BL/CH401 Lecture 16A -- Enzyme Mechanism - Examples

Part I. Introduction

Determination of Enzyme Mechanisms - A General Approach

1. Purify the enzyme to homogeneity and determine its amino acid sequence and 3-D structure. Compare these basic structural properties of the enzyme to other know amino acid sequences using the computer databases like GenBank maintained by the National Center for Biotechnology Information at NIH (using computer tools like 'ENTREZ' or 'BLAST' - go to HTTP://www.ncbi.nlm.nih.gov/ on the WEB to learn about this database), like Swiss-Prot in Switzerland which has a collection of protein sequences in its database (go to http://expasy.hcuge.ch/sprot/sprot-top.html) and to other protein 3-D structures using 'The Protein Data Base' maintained by Brookhaven National Laboratory (to learn more about protein 3-D structures go to the PDB via HTTP://pdb.pdb.bnl.gov/) or compare your 3-D structure to the PDB using a computer in Germany (go to DALI at http://www.embl-heidelberg.de/dali/dali.html). Finding amino acid sequences and 3-D structures related to the enzyme you are studying will be very helpful in identifying invariant amino acid residues important in the enzyme's structure and functionality.

2. Study the kinetics and substrate specificity of the enzyme and identify inhibitors.

3. Identify key functional amino acid side chains and do 'site-directed mutagenesis' using the cloned gene to confirm the importance of these key amino acid residues. Are they essential for catalytic activity? Are they important for substrate binding? Are they important for stability of the folded native state of the enzyme?

4. Make hypothesis of the chemical events and bond rearrangements occurring during catalysis. Test this hypothesis by 'site-directed mutagenesis' and methods to identify 'intermediates' in catalysis.

These two lectures will illustrate the results obtained in the studies of Enzyme Mechanisms by giving examples for specific enzymes, including an exploration of 'The Active Site' of some specific enzymes. The lectures are divided into 5 topic areas as shown below:

A. pH Effects in Active Sites

Why is chymotrypsin most active at pH 8? Why is ribonuclease more active at pH 6?

B. Metal Ion Assisted Catalysis

How do Zn-Proteases catalyze peptide bond hydrolysis?

C. Electron Transfers to/from NADH/NADPH

How is stereospecifity achieved in dehydrogenases? Can an enzyme's disulfide bond be redox reactive?

D. Beta Barrel Enzymes

How does the 'perfect enzyme' work? How does the most abundant enzyme on earth work?

E. An Enzyme from a Thermophilic Organism

How do enzymes remain stable and function at high temperature?

Part II. pH Effects in Active Sites

Why does the activity of enzymes vary with pH?

Changing the pH alters the ionization state of amino acid side chains that ionize:

Lys, Arg, His, Asp and Glu

So, if ionic bonds are important to structural stability then the shape of the enzyme will change and the functionality of the enzyme will change. This is a general phenomena - related to the overall 3-D structure of all enzymes.

