Beilstein-Institut ESCEC, September 23rd – 26th, 2007, Rüdesheim/Rhein, Germany

Considerations for the Specification of Enzyme Assays Involving Metal Ions

Richard Cammack[1*] and Martin N. Hughes[2]

[1]Pharmaceutical Sciences Research Division, King's College London, 150 Stamford Street, London SE1 9NH, UK

[2]Centre for Hepatology, Royal Free & University College London Medical School, Royal Free Campus, Rowland Hill Street, Hampstead, London NW3 2PF, UK

E-Mail: *richard.cammack@kcl.ac.uk

Received: 25th July 2008 / Published: 20th August 2008

Introduction

The recommendations of the STRENDA Commission (Version 1.2 June 16th, 2006) of standard requirements for reporting enzyme activity data (http://www.strenda.org/) include the proposal that the specification of assay conditions should include any metal salts to be added. They also require the definition of some other parameters which, as will be seen later, may have a bearing on the activity of metal ion-dependent enzymes. These include assay pH, buffer type and concentrations, and other assay components such as EDTA or dithiothreitol that will coordinate to metal ions [1, 2].

This chapter is intended to provide a guide to issues that are relevant to the determination of accurate kinetic data for the reactions of metal-dependent enzymes. Of particular importance are factors relating to the speciation and availability of metal ions in the assay medium. The interaction of the metal ions in the added metal salts with compounds present in the medium may result in the formation of a number of metal-ligand complexes. These may activate the enzyme to different extents at different rates. In extreme cases, metal ions may be precipitated out of solution and be unavailable to function in enzyme activation. We will further discuss the relevance of the metal ions in modelling the activity of the enzyme in the cell.

Essential Metallic Elements in Enzymes and Other Proteins

Eleven metallic elements are known to be essential for most types of living cells; two of these occur in Group 1 of the Periodic Table of the Elements, two in Group 2 and the remaining seven are d block elements (Fig. 1). Another four metals are, or may be, necessary for some organisms. Many of these metals are required for the activity of enzymes. The function of the metal ion may be catalytic, structural or regulatory.

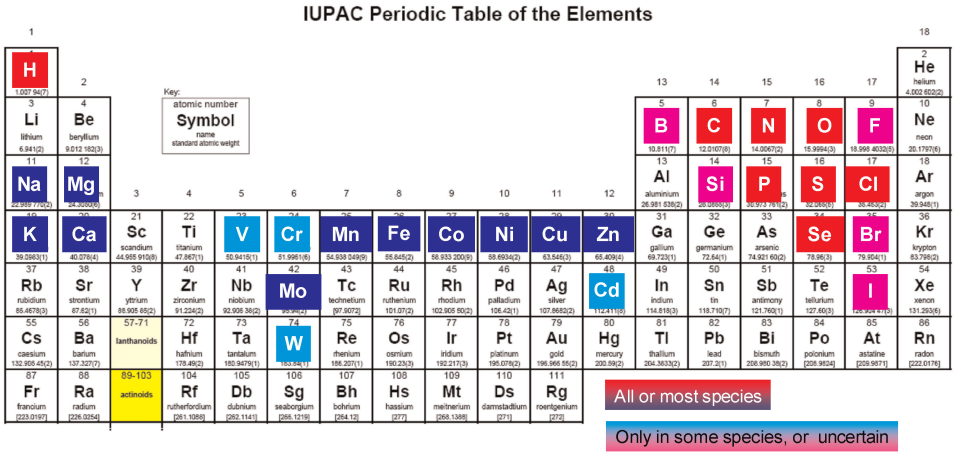

Figure 1. The periodic table, showing metals and non-metals that are required for life

Generally, according to the rules of the EC classification, enzymes that differ in their metal content or requirement have the same EC number if they catalyse the same reaction. However for some enzymes, the "Comments" field of the EC list specifies the presence of a metal; in other cases it may note a requirement for a metal ion for activity. Examples are given in Table 1.

Table 1. Examples of ways in which a metal requirement is specified in the EC List of enzymes.

"A copper protein"	EC 1.14.17.1 dopamine β-monooxygenase
"A zinc metallopeptidase"	EC 3.4.15.4 peptidyl-dipeptidase B
"Requires magnesium"	EC 6.3.1.8 glutathionylspermidine synthase
"Requires Ca^{2+}"	EC 2.7.11.18 myosin-light-chain kinase

Much more detailed information about metal ion requirements may be found in databases, notably BRENDA (http://www.brenda-enzymes.info/). Many enzymes contain metal ions in their structures, which can be detected by analytical methods such as particle-induced x-ray

emission (PIXE). The metal ions may be essential for activity or maintenance of protein structure. Occasionally they may be adventitiously bound to the protein, for example if a His-tag is used to aid purification, and the metal ion is bound to it [3].

