

Improvements in Molecular Mechanics Sampling and Energy Models

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ABSTRACT

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The process of bringing drugs to market continues to be a slow and expensive affair. And despite recent advances in technology, the cost both in monetary terms and in terms of time between target identification and arrival of a new drug on the market continues to increase.

High throughput screening is a first step towards testing a large number of possible bioactive compounds very quickly. However, the space of possible small molecules is limitless, and high throughput screening is limited both by the size of available libraries and the cost of running such a large number of experiments. Therefore, advancements in computational drug screening are necessary in order to maintain the current rate of progress in modern medicine.

Computational drug design, or computer assisted drug design, offers a possible way of addressing some of the shortfalls of conventional high throughput screening. Using computational methods, it is possible to estimate parameters such as binding affinity of any small molecule, even those not currently present in any small molecule library, without having to first invest in the often slow and expensive process of finding a synthetic pathway. Computational methods can be used to screen similar molecules, or mutations in small molecule space, seeking to increase binding affinity to the protein target, and thereby efficacy, while simultaneously minimizing binding affinity to other proteins, decreasing cross reactivity, and reducing toxicity and harmful side effects.

Computational biology methods of drug research can be broadly classified in a number of different ways. However, one of the most common classifications is according to the methods used to identify possible drug compounds and later optimize those leads. The first broad category is informatics or artificial intelligence based approaches. In these approaches, artificial intelligence methods such as neural networks, support vector machines, and qualitative structure-activity relationships (QSAR) are used to identify chemical or structural properties that contribute heavily

to binding affinity. The next category, ligand based approaches, is very useful when there are a large number of known binders for a specific family of proteins. In this approach, the ligands are clustered using a metric of chemical similarity and new compounds which occupy a similar chemical space are likely to also bind strongly with the protein of interest. The final class of methods of computational drug design, and the method explored in this thesis, is the diverse class known as structural methods. These approaches in the most general sense make use of a sampling method to sample a number of protein, or protein-small-molecule interaction conformations and an energy model or scoring function to measure dimensions which would be very difficult and or expensive to measure experimentally.

In this thesis, a number of different sampling methods that are applicable to different questions in computational biology are presented. Additionally, an improved algorithm for evaluating implicit solvent effects is presented, and a number of improvements in performance, reliability and utility of the molecular mechanics program used are discussed.

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Chapter 1

Introduction

1.1 Drug Development

1.1.1 Costs of Drug Development

The process of bringing new drugs to market is a long and expensive affair. At the least, it is necessary to identify a possible target molecule, find a small molecule with promising binding characteristics to that target, and is additionally neither toxic nor a strong binder to the wide variety of other proteins necessary for regular cellular function. These small molecules are then varied to maximize binding affinity to the target molecule, while attempting to simultaneously minimize cross reactivity. Finally, after this process, these drug compounds are rigorously tested through clinical trials.

Information about both the costs and time necessary to bring a drug to clinical trials are less available than statistics for drug molecules reaching clinical trials. As such, there is much debate over the average cost and time investment needed to develop a new drug. The final costs necessary for the entire process range from ~400 million US Dollars per new chemical entity to as much as ~2 billion USD. Estimates for the time required also vary significantly, but many estimates place the time required at around 10 years from target identification to an approved drug entering the market. One of the largest factors affecting the average cost of each new drug compound is the low success rate in clinical trials for compounds that have been under active research for a number of years. Because clinical trials are lengthy and expensive and and of themselves, but so too is

the process leading to clinical trials, effectively screening these compounds earlier in the pipeline has the potential to significantly decrease the average cost of each new drug molecule [Adams and Brantner, 2006].

