

# Chapter 9

Catalytic strategy:

Proteases

Carbonic Anhydrase

Restriction enzymes

Myosin

# Basic catalytic principles

- **Covalent catalysis:**
  - Active site contains a reactive group that becomes transiently attached to the substrate
- **Acid-Base catalysis:**
  - Other molecule than water play a role of a proton donor/acceptor
- **Catalysis by approximation**
  - The enzyme brings two substrates together in an orientation that facilitates catalysis.
- **Metal Ion Catalysis**
  - Metal ions function in a number of ways, including serving as an electrophilic catalyst.

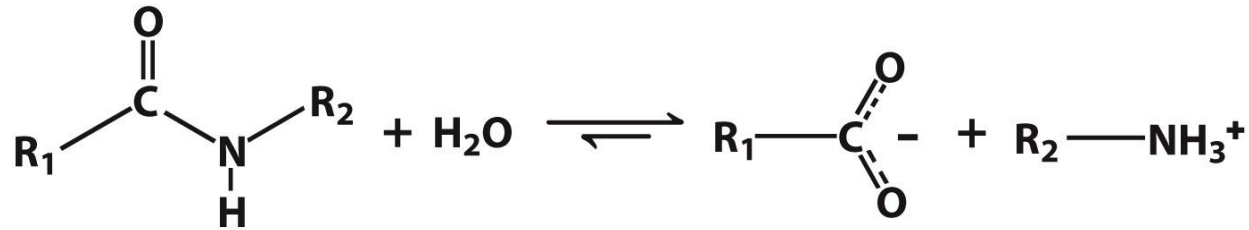
# Proteasis

Proteases cleave proteins by hydrolysis.

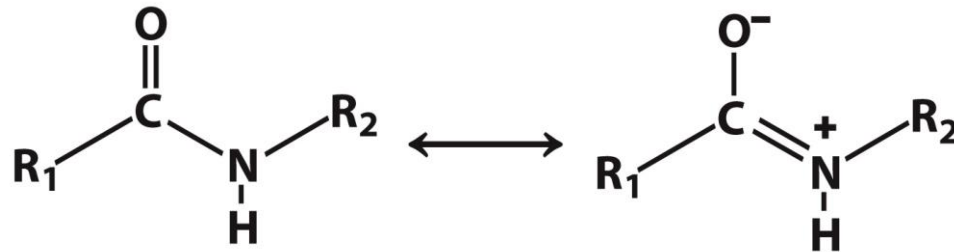
Cleavage of proteins and peptides is important for amino acids recycling and for digestion of proteins found in diet.

Protein hydrolysis is **exergonic** but **kinetically very slow**.

The planarity of the peptide bond accounts for the resistance to hydrolysis.



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Carbonyl carbon is less electrophilic than carbonyl carbon in carboxylate esters

# Chemotrypsin

- a proteolytic enzyme secreted by the pancreas that hydrolyzes peptide bonds selectively on the carboxyl side of large hydrophobic amino acids.

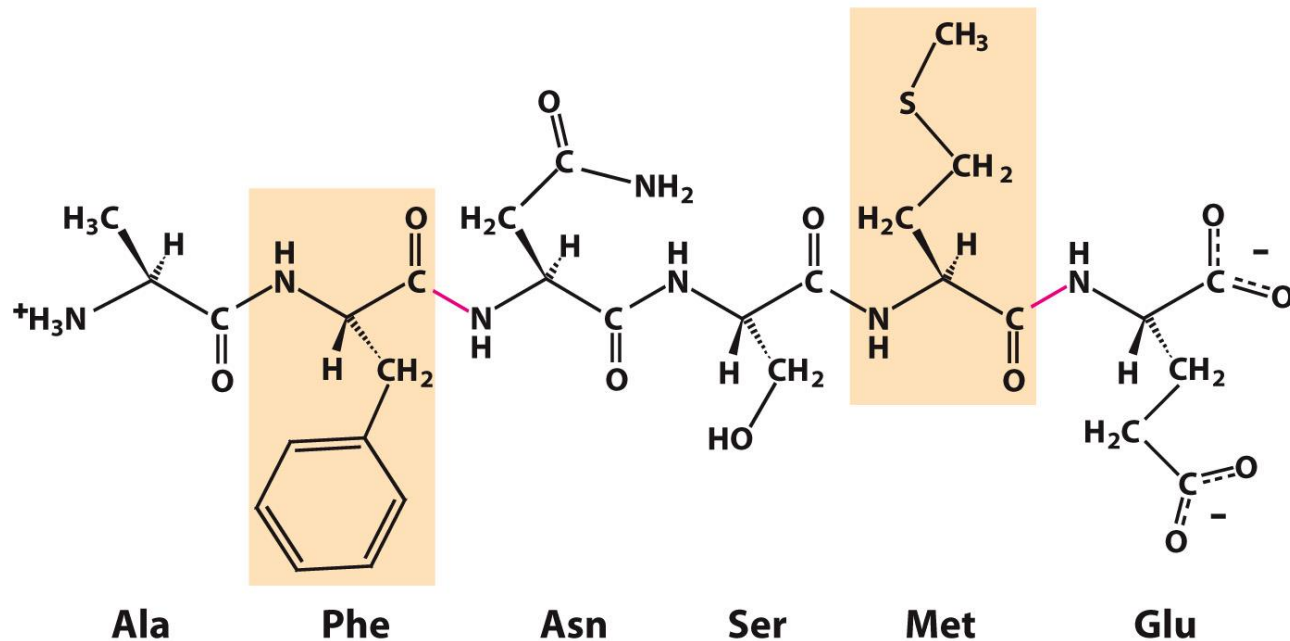


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In the process of catalysis, serine 195 becomes a nucleophile and attacks the carbonyl group of the protein substrate.

The group-specific reagent diisopropylphosphofluoridate (DIPF) modifies only serine 195, one of 28 serine residues in chymotrypsin, and inhibits the enzyme.

Ser195 is an example of covalent catalyst

its sidechain is highly reactive and play central role in enzymatic reaction

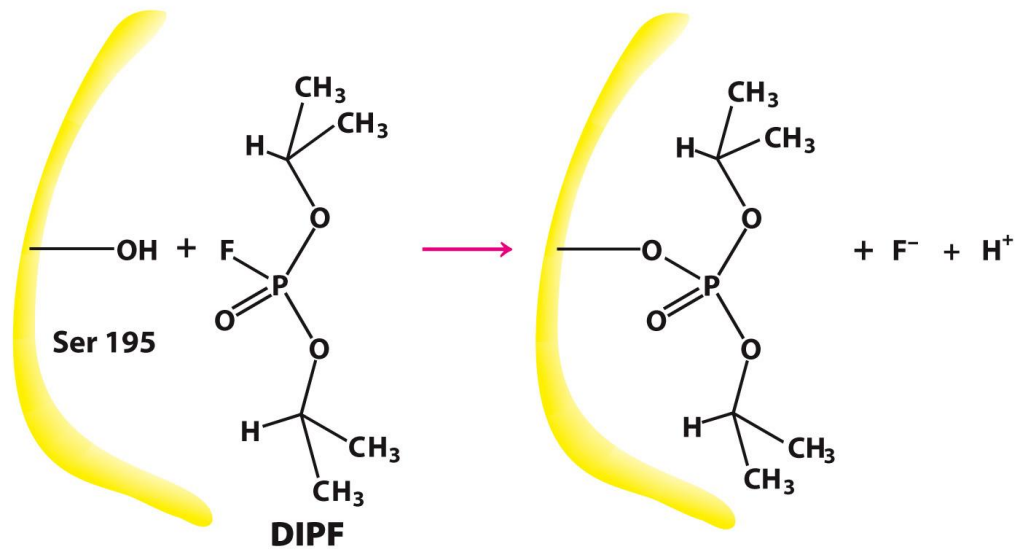


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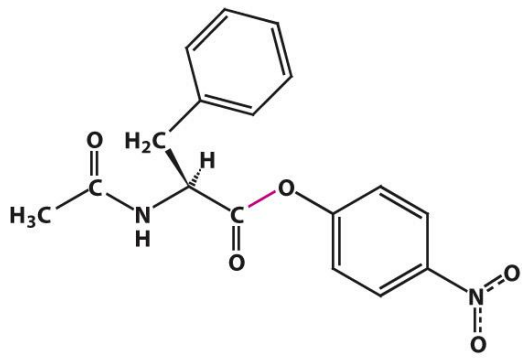
# Chymotrypsin catalysed cleavage of the peptide bond is a two step reaction

Chromogenic substrates generate colored products, facilitating enzymatic studies.

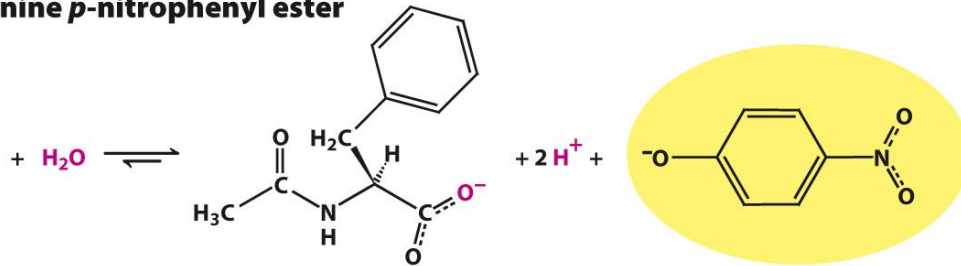
*N*-Acetyl-L-phenylalanine-*p*-nitrophenyl ester is a chromogenic substrate for chymotrypsin.

Studies with the chromogenic substrate revealed that catalysis by chymotrypsin occurs in two stages: a rapid step (pre-steady state) and a slower step (steady state).

The steps in catalysis are explained by the rapid formation of an acyl-enzyme intermediate and a slower release of the acyl component to regenerate free enzyme.



**N-Acetyl-L-phenylalanine *p*-nitrophenyl ester**



***p*-Nitrophenolate**

Two step reaction:

**Pre-steady state step:** acyl group of the substrate is covalently attached to the enzyme resulting in the fast release of *p*-nitrophenolate

**Second step:**

the acyl –enzyme intermediate is hydrolyses releasing the carboxylic acid component and regenerating free enzyme

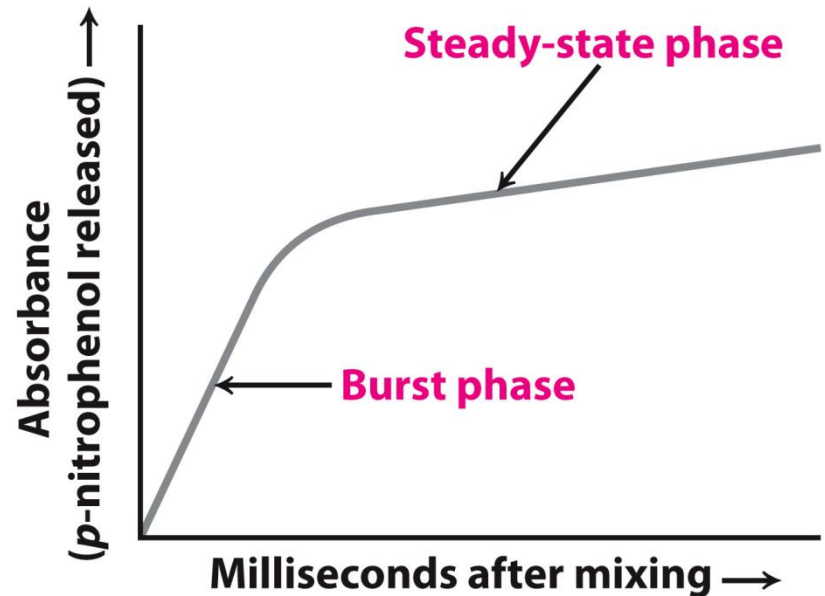
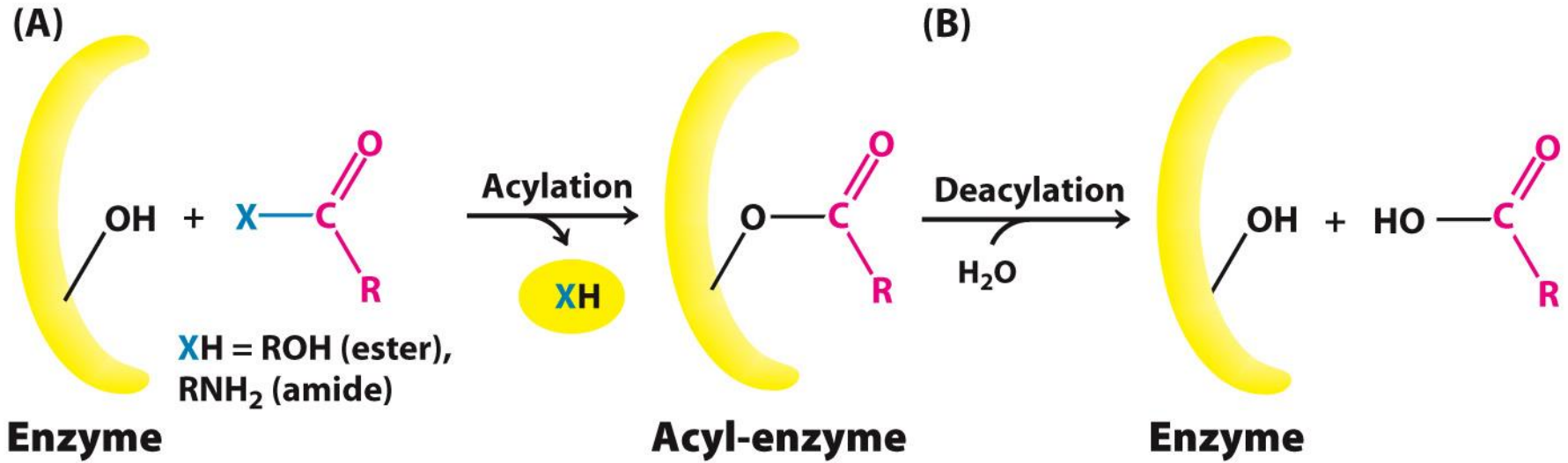


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Hydrolysis by chymotrypsin takes place in two phases:  
(A) acylation to form the acyl-enzyme intermediate  
(B) deacylation to regenerate the free enzyme.



