CROATICA CHEMICA ACTA CCACAA **71** (3) 659–672 (1998)

ISSN-0011-1643
CCA-2518 *Conference Paper*

Structural Studies on the Interactions between Metal Ions and Biological Macromolecules*

Pierluigi Orioli

Department of Chemistry, University of Florence, Via Capponi 7, 50121 Florence, Italy (e-mail: orioli@risc.lrm.fi.cnr.it)

Received February 3, 1998; revised July 13, 1998; accepted July 15, 1998

The essential role of metal ions in biology is stressed by structural investigations on two metalloenzymes: human carbonic anhydrase (CA) and Cu, Zn superoxide dismutase (SOD). The X-ray structure of CA complex with histamine affords a satisfactory explanation of the mechanism of activation of CA. The high resolution (1.02 Å) crystal structure of a mutant monomeric Cu, Zn superoxide dismutase gives relevant information on the mechanism of the catalytic activity of the native dimeric enzyme and on the reduced activity of the monomer.

INTRODUCTION

Metal ions play a crucial role in many biological processes such as: catalytic activity of metalloenzymes, regulation of nucleic acids replication, pharmacological activity of many metal complexes, *etc*.. Structural studies by X-ray diffraction can be of great help for understanding the mechanism of these processes.

Our group has been recently involved in the study of some of these processes: the activation of human carbonic anhydrase by some small molecules[1] and the mechanism of the catalytic activity of Cu,Zn superoxide dismutase.[2]

---

* Based upon the plenary lecture presented at the Sixth Croatian-Slovenian Crystallographic Meeting, Umag, Croatia, June 19 - June 21, 1997.

CARBONIC ANHYDRASE

Carbonic anhydrase (CA, EC 4.2.1.1) is a zinc enzyme which catalyzes the reversible interconversion between CO_2 and the bicarbonate ion:[3,4]

$$CO_2 + H_2O \Leftrightarrow HCO_3^- + H^+$$

Seven distinct isozymes are presently known in higher vertebrates,[5] though their physiological function is not completely known. In this paper I will mainly focus on the human isozyme II whose crystal structure has been refined at a resolution of 1.54 Å.[6] The enzyme molecule has three layers of secondary structure, the central one being an extensive β-sheet (Figure 1). The active center is a deep cavity containing the zinc ion, which is attached to the central β-structure through three histidyl residues (residues 94, 96 and 119) . A water molecule, Wat 263, which ionizes to OH^- with a pK_a near 7, completes the tetrahedral coordination sphere of the zinc ion (Figure 2). Distances and angles around the zinc ion are reported in Table I.

The Reaction Mechanism and Proton Transfer

According to the generally accepted »zinc-hydroxide mechanism«,[7] the zinc bound OH^- reacts with CO_2 to form HCO_3^-, which is then displaced

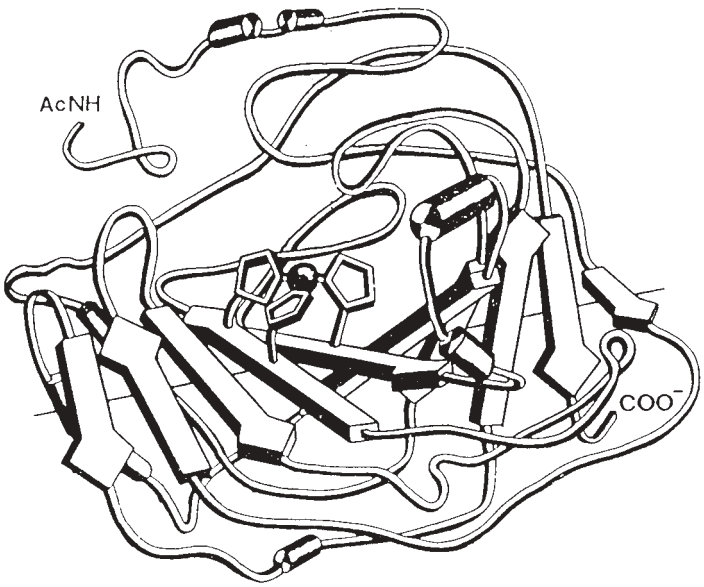


Figure 1. Schematic view of the CA II structure. The Zn(II) ion and three imidazolic residues of coordinated histidines are shown.