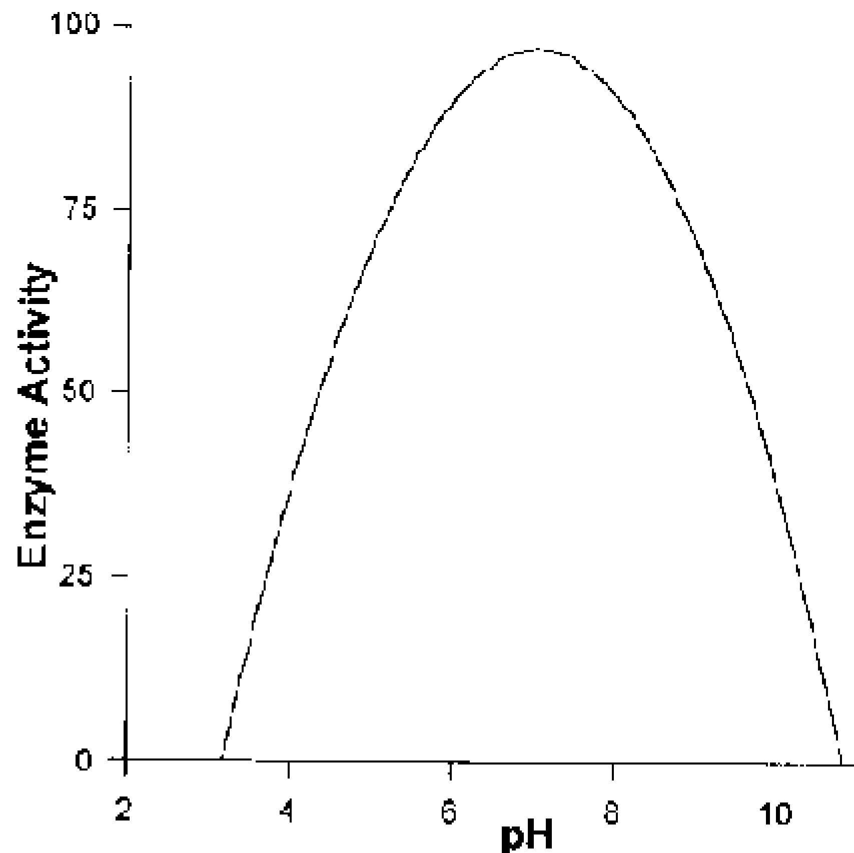

Figure 1. pH optimum of an enzyme shown as the classic 'bell-shaped' curve.

Why is chymotrypsin most active at pH 8? What if AA side chains that ionize are involved in catalysis?

For example, the mechanism of serine proteases, which was described in some detail in lecture

15B, involves Asp-102 and His-57. A ball-and-stick model of the active site of chymotrypsin is shown below:

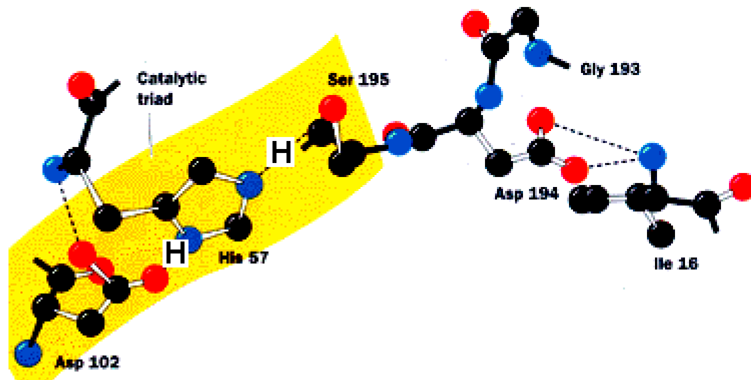

Figure 2. Catalytic residues in the active site of chymotrypsin with the 'catalytic triad' of Asp-102, His-57 and Ser-195 highlighted.
Figure from Voet's Biochemistry, copyright ©1990, John Wiley & Sons, Inc.

Protonation of Asp-102 and His-57 would block enzyme activity at pH below the pK of Asp (~4). But the pK of His-57 in chymotrypsin is ~6.8, a bit higher than a normal His (~6). So, we expect chymotrypsin to be most active at pH above 7. This is illustrated below with kinetic data collected on chymotrypsin.

