# L. LEWIS ACID CATALYSIS

Background

Twenty amino acids is not enough. The breadth of chemistry handled by enzymes requires that additional chemical species be employed in catalysis. So-called **cofactors** are non-amino acid components of enzymes that may be either associated or bonded to proteins and contribute to rate acceleration. Roughly one-third of all enzymes studied to date employ metal ion cofactors. Metal cations can play one of two roles; they may act as Lewis acids or as redox reagents. As Lewis acids, they function in a role similar to general acids, stabilizing negative charge in the enzyme active site. This chapter will focus on Lewis acid catalysis in reactions that employ water as a nucleophile. Lewis acid catalysis can be observed to play a role in either (a) activating water as a nucleophile or (b) activating the electrophile for nucleophilic attack.

Activation of Water as a Nucleophile

As noted earlier, water is a poor nucleophile due to the build up of positive charge on the oxygen that accompanies attack upon an electrophile. General base catalysis overcomes that issue by promoting deprotonation of the water molecule as it attacks. Another approach is to stabilize the hydroxide ion within the enzyme active site.

While the metal ion itself is a Lewis acid (electron pair acceptor), the hydrated metal ion is a Bronsted acid (a proton donor) according to Equation L.1.

$$M(H_2O)_6{}^{n+} \Leftrightarrow M(H_2O)_5(OH)^{(n-1)+} + H^+ \qquad\qquad Eq\ L.1$$
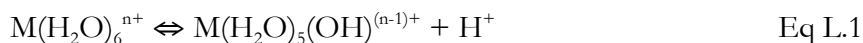
In this instance Lewis acidity and Bronsted acidity are linked. The capacity of the metal cation to stabilize negative charge from a hydroxide (hydroxo in inorganic-speak) ligand is a reflection of the Lewis acidity of the cation. Another way of saying it is that the stability of the conjugate base in Eq. L.1 is a function of the Lewis acidity of the metal cation. In general, one finds that the Bronsted acidity of hydrated metal cations relates to charge and ionic radius (Table L.1). The more highly charged the cation, the more acidic its bound water ligand(s), and the shorter the metal-ligand bond, the better the metal ion is at stabilizing the hydroxide ion relative to water.

Table L.2. Acidities of hydrated metal ions.

Ion	Radius(Å)	pK$_a$
K$^+$	1.52	14.5
Na$^+$	1.16	14.2
Ca^{2+}	1.14	12.8
Mg^{2+}	0.86	11.4
Mn^{2+}	0.97	10.6
Cd^{2+}	1.09	10.2
Fe^{2+}	0.92	9.5
Co^{2+}	0.88	9.6
Zn^{2+}	0.88	9.0
Fe^{3+}	0.78	2.2

With the exception of the ferric ion, none of these complexes has a pK_a below 7, indicating that all solvent ligands are chiefly in the protonated state at pH 7 for hydrated metal cations. However, as we've seen in other instances, enzyme active sites can "tune" the pK_a of an acid via additional environmental influences on the relative stability of the conjugate acid and base, so there are numerous instances in which metal bound waters have pK_a values near neutrality, providing a significant concentration of hydroxide in the active site.

Activation of the Electrophile

The ability of a Lewis acid to activate an electrophile is relatively familiar from organic chemistry, and is more common sensical since it is directly analogous to general acid catalysis. During nucleophilic attack electrons are displaced from the electrophilic atom and some positive charge may be used to stabilize them. The most abundant example of this can be found in phosphoryl transfer reactions from ATP, which is almost always found as a Mg^{2+}-ion chelate in the cell. The Mg^{2+} ion acts to stabilize growing negative charge on the transition state during the transfer reaction, lowering the free energy of activation (Figure L.1).
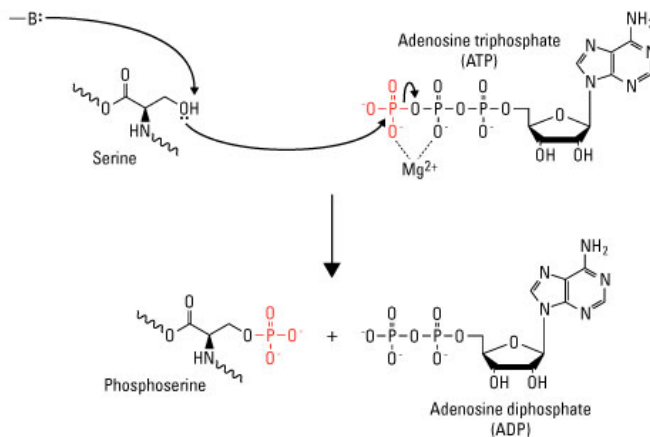

