Dalton Perspectives

The Emergence of Trinuclear Constellations at Metallobiosites

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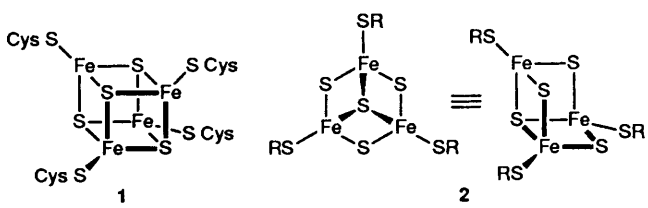
'Whereas the synthesis of new clusters is an everyday occurrence in chemistry, the discovery of a new metal cluster in biology is a signal event.'[1] Recently there has been a growing awareness of the involvement of trinuclear constellations of metal atoms at the active sites of biomolecules as structural information concerning these sites has been made available.

'Trinuclear constellations! Why constellations?'
'Constellation is a word that has been used by protein crystallographers to describe a cluster of closely spaced metal ions present in a metalloprotein or metalloenzyme.'
'So what's wrong with cluster—a trinuclear cluster?'
'Well cluster has acquired a particular meaning in chemistry. A metal-atom cluster, or cluster as it is usually called, is defined as a group of two or more metal atoms in which there are substantial and direct bonds between the metal atoms; you know, species such as $[Re_3Cl_{12}]^{3-}$ *and* $Os_5(CO)_{12}$.'[2]
'So a trinuclear cluster of metal atoms might be alright but a cluster is not. It all sounds like Humpty Dumpty to me.'†
'Well, as one who has recently been taken to task for using triangulo to describe a triangle of copper atoms in a co-ordination compound I think that it's better to avoid any problem of when is a cluster a cluster and use constellation. After all constellation is quite graphic, the heavy metal atoms standing out against the light atom background of proteinaceous material.'
'OK. But what about iron–sulfur clusters?'
'Well strictly no, but they are clusters of metals and non-metals within bonding distance of each other, and the usage is well understood and accepted.'

Iron–Sulfur Proteins

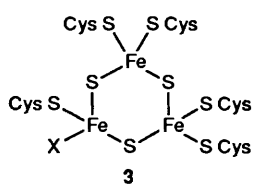
Iron–sulfur proteins are perhaps the most important class of proteins that mediate electron transfer; they are widely distributed in nature and can be found in anaerobic, aerobic and photosynthetic bacteria, algae, fungi, higher plants and mammals. The prosthetic group is non-haem iron bound to the peptide chain *via* cysteinyl sulfur bonds and having, with the exception of the rubredoxins, inorganic sulfide (S*) present. The more familiar Fe–S proteins are the ferredoxins containing 2Fe–2S* and 4Fe–4S* centres; the essential structural features of these sites were elucidated some twenty years ago and showed that the 4Fe–4S* exists as a cuboidal cluster **1**.[3]

A new class of Fe–S cluster, 3Fe–4S*, has now emerged and it has been remarked that 'the pleasure of watching the chemistry and biochemistry of the cuboidal 3Fe–4S* cluster **2** and its derivatives unfold is enhanced by the realisation that its occurrence was totally unpredicted.'[1] Comprehensive accounts of the discovery and of the properties of 3Fe–4S* clusters have been presented.[1,4–6]



1 **2**

Following initial observations that aerobically purified ferredoxins often retained a nearly isotropic EPR signal at $g \approx 2.01$, spectroscopic and crystallographic studies on *Desulfovibrio gigas* Fd II and *Azobacter vinlandii* Fd I led to the conclusion that these proteins contained a three iron atom cluster. The early structural studies were controversial, with interpretation of the X-ray data for the cluster in *A. vinlandii* leading to the suggestion that the cluster was in the form of a six-membered 3Fe–3S* ring **3**.[7] Two iron atoms were each



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ligated by two terminal cysteinyl sulfur atoms and the third iron atom was ligated by one such sulfur atom and a second ligand (X), possibly a water molecule or a hydroxide anion. More extensive X-ray studies have now established that it is the cuboidal cluster form **2** that is present in this protein,[8] in *D. gigas* Fd II (Fig. 1),[9] and in the C20A mutant of *A. vinlandii* Fd I.[10] The cluster is also found in the inactive form of pig heart aconitase (Fig. 2);[11] aconitase is involved in a stereospecific dehydration/rehydration reaction which converts citrate to isocitrate *via cis*-aconitate in the second and third steps of the tricarboxylic acid cycle.

The clusters are dimensionally similar; for example a comparison of those from aconitase and *A. vinlandii* Fd I shows that the positions of the Fe and S* atoms agree to within 0.09 Å. The 3Fe–4S* portions of the tri- and tetra-nuclear iron–sulfur clusters are also very similar, hence the use of the descriptor voided cube for the 3Fe–4S* cluster.

The 3Fe–4S* clusters have now been found in a number of proteins and enzymes and the similarity in the magnetic circular dichroism and Mössbauer spectra of these clusters suggests that

† 'When I use a word,' Humpty Dumpty said, 'it means just what I choose it to mean . . . neither more or less.' Lewis Carroll, in *Through the Looking Glass*.

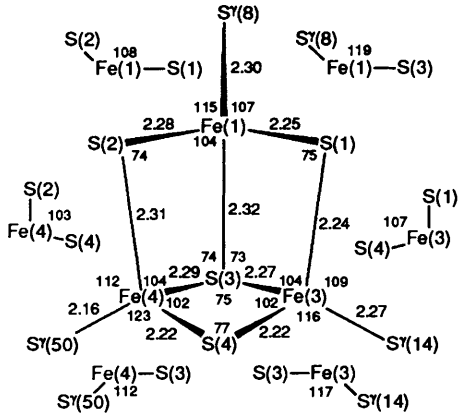


Fig. 1 The structure of the trinuclear site in *D. gigas* (reproduced with permission from The American Chemical Society[9a]); distances in Å, angles in °

