CHAPTER 1

WHY ENZYMES AS DRUG TARGETS?

COPYRIGHTED MATERIAL

KEY LEARNING POINTS

- Enzymes are excellent targets for pharmacological intervention, owing to their essential roles in life processes and pathophysiology.
- The structures of enzyme active sites, and other ligand binding pockets on enzymes, are ideally suited for high-affinity interactions with drug-like inhibitors.

Medicine in the twenty-first century has largely become a molecular science in which drug molecules are directed toward specific macromolecular targets whose bioactivity is pathogenic or at least associated with disease. In most clinical situations the most desirable course of treatment is by oral administration of safe and effective drugs with a duration of action that allows for convenient dosing schedules (typically once or twice daily). These criteria are best met by small molecule drugs, as opposed to peptide, protein, gene, or many natural product-based therapeutics. Among the biological macromolecules that one can envisage as drug targets, enzymes hold a preeminent position because of the essentiality of their activity in many disease processes, and because the structural determinants of enzyme catalysis lend themselves well to inhibition by small molecular weight, drug-like molecules. Not surprisingly,

Evaluation of Enzyme Inhibitors in Drug Discovery: A Guide for Medicinal Chemists and Pharmacologists, Second Edition. Robert A. Copeland.
© 2013 John Wiley & Sons, Inc. Published 2013 by John Wiley & Sons, Inc.

enzyme inhibitors represent almost half the drugs in clinical use today. Recent surveys of the human genome suggest that the portion of the genome that encodes for disease-associated, "druggable" targets is dominated by enzymes. It is therefore a virtual certainty that specific enzyme inhibition will remain a major focus of pharmaceutical research for the foreseeable future. In this chapter we review the salient features of enzyme catalysis and of enzyme structure that make this class of biological macromolecules such attractive targets for chemotherapeutic intervention in human diseases.

1.1 ENZYMES ARE ESSENTIAL FOR LIFE

In high school biology classes life is often defined as "a series of chemical reactions." This popular aphorism reflects the fact that living cells, and in turn multicellular organisms, depend on chemical transformations for every essential life process. Synthesis of biomacromolecules (proteins, nucleic acids, polysaccarides, and lipids), all aspects of intermediate metabolism, intercellular communication in, for example, the immune response, and catabolic processes involved in tissue remodeling, all involve sequential series of chemical reactions (i.e., biological pathways) to maintain life's critical functions. The vast majority of these essential biochemical reactions, however, proceed at uncatalyzed rates that are too slow to sustain life. For example, pyrimidines nucleotides, together with purine nucleotides, make up the building blocks of all nucleic acids. The de novo biosynthesis of pyrimidines requires the formation of uridine monophosphate (UMP) via the decarboxylation of orotidine monophosphate (OMP). Measurements of the rate of OMP decarboxylation have estimated the half-life of this chemical reaction to be approximately 78 million years! Obviously a reaction this slow cannot sustain life on earth without some very significant rate enhancement. The enzyme OMP decarboxylase (EC 4.1.1.23) fulfills this life-critical function, enhancing the rate of OMP decarboxylation by some 10^{17}-fold, so that the reaction half-life of the enzyme-catalyzed reaction (0.018 seconds) displays the rapidity necessary for living organisms (Radzicka and Wolfenden, 1995).

Enyzme catalysis is thus essential for all life. Hence the selective inhibition of critical enzymes of infectious organisms (i.e., viruses, bacteria, and multicellular parasites) is an attractive means of chemotherapeutic intervention for infectious diseases. This strategy is well represented in modern medicine, with a significant portion of antiviral, antibiotic, and antiparasitic drugs in clinical use today deriving their therapeutic efficacy through selective enzyme inhibition (see Table 1.1 for some examples).

Although enzymes are essential for life, dysregulated enzyme activity can also lead to disease states. In some cases mutations in genes encoding enzymes can lead to abnormally high concentrations of the enzyme within a cell (overexpression). Alternatively, point mutations can lead to an enhancement of the specific activity (i.e., catalytic efficiency) of the enzyme because of structural

TABLE 1.1 Selected Enzyme Inhibitors in Clinical Use or Trials

Compound	Target Enzyme	Clinical Use
Acetazolamide	Carbonic anhydrase	Glaucoma
Acyclovir	Viral DNA polymerase	Herpes
Amprenavir, indinavir, nelfinavir, ritonavir, saquinavir	HIV protease	AIDS
Allopurinol	Xanthine oxidase	Gout
Argatroban	Thrombin	Heart disease
Aspirin	Cyclooxygenases	Inflammation, pain, fever
Amoxicillin	Penicillin binding proteins	Bacterial infection
Captopril, enalapril	Angiotensin converting enzyme	Hypertension
Carbidopa	Dopa decarboxylase	Parkinson's disease
Celebrex, Vioxx	Cyclooxygenase-2	Inflammation
CI-1040, PD0325901	MAP kinase kinase	Cancer
Clavulanate	β-Lactamase	Bacterial resistance
Digoxin	Sodium, potassium ATPase	Heart disease
Efavirenz, nevirapine	HIV reverse transcriptase	AIDS
Epristeride, finasteride, dutasteride	Steroid 5α-reductase	Benign prostate hyperplasia, male pattern baldness
Fluorouracil	Thymidylate synthase	Cancer
Leflunomide	Dihydroorotate Dehydrogenase	Inflammation
Lovastatin and other statins	HMG-CoA reductase	Cholesterol lowering
Methotrexate	Dihydrofolate reductase	Cancer, immunosuppression
Nitecapone	Catechol-*O*-methyltransferase	Parkinson's disease
Norfloxacin	DNA gyrase	Urinary tract infections
Omeprazole	H^+, K^+ ATPase	Peptic ulcers
PALA	Aspartate Transcarbamoylase	Cancer
Sorbinol	Aldose reductase	Diabetic retinopathy
Trimethoprim	Bacterial dihydrofolate reductase	Bacterial infections
Viagra, Levitra	Phosphodiesterase	Erectile dysfunction

Source: Adapted and expanded from Copeland (2000).

changes in the catalytically critical amino acid residues. By either of these mechanisms, aberrant levels of the reaction product's formation can result, leading to specific pathologies. Hence human enzymes are also commonly targeted for pharmacological intervention in many diseases.

Enzymes, then, are attractive targets for drug therapy because of their essential roles in life processes and in pathophysiology. Indeed, a survey

reported in 2000 found that close to 30% of all drugs in clinical use derive their therapeutic efficacy through enzyme inhibition (Drews, 2000). More recently Hopkins and Groom (2002) updated this survey to include newly launched drugs and found that nearly half (47%) of all marketed small molecule drugs inhibit enzymes as their molecular target (Figure 1.1). Worldwide sales of small molecule drugs that function as enzyme inhibitors exceeded 65 billion dollars in 2001, and this market was expected to grow to more than 95 billion dollars by 2006 (see Figure 1.2). Some contraction of the worldwide market has occurred due to withdrawal of several products since 2005. Revised forecasts suggest that the worldwide market will now grow at a rate of about 6.7% as of 2005 (Business Communications Company, Inc., 2006, "Enzyme Inhibitors with Broad Therapeutic Application").

