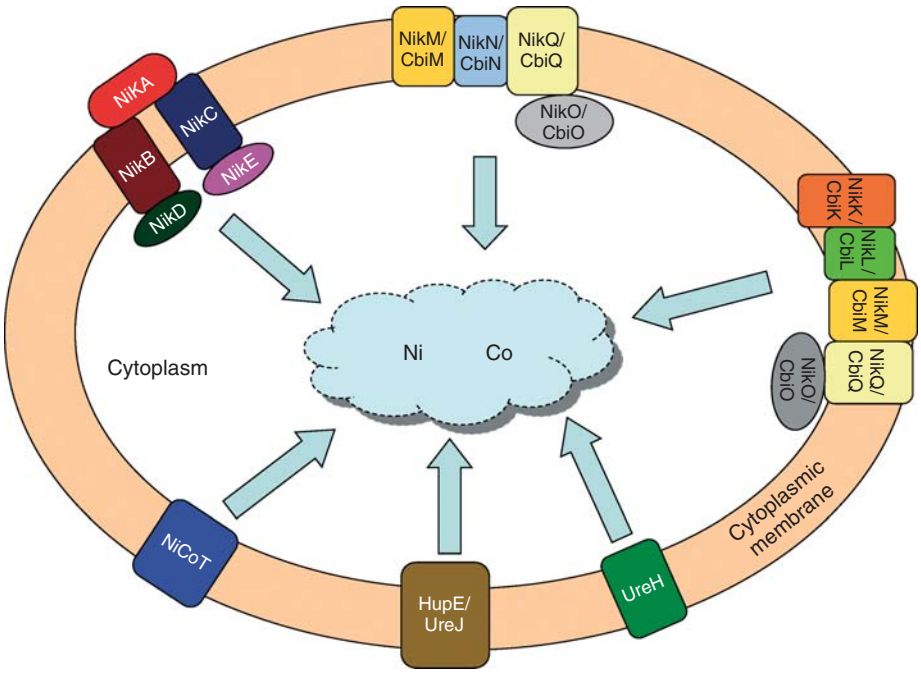

Figure 4 Schematic representation of Ni/Co transporters in prokaryotes. Known prokaryotic Ni/Co transport systems include NikABCDE, Nik/CbiMNQO, Nik/CbiKMLQO, NiCoT, HupE/UreJ, and UreH

preferably absorbed passively through a cation transport system; chelated Ni compounds are taken up through secondary, active-transport-mediated systems, using transport proteins such as permeases. Insoluble Ni compounds primarily enter plant root cells through endocytosis.[144] The Ni transport and retranslocation processes are strongly regulated by metal-ligand complexes (such as nicotianamine, His, and organic acids) and by some proteins that specifically bind and transport Ni. On the other hand, some suppressors of heavy metal toxicity, such as COT1 and GRR1, were identified in *S. cerevisiae*, which may decrease the cytoplasmic concentration of metal ions including Co and Zn.[145]

In *E. coli*, Ni trafficking is controlled by the Ni-dependent transcriptional regulator NikR (see ***NikR: Mechanism and Function in Nickel Homeostasis***), whose gene is often located immediately next to its major target, the *nikABCDE* operon. In addition, NikR-dependent regulation was also found for some other Ni transporters including NikMNQO and NiCoT, as well as several Ni-dependent metalloenzymes such as Ni–Fe hydrogenase (see ***[NiFe]-Hydrogenase Cofactor Assembly***).[27,146] The NikR-binding site contains an inverted repeat sequence and is present upstream of Ni-associated genes. In contrast to other NikR proteins, a recent study found that NikR from *Helicobacter pylori* is a pleiotropic regulator that, depending on the target gene, acts as an activator or a repressor, implying hierarchical regulation of the NikR-related Ni response in this organism.[147]

4.2 Nickel-Dependent Metalloenzymes

The major Ni-dependent metalloenzymes include urease, Ni–Fe hydrogenase, carbon monoxide dehydrogenase (CODH), acetyl-coenzyme A (CoA), decarbonylase/synthase (CODH/ACS), superoxide dismutase SodN, and methyl-coenzyme M reductase (MCR). Other Ni-containing proteins found in certain organisms appear to bind different metals. For example, glyoxalase I (GlxI) binds Ni in *E. coli* and human parasites *Leishmania* (e.g., *Leishmania major*) and *Trypanosoma* (e.g., *Trypanosoma cruzi*), but it binds Zn in *Pseudomonas putida*, yeast, and humans.[148,149] Such proteins are not discussed here. To date, urease is the only known Ni-dependent enzyme in eukaryotes.