The affinity with which metal ions bind to an enzyme varies greatly. Metal ions such as zinc and iron are usually tightly bound, and remain in position during isolation of the enzyme. However in some cases the metal ion can dissociate with loss of activity. In this case, addition of metal salts may reconstitute the protein and restore activity, though, as discussed later, this may not necessarily lead to a duplication of the normal environment of the metal in the cell.

The distinction between essential metal ions that are firmly bound to the enzyme, and those that can dissociate from the enzyme after the reaction cycle, is somewhat analogous to that between *prosthetic groups* such as pyridoxal phosphate which remain with the enzyme, and *cosubstrates* such as coenzyme A, which dissociate.

The requirement for loosely-bound metal ions in the activity of enzymes has often been inferred from observing the effects of added metal salts on the rate of reaction. A range of different salts may be incubated with the enzyme before assay, or included in the assay medium. Sometimes more than one element will confer activity, while others are inhibitory. Alternatively, the presence of metal ions may be inferred from the effects of treating the enzyme with chelating agents. Chelators display some degree of specificity for binding of a particular type of metal ion. A well-known example of the use of specific chelators to establish a role for a specific metal ion is the siderophore desferrioxamine, a specific and strongly binding chelator (log $K_d \approx 31$) for Fe^{3+}. Desferrioxamine cannot penetrate into an iron-containing enzyme such as ribonucleotide reductase, and can complex Fe(III) only when it dissociates from the protein [4]. More rapid complexation may be achieved with small ligands such as cyanide or nitric oxide, or the hydroxypyridones, [5] which can penetrate to some extent into the protein. Loss of iron from the enzyme may be facilitated by changing the protein conformation, e. g. by reduction of Fe(III) to Fe(II), or by lowering the pH. The rate of chelation of the metal ion depends on the spontaneous dissociation of the metal ion from its binding site, rather than on the thermodynamic stability of the final complex.

Properties of Metal Ions

Oxidation states

The oxidation state of a metal in a complex may be defined as the effective charge left on the metal ion when the ligands attached to the metal centre have been removed in their normal charged form. Thus, in aqueous solution, for a cation such as $Fe(H_2O)_6^{2+}$, the oxidation state is Fe(II), and the cation may be described by its charge number, i. e. iron(2+) or Fe^{2+} [6]. It

should be noted that while oxidation states of metal ions in complexes may usually be calculated straightforwardly, some "non-innocent" ligands may not allow this to be done. An example is nitric oxide, as this molecule may be bound to the metal centre as either NO^+ or NO^-. In complex structures such as iron-sulfur clusters, the oxidation state is more difficult to define.

"Hard" versus "soft" metal ions

A useful guide to the nature and extent of metal-ligand binding comes from the concept of hard and soft acids and bases [7]. Chemically-hard metal ions (acids) are small and not easily polarized, while soft metal ions are large and easily polarized. Ligands with highly electronegative donor atoms (O or N centres) are hard bases, while polarisable ligands such as those with sulfur donors are soft bases. In general stable complexes are formed between hard acids and hard bases and between soft acids and soft bases (Fig. 2).

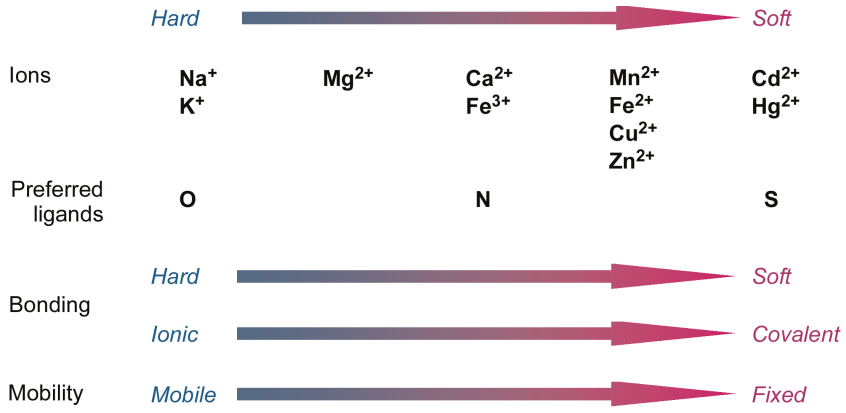

Figure 2. General properties of "hard" and "soft" metals and ligands.

There is an important gradation of properties between the sets of metals comprising (a) the Group 1 metal ions Na^+ and K^+, where the metal ions bind reversibly and weakly to the protein, (b) Group 2 metal ions Mg^{2+} and Ca^{2+} (and the transition metal Mn^{2+}), which bind more strongly than do Na^+ and K^+, and (c) the transition metal ions and Zn^{2+}, where the metal ion is bound firmly to the protein. In general, the "hard" metal ions such as K^+ and Mg^{2+} are considered to be freely mobile in the aqueous phase. Salts of the hard metal ions are added to the assay medium. By contrast soft metals form stronger, covalent interactions with their ligands. These complexes are more stable kinetically, and so the metal ion is not readily released, and so they do not usually need to be added to the assay medium.