The attractiveness of enzymes as drug targets results not only from the essentiality of their catalytic activity but also from the fact that enzymes, by their very nature, are highly amenable to inhibition by small molecular weight, drug-like molecules. Because of this susceptibility to inhibition by small molecule drugs, enzymes are commonly the target of new drug discovery and design efforts at major pharmaceutical and biotechnology companies today; my own informal survey suggests that between 50 and 75% of all new drug-seeking efforts at several major pharmaceutical companies in the United States are focused on enzymes as primary targets.

While the initial excitement generated by the completion of the Human Genome Project was in part due to the promise of a bounty of new targets for drug therapy, it is now apparent that only a portion of the some 30,000 proteins encoded for by the human genome are likely to be amenable to small molecule drug intervention. A recent study suggested that the size of the human "drug-

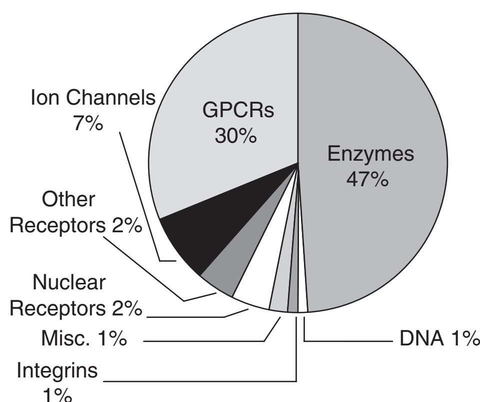

Figure 1.1 Distribution of marketed drugs by biochemical target class. GPCRs = G-Protein coupled receptors.
Source: Redrawn from Hopkins and Groom (2002).

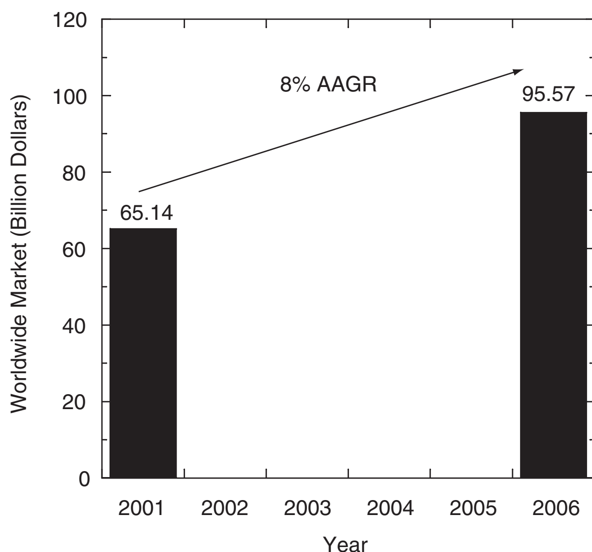

Figure 1.2 Worldwide market for small molecule drugs that function as enzyme inhibitors in 2001 and projected for 2006. AAGR = average annual growth rate.
Source: Business Communications Company, Inc. Report RC-202R: New Developments in Therapeutic Enzyme Inhibitors and Receptor Blockers, www.bccresearch.com.

gable genome" (e.g., human genes encoding proteins that are expected to contain functionally necessary binding pockets with appropriate structures for interactions with drug-like molecules) is more on the order of 3000 target proteins (i.e., about 10% of the genome), a significant portion of these being enzymes (Hopkins and Groom, 2002). As pointed out by Hopkins and Groom, just because a protein contains a druggable binding pocket does not necessarily make it a good target for drug discovery; there must be some expectation that the protein plays some pathogenic role in disease so that inhibition of the protein will lead to a disease modification. Furthermore the same study estimates that of the nearly 30,000 proteins encoded by the human genome, only about 10% (3000) can be classified as "disease-modifying genes" (e.g., genes that, when knocked out in mice, effect a disease-related phenotype). The intersection of the druggable genome and the disease-modifying genome thus defines the number of bona fide drug targets of greatest interest to pharmaceutical scientists. This intersection, according to Hopkins and Groom (2002), contains only between 600 and 1500 genes, again with a large proportion of these genes encoding for enzyme targets.

The "druggability" of enzymes as targets reflects the evolution of enzyme structure to efficiently perform catalysis of chemical reactions, as discussed in the following section.

1.2 ENZYME STRUCTURE AND CATALYSIS

From more than a thousand years of folk remedies and more recent systematic pharmacology, it is well known that compounds that work most effectively as drugs generally conform to certain physicochemical criteria (Table 1.2). To be effective in vivo, molecules must be absorbed and distributed, usually permeate cell membranes to reach their molecular targets, and be retained in systemic circulation for a reasonable period of time (i.e., pharmacokinetic residence time). These and other necessary biological features of small molecule drugs are dictated by the physicochemical nature of the drug molecules. Over the years there have been a number of published surveys that relate specific physicochemical properties of small molecules to their utility as therapeutic agents (Ajay et al., 1998; Lipinski et al., 2001; Veber et al., 2002; Vieth et al., 2004; Keller et al., 2006). With respect to orally administered small molecule drugs, a specific set of physicochemical features is commonly articulated as important for success; these are summarized in Table 1.2. Generally, drug molecules need to be relatively small, with molecular weights less than 1000 Da and preferably less than or equal to 500 Da. Drug molecules are generally hydrophobic, but very often contain polarizable groups at precise locations within the molecule. Hence, drug molecules typically contain a number of specifically oriented heteroatoms and hydrogen-bond donors (for more details on chemical features of drug-like molecules, see Ajay et al., 1998; Lipinski et al., 1997; Veber et al., 2002). Note that these "rules" of chemical structure for drug molecules are significantly relaxed, and sometimes altered completely in the case of natural products (Clardy and Walsh, 2004). Nevertheless, even in the case of natural products, target binding affinity and in vivo delivery are dictated largely by specific physicochemical properties of the drug molecule.

Accepting the premise that drug molecules conform to specific stereochemical, electrostatic, hydrophobic, and other physiochemical properties, it follows that drug targets must contain binding pockets for these molecules that demonstrate structural and electronic complementarity to the small

TABLE 1.2 Some Physicochemical Properties of Drug-like Molecules

Molecular Property	Typical Value
Molecular weight	≤500 Da
cLog(P)	≤5
Number of H-bond donors	≤5
Sum of N and O atoms	≤10
Polar surface area	≤140 Å2
Rotatable bonds	≤10

Sources: Data from Lipinski et al. (2001), Veber et al. (2002), and Keller et al. (2006).

molecule drugs. Thus a "druggable target" is one that contains a "druggable binding pocket" as part of its three-dimensional structure, and a druggable binding pocket conforms to specific structural and chemical requirements.

The features that make a binding pocket on a protein "druggable" have been reviewed by several authors (Liang et al., 1998; Hajduk et al., 2005). Generally, drug binding pockets are cavities or clefts along the protein surface, with small molecular volumes (relative to that of the entire protein) of around 1000 $Å^3$ (Liang et al., 1998). Estimates of the volume relationship between a ligand binding pocket and the overall protein have suggested that the ligand binding pocket constitutes around 1–5% of the total volume of the protein molecule (Liang et al., 1998). Drug binding pockets tend to display a large surface area to volume ratio, a factor referred to as surface roughness (Pettit and Bowie, 1999) and which reflects the stereochemical uniqueness of the binding pockets; by having a large surface area to volume ratio, the potential for favorable van der Waals interactions between the pocket and ligand is enhanced.

