INORGANIC BIOCHEMISTRY

Ivano Bertini, Marco Salomone-Stagni, and **Paola Turano**
University of Florence, Italy

Keywords: Bioinorganic chemistry, metal cofactors, metalloproteins, metalloenzymes, model compounds

Contents

Summary

The elements of biological relevance are highlighted within the periodic table. Historical remarks on the discovery of metal ions in biological systems are provided and the fundamental concepts of metal cofactor, special metal cofactor, and metalloprotein are defined. The main structural and functional features of metal centers in proteins are reviewed, and the main strategies used to obtain model compounds for protein metal centers are briefly summarized.

1. Introduction

Living organisms consist of proteins, DNA, RNA, lipids, carbohydrates, and a number of metabolites of different types. Carbon, hydrogen, nitrogen, oxygen, phosphorus, and

sulfur are the six bulk constituents of their molecular structures. However, other twenty elements, generally considered "inorganic" are also essential for life. They are highlighted in the periodic table of Figure. 1. Their behavior within the biological context is the subject of Inorganic Biochemistry (or Bioinorganic Chemistry or Biological Inorganic Chemistry). Also relevant to the discipline are studies on model compounds aimed at the characterization of the geometric and electronic structure as well as the reactivity of biological metal sites.

2. Historical Background

Studies on chemical compounds of bioinorganic interest commenced in the early 19th century when the colored pigments of leaves and blood attracted the attention of scientists. In 1837-1838 J. J. Berzelius identified the leaf pigments, whereas in 1869 F. Hoppe-Seyler identified the blood pigment that he named hemoglobin and demonstrated the presence of iron. Further studies led to the identification of the most widespread heme proteins. In the period 1880-1890 C.A. MacMunn observed what he called histohematins or myohematins, later "re-discovered" by D. Keilin and re-named cytochromes. In the year 1897 K.A.H. Mörner was able to identify spectroscopically the myochrome, later called myoglobin. Insight into the chemical similarities between the two classes of pigments and into the presence and nature of metal ions inside them, however, came only after the beginning of the 20th century. Important studies, notably by C.A. Schunck and L.P. Marchlewski, ultimately established that the haematoporphyrin extracted from blood and the phylloporphyrin from leaves were pigments built upon the same basic tetrapyrrole. R. M. Willstätter was awarded the Nobel Prize in Chemistry in 1915 for proving with complete evidence that magnesium is an integral part of chlorophyll, where it is held "in a manner which is very similar to the way in which iron is held in haemoglobin". H. Fisher was awarded the Nobel Prize in Chemistry in 1930 for his researches into the constitution of hemin and chlorophyll and especially for his synthesis of hemin.

