FIGURE 14.25 A detailed mechanism for the chymotrypsin reaction. Note the low-barrier hydrogen bond (LBHB) in (c) and (g).
one contributed by each subunit (Figure 14.32). In the homodimer, the active site is covered by two identical "flaps," one from each subunit, in contrast to the monomeric aspartic proteases, which possess only a single active-site flap. Enzyme kinetic measurements by Thomas Meck and his collaborators at SmithKline Beecham Pharmaceuticals have shown that the mechanism of HIV-1 protease is very similar to those of other aspartic proteases.

**Lysozyme**

Lysozyme is an enzyme that hydrolyzes polysaccharide chains. It ruptures certain bacterial cells by cleaving the polysaccharide chains that make up their cell wall. Lysozyme is found in many body fluids, but the most thoroughly studied form is from hen egg whites. The Russian scientist P. Laschtchenko first described the bacteriolytic properties of hen egg white lysozyme in 1909. In 1922,
Alexander Fleming, the London bacteriologist who later discovered penicillin, gave the name lysozyme to the agent in mucus and tears that destroyed certain bacteria, because it was an enzyme that caused bacterial lysis.

As seen in Chapter 7, bacterial cells are surrounded by a rigid, strong wall of peptidoglycan, a copolymer of two sugar units, N-acetylmuramic acid (NAM) and N-acetylgalactosamine (NAG). Both of these sugars are N-acetylated analogs of glucosamine, and in bacterial cell wall polysaccharides, they are joined in β(1→4) glycosidic linkages (Figure 14.31). Lysozyme hydrolyzes the glycosidic bond between C-1 of NAM and C-4 of NAG, as shown in Figure 14.33, but does not act on the β(1→4) linkages between NAG and NAM.

Lysozyme is a small globular protein composed of 129 amino acids (14 kD) in a single polypeptide chain. It has eight cysteine residues linked in four disulfide bonds. The structure of this very stable protein was determined by X-ray crystallographic methods in 1965 by David Phillips (Figure 14.34). Although X-ray structures had previously been reported for proteins (hemoglobin and myoglobin), lysozyme was the first enzyme structure to be solved by crystallographic (or any other) methods. Although the location of the active site was not obvious from the X-ray structure of the protein alone, X-ray studies of lysozyme-inhibitor complexes soon revealed the location and nature of the active site. Since it is an enzyme, lysozyme cannot form stable ES complexes for structural studies, because the substrate is rapidly transformed into products. On the other hand, several substrate analogs have proved to be good competitive inhibitors of lysozyme that can form complexes with the enzyme stable enough to be characterized by X-ray crystallography and other physical techniques. One of the best is a trimer of N-acetylgalactosamine, (NAG)₃ (Figure 14.35), which is hydrolyzed

FIGURE 14.31 HIV mRNA provides the genetic information for synthesis of a polyprotein. Proteolytic cleavage of this polyprotein by HIV protease produces the individual proteins required for viral growth and cellular infection.

Biochemistry & Now ACTIVITY
FIGURE 14.32 (left) HIV-1 protease complexed with the inhibitor Crizivan (red) made by Merck. The flaps (residues 46–55 from each subunit) covering the active site are shown in green, and the active-site aspartate residues involved in catalysis are shown in white. (right) The close-up of the active site shows the interaction of Crizivan with the carboxyl groups of the essential aspartate residues. Test yourself on the concepts in this figure at http://chemistry.brookscole.com/ggb3
Protease Inhibitors Give Life to AIDS Patients

Infection with HIV was once considered a death sentence, but the emergence of a new family of drugs called protease inhibitors has made it possible for some AIDS patients to improve their overall health and extend their lives. These drugs are all specific inhibitors of the HIV protease. By inhibiting the protease, they prevent the development of new virus particles in the cells of infected patients. Clinical testing has shown that a combination of drugs—including a protease inhibitor together with a reverse transcriptase inhibitor like AZT—can reduce the human immunodeficiency virus (HIV) to undetectable levels in about 40% to 50% of infected individuals. Patients who respond successfully to this combination therapy have experienced dramatic improvement in their overall health and a substantially lengthened life span.