The average cost of identifying a new drug molecule and gaining approval for that molecule is actually growing at a rate greater than inflation. It is not just the rapidly growing costs of drug development that are alarming, but that these costs are increasingly ineffective and inefficient. The number of new drugs introduced during the period 2005-2010 was actually 50% fewer than the number introduced during the previous five years. This decreased rate of drug discovery is disheartening because new drug compounds have been shown to have important impacts on both longevity and quality of life. In fact, during the 14 year period from 1986 to 2000, 40% of the two year increase in life expectancy can be accounted for by the effect of new drugs introduced during that period [Paul *et al.*, 2010].

The expected period of time that a candidate drug compound will spend in clinical trials is approximately nine to fourteen years [DiMasi *et al.*, 2003; Paul *et al.*, 2010]. During the period from 1981 to 1990, the rate of approval of potential drugs decreased, as did that of self-originated drugs, or those drugs that were originally identified by a pharmaceutical research company. Of potential drug compounds reaching clinical trials, only 10% will finally be approved as new drugs [DiMasi, 2001; Paul *et al.*, 2010]. Of potential drug compounds entering clinical trials that fail to be approved as new drugs, approximately 60% will be abandoned or fail during phase II clinical trials [DiMasi, 2001], which test the efficacy of a drug. This is generally viewed as a failure to find a small molecule with sufficiently high binding affinity to the target protein. Thirty percent of potential drug compounds entering clinical trials will fail in stage I, either because they are poorly tolerated, toxic to humans or cause side effects [DiMasi, 2001]. Each of these is a potential indicator of cross reactivity with proteins other than the target molecule.

Finally, approximately 20% of potential drug compounds entering clinical trials will fail in stage III [DiMasi, 2001]. These drugs fail for a variety of reasons, though ineffectiveness is frequently cited as a reason. All told, efficacy accounts for 37.6% of all drugs that are abandoned after reaching clinical trials, making it the single largest contributing factor to the failure of these compounds to eventually receive approval as new drugs. Other factors include safety, and economics [DiMasi, 2001].

Computationally screening these compounds earlier in the process has the potential of reducing the attrition rate at this point in the process. Additionally, increasing the affinity for the target itself can allow for lower dosages, which can increase survival of the drug candidate through phase II clinical studies.

For new chemical entities introduced in the 1990's, the cost of research and development is increasing at a rate 7.4% above inflation. Rates for the 2000's are not yet available or are only now becoming available due to the long lead time between introduction of a new chemical entity and that new chemical entity becoming an approved drug. During the period from 1985 to 2000, the rate of spending on research and development increased at approximately twice the rate of introduction of new chemical entities. Although the largest factors in determining this cost are the costs during clinical trials, significant amounts are also spent earlier in the drug discovery pipeline, such as target identification, lead identification, and lead optimization. Improved computational techniques are generally viewed as possible means of decreasing costs or times associated with the earlier steps in the process. However, by increasing the fraction of leads that survive the screening process, techniques that help identify and optimize lead molecules can have a very large effect on the cost of each new molecular entity. Clinical trials consist of six sometimes overlapping stages, denoted 0 to V, though stages I to III are where the majority of drug molecules are abandoned. Of the candidate compounds that enter clinical trials, only approximately 20% will finally be approved as drugs [DiMasi *et al.*, 2003]. Using computational methods to improve efficiency of pre-clinical drug development has the potential to not reduce the cost of developing new drugs, by ensuring that money spend in clinical trials is more likely to lead to marketable drugs. Thus, the impact of computational drug design can reduce the costs of both the pre-clinical and clinical stages of drug development.

Since 1950, the number of new chemical entities introduced per billion dollars has decreased by 50% every 9 years. Possible problems cited as contributing to this decrease in efficiency include:

1. the ready availability of high quality and effective generic drugs as treatment options for many diseases,
2. decreased risk tolerance among regulatory agencies,
3. increased spending and personnel without understanding underlying relationships between

spending and personnel and discovery of new compounds, and the long period of time between beginning research on a drug target and finally gaining approval for a new drug compound, and

4. systematic overestimation of the efficacy of high throughput screening techniques relative to more classical techniques such as clinical science, and animal screening [Scannell *et al.*, 2012].