Figure L.1. $Mg^{2+} \bullet ATP$ chelate participating in a phosphoryl transfer reaction to serine. (Taken from http://www.piercenet.com/method/phosphorylation)

Nitrile Hydratase

The conversion of nitriles to amide (Figure L.2) is accompanied with a fairly significant free energy barrier. The standard protocol in organic texts is to heat the nitrile in the presence of concentrated strong acid or strong base for several hours. A gentler approach is to use the bacterial enzyme, nitrile hydratase (NHase), which catalyzes the same reaction much more rapidly at moderate temperatures. This approach is used in the industrial preparation of acrylamide (95,000 tons/yr).
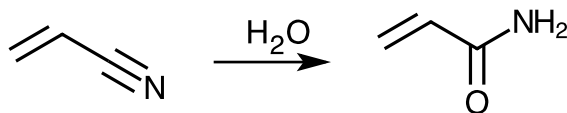

Figure L.2. Hydrolysis of acrylonitrile to acrylamide.

NHases possess an unusual metal binding site in their active site that contains either Fe^{3+} or Co^{3+} (the big guns in Lewis acid land). The metal cation receives four of the six ligand interactions from a tripeptide sequence CysSerCys, where the two amide groups are deprotonated to donate a lone pair from the nitrogens and each cysteine donates a lone pair as well in four interactions that occupy a single plane in the octahedral coordination geometry (Figure L.3). In addition a cysteinate and solvent molecule occupy axial positions across from each other. As a final surpise, the two cysteine residues in the CSC sequence are oxidized to a sulfinate ($-SO_2^-$) and a sulfenate ($-SO^-$). Lone pairs from the sulfur atoms of the groups are donated to the metal cation, but it is unusual to see these oxidation states for cysteine in a functional enzyme.
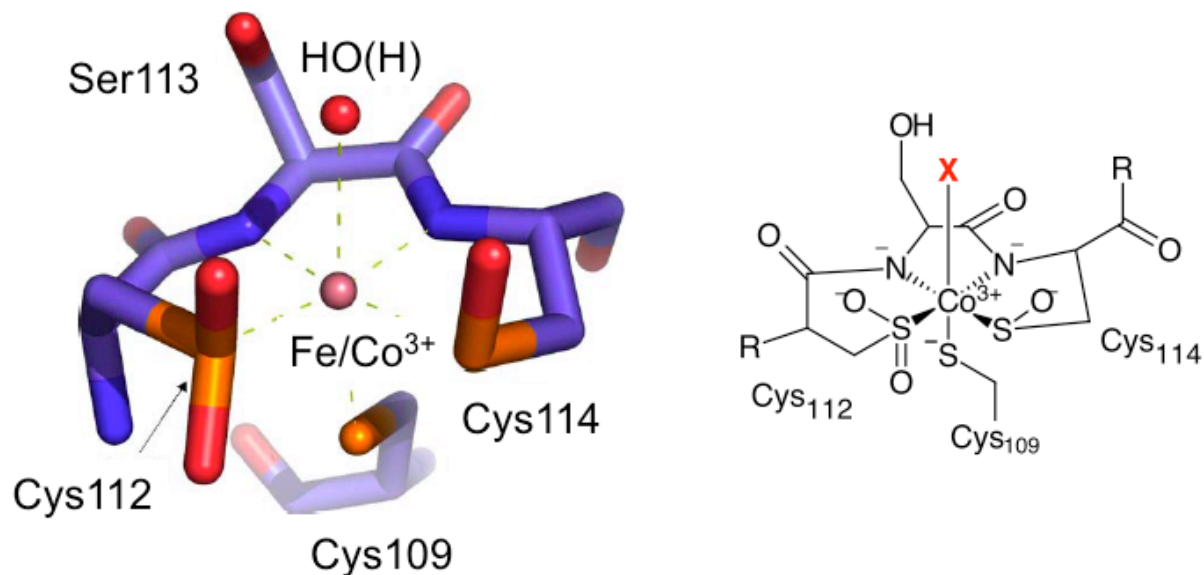

Figure L.3 Metal binding site of nitrile hydratase.