Urease, a Ni-dependent metalloenzyme, has been found in plants, some bacteria, and fungi. It catalyzes the hydrolysis of urea into ammonia and carbon dioxide. Although the amino acid sequences of plant and bacterial ureases are closely related, some biological activities differ significantly. Plant ureases but not bacterial ureases possess insecticidal properties independent of their ureolytic activity. The Ni active site appears to be particularly conserved, based on the crystal structures of different ureases.[150]

Ni–Fe hydrogenases are mainly utilized for the oxidation of dihydrogen to protons and electrons. This reversible reaction is based on a complex interplay of metal cofactors including the Ni–Fe active site and several Fe–S clusters.[151] The Ni–Fe active site is located in the inner part of the enzyme; therefore a number of pathways are involved in the

catalytic reaction route, which consist of an electron transfer pathway, a proton transfer pathway, and a gas-access channel. The N-terminal RxCGxC and the C-terminal DPCxxC motifs have been proposed to be involved in the ligation of Ni.

Another Ni–Fe enzyme, CODH, is a key player in the global carbon cycle and carries out the interconversion of the environmental pollutant CO and the greenhouse gas CO_2. It has been found in both archaea and bacteria. The active site responsible for this important chemistry, designated the C-cluster, is a complex Ni-, Fe-, and S-containing center.[152] The high resolution structure of CODH from *Carboxydothermus hydrogenoformans* in three states demonstrated the mechanism of CO oxidation and CO_2 reduction at its active site.[153]

In acetogenic bacteria and methanogenic archaea, CODHs are bifunctional enzymes that perform both the reversible CO-oxidation reaction and the synthesis or degradation of CoA and, therefore, are named CODH/ACS. Both catalytic clusters require Ni for catalysis.[154]

SODs are enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. On the basis of the metal cofactor they harbor, SODs can be classified into four groups: Fe SOD, Mn SOD, Cu–Zn SOD, and Ni SOD. The Ni-containing SOD has been isolated from several *Streptomyces* species and from cyanobacteria. A nine-residue motif (His-Cys-X-X-Pro-Cys-Gly-X-Tyr) appears to be essential for metal binding and catalysis.[155]

All biologically produced methane is formed by methanogenic archaea through the catalytic action of MCR. In some uncultured methanotrophic archaea, anaerobic methane oxidation also appears to be catalyzed by the same enzyme, or an isozyme of MCR. MCR catalyzes the conversion of methyl-coenzyme M (methyl-SCoM) and *N*-7-mercaptoheptanoylthreonine phosphate (CoBSH) to methane and the CoB-SS-CoM heterodisulfide. Such catalysis requires a Ni hydrocorphin called coenzyme F_{430}, which is located at the base of a narrow hydrophobic well that accommodates the two substrates and shields the reaction from solvent.[156]

4.3 Vitamin B_{12} Transport and Biosynthesis

Co is an essential cofactor in vitamin B_{12}-dependent enzymes. Vitamin B_{12}, also called cobalamin, is a group of closely related polypyrrole compounds, such as cyanocobalamin, methylcobalamin, and deoxyadenosyl cobalamin. They are required for the lipid, carbohydrate, and protein metabolism of many organisms.

Vitamin B_{12} uptake is important for B_{12}-utilizing organisms that cannot synthesize the coenzyme. BtuFCD is the only bacterial B_{12} transport system described to date, which consists of a periplasmic B_{12}-binding protein BtuF and two transmembrane subunits BtuC and BtuD.[157] In Gram-negative bacteria, a TonB-dependent outer membrane receptor BtuB is also involved in B_{12} uptake and forms a complex with BtuFCD.[158] In mammals, three B_{12}-binding proteins (haptocorrin, intrinsic factor, and transcobalamin) and several specific receptors are involved in the process of intestinal absorption, plasma transport, and cellular uptake.[159] However, the mechanism of B_{12} transport in other eukaryotes, such as algae, is still unclear. It was suggested that algae acquire vitamin B_{12} through a symbiotic relationship with B_{12}-synthesizing bacteria, and that they require vitamin B_{12} primarily for methionine biosynthesis.[160]

