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X-ray diffraction studies on carboxypeptidase A complexes: the zinc stereochemistry

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CONTENTS

ABBREVIATIONS

BBP	5-amino-(*N*-*t*-butoxycarbonyl)-2-benzyl-4-oxo-6-phenyl-hexanoic acid
BFP	2-benzyl-3-formylpropanoic acid
BMBP	(–)2-benzyl-3-*p*-methoxybenzoylpropionic acid
BOP	5-benzamido-2-benzyl-4-oxopentanoic acid

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BZF	*N*-benzoyl-L-phenylalanine
BZS	L-benzylsuccinate
CPA	Carboxypeptidase A
Niprop	bis-(-*N*-isopropyl-salicylaldiminato)nickel(II)
PCI	potato inhibitor
Phe-P	S(+) (1-amino-2-phenyl-ethyl) phosphonic acid
Phe	phenylalanyl
TFP	2-benzoyl-4-oxo-5,5,5-trifluoropentanoic acid
Tyr	tyrosine
ZAA^{P}(O)F	O-[[(1R)-[[*N*-(phenylmethoxycarbonyl)-L-alanyl]amino]ethyl] hydroxyphosphinyl]-L-3-phenyllactate
ZFV^{P}(O)F	O-[[(1R)-[[*N*-(phenylmethoxycarbonyl)-L-phenylalanyl]amino]isobutyl]hydroxyphosphinyl]-L-3-phenyllactate
ZGP′	*N*-[[[(benzyloxycarbonyl)amino)]methyl]hydroxy-phosphinyl]-L-phenylalanine
ZAG^{P}(O)F	O-[[(1R)-[[*N*-(phenylmethoxycarbonyl)-L-alanyl]amino]methyl]hydroxyphosphinyl]-L-3-phenyllactate

Aminoacid abbreviations and numbering are as in ref. 13.

A. INTRODUCTION

(i) Early X-ray diffraction in Florence

In 1960, Holm and McKinney published a short paper in which the paramagnetism of Niprop was explained in terms of a polymeric structure, the nickel(II) ion being octahedrally coordinated [1]. Professor Sacconi was not convinced of this explanation because, he argued, the size of the isopropyl group would not allow the attainment of a polymeric structure. We then decided to determine the crystal structure of Niprop but, in common with other groups at that time in Italy, we could only perform two-dimensional electron density projections by hand calculation with the Beevers–Lipson strips. From the projections, it became apparent that the shortest Ni–Ni distance was around 7 Å, which ruled out the polymeric structure, but serious overlap of the electron density maxima did not allow the assignment of the nickel stereochemistry. At that time, Lingafelter in Seattle was already calculating beautiful three-dimensional electron density syntheses on an IBM 650 with programs written in collaboration with Brown and Jensen. We decided that it was time to go to the U.S. and to learn how to perform three-dimensional crystallography. To our great surprise we found that Fox, one of Lingafelter's students, had collected 3-D data on Niprop. It was then decided to determine the structure together and jointly publish the results. The paper appeared in 1963 [2] and was the first X-ray structure of a tetrahedral nickel(II) chelate complex.

Then came the exciting time of the first five-coordinate structures of divalent 3d metal complexes [3–4]. Five coordination, which seemed so unusual at that time, was later found to be quite common in biological systems such as metalloenzymes. By properly choosing the shape of a polydentate ligand, we were able to build around a metal atom tetrahedral, trigonal bipyramidal, square pyramidal and octahedral stereochemistries. The coordination polyhedra always showed large deviations from the regular geometries due to the steric constraints of the ligands and it was often impossible to assign them a specific geometry. Irregular stereochemistries became the rule, not the exception: we were on the road to the structures of metal complexes in biological systems.

(ii) A new perspective: the study of the mechanism of enzymatic catalysis by X-ray diffraction

In the last few years, a large amount of information about the mechanism of enzyme catalysis and enzyme inhibition has come from X-ray diffraction studies on single crystals of enzyme complexes with small ligands mimicking molecules involved in the catalysis process (substrates, transition state intermediates, products, etc.). One may wonder how it may be possible to get information about a dynamic process such as catalysis from a static object such as a crystal. However, by observing different static images representative of different points along the reaction coordinate, it is possible to work out the actual sequence of microevents that reflect the catalytic process. The images we need are those of the macromolecule bound to the type of ligands mentioned above. These studies are of great importance not only for the elucidation of the catalytic mechanism, but also for the design of enzyme inhibitors having pharmaceutical interest.

Among the enzymes whose catalytic activity has been studied by this technique, by far the most investigated is CPA. The structures of about 20 complexes of CPA with various ligands have been determined by X-ray diffraction and some excellent reviews have appeared recently [5,6]. In the present review we aim to investigate the stereochemistry of the zinc ion in these complexes and to underline the role of the metal in the catalysis mechanism and in the inhibition process. We shall start by describing briefly the technique, which may be of some interest to non-crystallographers and to crystallographers working on the structure of relatively small molecules.

