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Metallo-enzyme catalysis

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All organisms depend upon metallo-enzymes. The dependence arises from the inability of individual *organic* side-chains of proteins to activate molecules such as H_2, N_2, CH_4 and CO and their weakness in hydrolysing many simple compounds such as many peptides, phosphates, even urea .[1] The metal ion sites have been found to be 'designed' for selective uptake and catalytic activity.[1,2] In this article a few examples will be used to illustrate these points. For more details of all the examples see the reference at the end of this article to Messerschmidt *et al.* (2001)[3].

Introduction

It was known before there was any structural data that many enzyme catalytic sites, metal ions or organic groups, were specially reactive and that the metal ions had unusual spectroscopic and thermodynamic properties. Two ideas were put forward to explain the observations on the metallo-enzymes.

Williams (1956) (see ref. [2]) proposed that the ground state of the metal ion was unusual in that the protein controlled a particular structural disposition of the possible ligand binding groups so aiding catalysis. Later (in 1969), Vallee and Williams (see ref. [2]) describe this ground state condition of both metallo and other enzymes under the name of "entasis".

Independently Lumry (1956) (see ref. [2]) proposed that enzyme active sites generally were "racks" which produced a stretching adjustment of bonds in a protein on binding a substrate or a metal generating the activity of the enzyme. The name "rack" was taken up, somewhat confusingly, by Malmström (1964) (see ref. [2]) as a way of modifying Williams' proposal. Gray, Malmström and Williams (2001)[3] combined both concepts by describing the metal condition in the site as "constrained". In essence this postulate stated that a protein fold is "designed" to give the metal ligands their positions in space, entatic state if this is exactly so, and that selected small dynamic adjustment on metal binding, a rack, may occur such that the active site properties become appropriate for both selected uptake of and catalysis by the metal.

Copper proteins

A good example is the blue copper (type 1) sites, present in both some simple electron transfer proteins and complicated copper oxidases, Fig. 1. They have a closely fixed structure, essentially independent of copper oxidation state, which controls the physical redox potential and relaxation energy in order to facilitate electron transfer. Crystallographic data show that the constrained ligand site, seen in oxidised and reduced states, in synthesised enzymes with a variety of metal ions, and even in the apoprotein is

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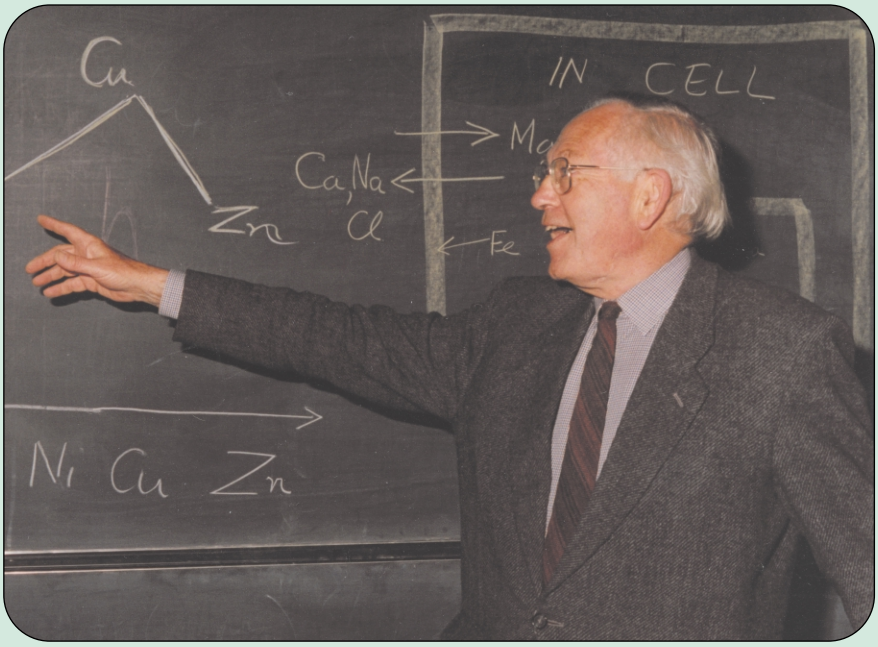

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Table 1 Some Major Features of Metallo-enzymes[1]

