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Molecular recognition: Models for drug design

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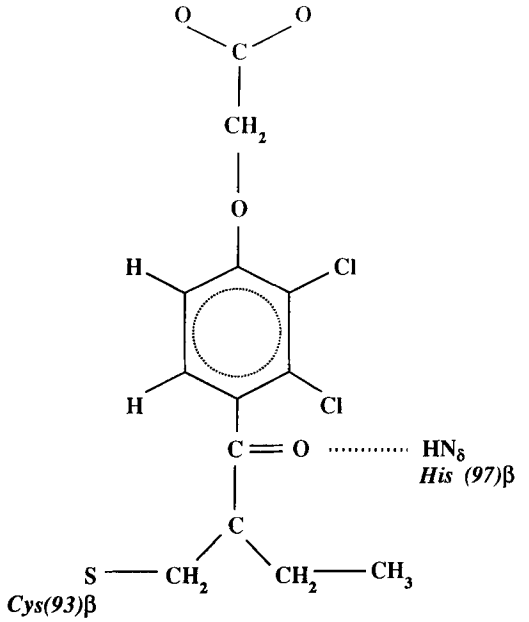
Abstract. The review takes examples, mostly from the recent literature, to illustrate how an understanding of physico-chemical properties and an appreciation of the molecular shape and electronic properties can lead to a better insight into molecular recognition processes. The techniques used to generate 3-dimensional structures of molecules and the influence this information has had on the drug design cycle, are briefly discussed.
Key words. Molecular recognition; modelling; NMR; structure; X-ray.

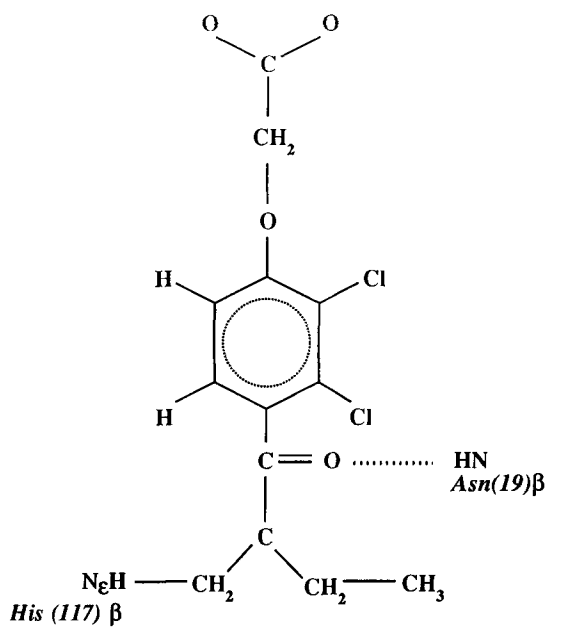
Introduction

The propagation of all life forms is dependent upon the recognition/response phenomenon. As described in other contributions to this multi-author review, molecular recognition plays a central role throughout the biological systems. It is the basis of the specificity seen during neurotransmitter response, cell cell, antigen antibody, substrate enzyme, and hormone receptor interactions. It is not a new concept, having occupied the minds of notable 19th century scientists like Pasteur and Ehrlich. Indeed, Ehrlich is widely credited with developing the concept of the pharmacophore. In a paper in 1900[25], he reasoned that toxins possessed two different combining groups, a hepatophore that bound the toxin to the cell and a toxophore that was responsible for the toxic action. Today, an acceptable definition of a pharmacophore would be that part of a molecule which contains a 3-dimensional pattern that exactly complements the shape and surface properties of the target protein.

The modern image of molecular recognition has developed as a direct result of the accessibility of 3-dimensional structural information and the means to visualise and interact with these structures. In 1965, Perutz and his colleagues published the first 3-dimensional picture of a protein structure[61], haemoglobin. This pioneering work on hemoglobin and subsequent work on related proteins led to an explanation, in molecular terms, of the way oxygen was transported in the blood stream. Even these early pictures were relevant to medicine because they provided a simple interpretation into the cause of the disease sickle cell anaemia. Haemoglobin is a tetramer consisting of two α chains, each of 141 amino acids, and two β chains, each of 146 amino acids. It is an allosteric protein in equilibrium between the oxy (R) structure and the deoxy (T) structure. The cause of the disease sickle cell anaemia can be traced to the 'natural' mutation of two glutamate residues to valine. The effect of these two mutations is to cause a disruption to the R/T equilibrium, in favour of the T-form, by creating a surface hydrophobic region which promotes the linear aggregation of haemoglobin and sickling of red blood cells. At least two approaches in drug design to alleviate this condition can be envisaged. First, compounds designed to alter the allosteric equilibrium in favour of the more soluble R-form, and second, compounds designed to alter the T-form in such a way as to destabilise the intermolecular interactions that lead to aggregation. Perutz[62] has pub-



(a)



(b)

Figure 1. One molecule of ethacrynic acid, ECA, (1), a potent antisickling agent, binds to each of the β chains of haemoglobin[61]. By crystallographic analysis, ECA has been shown to be covalently bound to a cystein residue, Cys-93, in one sub-unit, and to a histidine residue, His-117, in the second β subunit of haemoglobin.

lished a crystallographic analysis of the binding of five different drugs to human deoxyhemoglobin. These compounds are all known to affect either the R/T equilibrium or the gelling propensity of haemoglobin. The most potent antisickling compound, ethacrynic acid, ECA (**1**), has been shown to manifest its antigelling properties by binding to two different sites on haemoglobin (fig. 1).

All cell functions and membrane transport are mediated by a series of molecular events which are triggered by a highly specific molecular recognition mechanism. Individual recognition processes differ and, therefore, only principles can be generalised. The structural basis of protein-protein recognition, the ability of certain proteins to form specific stable complexes, have recently been reviewed[41,51]. Although all the mechanisms involved are not yet fully understood, there is no doubting the value of possessing an experimentally determined 3-dimensional structure, or theoretical model of the therapeutic macromolecule, to use in the drug design process[76].

Tools for structural information

3-Dimensional structural information now plays an integral role in the comprehensive understanding of molecular interactions. Over the last 25 years there has been an explosion in structural information generated by a combination of experimental and theoretical techniques. There are three sources of structural information available;

1) X-ray crystallography, which gives information over a slice in time,
2) the solution structures derived from NMR methods and
3) theoretical models, based upon mathematical principles.

Each provides a different but complementary perspective of molecular interactions. Recent advances in molecular biology and the consequent availability of large amounts of pure macromolecules have contributed to the attainment of a wealth of structural information, now becoming available from the two experimental techniques. Recent advances in computer technology have also directly benefited all three technologies to rapidly generate structural information.

1. X-ray diffraction

X-ray crystallography[73] can, in theory, define the 3-dimensional coordinates of any size of molecule, for which suitable crystals can be grown. The refinement of membrane proteins and complete viruses with a molecular mass of over 6 million Dalton are now available at atomic resolution. The major advance has been in the development of area detectors and high powered radiation sources. Both of these have contributed to reducing one of the bottlenecks; the time required for data collection has decreased from days and weeks to minutes and hours.