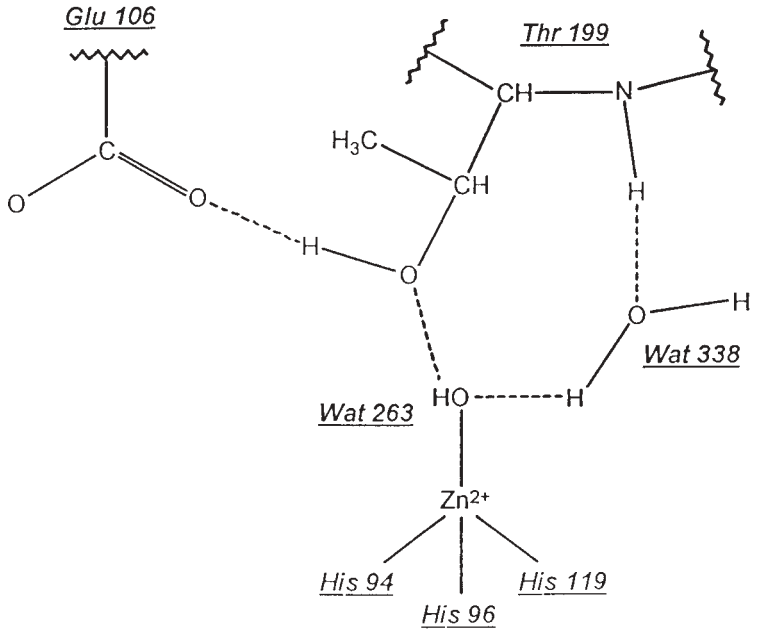


Figure 2. Coordination of the Zn(II) ion in the active site of CA II. Some important residues, involved in the catalytic process, are also shown.

TABLE I

Bond distances (Å) and bond angles (°) around the Zn(II) ion in native CA II.

Bond distances (Å)		Bond angles (°)	
Zn-Wat 263	2.05	Wat 263-Zn-Nε2 94	111.06
Zn-Nε2 94	2.10	Wat 263-Zn-Nε2 96	113.60
Zn-Nε2 96	2.12	Wat 263-Zn-Nδ1 119	112.94
Zn-Nδ1 119	2.11	Nε2 94-Zn-Nε2 96	103.92
		Nε2 94-Zn-Nδ1 119	115.32
		Nε2 96-Zn-Nδ1 119	99.17

from the metal ion by a water molecule, after Eq. (1). In order to regenerate the catalitically active form, a proton transfer reaction must occur, from the zinc bound water to the external medium, after Eq. (2). This is considered to be the rate limiting step and in isozyme CAII the step is assisted by His 64, located at the entrance to the active site cavity, which functions as a proton shuttle between the metal center and buffer molecules in the reaction medium.

$$EZn^{+2}\text{-}OH^- + CO_2 \Leftrightarrow EZn^{+2}\text{-}HCO_3^- \Leftrightarrow EZn^{+2}\text{-}OH_2 + HCO_3^- \quad (1)$$

$$EZn^{+2}\text{-}OH_2 \Leftrightarrow EZn^{+2}\text{-}OH^- + H^+ \quad (2)$$

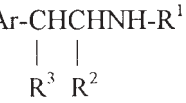
Indeed, in several crystal structures, the His 64 side chain has been found disordered in two orientations, one toward the inside and the other toward the outside of the active site cavity.[8]

Activation of CA

CA is one of the fastest enzymes known, with a maximal turnover rate for CO_2 hydration of ~106 s^{-1} at 25 °C, which is probably the reason why the activation of CA has not been much studied. In contrast, inhibition of CA has been widely investigated and several crystal structures of CA complexes with inhibitor molecules have been reported.[9] These studies have largely contributed to the elucidation of the reaction mechanism of CA and to the development of new drugs for several diseases, such as glaucoma, gastro-duodenal ulcers, hypertension, and so on.

In analogy with the inhibition studies, investigations on CA activators can contribute to a better understanding of the reaction kinetics and to the development of clinical agents for the treatment of some disorders caused by CAII deficiency, such as osteopetrosis, renal tubular acidosis and brain calcification.[10]

It has been shown that the molecular structure of CA activators must satisfy precise electronic and steric factors.[10] Particularly efficient are amines with the general formula:



Ar = aromatic/heterocyclic group;
$R^1 = R^2 = H, CH_3$; $R^3 = H, OH, COOH$

It is generally accepted that activators essentially increase the rate of the proton transfer to the bulk solvent, which is the rate limiting step, as mentioned above. Most activators in fact possess an amino group in their molecule, which can act as proton acceptor.[10] In the presence of activators, the reaction mechanism can be summarized as follows:

$$EZn^{+2}\text{-}OH_2 + A \Leftrightarrow [EZn^{+2}\text{-}OH_2\text{-}A] \Leftrightarrow [EZn^{+2}\text{-}OH^-\text{-}AH^+] \Leftrightarrow EZn^{+2}\text{-}OH^- + AH^+ \quad (3)$$

It is not known, however, whether activators bind in the active site of the enzyme or somewhere else and so far no crystallographic investigation

has been performed on CA-activator complexes. I wish to describe here the first crystal structure of one of such complexes, the adduct between histamine and CAII.