Metal ion	Major catalytic functions
Mg^{2+} (Ca^{2+})	Easy hydrolyses, phosphate transfer, (light capture, chlorophyll)
Zn^{2+}	Difficult hydrolyses; hydride transfer
Mn^{2+}	O_2-generation; some hydrolysases
Ni^{n+}	H_2 activation; urea hydrolysis; F-430 enzymes
Co^{n+}	B_{12}-enzymes for transformation of diols and other simple saccharides (ribose to deoxyribose)
Cu^{n+}	Oxidation of phenols, amino acids, sugars; *e*-transfer (outside cytoplasm)
Fe^{n+}	*e*-transfer; oxidation (hydroxylation); H-transfer (inside cytoplasm) N.B. haem units
Mo^{n+}	Oxygen atom transfer (pterin cofactor); N_2 activation

N.B. All these elements (in enzymes) are probably *essential for all life*, including human life.[1] The sites of binding are often of unexpected geometry before or during reaction.

Table 2 Some β-sheet and α-helical enzymes[1]

Largely β-sheet	Largely α-helical[a]
Cu-enzymes (oxidative)	Many heme-oxidases, *e.g.* cytochrome P-450
Cu/Zn, Superoxide dismutase	Many simple cytochromes (electron transfer)
Fe-ferredoxins (electron transfer)	Isopenicillin N-synthetase (hemerythrin)
Ni-urease-site	Methane mono-oxygenase
Zn-enzymes (hydrolytic)	Ribonucleotide reductase (haemocyanin, haemoglobin)

[a]Note also molecular machines[12,13] such as MgATP-synthetase,[13] Cytochrome Oxidase, and ATP-driven pumps[12] are largely helical. The Table is compiled from data in Handbook of Metalloproteins Vols. 1 and 2. A. Messerschmidt, R. Huber, T. Poulos and K. Wieghardt (eds) J. Wiley and Sons, New York, 2001.

due to the stiff β-sheet barrel fold of the protein which gives ligand coordination by three selected ligands, two histidines and a thiol, with a fourth and possibly a fifth ligand at long distances. Note that the site excludes water and its geometry, trigonal or approaching tetrahedral is not that seen in any model complex ion studies of copper(II) compounds. All the work of these and of many other authors is summarised in [ref. 3], and see Fig. 1. There are also similar structures of dimeric electron-transfer copper sites in enzymes, for example, cytochrome oxidase and nitrous oxide reductase, Fig. 1.

Continuing with the examination of the active site of the blue copper proteins, the idea of a constrained state has been challenged by theoreticians. Ryde[4] calculated that, allowing the four observed ligands now free (no protein or solvent) to collapse around the copper while minimising energy, their positions reproduced those seen by X-ray diffraction studies for at least three of the four ligands. This was a surprising result. However it remains the case that the protein structure provides the particular ligand geometry even in the apoprotein and that the complex forms in water by excluding all alternative ligands and water from the metal ion. The calculations are less predictive when dealing with redox potentials and relaxation energies for electron transfer which depend on large regions of the protein.

Three quite separate explanations have centred on the general suitability of these and similar proteins to make a path for electron transfer[5,6,7]: (1) the paths are of chemical bonds (2) the route follows the local physical protein electron densities (3) that there is a simple general distance dependence in an effectively uniform medium of fixed dielectric constant. Note in the last model all types of protein folds are as good as one another for electron transfer. I believe that no experiment yet separates the three within error limits. Associated long-range *proton* transfer in enzymes is not yet understood but this is a critical problem in the consideration of the copper site for O_2 reduction in cytochrome oxidase for example.

Before continuing notice it is not just the secondary structure of the fold that is critical but the way it holds the side-chains which ligate the metal ion and limits movement. Different secondary structures can then lead to similar catalytic sites and similar secondary structures can yield different catalytic sites as we show below. In particular β-sheet structures constrain sites more than do α-helices.[1]

A comparison between the above, type 1, copper sites for electron transfer and type 2 copper sites, which bind oxygen or superoxide in enzymes, shows that the copper is now usually bound by a different set of ligands, only histidines, and it has exchangeable *water* or a vacant coordination position. The enzymes are again β-sheet proteins. The symmetry of the site is a distorted square pyramid, with water or a vacancy in the weak axial position. Clearly this site is constrained to accept reactive substrates which come to it via an access channel. During the reaction cycle the copper may move about 1.0 Å. A third type 3, now dimeric, copper site, is found in many of these β-sheet enzymes such as caeruloplasmin and laccase where each copper(II) is in a constrained trigonal pyramid bound by three histidines with a bridging hydroxide ion. In the reaction cycle copper(I) becomes trigonal and open sided for reaction with O_2.

