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Carbonic Anhydrase: An Example of How the Cavity Governs the Reactivity at the Zinc Ion

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Carbonic Anhydrase: An Example of How the Cavity Governs the Reactivity at the Zinc Ion

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The recent availability of X-ray structures and new biophysical measurements have shed further light on the detailed mechanism of carbonic anhydrase and its inhibition. It is noted that in some instances the structural information obtained through X-ray analysis of single crystals and NMR measurements in solution disagree. We take these conflicting results as possible conformations close in energies, both of which can be used to design the enzymatic pathway.

Key Words: *carbonic anhydrase, CO_2 hydration, carbonate dehydration, zinc enzyme, enzyme inhibition*

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INTRODUCTION

The enzyme carbonic anhydrase (CA, E.C. 4.2.1.1) has been one of the most studied zinc enzymes,[1] and it appeared to be a typical case history in bioinorganic chemistry.[2] Still, recent crystallographic and NMR investigations succeeded in providing new insight into the reaction mechanism, and led us to appreciate even more deeply than before the design and role of the enzyme active site cavity. The catalyzed reactions is:

$$CO_2 + H_2O \rightleftarrows HCO_3^- + H^+ \qquad (1)$$

The enzyme requires zinc for activity and is a very efficient catalyst for the above reaction as indicated by the k_{cat} of 1.4×10^{-6} s^{-1} at 298 K for the isoenzyme II.[3]

The wealth of information coming from crystallographic studies of the enzyme and its complexes with several inhibitors and its implications on the mechanism have been recently reviewed.[4] Here we will first describe the proposed mechanism and will use data provided by X-ray crystallography and biophysical measurements to justify the structural features of the various reaction intermediates. The catalyzed reaction is very simple, but its pathway is unpredictable indeed!

THE pH DEPENDENT PROPERTIES OF THE ENZYME

The active site of the enzyme as it appears from the high resolution (1.54 Å) crystal structure of human CA II[5] is shown in Fig. 1. Zinc is coordinated to three histidines and sits at the bottom of a cavity exposed to the solvent. This crystal structure, obtained at pH 7.8,[5] shows that a solvent molecule is bound to the zinc. This ligand is termed Wat263. The enzyme in solution gives rise to the following acid–base equilibrium:

$$\text{—Zn—}OH_2 \rightleftarrows \text{—Zn—}OH^- + H^+ \qquad (2)$$

with a pK_a of about 5.5–8 depending on the isoenzyme. Therefore, the solvent donor observed in the X-ray structure at pH 7.8 is probably

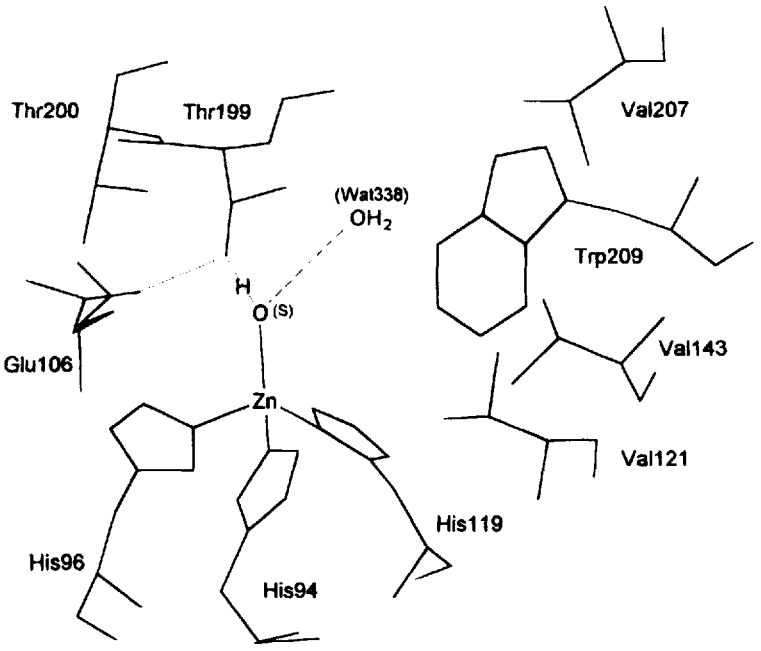

FIGURE 1 The active site of carbonic anhydrase as it appears in the crystal structure of the human II isoenzyme (5).