X-ray diffraction techniques are well suited to ordered systems and with today's computational capabilities the size of the structure is not a problem. The prerequisite is the availability of suitable crystals. One of the main drawbacks to this methodology is the fact that for peptide and protein structures large amounts (tens of milligrams) of pure material must be prepared before crystallisation can be attempted. With today's advances in molecular biology this has become less of a problem. For the crystallographer, obtaining suitable crystals for diffraction studies is only the first hurdle; thereafter, the successful solution of the phase problem depends upon finding at least two independent isomorphous heavy atom derivatives, if molecular replacement techniques cannot be used. Only after the unit cell has been successfully defined can the structure be refined and atomic coordinates derived.

Single crystal X-ray diffraction studies provide the most useful experimental data on 3-dimensional structures. Essentially the same crystallographic approach can be applied to solving small molecules like dopamine[6] or large biological structures, like viruses[67]. The result is a precise 3-dimensional picture of the atomic positions in the molecule. Over 70,000 organic structures are now available in the Cambridge Crystallographic Databank[2] and to this can be added the smaller number of protein structures deposited in the Brookhaven Protein Databank[7]. These two databanks, although containing only a fraction of all the crystal structures ever solved, nonetheless provide an invaluable starting point from which to study molecular recognition.

2. Nuclear Magnetic Resonance spectroscopy

Over the last 10 years Nuclear Magnetic Resonance (NMR) spectroscopy[21,87,88], has emerged as a powerful method for studies on the structure and dynamic properties of peptides and proteins in solution. Information on dynamic process, on the picosecond to second time scale, accessible by NMR studies complement the 'static' 3-dimensional structural information obtained from crystallography.

Well-resolved 3-dimensional structures of several small proteins[43,44] (less than 100 residues) have been obtained using 2D proton NMR techniques[87,88]. For larger proteins the conventional 2D techniques are not quite so suitable, because of problems in spectral overlap. The use of isotopically labelled proteins with N15 and C13, in conjunction with the development of 3D and 4D experiments[15] has meant that proteins of 150–300 amino acids can now be studied and a 3-dimensional structure defined. Larger proteins than this cannot be studied today because of the physical limitations of the NMR technique; the slow rotational motion of large molecules such as proteins leads to broad resonances that give no detailed structural information.

3. Molecular modelling

Molecular modelling systems provide elegant and powerful tools for building, visualising, analysing and storing models that can be used to interpret structure-activity relationships. Models are built using mathematical tools based upon the principles of molecular mechanics[84] and quantum theory[66]. These methodologies are complementary. Molecular mechanics describe the molecular system in terms of classical forces, such as bond stretching, angle bending etc. Here, the molecule can be envisaged as a set of 'balls and springs' with a series of potential energy functions expressing the molecular force field as a sum of these functions, thus:

$$E_{\text{total}} = E_{\text{stretching}} + E_{\text{bending}} + E_{\text{dihedral}} + E_{\text{van der Waals}} + E_{\text{electrostatic}} + E_{\text{hydrogen bond}}$$

Each of the individual energy terms has a preferential equilibrium position (bond lengths, bond angles etc.) and an associated force constant which is either experimentally known or theoretically derived, the 'force field'. These force constants determine the energy penalty associated with an individual deviation from the desired equilibrium position. Two of the most extensively tested force fields are MM2[3], for hydrocarbons and a limited set of heteroatom containing functional groups, and AMBER[81,82] for peptides and nucleic acids.

One of the major disadvantages of the molecular mechanics approach is the difficulty in dealing with metals. Most of the available packages treat the metal-ligand interaction by defining a covalent bond between the metal and the ligand, or alternatively relying solely on electrostatic and van der Waals forces. The first method preordains the coordination and geometry of the metal centre while the second relies extensively on choosing an appropriate electrostatic model. These deficiencies have been addressed in a recent publication by Vedani[75], in which a more robust treatment of metal-ligand interactions is described. The new force field features a novel potential function describing the metal-ligand interactions and including variables for metal-ligand separation, symmetry at the metal centre, directionality of the metal-ligand bond and ligand-metal charge transfer parameters.

Quantum mechanics, including the semi-empirical and ab initio techniques depend on finding a solution to the Schroedinger equation to calculate a variety of properties, such as electron densities, atomic charge, dipole moments and orbital energies and populations. Quantum mechanics can provide more accuracy and allows the calculation of electronic effects which are impossible to determine in the molecular mechanics frame. The cost of this precision is that these calculations can consume vast quantities of computational resources. AMPAC[63] and MOPAC[63], are two semi-empirical packages containing programs like AM1[22] and MNDO[23], and along with the

GAUSSIAN series[29], are amongst the most popular programs for quantum mechanical calculations.

Molecular dynamics calculations are used to investigate the dynamic behaviour of molecules with time, for example the motion of a substrate as it enters the active site of an enzyme[30,35]. In this case, a solution is found to the classical Newtonian equations of motion (Newton's second law) given a potential energy function and its associated force field. There are a number of dedicated review articles[8,14,33,37] on the influence of molecular modelling on drug design and at least one comprehensive review on molecular modelling software[16].

The physical chemistry of molecular recognition

The rules governing all physical chemical processes can also be applied to molecular recognition. The binding energy, ΔG, is composed of two opposing terms. An energy component ΔH, and an entropic component $-T\Delta S$. It is a complex process involving solvation/desolvation, global changes in translation and rotational degrees of freedom as well as internal modifications to rotation and vibrational modes[27,45]. The process of binding requires that the drug and receptor molecule have a complementarity of shape and charge which leads to a negative interaction energy. Bimolecular complex formation is governed by the classical law relating the free energy of dissociation (free energy at constant pressure of Gibbs free energy) to an equilibrium constant K, thus

$$\Delta G = -RT\ln K$$

For example, morphine which binds to its receptor with an inhibitory constant of 5 nM, will according to the formula have a binding energy of some 47.6 kJ/mol, at a temperature of 25 °C. The equation goes on to demonstrate that for a small increase in binding energy of -6.3 kJ/mol, the binding constant will improve by a factor ten. In other words the design of small surface changes can result in dramatic improvements (or losses) in biological affinity. A typical structural alteration that might produce such a change in binding energy would be the formation of an additional hydrogen bond or hydrophobic interaction. Andrews[4] has taken the argument further and developed an empirical formula to define the 'goodness' of fit of functional groups with a receptor.