B. THE TECHNIQUE

(i) Crystallization of the complex

The first step of the method consists in obtaining crystals of the protein complexed with the ligand of interest. There are basically two approaches for this

purpose. The first, and less used, approach is simply to mix the protein and the ligand in solution and to try to crystallize the complex in conditions which should not be too different from those used to grow crystals of the native protein. The crystals so obtained should be checked for isomorphism with the crystals of the native protein (identical unit cell dimensions and space group symmetry), a condition which greatly simplifies the method. Unfortunately, this technique of "co-crystallization" sometimes does not give good results. The interactions of the ligand with the chemical groups at the protein surface, often result in lack of crystallization or lack of isomorphism with the native crystals.

The technique most often used for obtaining crystals of the complexes takes advantage of the presence of large channels of solvent between the molecules in the crystals of proteins. It is well known that solvent composition in protein crystals may vary from 30 to 80% and that the solvent molecules, which are responsible for the low diffracting power of the crystals, surround the protein molecules, filling the space between them. Therefore, when a ligand of interest is added to the mother liquor of protein crystals, the ligand molecule can diffuse through the solvent channels and reach the binding site or sites. This technique of "soaking" may require from one day to several weeks for maximum occupation of all the binding sites in the crystal [7].

(ii) The Fourier difference

In the following discussion, it is assumed that the structure of the native protein is known and that its atomic coordinates are available, otherwise its structure should first be determined by using the methods described in the literature [8].

It is well known that the electron density at a point x, y, z in the unit cell of a crystal can be obtained by a Fourier transformation:

$$\rho(x, y, z) = V^{-1} \sum_h \sum_k \sum_l \mathrm{F}(hkl) \exp(-2\pi i(hx + ky + lz))$$

where the coefficients F(hkl) are complex numbers called structure factors. From the diffraction intensities, the moduli of the structure factors can easily be obtained but their phases, which are indispensable for calculating the Fourier summation, can only be approximately calculated by rather complex procedures [8]. Accurate phases, however, can easily be calculated if well-refined atomic coordinates in the unit cell are known.

The method most often used to determine the structure of complexes between proteins and ligands, makes use of a Fourier transformation where the coefficients are the difference between the structure factors measured for the crystal of the complex and those calculated from the atomic coordinates of the native protein. Phases for this synthesis are calculated from the known structure of the native protein, on the assumption of isomorphism with the structure of the complex. The

contribution of the ligand atoms is neglected at this stage. The resulting electron density map will show all the differences between the structures of the native and of the complexed protein, i.e. not only the ligand bound to the protein, but also any part of the protein backbone or amino acid residues which have altered their position during the binding process. Figure 1 shows the active site region of the refined model of the CPA–BZS complex, where the electron density from a difference map has been superimposed.

If very accurate, high-resolution (2.0 Å or less) native protein phases can be calculated from well-refined structures and if the binding process does not dramatically alter the native structure, the ligand will readily be visualized in the electron density map. A model of the ligand can be fitted in the electron density map using a computer-automated graphics procedure [9] and its atomic coordinates can be derived.

(iii) Refinement process

The last step of the method is the improvement of the atomic coordinates of the complex. This is usually performed by a non-linear least squares procedure, where the differences between observed and calculated structure factors are minimized.

Automatic least squares refinement of atomic parameters is routinely employed in crystal structure analysis, but for protein structures the number of parameters to be refined is exceedingly high compared with the amount of data available (observed structure factors). Therefore most computer programs dealing with protein structure refinement increase the number of observational equations by introducing stereochemical and energy restraints [10,11].

After several refinement cycles, each followed by inspection and rebuilding of

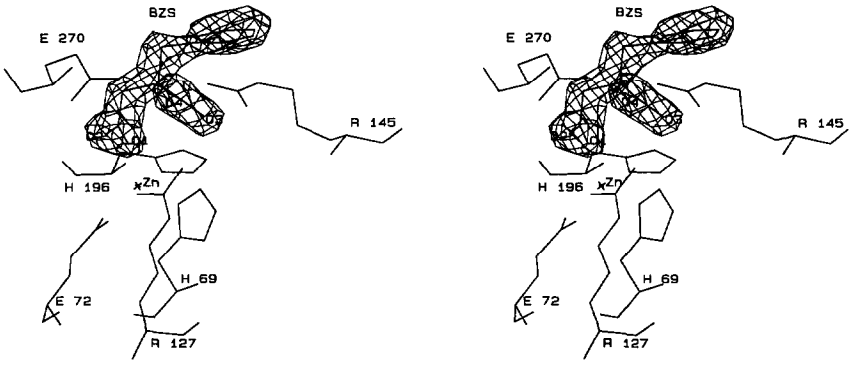


Fig. 1. Stereoview of the ΔF map of the CPA–BZS complex, calculated with coefficients and phases from the refined model. The coordinates of BZS where omitted from the calculation. The positive electron density is contoured at the level 4.5 σ.

the model, convergence can be attained to a structural model with estimated errors in the atomic coordinates of 0.1–0.2 Å*. Calculations of bond lengths and angles involving the ligand and the residues in the binding site can then be performed, so that the spatial relationships between the ligand and the protein can be successfully visualized.