an OH^- ion. The estimated pK_a values mostly come from the activity profiles.[3] Estimates are also provided by the cobalt substituted enzyme through the pH dependence of its electronic absorption spectra.[6] When performing these measurements care must be taken not to have anions in solution, since they bind the metal ion of the enzyme acidic form and grossly alter the pK_a estimates.[7] Buffers can be used as long as the anionic species is bulky enough not to enter the active cavity. The metal coordinated OH^- group donates a hydrogen bond to the Oγ atom of Thr199, which in turn acts as a donor towards the deprotonated side chain of Glu106. The OH^- is involved in two further H-bonds with Wat318 and Wat338 (the latter called "deep water" and buried in the cavity, Fig. 1), completing a tetrahedron of hydrogen bonds staggered with respect to the one formed by the zinc ligands.

It is believed that the Zn–O–H···Thr199 network keeps the coordinated OH^- properly oriented for the nucleophylic attack on the CO_2 substrate.[8] In the acidic form water is probably the species bound to zinc. There have been suggestions that a second water molecule binds cobalt in Co(II)CA to some extent in certain isoenzymes.[9] Electronic spectra and electron relaxation times seem to indicate the presence of five coordinated species. Monte Carlo calculations on the native enzyme reach the same conclusion.[10] This suggestion, even if correct, is probably not meaningful for the mechanism, but it fits with the general scheme that a fifth binding site is available when the enzyme is in the acidic form. The negative OH^- group presumably binds tighter than H_2O and induces definite tetracoordination around zinc.

The pK_a of the equilibrium (2) depends critically on several factors: the actual charge on the zinc ion,[11] the presence of positive groups (His^+, Lys^+, Arg^+) in the cavity, the metal coordination number, and the hydrophobicity of part of the cavity which would favor a neutral zinc-donor ligand moiety.

CO_2 IN THE ACTIVE CAVITY

The role of the enzyme in attracting, and possibly activating, CO_2 has been discussed at length. It is reasonable to conceive that the apolar CO_2 molecule is attracted in the hydrophobic region of the active cavity. Such a region (in the human II isoenzyme) is determined by Val121, Leu141, Val143, Leu198, Val207, Trp209. ^{13}C NMR studies have been performed on $^{13}CO_2$ and $H^{13}CO_3^-$ in the presence of copper substituted CA.[12,13] Such a derivative is inactive and therefore suitable for monitoring the two substrate species separately. Even though HCO_3^- is bound to copper, and therefore may reduce the free volume available to CO_2, it seems that the concentration of CO_2 in the cavity is larger than in solution.[13] Molecular dynamics calculations indicate that two CO_2 molecules can be hosted in the cavity of the native enzyme.[14,15]

The recently determined crystal structures of two complexes of HCAII with the anionic inhibitors CN^- and NCO^- [16] provide further insight into the location of the CO_2 binding site. Indeed, the study of the binding of the NCO^- anion, which is isoelectronic and iso-

structural with CO_2, may provide pertinent information about the substrate binding site. Furthermore, being the cyanate a linear molecule with delocalized negative charges at its ends, it may resemble a transition state where the CO_2 molecule is polarized by the interaction with the cavity.[5]