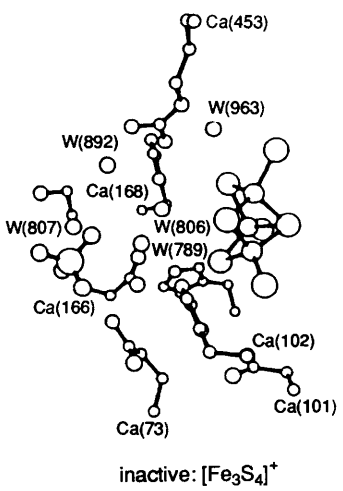
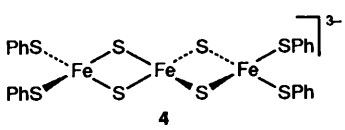


Fig. 2 The structure of the trinuclear site in aconitase (reproduced with permission from Professor C. D. Stout[11a])

they are all likely to have the voided cuboidal form.[1] It has been remarked that the ferredoxins from *D. gigas* Fd II and *Pyrococcus furiosus* may be regarded as prototypes for the 3Fe–4S* cluster, 'for which no chemical models have (yet) been synthesised.'[6] A model, $[Fe_3S^*_4(SPh)_4]^{3-}$ **4**, for the linear form of 3Fe–4S*, which is available in the unfolded form of inactive aconitase, has been synthesised and fully characterised.[12]



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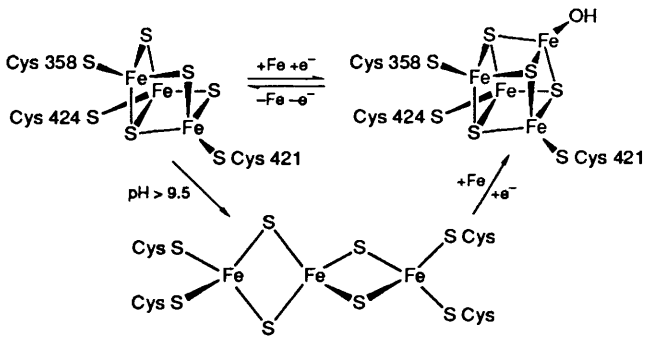
On the basis of their ability to undergo interconversion reactions the clusters can be divided into two classes.[13] The first type undergoes a reversible 3Fe–4S* to 4Fe–4S* conversion with minimal structural change because there is a labile non-thiol fourth ligand available in the 4Fe–4S* form, for example aconitase has a water molecule present. The cuboidal 3Fe–4S* form of aconitase is inactive but can be reactivated by the addition of Fe^{II} under anaerobic conditions followed by interconversion to the active cuboidal 4Fe–4S* centre (Scheme 1).[14,15] The second type, of which *A. vinlandii* Fd I is an example, is more deeply contained within the protein and so would require full denaturation of the protein prior to conversion. The role of the trinuclear clusters is open to some speculation. Some proteins appear to be functional only as the 3Fe form whereas for others the 3Fe form may be the



Scheme 1 Cluster interconversion in aconitase (reproduced with permission from the Federation of European Biochemical Sciences[15])

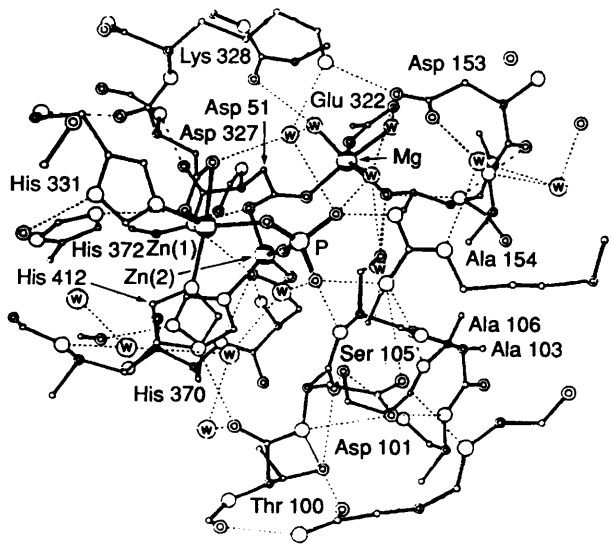


Fig. 3 The metal constellation in *Escherichia coli* alkaline phosphatase (reproduced with permission from Academic Press[18])

consequence of having a readily displaced ligand which is required for enzyme activity as found in aconitase. In *D. gigas* the ready interconversion between the two forms allows one protein to perform more than one role—*D. gigas* Fd I (4Fe–4S*) couples the phosphoroclastic reaction to hydrogenase and *D. gigas* Fd II (3Fe–4S*) serves an an electron donor to sulfite reductase.[16]

The availability of 3Fe–4S* ⟶ 4Fe–4S* interconversions raises the possibility of introducing further metal atoms to produce more complex clusters with new functions. It is also possible to consider the introduction of heteroatoms into the void in the trinuclear cluster.[1] This has been achieved with divalent metals such as Co, Ni, Zn and Cd; although this addition can be applied to a cluster in a protein it has not yet been detected in nature.

'And the other trinuclear sites, they all seem to depend upon protein-based donors and the metals are certainly not within bonding distance of each other.'

'Well this is where we introduce constellations, and some intriguing questions—but not necessarily any answers.'

Alkaline Phosphatase

Alkaline phosphatase is a dimeric metalloenzyme having phosphomonoesterase activity. It was the first zinc enzyme to be discovered in which there are three metal atoms (two zinc and one magnesium, or in the absence of magnesium a third zinc), at the active site.[17] The X-ray crystal structure of native *Escherichia coli* alkaline phosphatase complexed with inorganic phosphate (the E·P complex) has been determined at 2.0 Å