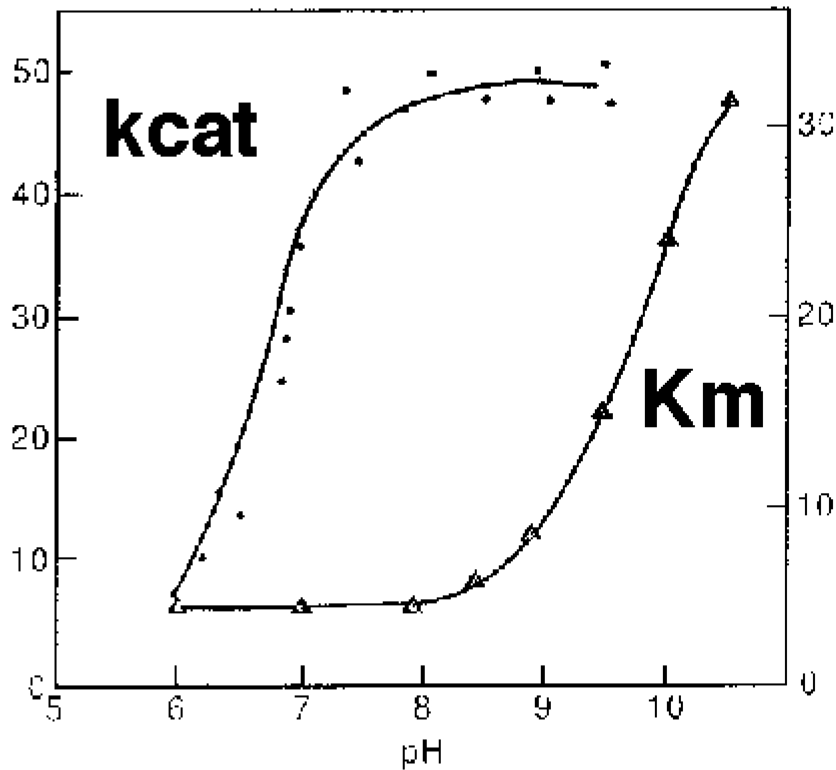

Figure 3. The impact of pH variation on the kcat (catalytic constant) for chymotrypsin (left hand scale) and on the Km for an artificial substrate (high hand scale).
Figure from Zubay et al., Principles of Biochemistry, copyright ©1995 Wm. C. Brown Comm.,

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This diagram clearly shows that as the pH is raised from 6 the kcat increases until a pH of about 8 is reached, where His-57 would be fully de-protonated.

A second effect of pH on chymotrypsin activity results from a change in substrate binding. When chymotrypsin is activated by proteolysis in the intestine (as shown below in Fig. 4) Ile-16 becomes an additional 'N-terminal residue' and its free amino acid group helps position Asp-194 and the protein's backbone for substrate binding.

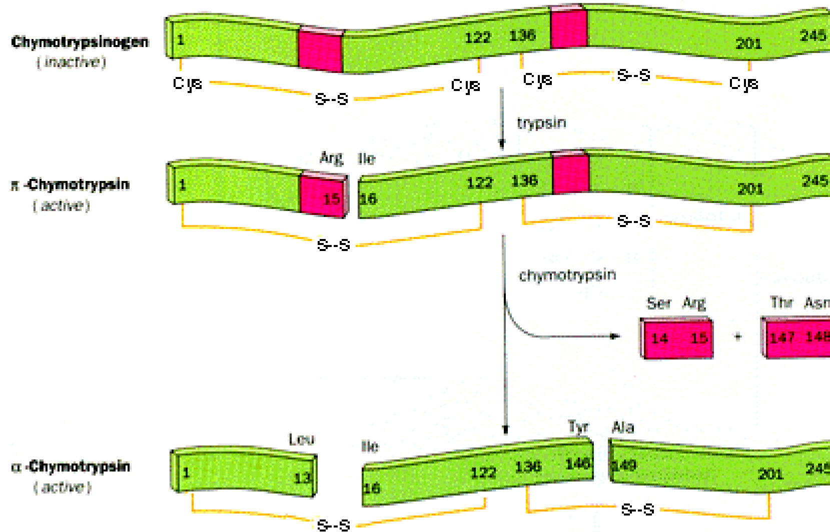


Figure 4. The proteolytic activation of chymotrypsin when the 'pro-enzyme' (called chymotrypsinogen, which is inactive) is processed in the intestine. Figure from Voet's Biochemistry, copyright ©1990, John Wiley & Sons, Inc.

During activation of chymotrypsin, two short sections of the amino acid backbone of the polypeptide are excised by tryptic and chymotryptic digestion (the latter being an example of self-digestion). However, the overall structure of chymotrypsin, in its activated forms which are called lambda-chymotrypsin (after tryptic digestion) and alpha-chymotrypsin (after the self digestion) is stabilized by two disulfide bonds which bridge the gaps in the backbone created by the removal of the short sections.

So titration of Ile-16 amino group (pK~9) leads to an increase in the Km at pH above 9 as shown in Figure 3 above. The Km increase means the substrate is more weakly bound at pH 9 and above. The net effect of pH on kcat and Km is that Chymotrypsin's pH optimum is 8, which is illustrated below with a plot of the specificity constant (ie kcat/Km) versus pH.