Structure of the CAII-Histamine Complex

The overall three-dimensional structure of the CAII-histamine complex is close to that of other published CA structures, the rms deviation between the backbone atoms of the complex and those of CAII (PDB code 2CBA) being only 0.19 Å. The histamine molecule is found at the entrance to the active site cavity, forming hydrogen bonds to amino acid side chains and to water molecules. (Figure 3) The Nδ1 and Nε2 of the histamine imidazole ring form hydrogen bonds with the side chains of Asn 62 and of Gln 92 and to Wat 152 (Table II). The relatively weak binding of histamine is consistent with its affinity constant which is in the millimolar range as well as with its role as activator. The entropic contribution due to the release of some water molecules provides additional contribution to the stability of the complex. This behaviour is quite different from that of the classical CA inhibitors, which bind to the metal in the active site replacing the zinc bound water.

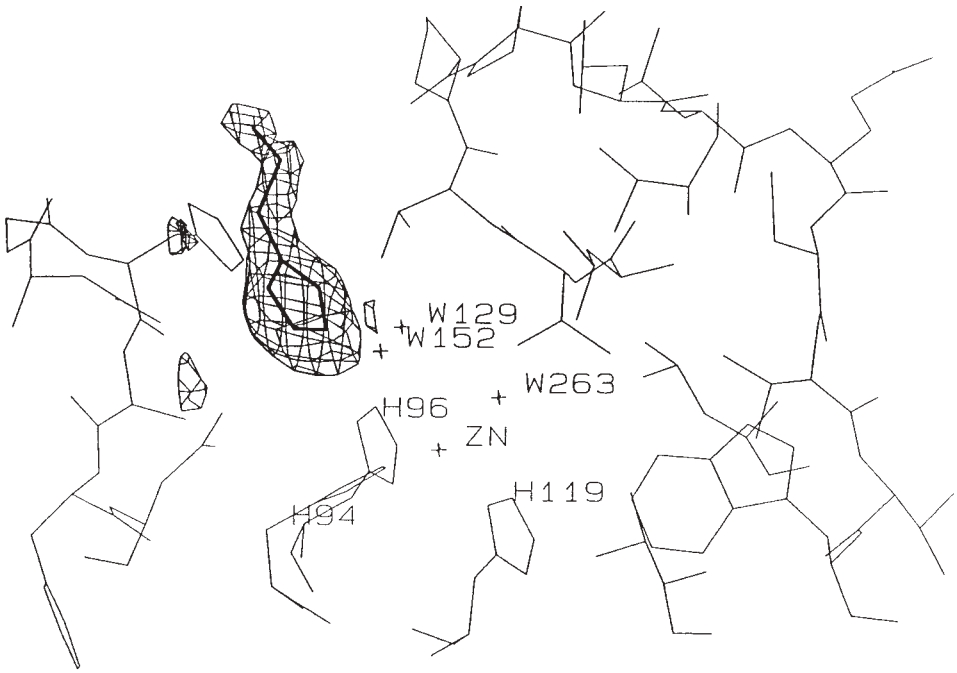


Figure 3. View of the histamine molecule as it appears in a $/F_o/ - /F_c/$ map not including the histamine contribution.

TABLE II

Shortest contact distances between the histamine molecule and CA II.

Histamine	CA II	Distances (Å)
Nε2 264	Wat 152	2.66
Nδ1 264	Wat 133	3.12
Nε2 264	Nε2 92	2.64
Nε2 264	Oδ1 67	3.47
Nε1 264	Nδ2 62	3.20