**Figure 9.5**

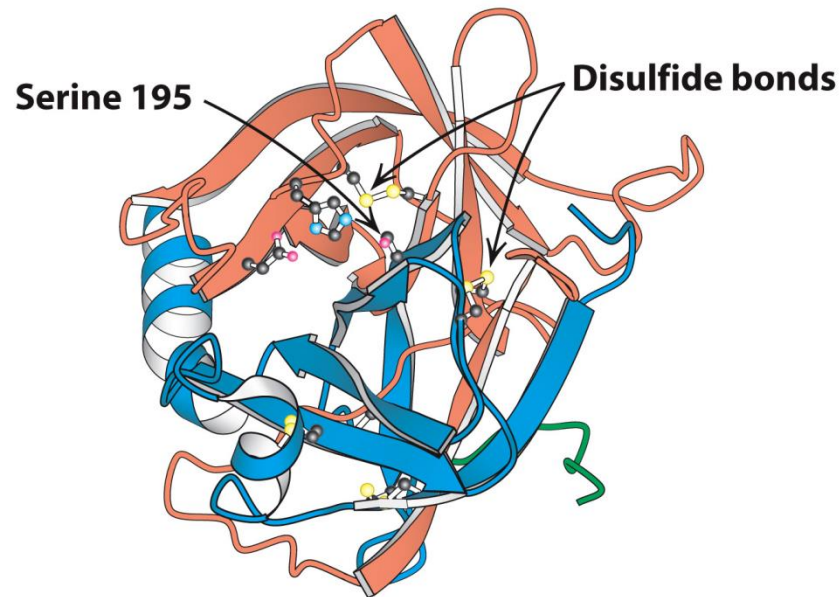
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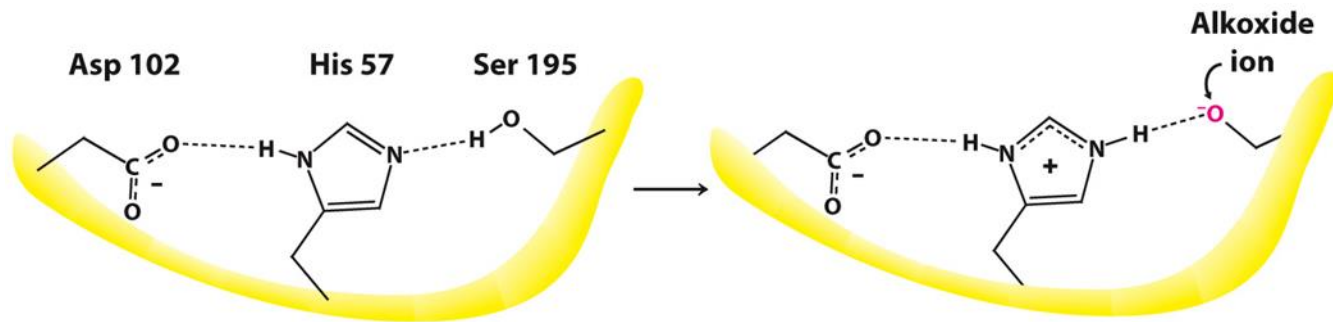


- Chymotrypsin is synthesized as single polypeptide (chymotrypsinogen) and activated (cleaved) into three polypeptide chains connected by a disulfide bridges
- The active center includes catalytic triad

## **Ser195 His 57 and Asp 102**

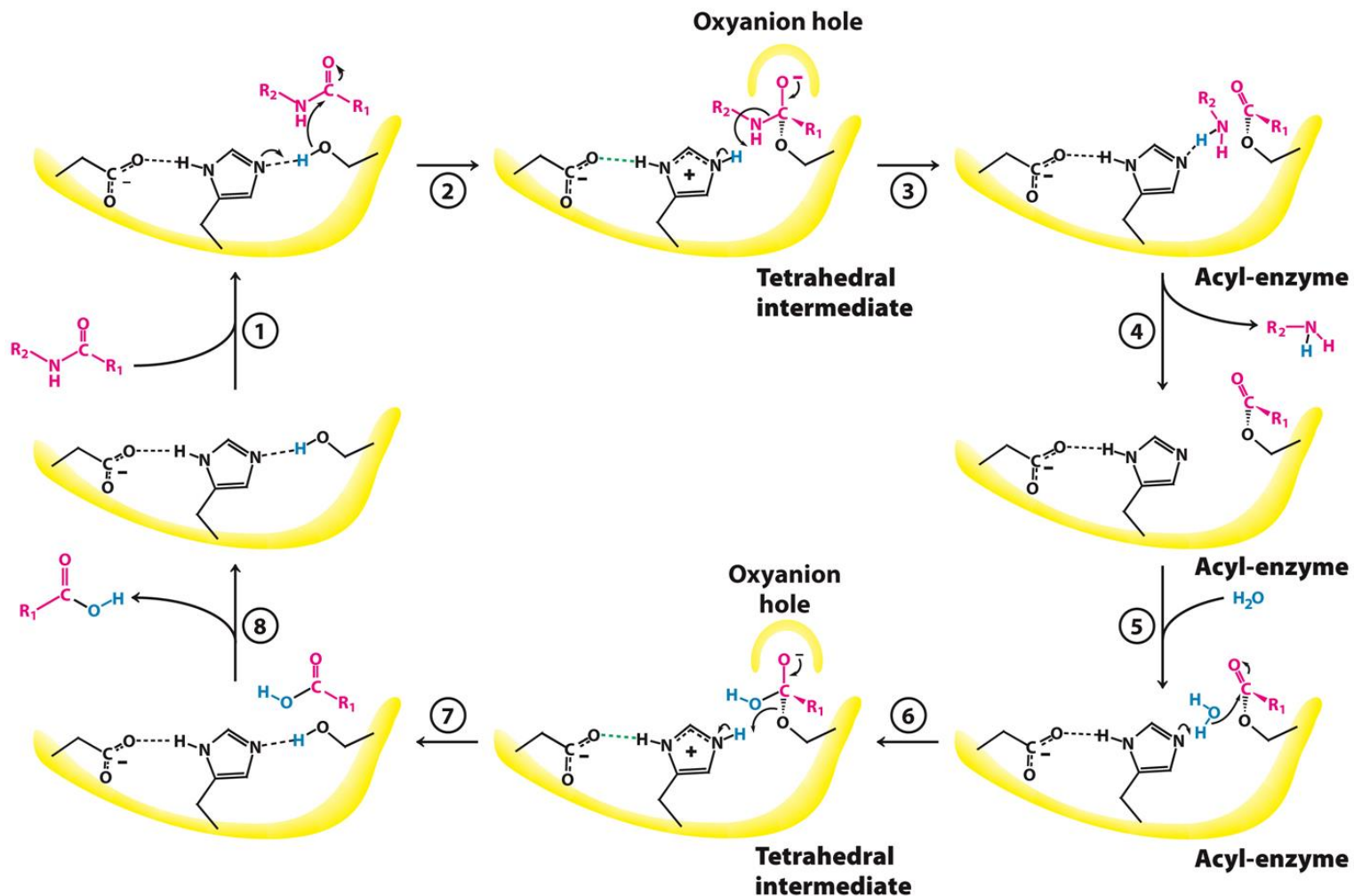


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During the catalytic reaction His57 accepts a proton from Ser 195 ; generating a n alkoxide ion that is a stronger nucleophile than the hydroxy group.

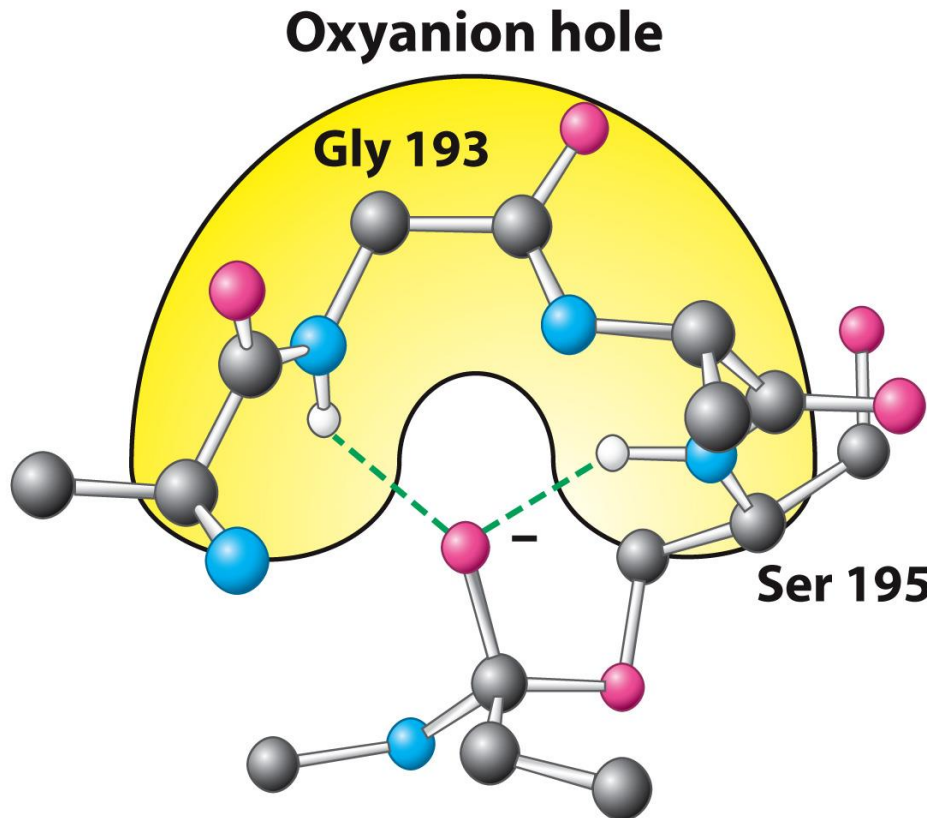


**Figure 9.8**  
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(1) substrate binding, (2) nucleophilic attack of serine on the peptide carbonyl group, (3) collapse of the tetrahedral intermediate, (4) release of the amine component, (5) water binding, (6) nucleophilic attack of water on the acyl-enzyme intermediate, (7) collapse of the tetrahedral intermediate; and (8) release of the carboxylic acid component. The dashed green lines represent hydrogen bonds.

# Oxanion hole

- a region of the active site, stabilizes the tetrahedral reaction intermediate.



The structure stabilizes the tetrahedral intermediate of the chymotrypsin reaction. Notice that hydrogen bonds (shown in green) link peptide NH groups and the negatively charged oxygen atom of the intermediate.

# Substrate specificity of chymotrypsin

The specificity of chymotrypsin is accounted for by the S1 pocket, a hydrophobic pocket that binds a hydrophobic residue on the substrate and positions the adjacent peptide bond for cleavage.

Some proteases have more complex specificity patterns. Residues on the amino-terminal side of the bond to be cleaved are labeled P1, P2, P3 and so forth, while those on the carboxyl side are labeled P1', P2' and so forth.

The corresponding sites on the enzyme are referred to as S1, S2 or S1', S2' and so forth.

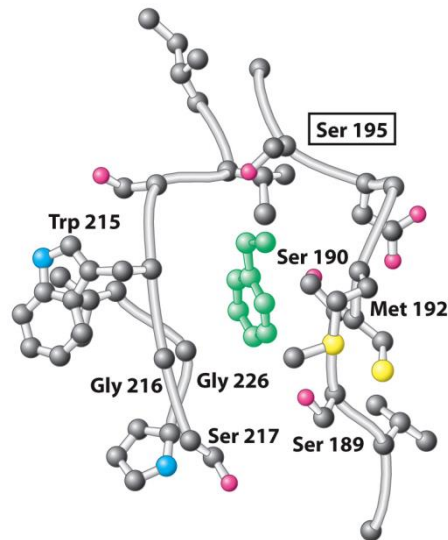
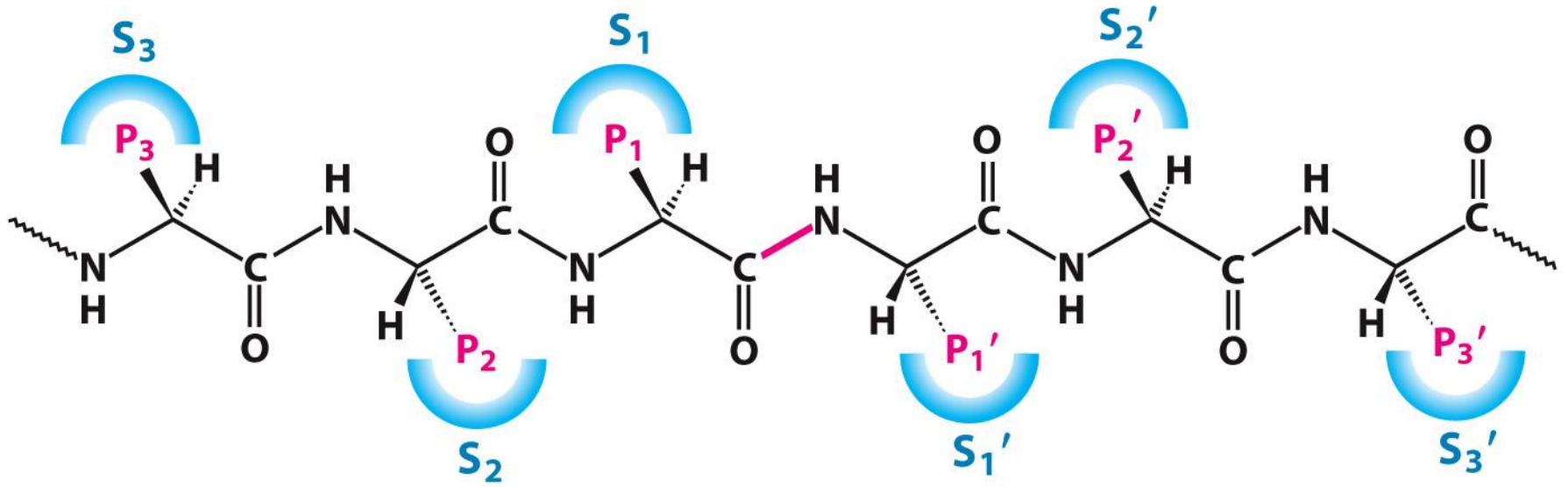


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**Figure 9.11**  
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# Catalytic triads in other proteases

Trypsin and elastase contains analogous catalytic triad residues and the catalytic mechanism is the same as in chymotrypsin

Differences are in substrate specificity; trypsin cleaves after positively charged residues whereas elastase cleaves after the residues with the small side chain