The distinction implicit in the activity of these groups of metals is essentially a consequence of the polarizing powers of their metal cation, that is, the charge/radius ratio. A metal cation of high charge and small size has a high density of positive charge. It thus has high

polarizing power and so the interaction between the metal ion and a ligand is likely to be strong. A metal ion of large size and low charge polarizes ligands only weakly and binds to a lesser extent to ligands. Thus, the doubly charged cations Mg^{2+} and Ca^{2+} interact more strongly with ligands than do the Group 1 cations Na^{+} and K^{+}, but weakly compared to the transition metal ions.

Ionic radii decrease from left to right across the Periodic Table, so that the complexing power of the divalent transition metal cations increases from left to right, as expressed by the well known Irving-Williams series for the stability of high-spin octahedral metal complexes, $Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} > Zn^{2+}$ [8]. In accord with this, formation constant data show that Cu^{2+} binds most strongly of all the divalent transition metal ions. Thus, it complexes glycine with approximately a thousand-fold greater affinity than does the $3d^{10}$ ion Zn^{2+}. The strength of binding of Cu(II) to ligands is rather unexpected if is just considered in terms of the electronic configuration of Cu(II) and its ligand field stabilization energy, but other factors also contribute to values of formation constants. In the case of Cu(II) the structural distortion resulting from the Jahn-Teller effect leads to Cu(II) having two long axial bonds and four short bonds in the plane. Thus the overall binding of ligands to Cu(II) is controlled by the strong binding of the first four ligands in the plane. However, Cu^{2+} cannot compete with Fe^{3+} for binding of glycine, in accord with their relative polarizing power as discussed above.

Common toxic metal ions such as those of Cd^{2+}, Pb^{2+} and Hg^{2+} are soft, and form very strong bonds with sulfur ligands in particular, and so they can displace essential metal ions from their binding sites on proteins. The bound form is usually inactive, but in some cases it may have some activity. In one remarkable case, cadmium seems to have been adopted as the catalytic metal in place of zinc, in the carbonic anhydrase of diatoms; this is presumably the result of the availability of the metal in the environment [9].

Ligand types

When discussing coordination chemistry in biology, it should be noted that there are two different conventions for the use of the term ‘‘ligand’’. In biochemistry, any group that is bound to a protein, including a metal ion, might be termed a ligand. In inorganic chemistry, and for the purposes of this chapter, a ligand is a group that coordinates to a metal ion. A range of ligand groups are available for binding metal ions in biology, from proteins, nucleic acids and carbohydrates, together with a variety of smaller, specialized ligands. These include carboxylate, thiolato, amino, imidazolato and phosphate groups.

Substantial collections of formation constants for various metal ions and ligands are available that allow the likely speciation of a metal ion in a solution containing various ligands to be assessed.

Chelate effect

The chelate effect refers to the greater stability of complexes between a transition metal ion and bidentate or multidentate ligands compared to complexes of that metal ion with monodentate ligands of similar chemical character. Thus, the bidentate ligand ethylenediamine ($NH_2CH_2CH_2NH_2$) will displace ammonia from a metal complex. The sites for binding metal ions in proteins will usually be multidentate, and the binding will be correspondingly stronger.

Coordination geometry

In addition to variations in polarizing power, different metal ions have different preferences for ligand type and coordination geometry, which provide proteins with further selectivity for binding particular metal ions. This is particularly important for proteins involved in transport and storage of metal ions, which must show a high level of selectivity for the appropriate metal ion. In enzymes, the binding geometry provided for the metal by a protein is often distorted from the ideal geometry normally associated with these metal ions, resulting in a fine tuning of the reactivity of the metal. This is the so-called "entatic state" of Vallee and Williams [10]. For example, binding sites for copper centres, which undergo oxidation-reduction during catalytic reaction, are intermediate in geometry between square planar (optimum for Cu(II)) and tetrahedral (optimum for Cu(I)). This is enforced by the protein and so limits the extent of reorganization that has to take place in the localized geometry of the metal ion during the redox reaction. In the "blue" copper centres, sulfur occurs as a soft ligand, which will tend to stabilize the lower oxidation state.

Roles for Metals in Biology

The biochemical functions of metal ions reflect their chemistry (see Figure 2). In general, some metal ions are an essential component of the enzyme-catalysed reaction, while others are found in special sites in the protein, for structural or other reasons. Generally, the ionic strength affects protein-protein interactions, but in some cases a particular metal ion is involved. In the crystal structures of enzymes, special sites are sometimes found to be occupied by Na^+ or K^+ or Mg^{2+} ions.