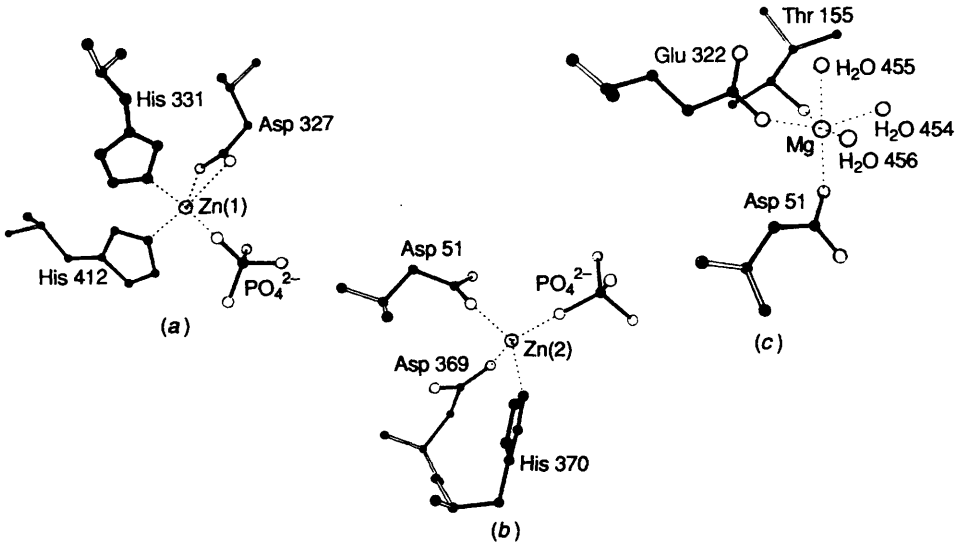


Fig. 4 The co-ordination spheres of the three metal ions in phosphate bound alkaline phosphatase; (*a*) Zn(1), (*b*) Zn(2) and (*c*) Mg (reproduced with permission from Academic Press [18])

resolution and shows that the three metal atoms are in close proximity (Fig. 3).[18] The intermetallic separations are d[Zn(1)···Zn(2)] = 3.94, d[Zn(2)···Mg] = 4.88 and d[Zn(1)···Mg] = 7.09 Å in one subunit and 4.18, 4.66 and 7.08 Å respectively in the second subunit. The co-ordination spheres of the metals are shown in Fig. 4.

The first zinc atom [Zn(1)] is five-co-ordinated by the imidazole nitrogen atoms of His 331 and His 412, both carbonyl oxygen atoms of Asp 327 and one of the phosphate oxygen atoms with an average metal–ligand distance of 2.07 Å; the co-ordination polyhedron at the metal is best described as pseudo-tetrahedral with both oxygen atoms of the chelating Asp 327 occupying one apex. It is not possible to exclude the possibility that there is an additional exchanging water molecule at this site, the presence of which has been indicated by NMR studies.[19]

The second zinc atom [Zn(2)] is tetrahedrally co-ordinated by one carboxyl oxygen from each of Asp 51 and Asp 369, the imidazole nitrogen from His 370 and one of the phosphate oxygen atoms; the average metal–ligand distance is 2.00 Å. The zinc atoms are bridged by the inorganic phosphate (at Zn–O distances of 2.12 and 2.23 Å). The carboxyl group of Asp 51 forms a bridge between Zn(2) and the Mg atom; the phosphate is also associated with the magnesium atom *via* one of the water molecules co-ordinated to that atom. The magnesium atom has a slightly distorted octahedral co-ordination environment with the second carboxyl group of Asp 51, one of the carboxyl oxygens of Glu 322, the hydroxyl of Thr 155 and three water molecules as the co-ordinating ligands. The average Mg–O distance is 2.12 Å.

Interestingly all three types of carboxylate group to metal co-ordination are found at the trinuclear site. The unidentate mode of bonding is exhibited, for example, by Asp 369 [to Zn(2)], the bidentate mode is found with Asp 327 binding Zn(1) and the bridging mode is found with Asp 51 bridging Mg and Zn(2). This phenomenon has also been noted in the structure of the non-haem diiron metalloenzyme ribonucleotide reductase.[20]

The structure of the Zn(1) site in native alkaline phosphatase has been probed by extended X-ray absorption fine structure (EXAFS) spectroscopy by studying two derivatives one of which has only the active site occupied, by zinc, and one in which site 1 is occupied by zinc and site 2 is occupied by cobalt.[21] The atom Zn(1) appears to be co-ordinated by two histidine nitrogen atoms and by four oxygen atoms at an average distance of 2.04 Å from the metal. These oxygen atoms appear to be made up from a bidentate carboxylate ligand and two water molecules. This relates well to the X-ray structural

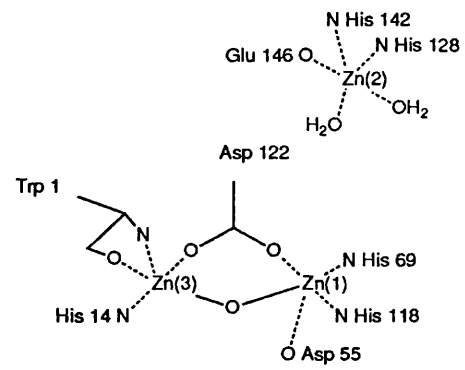


Fig. 5 The metal constellation in phospholipase C from *Bacillus cereus*

evidence, derived from the non-covalent phosphoenzyme (E·P) complex, for this site. Furthermore neither the ligands nor the co-ordination geometry at the site change when cobalt is introduced at the Zn(2) site.

Phospholipase C

The crystal structure of phospholipase C from *Bacillus cereus* shows that the enzyme is an all-helix protein with, in the crystalline state, three zinc atoms at the active site (Fig. 5).[22] This constellation of metal ions resembles that found in alkaline phosphatase. One zinc atom [Zn(2)] is five-co-ordinated by His 128, His 142, Glu 146 and two water molecules. The remaining zinc atoms [Zn(1) and Zn(3)] are doubly bridged by Asp 122 and by a molecule of water, or OH^-, with an intermetallic separation of 3.3 Å. The metal co-ordination is completed by interactions with His 69, Asp 55 and His 118 for Zn(1), and with His 14, and Trp 1 (*via* both the amino and peptide carbonyl groups which form a five-membered chelate ring as has been observed with many zinc–amino acid complexes),[23,24] for Zn(3).