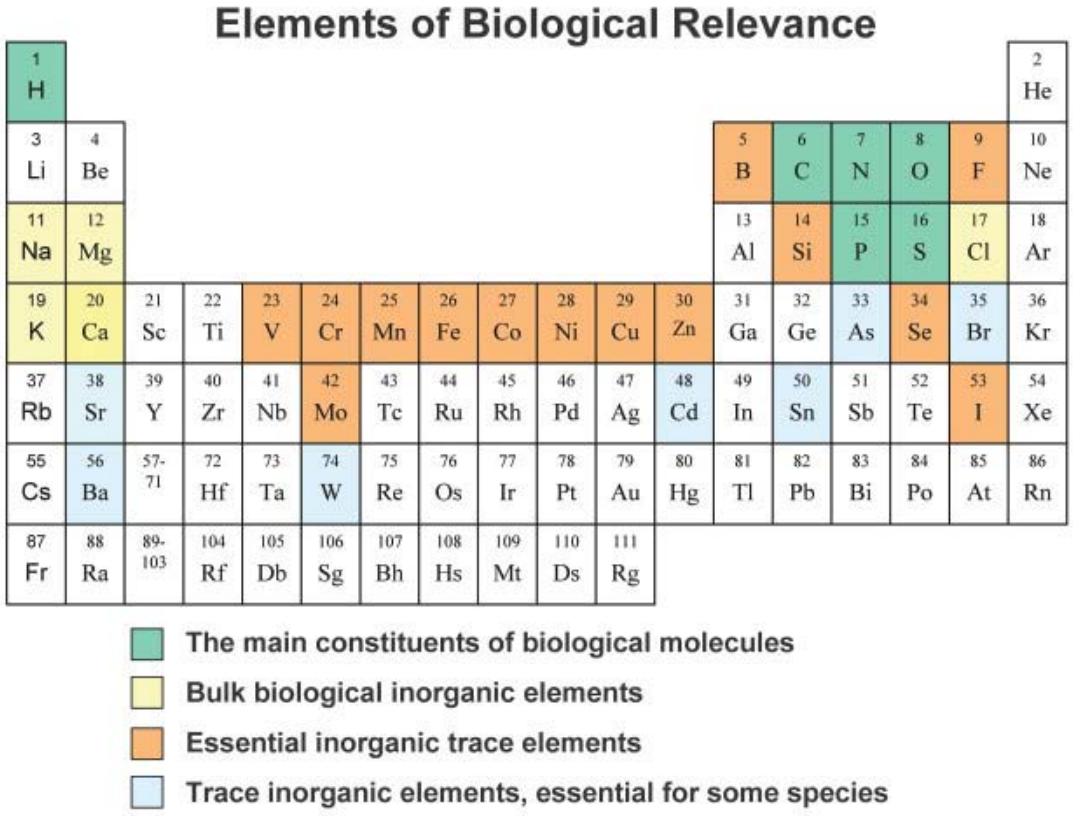


Figure 1: The elements of the periodic table that have biological relevance

Around 1930, added impetus was given to heme protein chemistry with the outstanding work of D. Keilin. He was largely responsible for the elucidation of the cytochrome (meaning cellular pigment) components of the mitochondrial respiratory chain. Thus, he first introduced the nomenclature of *a*-type, *b*-type and *c*-type cytochromes on the basis of the distinctive longest wavelength at which each of these types, under reducing conditions, had maximal absorbance. In 1936 horse heart cytochrome was obtained approximately 80% pure, and in 1939 close to 100%. In 1938 H. Theorell succeeded in showing that the porphyrin part of cytochrome was linked to the protein by means of two sulfur bridges from cysteine residues in such a way that the vinyl groups of the porphyrin were saturated and were converted to thioether groups. In 1941 H. Theorell and Å. Åkeson identified the imidazole side chain of a protein histidine as one of the two axial ligands of the heme iron in cytochrome.

A parallel story led to the discovery of the first enzymes. It was again Berzelius that first introduced the concept of biological catalyst: “This is a new force producing chemical activity and belonging as well to inorganic as organic nature ... I shall therefore, to use a derivation well-known in chemistry, call it the *catalytic force* of bodies” (from Jöns Jacob Berzelius, 1835 yearbook). The chemical nature of this force, however, remained elusive for one century. In 1926 R. M. Willstätter had to admit his inability to identify the chemical nature of enzymes. In the same year, J.B. Sumner announced the crystallization of a pure form of urease (now known to contain nickel). His idea that an enzyme is a protein was heavily opposed by the scientific community: it was ignored or

disbelieved by most biochemists but finally it brought him the Nobel Prize in Chemistry 1946.

Nevertheless, during the century between the first idea of Berzelius and the final results of Sumner, important enzymes were discovered. In the years 1880-1910 A. Macfadyen discovered the role of phosphatases in alcoholic fermentation, G. Bertrand discovered two copper-containing enzymes, laccase (that he named oxidase) and tyrosinase. In the early 1920s, T. Thunberg showed that the oxidation of a large number of organic compounds such as succinic acid is catalyzed by enzymes, each specific for its substrate, named dehydrases and later dehydrogenases by O. Wieland.