Consider the following example. Weber[79] has published a crystallographic analysis of the tetrameric vitamin-binding protein streptavidin and its ligand, biotin. This is an interesting system because the ligand binds with a dissociation constant of 10^{-14}, making it amongst the strongest known protein ligand interactions. From a comparison of the structures of streptavidin with and without biotin bound, it is apparent that the unusually high affinity streptavidin exhibits for biotin reflects the participation of many factors that cooperate to allow the formation of multiple hydrogen bonds between the protein and biotin. These factors include the displacement of strongly bound water to reveal a polar binding pocket. It is in this pocket that the ureido oxygen of biotin binds (fig. 2). The resulting oxyanionic form is then stabilised by an extended dipolar array. These effects are enhanced by the ordering of a flap region, whose function is thought to be the sequestering of the biotin from the surrounding aqueous environment. Substantial changes to the quaternary structure of the tetramer also result when the ligand is bound. All these interactions are hidden in the binding energy term. Simplistically, it

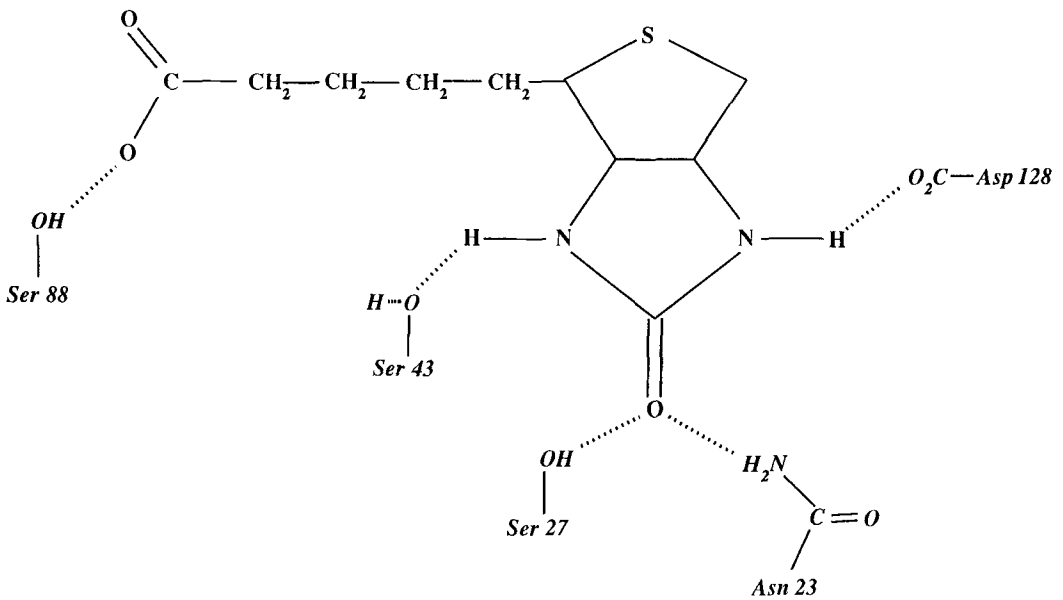


Figure 2. Some of the electrostatic interactions between biotin and streptavidin that gives rise to the high binding affinity[79].

would appear, from structural analysis, that the main contributions to the binding energy are enthalpic and derived from the increased number of hydrogen bonds that the bound biotin makes.

Contributions to the binding energy can be quantified as a minor van der Waals term and a dominant electrostatic term. A typical van der Waals contact between two hydrogen atoms, at their optimum separation distance of 2.0 Å, has an interaction energy of -0.08 kJ/mol. An optimum electrostatic interaction between hydrogen and oxygen or nitrogen (e.g. N–H...O or O–H...O or O–H...N), will have energies in the range -16.7 to -29.3 kJ/mol. Electrostatic effects, apart from making a significant contribution to the binding energy, are also involved in the orientation of molecules and the recognition processes that lead to binding [83]. These forces have been implicated in a variety of biologically important intermolecular interactions including drug orientation by DNA [65], macromolecular assembly [60], substrate binding and catalysis [77], and macromolecular complementarity with inhibitors, drugs and hormones [9, 65, 81]. Getzoff has developed algorithms that calculate the 3-dimensional electrostatic field around a macromolecule, and applied this approach to studies on Cu, Zn superoxide dismutase and its substrate [31].

One of the problems in the design approach is the difficulty in calculating the free energy of binding between two molecules in aqueous solution. The most recent mathematical technique which has been applied to ligand receptor interactions is the free energy perturbation method [36, 49]. This method takes advantage of the properties of a thermodynamic cycle to calculate the free energy differences associated with two similar drugs, A and B (fig. 3). In this case the free energy of solvation of either molecule is unknown. By computationally simulating the non-physical process, mutating drug A into drug B, in the gas phase and in solution, the difference in free energy of solvation, $\Delta\Delta G$, can be found. The same technique can be used to calculate differences in the binding free energy between various drugs, bound and unbound. A number of papers [5, 86] have reported favourable examples and the pitfalls for the method have been reviewed [34, 58].

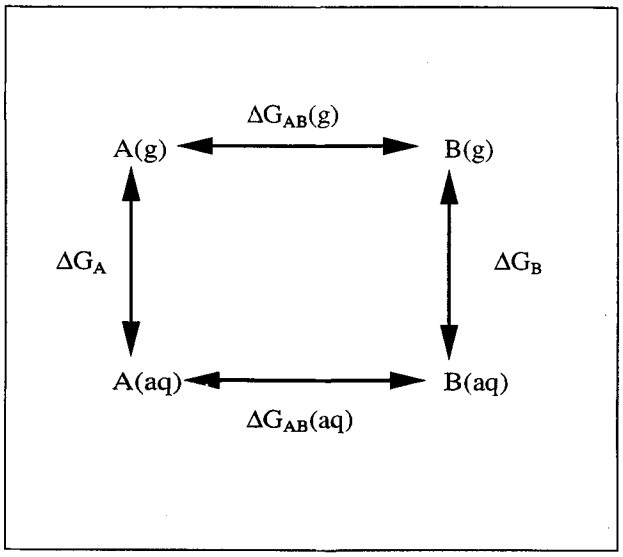


Figure 3. The thermodynamic cycle, as applied to a free energy calculation that results in the free energy of solvation for two molecules A and B. Similar cycles can be used to obtain the difference in binding energies of two ligands to a common receptor.

Drug design

Molecular modelling techniques blend the experimentally derived structural data with theoretical models to study recognition phenomena, develop structure-activity hypotheses and invent novel pharmaceutical compounds. In the past, the drug design process has ignored a complete 3-dimensional structural picture. Physicochemical properties and substituent constants that describe solubility properties, as well as steric and electronic effects, have been used to obtain correlations and to define structure activity relationships. Today, however, the new structural descriptors, volume and electrostatic components are becoming the preferred correlation criteria. The comparative field analysis, CoMFA [18], approach is an example of this trend.

This approach is usually applied to the cases where only 3-dimensional structures of the ligand is available. The typical situation, e.g. neurotransmitter molecules or small peptides, like hormones, is where structural information on the biological receptors (which usually happen to be membrane bound), is lacking. New crystallisation techniques have had to be developed in order to tackle the problem of growing crystals of membrane bound proteins. Henderson's [38] findings on bacteriorhodopsin, determined to a resolution of 3.5 Å, are amongst the first membrane proteins to be successfully analysed.