Phospholipase C is inhibited by inorganic phosphate (P_i) and so structural details on phosphate treated phospholipase C should give useful information on the location of the active site and on substrate binding. The crystal structure of phosphate-inhibited phospholipase C from *Bacillus cereus* shows that the substrate is closely associated with all three zinc atoms of the metal site with its oxygen atoms replacing two of the co-ordinated water molecules (Fig. 6).[25] One phosphate oxygen atom is bound to Zn(1) and Zn(3) at 1.96 and 2.24 Å respectively, and a second phosphate oxygen is bound to Zn(2)

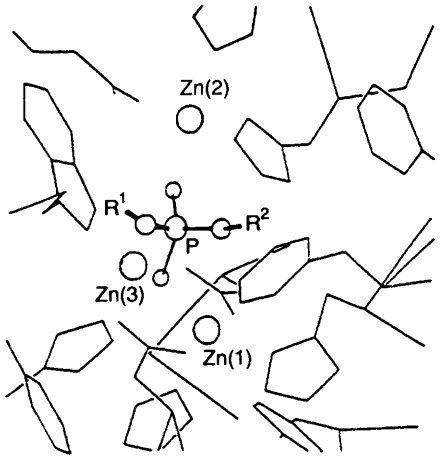


Fig. 6 A possible orientation of a phospholipid molecule in the active site of phospholipase C (reproduced with permission from Academic Press[25])

at 2.1 Å; this confirms the identification of the active site. The Zn(1)–O–Zn(3) bridge is now asymmetric as compared with the symmetric bridge found in phospholipase C itself.

P1 Nuclease

This nuclease is an endonuclease which hydrolyses single-stranded ribo- and deoxyribo-nucleotides. The crystal structure of the P1 nuclease from *Penicillium citrinium* at 2.8 Å has revealed that the spatial arrangement of the three zinc atoms at the active site contains a zinc pair [Zn(1) and Zn(3)] having *ca.* 3.2 Å separation with a third zinc atom [Zn(2)] some 5.5 Å distant [Zn(1)···Zn(2) 5.8, Zn(1)···Zn(3) 4.7 Å] thus resembling that found in phospholipase C (Fig. 7).[26,27] The atom Zn(1) is bound by the nitrogens from His 60 and His 116, two oxygen atoms from Asp 45 and Asp 120 and a water molecule; Asp 120 and the water molecule bridge Zn(1) and Zn(3). This latter zinc atom is also co-ordinated to a nitrogen atom from His 6 and a nitrogen and an oxygen from Trp 1. The atom Zn(2) is bound by two water molecules, an oxygen from Asp 153 and two nitrogens from His 126 and His 149. All of the zinc atoms are five-co-ordinated by two nitrogens and three oxygens. The active site is located within a molecular cleft and suggestions for the mode of substrate binding have been presented.

'These three zinc-containing enzymes have very similar trinuclear active sites, so are they all related?'
'In general terms the disposition of the metal atoms is similar but there is a difference between alkaline earth phosphatase and the other two enzymes.'

The sites in phospholipase C and P1 nuclease are all typical zinc sites built up from nitrogen and oxygen donors, whereas in alkaline phosphatase only two sites are of this form. The third site is an all-oxygen donor site and so this may explain its preference for magnesium. The aspartate group bridging pattern is also different as it bridges two zinc atoms and so defines a dinuclear zinc site in phospholipase C and P1 nuclease but bridges magnesium and zinc in alkaline phosphatase in which the two non-bridged zinc atoms are sufficiently close to provide a functional dinuclear pair bridged by the phosphate (Fig. 8).

'How are the constellations involved in enzyme activity and do the metals have structural or functional roles[28]?'
'That's an intriguing question. Some speculative mechanisms have been advanced involving one, two or three zinc atoms in the hydrolyses. Two-metal catalysis is the favoured route for alkaline phosphatase but maybe three-metal catalysis is more likely for the others.'

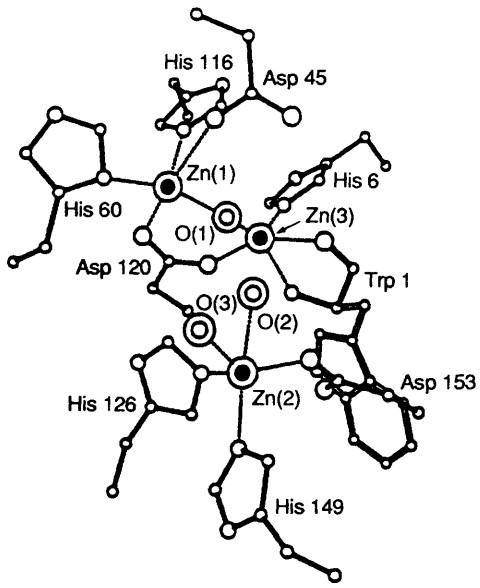


Fig. 7 The structure of the dinuclear centre from the P1 nuclease of *Penicillium citrinium* (reproduced with permission from Oxford University Press[27])

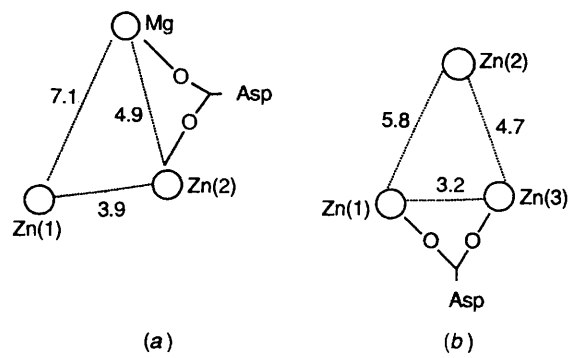
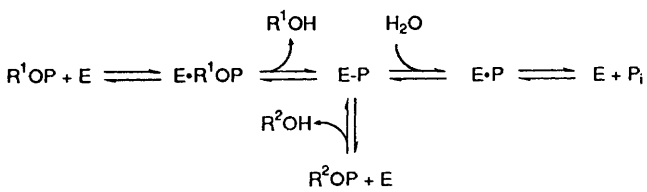


Fig. 8 Comparison of the triangular sites in alkaline phosphatase (*a*) and phospholipase C (*b*); distances in Å



