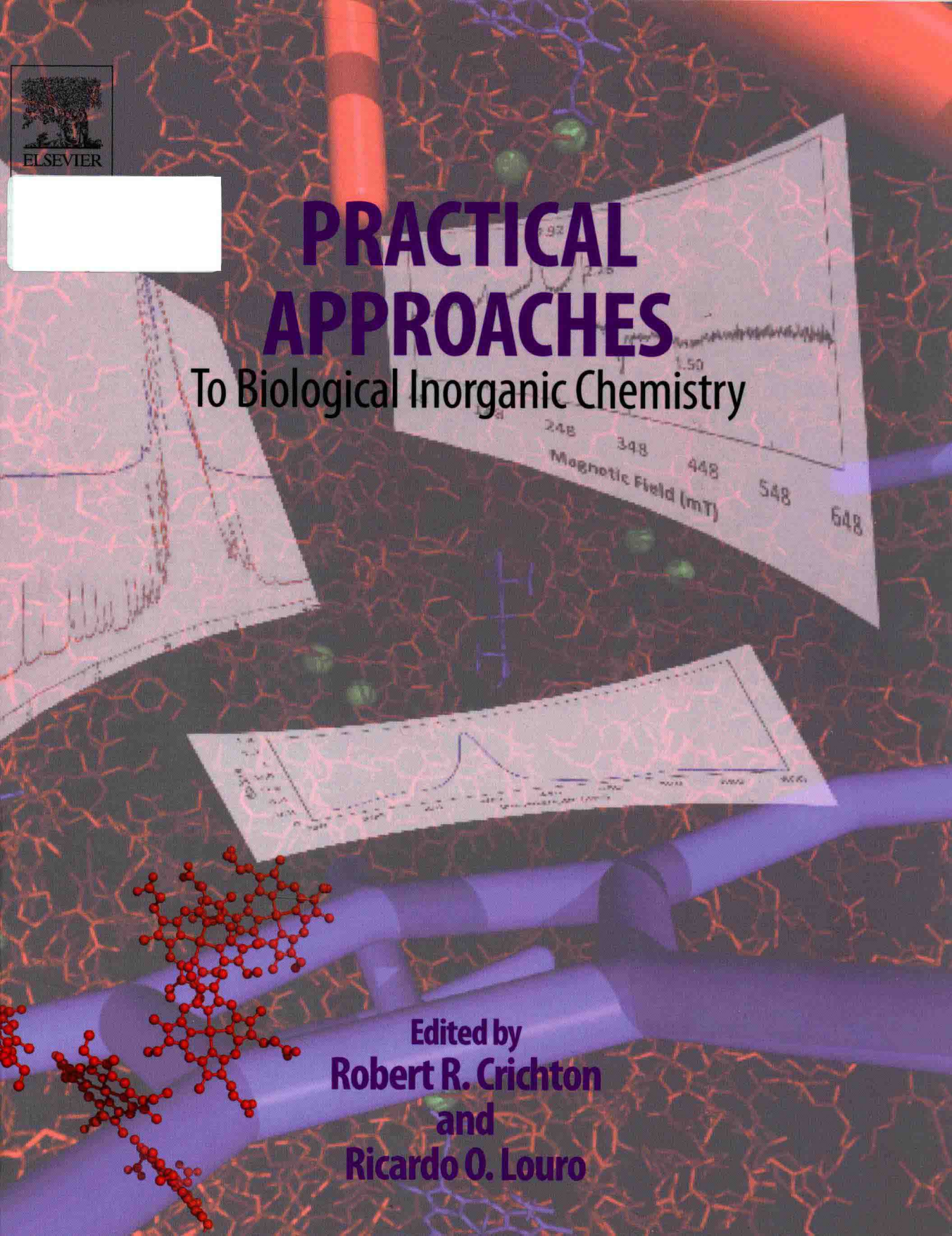
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Edited by
Robert R. Crichton
and
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Practical Approaches to Biological Inorganic Chemistry

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Practical Approaches to Biological Inorganic Chemistry

Preface

Shrouded in the mists of scientific antiquity (things move so quickly that even a decade or two seems a long time), in reality a little less than 30 years ago – the Federation of European Biochemical Societies, better known by its acronym FEBS, invited the Belgian Biochemical society to organise their annual Congress in Belgium. For the first time in the history of these meetings (since the inaugural Congress, in London in 1964), two half day symposia were organised on the subject of metalloproteins. At the end of the second of these, a group of what in those days were called inorganic biochemists met to enjoy a drink together in the bar of the Sheraton Hotel. The outcome was that two of those present, one of whom is co-editor of the present volume, together with Cees Veeger were entrusted with the task of organising a FEBS Workshop Course on Inorganic Biochemistry. The first of these was held at the Hotel Etap in Louvain-la Neuve at the end of April, 1985. The origins of this book can be traced back to the long series of Advanced Courses which have followed that pioneering start.