Among the B_{12}-utilizing prokaryotes, some species are able to synthesize vitamin B_{12} de novo. There are two distinct routes for B_{12} biosynthesis in bacteria: oxygen-dependent (aerobic) and oxygen-independent (anaerobic) pathways.[23,141] The aerobic pathway incorporates molecular oxygen into the macrocycle as a prerequisite to ring contraction, and more than 20 genes have been proposed to be involved in these processes. On the other hand, the anaerobic pathway uses the chelated Co ion to support ring contraction without oxygen. Both routes may have specific enzymes, such as CbiD, CbiG, and CbiK for the anaerobic route, as well as CobE, CobF, CobG, CobN, CobS, CobT, and CobW for the aerobic pathway.[141] Recently, an adenosyltransferase that catalyzes the final step in the assimilation of vitamin B_{12} was found to transfer B_{12} to methylmalonyl-CoA mutase in *Methylobacterium extorquens*. This finding suggests a general strategy for cofactor trafficking, which uses the final enzyme for delivering the cofactor to its user enzyme.[161] This process also needs a small G protein editor MeaB that gates B_{12} loading.[162]

4.4 Vitamin B_{12}-Containing Enzymes

Vitamin B_{12} is mainly found in three families of enzymes: adenosylcobalamin-dependent isomerases, methylcobalamin-dependent methyltransferases, and dehalogenases.[163,164] Each of these is then further divided into more subclasses (Table 3). Different chemical aspects of the cofactor are exploited during catalysis by isomerases and methyltransferases. Thus, the Co-carbon bond ruptures homolytically in isomerases, whereas it is cleaved heterolytically in methyltransferases. The reaction mechanism of dehalogenases is poorly understood.

Adenosylcobalamin-dependent isomerases are mainly found in bacteria, where they play important roles in fermentation pathways.[163] Members of this family include methylmalonyl-CoA mutase (MCM), isobutyryl-CoA mutase (ICM), ethylmalonyl-CoA mutase (ECM), glutamate mutase (GM), methyleneglutarate mutase (MGM), D-lysine 5,6-aminomutase (5,6-LAM), diol/glycerol dehydratase (DDH/GDH), ethanolamine ammonia lyase (EAL), and ribonucleotide reductase class II (RNR II).

MCM has been characterized in both bacteria and humans. It catalyzes isomerization of methylmalonyl-CoA to succinyl-CoA and is involved in key metabolic pathways.[165] The coordination of Co in vitamin B_{12} needs a His in the DXHXXG motif within the C-terminal B_{12}-binding domain.[166] Two homologs of MCM, ICM and

ECM, have also been found in a variety of bacteria. ICM catalyzes reversible interconversion of isobutyryl-CoA and *n*-butyryl-CoA and exists as a heterotetramer. It comprises a large subunit IcmA and a small subunit IcmB. IcmB contains DXHXXG B_{12}-binding motif.[167] ECM catalyzes transformation of ethylmalonyl-CoA to methylsuccinyl-CoA. It is distinct from the other two enzymes and defines a new subclade of B_{12}-dependent acyl-CoA mutases.[168]

GM catalyzes carbon skeleton rearrangement of (*S*)-glutamate to (2*S*,3*S*)-3-methylaspartate, the first step of the glutamate fermentation pathway. This enzyme consists of two subunits (designated GlmE and GlmS in *Clostridium cochlearium*) as an $\alpha_2\beta_2$ tetramer, whose assembly is mediated by B_{12}. The GlmS subunit is responsible for binding to B_{12}.[169]

In *Eubacterium barkeri*, a MGM was characterized which catalyzes equilibration of 2-methyleneglutarate with (*R*)-3-methylitaconate. It contains a highly conserved DXHXXG(X)(41)GG motif, which is essential for B_{12} binding.[170]

5,6-LAM catalyzes the reversible and nearly isoenergetic transformations Of D-lysine into 2,5-diaminohexanoate (2,5-DAH) and Of L-beta-lysine into 3,5-diaminohexanoate (3,5-DAH). The crystal structure of 5,6-LAM from *Clostridium sticklandii* revealed that it is predominantly a PLP-binding TIM barrel domain, with several additional alpha-helices and beta-strands at the N and C termini. These helices and strands form an intertwined accessory clamp structure that provides most of the interactions between the protein and adenosylcobalamin.[171]