C. ZINC COORDINATION CHEMISTRY

Divalent zinc, together with cadmium(II) and mercury(II), has a d^{10} electronic configuration and shows a large variety of coordination polyhedra in its complexes. The most common coordination numbers are four, five and six, with tetrahedral, trigonal bipyramidal (or square pyramidal) and octahedral stereochemistries, respectively [12]. The zinc coordination sphere has proven exceptionally flexible and easily adaptable to the demands of the ligands. In fact, in the absence of crystal field stabilization effects, the stereochemistry of the ligands around the metal ion is determined essentially by size, electrostatic forces and covalent bonding forces. Zinc coordination polyhedra therefore usually show large deviations from regular geometries. This has made the zinc ion particularly adaptable to the enzyme active sites, where the steric requirements of the protein secondary structure do not allow great mobility to the amino acids binding to the metal. In fact, among the first row transition metals, zinc is second only to iron in terms of abundance in biological systems, in spite of the fact that, due to the stability of its divalent oxidation state, zinc is never involved in oxido-reduction processes.

Other physico-chemical properties of zinc present important advantages in biology. Zinc is amphoteric and exists in both metal hydrate and hydroxide forms at pH values near neutrality. Furthermore, zinc can bind nitrogen, oxygen and sulphur donor atoms equally well, as shown by its numerous complexes with histidine, carboxylate and cysteine ligands in proteins [6].

D. ZINC STEREOCHEMISTRY IN CARBOXYPEPTIDASE A

(i) The structure of the active site

CPA is a metalloexopeptidase having a molecular weight of about 35 kD and containing one zinc ion per molecule. Its biological function is the hydrolysis of C-terminal amino acids from polypeptide substrates, with preference for those substrates which have large hydrophobic C-terminal side chains. The structure of the native enzyme has been determined by X-ray diffraction to a resolution of 1.54 Å [13]. In the active site, the zinc atom is coordinated by Oε1 and Oε2 of Glu72, Nδ1 of His69, Nδ1 of His196 and one water molecule (Wat571) (Fig. 2). Table 1 reports

* *R* factors, 0.14–0.19, are substantially larger than for smaller molecules.

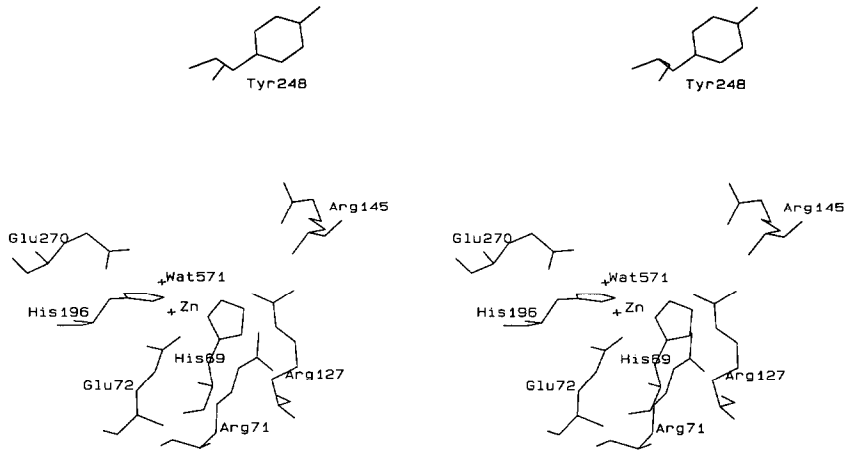


Fig. 2. Stereoview of the native CPA active site cavity. Coordinates are from ref. 13.

TABLE 1

Distances and angles of zinc coordination in native CPA assuming a trigonal bipyramidal (1) and a tetrahedral coordination (2)[a,b]

Distances (Å)			
Zn–Oε1(Glu72)	2.3		
Zn–Oε2(Glu72)	2.2		
Zn–Nδ1(His69)	2.1		
Zn–Nδ1(His196)	2.1		
Zn–O(Wat571)	2.0		
Angles (deg)			
Nδ1(His69)–Zn–O(Wat571)	122		
Nδ1(His69)–Zn–Nδ1(His196)	99		
Nδ1(His196)–Zn–O(Wat571)	96		
(1)		(2)	
Nδ1(His69)–Zn–Oε1(Glu72)	91	Nδ1(His69)–Zn–Oε(Glu72)	107
Nδ1(His69)–Zn–Oε2(Glu72)	121	Nδ1(His196)–Zn–Oε(Glu72)	129
Nδ1(His196)–Zn–Oε1(Glu72)	156	O(Wat571)–Zn–Oε(Glu72)	113
Nδ1(His196)–Zn–Oε2(Glu72)	101		
O(Wat571)–Zn–Oε1(Glu72)	98		
O(Wat571)–Zn–Oε2(Glu72)	111		
Oε1(Glu72)–Zn–Oε2(Glu72)	56		

[a] R.m.s. errors on atomic positions are between 0.15 and 0.20 Å.
[b] Oε is a dummy atom positioned halfway between Oε1 and Oε2.

distances and angles around the zinc atom. Inspection of the angular values shows that the zinc coordination polyhedron can be described as a distorted trigonal bipyramid. The deviations from the least squares plane through the equatorial atoms are: Zn = −0.23, Nδ1(69) = +0.08, O(Wat571) = +0.08, Oε2(72) = +0.07 (Å). Alternatively, by combining the two carboxylate oxygens of Glu72 into a hypothetical atom Oε, situated midway between the two atoms, the coordination polyhedron around zinc can be considered to be a distorted tetrahedron (Table 1).