At that very first Course, the pattern was established of organising lectures to introduce the subject and to present a theoretical background to the methods which could be used to study metals in biological systems, together with practical sessions in smaller groups. The final lectures were then devoted to specific examples. It is interesting, and perhaps not too surprising, that after an introduction to ligand field theory by Bob Williams, and metal coordination in biology by Jan Reedijk, X-ray, EPR, NMR, Mössbauer and EXAFS spectroscopy of metalloproteins were on the programme. The practicals included NMR, EPR and Mössbauer as well as Cees Veeger's favourite, biochemical analysis of Fe and S in Fe–S proteins. There was an evening lecture by Helmut Beinert (then on sabbatical in Konstanz) entitled 'Limitations of Spectroscopic Studies on Metalloproteins and Chemical Analysis of Metals in Proteins'. While the lecturers were shuffled around from year to year, Fred Hagen, Antonio Xavier, Alfred Trautwein, and Dave Garner represented the cornerstone of the spectroscopic part of the course over the early years.

Since then, over the period from 1985 until now we have organised some 20 courses, and trained over 800 students, most of whom were doctoral or post-doctoral students when they came on the course. It is a source of great pride and satisfaction that many of the former students still enjoy active and distinguished careers in the area of Biological Inorganic Chemistry, as we now call the subject. Even more rewarding are the number of former participants who now form the staff of the course, notably the other co-editor, who has also taken on the mantle of co-organiser of the most recent courses. Indeed, with the exception of Rob Robson, who taught the Molecular Biology lectures and practical for many years, the other authors contributing to this book, Frank Neese, Fred Hagen, Eckhard Bill, Martin Feiters, Christophe Leger and Margarida Archer are all alumni of the 'Louvain-la-Neuve' course.

Our intention in editing this volume is that it can serve as a starting point for any student who wants to study metals in biological systems. The presentations by the authors represent a distillation of what they have taught over a number of years in the advanced course. We begin with an overview of the roles of metal ions in biological systems, which we hope will serve as taster for the reader, who will find a much more detailed account in the companion work to this volume (Crichton, 2012). Thereafter, after an introduction to that most erudite of discipline (at least for non-inorganic chemists) ligand field theory, augmented by a good dose of how molecular orbital theory can predict the properties of catalytic metal sites. This leads naturally into a sequence which describes the physicochemical methods which can be used to study metals in biology, concluding with an overview of the application of the powerful methods of modern genetics to metalloproteins.

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The considerations expressed by that pioneer of analytical precision Helmut Beinert in his 1985 evening lecture in Louvain-la-Neuve are as relevant today as they were then. Use as many techniques as possible to analyse your sample – the more information from different approaches you have, the better we will understand your protein. Do not waste expensive and sensitive methods on shoddy impure samples, and conversely do not employ primitive technical means to analyse highly purified samples, which have required enormous investment to obtain them. And above all recognise that the key to metalloprotein characterisation is collaboration. Do not think you can simply phagocytise a technique from the laboratory of a colleague who knows the method inside out – it is much richer to collaborate, incorporating his or her know-how into your research. And you will be the richer for it.

Bonne chance, good luck, boa sorte – and we look forward to greet you on one of the courses which will, we hope, continue into the future. Hopefully, this little introductory text will not only whet your appetite, but help you to find your way about the myriad practical methods which can be used to study metals in biological systems.

Robert R. Crichton and Ricardo O. Louro
Louvain-la-Neuve, July, 2012

Contents

Chapter 1

An Overview of the Roles of Metals in Biological Systems

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Chapter Outline

INTRODUCTION: WHICH METALS IONS AND WHY?

In the companion book to this one, 'Biological Inorganic Chemistry 2nd edition' (Crichton, 2011), we explain in greater detail why life as we know it would not be possible with just the elements found in organic chemistry – namely carbon, oxygen, hydrogen, nitrogen, phosphorus and sulfur. We also need components of inorganic chemistry as well, and in the course of evolution nature has selected a number of metal ions to construct living organisms. Some of them, like sodium and potassium, calcium and magnesium, are present at quite large concentrations, constituting the so-called 'bulk elements', whereas others, like cobalt, copper, iron and zinc, are known as 'trace elements', with dietary requirements that are much lower than the bulk elements.