Four of the protease inhibitors approved for use in humans by the U.S. Food and Drug Administration are shown below: Crixivan by Merck, Invirase by Hoffman-LaRoche, Norvir by Abbott, and Viracept by Agouron. These drugs were all developed from a "structure-based" design strategy; that is, the drug molecules were designed to bind tightly to the active site of the HIV-1 protease. The backbone OH-group in all these substances inserts between the two active-site carboxyl groups of the protease.

In the development of an effective drug, it is not sufficient merely to show that a candidate compound can cause the desired biochemical effect. It must also be demonstrated that the drug can be effectively delivered in sufficient quantities to the desired site(s) of action in the organism and that the drug does not cause undesirable side effects. The HIV-1 protease inhibitors shown here fulfill all of these criteria. Other drug candidates have been found that are even better inhibitors of HIV-1 protease in cell cultures, but many of these fail the test of bioavailability—the ability of a drug to be delivered to the desired site(s) of action in the organism.

Candidate protease inhibitor drugs must be relatively specific for the HIV-1 protease. Many other aspartic proteases exist in the human body and are essential to a variety of body functions, including digestion of food and processing of hormones. An ideal drug thus must strongly inhibit the HIV-1 protease, must be delivered effectively to the lymphocytes where the protease must be blocked, and should not adversely affect the activities of the essential human aspartic proteases.

A final but important consideration is viral mutation. Certain mutant HIV strains are resistant to one or more of the protease inhibitors, and even for patients who respond initially to protease inhibitors it is possible that mutant viral forms may eventually arise and thrive in the infected individual. The search for new and more effective protease inhibitors is ongoing.
hydrophobic and van der Waals interactions with (NAG)$_6$, as well as the normal substrate. The absence of charged groups on (NAG)$_6$ precludes the involvement of electrostatic interactions with the enzyme. Comparisons of the X-ray structures of the native lysozyme and the lysozyme--(NAG)$_6$ complex reveal that several amino acid residues at the active site move slightly upon inhibitor binding, including Trp$^{65}$, which moves about 0.75 Å to form a hydrogen bond with a hydroxymethyl group (Figure 14.57).

Model Studies Reveal a Strain-Induced Destabilization of a Bound Substrate on Lysozyme

One of the premises of lysozyme models is that the native substrate would occupy the rest of the crevice or depression running across the surface of the enzyme, because there is room to fit three more sugar residues into the crevice and because the hexamer (NAG)$_6$ is in fact a good substrate for lysozyme (Table 14.4). The model-building studies refer to the six sugar residue-binding subsites in the crevice with the letters A through F, with A, B, and C representing the part of the crevice occupied by the (NAG)$_6$ inhibitor (Figure 14.57). Modeling studies clearly show that NAG residues fit nicely into subsites A, B, C, E, and F of the crevice but that fitting a residue of the (NAG)$_6$ hexamer into site D requires a

Table 14.4

<table>
<thead>
<tr>
<th>Oligosaccharide</th>
<th>Rate Constant, $k_{cat}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NAG-NAM)$_3$</td>
<td>0.5</td>
</tr>
<tr>
<td>(NAG)$_6$</td>
<td>0.25</td>
</tr>
<tr>
<td>(NAG)$_5$</td>
<td>0.033</td>
</tr>
<tr>
<td>(NAG)$_4$</td>
<td>$7 \times 10^{-5}$</td>
</tr>
<tr>
<td>(NAG)$_3$</td>
<td>$8 \times 10^{-6}$</td>
</tr>
<tr>
<td>(NAG)$_2$</td>
<td>$2.5 \times 10^{-6}$</td>
</tr>
</tbody>
</table>

FIGURE 14.35 (NAG)$_6$, a substrate analog, forms stable complexes with lysozyme.

FIGURE 14.36 The lysozyme-enzyme-substrate complex.