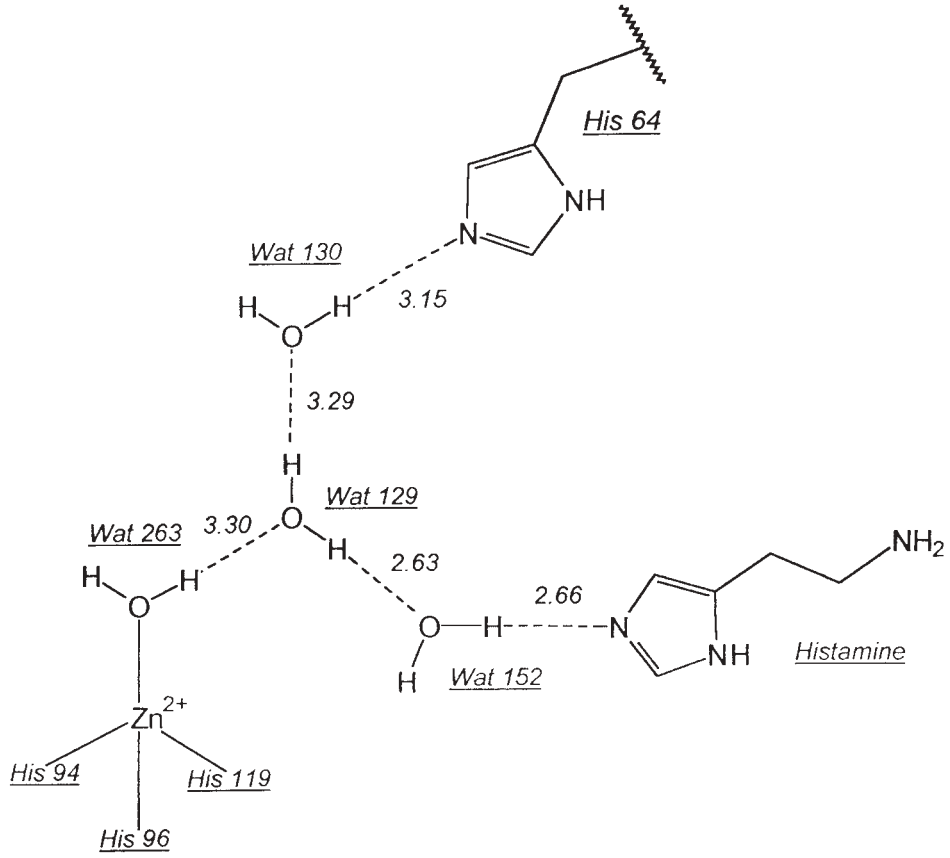


Figure 4. The hydrogen bonding pathways linking the zinc bound water to histamine and His 64.

Interestingly enough, His 64, which is often found disordered in two orientations, as mentioned above, in agreement with its probable role as pro-

ton shuttle, is found ordered in the present structure, oriented toward the inside of the cavity.

The stereochemistry around the zinc ion appears unaltered and bond lengths and angles are identical with those of the native enzyme, with the exception of the water molecule, which appears slightly removed from the metal (Zn-O 2.49 Å). This seems to be due to partial occupancy of the azide ion used in the crystallization solution, and to the consequent difficulty in locating the water molecule in the electron density maps, rather than to the effect of the complex formation.

The Molecular Activation Mechanism

Figure 4 shows the hydrogen bonding pathway linking the zinc bound water to the histamine molecule and to His 64. The pathway involving one histamine molecule provides an alternative route for proton release besides the His 64 pathway, which is believed to be the route for the proton in the native enzyme. The mere availability of more than one pathway for the proton to leave the active site appears to be a satisfactory explanation for the activatory property of histamine toward CAII

The ability of histamine to easily leave the active site and act as a second proton shuttle seems to fit perfectly the activation mechanism.

HUMAN MONOMERIC MUTANT OF Cu,Zn SUPEROXIDE DISMUTASE (M2-Q133SOD)

Among the several classes of superoxide dismutases, cytoplasmic Cu,Zn SODs are found both in bacteria and in eukaryotic organisms. These enzymes catalyze the dismutation of the toxic superoxide anion by converting it to dioxygen and hydrogen peroxide:[11]

$$\text{E-Cu(II)} + O_2^- + H^+ \Leftrightarrow \text{EH-Cu(I)} + O_2$$
$$\text{EH-Cu(I)} + O_2^- + H^+ \Leftrightarrow \text{E-Cu(II)} + H_2O_2$$

$$2O_2^- + 2H^+ \Leftrightarrow O_2 + H_2O_2$$

These enzymes occur essentially as dimers of identical subunits, related in the solid state by a non crystallographic 2 fold axis. Each subunit contains a catalytic copper ion bound to four histidine residues, one of which is a bridging ligand to a structural zinc ion. The coordination of the zinc ion is completed by an aspartate and two more histidine residues[12] (Figure 5). Substitution of copper is associated with a complete loss of the catalytic activity, whereas zinc can be easily replaced by several cations.