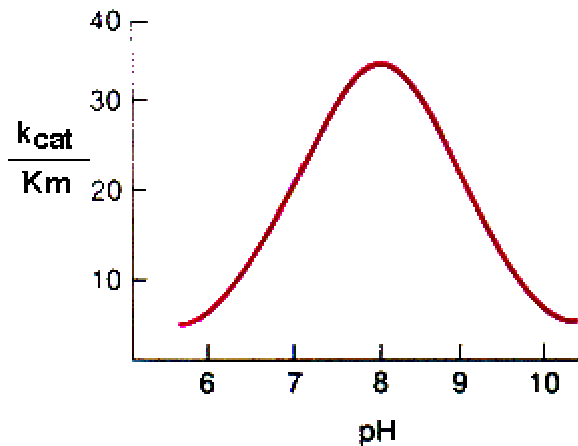


Figure 4A. Plot of kcat/Km (specificity constant) for chymotrypsin versus pH.
Figure from Zubay Biochemistry, 2nd Ed., 1988

Another example of pH effects at active site is found with ribonuclease (RNase) - an enzyme which degrades RNA. Why is ribonuclease most active at pH 6? Two His (His-12 and His-119) are involved in catalysis of RNA breakdown by RNase.

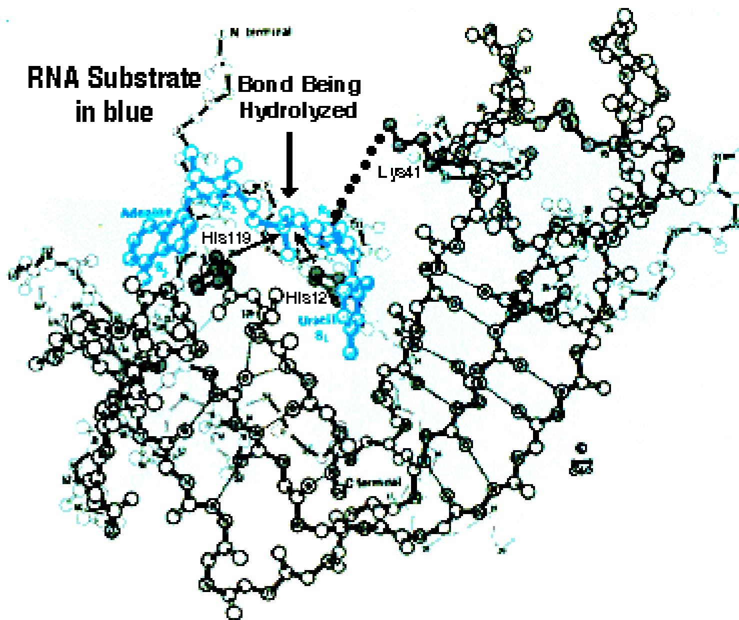


Figure 5. Model of a substrate molecule bound in the active site of RNase. The positions of His-12 and His-119 in the 3-D structure of RNase are shown in relation to the bond of the substrate to be hydrolyzed.
Figure from Zubay et al., Principles of Biochemistry, copyright ©1995 Wm. C. Brown Comm., Inc.

So it makes sense that RNase is most active at pH 6 since this is the pK of His. But one His must be protonated and one His unprotonated for the suggested mechanism. NMR expts have shown

that one His has a pK of 5.4 and the other has a pK of 6.4.

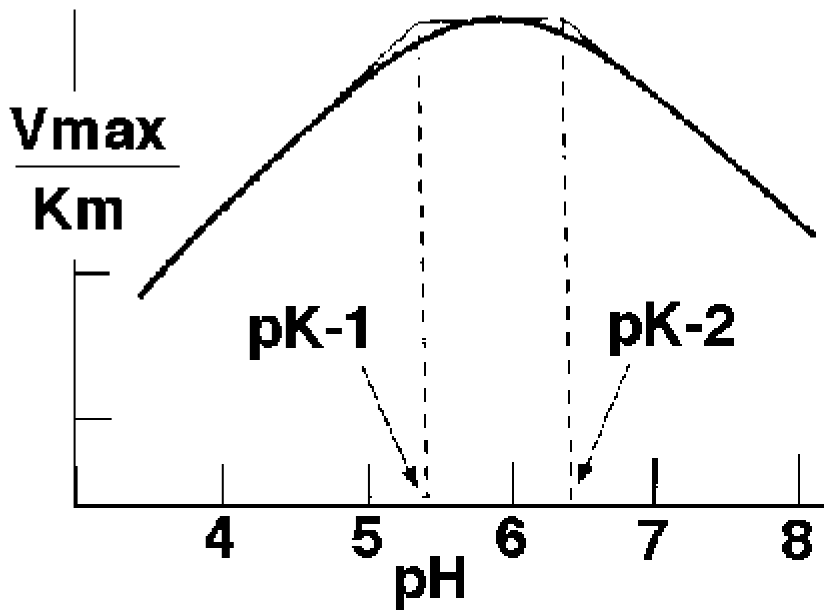


Figure 6. pH optimum of ribonuclease.