GDH and DDH are homologous isofunctional enzymes that catalyze elimination of water from glycerol and 1,2-propanediol to the corresponding aldehyde. Structural analysis of different GDH and DDH proteins revealed residues that may be important for substrate preference and specificity of protein–protein interactions.[172]

B_{12}-dependent EAL acts on both enantiomers of the substrate 2-amino-1-propanol. Several structures have been solved for this class of enzymes. Computational modeling of EAL from *Salmonella typhimurium* revealed that this enzyme may have a similar TIM-barrel fold as DDH and GDH, which contains the active site of the coenzyme B_{12}.[173]

RNR II catalyzes the formation of an essential thiyl radical by homolytic cleavage of the Co-C bond in their adenosylcobalamin cofactor. These enzymes are mainly found in bacteria and also in some of their phages. They use an adenosylcobalamin cofactor that interacts with a Cys residue to form the reactive Cys radical needed for the reduction of ribonucleotides.[174]

Methylcobalamin-dependent methyltransferases play important roles in amino acid metabolism as well as in carbon metabolism and CO_2 fixation. These enzymes can be divided into two subgroups: one subgroup, namely methionine synthase (MetH), catalyzes the final step in the regeneration of Met from homocysteine, while the other binds a variety of simple substrates such as methanol (MtaB), methylated amines (MttB, MtbB, and MtmB), methylated thiols (MtsB), methoxylated aromatics (MtvB), and methylated heavy metals.[163,175]

MetH is the most intensively studied B_{12}-dependent methyltransferase. It has different regions for binding homocysteine, methyltetrahydrofolate, B_{12}, and adenosylmethionine. The crystal structure of a B_{12}-containing fragment of MetH from *E. coli* revealed that the His residue in the DXHXXG motif is essential for Co binding.[176] Other B_{12}-dependent methyltransferases are designated as Mtx, where *x* represents the methyl donor (e.g., a, methanol; v, vanillate; m, methylamine; b, dimethylamine). These methyltransferases, which are important for energy metabolism and in cell carbon synthesis in anaerobic microbes, consist of subunits: Mt_A methylates CoM, Mt_B methylates the corrinoid protein, and Mt_C is the corrinoid protein containing B_{12}.

The majority of the known B_{12} reductive dehalogenases belong to the CprA/PceA family. These are single-polypeptide membrane-associated anaerobic enzymes that are synthesized as preproteins with a cleavable twin arginine translocation peptide signal. They contain one corrinoid and two Fe-S clusters as cofactors.[177] The reaction mechanism of dehalogenases remains unclear.

Co utilization is restricted in eukaryotes as only three B_{12}-dependent enzymes, MCM, MetH, and RNR II, have been reported.

4.5 Other Cobalt-Binding Enzymes

Several noncorrin-Co-containing enzymes have been isolated in certain organisms, such as methionine aminopeptidase (*S. typhimurium*), prolidase (*Pyrococcus furiosus*), nitrile hydratase (*Rhodococcus rhodochrous*), aldehyde decarbonylase (*Botryococcus braunii*), glucose isomerase (*Streptomyces albus*), and methylmalonyl-CoA carboxytransferase (*P. shermanii*).[134] These enzymes are not strictly dependent on Co and may bind other metals (such as Fe and Zn) in place of Co in other organisms. Among them, only nitrile hydratase was suggested to have different sequence motifs for Co- and Fe-binding.[178]

4.6 Advances in Comparative Genomics of Nickel and Cobalt Utilization

Several studies have been conducted to investigate Ni and Co utilization in all three domains of life. An early bioinformatics study demonstrated a complex utilization of both transition metals in prokaryotes by examining Ni- and Co transport systems of different types in approximately 200 microbial genomes.[27] It appeared revealed that the Ni-/Co-transporter genes are often located near with either Ni-dependent or coenzyme B_{12} biosynthesis genes. Different Ni-/Co-transporter families had a mosaic distribution in examined organisms. In silico analyses identified Cbi/NikMNQO transport system (including Cbi/NikKMLQO) as the most

widespread Ni/Co transporters in prokaryotes. The Ni-responsive repressor NikR regulates many Ni uptake systems, though the NikR-binding signal is divergent in various taxonomic groups of bacteria and archaea, whereas a highly conserved RNA secondary structure (the regulatory B_{12} element or B_{12} riboswitch) regulates most of the candidate Co transporters. Another bioinformatics analysis of B_{12} metabolism also provided important insights into B_{12} utilization in prokaryotes.[141] The B_{12} element was found to be widely present upstream of B_{12} biosynthetic genes. Several candidate Co transporters and new proteins involved in B_{12} biosynthesis pathway, including chelatases and methyltransferases, were predicted. Interestingly, the B_{12} element was also found to regulate B_{12}-independent MetH and RNR isozymes in some bacteria.