In both the CN^- and NCO^- crystal structures, electron density, which can be interpreted as due to the anion, is found between the hydrophilic and the hydrophobic parts of the cavity.[16] In the resulting model the anion is not coordinated to the metal but replaces Wat338 and is located between 3.1 and 3.4 Å from the zinc ion and 2.2–2.3 Å from the zinc bound Wat263 molecule (Fig. 2A). The metal ion maintains its tetrahedral coordination geometry, apparently consistent with the conclusion from electronic spectroscopy.[6] Interestingly, ^{13}C and ^{15}N NMR spectroscopic measurements on the same adducts of Co and ^{67}Zn substituted HCAII have shown that the strong ligands CN^- and NCO^- are able to replace Wat263 in zinc coordination in solution (Fig. 2B).[17] Indeed ^{13}C and ^{15}N NMR spectroscopy probed the direct binding of NCO^- to the paramagnetic Co(II) ion. Under fast exchange conditions, a nucleus in the vicinity of the paramagnetic center senses the presence of the unpaired electrons, which provide efficient nuclear relaxation pathways and therefore shorten the nuclear T_1 and T_2 relaxation times. Measurements of the longitudinal relaxation time of the ^{13}C nucleus of the NCO^- anion bound to bovine CAII have ascertained the direct binding of the anion to the cobalt(II) ion. In the case of CN^-, the ligand exchange is slow and no ^{13}C signal can be observed. However, it is possible to detect such a signal in the case of the diamagnetic Zn derivative. In an experiment ^{67}Zn was used.[17] This isotope has $I = 5/2$ and a large quadrupolar moment, which is responsible for an additional contribution to the relaxation rates of the ^{13}C nucleus. The detection of a ^{13}C relaxation rate enhancement mediated by scalar ^{67}Zn–^{13}C coupling demonstrated the direct binding of the cyanide anion to zinc in CA in solution.

The above results of X-ray and NMR experiments raise a question about the possible origin of the observed difference between solution and solid-state behavior of the enzyme and about its possible mechanistic significance. The mechanism of action of enzymes needs many energetically close steps, and X-ray crystallography may have trapped either one of the several conformations in equilibrium in solution, or a conformation different from the solution structure. This can be

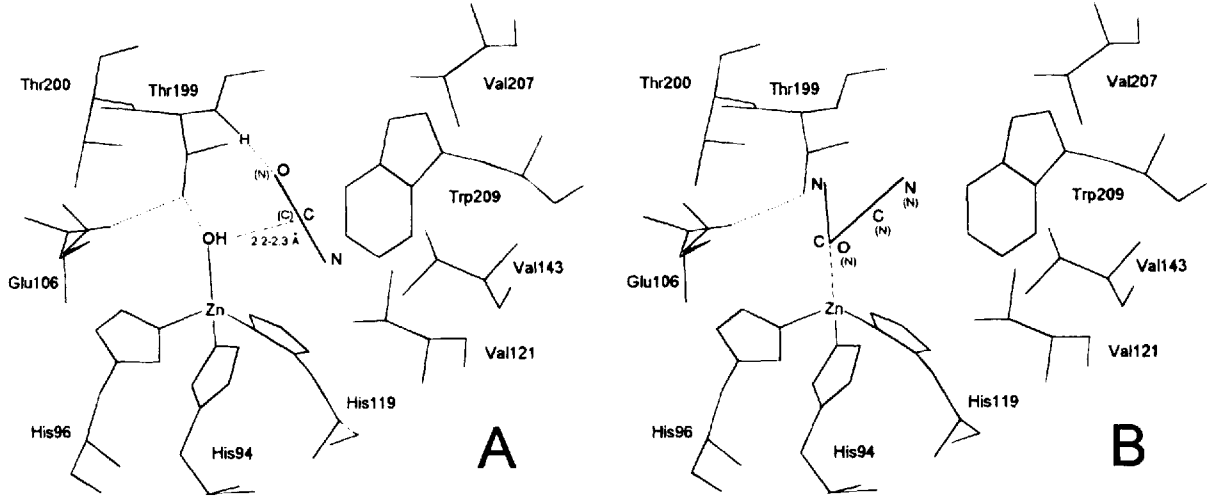

FIGURE 2 Schematic draw of the binding mode of NCO^- and CN^- as taken from the X-ray structure (A)(16) and as it results from NMR data (B)(17).

especially true in the case where small substrates are involved. CO_2 is a molecule with cylindrical symmetry, and does not have structural and chemical determinants able to induce the design of a highly specific binding site. Hence it is reasonable to think that several slightly different sites able to host a carbon dioxide molecule may exist in the active site cavity of the enzyme, and that equilibria may occur between states separated by small energetic barriers. As far as CN^- and NCO^- are concerned, it is possible that the two pictures (Figs. 2A and 2B) represent different states of the enzyme–inhibitor complex that are similar in energy. This hypothesis is corroborated by the observation of two different pH-dependent binding modes for the N_3^- ion, isoelectronic with NCO^- and CO_2, in the solid state (Figs. 2A and 2B).[18,19] Further considerations on the existence of multiple states will be made in the following sections.