FIGURE 14.34 The structure of lysozyme. Glu$^{35}$ and Asp$^{39}$ are shown in white.
FIGURE 14.37 Enzyme–substrate interactions at the six sugar residue-binding subsites of the lysozyme active site. (Illustration: Irving Geis. Rights owned by Howard Hughes Medical Institute. Not to be reproduced without permission.)
substantial distortion of the sugar (out of its preferred chair conformation) to
prevent steric crowding and overlap between atoms C-6 and O-6 of the sugar at
the D site and Ile\textsuperscript{38} of the enzyme. This distorted sugar residue is adjacent to the
glycosidic bond to be cleaved (between sites D and E), and the inference is made
that this distortion or strain brings the substrate closer to the transition state for
hydrolysis. This is a good example of strain-induced destabilization of an other-
wise favorably binding substrate (see Section 14.4). Thus, the overall binding
interaction of the rest of the sugar substrate would be favorable ($\Delta G < 0$), but
distortion of the ring at the D site uses some of this binding energy to raise the
substrate closer to the transition state for hydrolysis, an example of stabilization
of a transition state (relative to the simple enzyme-substrate complex). As noted in
Section 14.4, distortion is one of the molecular mechanisms that can lead to such
transition-state stabilization.

The Lysozyme Mechanism—A Classic Choice, and Recent Evidence

There are two mechanisms that would be consistent with the early X-ray struc-
tures of lysozyme and its model substrate complexes, and these two reactions rep-
resent a classic choice for the student enzymologist. In order to choose between
these two, consider the following evidence: Studies using \textsuperscript{18}O-enriched water
showed that the C\textsubscript{1}=O bond is cleaved on the substrate between the D and E
sites. Hydrolysis under these conditions incorporates \textsuperscript{18}O into the C\textsubscript{1} position of
the sugar at the D site, not into the oxygen at C\textsubscript{1} at the E site (Figure 14.38).
Model building studies place the cleaved bond approximately between protein
residues Glu\textsuperscript{39} and Asp\textsuperscript{35}. Glu\textsuperscript{39} is in a nonpolar or hydrophobic region of the pro-
tein, whereas Asp\textsuperscript{35} is located in a much more polar environment. Glu\textsuperscript{39} is pro-
tonated, but Asp\textsuperscript{35} is ionized (Figure 14.39).

In the lysozyme mechanism that was accepted for many years (Figure 14.39a),
Glu\textsuperscript{39} may act as a general acid, donating a proton to the oxygen atom of the
glycosidic bond and accelerating the reaction. Asp\textsuperscript{35}, on the other hand, stabil-
ilizes the carbocation (also called a carbonium ion or an oxocarbenium ion)
generated at the D site upon bond cleavage. Formation of the carbocation ion
may also be enhanced by the strain on the ring at the D site. Following bond
cleavage, the product formed at the E site diffuses away, and the carbocation
intermediate can then react with H\textsubscript{2}O from the solution. Glu\textsuperscript{39} can now act
as a general base, accepting a proton from the attacking water. The tetramer
of NAG thus formed at sites A through D can now be dissociated from the
enzyme. If this were indeed the true mechanism for lysozyme, the rate acceler-
ation afforded by lysozyme would be due to (1) general acid catalysis by Glu\textsuperscript{39};
(2) distortion of the sugar ring at the D site, which may stabilize the carbonium
ion (\textit{and the transition state}); and (3) electrostatic stabilization of the carbocation
by nearby Asp\textsuperscript{35}.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure14.38.png}
\caption{The \textit{C\textsubscript{1}=O} bond, not the \textit{O--C\textsubscript{1}} bond, is cleaved in the lysozyme reaction.
\textsuperscript{18}O from H\textsubscript{2}\textsuperscript{18}O is thus incorporated at the C\textsubscript{1} position.}
\end{figure}
The other possible mechanism for lysozyme (Figure 14.39b) involves an initial nucleophilic attack by the carboxylate anion of Asp\textsuperscript{52}, in an associative \( S_n^2 \) reaction, to form a covalent glycosyl-enzyme intermediate, a step that would occur with inversion of configuration. The enzyme carboxylate would then be displaced from the glycosyl-enzyme intermediate by water in a second step, which would also occur with inversion of configuration. The second step would involve Glu\textsuperscript{35} as a general base, withdrawing a proton from a water molecule in the active site to produce hydroxide in the transition state. The result of these two inversion reactions would be a net retention of configuration at the glycosyl C-1 position. This latter mechanism would involve Asp\textsuperscript{52} in covalent catalysis, with Glu\textsuperscript{35} acting as a general base.