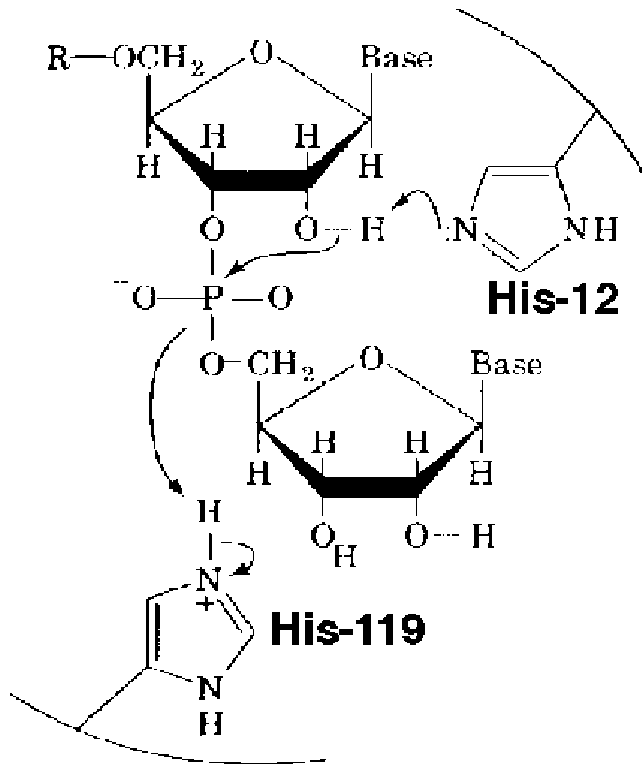
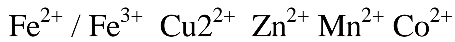


Figure 7. Suggested Catalytic Mechanism of Ribonuclease.

Figure 6 & 7 from Voet's Biochemistry, copyright ©1990, John Wiley & Sons, Inc.

Part III. Metal Ion Assisted Catalysis

Many enzymes are catalytically assisted by metal ions such as:



Other enzymes are 'activated' by metal ions such as:

Activation changes enzyme shape without the metal ion being involved in catalysis.

Endoproteases (ones that cut in the middle of a peptide chain) like thermolysin use Zn^{2+}. So do exoproteases (ones that chew in from the carboxy-terminal end) like carboxypeptidase. How do Zn-proteases catalyze peptide bond hydrolysis?

Thermolysin's Zn^{2+} coordinates the substrate's carbonyl while Glu-143 activates water, which attacks the substrate.