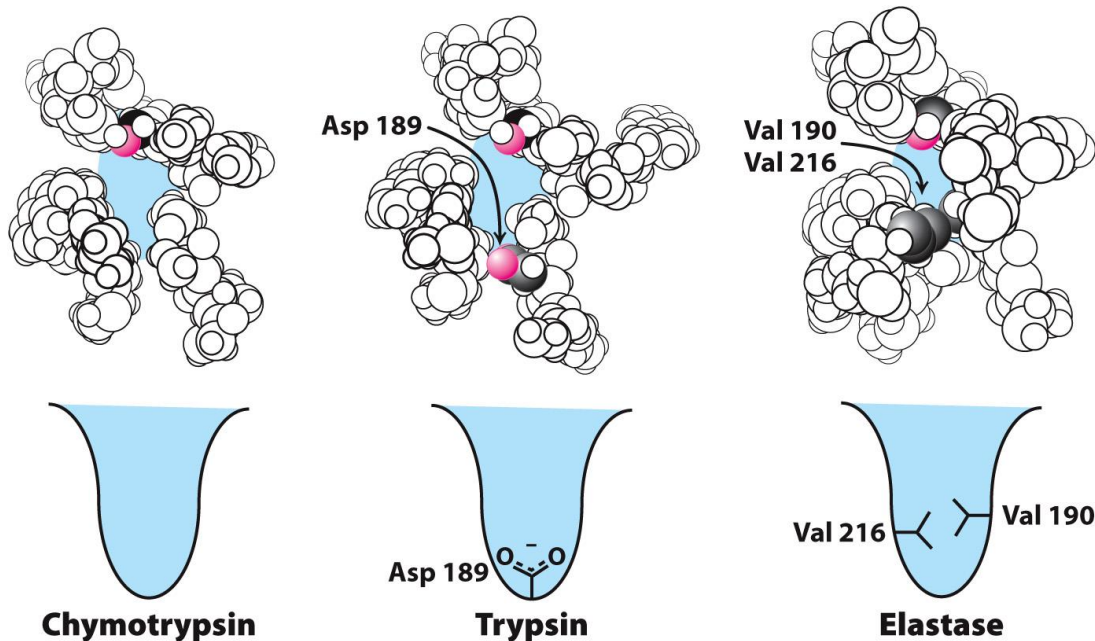


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# Other types of proteases

Not all proteases rely on an activated serine at the active site.

Cysteine, aspartate and metals are used to generate a nucleophile that attacks the carbonyl group of the peptide bond.

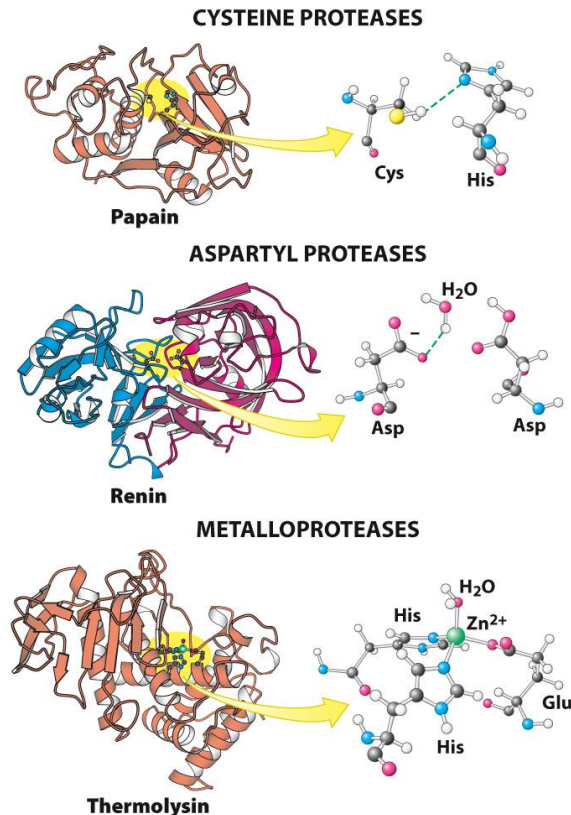


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## (A) CYSTEINE PROTEASES

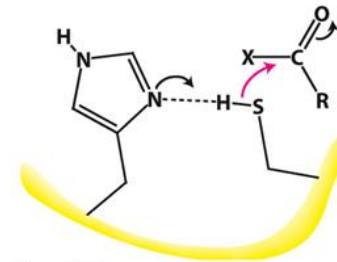
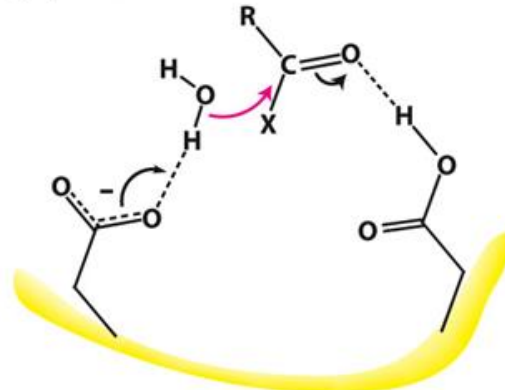
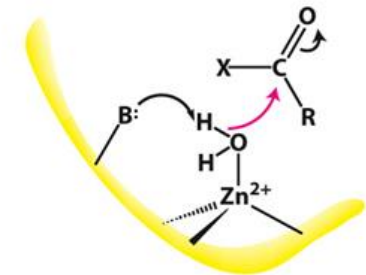


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## (B) ASPARTYL PROTEASES



## (C) METALLOPROTEASES

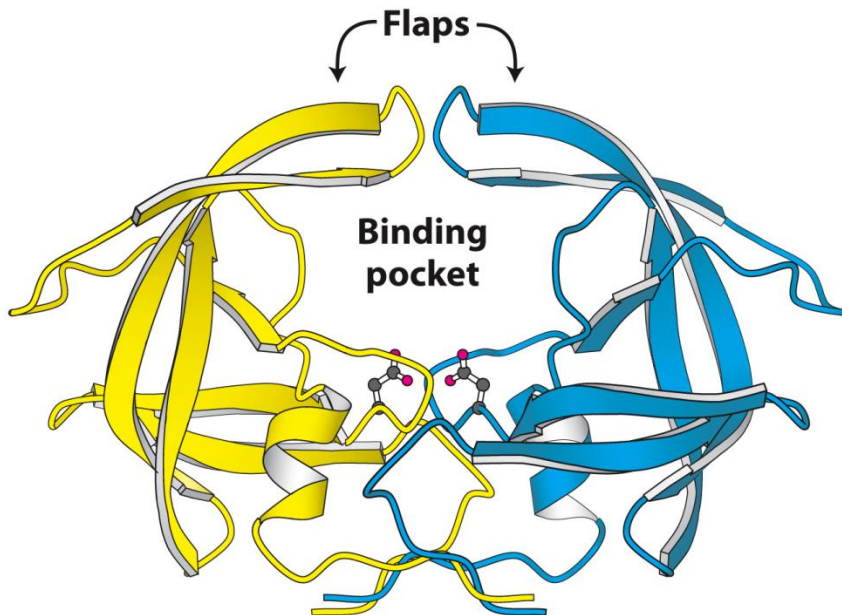




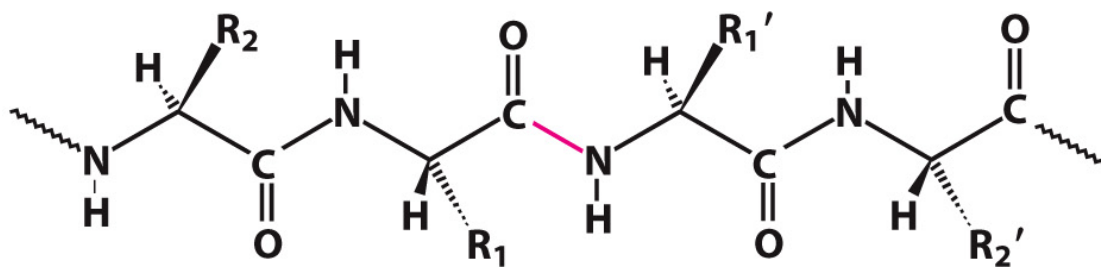
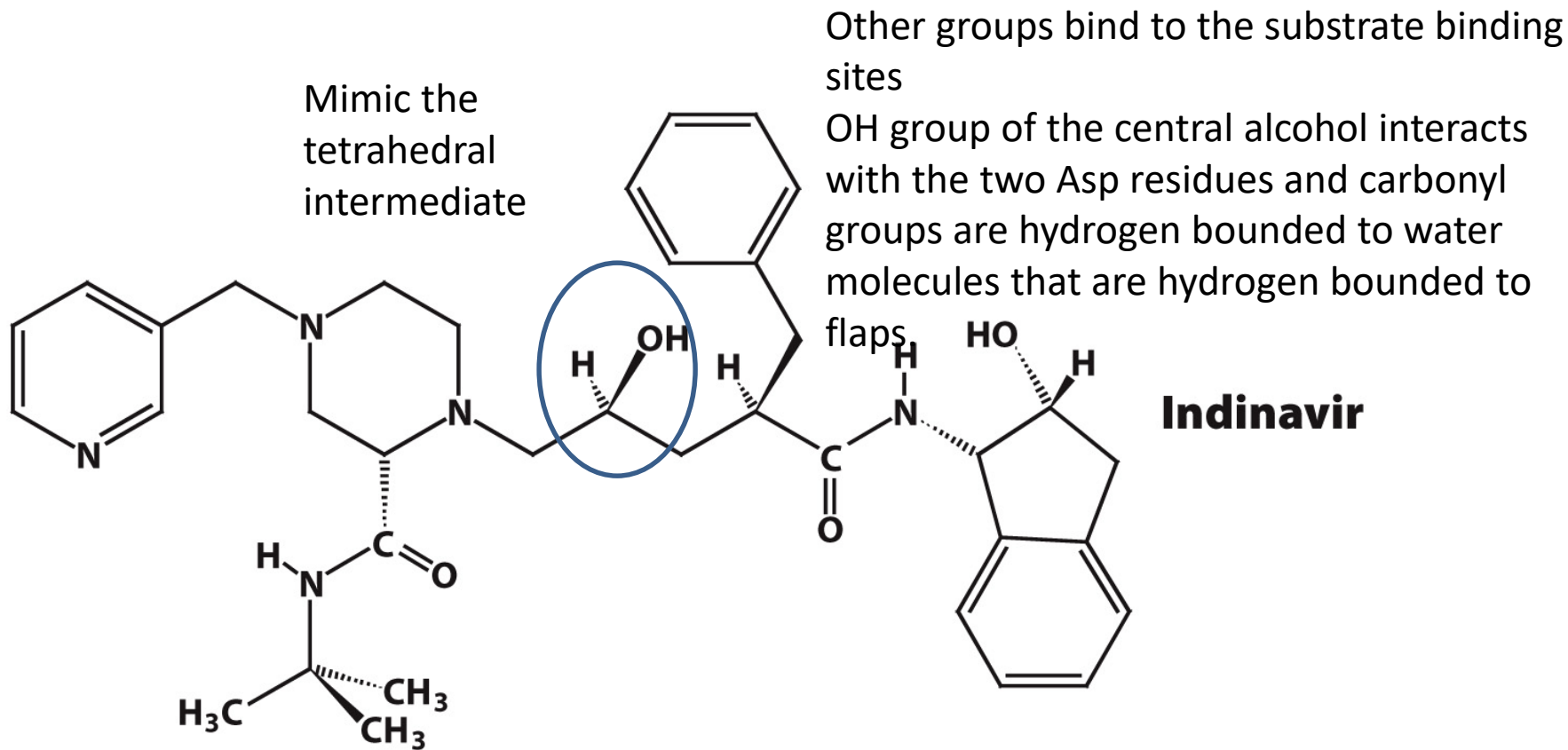
# Protease inhibitors are important class of drugs

Indinavir, an inhibitor of the HIV aspartyl protease, is an important drug used in the treatment of AIDS.

Indinavir is a substrate analog of the HIV protease that cleaves multidomain viral proteins into their active forms. Inhibition of the HIV protease prevent virus to become infectious



HIV protease, a dimeric aspartyl protease. The protease is a dimer of identical subunits, shown in blue and yellow, consisting of 99 amino acids each. Notice the placement of active site aspartic acid residues, one from each chain, which are shown as ball-and-stick structures. The flaps will close down on the binding pocket after substrate has been bound.