Sodium and potassium ions are usually present as free ions; K^+ has the higher concentration within cells, while Na^+ is present in higher concentrations as an extracellular cation. These ions make major contribution to the osmotic balance. In enzyme-catalysed reactions their functions include acting as counter-ions to negatively charged amino-acyl residues or phosphates. The compensation of electrostatic charge is necessary for the energetics of enzyme-catalysed reactions.

Magnesium ions are often required for complexes with substrates, especially nucleotides and nucleic acids. For reactions with nucleotides the ratio $[Mg^{2+}]/[NTP]$ is important. Mg is required in stoichiometric amounts with ATP, but in many enzymes, (e. g. pyruvate kinase [11]) excess Mg^{2+} is inhibitory, owing to the formation of Mg_2-ATP complex.

Calcium is well known to have a special role in regulation of metabolism e. g. protein kinases, protein phosphatases and certain peptidases. The intracellular concentration of ions fluctuates considerably in response to metabolic changes, e. g. in muscle contraction and nerve transmission. Ca^{2+} is fairly hard, so can bind and dissociate readily as the intracellular concentration of Ca^{2+} changes. Often it binds to calmodulin domains, which undergo structural changes on binding.

Zinc is the most common 3 d block metal ion in a typical animal or plant cell. Zn^{2+} is chemically softer than Mg^{2+}or Ca^{2+} and forms more stable complexes. Zinc is a component of hundreds of enzymes [12, 13]. Because of its strong Lewis acidity, Zn^{2+} may polarize a substrate or lower the pK_a of coordinated water, leading to the Zn-hydroxide pathway in hydrolases for example. It occurs in the catalytic centres, and in other parts of the protein where it stabilizes the protein structure. In yeast alcohol dehydrogenase for example there are two zinc ions, one catalytic and one structural; removal of the structural zinc does not affect catalysis, but renders the protein less stable [14].

Iron exists in heme and nonheme proteins, including many oxidoreductases. It has special roles such as binding gases such as O_2, NO or CO. Iron is assembled into complex metalloclusters, sometimes with other metals such as nickel, molybdenum or vanadium, in enzymes for the metabolism of H_2 or N_2 [15]. Iron metabolism is complex because of the different properties of its oxidation states. Fe(II) and Fe(III) are the most common; Fe(IV) may be formed in oxidases and peroxidases by reaction of Fe(III) with O_2 or H_2O_2.

Transition metal ions such as Fe(II) are known to be cytotoxic under aerobic conditions, forming the reactive oxygen species superoxide and hydroxyl radical [16]. An example is the Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH$$

In the cell, the concentration of free iron ions is maintained at a very low level, except under pathogenic conditions such as chronic iron overload. Some nonheme iron oxygenases use the oxygen chemistry of iron to catalyse the hydroxylation of their substrates. Since the substrate and reactive oxygen species must bind simultaneously to the iron, the iron sites in these enzymes have a rather open configuration, often with only three ligands from the protein, for example by the triad His_2Asp [17]. The harder Fe(III) may tend to dissociate from the enzyme during purification steps, such as column chromatography, that involve dilution [18]. Some nonheme iron-containing oxygenases are routinely assayed with iron

salts added to the reaction medium. Although the oxidation state of the iron in these enzymes is often Fe(III), Fe^{3+} salts will hydrolyze and precipitate at neutral pH, forming the mineral ferrihydrite [19]. In order to keep Fe(III) in solution it is necessary to have a tight, soluble complex such as Fe(III) citrate, where the iron may be unavailable for interaction with enzymes. Therefore, it is preferable to add Fe^{2+} salts, as a freshly prepared solution, under anaerobic conditions.

Copper: Cu^{2+} is a good Lewis-acid catalyst, but is seldom used as such by enzymes, presumably because of the possibility of forming reactive oxygen species in a similar way to iron. It tends to bind strongly to the peptide chains of proteins, as in the biuret reaction. Copper is found in a considerable number of oxidoreductases. In the rare cases where it needs to be added to an enzyme assay, it is usually provided as the cupric Cu^{2+} salt. In the cell, it is transported by metallochaperones in the cuprous state, Cu^{+}.

Metal Ion Homeostasis in the Cell

In a mixture of compounds including metal ions in aqueous solution, the distribution of ions is driven by simple thermodynamic equilibria. The outcome of this is the ''speciation'' of metal ions. The ''free'' metal ion, M^{n+}(aq) is in equilibrium with each protein or a small-molecule ligand L, with binding constant, K_d. It was assumed for a long time that a similar equilibrium existed in the cell. This implied that the cell buffers the concentrations of the free metal ions, by analogy with a pH buffering system, so that the free metal concentration would remain relatively constant as total metal concentration changed. A development from this was the concept of the ''labile metal pool'', or ''biologically available metal pool'' instead of free metal pool. This implies that metals complex with abundant small molecules in the cytoplasm, but are still available to sites of stronger complexing power.