The distribution of Ni and Co utilization traits at the level of both transporters and metalloproteomes have been recently carried out in more than 700 organisms.[94,179] Consistent with previous results, both metals were found to be widely used in prokaryotes. In bacteria, urease and MetH were the most widely used Ni- and Co-containing proteins, respectively. However, they were very rare or absent in archaea. The majority of prokaryotic organisms contained one to four Ni- and Co-dependent metalloproteins. The largest Ni-dependent metalloproteome was reported in *Deltaproteobacterium* MLMS-1 (16 Ni-binding enzymes) and *Dehalococcoides* sp. CBDB1 contained the largest B_{12}-dependent metalloproteome (35 B_{12}-binding enzymes). Similar to Mo utilization, reduced Ni or Co utilization may be associated with obligate intracellular parasites and endosymbionts.[179]

In eukaryotes, the number of Ni transporters and that of Ni/B_{12}-dependent metalloproteins are quite limited. There are very few organisms that can utilize both metals. The majority of Ni-utilizing organisms are fungi (except saccharomycotina) and plants. NiCoT and urease were the most widely used Ni transporter and Ni protein, respectively. On the other hand, B_{12}-utilizing organisms are mainly found in animals, and MetH was the most widespread B_{12}-dependent enzyme in eukaryotes. Very small Ni and Co metallopproteomes were present in examined eukaryotic genomes (say, 1-3 B_{12}-dependent enzymes).

5 OTHER METALS

5.1 Zinc Metalloproteomes

Zn is thought to be essential for all forms of life and was considered as a key element in the origin of life.[180] Since Zn uptake, storage, homeostasis, and user proteins have been discussed in many other reviews, here we focus only on comparative analysis of Zn-dependent metalloproteomes.

On the basis of known Zn-binding domains and patterns, the Zn proteomes in several organisms from the three domains of life have been investigated via computational approaches.[93] Zn-binding proteins are widespread in all living organisms. It was found that the number of Zn-binding proteins in an organism correlated with the proteome size. However, Zn-binding proteins appeared to be enriched in the hyperthermophilic prokaryotes, probably due to an increased use of Zn to enhance the structural stability of proteins by these organisms. The majority of prokaryotic Zn-binding protein families could be detected in eukaryotes. On the other hand, three-quarters of eukaryotic Zn-dependent metalloproteomes comprise proteins found only in eukaryotes.

Functional diversification was also observed between prokaryotic and eukaryotic Zn-dependent proteomes. Most prokaryotic Zn proteins are enzymes, whereas eukaryotic Zn proteins almost equally perform enzymatic catalysis and transcription regulation. This may indicate that Zn has been utilized in the active site of enzymes before the split of the three domains of life. On the other hand, Zn-binding transcription factors normally containing Zn-finger are almost exclusively a privilege of eukaryotes. This observation suggests that Zn-binding transcription factors have become essential for regulating complex processes in higher organisms.

Such a functional difference is associated with with the organization of Zn-binding patterns. Zn-binding patterns with four ligands are related to the stability of protein structure, and the patterns with three ligands are mostly catalytic sites.[181] Residues within these patterns are quite different. It has been reported that 97% of human Zn proteins have a structural Zn site with at least one Cys, and 40% have four Cys residues.[182] In addition, nearly one-third of human Zn proteins with three-ligand Zn-binding pattern contain three His residues. Similar Zn-binding sites were observed for a variety of transcription factors, mostly composed by Cys and His. The differentiation of the catalytic Zn-binding sites could be the result of evolutionary processes that led to the development of different enzymatic reactions.[183] Recently, a potential correlation between Zn-finger proteins and Zn hydrolytic enzymes was suggested during evolution associated with environmental changes.[184]