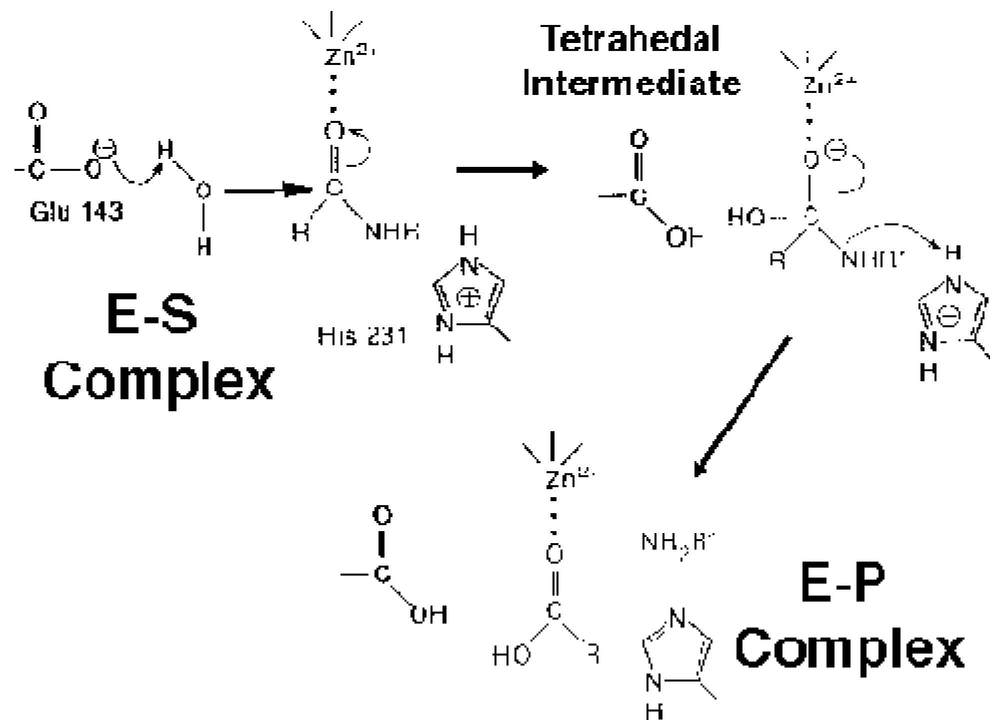


Figure 8. Model catalytic mechanism for peptide bond hydrolysis as catalyzed by the endoprotease thermolysin, which is an enzyme found in some laundry detergents where it is used to help remove protein stains from your clothes.

Carboxypeptidase works in a similar way.

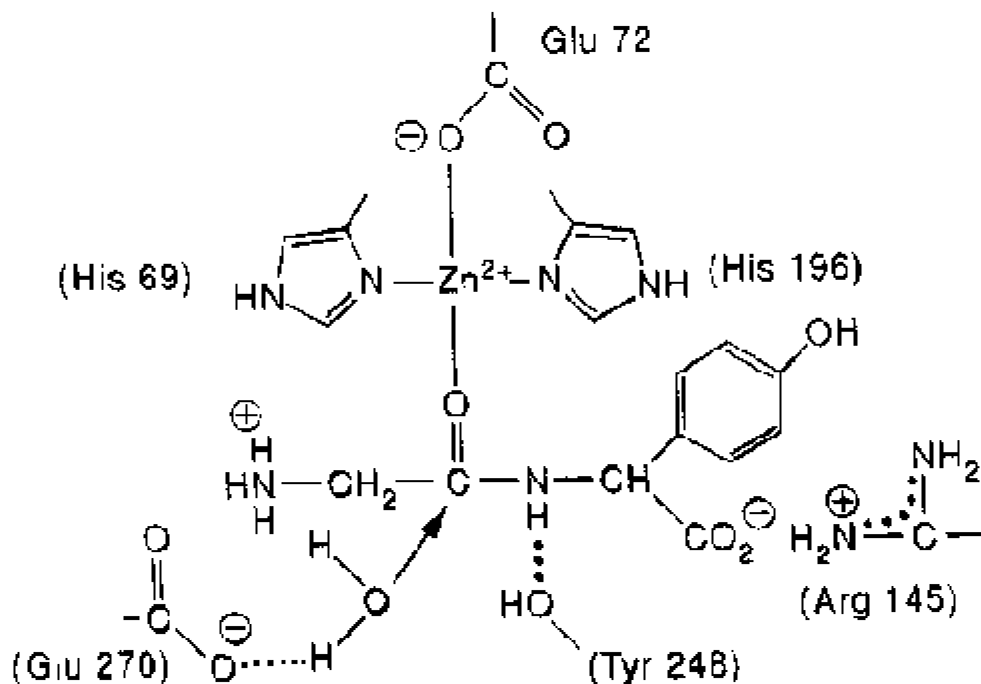


Figure 9. Model of the active site of the exoprotease carboxypeptidase showing the role of Zn^{2+} in activating the substrate molecule by binding the carbonyl oxygen, which would make the peptide bond more easily hydrolyzed.

So these proteases depend more on activation of the substrate in a way more like the H^+ catalysis of ester hydrolysis, rather than generating a very strong nucleophile (i.e. Ser-194) like in the serine proteases.