An example is the distribution of zinc in a bacterium such as *E. coli*. In the growth medium, the zinc concentration is approximately 100 nM. It is actively transported into the cell. The total amount of zinc corresponds to a concentration of approximately 0.2 mM, but this is bound to proteins and not freely available. The level of available zinc is controlled by the expression of the genes for two transporters: Zur, which is responsible for uptake, and ZntR, which is responsible for efflux. The expression is in turn controlled by metalloregulatory proteins binding to DNA. In consequence, the concentration of ''available'' zinc is around 1 femtomolar [20]. An even more extreme example is copper, for which the concentration of free ions in the cell has been estimated to be in the zeptomolar range, around 2.10^{-21} M [21].

Table 2. Enzyme assays that require activation by a metal ion

Enzyme	Metal ion	Conditions	Ref
EC 1.14.12.11 Toluene dioxygenase	Fe^{2+}	0.36 mM Fe^{2+} in assay medium	[33]
EC 2.7.1.40 Pyruvate kinase	Mg^{2+}	10 mM Mg^{2+}, 2 mM ADP	[34]
ADAM8 peptidase	Zn^{2+}	Pre-incubate with 0.1 mM Zn^{2+}	[35]
EC 1.14.17.3 Peptidylglycine monooxygenase	Cu^{2+}	Pre-treat with 25 µM Cu^{2+}	[36]

However, when metal ions such as iron, copper or zinc are added to enzyme assays, much higher concentrations, typically micromolar to millimolar, are found to be optimal (Table 2). This immediately suggests that the binding equilibria in the enzyme assay do not reflect the conditions in the cell, where there are specific systems for import, trafficking and storage of metal ions. These have become apparent from the discovery of genes, in addition to the structural genes for the enzyme proteins, which are required for expression of the metalloenzymes in their active forms. The insertion of metal ions such as iron, zinc or copper is a post-translational modification, which requires the presence of ion transporters, storage proteins, and ‘‘metallochaperones’’ [22]. The term ‘‘metallochaperones’’ applies to proteins with a variety of functions [23, 24]. A principal function is to act as a type of ‘‘escort protein’’, which selectively binds a particular metal, and sequesters it inside the cell. Metallochaperones protect metal ions from adventitious binding to metabolites and macromolecules and, in doing so, they protect the cell from potential damage, for example from radicals produced from reaction of transition metal ions with O_2 and H_2O_2. By means of specific protein-protein interactions, the metallochaperones accept the ions only from an appropriate donor such as a membrane transporter, and transfer them to a specific acceptor such as an apoenzyme. Thus, they ensure that the metal ions are delivered efficiently to their targets, while the intracellular concentration of free metal ions is kept extremely low (Fig. 3). Like the chaperonins that assist protein folding, the metallochaperone may partly unfold and refold the donor and/or acceptor proteins to assist the release and binding of metal ions. They can ensure that the metal ion is only transferred to the right protein when it is in the right state [25, 26]

For copper, a number of proteins involved in trafficking have been identified and characterized. There are different systems for delivery of copper to Cu/Zn superoxide dismutase, cytochrome oxidase, ceruloplasmin and other enzymes.

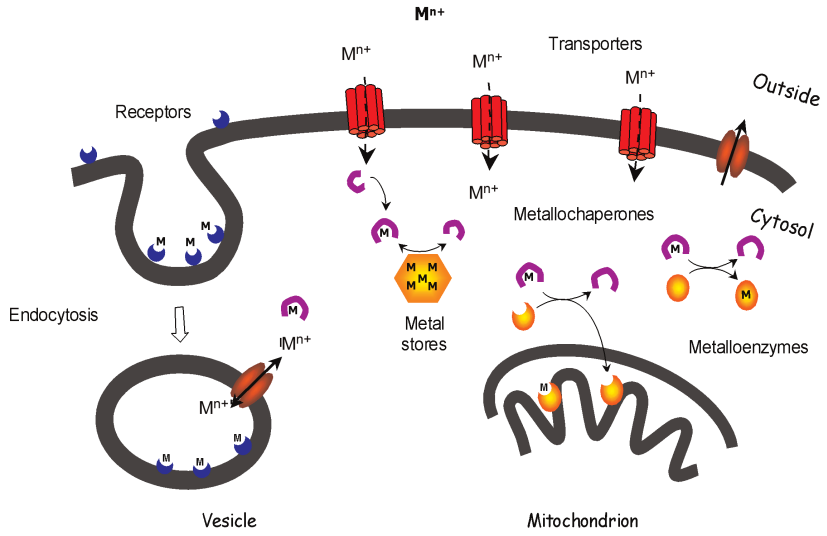

Figure 3. General model of intracellular transport, trafficking and delivery of metal ions such as Fe, Cu and Zn. Free metal ions are bound by, and transferred between various transporters, metallochaperones and storage proteins. They may also be stored in intracellular organelles such as the Golgi apparatus, vacuoles or plastids. The concentration of the free unbound metal ion is generally extremely low, though it may still be accessible to chelators.