In the resting state of the enzyme (no substrate bound) it is unclear as to whether the solvent ligand is a water or hydroxide, but the mechanism for nitrile hydrolysis was long unclear. Two hypotheses had long co-existed. Either the enzyme uses the metal bound hydroxide as a nucleophile or the substrate displaces the solvent ligand and coordinates the nitrile nitrogen, thereby activating the nitrile as an electrophile. (Figure L.4).
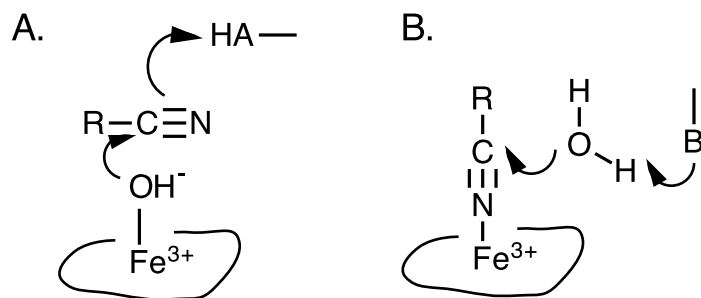

Figure L.4. Two hypotheses for the mechanism of nitrile hydratase. (A) Metal-bound hydroxide acts as an active site nucleophile, or (B) Metal-bound nitrile is activated as an electrophile.

Strong evidence for the mechanism that invokes a metal-bound nitrile (Figure L.4B) was uncovered by the Holz group.[1] The problem is capturing the E•S complex of NHase during catalysis. Hydration proceeds rapidly (k_{cat} = 125 s^{-1}) and it is difficult to obtain a large steady state concentration of the nitrile-bound from of the enzyme. Stopped flow kinetics, however, provides an alternate approach. By mixing large concentrations of the enzyme (330 μM) with high concentrations of the substrate methylacrylonitrile (190 μM) one rapidly forms a high concentration of the E•S complex. In stopped flow kinetics, the mixed solution is constantly being created and then being removed from the sample cell as it flows out. When the flow stops, only then does the mixed solution begin to age in the sample cell. Thanks to the wonders of modern electronics, it is possible to collect dozens of full visible spectra within a time frame of 0.005 to 0.5 s (Figure L.5A). Knowledge of the spectrum of the free enzyme as well as the kinetic mechanism employed by the enzyme (which includes the product-bound form in a reasonably high concentration) permits deconvolution of the spectra into its constitutive compoments.
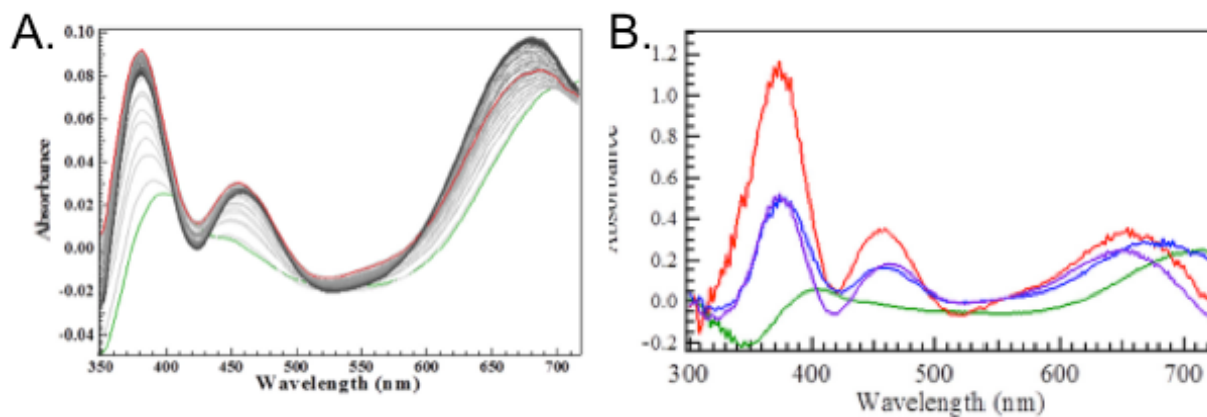

Figure L.5. Stopped flow investigation of reaction initiated by mixing NHase and substrate (methylacrylonitrile). (A) The time course rapidly rises to produce a great deal of the enzyme bound intermediate (in red) which then decays slowly to give spectrum of the free enzyme in green. (B) The spectra are deconvoluted to show the presence of two intermediates (blue and red) as well as the product bound enzyme (purple) and free enzyme (green). Taken from ref. 1.