In the same years, a growing interest in aerobic respiration developed. During the period 1910-1920 F. Battelli and L. Stern studied the oxidation of a number of substances by molecular oxygen in the presence on animal tissues. In the course of this research they were able to demonstrate the inhibitory effect of cyanide and interpreted their results as indicating the presence of an enzyme, sensitive to the presence of CN^-, that they called indophenol oxidase. In the following decade, two different interpretations were provided on the mechanism of action of this enzyme. Accordingly to O. Wieland and T. Thunberg, the first step of biological oxidation should consist of the activation of hydrogen atoms, otherwise inert, so that they can react with molecular oxygen. O.H. Warburg, impressed by the presence of iron in respiring cells and the ability of cyanide both to combine with iron and to inhibit cell respiration, proposed that the fundamental process is the activation of molecular oxygen by an iron-containing respiratory enzyme (atmungsferment). It was the merit of A. Szent-György (Nobel Prize in Medicine 1937) to reconcile the two views and to demonstrate that the two processes are complementary to each other and that, in the muscle cells, it is activated oxygen that activates hydrogen. Keilin's paper in the *Proceedings of the Royal Society* in 1925 with the title "On cytochrome, a respiratory pigment, common to animals, yeast, and higher plants" marked the beginning of studies of what Warburg later called the respiratory chain. He made it clear that the electrons derived from the activation of the hydrogen atoms by the dehydrogenase are transferred via three hemoproteins, which he named cytochromes *a*, *b*, and *c*, to an oxygen-activating oxidase. He did not name the oxidase in his 1925 paper, but in 1927 identified it, on the basis of its sensitivity to cyanide, as Battelli and Stern's indophenol oxidase and, on the basis of its sensitivity to both cyanide and carbon monoxide, with Warburg's atmungsferment. Keilin was able to establish that the first electron acceptor is cytochrome b, that there are two types of c-cytochromes (and he called the second c1), and that there are two a-type components, named cytochrome a and cytochrome a3, the latter having the properties reported for the Warburg's atmungsferment. Warburg refused to accept the suggested role of the cytochromes. This became one of the great controversies of the 1930s, and it was only later on that the respiratory enzyme was named cytochrome c oxidase, in recognition of Keilin's fundamental studies. Finally, the presence of copper in this enzyme was established in 1939 by D. Keilin and E. F. Hartree.

Another emblematic story of the development of scientific knowledge in bioinorganic chemistry is that of the definition of the biological role of zinc. The essential role of zinc in living organisms was established in the second half of the 19th century. Around 1940, D. Keilin and T. Mann reported the ubiquitous presence of zinc in human organs and

tissues. At the same time, new enzymes, later found to be zinc-enzymes, were discoverd: carbonic anhydrase was identified by N.U. Meldrum and F. J. W. Roughton in 1933; in 1936 alcohol dehydrogenase was purified from yeast and crystallized by E. Negelein and H.J. Wulf; carboxypeptidase was identified by M. L. Anson in 1937. However, it is only with the work of B.L. Vallee around 1950 that the presence of zinc was revealed to be essential for the catalytic function of these enzymes.

A milestone in the approach for studying proteins was established with the first X-ray crystal structure determinations of proteins. In 1960 M. F. Perutz published the crystal structure of hemoglobin. In the same year, J.C. Kendrew published the crystal structure of myoglobin. For these results, they were awarded the Nobel Prize in Chemistry 1962. It was the beginning of a new era: from then on the knowledge of the three dimensional structure of proteins became central to the comprehension of their function.

3. The Philosophy of Model Chemistry

Following the discovery of metal centers in metalloproteins, chemists started trying to synthesize compounds that mimicked their spectroscopic and functional properties. The guiding idea was that the synthesis and characterization of a compound with spectroscopic properties similar to those of a given metal center would shed light on the chemical nature and reactivity of the real biological system. The main advantage of studying the model compound instead of the protein itself was related to the much smaller size of the former and its suitability for biophysical studies. From the inorganic point of view the biological metal centers often represented interesting synthetic challenges and thus were relevant for science beyond their importance as model compounds. The chemistry of biomimetic model compounds has flourished since the ‘70s. However, nowadays, the advancements in spectroscopic, structural and biological tools make easier than before to study metal centers directly within their biological context. As a consequence, the impact of model compounds has diminished.