Scheme 2

A mechanistic proposal for phosphomonoesterase activity has been presented which suggests that it functions through a phosphoseryl intermediate to produce free inorganic phosphate, or to transfer the phosphoryl group to other alcohols (Scheme 2; E = enzyme).[29] The left hand side of the scheme shows the formation of the phosphoseryl intermediate (E-P) and the right hand side shows the hydrolysis of it to release inorganic phosphate (P_i). If the alcohol R^2OH is added it is then possible to effect transfer of phosphate from R^1OP to yield R^2OP. The degree to which the transfer of phosphate to serine is associative or dissociative is not known; diester transfer is generally thought to be associative,[30] proceeding *via* a pentacovalent phosphorus intermediate, whereas monoester transfer (metal-catalysed) may be more dissociative.[31]

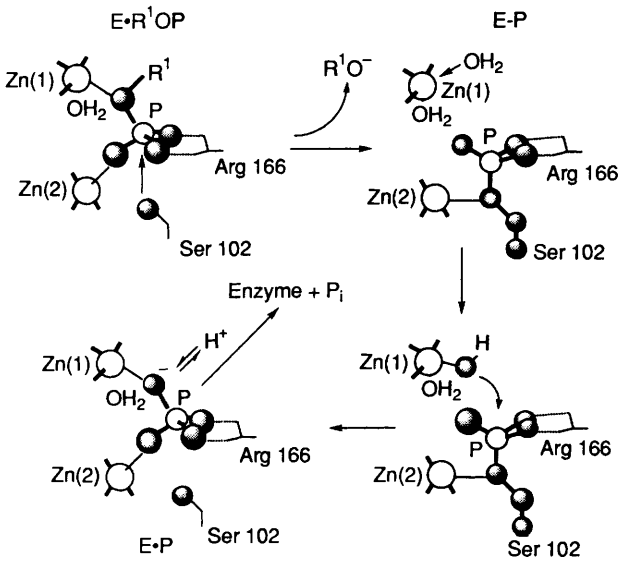


Fig. 9 Mechanistic proposal for alkaline phosphatase (after ref. 32)

The X-ray results from the phosphate complex with alkaline phosphatase show that the two of the phosphate oxygens form a bridge between Zn(1) and Zn(2), the distance between the two zinc atoms helping to stabilise the intermediate and also to permit any molecular motions required during the reaction. The remaining phosphate oxygen atoms are involved in a hydrogen-bonding network which involves association with the magnesium atom *via* one of the water molecules co-ordinated to that atom and the guanidinium group of Arg 166. This brings the Ser 102 residue which is to be phosphorylated during the phosphate hydrolysis into the required apical position to initiate a nucleophilic attack on the phosphorus.

If this is extrapolated to include the substrate phosphomonoester (R^1OP) the reaction scheme shown in Fig. 9 can be envisaged.[32] The dianion of the phosphomonoester forms $E{\cdot}R^1OP$ in which the ester oxygen co-ordinates to Zn(1); a second phosphate oxygen co-ordinates to Zn(2) and the remaining phosphate oxygens are hydrogen-bonded to the guanidinium group of Arg 166. The Ser 102 acts as a nucleophile and would occupy a position opposite to the R^1O^- leaving group in a five-co-ordinate intermediate. Upon formation of E-P a water molecule can co-ordinate to Zn(1) in the position vacated by R^1O^-; at alkaline pH this water loses a proton to give Zn–OH which acts as a nucleophile for the hydrolysis of the phosphoseryl ester. The E•P complex forms and this is followed by dissociation of the phosphate from this complex.

The two metal atoms therefore activate the oxygens of the water and Ser 102 and also stabilise the appropriate leaving groups. Although the magnesium site does not appear to play any direct role in the catalysis steps it is known that its presence does enhance the catalytic ability of the enzyme.[18] It may therefore be that the magnesium plays a structural role by moderating the necessary environment for catalytic activity through the hydrogen-bonded network associated with the magnesium co-ordinated water molecules.

Such two-metal-assisted catalysis might be a more general phenomenon; phospholipase C[22,33] and the exonuclease site of the large fragment of DNA polymerase I[34,35] both have available similar dinuclear sites and carry out related hydrolytic reactions with phosphodiester substrates. However the information recovered from the crystal-structure studies on phosphate-inhibited phospholipase C show that an interaction with all three zinc atoms can take place and so raises the possibility that all three could be involved in the catalytic process. A molecular modelling study of substrate–enzyme interactions in phospholipase C has led to the suggestion that the phospholipid would be bound through its phosphate

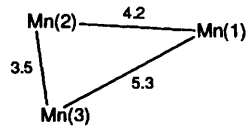


Fig. 10 Schematic representation of the manganese cluster from the enzyme–Mn_3–$(P_i)_2$ complex of SCE1-PPase from *Saccharomyces cerevisiae*; distances in Å

oxygen atoms to Zn(2) as well as providing a single atom bridge between Zn(1) and Zn(3). Hydrolysis is then proposed to occur *via* nucleophilic attack of water activated by a proximal acidic residue from the protein; the trinuclear centre can so be envisaged as providing a template for the phosphate co-ordination prior to the hydrolysis step.

The metal constellation in P1 nuclease is located at the bottom of a substrate binding cleft leading to a relatively inaccessible zinc pair and a more exposed single zinc atom. The co-ordination of the isolated zinc [Zn(2)] resembles those found for active site zinc atoms, that is three amino-acids and at least one water molecule.[36] The single, and more accessible, zinc is therefore proposed as being active in catalysis with the zinc pair playing an important role in the stabilisation of the folding of P1 nuclease.

'So each constellation may function differently; as a two-metal catalytic centre with a third structural component, as a trinuclear template to position the substrate and as a single-atom catalytic centre with a dinuclear structural component.'

'Yes. Other two-metal mechanistic paths, with small variations, have also been proposed and so it becomes clear that unravelling the relationship between structure and mechanism in these metalleoenzymes provides a major challenge.'

'What about other metals? Are any other metals involved in constellations?'