Just six elements – oxygen, carbon, hydrogen, nitrogen, calcium and phosphorus – make up almost 98.5% of the elemental composition of the human body by weight. And just 11 elements account for 99.9% of the human body (the five others are potassium, sulfur, sodium, magnesium and chlorine). However, between 22 and 30 elements are required by some, if not all, living organisms, and of these are quite a number are metals. In addition to the four metal ions mentioned above, we know that cobalt, copper, iron, manganese, molybdenum, nickel, vanadium and zinc are essential for humans, while tungsten replaces molybdenum in some bacteria. The essential nature of chromium for humans remains enigmatic.

Just why these elements out of the entire periodic table (Figure 1.1) have been selected will be discussed here. However, their selection was presumably based not only on suitability for the functions that they are called upon to

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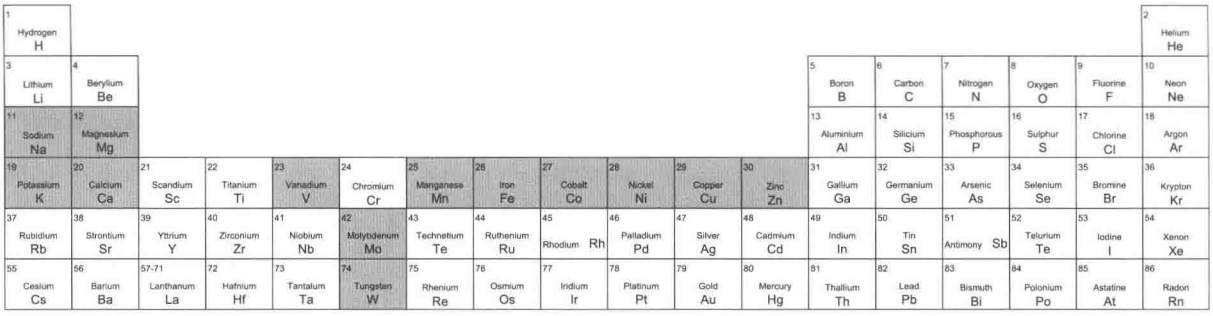

FIGURE 1.1 An abbreviated periodic table of the elements showing the metal ions discussed in this chapter.

play in what is predominantly an aqueous environment, but also on their abundance and their availability in the earth's crust and its oceans (which constitute the major proportion of the earth's surface).

The 13 metal ions that we will discuss here fall naturally into four groups based on their chemical properties. In the first, we have the alkali metal ions Na^+ and K^+. Together with H^+ and Cl^-, they bind weakly to organic ligands, have high mobility, and are therefore ideally suited for generating ionic gradients across membranes and for maintaining osmotic balance. In most mammalian cells, most K^+ is intracellular, and Na^+ extracellular, with this concentration differential ensuring cellular osmotic balance, signal transduction and neurotransmission. Na^+ and K^+ fluxes play a crucial role in the transmission of nervous impulses both within the brain and from the brain to other parts of the body.

The second group is made up by the alkaline earths, Mg^{2+} and Ca^{2+}. With intermediate binding strengths to organic ligands, they are, at best semi-mobile, and play important structural roles. The role of Mg^{2+} is intimately associated with phosphate, and it is involved in many phosphoryl transfer reactions. Mg-ATP is important in muscle contraction, and also functions in the stabilisation of nucleic acid structures, as well as in the catalytic activity of ribozymes (catalytic RNA molecules). Mg^{2+} is also found in photosynthetic organisms as the metal centre in the light-absorbing chlorophylls. Ca^+ is a crucial second messenger, signalling key changes in cellular metabolism, but is also important in muscle activation, in the activation of many proteases, both intra- and extracellular, and as a major component of a range of bio-minerals, including bone.

Zn^{2+}, which is arguably not a transition element,[1] constitutes the third group on its own. It is moderate to strong binding, is of intermediate mobility and is often found playing a structural role, although it can also fulfil a very important function as a Lewis acid. Structural elements, called zinc fingers, play an important role in the regulation of gene expression.