Other residues in the active site which should be mentioned because they play an important role in the catalytic mechanism are Arg127, Arg145, Tyr248 and Glu270 (Fig. 2).

(ii) The role of zinc in the catalytic mechanism

The hydrolytic mechanism of CPA has been the subject of intense debate and presently two basic pathways are considered: (a) an anhydride pathway [5,14–16], the carbon atom of the scissile peptide bond being attacked by the carboxylate of Glu270 (Fig. 3(a)) or (b) a promoted water pathway [17–19], involving the attack of a water molecule promoted by zinc and assisted by Glu270 (Fig. 3(b)). Both pathways have recently been discussed and it has been shown that results from X-ray crystallographic studies of CPA–ligand complexes favour the promoted water mechanism [5,6]. However, possible evidence supporting the acyl pathway for some substrates, e.g. cinnamoyl esters, has also been considered [5].

In each pathway, the zinc ion seems to play a twofold role: (a) the formation of a precatalytic interaction with the carbonyl group, which would polarize the peptide bond and make it more susceptible to nucleophilic attack and (b) the stabilization of a proteolytic tetrahedral intermediate by covalently binding the diol oxygen in the transition state (Fig. 3). In addition, zinc promotes the nucleophilicity of the water molecule by lowering its pK_a to 7 [20]. Further enhancement of the water nucleophilic character is caused by a hydrogen bond with Glu270. However, the precatalytic role has been questioned by several authors who have shown that much evidence favours an outer sphere mechanism involving Arg127 for the interaction with the carbonyl bond [5].

E. ZINC STEREOCHEMISTRY IN CARBOXYPEPTIDASE A COMPLEXES

Examination of the structures of CPA complexes determined by X-ray diffraction shows that zinc stereochemistry is not largely affected by the binding of small molecules. In most cases, the water molecule bound to zinc is simply replaced by the donor atom(s) of the incoming ligand. The positions of the nitrogen donors of His69 and His196 and the oxygens of Glu72 are essentially unaltered (Fig. 4). A slight tendency of Glu72 to change its coordination to zinc from bidentate to unidentate upon binding of new ligands, can be noted. However, this is also achieved by the

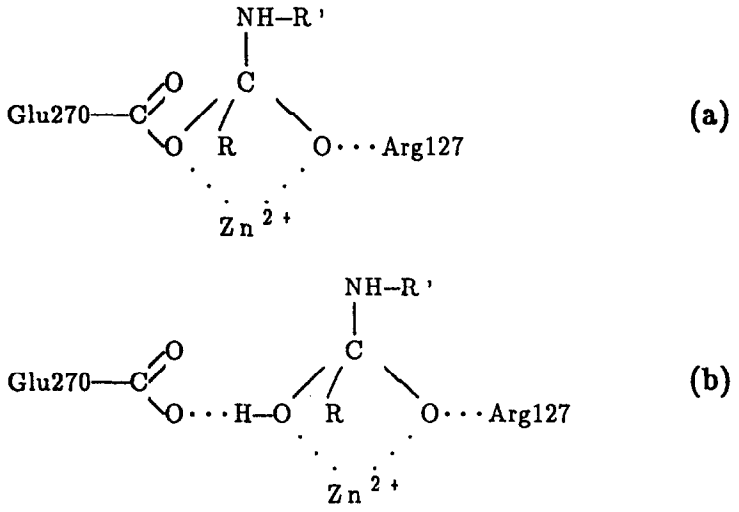


Fig. 3. Sketch showing the reaction intermediates of the two proposed pathways of CPA hydrolytic mechanism: (a) anhydride pathway; (b) promoted water pathway.

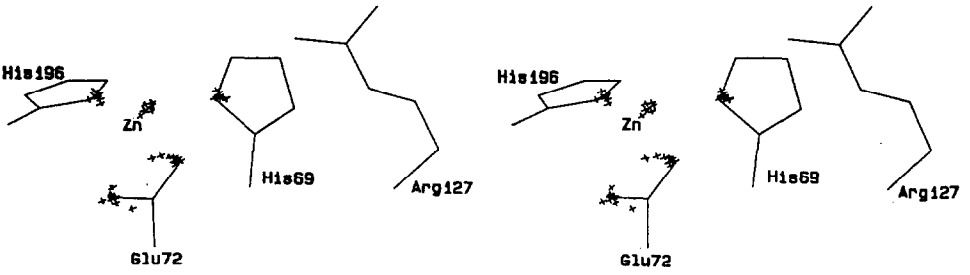


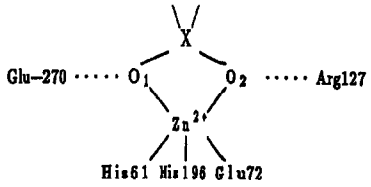
Fig. 4. Stereoview of the zinc coordination in native CPA active site with superimposed the scatter plot of His69 Nδ1, His196 Nδ1, Glu72 Oε1 and Oε2, from 10 structures of CPA complexes. Notice the random distribution of Nδ1 position in contrast to the systematic trend displayed by the zinc and Glu72 oxygens atoms.