For many years, the dissociative, noncovalent mechanism (Figure 14.39a) was the favored choice for lysozyme, and also for other enzymatic reactions that cleave glycosidic linkages with net retention of configuration. However, recent experiments by Stephen Withers and his colleagues provide convincing evidence.
for a covalent Asp$^{32}$-substrate intermediate and the pair of associative S$_{n}$2 mechanisms shown in Figure 14.39b. The challenge for Withers and his colleagues was to find conditions in which the rate of formation of the covalent intermediate would be faster than its rate of breakdown. They used a mutant lysozyme (in which Glu$^{32}$ is replaced by Gln) and several substrate analogs to prepare covalent enzyme–substrate complexes that could be observed unambiguously by electrospray ionization mass spectrometry (Figure 14.40). Then they succeeded in crystallizing the mutant lysozyme in a covalent complex with a difluorinated substrate analog (Figure 14.41). The X-ray diffraction structure clearly shows the covalent bond between Asp$^{32}$ and the C-1 carbon of the sugar in the D position in the active site. The structures in Figure 14.41 also show that carbon C-1 is located above the sugar ring in the noncovalent enzyme–substrate complex. Formation of the covalent complex involves an electrophilic migration of the C-1 carbon from above the ring plane to below the ring plane, where it approaches to within 1.6 to 1.8 Å of the Asp$^{32}$ oxygen—close enough to form a covalent bond.
Caught in the Act! A High-Energy Intermediate in the Phosphoglucomutase Reaction

Because the transition states of enzyme-catalyzed reactions are imagined to have lifetimes on the order of a bond vibration \((10^{-13}\ \text{sec})\), it has long been assumed that it would not be possible to see a transition state in the form of a crystal structure solved by X-ray diffraction. However, Debra Dunaway-Mariano and Karen Allen and their colleagues have crystallized phosphorylated \(\beta\)-phosphoglucomutase at low temperature in the presence of \(\text{Mg}^{2+}\) and either glucose-1-phosphate or glucose-6-phosphate and have observed a stable pentacoordinate phosphorane that looks very much like the transition state anticipated for the phosphoryl transfer carried out by this enzyme. The most likely mechanisms for a phosphoryl transfer reaction are shown in the accompanying figure: (a) is a dissociative mechanism involving an intermediate metaphosphate, with expected apical P-O distances of 0.35 nm or more. (b) is an \(S\text{N}_{2}\)-like, partly associative mechanism, with apical P-O distances of 0.19 to 0.21 nm and bond orders of 0.5. A fully-associative mechanism would have apical P-O distances of 0.166 to 0.176 nm. (c) The crystal structure of phosphoglucomutase shows a trigonal bipyramidal oxyporphorane with P-O distances of 0.2 and 0.21 nm and calculated bond orders of 0.24 to 0.45. The structure is remarkably similar to what would be expected for the transition state of a partly associative mechanism. Is this the transition state, trapped in a crystal? The crystals were frozen at liquid nitrogen temperature \((77\ \text{K})\), and the X-ray diffraction data were collected at 93 K. Because we imagine that a true transition state has a lifetime too short to be observed in this way, we may surmise that what is a transition state at physiological temperature is a stable intermediate at very low temperature.

\[\begin{align*}
\text{(a) Dissociative} & \\
\end{align*}\]

\[\begin{align*}
\text{Tetrahedral P} & \quad \text{Planar} & \quad \text{Tetrahedral P} \\
\end{align*}\]

\[\begin{align*}
\text{(b) Partly associative} & \\
\end{align*}\]

\[\begin{align*}
\text{(c) Crystal structure} & \\
\end{align*}\]

with this residue. The mass spectrometry and X-ray diffraction data provide unequivocal evidence that the mechanism of hen egg white lysozyme involves a covalent intermediate, as portrayed in Figure 14.89b.