The ideal situation is where there is structural data on both the target protein and ligand, and where preferably data on both the native and complexed forms are available. The object is to use this information and to understand the reasons for binding and then to use this knowledge to design novel agonist/antagonists, when the target is a receptor, or an inhibitor if the target is an enzyme. There is a major drug design problem when dealing with the large peptides and small proteins, between 10 and 40 amino acid residues. First, there are usually no structural data available on the receptor and second, peptides of this length are unconstrained and are extremely conformationally flexible. Calcitonin, a 32 amino acid hormone, is such an example. NMR and molecule dynamics techniques [80] have been used to demonstrate that in solution, at least 1/3 of the molecule is too conformationally mobile to have any secondary structural characteristics associated with it.

Occasionally smaller systems, like the well-studied case of the cyclic undecapeptide cyclosporin A, can be crys-

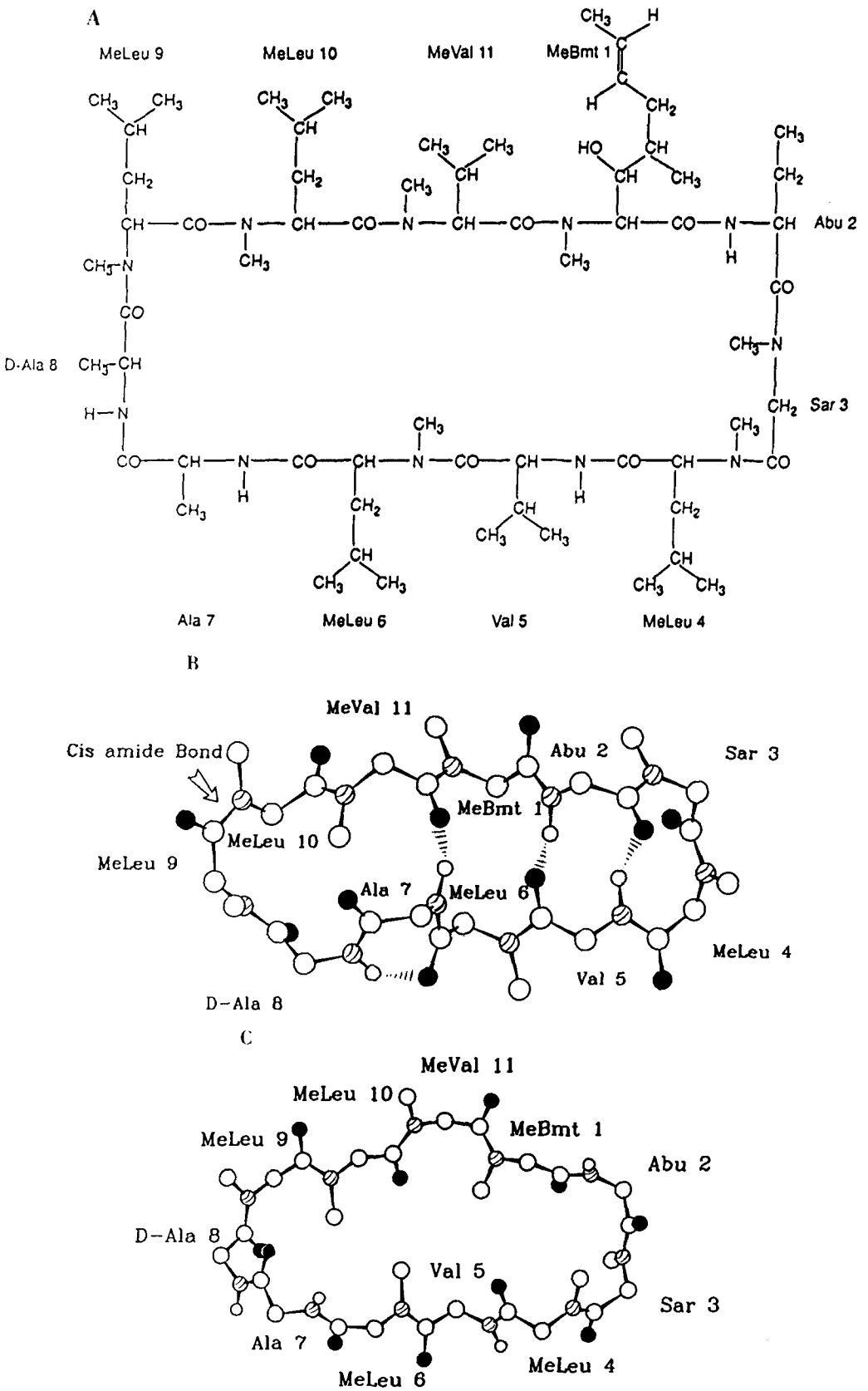


Figure 4. Structures of cyclosporin A, CsA. *A* Chemical formula of CsA, *B* the backbone of CsA determined by NMR[48], and highlighting intramolecular hydrogen bonds. This backbone conformation is similar to that structure determined by X-ray diffraction[48]. *C* The solution structure of CsA when complexed to cyclophilin[78]. The bound conformation is all 'trans' in the peptide backbone, the other major difference is a frame shift in the position of the β-turn resulting in a different juxtaposition of the side chains in the bound and unbound forms.

tallised[48]. It has also been found that the solution structure of cyclosporin A, in nonaqueous solvents, is comparable to the crystal conformation[48] (for crystals grown from organic solvents) (fig. 4). In the absence of all other structural evidence, this conformation has been used as a basis for the design of cyclosporin analogues. Recently, however, the biological relevance of these structures has been called into question. Cyclosporin is now known to exist in a dramatically different conformation when bound to cyclophilin, its putative protein receptor[78]. This is not the only exception. Another dramatic example is the conformation of the macrocycle FK506 (fig. 5). Karuso et al.[42] have shown, in solution, that an equilibrium exists between two conformations, where the two conformations bear a cis/trans relationship around the diketopipecolinyl moiety. The crystal structure reported by Albers and co-workers[1] has a cis configuration at this centre. A comparison of the two structures, one from NMR the other from X-ray, both of which contain the cis amide bond, shows they are quite different in the conformation of the macrocyclic ring. These three experimentally derived conformations apparently have little relevance to the conformation adopted by this macrocycle when complexed to a biological macromolecule. In a recent paper, Van Duyne et al.[24] have shown that none of these unbound structures is comparable to the structure FK506 adopts on binding to its putative protein receptor macrophilin (FKBP). The dissociation constant for FK506 binding to FKBP is 0.4 nM. This binding constant is a reflection of the existence of four direct electrostatic interactions between ligand and receptor as well as a number of smaller contributions from van der Waals contacts; approximately 50% of FK506 is buried into a very shallow cleft on FKBP.