Nitrile hydratase is often purified with a nitric oxide (NO) ligand instead of the solvent molecule at position X in Figure L.3. It is know that this complex, in which the nitrogen donates a lone pair to the metal ion, has a peak at 375 nm. Thus the intermediates that appear in the spectra that are assigned to intermediates provide confirming evidence for a nitrogen lone pair donation to iron (see the red and blue traces in Figure L.5B). These spectra are the first direct evidence of nitrile binding to the metal ion.

However, other evidence exists to support the role of the Fe^{3+}/Co^{3+} ion in electrophile activation. It turns out that NHase can hydrolyze isonitriles as well as nitriles, albeit very, very slowly (Figure L.6).[2] In a crafty crystallographic experiment, NHase was purified in complex with NO, which can

[1] Gumataotao et al. (2013) *J. Biol. Chem.* **288**, 15532-15536.

[2] Hashimoto et al. (2008) *J. Biol. Chem.* **283**, 36612-36623.

be photolyzed away from the metal ion center by visible light. When crystals are illuminated with a laser, the enzyme active site is evacuated by NO and slowly (over 2 hours) replaces with the isonitrile Figure L.7). Amazingly, about five hours later, there is strong evidence for a water molecule being added to the carbon of the isonitrile group (see arrow, Figure L.7).
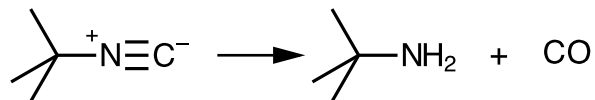

Figure L.6. Hydrolysis of t-butylisonitrile is hydrolyzed slowly to yield t-butylamine and carbon monoxide.
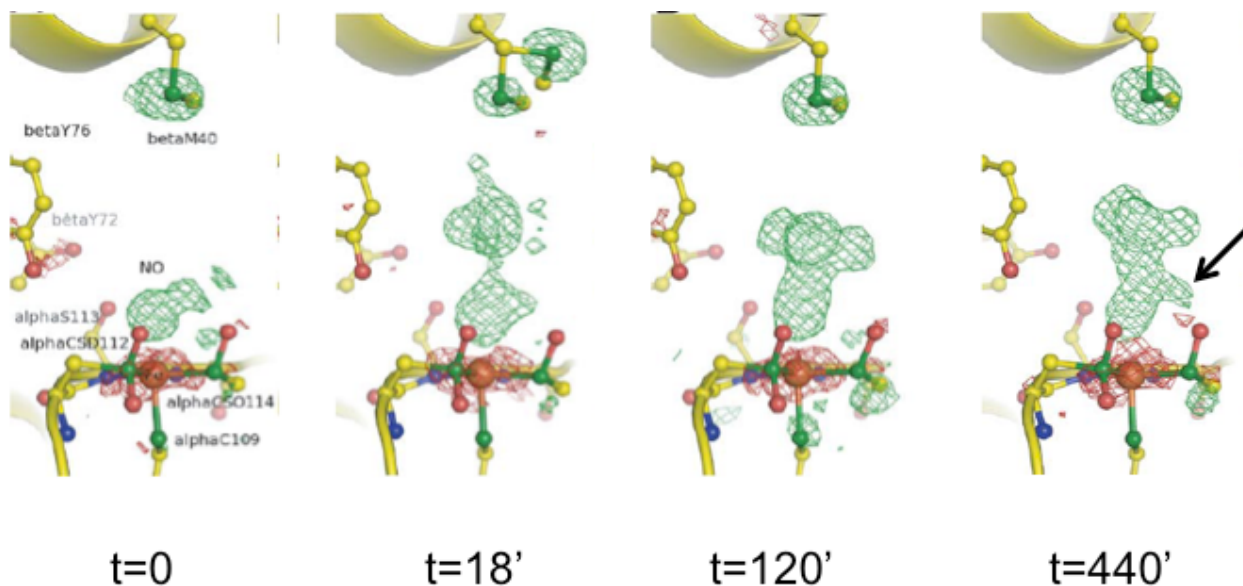

Figure L.7. Time-resolved crystallographic analysis of t-butylisonitrile hydrolysis in the NHase active site. At t=0, the enzyme has NO bound, but by 120 minutes the density for the substrate is clearly visible. Past 7 hours (440 minutes) a blob of electron density enters from the side (see arrow), presumably marking the beginning of hydrolysis. From ref. 2.