Following the crystallographic results, Liljas and co-workers[5,20] have proposed that the cyanate and cyanide binding site as found in the crystal structures of the respective HCAII adducts may represent a model of productive CO_2 binding to the enzyme. The substrate molecule bound as depicted in Fig. 2A is properly positioned to undergo nucleophylic attack by the zinc bound hydroxide group, which is in turn oriented by the hydrogen bond donated by Thr199. The putative hydrogen bond with the protonated amide nitrogen of Thr199 and the electrostatic interaction with the zinc ion would have the role of polarizing the CO_2 molecule activating it towards the nucleophylic attack.

THE FIRST PENTACOORDINATE INTERMEDIATE

Upon attack of the OH^- nucleophile on the carbon dioxide molecule, a pentacoordinate adduct with HCO_3^- anion is formed. Bicarbonate itself could not be located in the active cavity by X-ray techniques because of its low affinity for zinc. The possible occurrence of a pentacoordinate reaction intermediate is suggested by the X-ray structure of the bicarbonate adduct with the Thr200 → His–HCAII mutant (Fig. 3).[21] In this structure the bicarbonate anion is found bound to zinc at 2.2 Å replacing the coordinated Wat263 with the protonated oxygen donating a hydrogen bond to Oγ of Thr199. A

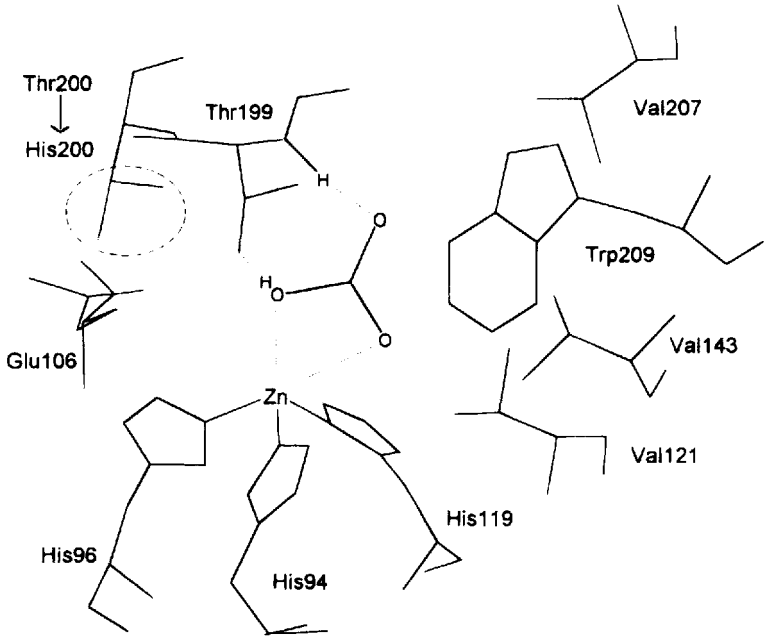

FIGURE 3 Binding mode of HCO_3^- to CA inferred from the crystal structure of the Thr200 → His–HCAII mutant.