'Certainly, there's manganese in inorganic pyrophosphatases—in fact this observation led to the comment that "the basic phenomenon of phosphate hydrolysis catalysed by two metal ions and perhaps modulated by a third may well prove to be a general one."[37]*—and copper in blue "oxidases". There's also the metal centres in metallothioneins.'*

Inorganic Pyrophosphatases

The inorganic pyrophosphatases ECO-PPase (from *E. coli*) and SCEI-PPase (from the yeast *Saccharomyces cerevisiae*), which hydrolyse inorganic pyrophosphate (PP_i) to inorganic phosphate (P_i), are known to include at least three metal atoms in close proximity.[37–39] From a mechanistic viewpoint the presence of magnesium cations leads to the highest activity, but zinc, cobalt and manganese also confer appreciable activity to the enzyme.[40] The structure has recently been reported of an enzyme–Mn_3–$(P_i)_2$ complex of the SCE1-PPase from *Saccharomyces cerevisiae*.[39] Three manganese atoms are in close proximity and separated by 3.5, 4.2 and 5.3 Å respectively, and there is a fourth manganese atom in close proximity (Fig. 10). The donors at the site are from glutamate, aspartate, tyrosine and water molecules; all three manganese atoms are bound to one phosphate with a second phosphate bound only at one manganese atom. This positioning of the metals has led to the suggestion that a two-metal catalysed hydrolysis might occur of the type discussed above but it might be more likely that a three-atom process occurs with the two oxygen atoms from one phosphorus of PP_i bridging the manganese pair [Mn(2), Mn(3)] and an oxygen from the second phosphorus binding to the isolated manganese atom together with a water molecule which might then be activated for nucleophilic attack.[37]

Metallothioneins

Although the six-membered ring structure proposed for the ferredoxins has been discounted a related structure has been

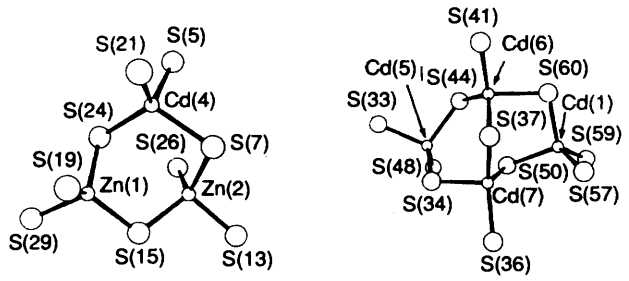


Fig. 11 The structures of Zn_2Cd- and Cd_4-constellations in rat liver metallothionein (isoform 2) (reproduced with permission from Academic Press[47])

detected in metallothioneins. Mammalian metallothioneins are small (*ca.* 61 amino acids) cysteine rich (*ca.* 30%) proteins which can bind seven essential, Cu^{I} and Zn^{II}, or non-essential/toxic, Cd^{II} and Hg^{II}, metals per polypeptide.[41] The biological role is not clear but it is likely that they are involved in metal metabolism–metal storage and transfer, and the detoxification of toxic metals. The metallothionein from *Neurospora crassa* appears to play a role in the copper metabolism of the fungus.[42]

The use of NMR spectroscopy as a technique for determining protein structure has developed rapidly and has seen application in the study of the structures of metallothioneins.[43] Two-dimensional [^{113}Cd–^{1}H] NMR studies of Cd_7 metallothioneins in solution have led to the determination of three-dimensional structures for the metallothioneins from rat liver (isoform 2),[44] rabbit liver (isoform 2a)[45] and human liver.[46] All three proteins adopt a similar conformation in solution, and there are two distinct metal clusters present. The seven Cd atoms are bound in clusters of three and four metal atoms held together by terminal and bridging cysteine residues.

The crystal structure of the Cd_5Zn_2 metallothionein from rat liver (isoform 2) confirms the predictions from NMR and shows the presence of a Zn_2Cd cluster and a Cd_4 cluster (Fig. 11).[47] Each metal atom is tetrahedrally co-ordinated and the six-membered ring shows distortions from an ideal chair conformation.

The determination of the three-dimensional structure of the metallothionein from *N. crassa* by two-dimensional NMR has been hampered by the scarcity of long range ^{1}H–^{1}H nuclear Overhauser effects.[48] In order to elucidate the structure of the yeast copper metallothionein (*Saccharomyces cerevisiae*) an isomorphic replacement of copper by silver was carried out.[42] This was based on the similarity of the co-ordination chemistries of the metals and the availability of $I = \frac{1}{2}$ for Ag^{I}. An analysis of the silver substitute by NMR spectroscopy revealed the presence of seven ^{109}Ag nuclei connected to ten cysteines with at least eight bridging ligands. A full three-dimensional structural analysis for the Ag^{I} isomorph, and hence the Cu^{I} form, is in progress.

Blue 'Oxidases'

Laccase (M_r *ca.* 65 000) is the simplest member of this family and contains four copper(II) atoms (one Type 1, one Type 2 and two Type 3).[49] [Copper(II) atoms present at copper-containing biosites have been classified according to their spectroscopic properties as Type 1 (or blue), which has high absorption in the visible region ($\varepsilon > 3000$ dm^3 mol^{-1} cm^{-1} at 600 nm) and an EPR spectrum with $A_{\parallel} < 95 \times 10^{-4}$ cm^{-1}; Type 2 (or normal), which has limited absorption and an EPR spectrum typical of small molecule copper(II) complexes ($A_{\parallel} > 140 \times 10^{-4}$ cm^{-1}) and Type 3, which has a strong absorption in the near UV region ($\lambda_{max} = 330$ nm), no EPR signal and is believed to consist of a pair of antiferromagnetically coupled copper(II) ions.[49–52]] Dimeric ascorbate oxidase contains eight copper(II) atoms and it was suggested that it was a dimer of two identical laccase-like subunits.[53]

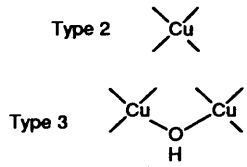


Fig. 12 Proposed triangular copper site in *Rhus vernicifera* laccase

Cumulative spectroscopic and azide bonding studies on *Rhus vernicifera* laccase led to the proposal that the Type 2 and Type 3 centres defined a trinuclear copper cluster site (Fig. 12).[54–56]