Very intriguingly there are α-helical proteins which generate virtually the same type 3 copper sites. One of them is the oxygen carrier, haemocyanin, a Cu(I) protein, which transports O_2. Now however binding of O_2 causes a conformation change and the multi-subunit protein is allosteric and binding is cooperative. An enzyme with this site is α-helical catechol oxidase but it undergoes smaller protein changes. A mono-copper with this site also is found in α-helical cytochrome oxidase paired with a haem a_3 instead of a second copper for oxygen activation. Water and protons must leave and enter all the above oxidase sites in the reaction cycle: Details of all these copper sites and some others of rarer occurrence are given in the references section, see Messerschmidt *et al.* (2001).

Iron proteins

Here we shall not describe in detail the iron-sulfur proteins used in simple electron transfer reactions. These proteins are largely β-sheet and a single Fe or a cluster of Fe/S is bound by thiolates. The high-spin iron has a variety of structures from very closely to distorted tetrahedral. The parallels with type 1 copper sites are clear. There is also a series of haem-proteins, cytochromes *a*, *b* and *c*, which are simple electron transfer proteins but they are α-helical with fixed octahedral low-spin iron sites. There is often small adjustment internally of the protein on redox reaction.[1] This is more noticeable in the cytochromes c′ which like the O_2-binding haemoglobins[8] have α-helical multi-subunit structures and give allosteric binding of CO. Amongst these cytochromes c′ there is a great variety of from 5 to 6 coordination depending on the closeness of approach of a sixth, methionine, ligand. Each protein constrains the site differently, gives different binding properties and the Fe(III) ion is seen in all three of its spin states often in equilibrium.[1]

The theme that some α-helical proteins and their active sites are open to larger adjustments within the fold is illustrated very well by the P-450 cytochromes for the oxidation of inert hydrocarbons such as camphor and sterols, Fig. 2.[9] Here the three substrates, an organic molecule, electrons, and oxygen are added in this necessary order. The site is largely similar to that in many haem proteins with a sixth