What these studies have illustrated is the absolute necessity of using biologically relevant structural models in the drug design process. In the two cases highlighted here, renewed synthetic effort will no doubt attempt to capitalise upon the improved understanding of the molecular recognition process which these spectroscopic techniques have highlighted. 3-Dimensional models are a prerequisite for structure-based drug design. There is plenty of structural data on ligands, unfortunately, structural information on ligand-receptor complexes is rare. Our understanding of the relationship between the conformations adopted by the bound and unbound ligand is still too naive to be confident in using the conformation of the unbound ligand, in the drug design process.

The ideal cases

Perhaps the best understood class of protein structures are the proteolytic enzymes, especially the serine and aspartic proteases. In general, the active site of these proteases is characterised by an extensive series of hydrogen bonds formed between the backbone amide bonds of the substrate and those of the enzyme. In addition inter-

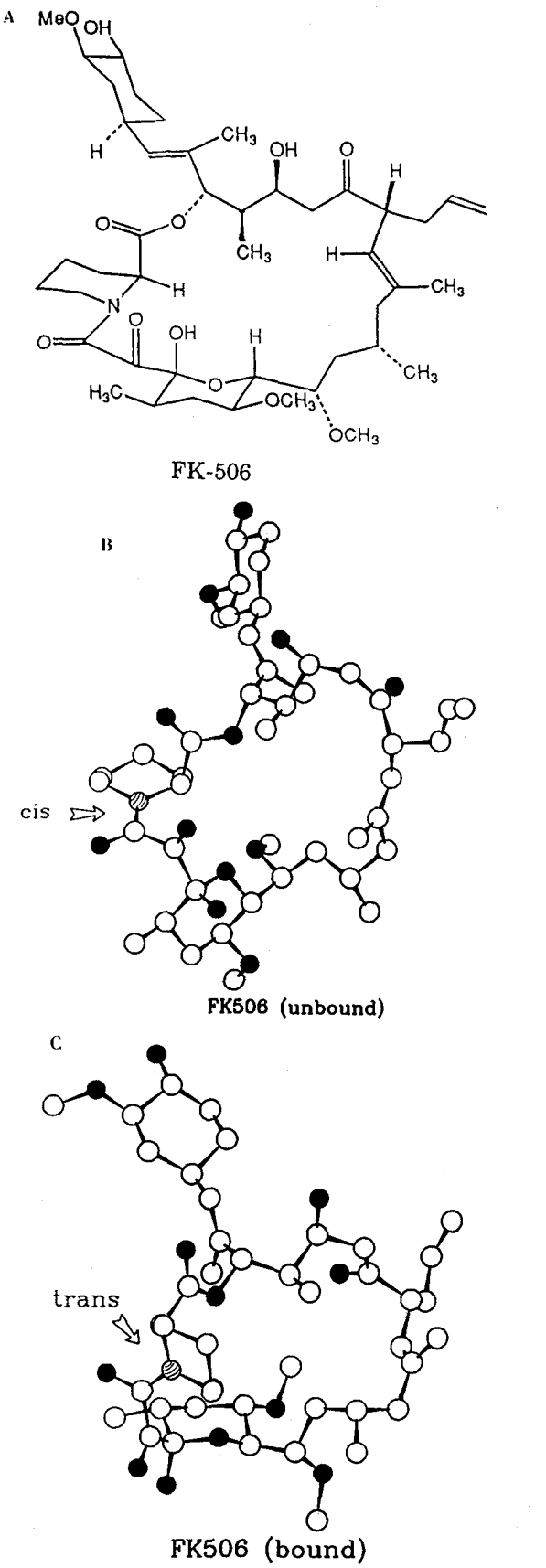


Figure 5. A schematic representation of *A* FK506, *B* the unbound conformation as defined by X-ray analysis[1], *C* the conformation of FK506 bound to FKBP[24].

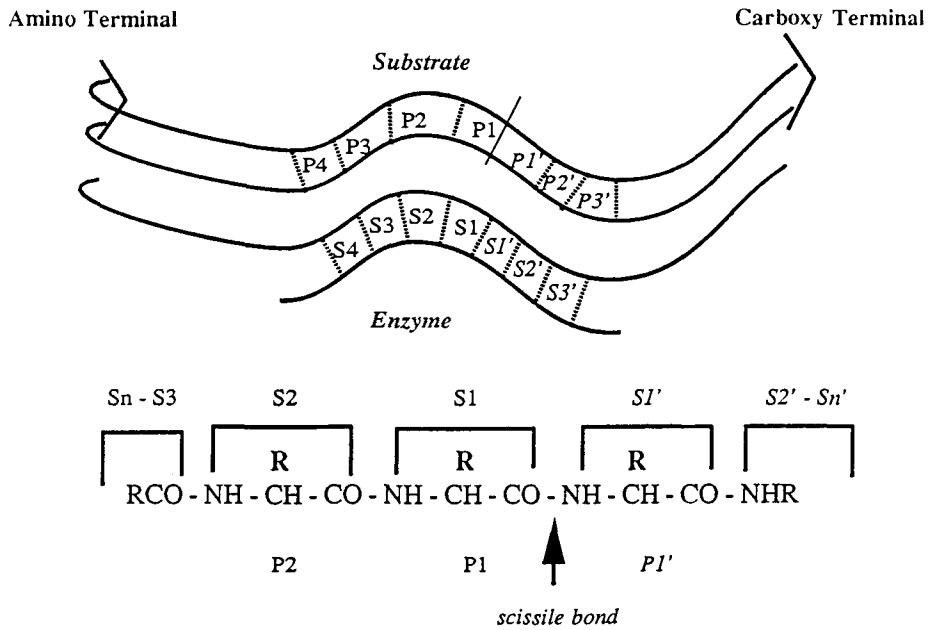


Figure 6. Schechter and Berger's classification of the protease active site.

actions between the side chains of the substrate and well-defined pockets on the enzyme impart specificity to the individual enzymes. A convention has arisen, first proposed by Schechter and Berger[69], that classifies these interactions (fig. 6).

The serine proteases are a class of enzymes that have been well studied because they are obvious targets in controlling many diseases. All known serine proteases have been shown to act via the same mechanism involving a triad of amino acids, His-57, Asp-102 and Ser-195 (chymotrypsin numbering) even when overall homology is low. In all crystallised examples of serine proteases this catalytic triad can be overlaid on each other with a very small mean deviation. These enzymes achieve their substrate selectivity by altering the shape and electronic characteristics of the binding cleft, which is a consequence of amino acid mutations in the overall sequence derived from a common ancestor.

18 crystal complexes of the elastase family of serine proteases have been published and reviewed[12, 55, 74]. A comparison of the native and complexed enzymes show only minor structural alterations to the enzyme resulting from ligand complexation. Elastases are responsible for the cleavage of connective tissue and are essential for phagocytosis and defense mechanisms preventing infectious diseases. However, if not controlled by circulating plasma inhibitors like α1-antitrypsin and α2-macroglobulin, a rampant elastase will give rise to the disease state known as emphysema. Low molecular weight inhibitors[64] have been designed using the available crystallographic evidence and are now being evaluated as possible drugs to combat emphysema. Inhibitor design has taken advantage of the fact that the active site in this class of proteases is well defined. Hydrogen bonds form an antiparallel β-pleated sheet between the enzyme and the substrate and a covalent contact in the S^1 enzyme pocket. The Merck group[54] has found that β-lactam compounds are inhibitors of elastase, via an acylation mechanism. Subsequently, it was also reported that isocoumarins[64] inhibit elastase via an acylation mechanism, forming covalent bonds to two of the residues, Ser-195 and His-57, of the catalytic triad.