The other eight transition metal ions, Co, Cu, Fe, Mn, Mo, Ni, V and W form the final group. They bind tightly to organic ligands and therefore have very low mobility. Since they can exist in various oxidation states, they participate in innumerable redox reactions, and many of them are involved in oxygen chemistry. Fe and Cu are constituents of a large number of proteins involved in electron transfer chains. They also play an important role in oxygen-binding proteins involved in oxygen activation as well as in oxygen transport and storage. Co, together with another essential transition metal, Ni, is particularly important in the metabolism of small molecules like carbon monoxide, hydrogen and methane. Co is also involved in isomerisation and methyl transfer reactions. A major role of Mn is in the catalytic cluster involved in the photosynthetic oxidation of water to dioxygen in plants, and, from a much earlier period in geological time, in cyanobacteria. Mo and W enzymes contain a pyranopterindithiolate cofactor, while nitrogenase, the key enzyme of N_2 fixation contains a molybdenum–iron–sulfur cofactor, in which V can replace Mo when Mo is deficient. Other V enzymes include

1. IUPAC defines a transition metal as "an element whose atom has an incomplete d sub-shell, or which can give rise to cations with an incomplete d sub-shell."

haloperoxidases. To date no Cr-binding proteins have been found, adding to the lack of biochemical evidence for a biological role of the enigmatic Cr.

SOME PHYSICOCHEMICAL CONSIDERATIONS ON ALKALI METALS

Before considering, in more detail, the roles of the alkali metals, Na^+ and K^+, and the alkaline earth metals, Mg^{2+} and Ca^{2+}, it may be useful to examine some of their physicochemical properties (Table 1.1). We can observe, for example that Na^+ and K^+ have quite significantly different unhydrated ionic radii, whereas, the hydrated radii are much more similar. It therefore comes as no surprise that the pumps and channels which carry them across membranes, and which can easily distinguish between them, as we will see shortly, transport the unhydrated ions. Although not indicated in the table, it is clear that Na^+ is invariably hexa-coordinate, whereas K^+ and Ca^{2+} can adjust to accommodate 6, 7 or 8 ligands. As we indicated above, both Na^+ and K^+ are characterised by very high solvent exchange rates (around 10^9/s), consistent with their high mobility and their role in generating ionic gradients across membranes. In contrast, the mobility of Mg^{2+} is some four orders of magnitude slower, consistent with its essentially structural and catalytic. Perhaps surprisingly, Ca^{2+} has a much higher mobility (3×10^8/s), which explains why it is involved in cell signalling via rapid changes on Ca^{2+} fluxes.

The selective binding of Ca^{2+} by biological ligands compared to Mg^{2+} can be explained by the difference in their ionic radius, as we pointed out above. Also, for the smaller Mg^{2+} ion, the central field of the cation dominates its coordination sphere, whereas for Ca^{2+}, the second and possibly even the third, coordination spheres have an important influence resulting in irregular coordination geometry. This allows Ca^{2+}, unlike Mg^{2+} to bind to a large number of centres at once.

The high charge density on Mg^{2+} as a consequence of its small ionic radius ensures that it is an excellent Lewis acid in reactions notably involving phosphoryl transfers and hydrolysis of phosphoesters. Typically, Mg^{2+} functions as a Lewis acid, either by activating a bound nucleophile to a more reactive anionic form (e.g. water to hydroxide anion), or by stabilising an intermediate. The invariably hexacoordinate Mg^{2+} often participates in structures where the metal is bound to four or five ligands from the protein and a phosphorylated substrate. This leaves one or two coordination positions vacant for occupation by water molecules, which can be positioned in a particular geometry by the Mg^{2+} to participate in the catalytic mechanism of the enzyme.

NA^+ AND K^+ – FUNCTIONAL IONIC GRADIENTS

How, we might ask, do the pumps and channels responsible for transport across membranes distinguish between Na^+ and K^+ ions? Studies over the last 50 years or so of synthetic and naturally occurring small molecules which bind ions have established the basic rules of ion selectivity. Two major factors appear to be of capital importance,

TABLE 1.1 Properties of Common Biological Cations

Cation	Ionic radius (Å)	Hydrated radius (Å)	Ionic volume (Å^3)	Hydrated volume (Å^3)	Exchange rate (sec^{-1})	Transport number
Na^+	0.95	2.75	3.6	88.3	8×10^8	7–13
K^+	1.38	2.32	11.0	52.5	10^9	4–6
Mg^{2+}	0.65	4.76	1.2	453	10^5	12–14
Ca^{2+}	0.99	2.95	4.1	108	3×10^8	8–12

(From Maguire and Cowan, 2002).

namely the molecular composition and the stereochemistry (essentially the size) of the binding site. Synthetic molecules have been created which selectivity bind Li^+ (radius 0.60 Å), Na^+ (0.95 Å), K^+ (1.35 Å) and Rb^+ (radius 1.48 Å) by simply adjusting the cavity size to match the ion (Dietrich, 1985). Now that we have the crystal structures of membrane transport proteins, we can begin to understand how ion selectivity is accomplished (MacKinnon, 2004; Gouax and MacKinnon, 2005). The Na^+-selective binding sites in the Na^+-dependant leucine transporter LeuT and the K^+-selective binding sites in the K^+ channel have been determined, providing a direct comparison of selectivity for Na^+ and K^+. The Na^+ and K^+ ions are completely dehydrated, both the Na^+ and the K^+ sites contain oxygen ligands, but by far the most important factor distinguishing Na^+ and K^+ sites is the size of the cavity formed by the binding site, which agrees well with the rules already learned from host/guest chemistry. What determines alkali metal cation selectivity, similar to that observed in ion binding by small molecules, is that the protein selects for a particular ion, Na^+ or K^+, by providing an oxygen-lined binding site of the appropriate cavity size.