The high failure rates during clinical trials have been identified as one of the most critical factors in determining the overall costs of drug development [Bleicher *et al.*, 2003].

1.1.2 Computer Assisted Drug Design

The ultimate goal of computer assisted drug design is to improve rational drug design by exploiting the continuously increasing processing power available both in high performance super computers as well as in single workstations. Researchers through application of computational methods to drug design seek to supplement their ability to quickly examine a large number of possible interactions or gain insights that would be much more difficult, both in terms of time and expense, to obtain through biochemical experiments. Different classes of programs have been developed to help solve each of the three distinct steps in the pre-clinical stages of drug development:

1. Hit Identification – the process of screening a large small molecule database, containing up to one million and sometimes more small molecules, to identify small molecules that bind a given target protein, or hits. These hits are usually small molecules with a target binding affinity on the order of micromolars.
2. Hit to lead optimization – the process of modifying these hit molecules, either by substitution or addition of chemical moieties or mixing and matching substructures between given hits, to produce compounds with higher binding affinities than the initial hit compounds. Hit to lead optimization seeks to improve the micromolar binding affinity of hit compounds to nanomolar affinity or better.
3. Lead Optimization – the final step of modifying lead compounds to increase “druglikeness” to ensure that the molecule is sufficiently soluble, well tolerated, and does not disrupt regular cellular function.

1.1.2.1 Hit Identification

The earliest form of hit identification experiments were animal screens, where mutant animals were studied to find the specific gene or protein involved in a specific phenotype. This type of experiment relies on careful genetic controls and breeding, but also some element of luck in observing a relevant phenotype in the first place. “Brute force” animal screens have since been improved with extensive mutation libraries and exhaustive non-lethal mutation libraries for organisms such as yeast and *Escherichia coli*. Even so, these screens are slow, often taking three years or longer, and any such studies in mammalian model organisms, like mice, quickly become extremely expensive. Furthermore, these screens can be error prone, as performing a large number of repetitive experiments causes even the most fastidious of scientists to lose focus. High-throughput screening seeks to supplement the human factor with robots, which are capable of performing similar experiments with greater speed and fewer errors. With the help of this automation it is possible to test the interactions of as many as 100 million different reactions per day [Agresti *et al.*, 2010]. However, the high initial cost of high-throughput screening equipment as well as the cost of the small molecule libraries necessary for screening are often prohibitive even to large research institutions. In order to make this sort of experiment available to a larger number of institutions, some research institutions have instituted means of sharing this equipment, through high-throughput screening as a service type arrangements [HTSRC, 2004; MSSR, 2006].

The direct computational equivalent to high-throughput screening is virtual screening, where a library of small molecules is computational “docked” into the active site of the target protein, and some scoring metric is used to identify possible binders. In this sort of computational screen, the problem of the cost of small molecule libraries is essentially solved, as there are readily available libraries, some of which are free, of drug-like small molecules for use in virtual screening programs. For example, ZINC is a free database that provides a library of over seven-hundred thousand commercially available small molecules in a number of different file formats for use in virtual screening [Irwin and Shoichet, 2005].

Another possibility for hit identification *in silico* is through fragment assembly methods. These methods seek to identify conserved moieties in various binding compounds and assemble a high affinity binder by joining together these moieties in a single compound which collects the binding

affinities of each of its constituent parts [Jorgensen *et al.*, 2006; Jorgensen, 2004; Jorgensen, 2009].

The first published study using computational docking dates to 1982, by Irwin Kuntz, describing a program that would later go on to become the well known DOCK program [Kuntz *et al.*, 1982]. Generally, docking consists of a method of quickly screening possible protein-small-molecule interaction conformations. An emphasis is placed on the computational cost of evaluating the energy function over accuracy, as the poses generated by this step are usually fed into structural refinement programs for further sampling and more accurate estimation of energies. For example, in the original Kuntz study, the system only had six degrees of freedom on which to sample – three translational and three rotational degrees of freedom for the ligand with the protein held fixed. Along with a hard sphere collision model, this provided a sufficiently selective screen to identify the native binding geometry of the heme group to myoglobin as well as thyroid hormone analogs to prealbumin [Kuntz *et al.*, 1982].