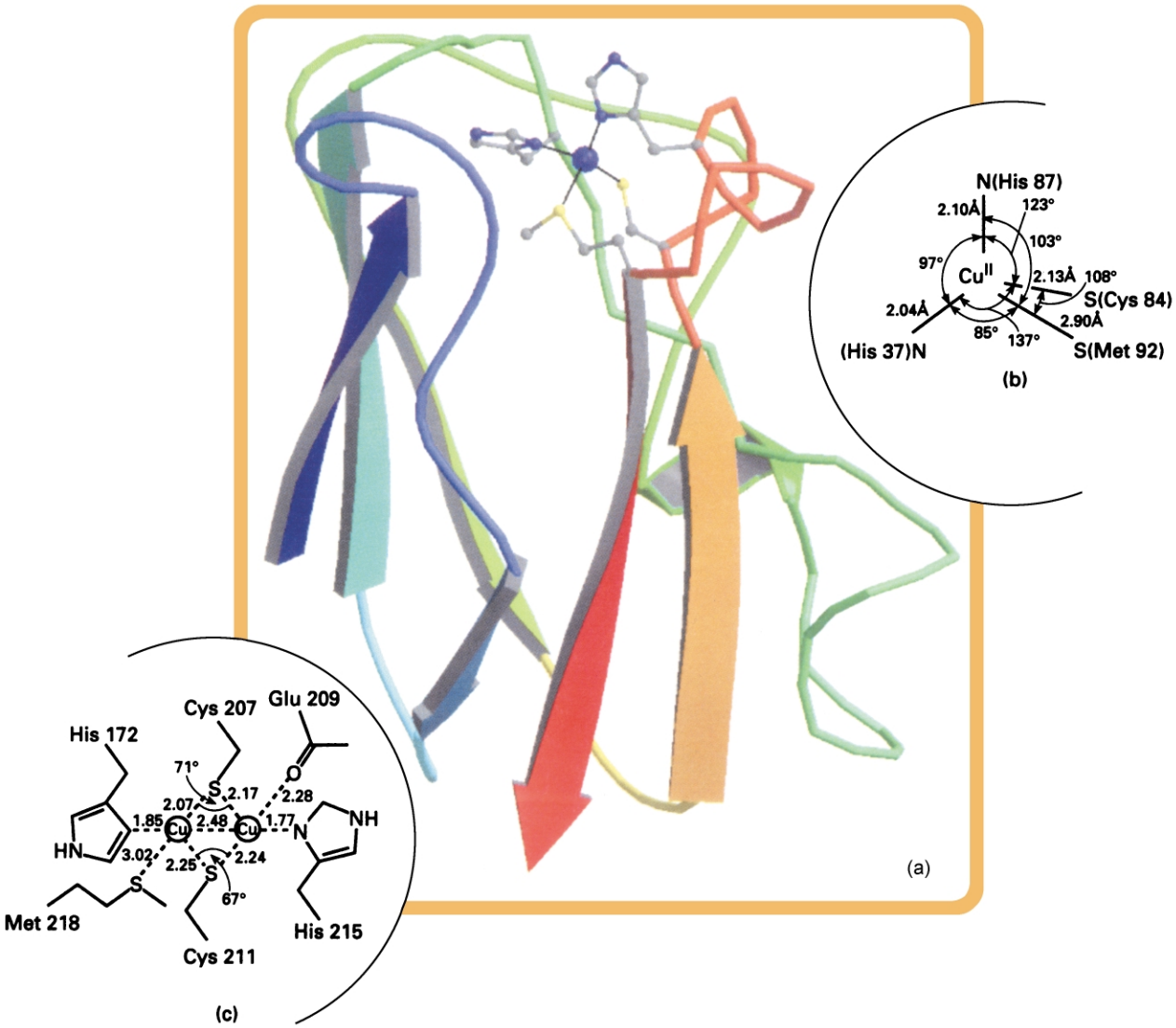

Fig. 1 (a) The ribbon structure of a blue copper protein showing the β-sheet structure, arrows, and the site of the copper (printed with permission, see reference to Messerschmidt *et al.*). This constrained structure is present in many complicated oxidases and catalyses *selective* electron transfer. (b) Details of its rigid distorted tetrahedral (or trigonal) site, note the long-bond to Met-92. (c) A dimeric copper site found in some oxidases which has the possibility of acting as a two-electron condenser for passing charge. Note that a β-sheet holds the ligands in virtually fixed positions. Such rigidity generates selectivity of metal and substrate binding and uptake.

water ligand, but has a fifth cysteine thiolate ligand. The iron is initially in the low-spin ferric state.[7] The cycle then includes switches of oxidation state, Fe(III) to Fe(II), after organic substrate binding and with loss of water, before the high spin Fe(II) binds oxygen with a change to a low spin state. While these changes occur the protein undergoes conformation changes and the iron atom moves into the porphyrin plane. During the reaction cycle an oxo–Fe complex, FeO, is seen after introduction of further electrons and loss of water. The protein cycles through the oxidation states of iron while the substrate is hydroxylated and protects the protein from attack by the active oxygen intermediates. In somewhat parallel ways the haem of peroxidase goes through a cycle of constrained states but here the haem porphyrin ring also cycles through a radical condition as iron goes through oxidation states including FeO. There is a film of this catalytic cycle.[10] Hydroxylation by a second series of non-haem, non Fe/S, α-helical proteins containing high-spin iron goes through a somewhat parallel set of reactions. The Fe(II) is here constrained in a distorted octahedron of two histidines, a carboxylate and 3 H_2O molecules but the FeO state is pentacoordinate.[11] Further details of iron enzymes are given at the end of this article, Messerschmidt *et al.* (2001).[3]

A feature of the combination of the relative mobility of α-helices and the relative rigidity of β-sheets is that they combine units well-known in man's machines of moving rods and fixed platforms.[12] Some enzymes are therefore called molecular machines.[12,13]

Organic free radicals

An unexpected feature amongst some metallo-enzymes has been the finding that their reactions are dependent upon both constrained metal ions and protein side-chain organic units which are seen as quite stable free radicals.[1,2,14] For example several reactions of saccharides involve H-atom transfer using Cu, Co, or Fe enzymes and organic radicals. In some cases such as ribonucleotide reductase class II and propane-diol-dehydratase, vitamin B_{12} is involved. The active state of the metal (cobalt(II)) and the free radical are produced only when the substrate binds which is a good example of small conformation changes generating