Mammalian cells maintain a high intracellular K^+ (around 140 mM) and low intracellular Na^+ (around 12 mM) through the action of the Na^+, K^+-ATPase present in the plasma membrane. The overall reaction catalysed is:

$$3Na^+(in) + 2K^+(out) + ATP + H_2O \Leftrightarrow 3Na^+(out) + 2K^+(in) + ADP + P_i$$

The extrusion of three positive charges for every two which enter the cell, results in a transmembrane potential of 50–70 mV, which has enormous physiological significance, controlling cell volume, allowing neurons and muscle cells to be electrically excitable, and driving the active transport of important metabolites such as sugars and amino acids. More than one-third of ATP consumption by resting mammalian cells is used to maintain this intracellular $Na^+ - K^+$ gradient (in nerve cells this can rise to up to 70%).

This thermodynamically unfavourable exchange is achieved by ATP-mediated phosphorylation of the Na^+,K^+-ATPase followed by dephosphorylation of the resulting aspartyl phosphate residue, which drives conformational changes that allow ion access to the binding sites of the pump from only one side of the membrane at a time. The ATPase exists in two distinct conformations, E_1 and E_2, which differ in their catalytic activity and their ligand specificity (Figure 1.2). The E_1 form, which has a high affinity for Na^+, binds Na^+, and the $E_1.3Na^+$ form then reacts with ATP to form the "high-energy" aspartyl phosphate ternary complex $E_1 \sim P.3Na^+$. In relaxing to its "low-energy" conformation E_2-P, the bound Na^+ is released outside the cell. The E_2-P, which has a high affinity for K^+, binds $2K^+$, and the aspartyl phosphate group is hydrolysed to give $E_2.2K^+$, which then changes conformation to the E_1 form, releasing its $2K^+$ inside the cell. The structures of a number of P-type ATPases, including the Na^+ - K^+-ATPase and the Ca^{2+} ATPase of the Sarcoplasmic reticulum have been determined and are shown in Figure 1.3.

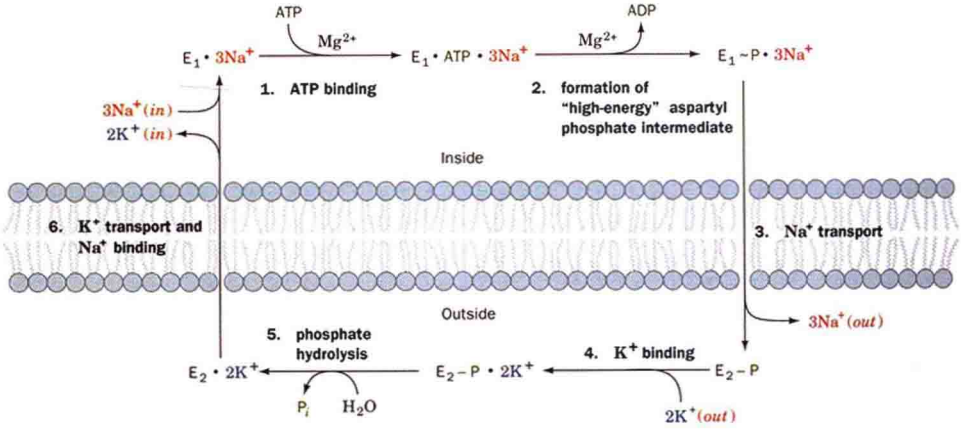

FIGURE 1.2 A model for the active transport of Na^+ and K^+ by the Na^+-K^+-ATPase.