movement of the zinc ion. The rigidity of the ligand positions around zinc must be mainly attributed to the strong β-pleated sheet fold of the CPA secondary structure, which greatly controls the stereochemistry around the metal.

Inspection of Table 2 shows that the incoming ligand always binds the zinc atom in an asymmetric fashion, which can be described in most cases in terms of a slight clockwise rotation of the carbon (or phosphorus) tetrahedron viewed as in the sketch of Table 2. This rotation, which brings O2 closer to the zinc atom, favours the formation of a strong hydrogen bond between O1 and Glu270. In a few cases, the rotation of the tetrahedron is anti clockwise, with consequent tightening of the O2···Arg127 hydrogen bond (ZGP' (hydr.), ZGP', Phe-P and PCI (hydr.)). However, in these cases, the ligands bind anomalously in the active site cavity. In fact these

TABLE 2

Ligand–zinc interactions in CPA complexes
Distances are in Å[a]



Ligand	Glu270···O_1	Zn–O_1	Zn–O_2	O_2···Arg127	K_i (m)	Ref.
Carbonyl hydrates						
BFP	2.4	2.7	2.5	3.6	4.8×10^{-10}	29
TFP	2.6	3.4	2.6	3.2	2.0×10^{-7}	30
BOP	2.6	2.9	2.5	3.2	4.8×10^{-5}	31
BBP	2.6	2.6	2.1	3.3	6.7×10^{-7}	32
Phosphates						
ZGP'(Hydr.)	3.4	2.2	3.3	2.7	—	21
ZGP'	2.6	2.4	3.5	2.6	8.0×10^{-8}	22
Phe-P	3.4	2.2	3.3	2.7	3.0×10^{-5}	23
$ZAG^{P}(O)F$	2.4	3.2	1.9	3.0	7.1×10^{-10}	33
$ZAA^{P}(O)F$	2.2	3.1	2.2	2.8	3.0×10^{-12}	34
$ZFV^{P}(O)F$	2.4	3.0	2.2	2.8	1.1×10^{-14}	33
Carboxylates						
PCI (Hydr.)	4.5	1.8	3.2	4.3	5.0×10^{-9}	24
BZF (Hydr.)	2.5	2.7	2.2	3.3	8.8×10^{-5}	35
BZS	2.5	2.7	2.2	3.3	$0.4–1.1\times10^{-6}$	36

[a]The upper level of r.m.s. errors on atomic positions is 0.2 Å.

ligands, or portions of them, occupy the S_1 hydrophobic cleft which is usually reserved for the P1 aromatic side chains of substrates [21–24].

This shows clearly that the stereochemistry around a metal ion in enzymes is also conditioned by the interaction between the ligands and the active site cavity. Considering the asymmetric binding of the exogenous ligand and the tendency of Glu72 to assume a monodentate coordination, it appears that in CPA complexes, although six ligands are within 3.5 Å from the metal, the zinc atom tends to be four-coordinated with a distorted tetrahedral stereochemistry.

In a few cases, the incoming molecule does not bind to zinc, although it binds in the active site. These include the amino acids D-phenylalanine and D-tyrosine whose carboxylate group salt links with Arg145 and whose α-amino group salt links with Glu270. Breaking of the Glu270 hydrogen bond with the native zinc-bound water molecule allows a significant shift (~1.0 Å) of the water molecule with a slight

increase of the $Zn-H_2O$ distance to 2.3 Å [25]. This is in agreement with the reported facile replacement of the water molecule by anions [26].

The ketonic substrate analogue BMBP also does not bind to zinc and lends strong support for an outer sphere mechanism of the peptide bond polarization [27]. In fact the carbonyl oxygen corresponding to the scissile carbonyl of an actual substrate forms a bifurcated hydrogen bond (2.9 Å to each terminal nitrogen) with Arg127.

All the other small ligands forming complexes with CPA whose structure have been determined by X-ray analysis, bind to the metal. According to the nature of their donor groups, they can be grouped into three main classes: (a) carbonyl ligands, (b) phosphate ligands, and (c) carboxylate ligands. We shall now discuss their characteristics with the focus on zinc stereochemistry, rather than on the functional groups. Ternary complexes will also be discussed briefly.