Site specific mutation has been widely used to study the role of residues for the catalytic activity. Replacement of the two hydrophobic residues Phe50 and Gly51 located at the dimer interface with two hydrophilic glutamate residues, gives a soluble, stable monomeric species of human SOD, almost deprived of superoxide dismutase activity.[13] A further modification, obtained by replacing the Glu133 residue at the entrance of the active cavity by a glutamine residue resulted in a 2–3 fold increase of the catalytic activity.

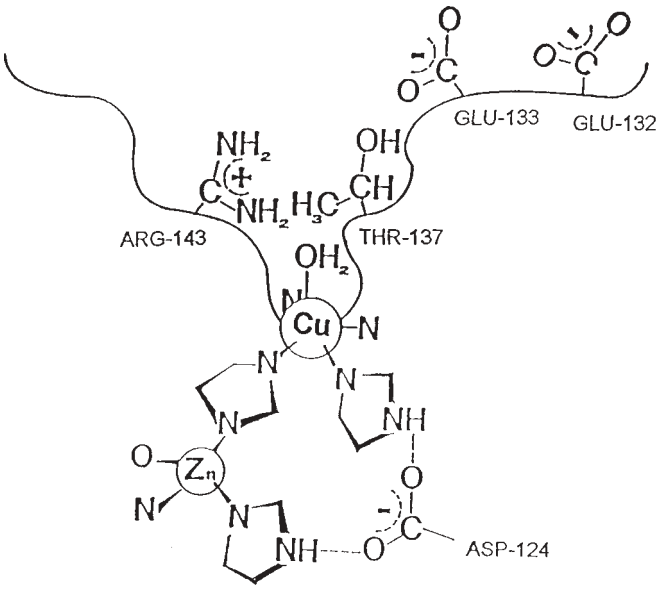


Figure 5. Schematic view of the active site of native SOD, showing also some relevant residues playing important roles in the catalytic mechanism.

The knowledge of the spatial relationships among these charged residues and its correlation with the catalytic activity of the mutants can be of great importance for the elucidation of the molecular mechanism of the activity of the dimeric native enzyme. A further reason of interest for the determination of the structure of monomeric SOD is the possibility to obtain a high resolution structure for the enzyme, in view of its reduced complexity, which may reveal interesting details in the active site conformation.

Description of the Structure

The structure reported here is the first high resolution structure for Cu,Zn Superoxide Dismutase and also the first structure for monomeric SOD. The final model contains 1166 protein atoms, 284 water molecules,

two catalytic metal atoms and 8 cadmium ions. The structure reveals some interesting differences with either subunit of the native enzyme.

The electron density is well defined in the β-sheet regions of the molecule, but there are many loop regions that are poorly defined. Most of the disordered or double conformation residues are lysines or residues at the surface of the enzyme, which were implied in intermolecular contacts with the other subunit. Arg 143, which is the residue responsible for the correct orientation of the superoxide ion, is highly disordered. This might be partly responsible for the diminished activity of the monomer.

One interesting aspect of this structure is the presence of cadmium atoms, which were introduced into the crystallization medium in order to obtain ordered crystals. The cadmium atoms, are located at the protein surface and are coordinated by side chain atoms usually glutamate or aspartate. The octahedral coordination is completed by solvent molecules or chloride anions. The cadmium ions mediate contacts between molecules inside the crystal and this can be the reason for their efficiency in promoting the formation of high resolution diffracting crystals.

The coordination around the Zn^{+2} ion is quite similar to that found in the native human enzyme (Table III and Figure 6).

The copper site shows some unexpected features. The presence of two electron density peaks at the 10.0 σ electron density level, 1.6 Å apart from

TABLE III

Bond distances (Å) and bond angles (°) around the Zn(II) ion in native SOD and in the M2-Q133SOD mutant.

	M2-Q133SOD	Native SOD
Bond distances (Å)		
Zn-Nδ1 His 63	2.00	2.10
Zn-Nδ1 His 71	2.03	2.06
Zn-Nδ1 His 80	2.01	2.04
Zn-Oδ1 Asp 83	1.97	1.92
Bond angles(°)		
Nδ1 His 63-Zn-Nδ1 His 71	106.9	104.9
Nδ1 His 63-Zn-Nδ1 His 80	112.3	120.4
Nδ1 His 63-Zn-Oδ1 Asp 83	104.4	116.8
Nδ1 His 71-Zn-Nδ1 His 80	119.8	112.4
Nδ1 His 71-Zn-Oδ1 Asp 83	97.6	84.8
Nδ1 His 80-Zn-Oδ1 Asp 83	116.0	117.7