Here we aim to presenting a brief overview of some of the seminal contributions of models in bioinorganic chemistry. This survey surely neglects many researchers, but it is intended to provide a few notable examples and does not aim at completeness.

The literature on iron-porphyrin model compounds is immense and entire books or series of books are dedicated to this topic. From the chemical point of view, the high level of interest in metalloporphyrins is justified by their behavior as complex physiochemical systems. The relevance of iron porphyrins as models is further enhanced by the variety of biological functions of heme proteins. The minor role of peripheral substituents in the active site chemistry of heme proteins legitimizes the initial model approach, which relied on the synthetically convenient tetraphenylporphyrin and octaethylporphyrin. Initial studies demonstrated that the spin state and stereochemistry of the iron(III) and iron(II) centers is controlled by the nature and number of axial ligands. The most convincing application of the above findings was the prediction of the stereochemical effects of the binding of molecular oxygen to hemoglobin. The dioxygen affinities of myoglobin and both the low-affinity and high-affinity states of hemoglobin have been reproduced by model iron(II) porphyrin complexes with imidazole as a fifth ligand. Because these simple iron(II) porphyrins

irreversible oxidize when exposed to dioxygen, picket fence porphyrins were later developed, whose steric hindrance inhibits the oxidation process. These porphyrin complexes were found to bind O_2 with the same affinity as do hemoglobin and myoglobin. The same model compounds were found to bind CO with significantly higher affinity than do normal heme proteins. This was attributed to the steric features of the binding site of the proteins that distorts the Fe-CO geometry and lowers the CO affinity. High-valent iron(IV)-oxo heme intermediates are characterized by a rich oxidative chemistry in heme enzymes. Many efforts have been devoted to the understanding of P450-catalyzed oxygenation of hydrocarbons as well as to the recognition of the intermediate species in the catalytic cycles of heme enzymes like P450 and peroxidases. The role of the proximal ligand, proximal- and distal-binding site environments, porphyrin macrocycle distortions from planarity can all contribute to the nature and efficiency of the catalytic chemistry of these high-valent intermediates. Recently the model chemistry approach has been coupled to computational investigations focused on the electronic description of the possible intermediates and reaction pathways. Porphyrins have also been incorporated into micellar or bilayer structures in order to induce regioselectivity and controlled substrate access to the porphyrin metal ion.

The heme-copper binuclear site at the active site of cytochrome c oxidase has attracted the attention of a number of synthetic bioinorganic chemists. The earliest and most common biomimetic efforts focused on the oxidized or "resting state" form of this center possessing a porphyrinate-iron(III) and copper(II). More recent efforts have been directed at modeling the reduced state and at O_2 and CO (as dioxygen surrogate) chemistry. Synthetic models have also been developed to reproduce the characteristic features of the binuclear Cu_A electron-transfer center. The first example of dithiolate-bridging that closely mimics the Cu_A geometry and other spectroscopic features was reported in 1996.

For the oxidized copper center of blue copper proteins, the most challenging aspect for understanding the site was the presence of unique spectral features, i.e. the strong charge-transfer band at approximately 600 nm and the narrow hyperfine coupling in the EPR spectrum. Trying to explain these features required a deep understanding of the spectroscopic properties of copper(II) in different coordination environments. A couple of structurally defined complexes now exist that model key geometric and electronic features of the blue copper site.

The synthetic analogue approach to the metal sites in iron-sulfur proteins was initiated in the early 1970s and has the merit to have provided fundamental insights on the nature of the chemical bonds in metal clusters and on the spectroscopic features of the different oxidation states. Many efforts were also devoted to synthesis and characterization of model compounds for the non-heme non-iron-sulfur proteins.