second bicarbonate oxygen is bound to zinc at 2.5 Å, and the third one is engaged in a hydrogen bond with the amide nitrogen of Thr199. This type of structure may be by itself consistent with the equilibrium between four and five coordinated species resulting from the electronic spectroscopy of the Co(II)–HCAII adducts with bicarbonate[22] (see, however, the following section). Direct binding to zinc of the protonated bicarbonate oxygen may explain the absence of the isotope effect in the enzyme kinetics. Håkansson *et al.*[5] have pointed out that this type of binding would not have been possible without the assistance of the enzyme molecule, as carboxylate groups would bind the free aquo-zinc either as monodentate or bidentate ligands but always with the negatively charged oxygen atoms close to the metal. It has also been pointed out that this kind of binding destabilizes the bicarbonate molecule, promoting the acid cleavage of the C–OH bond which results in originating the CO_2 molecule in the reverse dehydration reaction.[5]

THE SECOND PENTA-(ESA?)COORDINATE INTERMEDIATE

In addition to the case of Thr200 → His–HCAII mutant, two other different binding modes for HCO_3^- have been observed in the cobalt(II) derivative of HCAII[23] and in the Thr199 → Ala–HCAII mutant.[24] If these binding modes are read in sequence, as summarized in Fig. 4, a multistep bicarbonate dissociation pathway could be envisaged. The tendency of HCO_3^- to bind the metal in a bidentate fashion as in Figs. 4A and 4B is consistent with suggestions from ^{13}C NMR studies on the Mn derivative complex.[12,25] It has also been suggested that the different abilities to catalyze the reaction by different metal substituted enzymes depend on the affinity of HCO_3^- for the metal ion and finally on its bidentate behavior.[26] The presence of a monodentate bicarbonate intermediate with a coordinated water molecule (Fig. 4C) is further supported by NMR data relative to complexes of CA with weak donors like ClO_4^- or NO_3^-, $HCOO^-$ and CH_3COO^- which are indicative of pentacoordination with retention of water,[27–29] and by the X-ray analysis of the HCAII–NO_3^-, –$HCOO^-$ and –CH_3COO^- complex[5,30,31] shown in Fig. 5. In the NO_3^- complex in the solid state a very long bond is observed between a nitrate oxygen and zinc (2.8 Å), while a second nitrate oxygen is hydrogen bonded to the metal coordinated Wat263. No hydrogen bonds are present between the nitrate and the amide nitrogen of Thr199. The HCO_3^- binding mode depicted in Fig. 4C can be originated by the breaking of the Zn–OH–CO_2 bond and of the hydrogen bond between bicarbonate and the N–H of Thr199 upon the addition of the incoming water molecule. The loss of the latter hydrogen bond may destabilize the bicarbonate molecule promoting its detachment.

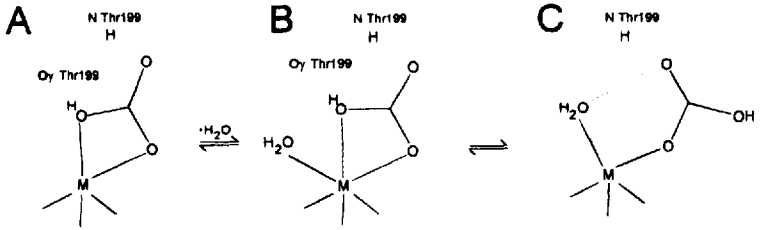

FIGURE 4 A possible bicarbonate detachment pathway.