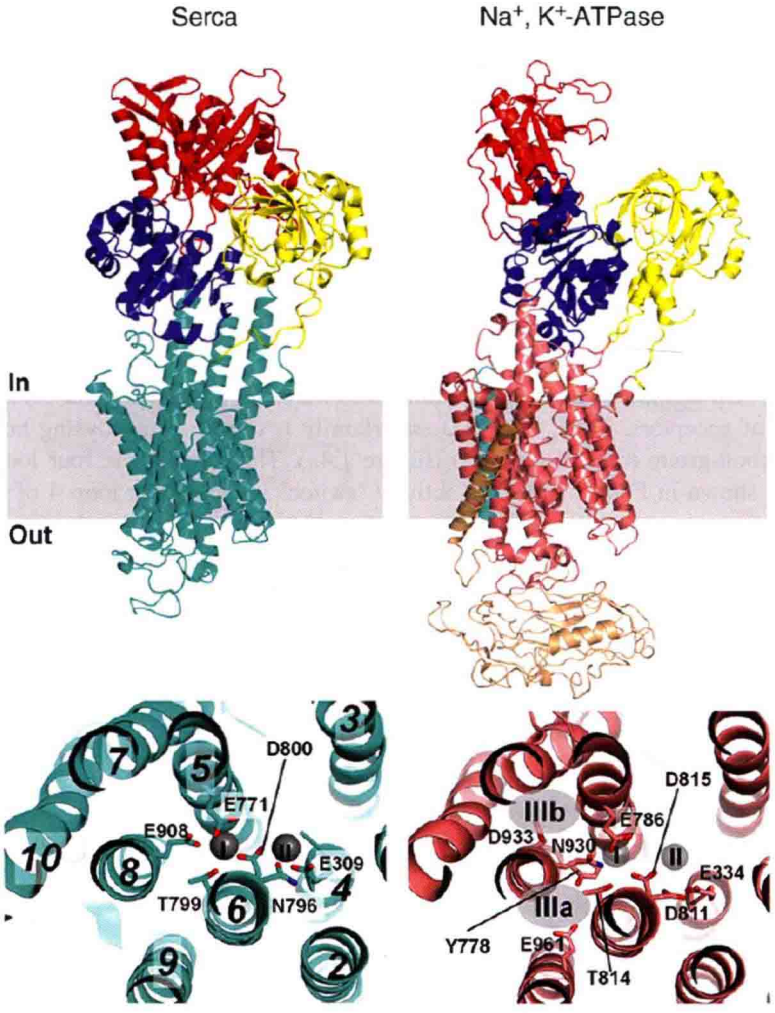

FIGURE 1.3 Overall structures and ion-binding site architectures of two P-type ATPases, rabbit sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) and pig Na^+,K^+-ATPase. The upper panel depicts rabbit SERCA (E1 Protein Data Base [PDB] entry 1T5S) and pig Na^+-K^+-ATPase (E2:Pi, PDB entry 3KDP). N-, P-, and A-domains are coloured red, blue and yellow, respectively; the β-subunit and γ-subunit of Na^+,K^+-ATPase wheat and cyan. The lower panel depicts the ion-binding sites, viewed approximately perpendicular to the membrane plane from the extracytoplasmic side, in the E1 state. Ion liganding residues are shown as sticks, transmembrane helices and calcium ions in SERCA are indicated by numbers and grey spheres, respectively, and the sites superposed as transparent spheres onto the Na^+,K^+-ATPase model. Putative binding sites for the third sodium ion in the Na^+,K^+-ATPase are indicated as grey ellipses. (From Bublitz et al., 2010. *Reproduced Copyright 2010 with permission from Elsevier).*

MG^{2+} – PHOSPHATE METABOLISM

The intracellular concentration of free Mg^{2+} is about 5×10^{-3} M, so that although Mg^{2+}-binding to enzymes is relatively weak (K_a not more than $10^5 M^{-1}$) and most Mg^{2+}-dependent enzymes have adequate local concentrations of Mg^{2+} for their activity. Mg^{2+} is the most abundant divalent cation in the cytosol of mammalian cells, binds strongly to ATP and ADP, and is therefore extensively involved in intermediary metabolism and in nucleic acid metabolism. However, like Zn^{2+}, it is a difficult metal ion to study, since it is spectroscopically silent, with the consequence that many spectroscopic studies on Mg^{2+} enzymes utilise Mn^{2+} as a replacement metal ion.

Of the five enzymes selected in the Enzyme Function Initiative, recently established to address the challenge of assigning reliable functions to enzymes discovered in bacterial genome projects, but for which functions have not yet been attributed (Gerlt et al., 2011), three of them are Mg^{2+}-dependent. We discuss two of them briefly here.