Another example of metal ion assisted enzyme catalysis is carbonic anhydrase, which catalyzes the hydration of carbon dioxide leading to the formation of bicarbonate. Carbonic anhydrase is an enzyme with a very high catalytic rate, one of the highest known. Carbonic anhydrase is used to balance the pH in the kidney during filtration of urine from the serum. In this case, a Zn^{2+} is bound by 3 His side chains of the enzyme and a water molecule is also bound to the metal ion, as shown below:

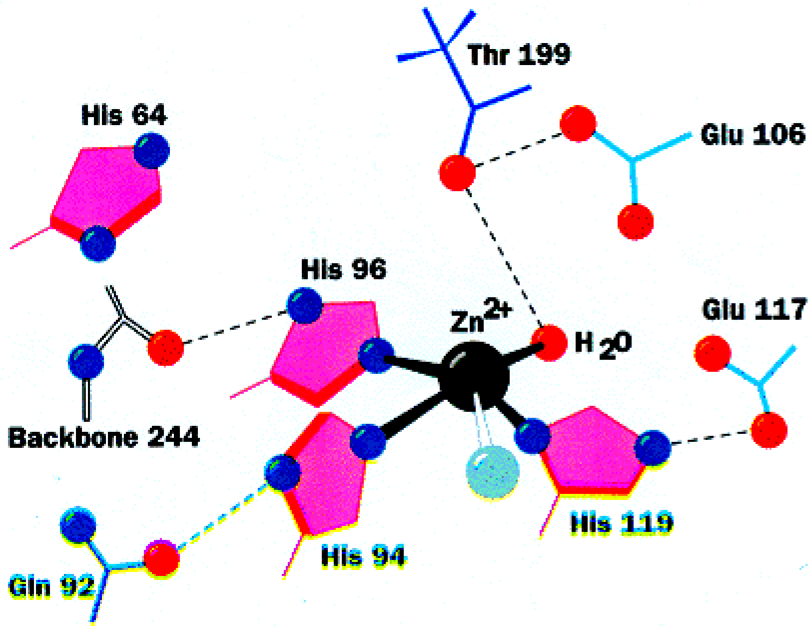


Figure 10. The active site of carbonic anhydrase. Figure from Voet's Biochemistry, copyright ©1990, John Wiley & Sons, Inc.

The role of Zn^{2+} in the carbonic anhydrase active site has been suggested to help activate the bound water molecule to generate a hydroxide ion which can attack carbon dioxide to directly make bicarbonate. The Zn2+ is assisted in removing a proton from water by nearby acidic side chains (Glu-106 or Glu-117 as shown above in the model of carbonic anhydrase's active site - see Fig. 10). The proposed catalytic mechanism of carbonic anhydrase is shown below:

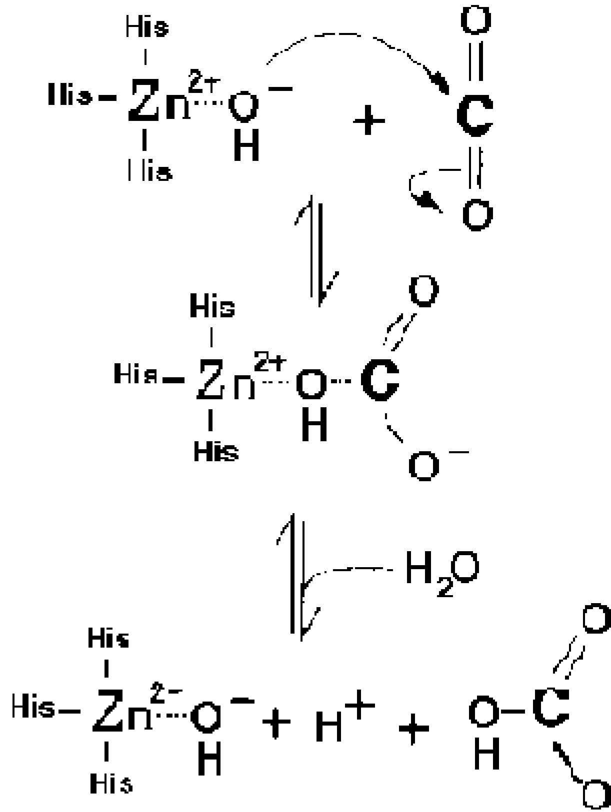


Figure 11. The catalytic mechanism of carbonic anhydrase emphasizing the role of Zn^{2+}. Figure from Voet's Biochemistry, copyright ©1990, John Wiley & Sons, Inc.

After the gaseous carbon dioxide is attacked by the hydroxide ion bound to Zn2+ and the intermediate forms, a water molecule displaces the bicarbonate and replaces the hydroxide ion bound to Zn^{2+}. In this process, a proton is generated which can be used to balance the pH during the function of the kidney.

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