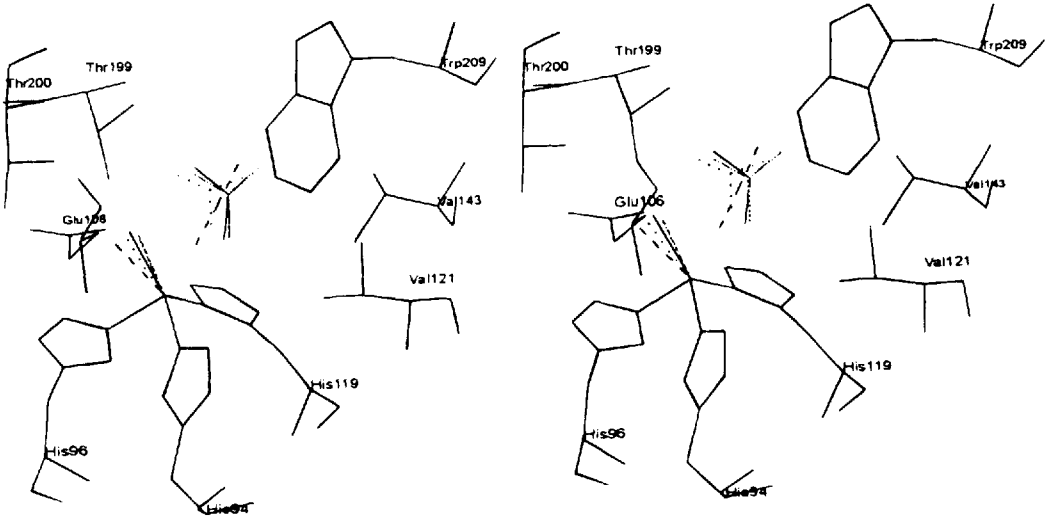

FIGURE 5 Stereo view of the different anions bound to the CA active site. The binding sites of nitrate (· · ·), tiocyanate (---), formate(——) and acetate (· - · -) are shown.

THE RATE DETERMINING PROTON RELEASE STEP

For a long time it has been recognized that the rate limiting step of the CA catalyzed reaction must be the proton release to the solution necessary to restore the zinc bound hydroxyl nucleophile;[3] however, the proton transfer from an enzyme group with a pK_a around 7 to bulk water is at least two orders of magnitude slower than the turnover number for CO_2 hydration (10^6 sec^{-1}). It has been proposed that buffers are better proton acceptors than water, and it has been shown that buffers do enhance the turnover.[3] Kinetics measurements at high buffer concentrations show the presence of an isotope effect consistent with the presence of an intramolecular proton transfer as the rate limiting step.[3] A residue able to act as a proton shuttle between the enzyme and the bulk solution in HCAII is His64. Indeed His64 has a pK_a similar to Wat263 and is connected to it by a hydrogen bonding network. Furthermore the side chain of this residue is observed in several crystal structures as disordered over two distinct conformations, one pointing towards the cavity and the second one towards the solvent, suggesting that through a similar movement a proton can be transferred in and out of the active site at the expected rate.[5,32]

ANION BINDING

The crystallographic studies of the complexes of $HCOO^-$,[5] CH_3COO^- [31] and HSO_3^- [5] with HCAII, and of HCO_3^- with cobalt(II) substituted[33] and with the Thr200 → His mutant of HCAII[21] discussed above have evidenced that anionic oxygen bearing molecules have a binding site in the hydrophobic region of the active site cavity. In every case one of the ligand oxygen atoms is coordinated to zinc and a second one is within hydrogen bond distance of the amide nitrogen of Thr199. It must be noticed that the position of two oxygen atoms from the formate, bicarbonate and bisulphite structures are nearly coincident with the nitrogen and oxygen atoms of the cyanate structure (compare Figs. 2A and 4C). NMR studies on the formate and acetate ligands on the cobalt(II) substituted BCAII have obtained similar results.[28,34] Using the effect of the paramagnetic center on the relaxation time of the carboxylate carbon atom it is possible to

determine the distances of the carbon atom from the metal. In the case of acetate and formate the obtained distances are 3.1 and 2.9Å, respectively. These values are somewhat shorter than those found for the formate anion in the solid state. Furthermore, the irradiation of the proton resonance of the inhibitor yields significant information for the identification of the neighboring protein residues. The latter could be assigned through 2D-NOESY experiments[28]: the methyl group of the acetate and the proton of the formate are oriented towards the hydrophobic portion of the cavity, in particular towards the Val143 and Val207 residues.