Ligand binding pockets are usually designed to exclude bulk solvent, and are generally composed of hydrophobic amino acids. Nevertheless, the pockets may contain highly ordered water molecules, incorporated as part of a specific architectural motif to participate in ligand interactions (see, for example, Figure 1.5). This exclusion of bulk water favors the formation of stronger hydrogen bonds and other electrostatic interactions between the protein and the ligand. Complementary to the drug molecules themselves, these pockets also often contain specific loci for hydrogen bonding, salt bridge formation, and other noncovalent, electrostatic interactions between the binding partners. The combination of electrostatic determinants of binding, the general hydrophobicity of the pockets, and surface roughness make for significant surface complexity in drug binding pockets (Hajduk et al., 2005).

Druggable binding pockets on protein surfaces have largely evolved to bind physiologically relevant small molecular weight ligands, such as nucleotide analogs (e.g., ATP, GTP, NADH), amino acids, steroid hormones, metabolites, peptides, cofactors (e.g., flavins, hemes), and the like. The interactions of these natural ligands with the protein binding site typically effects a change in the biological activity of the target protein. For example, binding of the physiologic agonist (a ligand that stimulates the biological activity of a receptor) to a G-protein coupled receptor (GPCR) on the surface of a cell elicits a conformational transition of the receptor, often leading to post-translational modification of cytosolic domains of the receptor protein. These post-translational modifications lead to recruitment and/or activation of various proteins, thus initiating cellular signal transduction cascades that are critical for a number of cellular activities, such as cell proliferation, mobility, and programmed cell death.

In the organism, the extent and duration of signal transduction—hence the interactions between the receptor and ligand—need to be responsive to the changing needs and environment of the cell. This need for facile responsiveness

at the receptor level is facilitated by three characteristics of protein interactions with physiologic ligands:

1. They are reversible.
2. They display moderate binding affinity (typically in the μM to mM range).
3. They are modulated by changes in the local concentration of ligand.

All of these properties are dicated by equilibrium binding between the protein receptor and the ligand, as discussed in more detail in Chapters 2 and 3 and Appendix 2. Hence, the elements of molecular recognition between proteins and their physiologic ligands are largely mediated through the cumulative effects of multiple, weak, reversible chemical forces, such as hydrogen bonds, salt bridges, van der Waals forces, and hydrophobic forces (Copeland, 2000). This is exemplified in Figure 1.5 where we illustrate the collective interactions between the enzymatic active site of dihydrofolate reductase and its substrate dihydrofolate. These same weak, noncovalent chemical forces typically also form the structural determinants of interaction between protein binding sites and drug molecules; this is also exemplified in Figure 1.5 where we see the same types of chemical interactions forming between the enzymatic active site of dihydrofolate reductase and the drug methotrexate.

Thus, the best molecular targets for drug intervention are those containing a relatively small volume, largely hydrophobic binding pocket that is polarized by specifically oriented loci for hydrogen bonding and other electrostatic interactions and that is critical for the biological function of the target (Liang et al., 1998). These criteria are well met by the structures of enzyme active sites and additional regulatory allosteric binding sites on enzyme molecules.

The vast majority of biological catalysis is performed by enzymes, which are proteins composed of polypeptide chains of amino acids (natural peptide synthesis at the ribosome, and a small number of other biochemical reactions are catalyzed by RNA molecules, though the bulk of biochemical reactions are catalyzed by protein-based enzymes). These polypeptide chains fold into regular, repeating structural motifs of secondary (alpha helices, beta pleated sheets, hairpin turns, etc.) and tertiary structures (see Figure 1.3). The overall folding pattern, or tertiary structure of the enzyme, provides a structural scaffolding that presents catalytically essential amino acids and cofactors in a specific spacial orientation to facilitate catalysis. As an example, consider the enzyme dihydrofolate reductase (DHFR), a key enzyme in the biosynthesis of deoxythymidine and the target of the antiproliferative drug methotrexate and the antibacterial drug trimethoprim (Klebe, 1994; Copeland, 2000). The bacterial enzyme has a molecular weight of around 180,000 (162 amino acid residues) and folds into a compact globular structure composed of 10 strands of beta pleated sheet, 7 alpha helices, and assorted turns and hairpin structures (Bolin et al., 1982). Figure 1.4 shows the overall size and shape of the enzyme

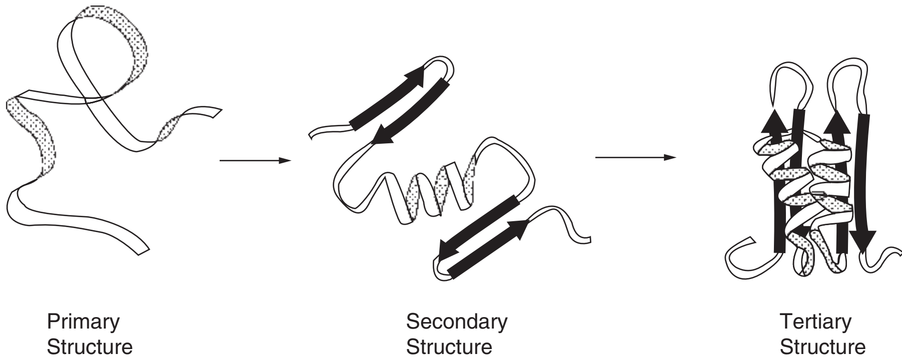

Figure 1.3 Folding of a polypeptide chain illustrating the hierarchy of protein structure from primary structure through secondary structure and tertiary structure.
Source: From Copeland (2000).

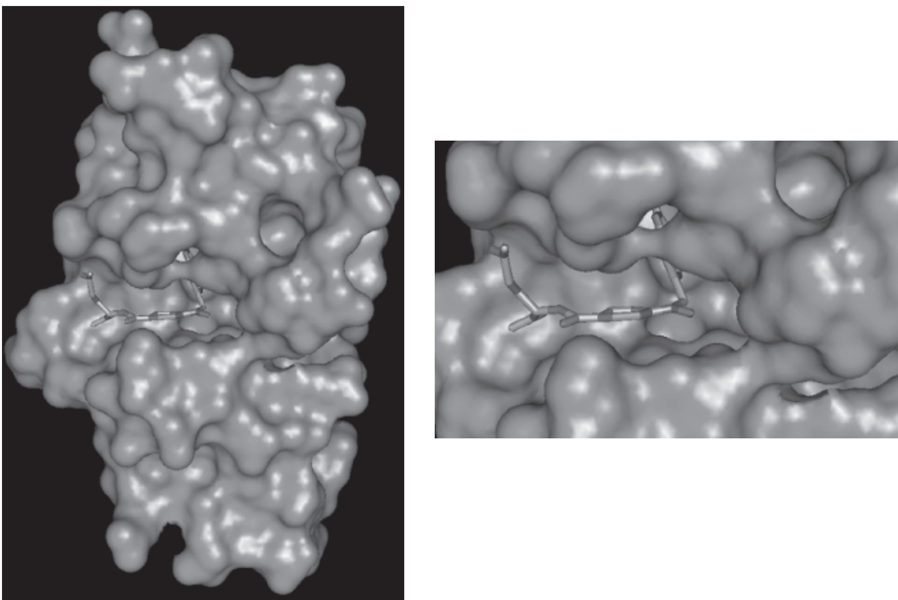

Figure 1.4 *Left panel*: Space filing model of the structure of bacterial dihydrofolate reductase with methotrexate bound to the active site. *Right panel*: Close-up view of the active site, illustrating the structural complementarity between the ligand (methotrexate) and the binding pocket. See color insert.
Source: Courtesy of Nesya Nevins.

molecule and illustrates the dimensions of the catalytic active site with the inhibitor methotrexate bound to it. We can immediately see that the site of chemical reactions—that is, the enzyme active site—constitutes a relatively small fraction of the overall volume of the protein molecule (Liang et al., 1998). Again, the bulk of the protein structure is used as scaffolding to create the required architecture of the active site. A more detailed view of the structure of the active site of DHFR is shown in Figure 1.5, which illustrates the specific interactions of active site components with the substrate dihydrofolate and with the inhibitor methotrexate. We see from Figure 1.5 that the active site of DHFR is relatively hydrophobic, but contains ordered water molecules and charged amino acid side chains (e.g., Asp 27) that form specific hydrogen bonding interactions with both the substrate and inhibitor molecules.