The plasma coagulation and complement family of serine proteases is another well-studied class. The structure of human α-thrombin complexed with D-Phe-Pro-Arg-chloromethyl-ketone, PPAC, has been solved to a resolution of 1.9 Å[11]. A structure of thrombin complexed to hirudin, a polypeptide thrombin inhibitor isolated from the saliva of leeches has been solved to a resolution of 2.3 Å[32, 68]. A comparison of the mechanisms of inhibition involving these two inhibitors is worthwhile.

The small organic compound, PPAC, binds to the active site of thrombin in the manner expected. The contact surface is small and restricted to the active site cleft. The catalytic triad attacks the chloromethyl function eliminating chloride and forming two covalent bonds to the inhibitor: a hemiketal formed by the hydroxy side chain of Ser-195 and a covalent link between the methylene and His-57 side chain. The arginine residue of the inhibitor sits in the S^1 pocket forming a strong salt bridge with Asp-189, and the remainder of the amide backbone of the tripeptide forms a series of hydrogen bonds with the enzyme (fig. 7). Bode[13] has published a study supporting this particular mechanism. Contrast this with an analysis of the hirudin-thrombin complex. Hirudin is a very specific thrombin inhibitor and has a rather unique in-

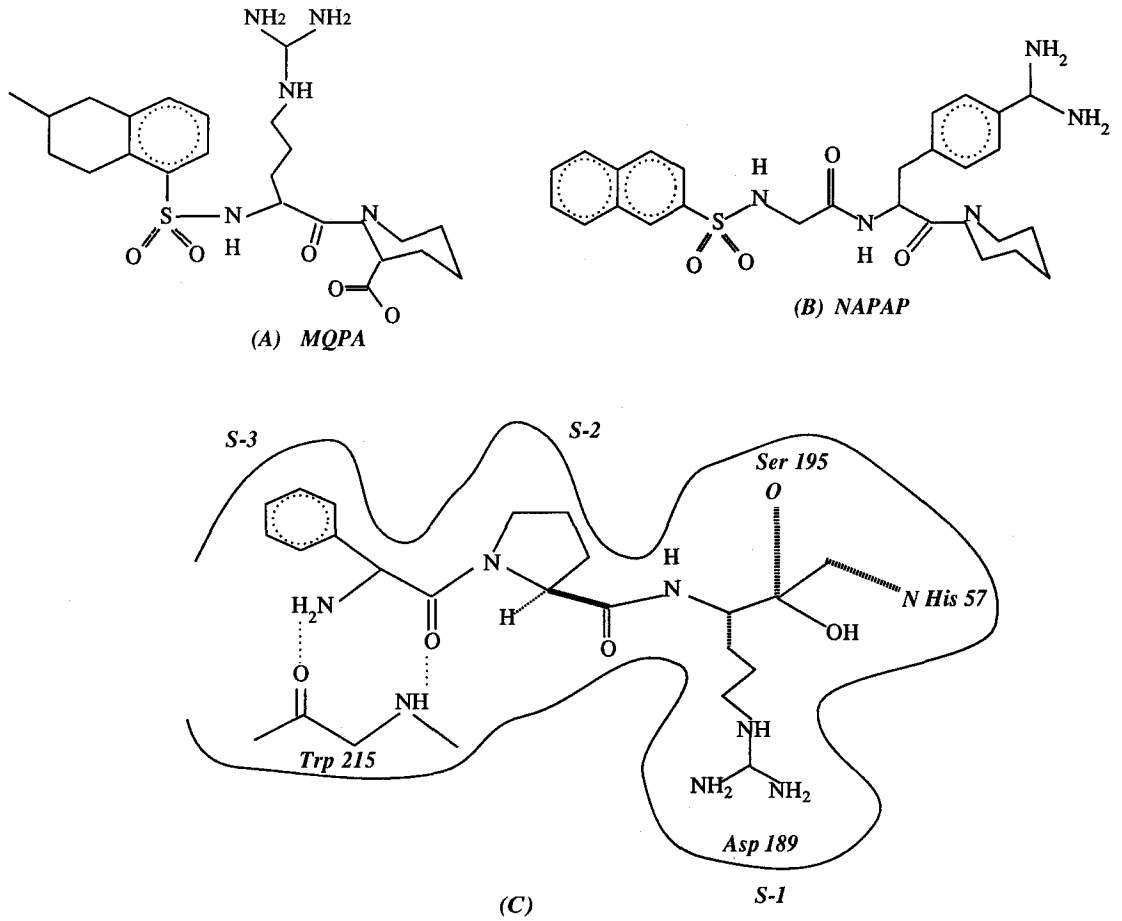


Figure 7. Two thrombin inhibitors: *A* MQPA and *B* NAPAP. *C* A schematic representation of the thrombin active site, with the small tripeptide inhibitor D-Phe-Pro-Arg-chloromethylketone (PPAC) bound.

hibitory mechanism. It does not interact with the catalytic triad but obtains its specificity and inhibitory action by throwing a blanket of amino acid residues over the active site, so that the contact surface area is around 1400 Å^2. Only the tri-peptide from the N-terminus of hirudin actually interacts with the active site. Val-1 binds to the S^2 pocket and Tyr-3 binds to the S^3 pocket; this in itself is unusual because the inhibitory chain runs in the opposite direction to the smaller classical inhibitors. Hirudin's inhibitory mechanism would be difficult to mimic a priori, and because of its large complementary interaction surface, would not make a good drug design target. However, it does illustrate the principle that small molecules can be designed to interact with and mimic the interactions and the biological consequences of an interaction between two large macromolecules.

Aspartic proteases

The renin angiotensin system has also been intensively studied. Renin, an aspartic acid protease causes the release of a decapeptide angiotensin I by proteolysis of the N-terminus of angiotensinogen, renin's only known substrate. The metallo-carboxydipeptidase, angiotensin converting enzyme, ACE, subsequently cleaves angiotensin I at the C-terminus producing angiotensin II (fig. 8). Angiotensin II triggers the responses leading to elevation of blood pressure. Enzyme inhibitors of both ACE and renin have been developed as antihypertensive agents useful in controlling blood pressure. However, only ACE inhibitors have so far been developed commercially.

A solution to the crystal structure of recombinant human renin was reported by two groups[47,71] in 1989. This structure, of deglycosylated recombinant human renin, validated the model proposed earlier by Blundell[10], who had predicted a theoretical model for renin by homology to other crystalline aspartic proteases like endothiapepsin[17] and penicillopepsin[40]. This theoretical model has been widely and successfully used to design potent in vitro inhibitors of renin. These inhibitors have never been further developed into pharmaceutical compounds because of weak in vivo activity, due to a poor bioavailability profile. It is not sufficient to consider the molecular recognition processes at the target site alone in the drug design cycle; biological availability must also be considered.