Zinc is required for hundreds of enzymes and over a thousand transcription factors and other regulatory proteins [12, 13]. Metallochaperones, transporters and other zinc-dependent factors have been identified, but their precise role in the insertion of Zn^{2+} is under investigation.

For iron, the systems are less well defined. It undergoes oxidation-reduction reactions as it is transported through the cell. Usually it is presented at the cell surface as Fe(III); it is reduced to Fe(II), and it is transported in this form through membranes, but for transport in the blood as transferrin, or storage in the cell in ferritin, it is oxidized again to the less toxic Fe(III) [27]. For iron, little is known about the precise role of metallochaperones, apart from a recent report of a selective iron metallochaperone for transfer to ferritin [28].

Relevant Parameters for Deriving Activity Data of Metal-Dependent Enzymes

A complete description of the activity of metalloenzymes in the cell would include the systems for insertion and maintenance of metal ions in enzymes. For assays of most enzymes containing metal ions such as zinc, iron or copper, it is not necessary to consider these processes because the metals are present in the isolated enzymes, and do not readily

dissociate. However, as already noted, the metal ion in some cases can dissociate, and has to be re-supplied in order for the enzyme to show activity. Some examples of enzyme assays where these metal ions are included are listed in Table 2.

In contrast to the situation in the cell, where the specific metal ion is often selected by protein-protein interactions between the apoprotein and a metallochaperone, *in vitro* it may be possible to insert different metal ions directly into the apoenzyme. If the non-physiological metal ion is inserted, the enzyme may be inactive, but in some cases several different metallic elements may restore some activity. In some cases they are inhibitory, but in others they may be active, and may change the specificity of the enzyme. Xylose isomerase (D-xylose ketol-isomerase; EC. 5.3.1.5), otherwise known as glucose isomerase, catalyzes the reversible isomerization of D-glucose and D-xylose to D-fructose and D-xylulose, respectively. The enzyme is widely used in the food industry because of its application in the production of high-fructose corn syrup. The enzyme has an absolute requirement for bivalent cations with ionic radii < 0.08 nm; Mg^{2+}, Co^{2+}, or Mn^{2+} are effective. There are two metal-binding sites, which have to be occupied for catalytic activity. Different metal ions in these sites change the specificity of the enzyme for sugars, as well as the stability of the protein [29, 30].

Additional parameters to be considered in assays of metalloenzymes may include the following factors, which relate largely to complications that arise from chemical interactions of the metal centre with components of the assay medium.

1. *Ligands in the medium that will bind to the metal ion that is necessary for activation of the enzyme under study.* Tables of formation constants will be helpful at this point. If ligands bind to the metal ion, then the metal may be present in the assay medium as several different complexes, possibly with different stoichiometry, overall charge, shape and lipophilicity. These different species may not activate the enzyme to the same extent.
2. *pH buffers that will bind the metal ion*. This is an important matter as the concentration of buffer may be relatively high compared to other components of the medium. Citrate buffers should be avoided if possible. The ‘‘Good buffers’’ are designed to avoid metal coordination, and should be preferred; at least the assay should be tried with one of these buffers [31].
3. *Precipitation by other constituents of the medium*. If phosphate, for example, is present in the medium, then metal ions may be lost from the solution by precipitation as metal phosphates. Tables of solubilities of inorganic compounds show that many common metal phosphates are very poorly soluble in water. For example, the solubilities of phosphates of Zn(II) and Fe(II) are very low, while the phosphate of Fe(III) is only slightly soluble.

4. *Precipitation as a function of pH*. Added transition metal and zinc ions in the assay medium may be precipitated as oxides and hydroxides at pH values over 6. The pK_a values of the aqua cations of Ni(II), Zn(II) and Cu(II) are 9.9, 9.0 and 8.0 respectively; precipitation normally begins to occur about $2-3$ pH units below the pK_a value of the aquo complex and, as the pH is raised, occurs in the order of the Lewis acidity of the metal ion, taking place first for Cu(II), the smallest ion. Precipitation is therefore much more pronounced for aquo cations $M(H_2O)_6^{3+}$ such as $[Fe(H_2O)_6]^{3+}$, as already noted.
5. *Chelating ligands such as citrate that are added to assay solutions, to ensure that precipitation does not occur*. It is important to check if this added ligand also binds the essential metal ion to an extent that lowers its bioavailability, leading to a decrease in rate constant for the enzyme reaction.
6. *Strong chelators, e.g. EDTA, that are added to scavenge heavy metals in the medium*. Examples were given by Boyce *et al.* [32]. These can also remove metal ions from proteins, as mentioned above.
7. In order to maintain low "free ion" concentrations, chelating agents with lower binding affinities can be used to buffer the metal ion concentration. For example, *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) was used by O'Halloran *et al.* to maintain femtomolar concentration of zinc [20].
8. *Reconstitution with a metal ion.* If this is necessary because a metal normally present in the enzyme has dissociated, the conditions used to restore the metal ion must be stated.
9. If the concentration of the metal ion such as calcium fluctuates with time, and with position in the cell, the activity of the enzyme should be explored over the appropriate range of concentrations.