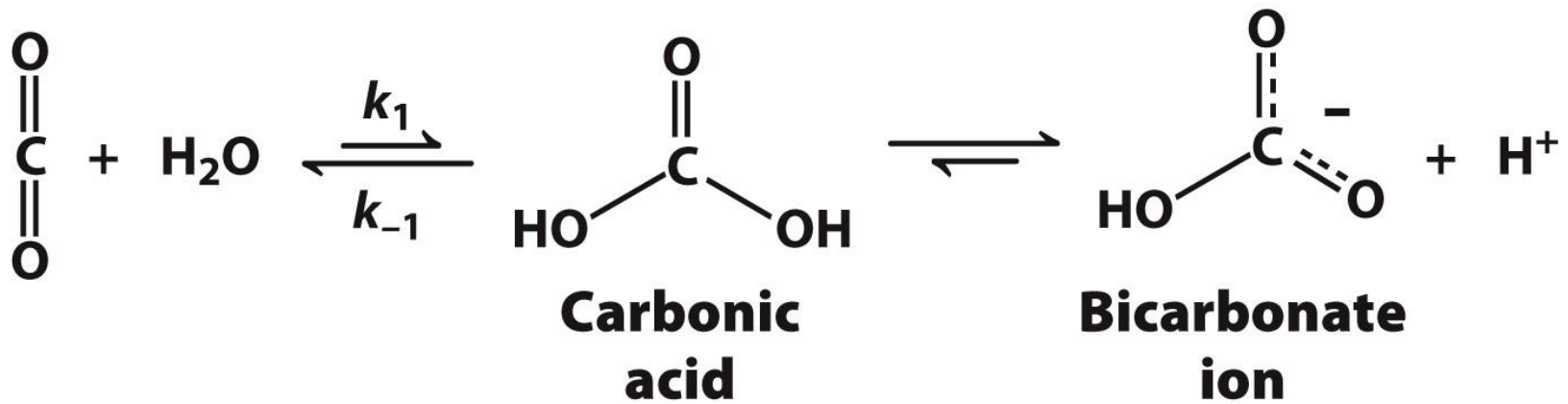
**Figure 9.19**

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# Carbonic anhydrase

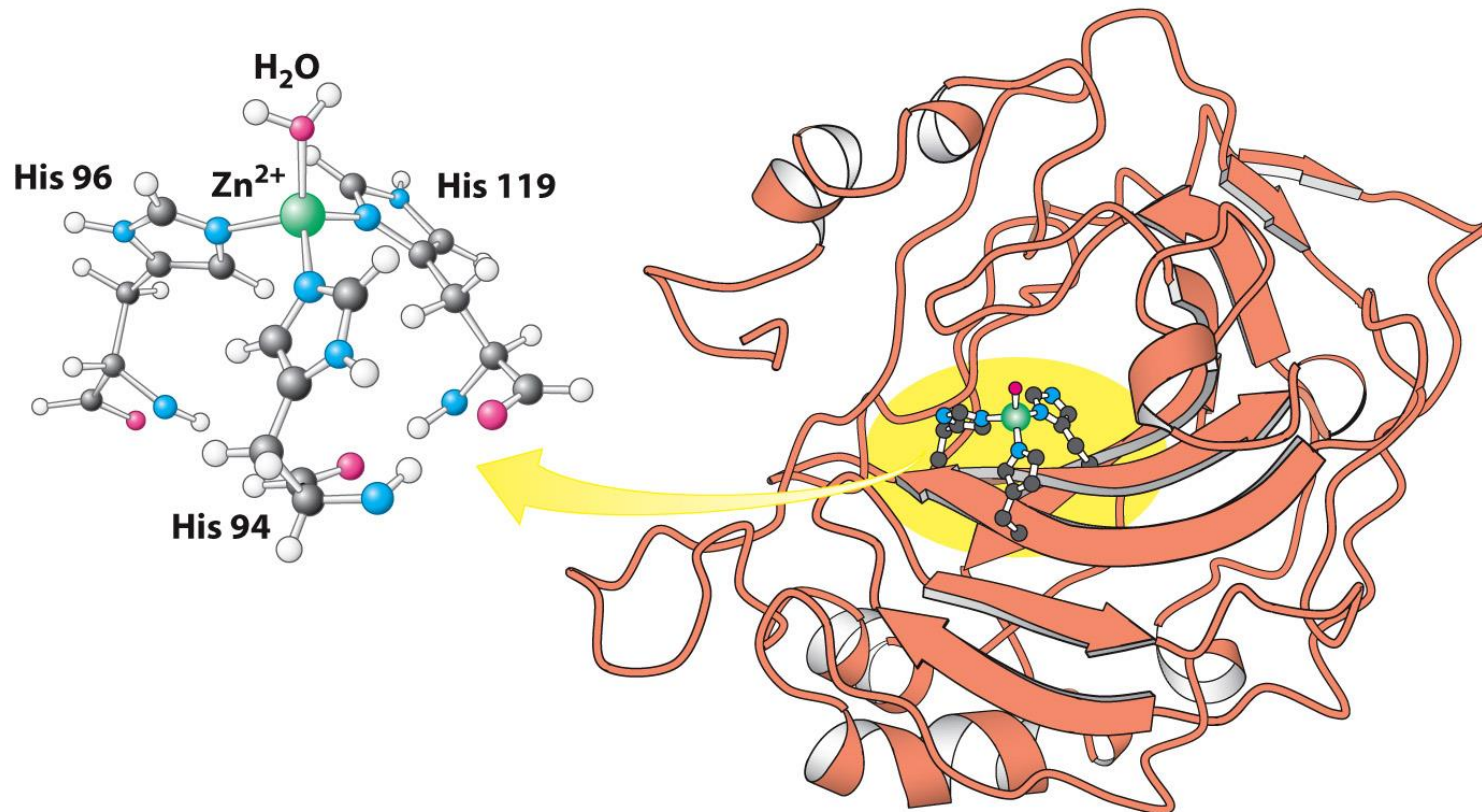
Carbon dioxide is an end product of aerobic metabolism. Carbon dioxide is converted into bicarbonate ion and a proton by the enzyme carbonic anhydrase. In the lungs, the bicarbonate is converted to CO<sub>2</sub> and exhaled.

Carbonic anhydrases play roles in the generation of the aqueous humor of the eye and a lack of carbonic anhydrase activity has been associated with osteopetrosis (excessive formation of dense bones) and intellectual disability.



The human genome contains at least seven genes encoding carbonic anhydrase and they are all homologs.

The  $\text{Zn}^{2+}$  is bound to 4 or more ligands.



**Figure 9.21**  
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The maximal rate of carbon dioxide hydration is attained at pH 8. Activity falls with a drop in pH and the titration curve suggests that an active site component with a  $pK_a$  of 7 is required.

$Zn^{2+}$  lowers the  $pK_a$  of water to 7, generating the potent nucleophile  $OH^-$ .

Adjacent to  $Zn^{2+}$  is a carbon dioxide binding site.

The  $OH^-$  attacks the  $CO_2$ , converting it to bicarbonate.

Studies with synthetic analog systems confirm the important role of  $Zn^{2+}$

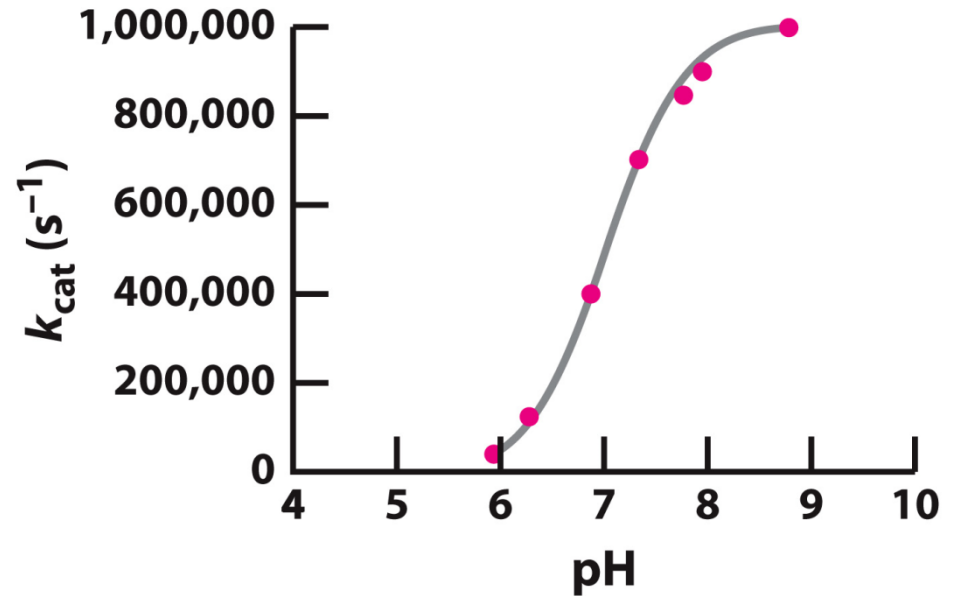
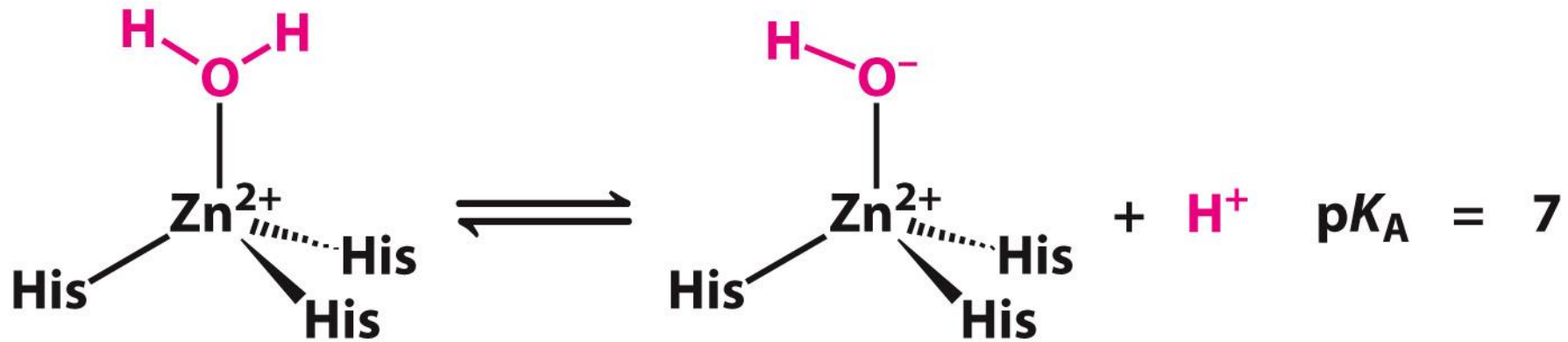


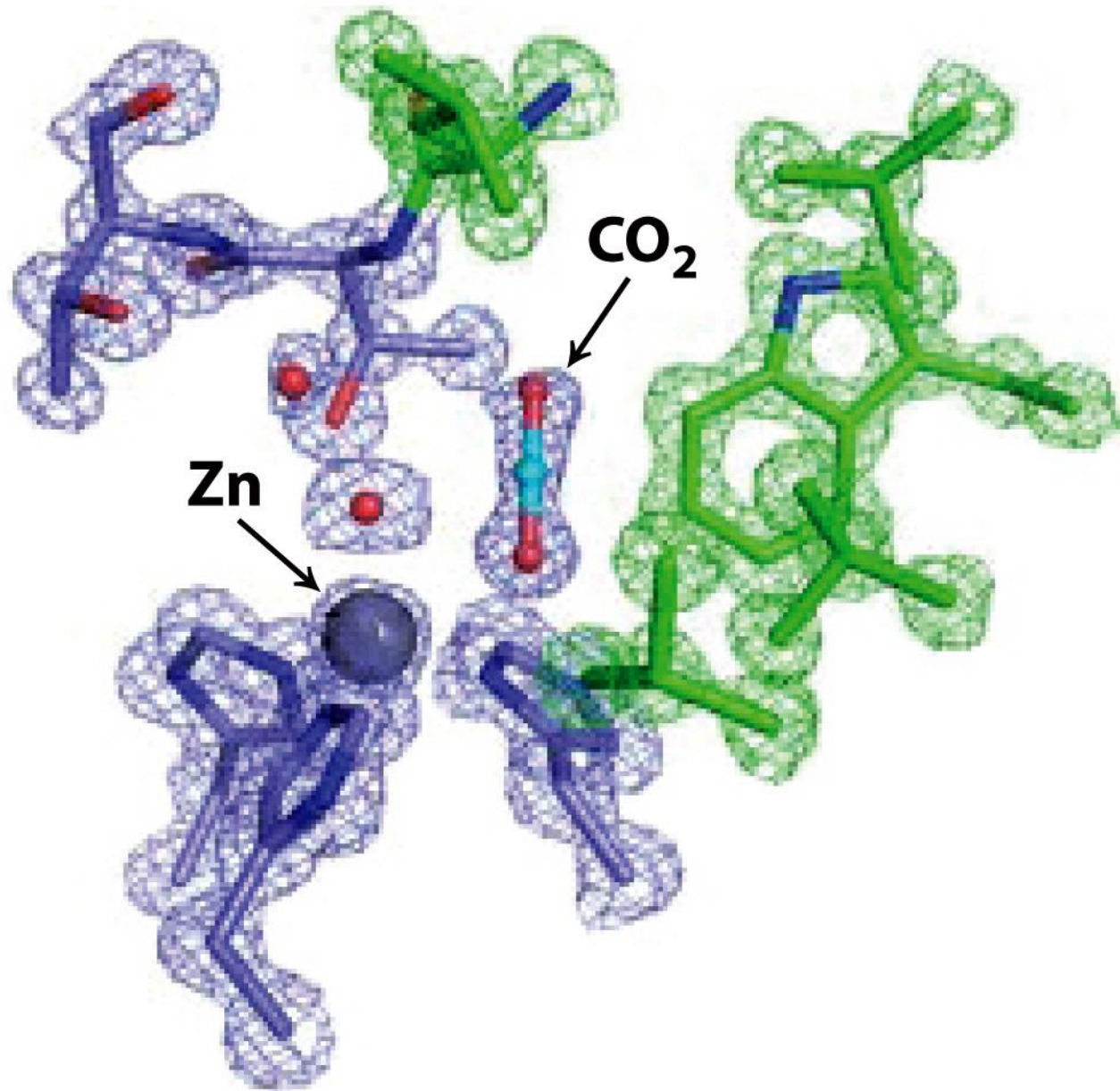
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**Figure 9.23**

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The pKa of zinc-bound water. Binding to zinc lowers the pKa of water from 15.7 to 7.