The active site of DHFR illustrates several features that are common to enzyme active sites. Some of the salient features of active site structure that relate to enzyme catalysis and ligand (e.g., inhibitor) interactions have been enumerated by Copeland (2000):

1. The active site of an enzyme is small relative to the total volume of the enzyme.
2. The active site is three-dimensional—that is, amino acids and cofactors in the active site are held in a precise arrangement with respect to one another and with respect to the structure of the substrate molecule. This active site three-dimensional structure is formed as a result of the overall tertiary structure of the protein.

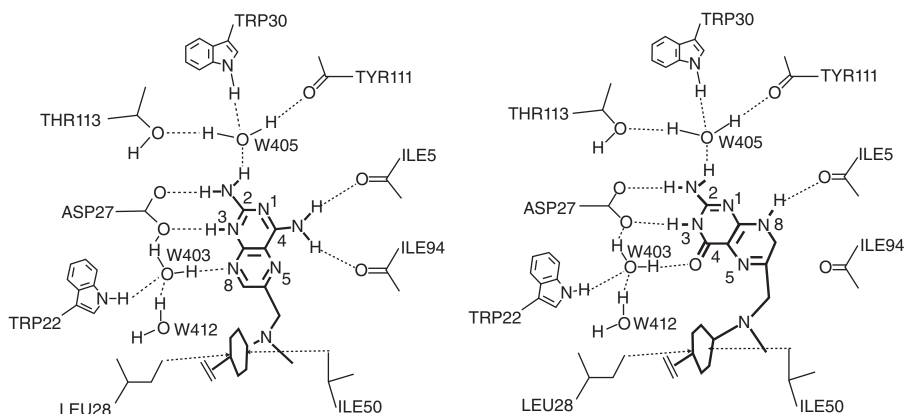

Figure 1.5 Interactions of the dihydrofolate reductase active site with the inhibitor methotrexate (*left*) and the substrate dihydrofolate (*right*).
Source: Reprinted from G. Klebe, *J. Mol. Biol.* 237, p. 224; copyright 1994 with permission from Elsevier.

3. In most cases the initial interactions between the enzyme and the substrate molecule (i.e., the initial binding event) are noncovalent, making use of hydrogen bonding, electrostatic, hydrophobic interactions, and van der Waals forces to effect binding.
4. The active site of enzymes usually are located in clefts and crevices in the protein. This design effectively excludes bulk solvent (water), which would otherwise reduce the catalytic activity of the enzyme. In other words, the substrate molecule is desolvated upon binding, and shielded from bulk solvent in the enzyme active site. Solvation by water is replaced by specific interactions with the protein (Warshel et al., 1989).
5. The specificity of substrate utilization depends on the well-defined arrangement of atoms in the enzyme active site that in some way complements the structure of the substrate molecule.

These features of enzyme active sites have evolved to facilitate catalysis by (1) binding substrate molecules through reversible, noncovalent interactions, (2) shielding substrate molecules from bulk solvent and creating a localized dielectric environment that helps reduce the activation barrier to reaction, and (3) binding substrate(s) in a specific orientation that aligns molecular orbitals on the substrate molecule(s) and reactive groups within the enzyme active site for optimal bond distortion as required for the chemical transformations of catalysis (see Copeland, 2000, for a more detailed discussion of these points). These same characteristics of enzyme active sites make them ideally suited for high-affinity interactions with molecules containing the druggable features described earlier (Taira and Benkovic, 1988).

An additional advantage of enzyme active sites as targets for drug binding is that it is only necessary for the bound drug to disrupt a small number of critical interactions within the active site to be an effective inhibitor. A macroscopic analogy for this would be inhibiting the ability of a truck to move by removing the spark plugs from the engine. While the spark plugs represent a small portion of the overall volume of the truck, and in fact a small portion of the overall volume of the active site (the engine) of the truck, they are nevertheless critical to the function of the truck. Removing the spark plugs, or simply filling the spark gap with grease, is sufficient to inhibit the overall function of the truck. In a like manner, a drug molecule need not fill the entire volume of the active site to be effective. Some enzymes, especially proteases and peptidases that serve to hydrolyze peptide bonds within specific protein or peptide substrates, contain extended active sites that make multiple contacts with the substrates. Yet the chemistry of peptide bond hydrolysis is typically dependent on a small number of critical amino acids or cofactor atoms that occupy a limited molecular volume. Hence small molecular weight drugs have been identified as potent inhibitors of these enzymes, though they occupy only a small fraction of the extended active site cavity. The zinc hydrolases offer a good example of this concept. The enzyme angiotensin converting

enzyme (ACE) is a zinc-dependent carboxypeptidase that plays a major role in the control of blood pressure by converting the decapeptide angiotensin I to the octapeptide angiotensin II (Ondetti and Cushman, 1984). Although the active site of the enzyme makes contacts along the polypeptide chain of the decapeptide substrate, the chemistry of bond cleavage occurs through coordinate bond formation between the carbonyl oxygen atom of the scissile bond and the active site zinc atom. Effective small molecule inhibitors of ACE, such as the antihypertensive drugs captopril and enalapril, function by chelating the critical zinc atom and thus disrupt a critical catalytic component of the enzyme's active site without the need to fill the entire volume of the active site cleft.

It is thus easy to see why targeting enzyme active sites is an attractive approach in drug discovery and design. However, it is important to recognize that the enzyme active site is not necessarily the only binding pocket on the enzyme molecule that may be an appropriate target for drug interactions. The catalytic activity of many enzymes is regulated by binding interactions with cofactors, metal ions, small molecule metabolites, and peptides at sites that are distal to the active site of chemical reactions. The binding sites for these regulatory molecules are generally referred to as allosteric binding pockets. Natural ligand binding at an allosteric binding pocket is somehow communicated to the distal enzyme active site in such a way as to modulate the catalytic activity of the enzyme. Ligands that interact with enzymes in this way can function as activators, to augment catalytic activity (positive regulation), or as inhibitors to diminish activity (negative regulation). Likewise drug molecules that interact with allosteric binding pockets on enzymes can attenuate enzymatic activity and thus produce the desired pharmacological effects of targeting of the enzyme molecule. Specific examples of this type of inhibition mechanism will be presented in subsequent chapters, and have been discussed by Copeland (2000) and by Copeland and Anderson (2001) (see also Wiesmann et al., 2004, for an interesting, recent example of allosteric inhibition of protein tyrosine phosphatase 1B as a potential mechanism for treating type 2 diabetes). Thus the presence of allosteric binding pockets adds to the attractiveness of enzyme molecules as drug targets by providing multiple mechanisms for interfering with enzyme activity, hence effecting the desired pharmacological outcome.