The implications of this experiment fully support the kinetics experiment – that the substrate binds directly to the metal cation, thereby providing a stabilizing interaction for the growth of negative charge that will take place during nucleophilic attack by water. Moreover, the pattern of density appears consistent with the sulfenate (-S-O⁻) acting as a general base for the activation of the nucleophilic water (Figure L.8).
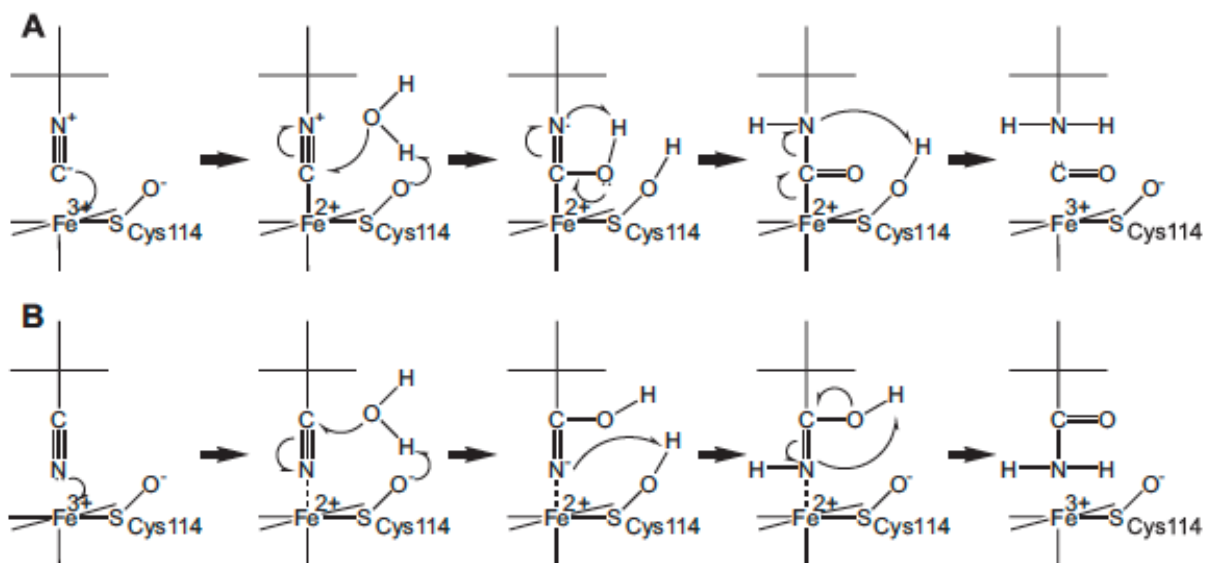
Figure L.8. (A) Proposed mechanism for hydrolysis of the isonitrile to an amine plus carbon monoxide, based on density maps shown in Figure L.7. (B) Extension of that mechanism to the true substrate, a nitrile. Obviously the proton migration pattern is speculative. From ref. 2. Note that in both mechanisms the sulfenate acts as a general base.

Carbonic Anhydrase

Nitrile hydratase is an example of an enzyme that uses Lewis acid catalysis to activate the electrophile. We'll now turn to carbonic anhydrase (CA) as an enzyme that uses its active site metal to activate the nucleophile. CA is a Zn^{2+} dependent enzyme that is commonly cited when someone is trying to impress you with how fast enzymes can be. It catalyzes the hydration of carbon dioxide to produce bicarbonate ion (and a proton; Eq. L.2) with a k_{cat} of 10^6 s^{-1}. This is among the fastest turnover numbers in enzymology, but is somewhat dampened by the speed of the uncatalyzed reaction, which isn't that bad (k_{uncat} 0.01 s^{-1}), but the 10^8 fold stabilization of the transition state relative to the substrate reflects a difference of roughly 11 $^{kcal}/_{mol}$. It operates at the diffusion limit, with a k_{cat}/K_m of 1.5 x 10^8 M^{-1}s^{-1}.