**Figure 9.24**

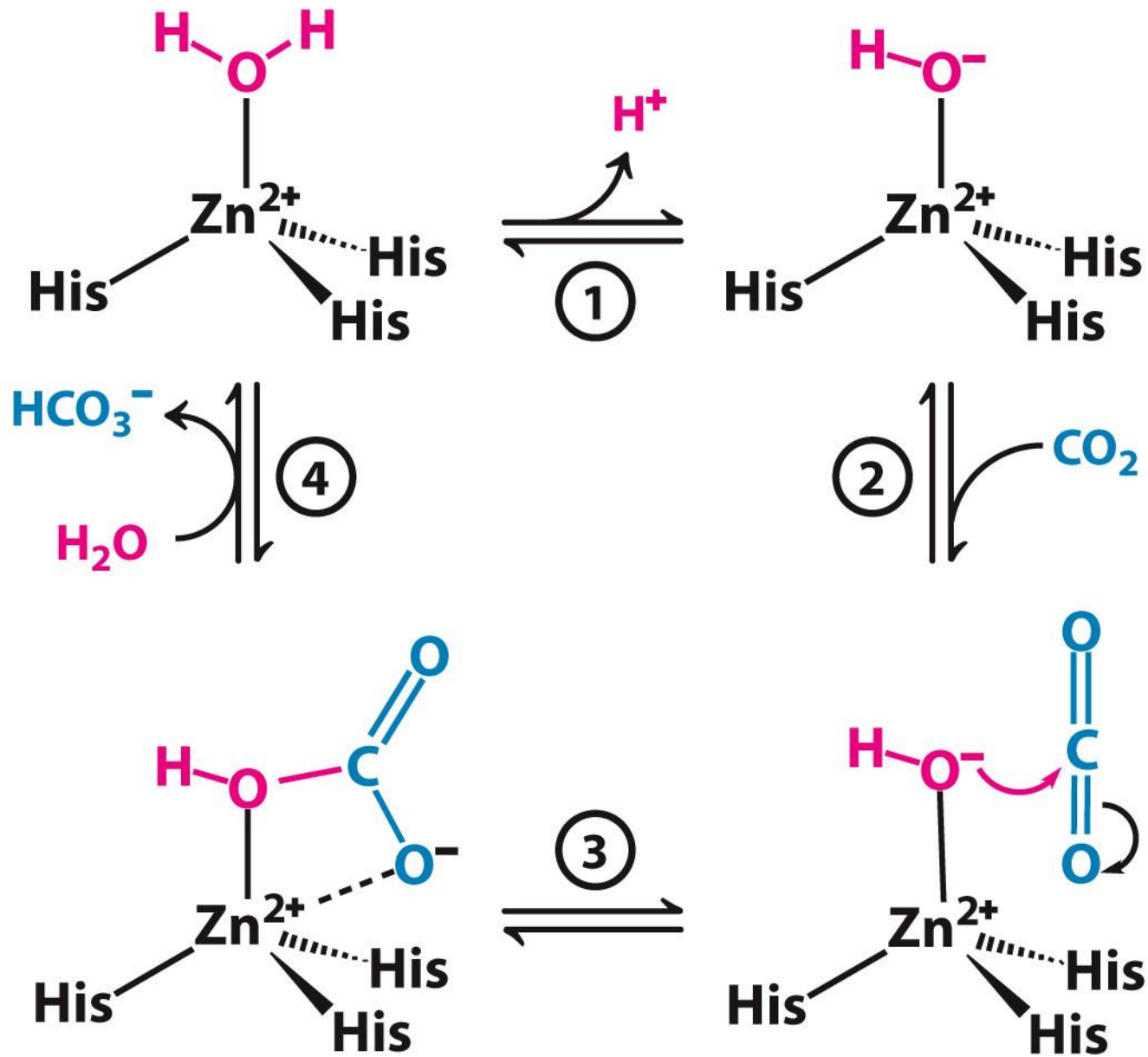
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A proposed mechanism for the hydration of carbon dioxide follows:

1.  $\text{Zn}^{2+}$  generates  $\text{OH}^-$  by facilitating the release of a  $\text{H}^+$  from water.
2.  $\text{CO}_2$  binds to the active site and is positioned to react with  $\text{OH}^-$ .
3. The  $\text{OH}^-$  attacks the  $\text{CO}_2$ , generating  $\text{HCO}_3^-$ .
4. The active site is regenerated with the release of  $\text{HCO}_3^-$  and binding of  $\text{H}_2\text{O}$ .

Studies with a synthetic analog system provide support for the proposed mechanism.



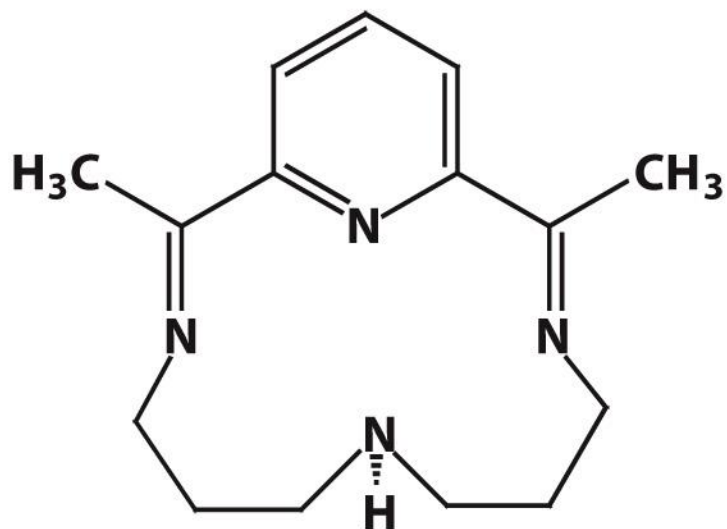


**Figure 9.25**

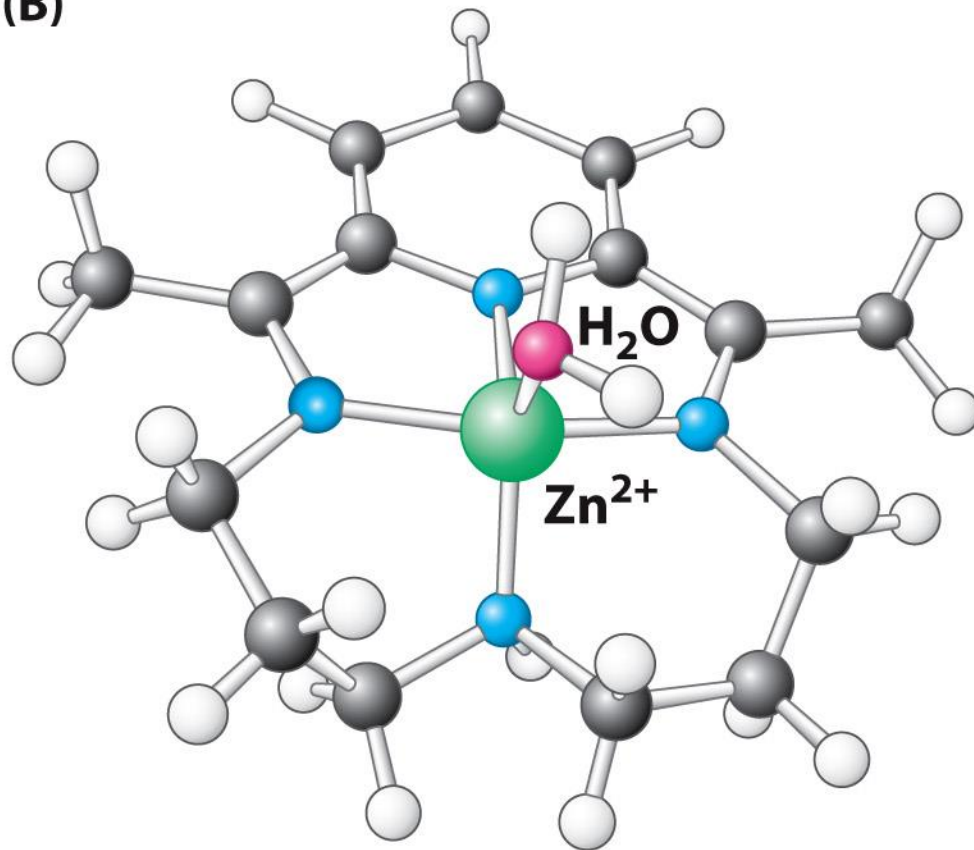
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**A synthetic analog model system for carbonic anhydrase.**

**(A)**



**(B)**



**Figure 9.26**

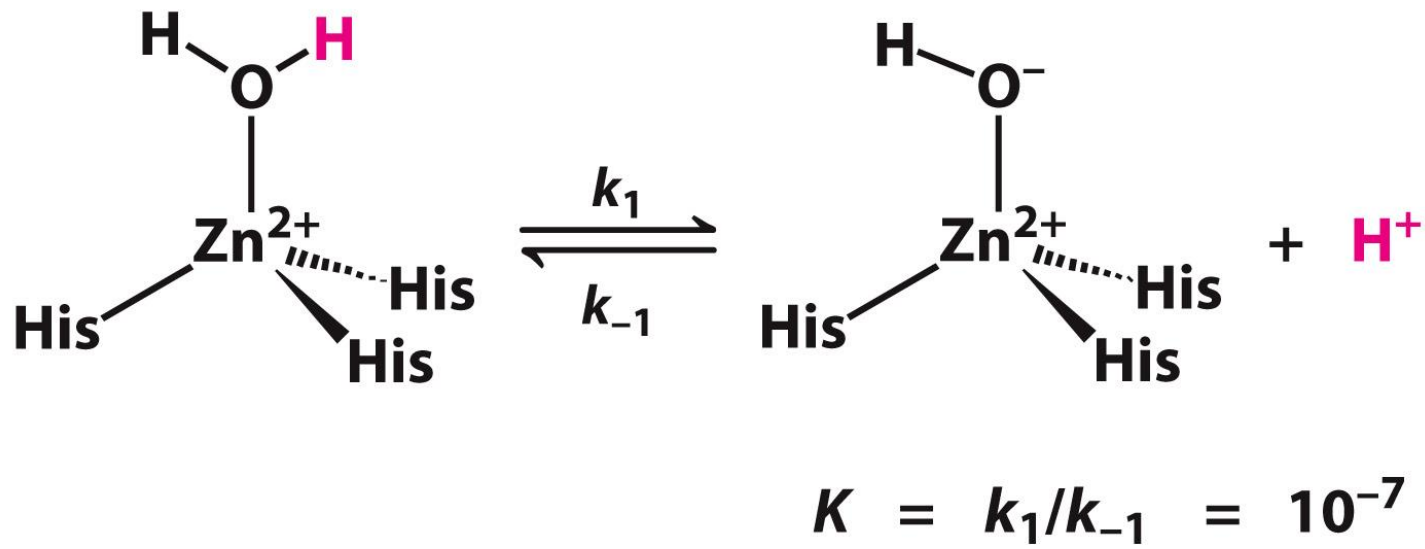
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The zinc complex of this ligand accelerates the hydration of carbon dioxide more than 100-fold under appropriate conditions.

The zinc-bound water molecule must lose a proton to begin the hydration reaction.

The backward reaction, the reprotonation of the  $\text{OH}^-$ , is prevented by the binding of the released proton by a molecule of buffer.

A histidine residue at the active site serves as a proton shuttle, transferring the released proton from water to the buffer.



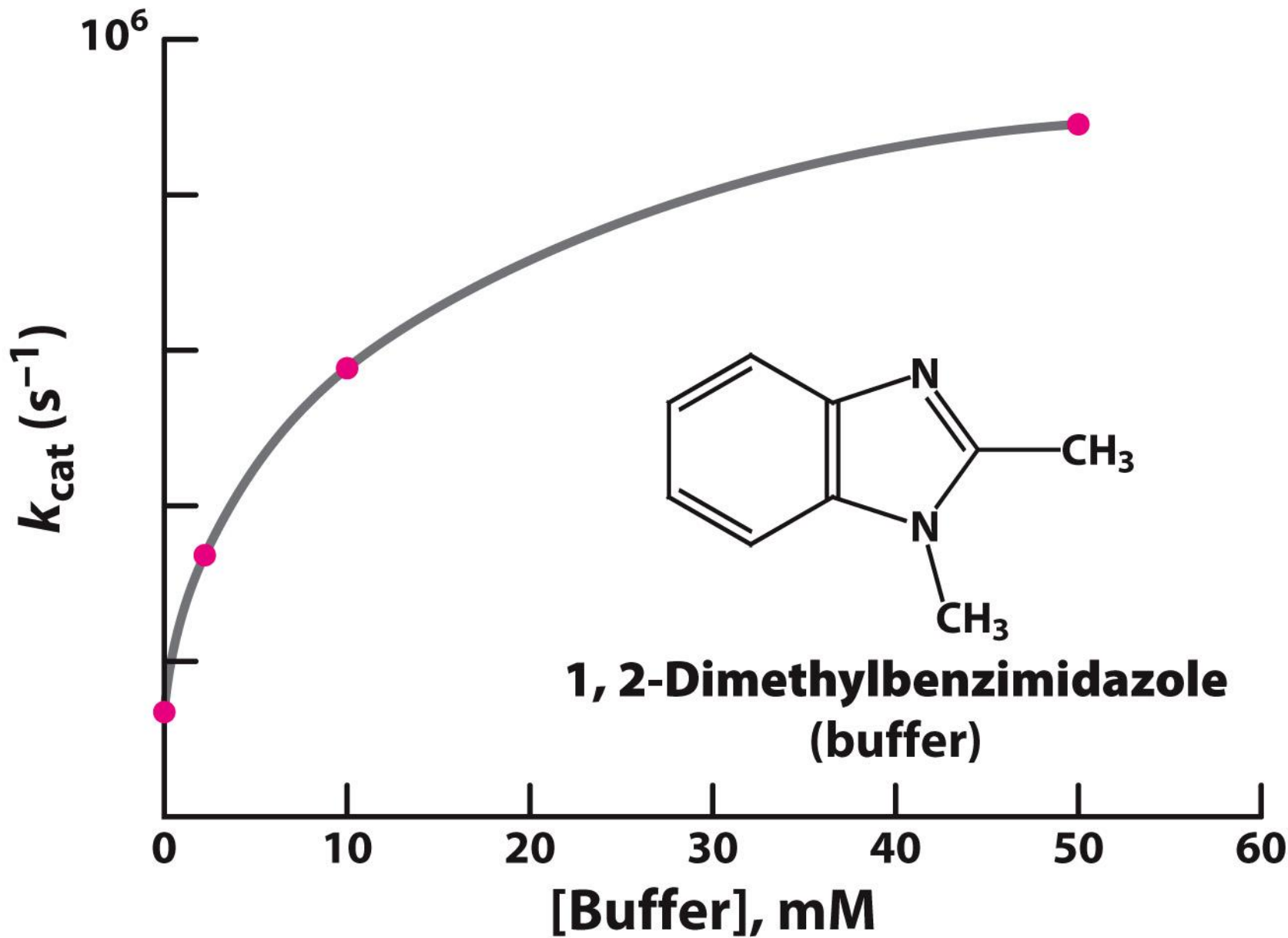
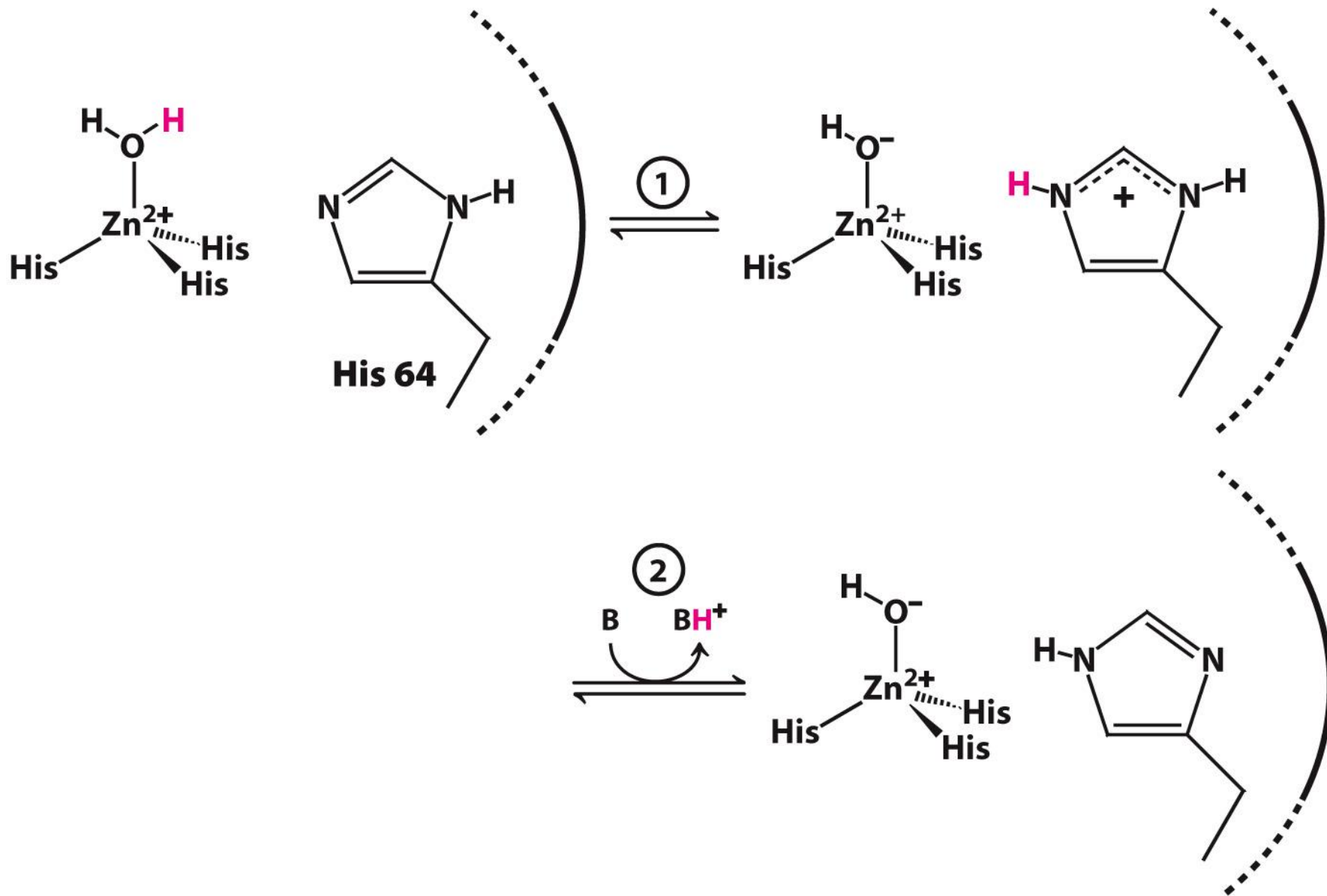