1.3 PERMUTATIONS OF ENZYME STRUCTURE DURING CATALYSIS

Enzymes catalyze chemical reactions; this is their biological function. To effectively catalyze the transformation of substrate molecules into products, the arrangement of chemically reactive groups within the active site must too change in terms of spatial orientation, bond strength and bond angle, and electronic character during the course of reaction. To effect these changes in the active site's structure, the overall conformation of the enzyme molecule must adjust, causing changes not only in the active site but in allosteric binding pockets as well.

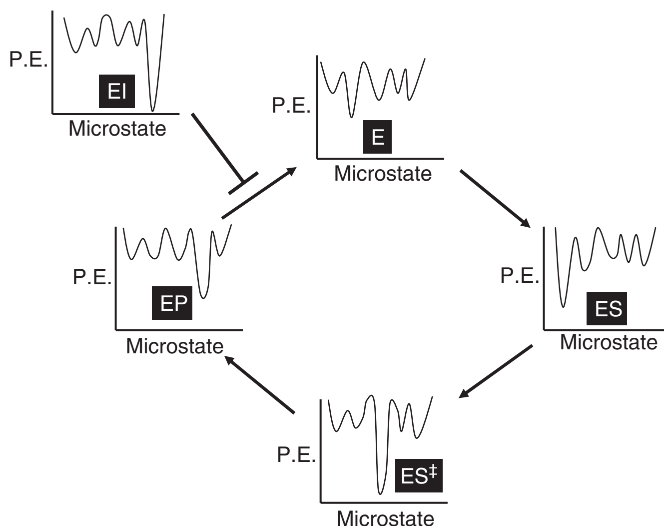

Figure 1.6 Schematic representation of the changes in protein conformational microstate distribution that attend ligand (i.e., substrate, transition state, product and inhibitor) binding during enzyme catalysis. For each step of the reaction cycle, the distribution of conformational microstates is represented as a potential energy (PE) diagram.

The overall globular structure of enzymes is marginally stabilized by a collection of weak intramolecular forces (hydrogen bonds, van der Waals forces, etc.; see Chapter 2). Individual hydrogen bonds and these other intramolecular forces are reversible and easily disrupted to effect a change in protein structure. As a result the structure of the free enzyme (i.e., without any ligand bound) is dynamic and actually represents a manifold of conformational substates, or microstates, that are readily interconvertable. Transitions among these microstates reflect electronic, translational, rotational, and mainly vibrational excursions along the potential energy surface of the microstate manifold (Figure 1.6). Ligands (e.g., substrate, transition state, product, or inhibitor) bind preferentially to a specific microstate, or to a subset of the available microstates, that represent the best complementarity between the binding pocket of that microstate(s) and the ligand structure (Eftink et al., 1983). The ligand binding event thus stabilizes a particular microstate (or subset of microstates) and thereby effects a shift in the distribution of states, relative to the free enzyme, toward greater population of a deeper, narrower potential well (i.e., a lower potential energy minimum). The depth of the potential well for the preferred microstate representative of the enzyme–ligand complex reflects the degree of stabilization of that state, which directly relates to the affinity of the ligand for that state. The deeper this potential well is, the greater is the energy barrier to interconversion between this microstate and the other

potential microstates of the system. Thus, as illustrated in Figure 1.6, a minimal enzyme catalytic cycle reflects a series of changes in microstate distribution as the enzyme binds substrate (*ES*), converts it to the transition state structure ($ES^{\ddagger}$), and converts this to the product state structure (*EP*). Inhibitor molecules likewise bind to a particular microstate, or subset of microstates, that best complements their structure. The highest affinity inhibitor binding microstate can occur anywhere along the reaction pathway of the enzyme; in Figure 1.6 we illustrate an example where the inhibitor binds preferentially to a microstate that is most populated after the product release step in the reaction pathway. If the resulting potential well of the enzyme–inhibitor complex microstate(s) is deep enough, the inhibitor traps the enzyme in this microstate, thus preventing the further interconversions among microstates that are required for catalysis.

Hence every conformational state of the active site and/or allosteric sites that is populated along the chemical reaction pathway of the enzyme presents a unique opportunity for interactions with drug molecules. This is yet another aspect of enzymes that make them attractive targets for drugs: enzymes offer multiple conformational forms, representing distinct binding site structures that can be exploited for drug interactions. One cannot know, a priori, which conformational state of the enzyme will provide the best target for drug interactions. This is why, as discussed in subsequent chapters, I believe that assays designed to screen for inhibitors of enzymes must rely on direct measurements of enzyme activity. Let us again consider the inhibition of DHFR by methotrexate as an illustrative example.

DHFR catalyzes the reduction of dihyrofolate to tetrahydrofolate utilizing an active site base and the redox cofactor NADPH as hydrogen and electron sources (Figure 1.7). The enzyme can bind substrate or NADPH cofactor, but there is kinetic evidence to suggest that the NADPH cofactor binds prior to dihydrofolate in the productive reaction pathway. The inhibitor methotrexate is a structural mimic of dihydrofolate (Figure 1.8). Measurements have been made of the equilibrium dissociation constant (K_d or in the specific case of an inhibitor, K_i) for methotrexate bound to the free enzyme and to the enzyme–NADPH binary complex. Methotrexate does make some specific interactions

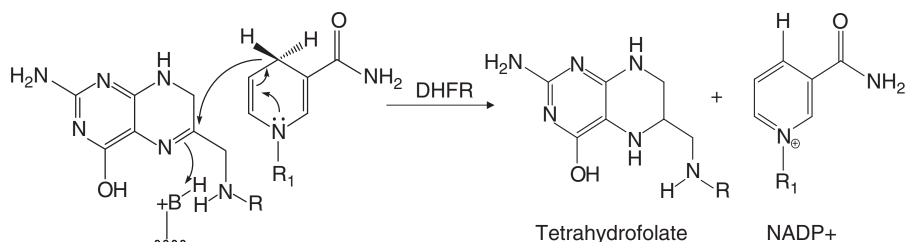

Figure 1.7 Chemical reaction catalyzed by dihydrofolate reductase.

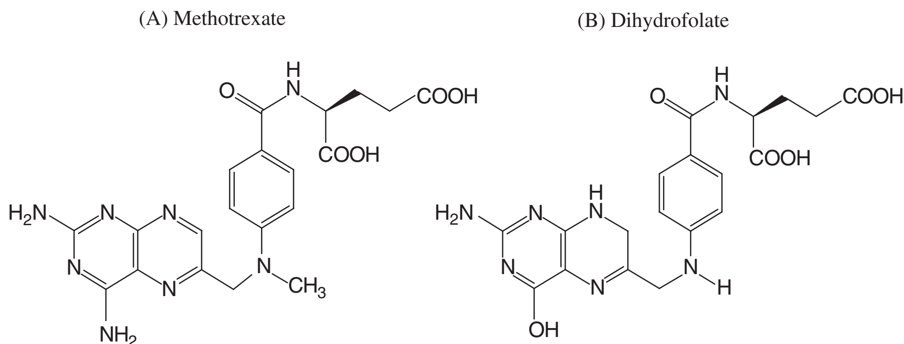

Figure 1.8 Chemical structures of (**A**) methotrexate and (**B**) dihydrofolate.

with the NADPH cofactor, but the binding of NADPH to the enzyme also modulates the conformation of the active site such that the K_i of methotrexate changes from 362 nM for the free enzyme to 0.058 nM (58 pM) for the enzyme–NADPH binary complex (Williams et al., 1979; see also Chapter 6). This represents an increase in binding affinity of some 6000-fold, or a change in binding free energy of 5.2 kcal/mol (at 25°C) for interactions of an inhibitor with a single, conformationally malleable, binding pocket on an enzyme!