The Protein Data Bank (PDB) is a commonly used source of structural information used in screens for hit compounds [Abola *et al.*, 1984]. The rate at which new structures are being deposited into the PDB is increasing on an annual basis, see figure 1.1. But new tools are necessary to draw meaningful insights, hopefully leading to new drugs, from this wealth of data.

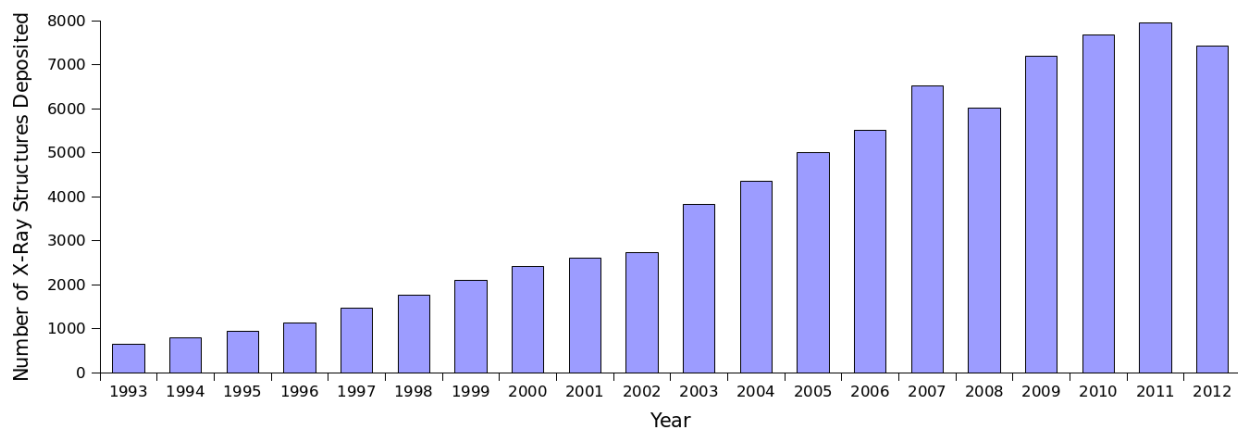


Figure 1.1: The rate at which new structures are deposited into the PDB over the last two decades. Due to a variety of improvements in the field of crystallography, this rate has been steadily increasing. Plot generated using data from the PDB [Berman *et al.*, 2003; Berman *et al.*, 2000].

For example, a recent advancement in the field of crystallography is “crystal-less” crystallography, in which small molecules are bound by a porous scaffold matrix. The regular structure of the matrix imparts a regular packing arrangement, necessary for interpreting diffraction patterns, onto the arrangement of small molecules. This has the potential to address one of the largest difficulties in obtaining quality structural data for proteins, which is that it is very difficult to purify and crystallize certain proteins [Inokuma *et al.*, 2013].

The number of target molecules of the set of all drugs currently available on the market consists of only about five-hundred proteins. The bottleneck in the introduction of new chemical entities is not virtual screening, but rather optimizing these hits into higher affinity leads (see 1.1.2.2) and eventually balancing the requirements across all characteristics to produce a new drug (see 1.1.2.3) [Bleicher *et al.*, 2003].