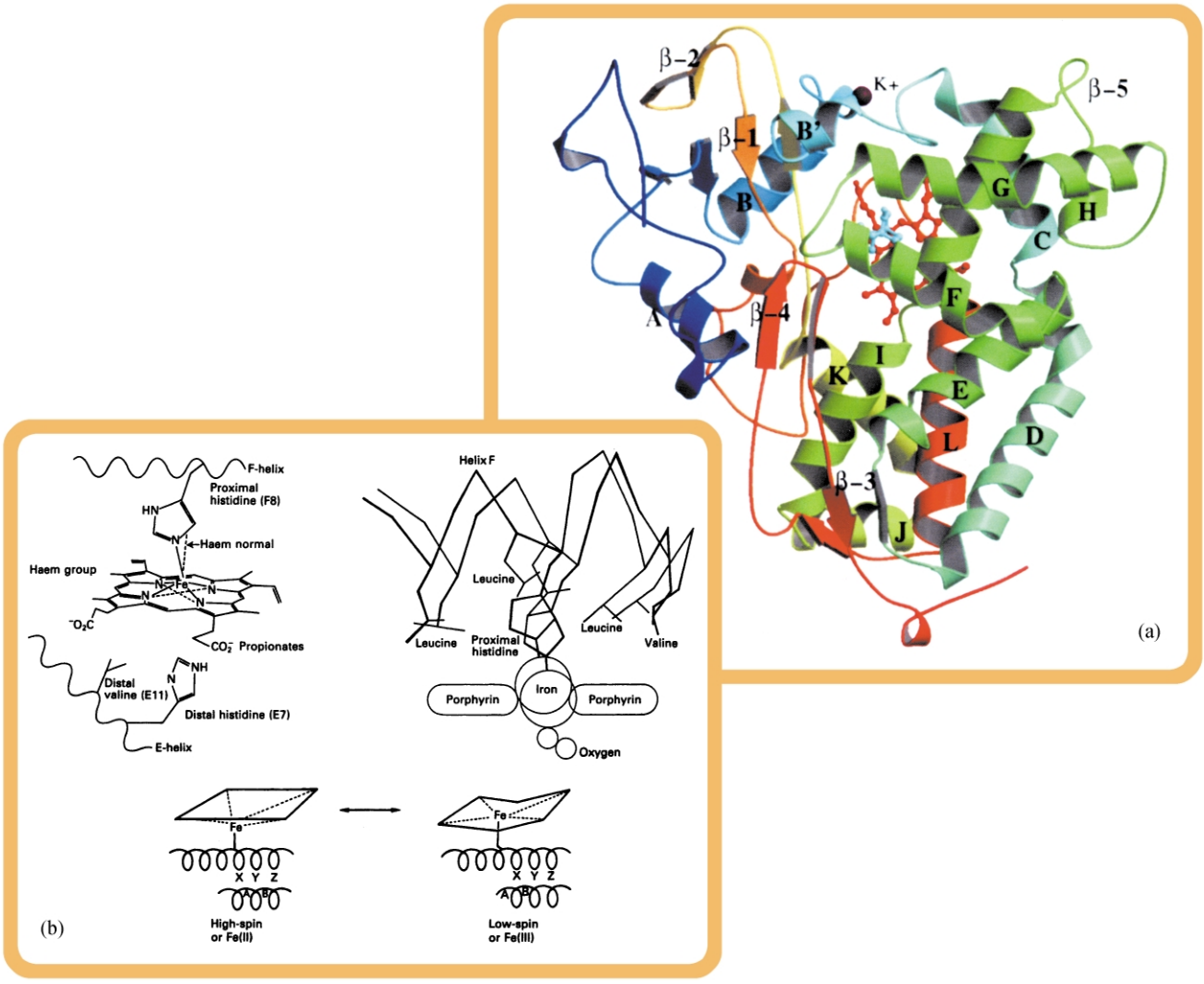

Fig. 2 (a) A picture of the enzyme cytochrome P-450 (cam), taken from Messerschmidt *et al.*,[3] with permission. The structure of the Fe(II) state is open sided. On accepting dioxygen the metal ion contracts, shortens bond-lengths and forces the helices to re-arrange, see also haemoglobin.[8] (b) The change of structure in haemoglobin is due to spin-state changes of the iron. Note that in all such haem enzymes the metal is held in constrained states by somewhat adjustable helices. This type of movement of helices is typical of molecular machinery.[12,13]

constrained states. An extreme case is that of ribonucleotide reductase class I which employs Fe–O–Fe and a tyrosine radical centre, or even an Fe/S centre and a glycine radical, not vitamin B_{12}. A very curious feature here is that the nucleotide active site is some 30 Å from the Fe–O–Fe centre, apparently too large for simple electron (plus proton) transfer. Theory, in the hands of Siegbahn,[14] appears to show that the necessary transfer of H between the sites occurs through long range, quite unusual, *H-atom* transfer. Several of his other theoretical studies indicate that even the well-trained practising chemist may not have considered the lowest energy reaction profile. Some other peculiar catalytic sites which require explanation are those in hydrogenases (Ni/Fe), oxygen evolution enzymes (Mn), nitrogenase (Fe/Mo), and nitrile dehydratase (low-spin Co/Fe).