Thus enzyme active sites (and often allosteric sites as well) adopt a variety of specific conformational states along the reaction pathway of the enzyme, as a direct consequence of their catalytic function. This has been exploited, for example, to identify and optimize nucleoside-analogue inhibitors and non-nucleoside inhibitors of the HIV reverse transcriptase. The nucleoside-analogue inhibitors bind in the enzyme active site, while the nonnucleoside inhibitors bind to an allosteric site that is created in the enzyme due to conformational changes in the polypeptide fold that attend enzyme turnover (see Furman et al., 2000, for an interesting review of how a detailed understanding of these conformational changes helped in the development of HIV reverse transcriptase inhibitors). Another illustrative example of this point comes from the examination of the reaction pathway of aspartyl proteases, enzymes that hydrolyze specific peptide bonds within protein substrates and that, as a class, are well-validated targets for several diseases (e.g., AIDS, Alzheimer's disease, and various parasitic diseases). From a large collection of experimental studies, a general reaction pathway can be described for aspartyl proteases that is illustrated, in terms of active site structure, in Figure 1.9. The resting or ground state of the free enzyme (E) contains two catalytically essential aspartic acid residues within the active site (from which this class of enzymes derives its name). One aspartate is present as the protonated acid, the other is present as the conjugate base form, and the two share the acid proton through a strong hydrogen-bonding interaction. The two aspartates also

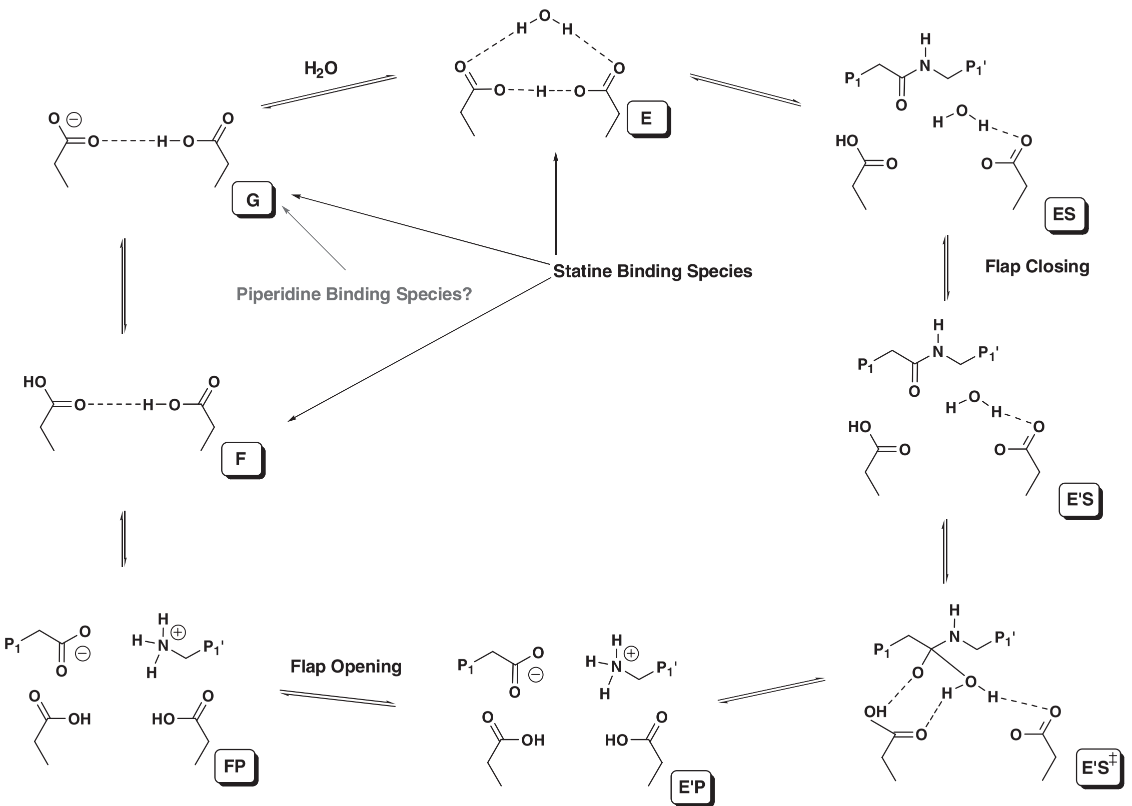

Figure 1.9 Reaction cycle for an aspartyl protease illustrating the conformational changes within the active site that attend enzyme turnover.
Source: Model based on experimental data summarized in Northrop (2001).

hydrogen bond to a critical active site water molecule. Substrate binding distrupts these hydrogen-bonding interactions, leading to the initial substrate encounter complex, *ES*. A conformational change then occurs as a "flap" (a loop structure within the polypeptide chain of the enzyme) folds down over the substrate-bound active site, creating a solvent-shielded binding pocket that is stabilized by various noncovalent interactions between the flap region and the substrate and other parts of the enzyme active site. The unique state derived from the flap's closing is designated $E'S$ in Figure 1.9 to emphasize that the structure of the enzyme molecule has changed. From here the active site's water molecule attacks the carbonyl carbon of the scissile peptide bond, forming a dioxy, tetrahederal carbon center on the substrate that constitutes the bound transition state of the chemical reaction ($E'S^{\ddagger}$). Bond rupture then occurs with formation of an initial product complex containing two protonated aspartates and cationic and anionic product peptides (state $E'P$). The flap region retracts, opening the active site (state *FP*) and allowing dissociation of product (state *F*). Deprotonation of one of the active site's apartates then occurs to form state *G* (note that the identity of the acid and conjugate base residues in state *G* is the opposite of that found in state *ES*). Addition of a water molecule to state *G* returns the enzyme to its original conformation (*E*). Initial attempts to inhibit aspartyl proteases focused on designing transition state mimics, based on incorportation of statine and hydroxyethylene functional groups into substrate peptides. The design strategy was based on the assumption that these inhibitors would interact with state E of the reaction pathway, expel the active site water, and create an enzyme-inhibitor complex similar to state $E'S^{\ddagger}$. A variety of kinetic and structural studies have revealed that these peptidic inhibitors likely bind to multiple states along the reaction pathway, possibly including states *E*, *F*, and *G*. Another class of piperidine-containing compounds has been shown to be potent inhibitors of some aspartyl proteases, such as pepsin and especially renin (Bursawich and Rich, 2000). Studies from Marcinkeviciene et al. (2002) suggest that these inhibitors interact not with the resting state of pepsin, but instead with the alternative conformational state *G*. This conclusion is consistent with X-ray crystallographic data showing that the piperidines induce an altered conformation of the aspartyl protease renin when bound to its active site (see Bursawich and Rich, 2000, for a review of these data).

The examples above serve to illustrate that the conformational dynamics of enzyme turnover create multiple, specific binding pocket configurations throughout the reaction pathway, each representing a distinct opportunity for drug binding and inhibition.