5.2 Iron Metalloproteomes

Fe is required by living organisms for a variety of purposes related to its favorable redox properties and rich coordination chemistry. Moreover, proteins can use a range of Fe-containing cofactors, including heme or Fe–S clusters. In humans, Fe is essential for the mitochondrial electron transport chain and nucleic acid synthesis. On the other hand, Fe's redox properties contribute to its toxicity, which produces reactive oxygen species (ROS) that are harmful to biological molecules. It is known that some Fe-binding proteins bind different metals and that some non-Fe-binding

metalloproteins may also bind Fe in certain organisms. For example, *Ferroplasma acidiphilum*, a chemoautotroph that grows optimally at pH 1.7 and gains energy by oxidizing ferrous iron and carbon by the fixation of carbon dioxide, was found to possess many Fe-binding proteins (86% of 189 investigated cellular proteins), including proteins that bind Zn and Mn in other organisms and proteins that are not identified as metalloproteins.[185] Similarly, organisms that can survive under Fe starvation conditions have also been reported.[8,186]

To date, several bioinformatics studies have been carried out for understanding ferroproteomes, at least partially, in some organisms. One preliminary study focused on the distribution of non-heme Fe proteins in several prokaryotes and eukaryotes, which was based on known non-heme Fe-binding patterns and protein domains.[26] It was found that modern organisms in all three domains of life inherited the majority of their Fe proteomes from the last universal common ancestor. Most non-heme Fe-binding proteins are involved in electron transfer or oxidoreductase activity, suggesting that Fe is the most used metal in redox reactions.[187] Fe–S clusters were found to be present in about 40% of non-heme Fe proteins, and their binding sites often consists of Cys residues. Recent structural analyses of the protein environment around non-heme Fe-binding sites revealed that at least 17% of the sites found in unrelated proteins are highly similar, and that functional variation across a large superfamily of Fe-dependent enzymes is associated with fine differences in Fe coordination within the active site.[188]

Biological systems rely on a variety of heme-containing proteins (or hemoproteins) to carry out a number of basic functions essential for their survival. Heme is a prosthetic group that consists of an Fe atom contained in the center of a large heterocyclic organic ring called a porphyrin. The majority of hemoproteins are involved in a variety of key biological processes, such as oxygen transport, catalysis, electron transfer/transport, sensory and defense. The utilization of heme requires complex machinery for its biosynthesis, insertion into hemoproteins, as well as uptake from external sources. Recently, a comparative study on heme biosynthesis and uptake systems was conducted in sequenced prokaryotes.[189] Their results allowed them to identify organisms capable of performing none, one, or both processes, based on the similarity to known system components. It appeared that many Gram-positive parasites or pathogens import heme from the host. Further analyses of sequences and structures involved in heme uptake suggest the presence of alternative modes of heme binding.

5.3 Other Metals

The bioinformatics approaches introduced here may be used to study other trace metals and identify the corresponding metalloproteomes. However, it is currently impossible to predict from genome sequence alone the numbers and types of metals an organism uses and their complete metalloproteomes because metal coordination sites are diverse and poorly recognized. Very recently, on the basis of high throughput mass spectrometry, and ICP-MS techniques, a comprehensive study has been carried out to characterize cytoplasmic metalloproteins from an exemplary microorganism *P. furiosus*, which showed that metalloproteomes are much more extensive and diverse than previously thought.[190] A bioinformatics approach was developed for the prediction of novel metalloproteins for several metals.[191] Further studies are needed for verification of these proteins as well as for the identification of additional metalloproteins.

6 IONOMICS

In the past several years, a new term, the ionome, has been introduced as an extension to the metallome. In general, the ionome is defined as all mineral nutrients and trace elements found in an organism.[192] The study of the ionome, ionomics, involves quantitative analyses of elemental composition in living systems using high throughput elemental profiling methods. This approach has been applied extensively in plants in both forward and reverse genetics studies, screening diversity panels, and modeling of physiological states.[193] It has been shown that ionomics has the potential to provide a powerful approach to not only functionally analyze genes and gene networks that directly control the ionome but also to examine more extended gene networks that control developmental and physiological processes that in turn affect the ionome indirectly.[31] In this section, we briefly describe the analytical and bioinformatics aspects of ionomics, as well as its recent application.