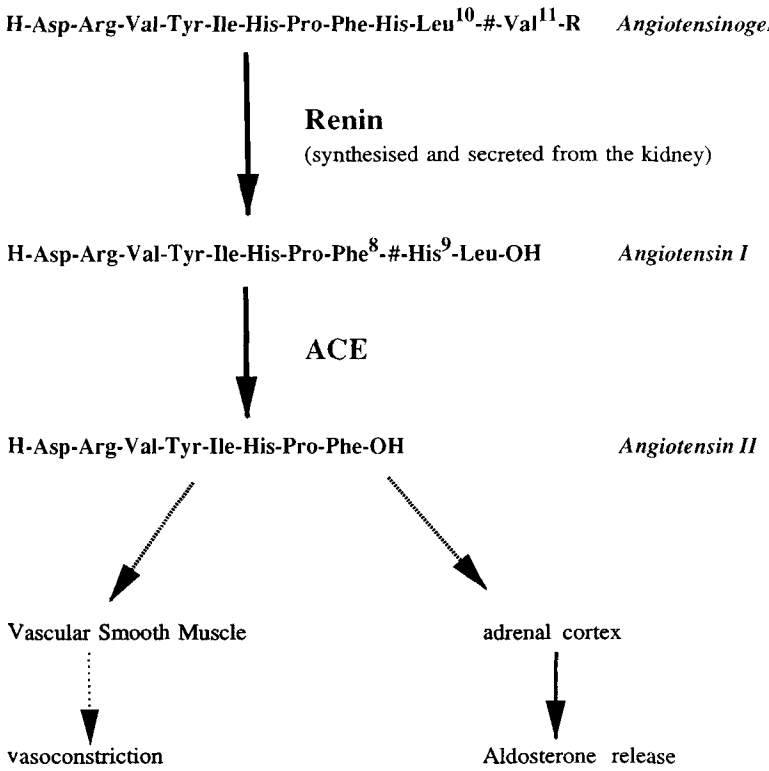


Figure 8. The renin and angiotensin pathway.

In the HIV-genome there are three major open reading frames, named gag, pol and env. These are initially translated into large polyprotein precursors that are subsequently processed to yield the mature structural proteins and enzymes of the virions. An aspartic protease, encoded by the virus itself is responsible for maturation of the gag and gag.pol precursors. Since this is an essential step in the retroviral life-cycle, HIV protease is a promising target for anti-retroviral therapy. In addition to processing viral proteins, HIV protease is able to cleave cellular proteins of the cytoskeleton. This may contribute to the pathogenesis observed during infection.

Pearl and Taylor[59] developed a hypothetical model for the retroviral protease in 1987, postulating that the protease functions as a dimer. Crystal structures of first a retroviral protease from Rous Sarcoma Virus[52] and then of HIV-1 protease[53,85] proved this initial hypothesis. Renin inhibitors were initially used to study the specifity of this protease which led to a number of crystallised complexes, the analysis of which showed that unlike other aspartic proteases, which only contained localized symmetry around the two domains that made up the active site, HIV-1 protease, in the native state, exhibits complete C2 symmetry. The potent Abbott[26] inhibitor A74704 (fig. 9) was designed to capitalise upon this two-fold rotational symmetry and thereby offer selectivity over other aspartic proteases. HIV-1 protease, typical of all other proteases binds the substrate in an extended conformation obtaining most of its binding energy from a series of hydrogen bonds between the backbone amide bonds (fig. 9).

As a final example of how 3-dimensional structural information can lead to a better understanding of molecular recognition and how this appreciation can lead to pharmaceutically useful agents, let us consider the case of dihydrofolate reductase, DHFR. Dihydrofolate reductase catalyses the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate. Inhibitors of DHFR will effectively block the biosynthesis of some amino acids and the purines and pyrimidine bases.

DHFR is one of the most studied and well-understood enzymes with at least 20 well-refined crystal structures from sources as varied as man to bacteria[50,56,72]. There is a high sequence homology (70–90%) between the vertebrate DHFR sequences which contrasts with the low (~30%) homology with their bacterial counterparts. Nonetheless, the overall protein architecture is conserved, with localized differences in binding pockets giving rise to the species selectivity exhibited by inhibitors. The difference in size of the binding pocket, smaller in bacterial DHFRs compared to vertebrate DHFRs, is responsible for the selectivity exhibited by these inhibitors. For example, the antibacterial trimethoprim, (TMP, fig. 10) binds some 3000 times more tightly to *E. coli* DHFR than it does to vertebrate DHFRs[19,39], primarily due to a better hydrophobic interaction between inhibitor and enzyme. Fleischman and Brooks[28] have reported a free energy study on the binding of TMP to the