$$CO_2(aq) + H_2O(l) \rightarrow HCO_3^-(aq) + H^+(aq) \qquad \text{Eq. L.2.}$$
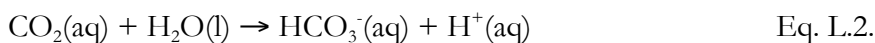
In the substrate free state, the active site Zn^{2+} adopts a tetrahedral geometry, bound by three histidine residues and (as it turns out) a bound hydroxide ion (Figure L.9). The pK_a of the zinc-bound water is 6.8, well below the pK_a of 9.6 listed for hexaaquozinc(II) in Table L.1. We'll get to that later. In relatively recent work, the CO_2 bound form of CA was obtained by exposing crystals to 10 atm of pressure of CO_2. In addition to raising the concentration of dissolved carbon dioxide, this also lowers the pH of the crystals since the reaction of CO_2 with water (Eq. L.2) generates acid. As a result, the zinc bound hydroxide is protonated and the enzyme is inactive.[3]

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[3] Sjöblom et al. (2009) *Proc. Natl. Acad. Sci. USA* **106**, 10609-10613.
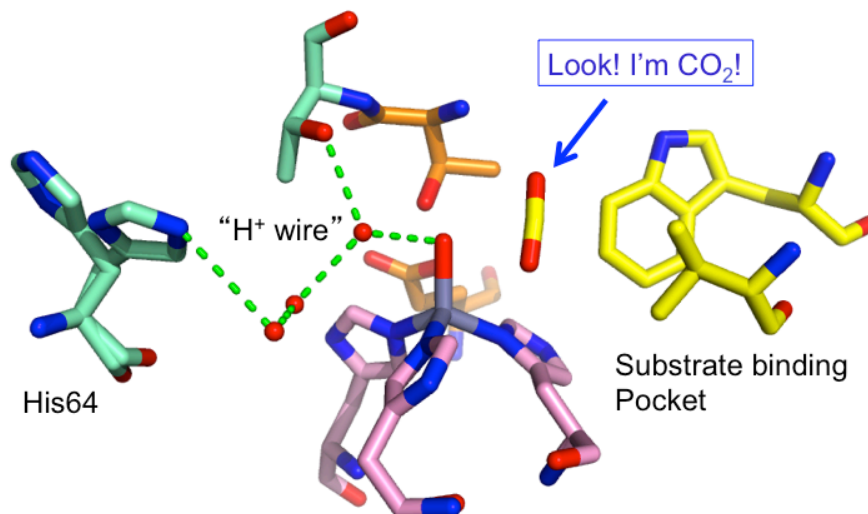
Figure L.9. Active site of carbonic anhydrase. The zinc binding residues are shown in pinc, while the CO_2 binding site is highlighted in yellow. Thr199 and His64 are part of the proton wire and shown in cyan.

The zinc-bound hydroxide is position with respect to the CO_2 substrate such that a lone pair on the oxygen is pointing directly at the electrophilic carbon – clearly predicting an attack. An unusual observation was made on crystals of CA containing CO_2. After repeated data collection runs on the same crystal, radiation damage of some form gradually leads to the formation of the bicarbonate product, still in the active site (Figure L.10). The geometry strongly suggests that the substrate receives no activation via the bound zinc, while the bicarbonate ion is coordinated only through the interaction of the oxygen derived from the nucleophile.
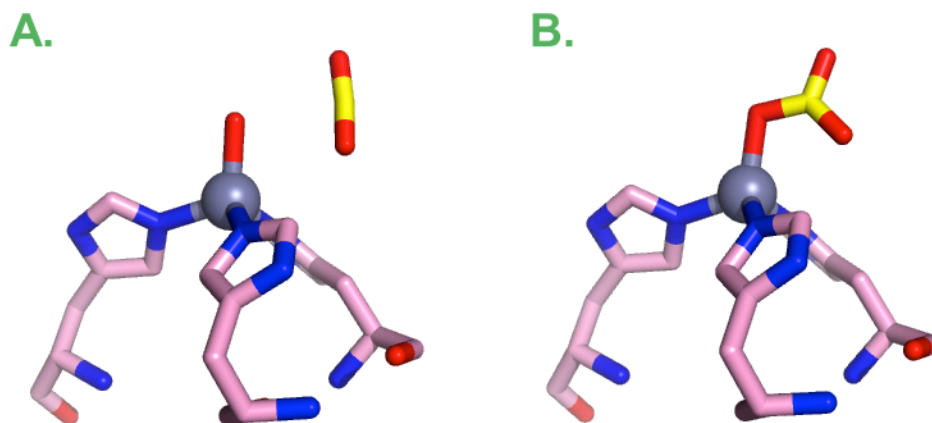

Figure L.10. (A) Active site of carbonic anhydrase with (A) bound CO_2 or (B) bound bicarbonate. The bicarbonate bound structure was derived from the same crystal as the CO_2-bound structure. Exposure to x-rays appears to promote the reaction.