Figure 9.29



**Figure 9.30**

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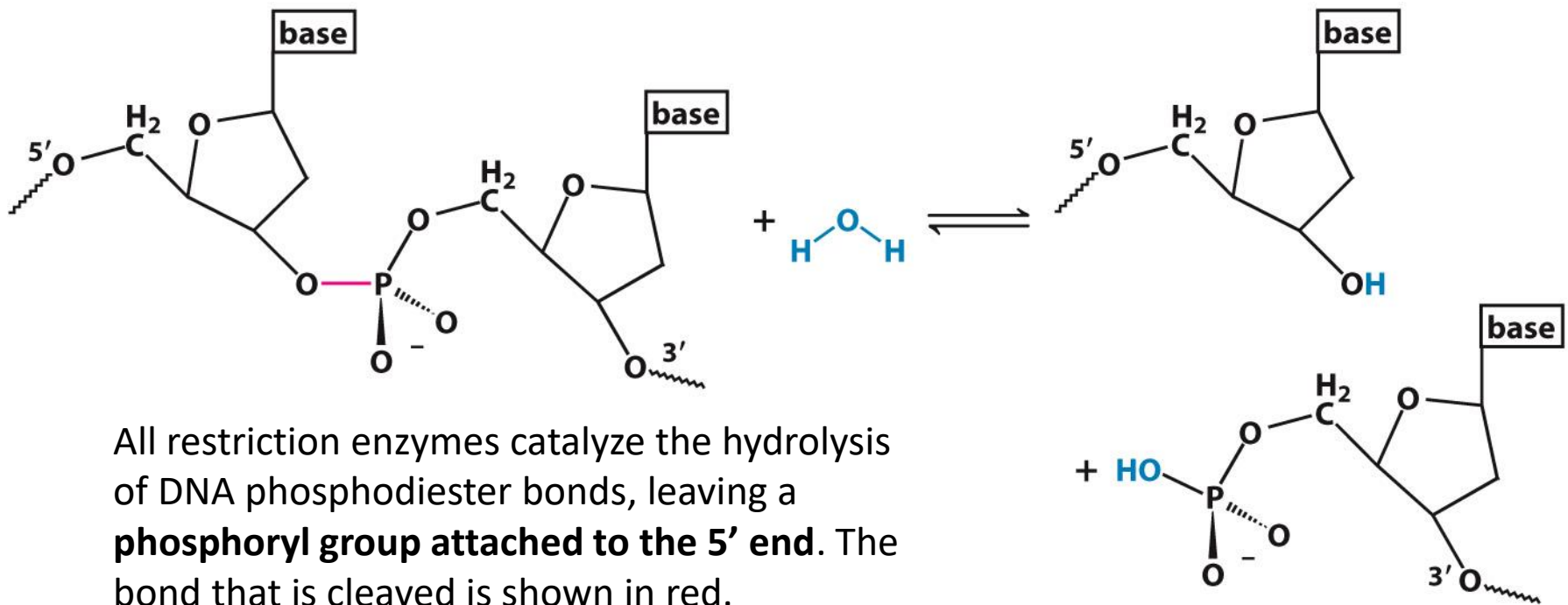
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# Restriction enzymes

Bacteria have restriction endonucleases (restriction enzymes) that degrade viral DNA.

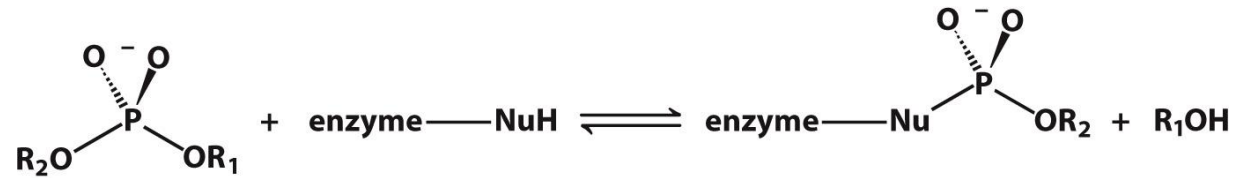
Type II restriction enzymes cleave within specific sequences of target DNA (cognate DNA), called recognition sequences or recognition sites.

Restriction enzymes do not degrade host DNA containing recognition sites.

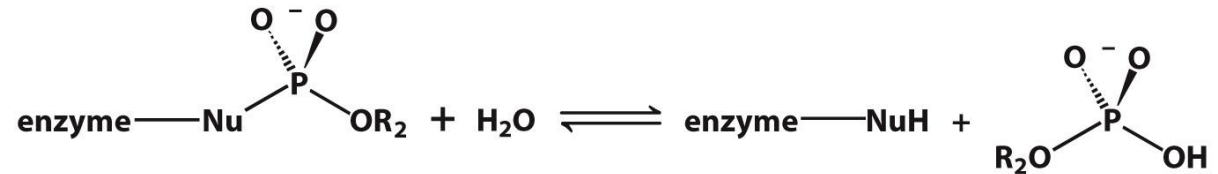


**Figure 9.31**

Restriction enzymes catalyze the hydrolysis of a phosphodiester bond. Two mechanisms are possible: One involving a covalent intermediate and the other direct hydrolysis.

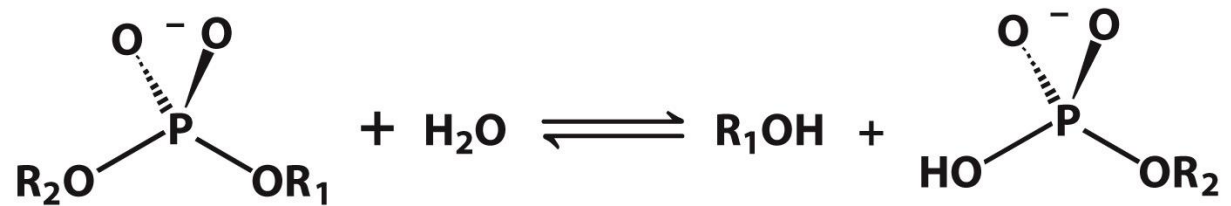


Mechanism 1  
(covalent intermediate)



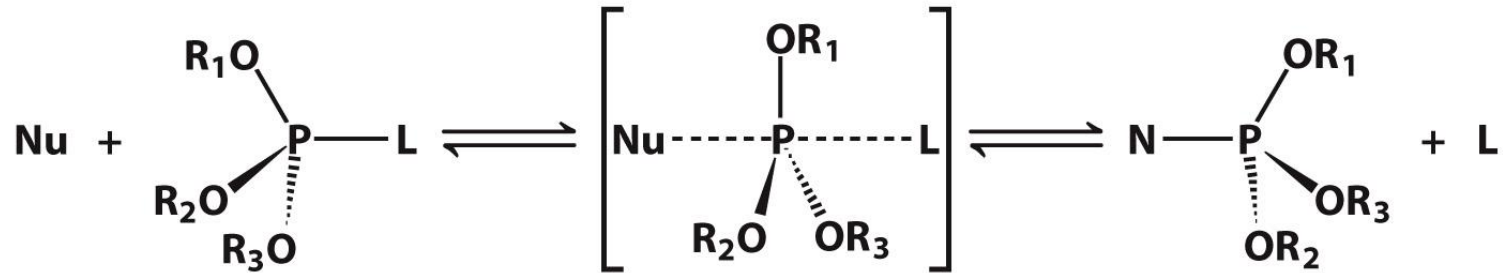
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Mechanism 2  
(direct hydrolysis)



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Each mechanism relies on an in-line displacement reaction.



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In mechanism 1, the stereochemical configuration of the phosphorous atom will be retained, while mechanism 2 will generate an inverted configuration.

Replacing an oxygen with a sulfur in the phosphodiester linkage to be cleaved established mechanism 2 as correct.



Multiple magnesium ions are required cofactors for restriction enzymes.

The magnesium may play a role in activating a water molecule that attacks the phosphorus atom in the cleavage reaction.

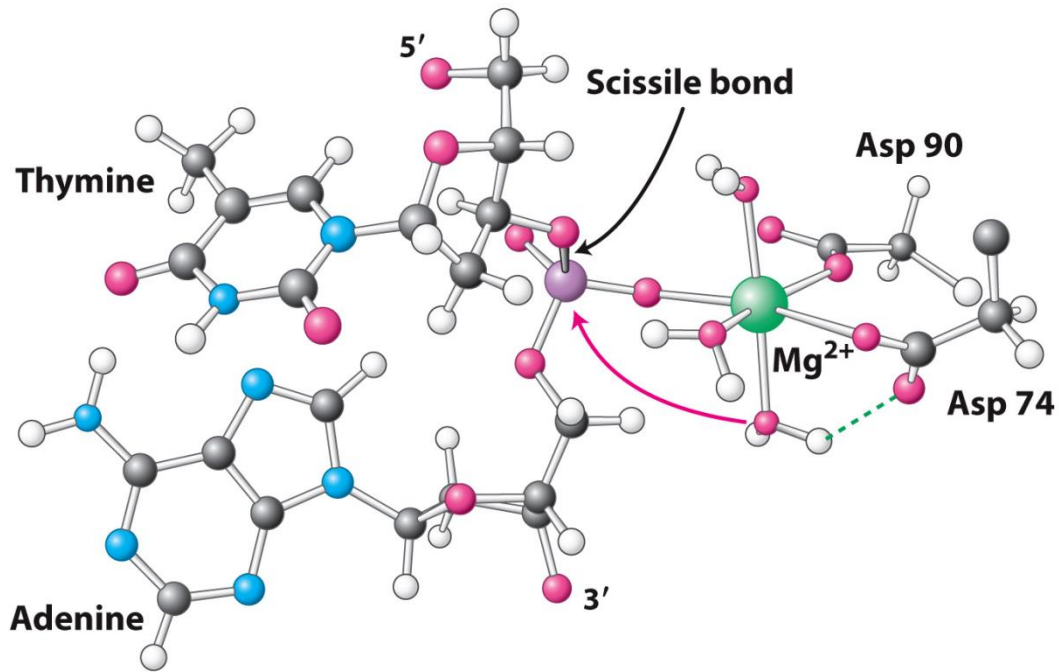


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**A magnesium ion-binding site in EcoRV endonuclease.** The magnesium ion helps to activate a water molecule and positions it so that it can attack the phosphorus atom.

Most restriction enzyme recognition sequences are inverted repeats, which display a twofold rotational symmetry.

Restriction enzymes themselves display a corresponding symmetry. The enzymes are dimers in which the subunits are related by twofold symmetry.

Binding of the enzyme to the recognition sequence distorts the DNA by introducing a kink.

Structure of the recognition site of EcoRV endonuclease.