(i) Carbonyl ligands

The pseudo-substrate glycyl-L-tyrosine replaces the water molecule and chelates to the zinc ion with the peptide carbonyl oxygen and the amino terminus (as the free base). A slight movement of 0.3 Å toward Arg127 of the zinc ion does not essentially alter the stereochemistry of His69, His196 and Glu72 bound to the metal [28]. However, this binding mode is not accessible for actual substrates, so the direct zinc–carbonyl interaction is probably a consequence of the chelate effect. The other carbonyl-containing ligands, whose complexes with CPA have been investigated by X-ray diffraction, bind as covalent hydrate adducts (Table 2). The chemistry of hydration has probably been performed by the enzyme with the formation of gem-diolate, analogous to the proteolytic tetrahedral intermediate. The two gem-diol oxygen atoms replace the water molecule and bind generally in asymmetric fashion to the zinc ion as mentioned above (Table 2). In addition, the zinc ion moves 0.2–0.3 Å toward Arg127 to accommodate the hydrate inhibitor. In spite of these alterations, the overall coordination polyhedron around the zinc ion changes relatively little. These complexes constitute the best steric and electronic analogues to the actual proteolytic tetrahedral intermediate.

(ii) Phosphate ligands

Derivatives containing the phosphinyl (PO_2^-) group (phosphonates, phosphonamidates, etc.) are among the strongest CPA binding inhibitors as shown by their inhibition constants (Table 2). They also bind to the zinc ion in asymmetric fashion by replacing the water molecule, with the two oxygen atoms involved in hydrogen bonds with Glu270 and Arg127. Again, the stereochemistry around Zn, as far as the rest of the ligands are concerned (His69, His196 and Glu72), is essentially unaltered.

Although the phosphinyl–zinc interaction gives only a partial, albeit important,

contribution to the overall stability of these complexes, it may be interesting to notice that the lowest K_i of ZFV^P(O)F corresponds to the smallest difference (0.8 Å) in the Zn–O distances (Table 2). The more symmetrical phosphinyl–zinc coordination in the CPA–ZFV^P(O)F complex certainly contributes to the greater stability of that complex [33].

Phosphate derivatives have been shown to be good transition state analogue of the CPA-catalyzed reaction and their extremely tight-binding nature is certainly connected with it [33]. The analogy between their mode of binding and that of gem-diolates therefore gives support to the general-base mechanism for CPA. These inhibitors are, in fact, unable to mimic the covalent intermediate of the acyl mechanism.

(iii) Carboxylate ligands

Only three structures of carboxylate ligands bound to zinc in CPA are reported in the literature. Two of them are observed as the result of the hydrolysis of CPA inhibitors, namely the structure of the complex between CPA and the 39-amino acid inhibitor PCI [24] and the structure of the cleaved substrate BZF [35]. The third example is given by the complex with the dicarboxylate inhibitor BZS [36]. In the first two cases, the carboxylate group is formed by the enzymatic cleavage of a peptide bond and the structures represent an enzyme–substrate–product complex. The third case represents an enzyme ground-state inhibitor complex. In these structures, the carboxylate group replaces the water molecule present in the native structure (Wat571) interacting with zinc either as essentially monodentate (PCI) or as bidentatate asymmetric ligand (BZF, BZS). In all three structures the carboxylate is observed to bind the metal in the syn stereochemistry. The preference of Lewis acids to interact with carboxylates adopting syn stereochemistry has recently been reviewed [2]. In this review, it was observed that the zinc ion markedly prefers to be in the plane of the carboxylate ligand, as expected for an optimal interaction with the sp^2 lone pair of the oxygen donor [2]. However, the three CPA complexes show an interesting deviation from this behaviour. In fact, the zinc ion always lies in the Glu72 carboxylate plane (as in every other CPA structure) while it is markedly out of the plane of the exogenous carboxylate group. The deviations are reported in Table 3.

In the PCI–CPA complex, Tyr248 donates a hydrogen bond to the non-coordinated oxygen atom of the inhibitor Val38 carboxylate group while in the cases of BZF and BZS complexes, it is the carboxylate group of Glu270 which is strongly hydrogen bonded to the metal-coordinated oxygen atom. For the BZS–CPA complex, there is experimental evidence that Glu270 carboxylate is the hydrogen bonding donor [36,37]. This interaction strongly polarizes the BZS C–O bond, enhancing the Lewis base properties of this carboxylate group.

TABLE 3
Deviations (Å) of zinc atom position from the carboxylate planes of Glu72 and of the exogenous carboxylate ligands[a]

Ligand	Glu72	Ex. ligand
PCI	0.4	0.9
BZF	0.2	1.0
BZS	0.1	1.0

[a]The upper level of r.m.s. errors on atomic positions is 0.2 Å.

(iv) Ternary complexes

The already discussed CPA–BZF and CPA–PCI complexes can be classified as ternary complexes, but here we want to draw attention to the CPA–L-phenylalanine–azide complex whose structure has recently been reported [38]. This particular ternary complex introduces discussion of an important aspect of CPA chemistry, namely the interaction of CPA with inorganic anions.