1.4 EXTENSION TO OTHER TARGET CLASSES

Although our discussion up to now has centered on the value of enzymes as targets for drug discovery, it is worth noting that many of the principles

described here and in subsequent chapters apply equally well to other target classes. For example, consider the GPCR target class.

Like enzymes, GPCRs bind small molecule ligands reversibly within a pocket intended for natural agonist (i.e., activating ligand) interactions. In response to agonist binding, GPCRs undergo conformational transitions akin to the changes in conformational state distribution described above for enzyme catalysis. The result of this ligand-induced redistribution of conformational states in GPCRs is changes in the conformation of cytosolic portions of the receptor molecule that expose loci for post-translational modification and resultant recruitment of other cytosolic proteins. Recruitment of G-proteins, for example, to the receptor is accompanied by nucleotide exchange and sets off a cascade of enzyme-catalyzed protein phosphorylation events that ultimately result in altered gene transcription. The cascade of biochemical reactions, starting with extracellular ligand engagement by the GPCR, intervening, sequential kinase phosphorylation, and ending with transcription factor recruitment and gene transcription, is referred to as a signal transduction cascade.

Aberrant signal transduction can lead to a variety of human diseases. Hence, pharmacological blockade of disease-associated signal transduction is a well-established means of disease intervention. Small molecule inhibitors (also referred to as antagonists) have been developed as drugs that bind to GPRCs within the natural agonist binding site, akin to competitive inhibitors that bind at enzyme active sites (such antagonists are also referred to as orthosteric to indicate the overlap of agonist and antagonist binding pockets). As with enzymes, GPCRs can also contain allosteric binding pockets that can be effectively targeted for drug interactions. Thus, there are many biochemical parallels between enzymes and GPCRs with respect to ligand binding, conformational dynamics, and drug interactions. Many of the quantitative biochemical methods that are germane to evaluation of enzyme inhibitors in drug discovery are thus equally applicable to the evaluation of pharmacological agonists and antagonists of GPCRs.

We have exemplified above the biochemical similarities between enzymes and GPCRs that translate into common pharmacological approaches to drug evaluation for these two target classes. The extension of the quantitative biochemical methods described in this text to GPCRs is merely an example of the larger extension of these methods to any target class for which ligand engagement is the antecedent of biological activity that can be modulated by pharmacological ligand (i.e., drug) intervention. Thus, the reader will find that much of the material presented in the following chapters, while focused on enzyme targets, will be of use broadly for receptors, ion channels, and other target classes of interest to drug discovery professionals.

1.5 OTHER REASONS FOR STUDYING ENZYMES

While the main focus of this chapter has been on enzymes as the primary molecular targets of drug action, it is worthwhile noting that the quantitative

evaluation of enzyme activity has other important roles in drug discovery and development, as will be discussed in detail in Chapter 10.

First, in addition to the primary target, related enzymes may need to be studied as "counterscreens" to avoid unwanted side effects due to collateral inhibition of the related enzymes. For example, suppose that we wish to inhibit the aspartyl protease of HIV as a mechanism for treatment of AIDS. Because the target is an aspartyl protease, we would wish to ensure that inhibitors that are taken forward to the clinic do not display significant side effects due to collateral inhibition of human aspartyl proteases, such as pepsin, rennin, and the cathepsins D and E. One might therefore set up in vitro assays to test compounds not only against the primary target enzyme but also against structurally or mechanistically related enzymes whose inhibition might create a liability in vivo. In such studies one wishes to compare the relative affinity of an experimental compound for the various enzymes. This is best done by determination of the K_i values for each enzyme, as described further in Chapter 5.

A second area of drug discovery and development in which enzyme reactions play a critical role is in the study of drug metabolism and pharmacokinetics. The elimination of xenobiotics, including drug molecules, from systemic circulation is driven by metabolic transformations that are entirely catalyzed by enzymes. These biotransformation reactions are divided into two general categories, phase I and phase II. Phase I reactions are used to increase the aqueous solubility of compounds to aid in their elimination. These reactions convert the parent drug to a more polar metabolite through oxidation, reduction, or hydrolysis reactions. Phase II reactions conjugate the drug or its metabolite to an endogenous substrate, such as glucuronic acid, sulfuric acid, acetic acid, or an amino acid, to again aid in its solubility and elimination.

The rate of drug disappearance from circulation (i.e., the pharmacokinetic half-life) is always measured in vivo in various animal species (including the human). However, it is common today for scientists to attempt to predict metabolic transformations of drug molecules by studying the interactions of the drugs with the transforming enzymes in vitro. For example, the cytochrome P450 family of hepatic enzymes commonly participates in the phase I oxidation of drug molecules. These enzymes can be studies in vitro in the form of liver slices, hepatocyte homogenates, and as isolated recombinant enzymes. Drug molecules can be utilized by these enzymes as substrates, leading to metabolic oxidation of the parent molecule. Different xenobiotics are recognized by different isozymes of the cytochrome P450 family. A quantitiative knowledge of the utilization of a drug by the different cytochrome P450 isozymes can be of great value in understanding the rate of drug transformations in patients, and in understanding differences in drug metabolism among individuals. For example, differences in expression levels of the various cytochrome P450 isozymes are seen between the genders and among different ethnic groups. Also certain disease states, or administration of certain drugs, can lead to induction of specific isozymes. Any of these differences can lead

to significant changes in drug metabolism rates that can have important clinical consequences in terms of both drug efficacy and safety.

Other drug molecules can behave as inhibitors of specific cytochrome P450s. Inhibition of cytochrome P450 isozymes can lead to a slowing down of the metabolism, hence unexpected accumulation, for drugs that would otherwise be metabolized by this route. Therefore untoward side effects, associated with the buildup of one drug, could occur if a patient were to receive a combination of that drug and a second drug that acted as a cytochrome P450 inhibitor. Therefore the information presented in this text is germane to studies of drug metabolism and pharmacokinetic as well as to the evaluation of compounds as inhibitors of an enzyme target.

It is also worth noting that some drugs utilize the activity of specific enzyme types to transform an inactive molecule to an active drug in vivo. The approach is commonly referred to as a "pro-drug" approach. In some cases the structural determinants of enzyme inhibition are incompatible with oral absorption, cell permeation, or some other critical component of drug action. In such cases it is sometimes possible to convert the problematic functionality to one that is compatible with absorption, permeation, and so on, and that can be transformed to the active functionality by enzymes within the body. For example, carboxylic acid groups can play an important role in forming strong interactions with charged residues and metal ions within the binding pocket of a target enzyme. Free carboxylic acids, however, are often not well transported across cell membranes and thus their in vivo effectiveness is limited. On the other hand, the charge-neutralized methyl and ethyl esters of carboxylates permeate cell membranes well. Thus one can often create a pro-drug of a carboxylate-containing molecule by forming the corresponding ester. Once the ester has entered the cell, it is acted upon by cellular esterases to liberate the active carboxylic acid. This approach was used with great success by the Merck group to deliver the active molecule enaliprilate (a carboxylic acid-containing inhibitor of angiotensin converting enzyme) in the form of an ethyl ester drug, enalipril. Pro-drug approaches like this are very common in human medicine (see Silverman, 1992, for more examples). A quantitative understanding of the processes involved in prodrug conversion could be of great value in drug optimization studies. Hence the types of evaluations of enzyme activity discussed in this book are directly relevant to the development of pro-drugs for use in human medicine.