The haloalkanoic acid dehalogenase superfamily (HADSF) (>32,000 nonredundant members) catalyse a diverse range of reactions that involve the Mg^{2+}-dependent formation of a covalent intermediate with an active site Asp. Despite being named after a dehalogenase, the vast majority are involved in phosphoryl transfer reactions (Allen and Dunaway-Mariano, 2004, 2009). While ATPases and phosphatases are the most prevalent, the haloacid dehalogenase (HAD) family can carry out many different metabolic functions, including membrane transport, signal transduction and nucleic-acid repair. Their physiological substrates cover an extensive range of both size and shape, ranging from phosphoglycolate, the smallest organophosphate substrate, to phosphoproteins, nucleic acids, phospholipids, phosphorylated disaccharides, sialic acids and terpenes.

In HAD enzymes, Asp mediates carbon-group transfer to water (in the dehalogenases) and phosphoryl-group transfer to a variety of acceptors. Thus, the HAD superfamily is unique in catalysing both phosphoryl-group transfer (top) and carbon-group transfer (bottom) (Figure 1.4a). The roles of the four loops that comprise the catalytic scaffold are shown in Figure 1.4b. The activity 'switch' is located on loop 4 of the catalytic scaffold (yellow) which positions one carboxylate residue to function as a general base for the dehalogenases and either two or three carboxylates to bind the Mg^{2+} cofactor essential for the phosphotransferases. CO represents the backbone carbonyl oxygen of the moiety that is two residues downstream from the loop 1 nucleophile (red). The side-chain at this position is also used as an acid-base catalyst by phosphatase and phosphomutase HAD members. Loop 2 (green) and loop 3 (cyan) serve to position the nucleophile and substrate phosphoryl moiety. Figure 1.4c presents a ribbon diagram of the fold supporting the catalytic scaffold of phosphonatase.

The members of another large superfamily of Mg^{2+} enzymes, the enolase superfamily (with more than 6000 nonredundant members) catalyse diverse reactions, including β-eliminations (cycloisomerisation, dehydration and deamination) and 1,1-proton transfers (epimerisation and racemisation). The three founder members of the family are illustrated by mandelate racemase, muconate lactonising enzyme and enolase (Figure 1.5). They all catalyse reactions in which the α-proton of the carboxylate substrate is abstracted by the enzyme, generating an enolate anion intermediate. This intermediate, which is stabilised by coordination to the essential Mg^{2+} ion of the enzyme, is then directed to different products in the enzyme active sites.

CA^{2+} AND CELL SIGNALLING

Calcium ions play a major role as structural components of bone and teeth, but are also crucially important in cell signalling. To prevent the precipitation of phosphorylated or carboxylated calcium complexes, many of which are insoluble, the cytosolic levels of Ca^{2+} in unexcited cells must be kept extremely low, much lower than that in the extracellular fluid and in intracellular Ca^{2+} stores. This concentration gradient gives cells the opportunity to use Ca^{2+} as a metabolic trigger – the cytosolic Ca^{2+} concentration can be abruptly increased for signalling purposes by transiently opening Ca^{2+} channels in the plasma membrane or in an intracellular membrane. These increases in intracellular free Ca^{2+} concentration can regulate a wide range of cellular processes, including fertilisation, muscle contraction, secretion, learning and memory and ultimately cell death, both apoptotic and necrotic.

Extracellular signals often act by causing a transient rise in cytosolic Ca^{2+} levels, which, in turn, activates a great variety of enzymes through the action of Ca^{2+}-binding proteins like calmodulin, as we will discuss in detail below: this triggers such diverse processes as glycogen breakdown, glycolysis and muscle contraction. In the phosphoinositide cascade (Figure 1.6), binding of the external signal (often referred to as the agonist[2] when it provokes a positive response) to the surface receptor R (step 1) activates phospholipase C, either through a G

2. Many drugs have been developed either as agonist or antagonists to receptor-mediated signalling pathways, e.g. β-blockers block the action of the endogenous catecholamines adrenaline (epinephrine) and noradrenaline (norepinephrine) on β-adrenergic receptors.