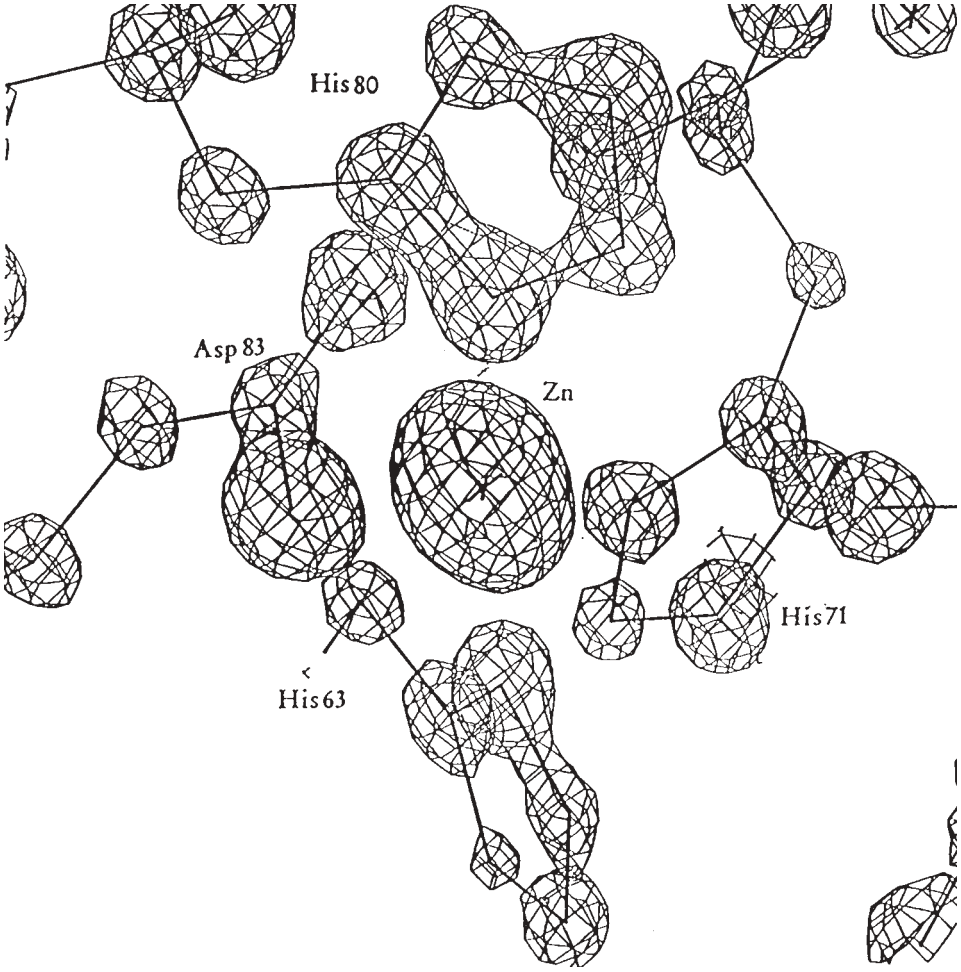


Figure 6. Electron density map of the zinc site in M2-Q133SOD, showing coordination around the Zn(II) ion.

each other in the Fo-Fc map, is consistent with two metal ions with fractional occupancy (Figure 7). The stereochemistry and the bond distances around them suggest the presence of one copper and one cadmium ion. The occupancy factors for these two metal ions refined to values of 0.41 and 0.45, respectively. The cadmium atom shows a distorted octahedral coordination with bond distances typical of cadmium(II) compounds (Table IV).

The other density peak can be clearly attributed to a copper ion. However, the coordination of this ion shows some differences relative to the copper ion of the native enzyme (Table V). Nε2 of the bridging His 63 is now at