It has been observed that the zinc-bound water molecule (Wat571) can be replaced by anions only by lowering the pH [39] or by chemical modification of Glu270 carboxylic group [40]. This observation suggests that the Wat571 coordination site becomes available to anionic ligands only if the hydrogen bond occurring between Wat571 and Glu270 is broken. Further spectroscopic and kinetic studies [26,41–43] on cobalt-substituted CPA showed that the binding of the products of the ester and peptide hydrolysis to the enzyme open that coordination site to anions, and that a strong synergistic effect exists in the binding of those two classes of protein ligands. Two structural studies were prompted by the above observations. The first one, the complex of CPA with the D-phenylalanine and D-tyrosine inhibitors [25], provides a structural basis for the mechanism of Wat571 substitution by anions. Such amino acid inhibitors do not bind to zinc, but in their interaction with the active site cavity they donate a strong hydrogen bond to the carboxylate group of Glu270. As a consequence, Wat571 is no longer hydrogen bonded to Glu270 and this loss of stabilization allows its substitution by other competing anionic ligands.

The second structure reports the ternary complex of CPA with L-phe and azide and gives a possible explanation of the observed synergistic effect. Two azide molecules are found bound in the active site cavity of the enzyme, one interacting electrostatically with Arg145 and the second replacing Wat571 in the zinc coordination sphere. Arg145 is proposed as the high affinity azide binding site observed in kinetic studies [26]. Figure 5 reports the structure of the ternary complex compared with the native CPA. The binding of azide to Arg145 causes the plane of the Arg145 guanidinium group to rotate in a position perpendicular to its orientation in the native enzyme and thus the whole side chain extends towards the interior of the active site cavity. This new conformation of Arg145 favours the binding of the

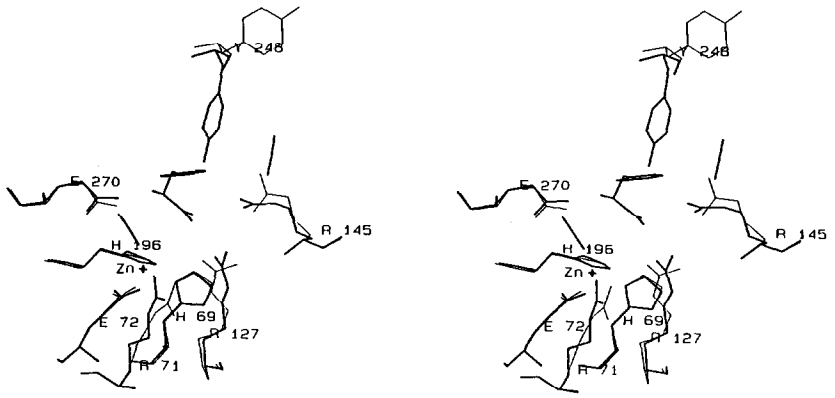


Fig. 5. Stereoview of the structure of the ternary complex CPA–L–phe–azide (thick bonds) superimposed on the structure of native CPA.

L-phenylalanine molecule, allowing its simultaneous interaction with Arg145 and Glu270. The latter hydrogen bond causes the breaking of the Glu270···Wat571 hydrogen bond, allowing replacement of the water by the second azide molecule.

It is interesting to notice also that, in other zinc enzymes such as carbonic anhydrase [44] and thermolysin [45], the zinc-coordinated water essential for catalysis is always found hydrogen bonded to a nearby residue. This hydrogen bond surely enhances the nucleophilicity of the water and also perhaps prevents enzyme poisoning by potentially coordinating anions abundant in the cellular medium, such as chloride, phosphate or sulphate.

F. CONCLUDING REMARKS

It should now be evident that the stereochemistry around a metal atom in a metalloenzyme is conditioned by a number of interactions which are absent in isolated complexes. Several examples have been discussed in the present review:

(a) the positions of protein residues bound to the metal can be greatly controlled by a rigid folding of the enzyme secondary structure (Fig. 4);

(b) the interactions between the hydrophobic part of an incoming ligand and the active site cavity can strongly affect its way of binding to the metal (Table 2);

(c) the formation of strong hydrogen bonds between the ligand and the protein residues in the vicinity of the metal can assist the coordination bond. As shown by Table 2, almost all exogenous ligands are at hydrogen bond distances from Glu270 and Arg127. A similar hydrogen bond network is also observed in thermolysin [45]; and

(d) in the carboxylate CPA complexes studied by X-ray diffraction the zinc ion is not coplanar with the incoming carboxylate group, which is in contrast with the trend observed for carboxylate ligands in small complexes [6] and for Glu72 (Table 3).

These results show that, in metalloenzymes, other important forces besides metal coordination act on the ligands and compete with the metal, contributing heavily to the overall stability of the complexes. Great caution should therefore be used in interpreting data from model complexes which are unable to take into account all these interactions.