6.1 Major Analytical Techniques and Resources for Ionomics

The majority of approaches for elemental analysis utilize the electronic properties of an atom (e.g., emission, absorption, and fluorescence spectroscopy). Among them, ICP-MS and X-ray fluorescence (XRF) are the most commonly used techniques. The goal of ICP is to ionize analyte atoms for their detection by either optical emission spectroscopy (ICP-OES, also known as atomic emission spectroscopy or ICP-AES) or ICP-MS. ICP-MS is a type of mass spectrometry that is capable of detecting metals and several nonmetals at concentrations as low as one part per trillion. This is achieved by ionizing the sample with inductively coupled plasma and then using a mass spectrometer to separate and quantify those ions. Compared to ICP-OES, ICP-MS allows for a smaller sample size owing to its greater sensitivity and has the ability to detect different isotopes of the same element. However, ICP-OES has the advantage of lower

cost and simplicity. In recent years, both ICP-OES and ICP-MS have been successfully used for large-scale ionomics studies. For example, Eide *et al*.[194] used ICP-OES to measure approximately 10 000 samples over 2 years in yeast, and others used ICP-MS to measure approximately 80 000 samples in *A. thaliana*.[192,195,196]

XRF is the emission of secondary or fluorescent X-rays from an atom that has been excited by the absorption of high energy X-rays or γ-rays. The emitted X-rays have energies characteristic of the atom, which can be used to detect and quantify specific elements in a complex mixture. In 1993, Delhaize *et al*.[197] used XRF for the multielement screening of more than 100 000 mutagenized *A. thaliana* seedlings to identify mutants with altered ionomes. A recent application of XRF in ionomics is the use of synchrotron-based micro-XRF as a rapid screening tool for the possible identification of *A. thaliana* seeds with mutant ionomic phenotypes.[198] In this work, *A. thaliana* seeds were arrayed in 5 × 3 blocks, and each block was scanned with a focused X-ray beam to quantify the relative content of Mn, Fe, Ni, Cu, Zn, K, and Ca of each seed in the block. Such a micro-XRF-based ionomics methodology holds great promise for the rapid screening of many thousands of seeds or other tissue samples. In addition, micro-XRF has been successfully used for two-dimensional imaging of the ionome in different biological samples.[199,200] Recently, XRF microtomography and confocal imaging were also used for quantitative imaging of the three-dimensional distribution of multiple elements in various plant samples.[201,202] These XRF imaging techniques have become powerful tools for understanding the fundamental biological processes that underlie the ionome.

Considering the large amount of data that an ionomics study produces, appropriate data management tools are very important. These tools should integrate defined workflow controls for genomic-scale data acquisition and validation, data storage and retrieval, and data analysis. The Purdue Ionomics Information Management System (PiiMS) is an example of such a workflow system, which allows open access for data mining and discovery.[195] This system (http://www.ionomicshub.org/home/PiiMS) provides integrated workflow control, data storage, and analysis to facilitate high throughput data acquisition, along with integrated tools for data search, retrieval, and visualization for hypothesis development. To promote rapid knowledge generation about the ionome and related genes and gene networks, it is also important that information obtained via experimental or bioinformatic approaches should be correctly annotated for further ionomic discovery. Currently, systems to allow researcher-driven annotation of genes with biological knowledge are very limited across all biological models. With the rapid growth in the number of novel metal-related genes and their functional data, new systems allowing for such systematized annotation are clearly needed.

6.2 Recent Application of Ionomics in Eukaryotes

In recent years, several high throughput ionomics studies have been carried out for plants and yeast, which illustrate the power of ionomics to identify new aspects of trace element homeostasis and how these data can be used to develop hypotheses regarding the functions of previously uncharacterized genes. For example, two studies that profiled trace elements in a large collection of *A. thaliana* mutants have identified novel functions of ferroportin and molybdenum transporters.[32,108] In another study, elemental content of a yeast knockout collection was analyzed using ICP-AES.[194] It included measurements of 13 elements and identified 212 strains that were outliers in at least one element from controls. Most of the mutants identified had defects in more than one element. Many mutants identified affected either mitochondrial or vacuolar function and these groups showed similar effects on the accumulation of many different elements.