The long absence of crystallographic information concerning this type of site was redressed with the publication of an X-ray diffraction study of the fully oxidised form of ascorbate oxidase from *Cucurbita pepo medullosa* (green zucchini).[57] This enzyme catalyses the four-electron reduction of dioxygen to water with the concomitant one-electron oxidation of the reducing organic substrate. Two crystal forms of the oxidase were analysed, one a dimer (M_r *ca.* 140 000) and one a tetramer (M_r *ca.* 280 000). Each subunit was found to have four copper atoms present bound as mono- and tri-nuclear species. The mononuclear copper is isolated from the trinuclear site by *ca.* 15 Å. It is of Type 1 being bound to two histidines, one cysteine and one methionine ligand thus resembling plastocyanin. The trinuclear site may be subdivided into a Type 2 copper and a pair of Type 3 copper atoms held in an approximately isosceles triangular array. The Type 2 copper which is 3.9 Å from one Type 3 copper and 4.0 Å from the second is co-ordinated to two histidine ligands and a water, or hydroxide, ligand. The Type 3 coppers are each co-ordinated by three histidine ligands and form a trigonal prism with an intermetallic separation of 3.4 Å; the X-ray data also indicates the existence of an oxo-, or hydroxo-bridging ligand. The identification of the trinuclear site thus provides confirmation of the earlier proposals from solution studies.

Further refinement of the structure at 1.9 Å resolution has led to intermetallic separations of 3.66 and 3.78 Å (Type 2–Type 3) and 3.68 Å (Type 3–Type 3) in subunit A and 3.69 and 3.90 Å (Type 2–Type 3) and 3.73 Å (Type 3–Type 3) in subunit B being reported.[58] The existence of an oxygen ligand at the centre of the copper triangle would give rise to five-ordination of both of the Type 3 copper atoms and square-planar geometry for the Type 2 copper atom,[54–56] and EPR studies on Type 2-depleted and reconstructed laccase have suggested that there is a central water molecule that could point towards the Type 3 pair and bridge with the Type 2 atom.[59] However no such oxygen atom has been seen in the X-ray structure and the difference Fourier map does not show any significant density that could correspond to such a molecule.

The way in which dioxygen interacts with the trinuclear copper centre is essential to an understanding of the catalytic mechanism. There are several options open to the dioxygen but none are yet confirmed (Fig. 13). The dioxygen may bind to the Type 2 copper in an end-on mode, or form internal *cis*-1,2-bridges between any pair of copper atoms together with a side-on bridge to the opposite copper. If the bridging hydroxide at the Type 3 is absent in the fully reduced form (as has been found at the Type 3 centre in deoxyhaemocyanin[60]) then dioxygen could form an external *cis*-1,2-bridge or a side-on $\eta^2:\eta^2$ bridge (as has now been found at the Type 3 centre in oxyhaemocyanin[61]). An external bridge involving the Type 2 copper however would sterically interfere with the histidine ligands. Dioxygen binding inside the triangular site would allow close interaction and rapid electron transfer to occur from all three copper atoms. Any negative charge which developed at the dioxygen, or oxygen intermediates, would be balanced by the metal ions and the protein so protected from oxygen radicals.

Studies of models based on the spatial structure of ascorbate oxidase have allowed an alignment of the amino acid sequences of laccase and ceruloplasmin to be made leading to the pro-

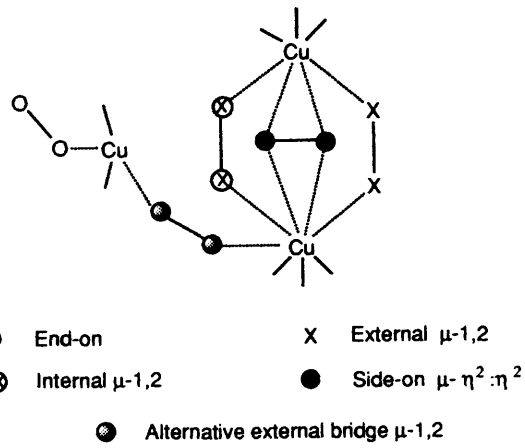
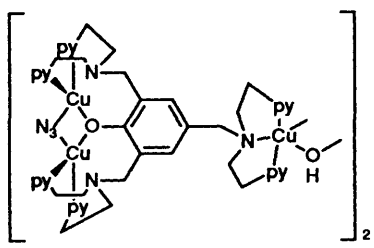
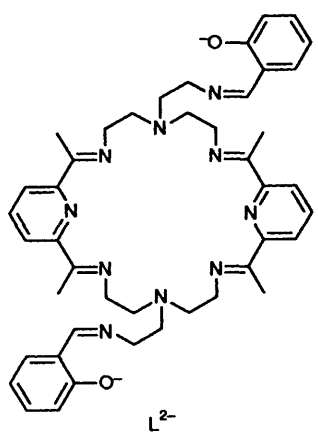


Fig. 13 Possible bonding modes for dioxygen at the trinuclear site in ascorbate oxidase (after ref. 59)



5; py = 2-pyridyl



L^{2-}

posal that laccase has one mononuclear copper atom and a trinuclear centre located similarly to those in ascorbate oxidase; ceruloplasmin has three mononuclear copper atoms and one trinuclear centre.[62]

'One more question. Models, are there any small molecule models for these sites?'
'At this point some but not a lot. So far knowledge of the sites has been heavily X-ray dependent and so any model will be of a corroborative nature.[28] The fascinating challenge is to produce a model which will confirm which of the metal sites is catalytic, which is structural and maybe which, if any, is redundant.'