In addition to pro-drug conversion to active species, there are also examples of marketed drugs for which the active molecule is the result of phase I metabolism (typically cytochrome P450-mediated transformation) of a parent compound. Acetaminophen, fexofenadine, cetirizine, and other marketed drugs represent examples of active metabolites, resulting from cytochrome P450-based transformations, that demonstrate superior pharmaceutical development properties relative to their parent compounds (Fura et al., 2004). Once again, a quantitative understanding of the enzymatic reactions leading to the active metabolite provides a rational approach to compound optimization for this drug discovery strategy as well.

We also note that enzymes are themselves used in clinical settings for a number of reasons. Enzymes form the basis of a number of diagnostic tests that are in current clinical use. The activity of specific enzymes is also being considered as potential biomarkers of disease modification in clinical trials for a variety of drug candidates. Enzymes are sometimes used directly as therapeutic agents themselves. For example, pancreatic enzymes are ingested to supplement the loss of those digestive enzymes in pancreatitis. Last, the genes that encode specific enzymes are being considered for use as therapeutic agents, especially for diseases associated with genetic-based loss of function for the cognate enzyme in patients. It is early days for these types of enzyme-based therapies. However, as this area of research matures, the application of quantitative studies of enzyme activity will clearly be critical to success.

The above-mentioned examples are but a few of the many applications in which quantitative studies of enzyme–ligand interactions are critical to the drug discovery and development process. Hence the reader is encouraged to consider the material in this text not only in the context of inhibition of a primary molecular target, but throughout the many steps in the development of a drug candidate for clinical application.

1.6 SUMMARY

In this chapter we have described some of the features of enzyme structure and reaction pathway that make enzymes particularly attractive targets for drug discovery and design efforts. These features include the following:

- Active sites amenable to binding drug-like molecules.
- Potential allosteric sites that offer additional avenues for drug interactions with functionally critical binding pockets.
- Conformational variation in binding sites that attend catalysis and offer a multiplicity of distinct opportunities for drug interactions with the target molecule in a manner leading to abolition of biological function.

A final feature of enzymes that contributes to their attractiveness as drug targets is historic precedence. Through trial and error and through more modern attempts at systematic pharmacology, enzymes emerge over and over again as preferred targets. As illustrated by the small sampling in Table 1.1, many enzymes have been successfully targeted for drug interactions in human medicine.

Having established in this chapter the desirability of enzymes as molecular targets for pharmacotherapy, we will now turn our attention to the experimental evaluation of drug–enzyme interactions. In the chapters that follow we introduce the reader to some of the salient features of enzyme catalysis as they relate to the proper development of activity assays with which to assess

inhibitor action. We then present a discussion of reversible inhibitor interactions with enzymes, and the quantitative analysis of these interactions. In subsequent chapters we discuss practical aspects of developing activity assays for high-throughput screening and for postscreening lead optimization and the establishment of structure-activity relationships (SARs). In the final chapters of this text we focus on commonly encountered forms of inhibition that do not conform to classical modes of reversible inhibition. Appropriate methods for the proper quantitative evaluation of these forms of inhibition will be presented.

REFERENCES

Ajay, Walters, W. P., and Murcko, M. A. (1998), *J. Med. Chem.* **41**: 3314–3324.

Bolin, J. T., Filman, D. J., Matthews, D. A., Hamlin, R., and Kraut, J. (1982), *J. Biol. Chem.* **257**: 13650–13662.

Bursawich, M. G., and Rich, D. H. (2000), *J. Med. Chem.* **45**: 541–558.

Clardy, J., and Walsh, C. T. (2004), *Nature* **432**: 829–837.

Copeland, R. A. (2000), *Enzymes: A Practical Introduction to Structure, Mechanism and Data Analysis*, 2nd ed., Wiley, New York.

Copeland, R. A., and Anderson, P. S. (2001), in *Textbook of Drug Design and Discovery*, 3rd ed., P. Krogsgaard-Larsen, T. Liljefors, and U. Madsen, eds., Taylor and Francis, New York, pp. 328–363.

Drews, J. (2000), *Science* **287**: 1960–1964.

Eftink, M. R., Anusien, A. C., and Biltonen, R. L. (1983), *Biochemistry* **22**: 3884–3896.

Fura, A., Shu, Y.-Z., Zhu, M., Hanson, R. L., Roongta, V., and Humphreys, W. G. (2004), *J. Med. Chem.* **47**: 4339–4351.

Furman, P. A., Painter, G. R., and Anderson, K. S. (2000), *Curr. Pharmaceut. Design* **6**: 547–567.

Hajduk, P. J., Huth, J. R., and Fesik, S. W. (2005), *J. Med. Chem.* **48**: 2518–2525.

Hopkins, A. L., and Groom, C. R. (2002), *Nature Rev. Drug Discov.* **1**: 727–730.

Keller, T. H., Pichota, A., and Yin, Z. (2006), *Curr. Opin. Chem. Biol.* **10**: 357–361.

Klebe, G. (1994), *J. Mol. Biol.* **237**: 212–235.

Liang, J., Edelsbrunner, H., and Woodward, C. (1998), *Protein Sci.* **7**: 1884–1897.

Lipinski, C., Lombardo, F., Doming, B., and Feeney, P. (1997), *Adv. Drug Deliv. Res.* **23**: 3–25.

Lipinski, C. A., Lombardo, F., Dominy, B. W., and Feeney, P. J. (2001), *Adv. Drug Del. Rev.* **46**: 3–26.

Marcinkeviciene, J., Kopcho, L. M., Yang, T., Copeland, R. A., Glass, B. M., Combs, A. P., Fatahatpisheh, N., and Thompson, L. (2002), *J. Biol. Chem.* **277**: 28677–28682.

Northrop, D. B. (2001), *Acc. Chem. Res.* **34**: 790–797.

Ondetti, M. A., and Cushman, D. W. (1984), *CRC Critical Rev. Biochem.* **16**: 381–411.

Pettit F. K., and Bowie, J. U. (1999), *J. Mol. Biol.* **285**: 1377–1382.

Radzicka, A., and Wolfenden, R. (1995), *Science* **267**: 90–93.

Taira, K., and Benkovic, S. J. (1988), *J. Med. Chem.* **31**: 129–137.

Veber, D. F., Johnson, S. R., Cheng, H.-Y., Smith, B. R., Ward, K. W., and Kopple, K. D. (2002), *J. Med. Chem.* **45**: 2615–2623.

Vieth, M., Siegel, M. G., Higgs, R. E., Watson, I. A., Robertson, D. H., Savin, K. A., Durst, G. L., and Hipskind, P. A. (2004), *J. Med. Chem.* **47**: 224–232.

Warshel, A., Åquist, J., and Creighton, S. (1989), *Proc. Nat. Acad. Sci. USA* **86**: 5820–5824.

Wiesmann, C., Barr, K. J., Kung, J., Zhu, J., Erlanson, D. A., Shen, W., Fahr, B. J., Zhong, M., Taylor, L., Randal, M., McDowell, R. S., and Hansen, S. K. (2004), *Nature Struct. Mol. Biol.* **11**: 730–737.

Williams, J. W., Morrison, J. F., and Duggleby, R. G. (1979), *Biochemistry* **18**: 2567–2573.