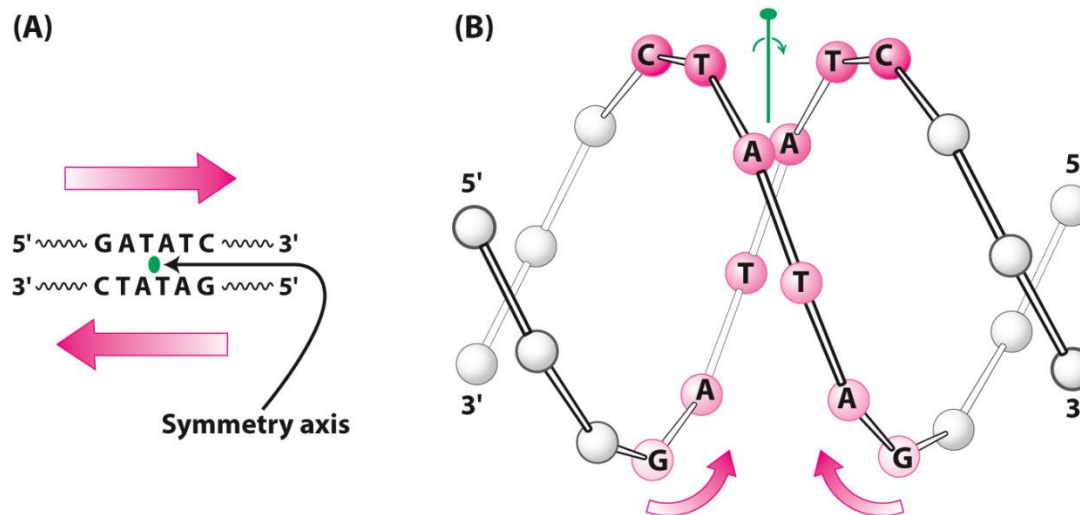
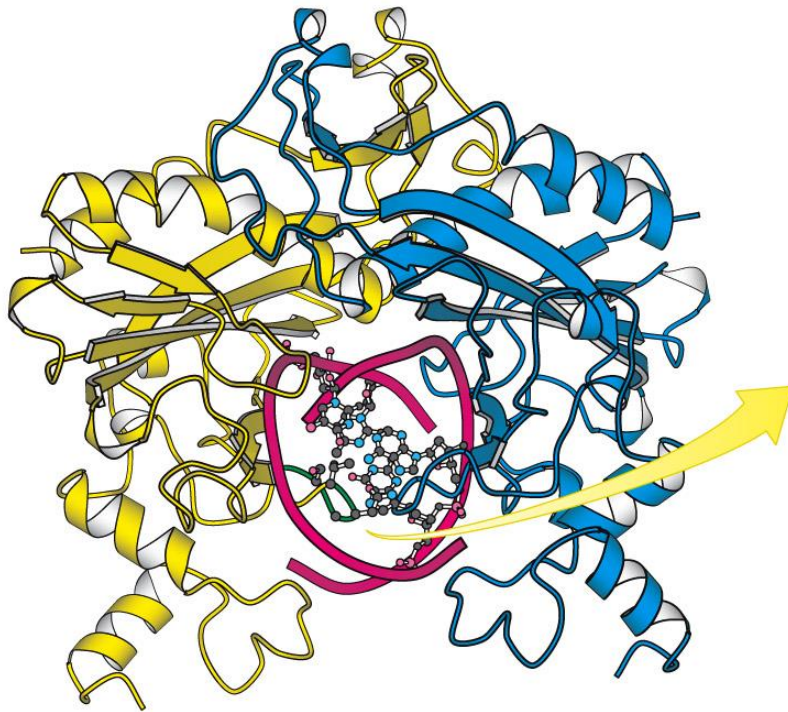
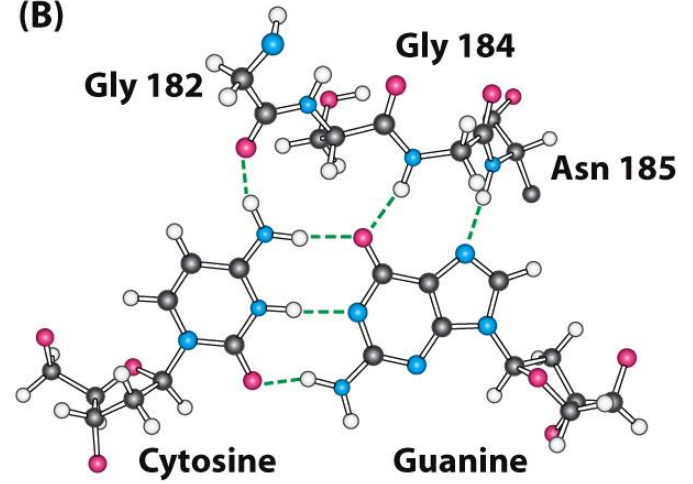


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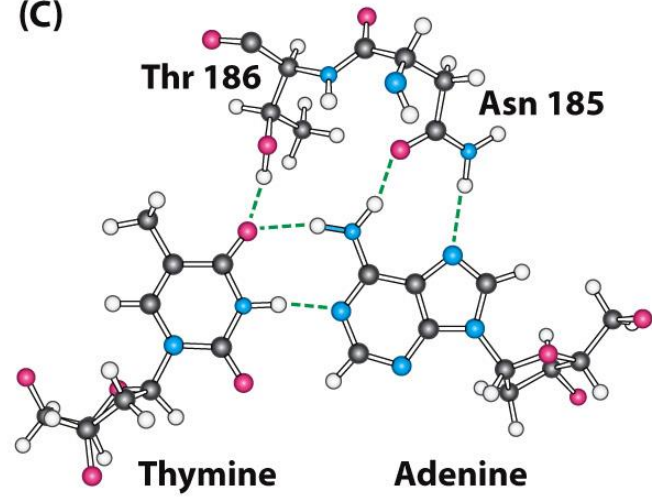
(A)



(B)



(C)



**Figure 9.36**

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The structure of noncognate DNA is not distorted by the enzyme.

Distortion is crucial for catalysis because it locates the phosphoanhydride linkage near the magnesium, which is crucial for hydrolysis.

The distorted DNA makes additional contacts with the enzyme, thereby increasing the binding energy

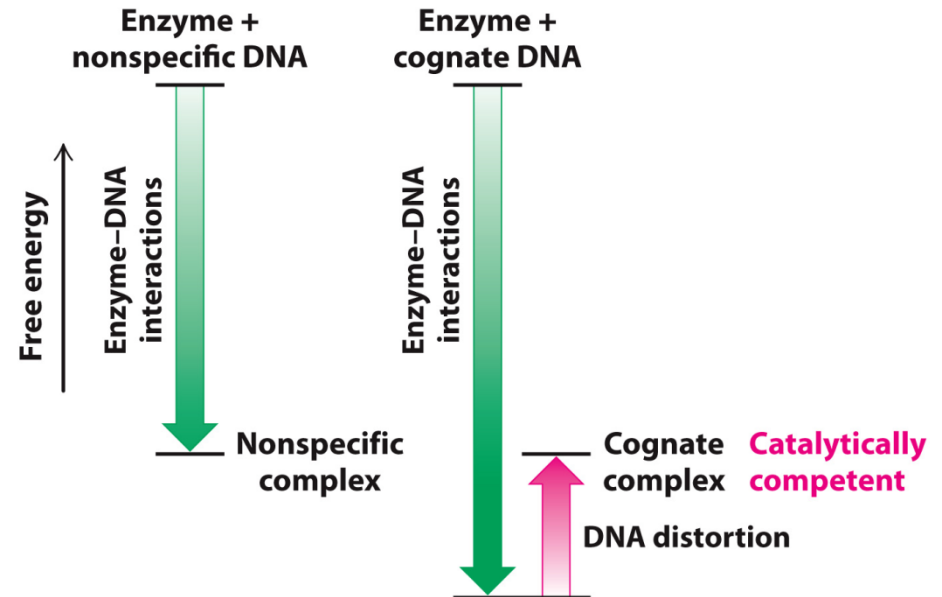
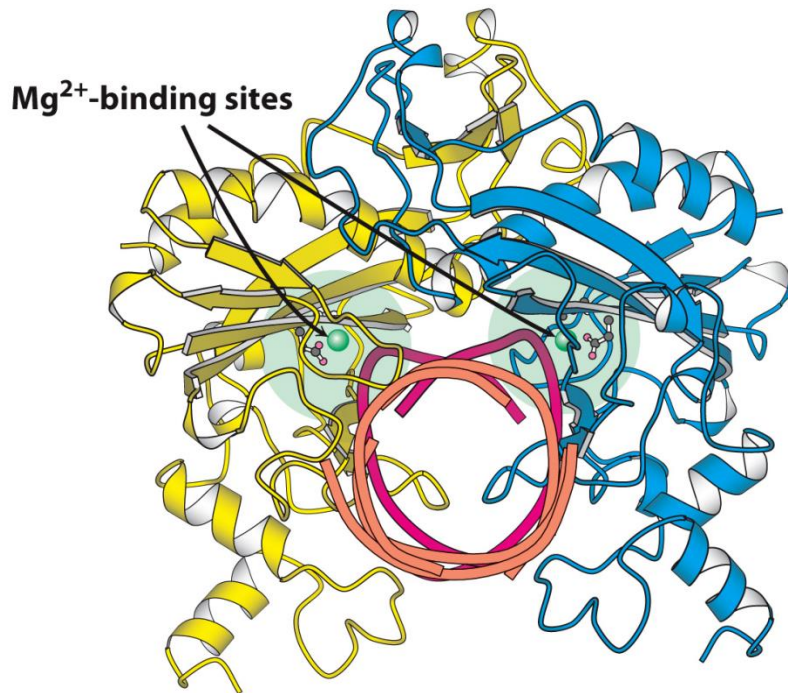
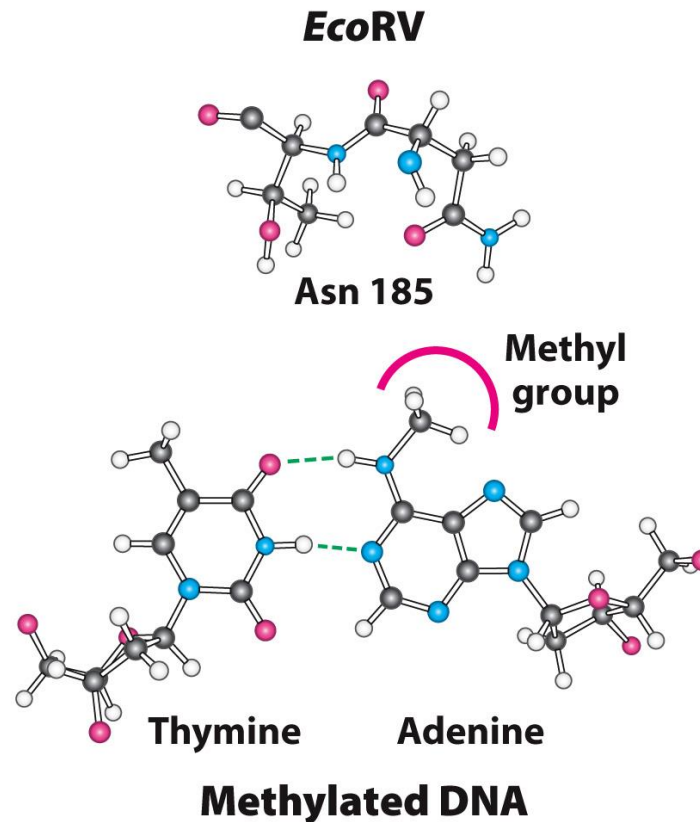


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Complex with nonspecific DNA does not bind Mg<sup>2+</sup> whereas complex with cognate DNA bind Mg<sup>2+</sup>

Recognition sites in the host DNA are modified by the addition of a methyl group, a reaction catalyzed by methylases.

Methylation prevents hydrolysis by restriction enzymes by preventing the formation of contacts necessary to distort the DNA.



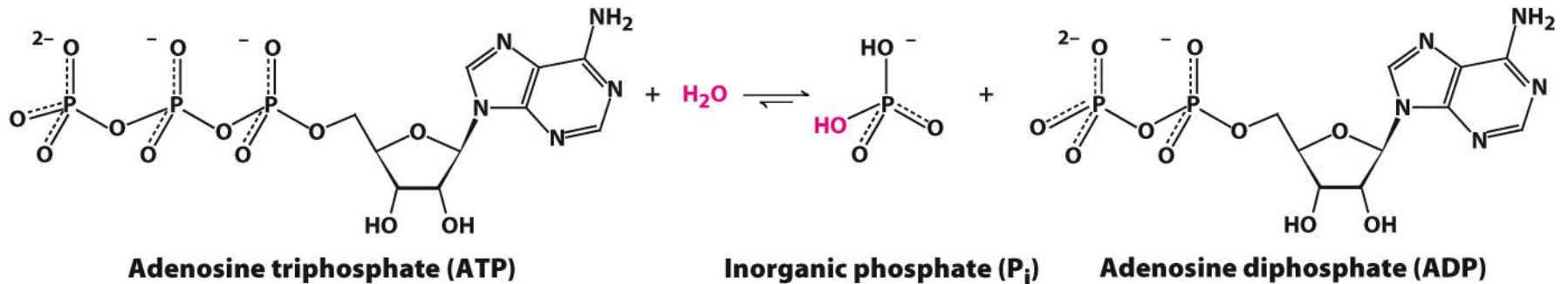
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# Myosin