Conclusions

In this article we have attempted to bring together and discuss the factors that underlie the accurate determination of enzyme kinetic parameters in cases where metal ions are involved. Recently there have been advances in the understanding of the assembly of complex metal ion clusters such as iron-sulfur clusters [33]. Each of these processes may require a highly-organized sequence of scaffolds and other accessory proteins [33]. These represent metabolic pathways in their own right. For future studies of metabolic reconstruction, it might be necessary to consider their involvement in enzyme activity.

References

[1] Apweiler, R., Cornish-Bowden, A., Hofmeyr, J.-H.S., Kettner, C., Leyh, T.S., Schomburg, D. and Tipton, K. (2005) The importance of uniformity in reporting protein-function data. *Trends Biochem. Sci.* **30**:11 – 12.

[2] Kettner, C. (2007) Good publication practice as a prerequisite for comparable enzyme data? *In Silico Biology.* **7**, S 57-S 64.

[3] Cammack, R. (2007) EPR spectroscopy in genome-wide expression studies. In *Spectral Techniques in Proteomics* (Sem, D. S., ed.). pp. 391 – 405, Taylor & Francis, London.

[4] Cooper, C.E., Lynagh, G.R., Hoyes, K.P., Hider, R.C., Cammack, R. and Porter, J.B. (1996) The relationship of intracellular iron chelation to the inhibition and regeneration of human ribonucleotide reductase as studied by EPR spectroscopy. *J. Biol. Chem.* **271**:20291 – 20299.

[5] Kayyali, R., Porter, J.B., Liu, Z.D., Davies, N.A., Nugent, J.H., Cooper, C.E. and Hider, R.C. (2001) Structure-function investigation of the interaction of 1-and 2-substituted 3-hydroxypyridin-4-ones with 5-lipoxygenase and ribonucleotide reductase. *J. Biol. Chem.* **276**:48814 – 48822.

[6] Connelly, N.G., Damhus, T., Hartshorn, R.M. and Hutton, A.T. (2005) Nomenclature of Inorganic Chemistry, IUPAC Recommendations 2005. RSC Publishing, London.

[7] Pearson, R.G. (1963) Hard and Soft Acids and Bases. *J. Am. Chem. Soc.* **85**:3533 – 3539.

[8] Housecroft, C. and Sharpe, A.G. (2007) *Inorganic Chemistry.* Prentice Hall, New Jersey.

[9] Xu, Y., Feng, L., Jeffrey, P.D., Shi, Y. and Morel, F.M.M. (2008) Structure and metal exchange in the cadmium carbonic anhydrase of marine diatoms. *Nature* **452**:56 – 61.

[10] Vallee, B.L. and Williams, R.J.P. (1968) Metalloenzymes – Entatic Nature of Their Active Sites. *Proc. Natl. Acad. Sci. U. S. A.* **59**:498 – 505.

[11] Holmsen, H. and Storm, E. (1969) Adenosine Triphosphate Inhibition of Pyruvate Kinase Reaction and Its Dependence on Total Magnesium Ion Concentration. *Biochem. J.* **112**:303 – 316.

[12] Vallee, B.L. and Auld, D.S. (1990) Zinc Coordination, Function, and Structure of Zinc Enzymes and Other Proteins. *Biochemistry* **29**:5647 – 5659.

[13] Maret, W. (2005) Zinc coordination environments in proteins determine zinc functions. *J. Trace Elem. Med. Biol.* **19**:7 – 12.

[14] Magonet, E., Hayen, P., Delforge, D., Delaive, E. and Remacle, J. (1992) Importance of the Structural Zinc Atom for the Stability of Yeast Alcohol-Dehydrogenase. *Biochem. J.* **287**:361 – 365.

[15] Rees, D.C. (2002) Great metalloclusters in enzymology. *Annu. Rev. Biochem.* **71**:221 – 246.

[16] Halliwell, B. and Gutteridge, J.M.C. (1992) Biologically Relevant Metal Ion-Dependent Hydroxyl Radical Generation – an Update. *FEBS Lett.* **307**:108 – 112.

[17] Koehntop, K.D., Emerson, J.P. and Que, L. (2005) The 2-His-1-carboxylate facial triad: a versatile platform for dioxygen activation by mononuclear non-heme iron(II) enzymes. *J. Biol. Inorg. Chem.* **10**:87 – 93.