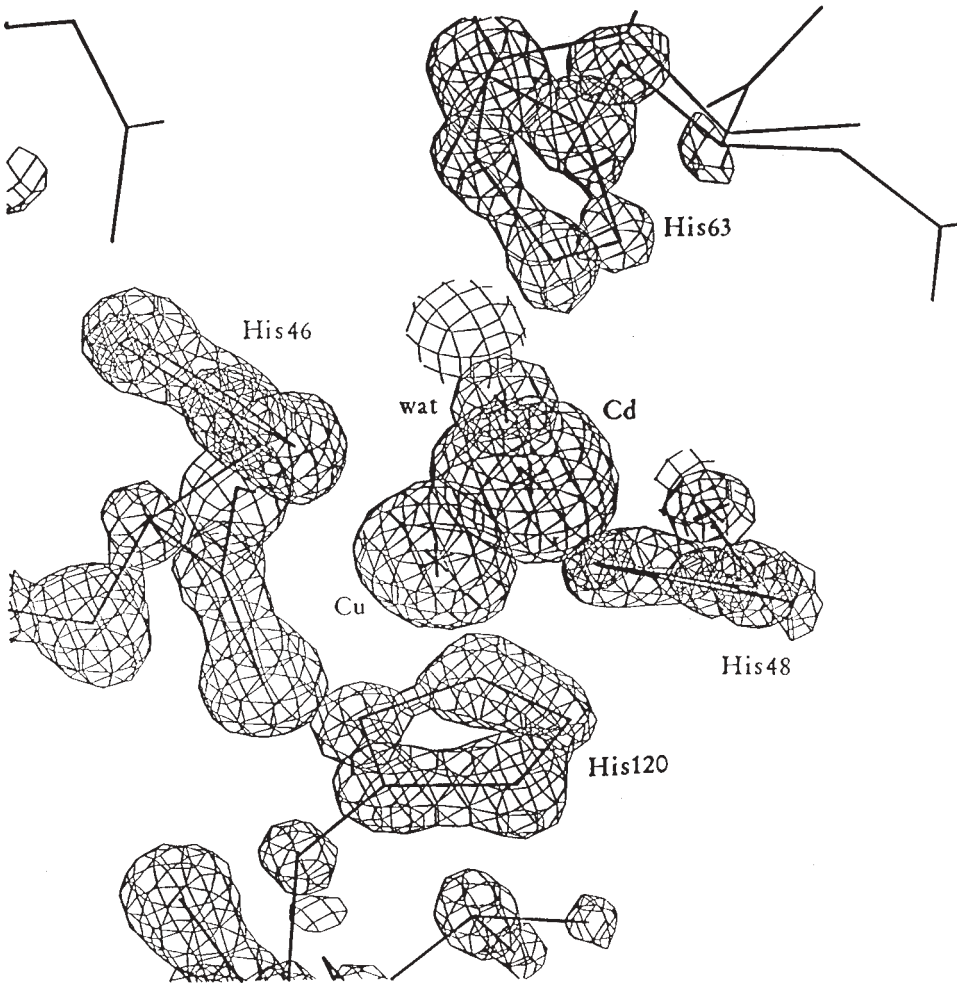


Figure 7. Electron density map of the copper site in M2-Q133SOD, showing coordination around the Cu(II) and Cd(II) ions.

3.31 Å from the copper ion, due to rotation of the imidazolic ring and to copper displacement of about 1Å with respect to the position in the native enzyme. This results in a trigonal coordination around the copper ion with roughly planar geometry.

The most interesting aspect of this structure is the breaking of the histidine bridge between the two active site metals with the consequent trigonal coordination around the copper ion.

In the mechanistic hypothesis proposed by Tainer,[14] the bridge formed by His 63 between the two metal ions is broken in the reduced form of the

TABLE IV

Bond distances (Å) and bond angles (°) around the Cd(II) ion in the active site of M2-Q133SOD.

Bond distances (Å)	
Cd-Nδ1 His 46	2.49
Cd-Nε2 His 48	2.39
Cd-Nε2 His 120	2.27
Cd-Nε2 His 63	2.27
Cd-O Wat 10	2.39
Cd-O Wat 58	2.59
Bond angles (°)	
Nδ1 His 46-Cd-Nε2 His 48	103.90
Nδ1 His 46-Cd-Nε2 His 120	87.27
Nδ1 His 46-Cd-Nε2 His 63	86.03
Nδ1 His 46-Cd-O Wat 10	90.02
Nδ1 His 46-Cd-O Wat 58	165.90
Nε2 His 48-Cd-Nε2 His 120	91.78
Nε2 His 48-Cd-Nε2 His 63	100.31
Nε2 His 48-Cd-O Wat 10	165.58
Nε2 His 48-Cd-O Wat 58	90.17
Nε2 His 63-Cd-Nε2 His 120	167.28
Nε2 His 63-Cd-O Wat 10	84.15
Nε2 His 63-Cd-O Wat 58	102.70
Nε2 His 120-Cd-O Wat 10	85.06
Nε2 His 120-Cd-O Wat 58	93.35
O Wat 10-Cd-O Wat 58	76.01

enzyme, the Cu(I) being tricoordinated, in agreement with a large body of experimental evidence collected in solution for this form. In contrast to these results, the crystal structure of the reduced form of the enzyme showed for the two metal centers a coordination similar to the oxidized form.[2]

On the other hand, a trigonal coordination for the Cu(II) ion has been recently observed in the structure of a new form of CuZnSOD from yeast.[15] It appears therefore that the Cu-Nε2 bond with His 63 is rather weak and that packing effects can easily break it, giving the metal ion, independently of its oxidation state, a planar trigonal coordination.