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REFERENCES

1 R.H. Holm and T.M. McKinney, J. Am. Chem. Soc., 82 (1960) 5507.
2 M.R. Fox, E.C. Lingafelter, P.L. Orioli and L. Sacconi, Nature, 197 (1963) 1104.
3 L. Sacconi, Pure Appl. Chem., 17 (1968) 95.
4 P.L. Orioli, Coord. Chem. Rev., 6 (1971) 285.
5 D.W. Christianson and W.N. Lipscomb, Acc. Chem. Res., 22 (1989) 62.
6 D.W. Christianson, Adv. Protein Chem., 42 (1991) 281.
7 A. McPherson, Cryst. Rev., 1 (1987) 191.
8 L. Johnson and T. Blundell, Protein Crystallography, Academic Press, New York, 1976.
9 T.A. Jones, in D. Sayre (Ed.), Computational Crystallography, Clarendon Press, Oxford, 1982, p. 30.
10 W.A. Hendrickson and J.H. Konnert, Acta Crystallogr. Sect. A, 36 (1980) 344.
11 A.T. Brunger, X-PLOR Manual, Yale University, New Haven, CT, 1990.
12 F.A. Cotton and G. Wilkinson, Advanced Inorganic Chemistry, 4th edn., Wiley, New York, 1980.
13 D.C. Rees, M. Lewis and W.N. Lipscomb, J. Mol. Biol., 168 (1983) 367.
14 M.W. Makinen, L.C. Kuo, J. Dynosky and J.J. Jaffer, J. Biol. Chem., 254 (1979) 356.
15 S.J. Hoffman, S.S. Chu, H. Lee, E.T. Kaiser and P.R. Caley, J. Am. Chem. Soc., 105 (1983) 6971.
16 J. Suh, W. Cho and S. Chung, J. Am. Chem. Soc., 107 (1985) 4530.
17 D.L. Warnick and R. Breslow, J. Am. Chem. Soc., 98 (1976) 259.
18 B.L. Vallee, D.S. Auld and A. Galdes, Biochemistry, 25 (1986) 646.
19 W.N. Lipscomb and H. Kim, Biochemistry, 19 (1990) 5546.
20 J.J. Groves and J.R. Olson, Inorg. Chem., 24 (1985) 2715.
21 D.W. Christianson and W.N. Lipscomb, J. Am. Chem. Soc., 108 (1986) 545.
22 D.W. Christianson and W.N. Lipscomb, J. Am. Chem. Soc., 110 (1988) 5560.
23 S. Mangani, P. Carloni and P. Orioli, Eur. J. Biochem. 203 (1992) 173.
24 D.C. Rees and W.N. Lipscomb, J. Mol. Biol., 160 (1982) 475.
25 D.W. Christianson, S. Mangani, G. Shoham and W.N. Lipscomb, J. Biol. Chem., 264 (1989) 12849.
26 R. Bicknell, A. Shöffer, I. Bertini, C. Luchinat, B.L. Valle and D.S. Auld, Biochemistry, 27 (1988) 1050.
27 D.W. Christianson, L.C. Kuo and W.N. Lipscomb, J. Am. Chem. Soc., 107 (1985) 8281.
28 D.W. Christianson and W.N. Lipscomb, Proc. Natl. Acad. Sci. U.S.A., 83 (1986) 7568.
29 D.W. Christianson and W.N. Lipscomb, Proc. Natl. Acad. Sci. U.S.A., 82 (1985) 6840.

30 D.W. Christianson and W.N. Lipscomb, J. Am. Chem. Soc., 108 (1986) 4998.
31 D.W. Christianson, P.R. David and W.N. Lipscomb, Proc. Natl. Acad. Sci. U.S.A., 84 (1987) 1512.
32 G. Shoham, D.W. Christianson and D.A. Oren, Proc. Natl. Acad. Sci. U.S.A., 85 (1988) 683.
33 H. Kim and W.N. Lipscomb, Biochemistry, 30 (1991) 8171.
34 H. Kim and W.N. Lipscomb, Biochemistry, 29 (1990) 5546.35.
35 D.W. Christianson and W.N. Lipscomb, J. Am. Chem. Soc. 109 (1987) 5536.
36 S. Mangani, P.L. Orioli and P. Carloni, J. Mol. Biol., 223 (1992) 573.
37 A.R. Palmer, P.D. Ellis and R. Wolfenden, Biochemistry, 21 (1982) 5056.
38 S. Mangani and P.L. Orioli, Inorg. Chem., 31 (1992) 365.
39 R.S. Stephens, J.E. Jentoft and R.G. Bryant, J. Am. Chem. Soc., 96 (1974) 8041.
40 K.F. Geoghegan, B. Holmquist, C.A. Spilburg and B.L. Vallee, Biochemistry, 22 (1983) 1847.
41 I. Bertini, G. Lanini, C. Luchinat and R. Monnanni, Inorg. Chim. Acta, 107 (1985) 153.
42 I. Bertini, C. Luchinat, L. Messori, R. Monnanni, D.S. Auld and J.F. Riordan, Biochemistry, 27 (1988) 8318.
43 I. Bertini, R. Monnanni, G.C. Pellacani, M. Sola, B.L. Vallee and D.S. Auld, J. Inorg. Biochem. 32 (1988) 8318.
44 S.J. Dogson, R.E. Tashion, G. Geos and N.D. Carter, The Carbonic Anhydrase, Plenum, New York, 1991.
45 B.W. Matthews, Acc. Chem. Res., 21 (1988) 333.