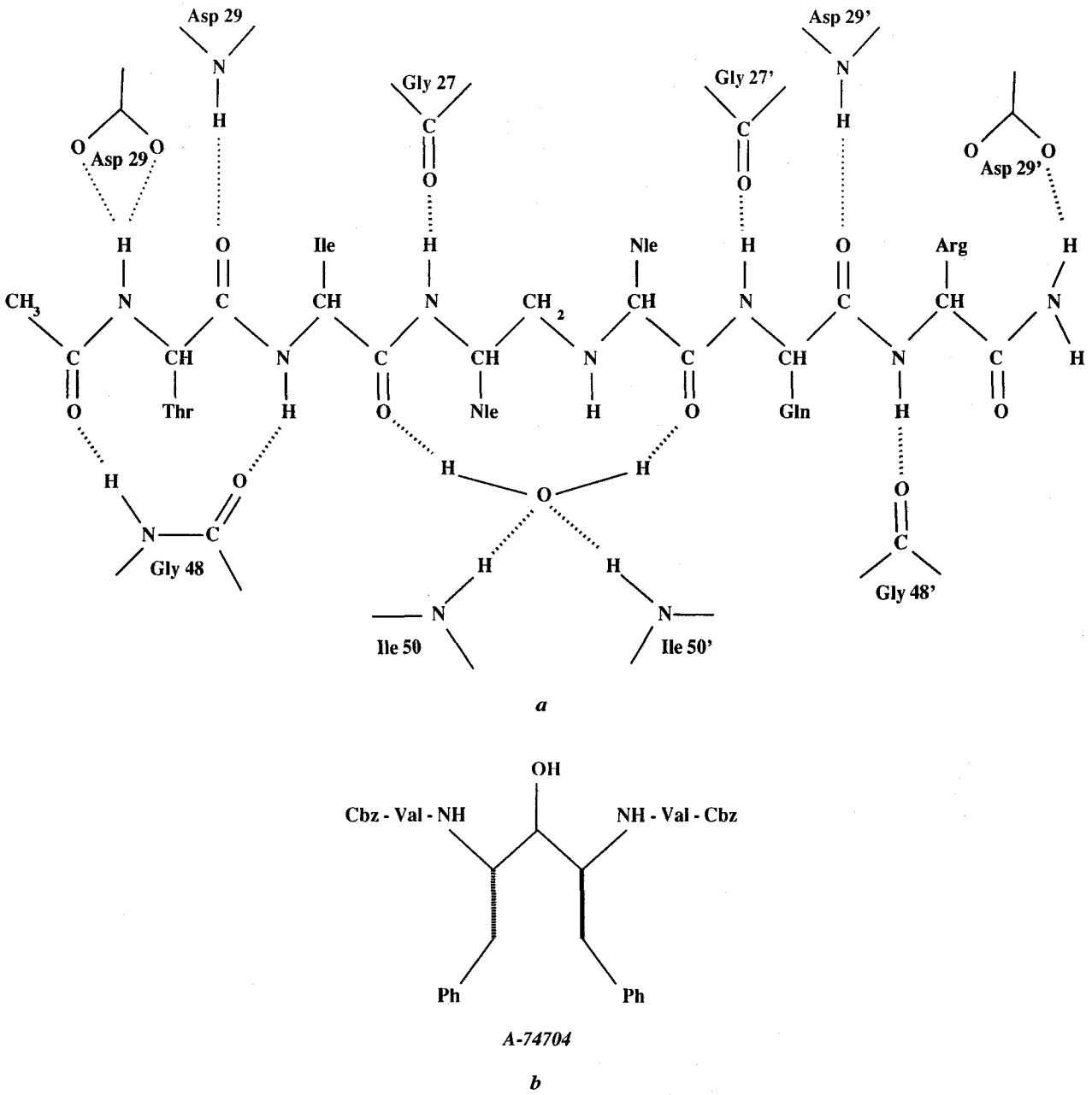
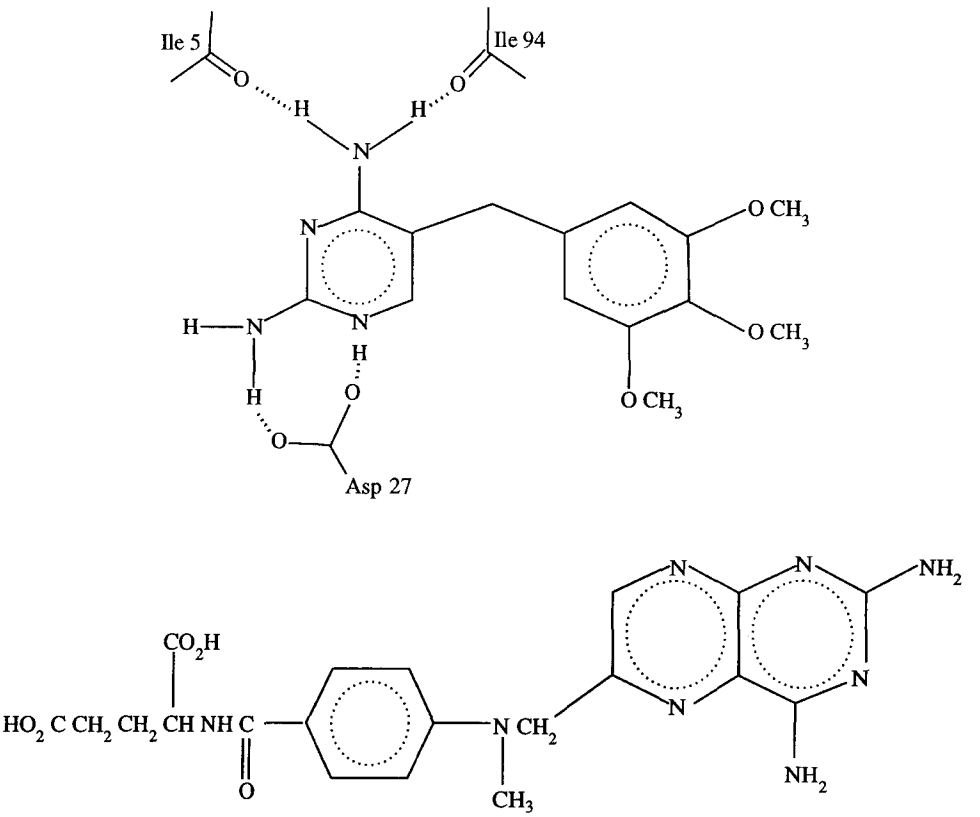


Figure 9. *a* Typical of the extended hydrogen bond pattern found between acid proteases and their ligands, is that of the inhibitor Thr-Ile-Nle-Ψ[CH_2—NH]-Nle-Gln-Arg[53], complexed with HIV-1 protease. *b* The C2 symmetric Abbott inhibitor.

binary complex of DHFR.NADPH. These calculations imply that the entropic contributions and desolvation thermodynamics play a critical role in determining binding energies. This paper supports the earlier finding reported by Osguthorpe et al.[57] whose attempts to quantify the binding energies by theoretical means, led to the conclusion that van der Waals interactions between 5 amino acid residues on the enzyme, defining part of the active site, and the ligand contributed some 12.4 kcal/mol to the binding energy. Using this vast wealth of structural and theoretical information, novel trimethoprim analogues have been designed which exhibit higher selectivity, 50,000 times, for the bacterial enzymes. This has been achieved by modifying the steric requirements and as a consequence altering the hydrophobic properties, of trimethoprim[46].

Methotrexate (MTX, fig. 10), one of a number of DHFR inhibitors with important clinical indications, predates the modern era of drug design, having been introduced as an antineoplastic agent in 1948. Until the complex of DHFR.MTX was finally solved, it had been assumed that the pteridine ring common to both folate and MTX,



b MTX

Figure 10. Schematic representations of *a* trimethoprim, TMP, and *b* methotrexate, MTX, two inhibitors of DHFR.

would bind to DHFR in the same orientation. However, with this crystal structure and in all subsequent crystal structures of DHFR-MTX complexes, the pteridine ring is rotated by 180° compared to the pteridine ring in the folate complex[39]. Crystallography highlighted this apparent paradox but fortunately also presented the data with which this enigma could be rationalised.

Overview

Molecular recognition is a ubiquitous feature of all biological processes. This review has tried to highlight the influence 3-dimensional structural information has had on our understanding of the molecular recognition phenomenon at the atomic level, and its consequent implication for the drug design process. Our present understanding of the subtle events which drive the molecular recognition path is innocent, and our database of knowledge too limited to be confident in predicting recognition processes. The examples chosen highlight both the naivity in our understanding of molecular interactions, and the enormous potential in utilising, both theoretically- and experimentally-derived, 3-dimensional structures in the drug design cycle. The thirst for structural information, allied to the ripening spectroscopic methods and improving mathematical techniques, will mean that more accurate predictions of where and how to favourably influence molecular recognition at the atomic level will become possible.

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