From the X-ray crystal structures on the SH^-, HCO_3^-, HSO_3^- and sulphonamide adducts, it seems that the hydrogen bond scheme (Wat263$\cdots$Oγ Thr199$\cdots$Oϵ Glu106) is the "device" used by the enzyme to discriminate between protonated and deprotonated donors since only the former seem to be able to replace the OH^- group in the zinc coordination.[5] In this way tetra- and penta-coordination may be selected.[4] The crystal structure of the adduct of HCAII with the HS^- [30] (Fig. 1) illustrates how the protonated anion is able to replace the zinc bound OH^- group. However, there are exceptions to this rule as demonstrated by the crystal structures of the complexes with azide (Fig. 2B)[18,19] and bromide[19] (not shown) and by the biophysical information in solution on CN^- and NCO^- derivatives. All of the latter class of anions are able to displace the zinc water without having hydrogen bond interactions with Thr199 Oγ. A further different way to replace Wat263 is shown by the complex with the bulk 1,2,4 triazole inhibitor[35] which occupies the Wat263 position because of its ability to simultaneously form hydrogen bonds with the side chain of Thr200 and with the amide nitrogen of Thr199 (not shown).

It is apparent that there are several possible arrangements of anions in the cavity depending on essentially three major factors:

(1) the strength of the coordination bond;
(2) the H-bond accepting and donating capability;
(3) hydrophobicity of some atoms of the ligand.

According to these driving forces, NO_3^-, $HCOO^-$ and CH_3COO^- anions behave in a similar way.[5,27–31] They are all located in the hydrophobic site of the cavity, with acetate and formate showing increasing hydrophobic interaction and at the same time increasing

metal affinity; as a result the water Wat263 moves more and more towards the opening of the cavity further away from the tetrahedral coordination position (Fig. 5).

The NCS^- anion is also located in the hydrophobic site, but is more strongly coordinated, as expected from its coordination chemistry (Fig. 5). The Wat263 is even further tilted.[36] NMR studies on the Co derivative show that in every case the z axis of the χ tensor bisects the angle CH_3(Thr199)–Co–CH_3(Thr200).[37]

Finally, the OH group of NCO_3^-, as observed in the cobalt(II) substituted enzyme (Fig. 4B), displaces Wat263 from its coordination position because of the ability to donate an H-bond to Thr199 Oγ. In this derivative, Wat263 shows the maximal tilt from its position originating a distorted octahedral geometry of the metal site.[23]

A COMMENT ON THE DISTANCES

The distances between the donor atom and zinc or cobalt in the various adducts are found to vary in a very large range. Values for the Zn–O distance of 2.8 Å and 2.4 Å, as found in the nitrate and in the case of the formate adducts, are quite unusual in coordination compounds. These data are even more striking if we consider that when we study the solution adducts via NMR we essentially find normal distances. As an extreme case, CN^- and NCO^- are found not bound in the crystalline state and bound in solution. Azide is found in both arrangements in the solid state. Even the structural differences between the HCO_3^- adducts of ZnCA and CoCA are instructive.

We are led to believe that a variety of structures can be obtained on the same or similar systems depending on the crystallization procedure and on the metal ion in CA. The overall protein folding provides the larger contribution to the protein stabilization energy. As outlined above, the various possibilities of anion binding must depend on (a) the energy of a weak coordination bond which is weakened by the H_2O substitution (or the coordination polyhedron expansion if the coordination number is increased); (b) the ability to donate H-bonds, whose energy may be fine-tuned by the protein folding; (c) hydrophobic interactions of the anion. The different anion binding modes are thus close in energy. In the crystallization process,

the protein may induce some changes in the active site cavity which stabilize a structure that is not necessarily maintained in solution. In this respect, the recent findings on the dimeric reduced Cu(I)–Zn–superoxide dismutase are enlightening. The two subunits are identical, but the coordination around copper is different in the two sites.[38] There is no evidence of heterogeneity in solution, indicating that the two subunits are either identical or made identical by fast fluxionality.[38] In the reduced proteins the bridge between Cu and Zn provided by a hystidinate group is broken in solution but present in the solid state. In other words, crystal packing forces may allow several structural arrangements around the metal ion in the solid state whereas in solution a different one can dominate. This idea is consistent with the notion that enzymes perform their function in a multistep fashion, where the energy barrier for each step is minimized. On the contrary, inorganic complexes may perform the job with a smaller number of steps, or even with a single step, of course with much smaller turnover numbers.[39]

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