Most disease implicated proteins are not targeted by current drugs and finding improved drugs for those proteins which are currently drug targets can be very difficult, and sometimes not productive. Therefore, new chemical entities frequently aim to target proteins that are currently not targeted by currently available drugs. Of the entire proteome, only ~30,000 proteins are regulated by small molecule binding, making them reasonable targets of drug action. A large number of these possible drug targets are not implicated in any disease. Due to this and a number of other factors, estimates of the total number of the proteins regulated by small molecule binding that are possible drug targets is much lower than 30,000. Frequently cited numbers for the number of possible drug targets in humans are six-hundred to fifteen-hundred, still significantly higher than the total number of targets exploited by current drugs [Imming *et al.*, 2006; Overington *et al.*, 2006]. Further, different families of cellular proteins are not equally likely to be targets of drugs. As of 2002, 47% of current drug targets are enzymes, followed by 30% being GPCR’s [Hopkins and Groom, 2002].

After identifying appropriate proteins as drug targets, focus is then turned to assessing the drug-likeness of candidate small molecules. A number of key characteristics are generally true of drug-like small molecules. These characteristics are often referred to as the “rule of five” [Lipinski, 2004; Hopkins and Groom, 2002]:

1. Five or fewer hydrogen bond donors,
2. 500 Da or less total molecular mass,

3. high lipophilicity,
4. number of nitrogen and oxygen atoms is not greater than 10 [Lipinski *et al.*, 1997].

Therefore, when screening small molecules libraries for hits, these criteria can help focus efforts on small molecules which are likely to have good absorption, distribution, metabolism, and excretion, characteristics, which are essential in effective drugs. These criteria are discussed further in Lead Optimization (1.1.2.3).

In addition to the drug-likeness of small molecules, another desirable criteria is some degree of flexibility of the lead compound. This is especially important as the location of heavy atoms in the target protein is frequently only known to an accuracy of ~ 0.4 angstroms and flexible substrates can alter their conformations in order to create better contacts with the protein structure, thereby increasing binding affinity. One of the earliest examples of the successful application of structure based drug design is the carbonic anhydrase inhibitor dorzolamide, in which many of these ideas were applied to find a drug with very high binding affinity [Greer *et al.*, 1994]. Through understanding the protein-ligand conformation and specific contacts, Greer *et al.* were able to modify a known substrate into an effective drug.

Despite advantages in speed and cost, computational screening has struggled to produce the same results as empirical screening, due to limitations in accuracy. However, more recently virtual screening has succeeded in producing hit rates greater than those from empirical screening techniques. Virtual screening has been used to identified leads which were later developed into the human immunodeficiency virus (HIV) protease inhibitor Viracept (figure 1.2) [Kaldor *et al.*, 1997], and the anti-influenza drug Relenza [von Itzstein *et al.*, 1993; Shoichet, 2004].

A number of challenges limiting the utility of docking programs have been identified:

1. The number of possible small molecules is essentially unbounded. However, only a very small fraction of these ligands are potential drug compounds. Limiting sampling to this subspace of small molecules is a challenging problem, but would significantly enhance accuracy and efficiency of virtual screening.
2. The number of conformations of ligand molecules rises exponentially with the number of internal degrees of freedom of the ligand. Sampling the huge conformational space of the ligand becomes extremely computationally expensive.