The geometry of the active site suggests a mechanism of action for CA (Figure L.11). Substrate binds to the hydroxo form of the enzyme, attack takes place and bicarbonate is displaced by water at the zinc. Then comes the slow step (surprisingly). Loss of the excess proton from the active site (see Eq. L.2) proceeds via exchange with solution via the "proton wire" (Figure L.9 and Figure L.11B). The wire operates by sequential proton exchange between three bound water molecules and His64 which is seen in structures to occupy two conformations within the crystal. It has been argued that the motion of His64 is responsible for loss of the excess proton to solution. Supporting data arises from mutation of Thr199 and His64 to alanine. Both mutations show substantial loss of activity with respect to the wild-type enzyme.
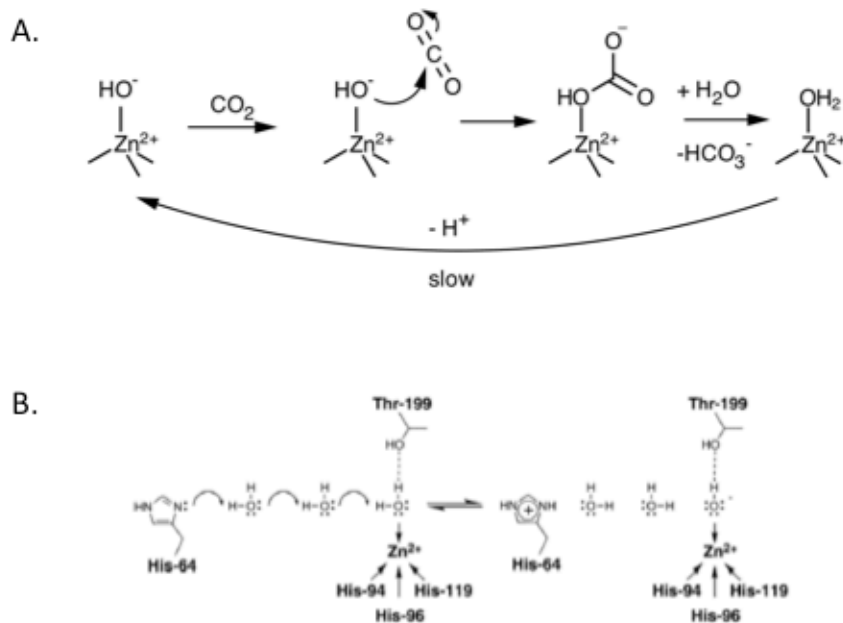

Figure L.11. (A) Mechanism of carbonic anhydrase. Note that the loss of a proton from the water bound state of the zinc ion is slow. (B) The proton wire transfers a proton from the zinc-bound water to His64, which can trade it then with solvent.

But now the real question. According to Table L.1, a zinc-bound water has a pK_a of 9.6, indicating that only about 0.1% of the bound solvent molecule will be deprotonated and therefore competent as a nucleophile at pH 7. In actuality, the water in the active site of CA has a pK_a of 6.8. A rationale can readily be offered for the difference. In $Zn(H_2O)_6^{2+}$, the zinc ion obtains lone pair donation from six oxygen atoms, each of which has a greater partial negative charge than a nitrogen in histidine (O-H bonds are more polar than N-C bonds). In addition there are six water ligands in solvated zinc, but only three histidine ligands in CA. Thus the active site Zn^{2+} might be viewed as "electron starved" in the enzyme active site, at least relative to when it is dissolved in solution. Thus the hydroxide will find greater electrostatic stabilization by the CA active site Zn^{2+} than a free Zn^{2+} in solution.