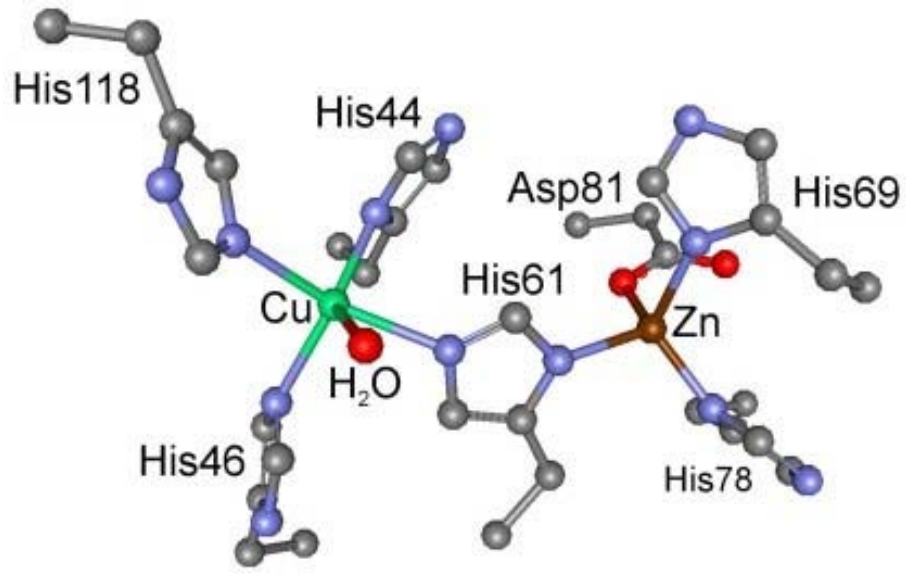
The recently discovered complex metal cofactors of enzymes involved in redox catalysis in biogeochemical cycles have attracted the attention of many researchers. Synthesis, structures, and reactivity of high-nuclearity Mo/Fe/S clusters, that model the

properties of the cofactors of nitrogenases, or of the Cu_Z center of nitrous oxide reductase, represent a focus of active research.

In addition to the preparation of synthetic models, chemists have largely employed metal substitution to gain information on the coordination environment of metalloproteins, taking advantage of the peculiar spectroscopic features of non-naturally occurring metal ions. One of the most common examples is the substitution of the spectroscopically silent zinc(II) in zinc-enzymes with the more suitable cobalt(II) or of calcium in calcium-binding proteins with lanthanides. We would like also to mention a peculiar example of metal substitution in multinuclear metal centers. Substitution of zinc in copper, zinc superoxide dismutase (see Figure. 2) has been performed providing a number of different derivatives that maintain high catalytic activity. Cu, Cu-SOD, Cu, Co-SOD, and Cu, Ni-SOD could serve as good models for the study of the molecular and mechanistic properties of the native enzyme by the use of different physical methods. In particular Cu, Co-SOD was employed for the NMR characterization of the protein active site by taking advantage of the magnetic interaction between the paramagnetic metals copper(II) and cobalt(II) through the bridging imidazolate. As a consequence, the slow electron relaxation rate of copper(II) is significantly enhanced through the coupling with cobalt(II), which enables the detection of sharp hyperfine shifted proton NMR signals of the metal ligands.

Substituting porphyrins in heme proteins is another method that has been used for many years to unravel the role of heme substituents in protein function and to gain information on the possible binding site of substrates in heme-enzymes.

In the last decade the use of models to unravel the features of natural metalloproteins has evolved from the above described purely inorganic approach to a more biologically-oriented methodology. This development was prompted by the intrinsic difficulties of the inorganic approach based on ligand design to mimic secondary coordination sphere effects and stereo and enantio-selectivity of the metal centers. In the novel approach biotechnological skills are used to engineer in natural protein scaffolds novel metal centers, making ample use of site directed mutagenesis. Such de novo design approach has been used e.g., to change the function of oxygen binding proteins into peroxidases, or of cytochromes into globins upon substitution of one or a few key residues. Engineering of entire protein fragments within another protein scaffold has been used to transform blue copper proteins into Cu_A centers.