The overall \(k_{\text{cat}}\) for lysozyme is about 0.5/sec, which is quite slow (Table 13.4) compared with that for other enzymes. On the other hand, the destruction of a bacterial cell wall may require hydrolysis of only a few polysaccharide chains. The high osmotic pressure of the cell ensures that cell rupture will follow rapidly. Thus, lysozyme can accomplish cell lysis without a particularly high \(k_{\text{cat}}\).
Summary

It is simply chemistry—the breaking and making of bonds—that gives enzymes their prowess. This chapter explores the unique features of this chemistry. The mechanisms of hundreds of enzymes have been studied in at least some detail.

14.1 What Role Does Transition-State Stabilization Play in Enzyme Catalysis? The energy barrier for the uncatalyzed reaction is the difference in energies of the S and X¹ states. Similarly, the energy barrier to be surmounted in the enzyme-catalyzed reaction, assuming that E is saturated with S, is the energy difference between ES and EX¹. Reaction rate acceleration by an enzyme means simply that the energy barrier between ES and EX¹ is less than the energy barrier between S and X¹. In terms of the free energies of activation, \( \Delta G_v > \Delta G_v^E \).

14.2 What Are the Magnitudes of Enzyme-Induced Rate Accelerations? Enzymes are powerful catalysts. Enzyme-catalyzed reactions are typically \( 10^7 \) to \( 10^{10} \) times faster than their uncatalyzed counterparts and may exceed \( 10^{16} \).

14.3 Why Is the Binding Energy of ES Crucial to Catalysis? The favorable interactions between the substrate and amino acid residues on the enzyme account for the intrinsic binding energy, \( \Delta G_v^E \). The intrinsic binding energy ensures the favorable formation of the ES complex, but if uncompensated, it makes the activation energy for the enzyme-catalyzed reaction unnecessarily large and wastes some of the catalytic power of the enzyme. Because the enzymatic reaction rate is determined by the difference in energies between ES and EX¹, the smaller this difference, the faster the enzyme-catalyzed reaction. Tight binding of the substrate deepens the energy well of the ES complex and actually lowers the rate of the reaction.

14.4 What Roles Do Entropy Loss and Destabilization of the ES Complex Play? Entropy is lost when two molecules (E and S) interact to form one molecule (the ES complex). Because \( \Delta S^E \) is negative for this process, the term \( -T \Delta S^E \) is a positive quantity, and the intrinsic binding energy of ES is compensated to some extent by the entropy loss that attends the formation of the complex. Destabilization of the ES complex can involve structural strain, desorption, or electrostatic effects. Destabilization by strain or distortion is usually just a consequence of the fact that the enzyme is designed to bind the transition state more strongly than the substrate.

14.5 How Tightly Do Transition-State Analogous Bind to the Active Site? Given a ratio \( k_a / k_c \) of \( 10^{12} \) and a typical \( K_r \) of \( 10^{-3} \) M, the value of \( K_r \) should be \( 10^{-15} \) M. This is the dissociation constant for the transition-state complex from the enzyme, and this very low value corresponds to very tight binding of the transition state by the enzyme. It is unlikely that such tight binding in an enzyme transition state will ever be measured experimentally, however, because the transition state itself is a "moving target."

14.6 What Are the Mechanisms of Catalysis? Enzyme reaction mechanisms involve covalent bond formation, general acid–base catalysis, low-barrier hydrogen bonds, metal ion effects, and proximity of reactants. Most enzymes display involvement of two of these or more in any given reaction.

14.7 What Can Be Learned from Typical Enzyme Mechanisms? The enzymes examined in this chapter—serine proteases, aspartic proteases, and lysozyme—all embody two or more of the rate enhancement contributions.