TABLE V

Distances (Å) and angles (°) around the Cu(II) in the active site of M2-Q133SOD.

	M2-Q133SOD	Native SOD
Distances (Å)		
Cu-Nδ1 His 46	1.99	2.07
Cu-Nε2 His 48	2.05	2.06
Cu-Nε2 His 120	2.20	2.07
Cu-Nε2 His 63	3.31	2.21
Angles (°)		
Nδ1 His 46-Cu-Nε2 His 63	–	72.9
Nδ1 His 48-Cu-Nε2 His 63	–	100.5
Nδ1 His120-Cu-Nε2 His 63	–	151.2
Nδ1 His 46-Cu-Nε2 His 48	144.6	131.9
Nδ1 His 46-Cu-Nε2 His 120	103.2	85.6
Nε2 His 48-Cu-Nε2 His 120	104.2	108.1

Acknowledgements. – The work reported here is the result of the effort of many people: M. Ferraroni, S. Mangani, A. Scozzafava, M.S. Viezzoli, L. Banci, G. Vernaglione, C. Supuran (Chemistry Department, University of Florence), W. Rypniewski, K.S. Wilson (EMBL Outstation, Hamburg).

REFERENCES

1. F. Briganti, S. Mangani, P. Orioli, A. Scozzafava, G. Vernaglione, and C. T. Supuran, *Biochemistry* **36** (1997) 10384–10392.
2. W. R. Rypniewski, S. Mangani, B. Bruni, P. Orioli, M. Casati, and K.S. Wilson, *J. Mol. Biol.* **251** (1995) 282–288.
3. I. Bertini and C. Luchinat, *Acc. Chem. Res.* **16** (1983) 272–277.
4. D. N. Silverman and S. Lindskog, *Acc. Chem. Res.* **21** (1988) 30–36.
5. D. Hewett-Emmett and R. E. Tashian, *Mol. Philogenet. Evol.* **5** (1996) 50–77.
6. K. Hakansson, M. Carlsson, L. A. Svensson, and A. Liljas, *J. Mol. Biol.* **227** (1992) 1192–1204.
7. S. Lindskog and A. Liljas, *Roum. Chem. Q. Rev.* **2** (1994) 243–257.
8. S. K. Nair and D. W. Christianson, *J. Am. Chem. Soc.* **113** (1991) 9455–9458.
9. D. W. Christianson and C. A. Fierke, *Acc. Chem. Res.* **29** (1996) 331–339.
10. C. T. Supuran, *Carbonic Anhydrase Activators,* in I. Puscas (Ed.), *Carbonic Anhydrase and Modulation of Physiologic and Pathologic Processes in the Organism*, Helicon, Timisoara, 1994, pp. 119–146.
11. I. Fridovich, *Adv. Enzymol.* **41** (1974) 35–97.
12. J. A. Tainer, E. D. Getzoff, K. M. Beem, J. S. Richardson, and D. C. Richardson, *J. Mol. Biol.* **160** (1982) 181–217.

13. I. Bertini, M. Piccioli, M. S. Viezzoli, and G. T. Mullenbach, *Eur. J. Biophys.* **23** (1994) 167–176.
14. J. A. Tainer, E. D. Getzoff, J. S. Richardson, and D. C. Richardson, *Nature* **306** (1983) 284–287.
15. N. L. Ogihara, H. E. Parge, P. J. Hart, M. S. Weiss, J. J. Goto, B. R. Crane, J. Tsang, K. Slater, J. A. Roe, J. S. Valentine, D. Eisenberg, and J. A. Tainer, *Biochemistry* **35** (1996) 2316–2321.

SAŽETAK

Strukturno proučavanje međudjelovanja metalnih iona i bioloških makromolekula

Pierluigi Orioli

Strukturna istraživanja dvaju metaloenzima: ljudske karboanhidraze (CA) i Cu, Zn-superoksid-dismutaze (SOD), istaknula su bitnu ulogu metalnih iona u biologiji. Zadovoljavajuće objašnjenje mehanizma aktivacije CA pruža kristalna struktura kompleksa CA s histaminom. Relevantnu informaciju o mehanizmu katalitičke aktivnosti nativnog dimernog enzima i o smanjenoj aktivnosti monomera daje kristalna struktura visoke rezolucije (1.02 Å) za mutant: monomernu Cu,Zn-superoksid-dismutazu.