To date, genome-wide analyses of trace elements in mammalian cells are thought to be too complex and have not yet been reported. Sun *et al*.[203] have used ICP-MS techniques to measure the plasma ions to investigate associations of ion modules/networks with overweight/obesity, metabolic syndrome, and type-2 diabetes in 976 middle-aged Chinese men and women. On the basis of mutual information analysis, they found that Cu and P always ranked the first two among three specific ion networks associated with the earlier-mentioned situations. Very recently, we developed a high throughput ICP-MS method for rapid element profiling (unpublished data) in HeLa cells. We applied it to mammalian cells and characterized a complete human genome siRNA library (more than 21 000 genes) transfected into HeLa cells. Each gene knockdown was analyzed for the elemental profiles of nine elements. Using these primary screen data, we carried out gene network analysis and performed a secondary screen, which revealed several targets involved in the metabolism of selenium as well as other trace elements. The top selenium hits included a selenoprotein, which we found to be the most abundant selenoprotein in human cells, and the key component involved in the insertion of selenium into proteins. We also identified a cluster of ribosomal proteins whose deficiency increased the amount of selenium in cells. Our approach also identified known regulators or users of several metals, and pointed to additional candidate proteins involved in trace element metabolism. This ionomic dataset should be useful for further research involving trace elements and may help uncover previously uncharacterized transport proteins and regulatory mechanisms.

7 CONCLUDING REMARKS

Bioinformatics provides a powerful tool for studying metal metabolism and the associated metalloproteomes. The strategies were mainly based on either identification of

metalloproteomes using known metal-binding motifs/patterns or investigation of metal utilization traits (e.g., specific transporters, regulators, cofactor biosynthesis components, and known metalloproteins). However, it is still very difficult to identify complete metalloproteomes for almost all metals. Nevertheless, bioinformatics studies, especially comparative genomics, have provided significant advances in identifying the general principles of utilization of metals in both prokaryotes and eukaryotes. In this article, we discussed how bioinformatics and systems level approaches can be used to analyze the function and evolution of metal utilization. We described recent progress that used computational methods to better understand the utilization of several essential transition metals. In the future, with the increased availability of genome sequences and improved experimental techniques for ionomics analyzes, bioinformatics and comparative genomics will play a significant role in studies on the utilization and evolution of metals.

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9 ABBREVIATIONS AND ACRONYMS

ABC = ATP-binding cassette; AO = aldehyde oxidase; AOR = aldehyde:ferredoxin oxidoreductase; Co = cobalt; CoA = coenzyme A; CODH = carbon monoxide dehydrogenase; CODH/ACS = acetyl-coenzyme A decarbonylase/synthase; CoM = coenzyme M; COX = cytochrome c oxidase; cPMP = cyclic pyranopterin monophosphate; Cr = chromium; Ctr = Cu transporter; Cu = copper; CuAO = Cu amine oxidase; Cu–Zn SOD = Cu–Zn superoxide dismutase; Cys = cysteine; DBM = dopamine β-monooxygenase; DDH/GDH = diol/glycerol dehydratase; DMSOR = dimethylsulfoxide reductase; EAL = ethanolamine ammonia lyase; ECM = ethylmalonyl-CoA mutase; EPR = electron paramagnetic resonance; Fe = iron; GAO = galactose oxidase; GlxI = glyoxalase I; GM = glutamate mutase; HCO = heme Cu oxidase; His = histidine; ICM = isobutyryl-CoA mutase; ICP-AES = inductively coupled plasma atomic emission spectroscopy; ICP-MS = inductively coupled plasma mass spectrometry; ICP-OES = inductively coupled plasma optical emission spectroscopy; 5,6-LAM = D-lysine 5,6-aminomutase; mARC = mitochondrial amidoxime-reducing component; MBD = metal-binding domain; MCM = methylmalonyl-CoA mutase; MCO = multicopper oxidase; MCR = methyl-coenzyme M reductase; Met = methionine; MetH = methionine synthase; MGD = molybdopterin guanine dinucleotide; MGM = methyleneglutarate mutase; Mn = manganese; Mo = molybdenum; MoBP = Moco-binding protein; Mop = Mo-binding protein; Moco = Mo cofactor; Ndh2 = NADH dehydrogenase 2; Ni = nickel; NiR = nitrite reductase; N_2OR = nitrous oxide reductase; NR = nitrate reductase; PHM = peptidylglycine R-hydroxylating monooxygenase; PiiMS = Purdue Ionomics Information Management System; pMMO = particulate methane monooxygenase; Pro = proline; ROS = reactive oxygen species; RNR II = B_{12}-dependent ribonucleotide reductase; Ser = serine; SO = sulfite oxidase; SodN = Ni-containing superoxide dismutase; Tyrp = tyrosinase-related proteins; V = vanadium; W = tungsten; XDH = xanthine dehydrogenase; XO = xanthine oxidase; XRF = X-ray fluorescence; Zn = zinc.

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