Synthetic analogues for the trinuclear site in ascorbate oxidase and the related 'blue' oxidases have recently been presented. One is a hexanuclear copper(II) complex, derived from a polypodal ligand, in which there are two approximately isosceles triangular arrays of copper(II) atoms present (**5**). Each array has a Type 3-like pair of copper atoms having a 3.11 Å separation supported by an endogenous phenoxo-bridge derived from the ligand; the third copper is distant from the pair by 7.78 and 7.46 Å respectively.[63]

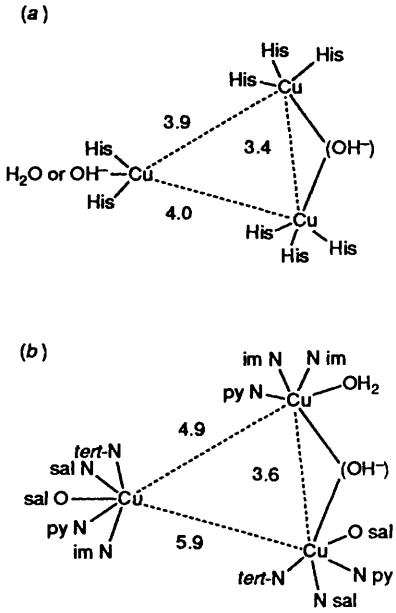
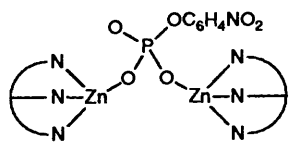


Fig. 14 A comparison of the *triangulo*-copper(II) sites in (*a*) ascorbate oxidase and (*b*) the model complex $[Cu_3(\mu\text{-}OH)L][ClO_4]_3\cdot 3H_2O$; distances in Å im N = iminenitrogen, py N = pyridyl nitrogen, sal O = salicyl oxygen, *tert* N = tertiary amine nitrogen

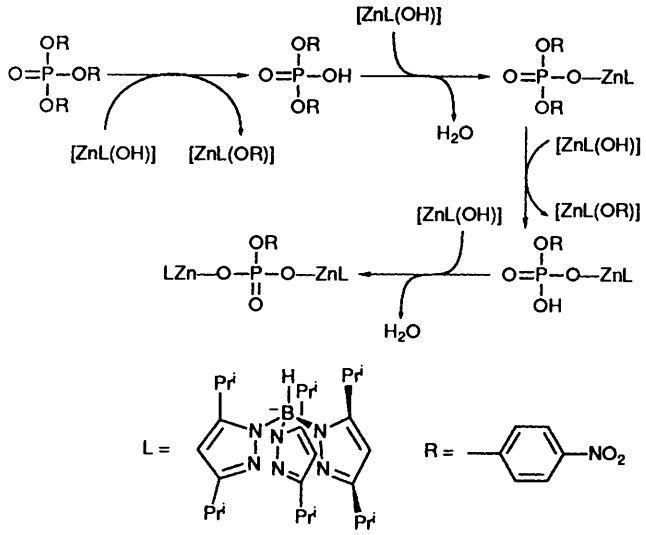


6

The trinuclear copper cluster of L^{2-}, $[Cu_3(\mu\text{-}OH)L][ClO_4]_3\cdot 3H_2O$, has been proposed as a first-generation model for the biosite cluster.[64] In this complex the three copper atoms are held within the ligand perimeter, and provide a Type 2-like copper atom and a Type 3-like centre. The Type 2 atom is 4.9 and 5.9 Å distant from the two copper atoms of the Type 3 centre. The two copper atoms of this centre are 3.6 Å apart and bridged by a hydroxy group the origin of which appears to be water from the reaction medium with the two copper(II) atoms acting in concert as a super acid pair to promote the generation of a nucleophile. The presence of the hydroxo bridge reinforces a statement made by Coughlin and Lippard[65] suggesting that the endogenous bridging protein ligand proposed for Type 3 biosites might simply be the hydroxide anion itself, generated from accompanying water molecules.

The trinuclear copper(II) complex cannot be claimed to provide a precise replication of the ascorbate oxidase clusters as the co-ordination spheres of the metal ions differ both in terms of the nature and the geometric arrangement of the donor atoms (Fig. 14). This probably arises because the small molecule derived ligand cleft has a greater degree of conformational freedom than the more highly defined proteinaceous clefts and so the need to design in features to constrain this mobility is apparent.

The structure of a dinuclear zinc centre bearing a phosphate bridge has been reported by Kitajima and co-workers[66] and related to the dinuclear centre in alkaline phosphatase. The reaction of hydroxo[tris(3,5-diisopropylpyrazol-1-yl)hydroborato]zinc(II), [ZnL(OH)], with mono(*p*-nitrophenyl) phosphate led to the cleavage of the P–O bond to give a dinuclear μ-phosphate monoester complex, $[LZn\{\mu\text{-}OP(O)(OC_6H_4NO_2)O\}ZnL]$ **6** together with a monomeric phosphate diester complex $[ZnL\{OP(O)(OC_6H_4NO_2)_2\}]$, and a phenoxo complex $[ZnL(OC_6H_4NO_2)]$ (Scheme 3). The structure of the dinuclear complex revealed that each zinc atom is in a distorted



Scheme 3 The phosphatase activity of the model complex [ZnL(OH)][66]

tetrahedral co-ordination geometry and that the zinc atoms are bridged by the phosphate which is strongly bonded in a *syn–anti* co-ordination mode. This separation is longer (5.1 Å) than that found in the metalloenzyme (3.9 Å) where the phosphate is found in a *syn–syn* co-ordination mode.

A phenoxy-bridged homodinuclear zinc complex in which the ligand 2,6-bis[bis(2-benzimidazolylmethyl)aminomethyl]-4-methylphenol provides a biomimetic N,O ligand sphere has been reported. In this complex both zinc atoms are co-ordinated by four ligand donors and an oxygen from a bridging benzoate; one of the zinc atoms is further co-ordinated by a water molecule. The intermetallic distance is 3.44 Å, very close to that of 3.3 Å reported for the enzyme. The presence of the water molecule, which might act as a nucleophile for any substrate, and the unusual unsymmetrical co-ordination of the two metals (five- and six-), has led to its being advanced as a structural model for transition state of the catalytic process of phospholipase C.[67]

Endword

'So that's it then?'

'No, not at all, it's very much a beginning. Trinuclear metal clusters and constellations have now been found at the active sites of a variety of biomolecules. As more information becomes available it will be possible to gain a better understanding of the structure-function relationships that relate to the diverse nature of the sites and their different functions—electron transfer, metal metabolism and detoxification, enzymatic activity. Establishing these relationships surely presents an intriguing and perhaps significant challenge both to the biological inorganic chemist and, as mentioned above, to those chemists interested in the modelling of metallobiosites.'

Acknowledgements

We thank the British Council whose support has given us the opportunity to engage in many stimulating conversations, and those who have given us permission to reproduce diagrams.

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Received 5th January 1993; *Paper* 3/00053B