Acid/base catalysis by zinc metallo-enzymes

Zinc is a strong Lewis acid and is used in enzymes which hydrolyse strong bonds[15] such as in peptides or to polarise substrates for hydride transfer, *e.g.* alcohol. Here the constraints on the site are readily seen both to select zinc and to activate a coordinated base. A good example is provided by the β-sheet section of the protein carbonic anhydrase, Fig. 3. In this case the constrained ground state geometry is usually four or five coordinate depending on pH using histidine-bonding with a water molecule and/or hydroxide as one ligand. The pathway of reaction on substrate, HCO_3, binding is now a complex succession of slightly adjusted ligated states, passing through both four and five coordination. It is of great interest that cobalt(II) only of the divalent ions in the first transition series is able to substitute for zinc and give an active enzyme. This accords with the ease with which both elements accept the same coordination numbers and stereochemistries.[16]

Conclusion

In conclusion the great advantage of metallo-enzymes for the study of mechanism lies in the ability to follow many states by fast spectroscopy. It is probably the case that this knowledge will allow a detailed comparison with modern theory which may not be as possible for organic enzyme sites where intermediates are more difficult to observe. Together with structures the physical data have confirmed the idea of constrained metal and/or organic groups in enzyme catalysts where "constrained" implies fixed structures with but small allowed *directed* adjustments, all of which are desirable for

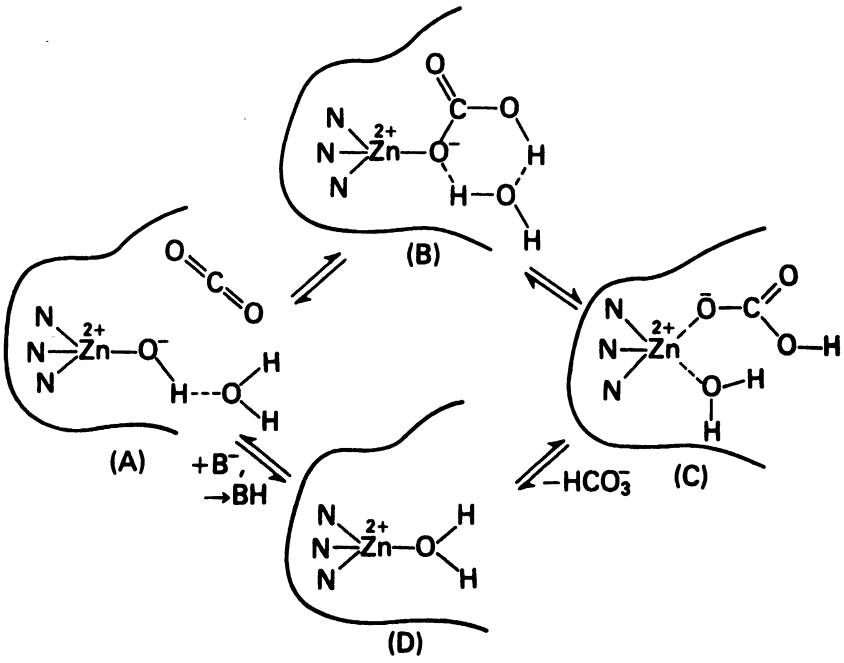

Fig. 3 A typical proposal for zinc ion catalysis of attack by water, here the production of bicarbonate from carbon dioxide in carbonic anhydrase. To generate the good attacking base, hydroxide, at pH = 7, the metal is open-sided, bound rigidly by three histidines (N) allowing one water molecule (hydroxide) in the site, contrast Fig. 1(b). The probable cyclic intermediate and the flow of protons via the base B is a part of the active site which in effect extends over a considerable part of the protein. Note the flexibility of zinc (4↔5 coordinate) and contrast copper in Fig. 1.[16]

catalysis or just substrate binding.

The subject matter of this article covers a vast literature so that the few references must be used to lead the reader to details on specific points of interest. For details of all structures and outlines of mechanisms described here see Messerschmidt *et al.*[3]

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