CuZn Superoxide Dismutase - Active Site
(PDB: 1CBJ; *Bos taurus*)

Figure 2: The active site of copper, zinc superoxide dismutase

-
-
-

Bibliography

Bertini I., Gray H.B., Stiefel E.I. and Valentine J.S. Eds. (2006) *Biological Inorganic Chemistry: Structure and Reactivity*, University Science Books [A modern textbook for courses in biological inorganic chemistry. It provides introductory information on the basic aspects of metalloproteins, as well as a detailed description of most metalloproteins within the context of their reaction pathways].

Bertini I., Sigel A. and Sigel H. Eds. (2001) *Handbook on metalloproteins*, Marcell Dekker Inc.[The text provides a detailed description of the metal centers in which the elements of biological relevance are encountered. It describes also the three-dimensional structure of proteins in which the metal centers are embedded, as well as the corresponding structure-function relationships].

Rees D.C. (2002) Great Metalloclusters in enzymology. *Annu. Rev. Biochem.* **71**, 221-246. [An excellent review of the most complex special metal cofactors and of the structure of the corresponding metalloproteins determined by X-ray crystallography]

Holm R.H. and Solomom E.I. Guest Eds. (2004) Thematic Issue: Biomimetic Inorganic Chemistry *Chem. Rev.* **104.** [An issue containing a series of reviews that provide a summary of the advances brought about by model inorganic chemistry to the problem of metal site elucidation.]

Lu Y., Berry S.M. and Pfister T.D. (2001) Engineering Novel Metalloproteins: Design of Metal-Binding Sites into Native Protein Scaffolds *Chem. Rev.* **101**, 3047-3080. [A competent review on the novel techniques for engineering metal sites in host protein scaffolds.]

For historical information refer to the Nobel Laureate lectures in Chemistry and Medicine available at http://nobelprize.org/.

Biographical Sketches

Ivano Bertini graduated in Chemistry at the University of Florence in 1964 and became full professor of Chemistry in 1975 at the same University [http://www.cerm.unifi.it/bertini.html]. He is Director of the Magnetic Resonance Center (CERM http://www.cerm.unifi.it/) of the University of Florence. His main research interests are the advancements in nuclear magnetic resonance spectroscopy, the expression and preparation of metalloproteins, their structural characterization and the investigation of their interactions with emphasis on understanding cellular processes at the molecular level. He has over 600 papers and many books. He has received the Chugaev Diploma of Kurnakov Institute of the Academy of Science, URSS, in 1981, the Golden Medal of the Magnetic Resonance Group of the Italian Chemical Society, in 1991, Prize Academia dei Lincei, Italy, in 1993, Bijvoet Medal, Utrecht, NL, in 1998, Sapio NMR Prize, Italy, in 1999, the Cannizzaro Medal of the Italian Chemical Society and the Basolo Medal in 2006. Amongst the special lectures: A.D. Little Lecturer at MIT, Cambridge, MS, USA, in 1997, E.L. Mütterties Lecturer at Berkeley, CA, USA, in 1997 and FECS lecturer, Athens in 2002. He has received three honorary doctorates. He is a member of the Accademia Nazionale dei Lincei and of the Academia Europaea.

Marco Salomone-Stagni graduated in Biology at the University of Bologna in 2005. During his degree thesis he studied the nickel containing enzyme urease and its associated chaperons using biochemical and bioinformatical approaches. Since 2006 he was awarded with a fellowship in the Magnetic Resonance Centre (CERM) of the University of Florence.

Paola Turano (http://www.cerm.unifi.it/turano) graduated in Chemistry at the University of Florence in 1989. She received her Ph.D. in Chemical Sciences in 1993 from the same University. In 2002 she became associate professor of chemistry of the University of Florence. She was awarded the Raffaello Nasini gold medal for Inorganic Chemistry of the Italian Chemical Society, 2003. Her research interests focus on the use of NMR for the structural characterization of metalloproteins and the study of the factors controlling their stability. Special attention is devoted to the methodological aspects related to the application of NMR to proteins containing paramagnetic metal ions.