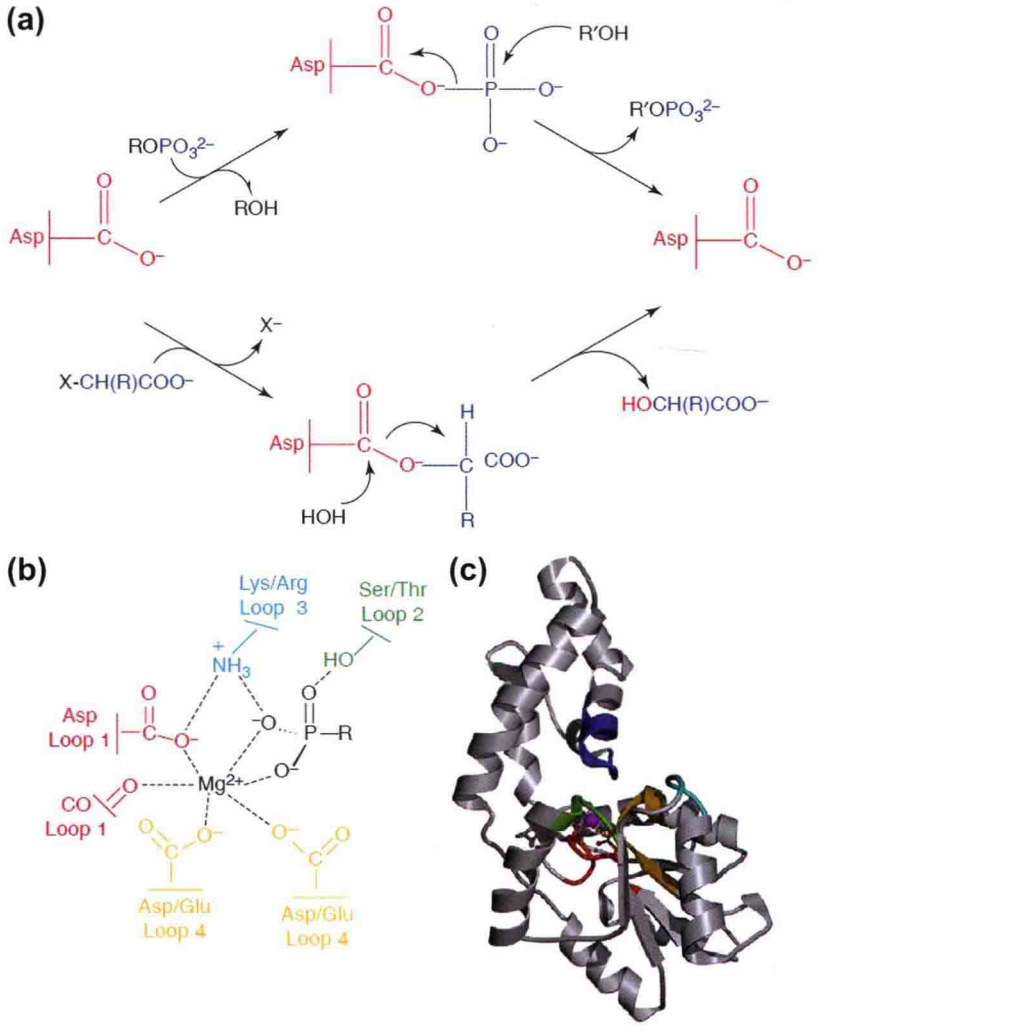

FIGURE 1.4 The catalytic scaffold in the haloacid dehalogenase (HAD) family of phosphotransferases. **(a)** In HAD enzymes, Asp mediates carbon-group transfer to water (in the dehalogenases) and phosphoryl-group transfer to a variety of acceptors. Thus, the HAD superfamily is unique in catalyzing both phosphoryl-group transfer (top) and carbon-group transfer (bottom). **(b)** Schematic of the roles of the four loops that comprise the catalytic scaffold. The activity 'switch' is located on loop 4 of the catalytic scaffold (yellow) which positions one carboxylate residue to function as a general base for the dehalogenases and either two or three carboxylates to bind the Mg^{2+} cofactor essential for the phosphotransferases. CO represents the backbone carbonyl oxygen of the moiety that is two residues downstream from the loop 1 nucleophile (red). The side-chain at this position is also used as an acid-base catalyst by phosphatase and phosphomutase HAD members. Loop 2 (green) and loop 3 (cyan) serve to position the nucleophile and substrate phosphoryl moiety. **(c)** Ribbon diagram (core domain: loop 1, red; loop 2, cyan; loop 3, green; loop 4, yellow; cap domain: specificity loop, blue) of the fold supporting the catalytic scaffold of phosphonatase (1FES). *(From Allen and Dunaway-Mariano, 2004. Copyright 2004, with permission from Elsevier).*

protein which uses the energy of guanosine triphosphate hydrolysis to liberate a subunit capable of activating the next partner in the cascade (2) or alternatively (not shown) by activating a tyrosine kinase. The activated phospholipase C, then hydrolyses phosphatidylinositol-4,5-bisphosphate (PIP_2) in the plasma membrane to $InsP_3$ (IP_3 in the figure) and diacylglycerol (DG) (3). $InsP_3$ stimulates the release of Ca^{2+}, sequestered in the endoplasmic reticulum (4), and this in turn activates numerous cellular processes through Ca^{2+}-binding proteins, such as

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