Couples ATP hydrolysis to a mechanical work

<http://www.nature.com/nature/journal/v468/n7320/full/nature09450.html#access>

<https://www.youtube.com/watch?v=KfEbuHCGIlo>

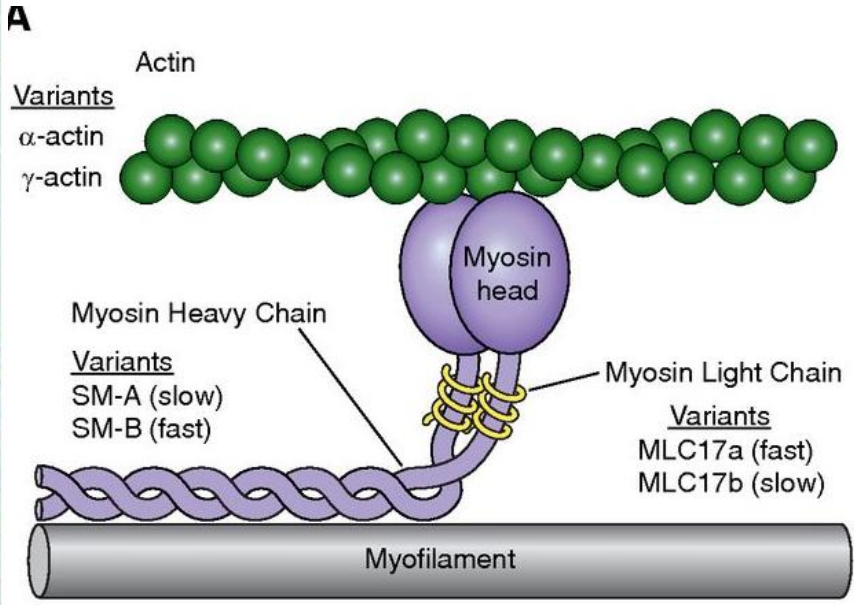
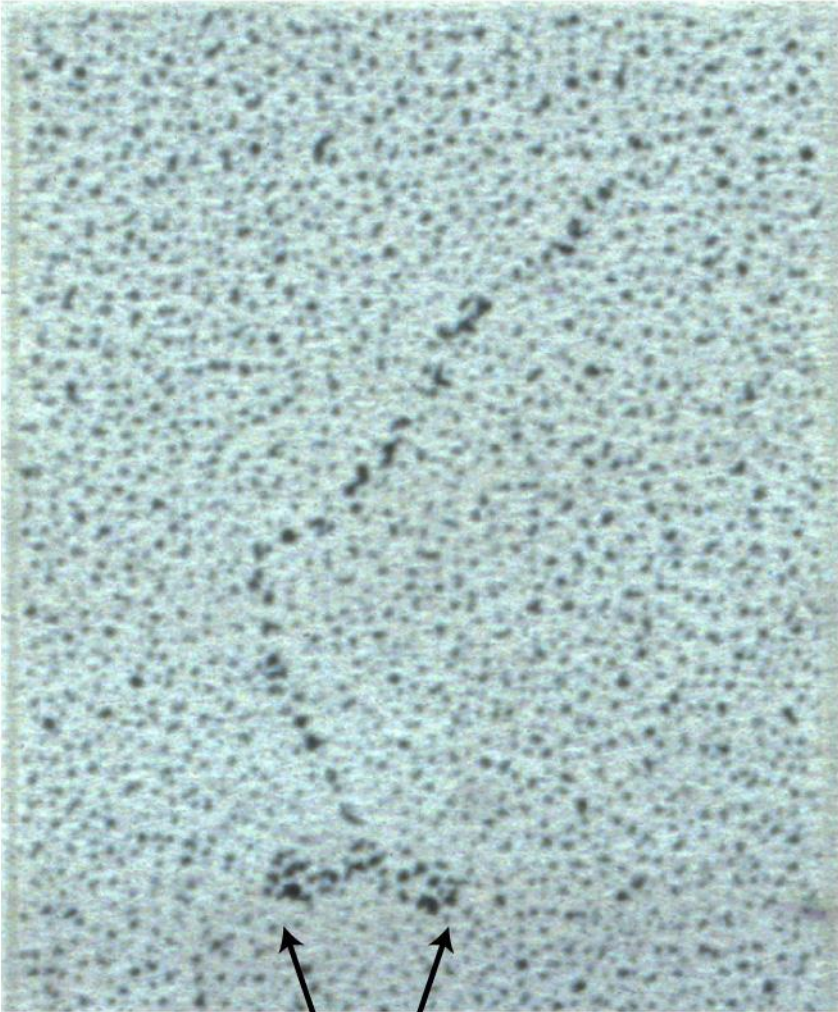


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# Structure of myosin

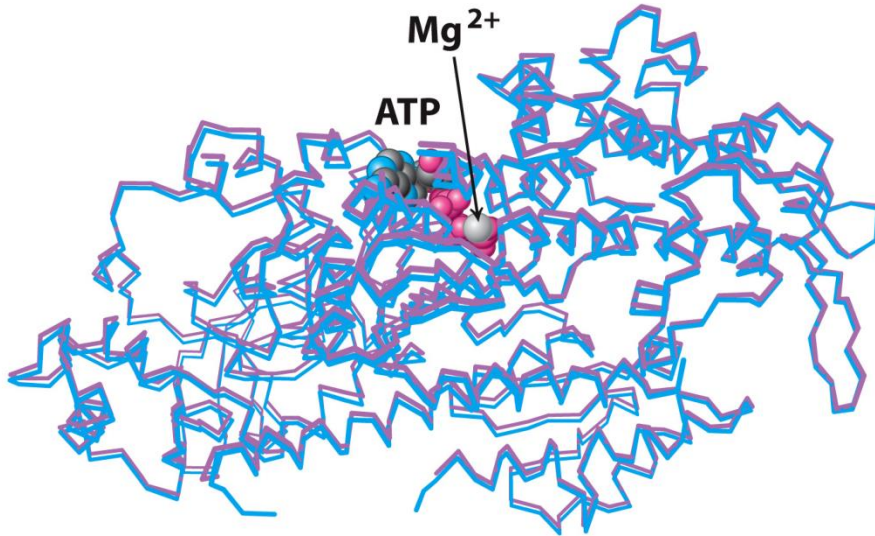


**Globular ATPase domains**

**Figure 9.42**  
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Courtesy of Dr. Paula Flicker, Dr. Theo Walliman, and Dr. Peter Vibert

ATP must be bound to  $Mg^{2+}$  or  $Mn^{2+}$  to function as a substrate for myosin.

Most enzymes that hydrolyze nucleoside triphosphates require the nucleotide to be in a complex with  $Mg^{2+}$  or  $Mn^{2+}$ .



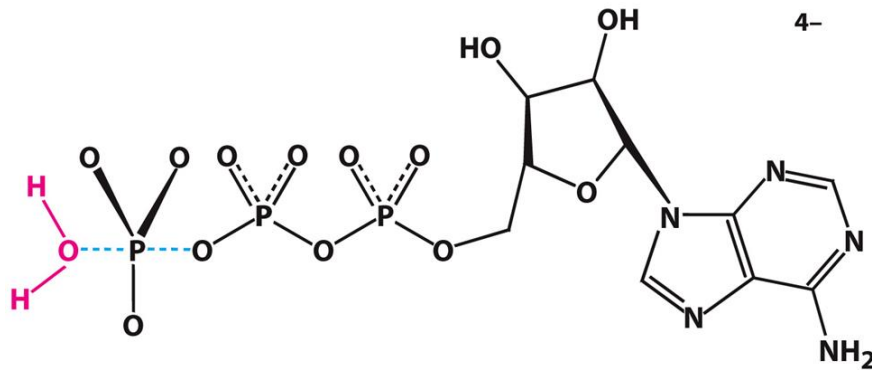
An overlay of the structures of the ATPase domain from *Dictyostelium discoideum* myosin with no ligands bound (blue) and the complex of this protein with ATP and magnesium bound (red).

**Figure 9.43**  
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The complex of the ATPase domain with bound ATP and  $Mg^{2+}$  is stable. It means that the enzyme has to undergo a conformational change to be enzymatically active



ATP hydrolysis by myosin proceeds through a pentacoordinate transition state that is too unstable to observe. Use of a vanadium-based substrate analog established the existence of such a transition state.



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The structure of the transition-state analog formed by treating the myosin ATPase domain with ADP and vanadate ( $\text{VO}_4^{2-}$ ) in the presence of magnesium. Vanadium ion is coordinated to 5 oxygen atoms. The positions of two residues that bind magnesium as well as Ser 236, a residue that appears to play a direct role in catalysis, are shown.

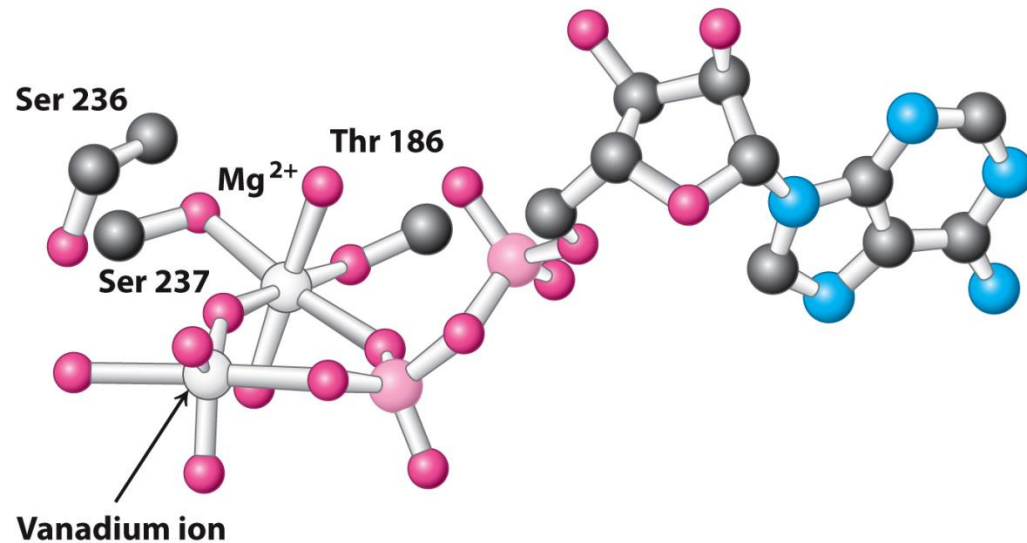
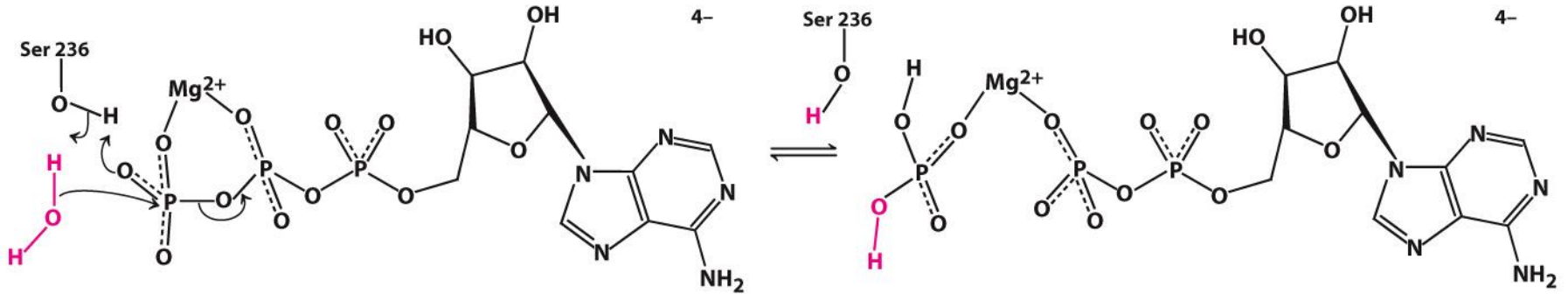


Figure 9.44  
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## Proposed mechanism of ATP hydrolysis by myosin



**Figure 9.45**

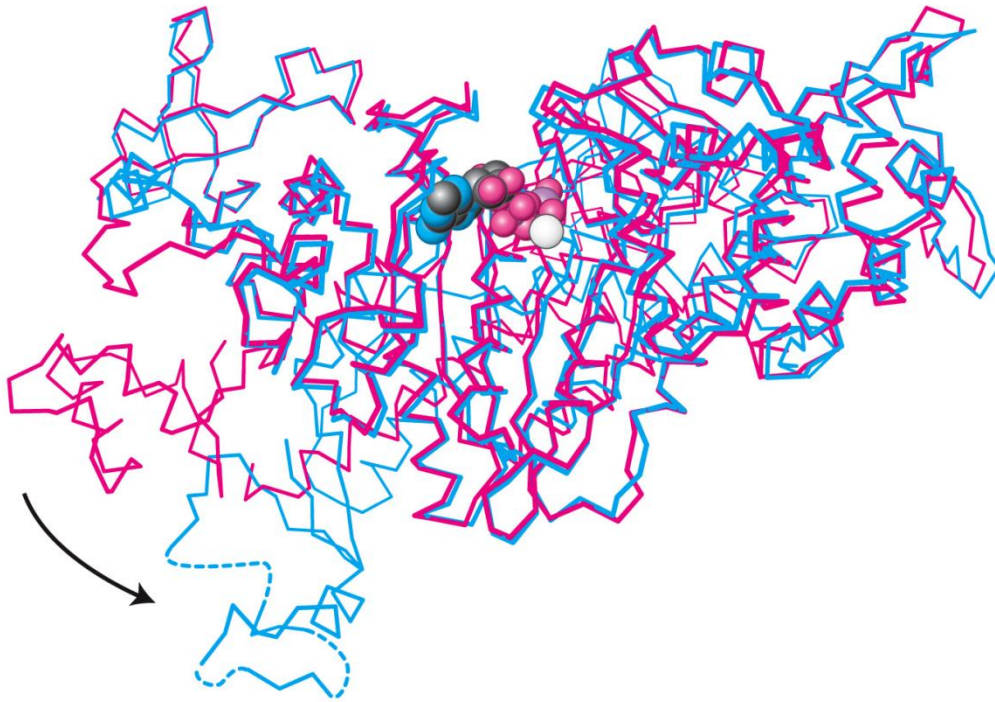
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ATP serves as a base by deprotonating the hydroxy group of Ser 236

Water molecule attacks  $\gamma$  phosphoryl group

Ser 236 facilitates deprotonation of water molecule

A comparison of the overall structures of the myosin ATPase domain with ATP bound (shown in red) and that with the transition-state analog ADP– vanadate (shown in blue). Notice the large conformational change of a region at the carboxyl-terminus of the domain, some parts of which move as much as 25 Å.

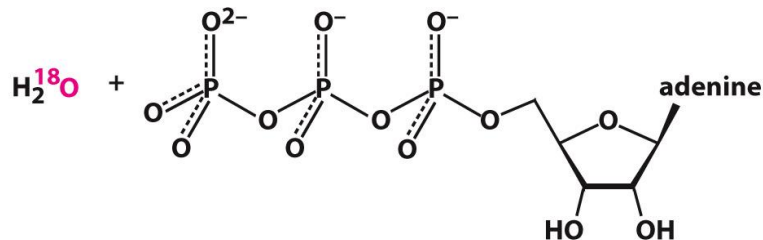


The relatively subtle conformational changes in the active site are amplified and lead to large rearrangement of the C-terminal domain that is coupled to other structures of myosin

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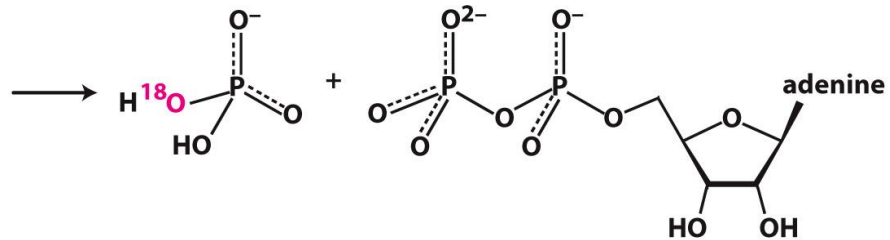
# ATP hydrolysis is reversible

ATP hydrolysis in the presence of  $\text{H}_2^{18}\text{O}$



Phosphate product should contain one oxygen from  $\text{H}_2\text{O}$

Analysis of the product showed 2 – 3 oxygens



Rate of hydrolysis is slow: 1 molecule per second

Hydrolysis is reversible leading to the reformation of ATP and a new hydrolysis step

Release of the product (phosphate group) is the rate limiting step

Slow release of the product is important for coupling conformational changes within ATPase domain to other processes