[18] Axcell, B.C. and Geary, P.J. (1975) Purification and some properties of a soluble benzene-oxidizing system from a strain of Pseudomonas. *Biochem. J.* **146**:173 – 183.

[19] Michel, F.M., Ehm, L., Antao, S.M., Lee, P.L., Chupas, P.J., Liu, G., Strongin, D.R., Schoonen, M.A.A., Phillips, B.L. and Parise, J.B. (2007) The structure of ferrihydrite, a nanocrystalline material. *Science* **316**:1726 – 1729.

[20] Outten, C.E. and O'Halloran, T.V. (2001) Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. *Science* **292**:2488 – 2492.

[21] Changela, A., Chen, K., Xue, Y., Holschen, J., Outten, C.E., O'Halloran, T.V. and Mondragon, A. (2003) Molecular basis of metal-ion selectivity and zeptomolar sensitivity by CueR. *Science* **301**:1383 – 1387.

[22] Finney, L.A. and O'Halloran, T.V. (2003) Transition metal speciation in the cell: Insights from the chemistry of metal ion receptors. *Science* **300**:931 – 936.

[23] Hausinger, R.P. (1997) Metallocenter assembly in nickel-containing enzymes. *J. Biol. Inorg. Chem.* **2**:279 – 286.

[24] O'Halloran, T.V. and Culotta, V.C. (2000) Metallochaperones, an intracellular shuttle service for metal ions. *J. Biol. Chem.* **275**:25057 – 25060.

[25] Luk, E., Jensen, L.T. and Culotta, V.C. (2003) The many highways for intracellular trafficking of metals. *J. Biol. Inorg. Chem.* **8**:803 – 809.

[26] Puig, S. and Thiele, D.J. (2002) Molecular mechanisms of copper uptake and distribution. *Curr. Opin. Chem. Biol.* **6**:171 – 180.

[27] Crichton, R. (2008) Iron Metabolism: From Molecular Mechanisms to Clinical Consequences. John Wiley & Sons, Chichester.

[28] Shi, H., Bencze, K.Z., Stemmler, T.L. and Philpott, C.C. (2008) A cytosolic iron chaperone that delivers iron to ferritin. *Science* **320**:1207 – 1210.

[29] Van Bastelaere, P., Vangrysperre, W. and Kersters-Hilderson, H. (1991) Kinetic Studies of Mg^{2+}-Activated, Co^{2+}-Activated and Mn^{2+}-Activated D-Xylose Isomerases. *Biochem. J.* **278**:285 – 292.

[30] Epting, K.L., Vieille, C., Zeikus, J.G., Kelly, R.M., Kelly, R.M., Zeikus, J.G. and Vieille, C. (2005) Influence of divalent cations on the structural thermostability and thermal inactivation kinetics of class II xylose isomerases. *FEBS J.* **272**:1454 – 1464.

[31] Good, N.E., Winget, G.D., Winter, W., Conolly, T.N., Izawa, S. and Singh, R.M.M. (1966) Hydrogen Ion Buffers for Biological Research. *Biochemistry.* **5**:467–477.

[32] Boyce, S., Tipton, K.F. and McDonald, A. (2004) Extending enzyme classification with metabolic and kinetic data: some difficulties to be resolved. In *Experimental Standard Conditions of Enzyme Characterizations* (Hicks, M.G. and Kettner, C., eds.). pp. 17 – 44, Beilstein Institut, Frankfurt

[33] Lill, R., Dutkiewicz, R., Elsasser, H.P., Hausmann, A., Netz, D.J.A., Pierik, A.J., Stehling, O., Urzica, E. and Muhlenhoff, U. (2006) Mechanisms of iron-sulfur protein maturation in mitochondria, cytosol and nucleus of eukaryotes. *Biochim. Biophys. Acta –Mol. Cell Res.* **1763**:652 – 667.

[34] Wackett, L.P. (1990) Toluene Dioxygenase from *Pseudomonas putida* F1. *Methods Enzymol.* **188**:39 – 45.

[35] Cardenas, J.M. (1982) Pyruvate-Kinase from Bovine Muscle and Liver. *Methods Enzymol.* **90**:140 – 149.

[36] Naus, S., Reipschlager, S., Wildeboer, D., Lichtenthaler, S.F., Mitterreiter, S., Guan, Z. Q., Moss, M.L. and Bartsch, J.W. (2006) Identification of candidate substrates for ectodomain shedding by the metalloprotease-disintegrin ADAM8. *Biol. Chem.* **387:**337 – 346.

[37] Jaron, S. and Blackburn, N.J. (2001) Characterization of a half-apo derivative of peptidylglycine monooxygenase. Insight into the reactivity of each active site copper. *Biochemistry* **40**:6867 – 6875.