Carol Fierke's group investigated this effect further and examined the role of amino acid substitutions in the coordination environment surrounding Zn^{2+} in CA (Figure L.12).[4,5] The choice of direct ligands to the zinc ion has a clear effect on pK_a (Table L.2). Of the three histidine residues that bind the zinc, His 94 and His119 were substituted with retention of enzyme activity. If either His94 or His119 is replaced with an anionic Asp residue, the zinc becomes less electron-starved, and the hydroxide less stabilized – as a result the pK_as of the H94D and H119D mutants have higher pK_as than wild type (9.6 and 8.6 vs. 6.8). On the other hand, if histidine is replaced with Asn or Gln mutant is prepared, a neutral ligating residue replaces another neutral ligating residue and the zinc remains relatively electron deficient. The pK_as of the H94N and H119Q mutant are 7.3 and 6.9 respectively, similar to wild type (Table L.2).

Interestingly, the pK_a of the bound water is affected by "second sphere" residues that H-bond to those histidines. His94 and His119 are H-bonded to Gln92 and Glu117 respectively. The relative quantity of negative charge on these residues effects the electron density donated to the zinc by a histidine residue. Three substitutions of Gln117 lead to three different results in pK_a. Q92E adds an anionic residue to the second sphere, ultimately pushing more electron density on zinc and stabilizing the hydroxide less. The pK_a of the zinc-bound water increases to 7.7. Replacing Gln with Leu actually removes electron pair donation of any kind to His94, reducing the amount that His94 can donate. The pK_a of the zinc bound water decreases to 6.4, because the zinc is more electron starved. By if Gln92 is replaced with Ala, there is a chance for water to enter the pocket and H-bond with His94, retaining the neutral lone pair donation that exists in the wild-type enzyme. The pK_a of the Q92A mutant is 6.8, the same as wild type.

Table L.2. Effects of amino acid substitutions on the affinity of CA for zinc, its activity and the pK_a of the bound water. Mutations in red add electron density to the zinc and thereby raise the pK_a of the water. Mutations in green retain similar levels of electron density donation and maintain roughly constant pK_a values. The mutation in blue decreases electron density donation and leads to a decrease in pK_a.

	K_d Zn^{2+} (nM)	k_{cat}/K_m ($M^{-1}s^{-1}$)**	pK_a
WT	0.004	2500	6.8
H94D	3300	365	9.6
H94N	200	370	7.3
H119D	25	830	8.6
H119Q	15000	1490	6.9
Q92A	0.018	965	6.8
Q92L	0.030	1750	6.4
Q92E	0.005	1362	7.7

[4] Kiefer et al. (1995) *J. Am. Chem. Soc.* **117**, 6831-6837.

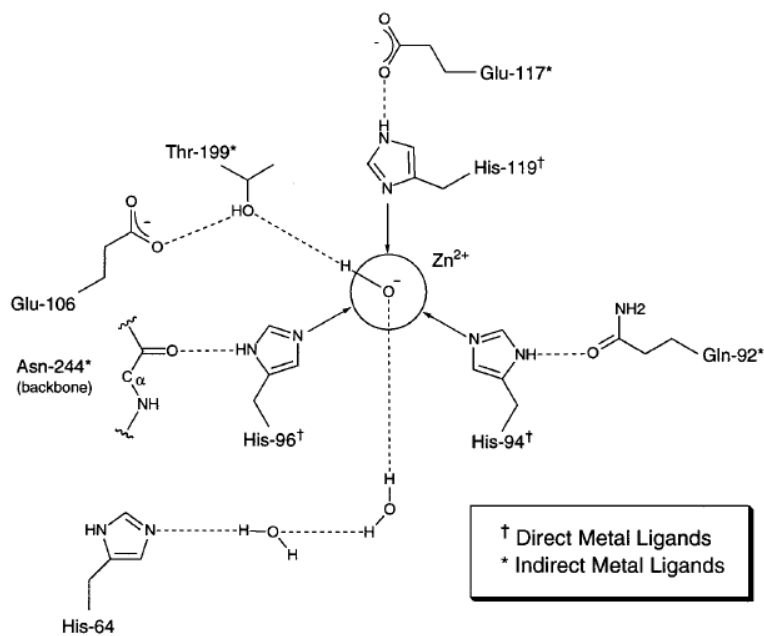[5] Lesburg et al. (1997) *Biochem.* **36**, 15780-15791.

Figure L.12. Diagram of the zinc binding site in carbonic anhydrase, showing direct metal ligands (His94, His96 and His119) and the second sphere residues (indirect ligands). Figure from ref. 5.