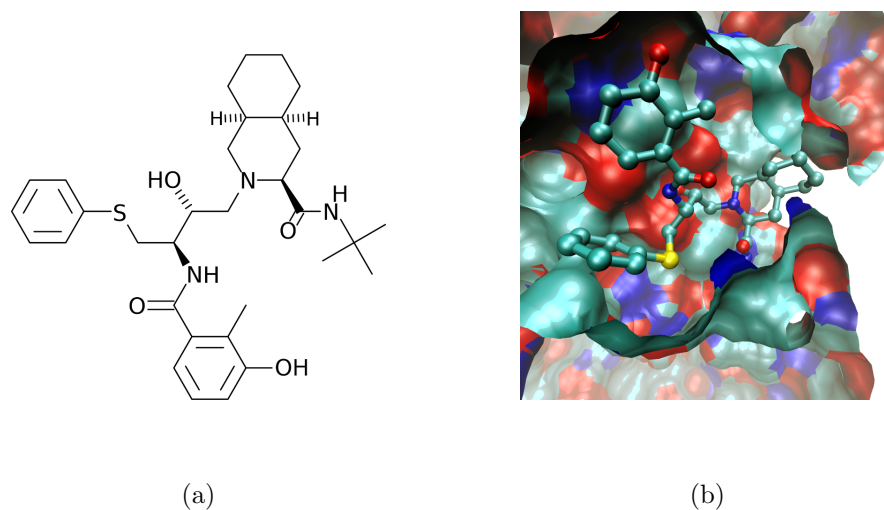


Figure 1.2: (a,b) The HIV protease inhibitor, nelfinavir, marketed under the name Viracept was originally identified using a computational docking screen. It has a very high binding affinity (2 nM) for HIV-1 protease, its target protein. (b) Here it is shown crystallized with multidrug variant (ACT) (V82T/I84V) of HIV-1 protease, PDBid 3EL5 [King *et al.*, 2012]. Generated with Visual Molecular Dynamics [Humphrey *et al.*, 1996] and [POV-Ray 3.6, 2004].

3. Accurately assessing or comparing the energy of different protein-ligand complexes or conformations [Shoichet, 2004]. This is because molecular mechanics force fields must make approximations about the molecular forces.

It has been found that introduced drugs are often very chemically similar the hit compounds from which they were derived [Proudfoot, 2002]. Therefore, in order to increase the diversity of drugs and find drugs that are able to treat new diseases, or diseases that have evolved resistance to current drugs, it may be necessary to either increase the size of screened databases or increase the possible diversity which might be introduced through the hit-to-lead step.

1.1.2.2 Hit-to-Lead Optimization

Hit compounds generally have a binding affinity for the target protein on the order of micromolar binding. The goals of hit-to-lead optimization are to further increase that affinity with the goal of eventually reaching binding affinities on the order of ~ 10 nanomolar or better, find other molecules with similar chemical characteristics to increase the size and diversity of the set of lead compounds,

and screen hit compounds for any obvious issues. At this stage of computational screening, more accurate energy models are required than for the initial screen [Jorgensen, 2004; Gohlke and Klebe, 2002; Jorgensen, 2009].

In the hit-to-lead stage, there are multiple methods used to convert hit compounds into multiple and chemically distinct lead compounds. First, pieces of multiple hit compounds can be joined to construct larger compounds, hopefully accumulating the attractive forces of each. Second, functional groups can be added or replaced through molecular growing and evolution techniques. Finally, a library can be searched by chemical similarity to the initial hit compound. In all cases, the potential lead compound is docked or grown in the known binding site of the protein target. Docking as a means of converting hit compounds to lead compounds is very similar to docking as a means of hit generation. However, in this case the small molecule library is restricted to chemical space surrounding hit compounds.

A popular program for building, or mutating, lead compounds is Biochemical and Organic Model Builder (BOMB) [Barreiro *et al.*, 2007]. BOMB can operate as either a hit identification program or as a hit-to-lead optimization method. Working to identify new compounds, BOMB starts with a number of different small “core” scaffolds and attempts to increase binding affinity by adding or replacing substituents to add favorable interactions while avoiding steric clashes. BOMB has been successfully used to evolve a hit compound that showed no inhibition of HIV reverse transcriptase (RT) into a potent non-nucleoside RT inhibitor with nanomolar level binding [Barreiro *et al.*, 2007].

After conversion of a hit compound into a number of possible leads, a scoring function is used to rank and identify lead compounds. This scoring function may be based either on statistical knowledge of similar structures or basic physical forces. A successful scoring function, be it knowledge-based or physical, must be able to successfully solve the classification problem of distinguishing strong binders from weak binders. For initial hit generation, a coarse grained energy function may be sufficient to differentiate ligands which bind strongly from those which do not bind at all. However, in order to convert hit compounds to lead compounds, it is necessary to use a more sensitive (and generally slower) energy model to accurately rank the binding affinity of different small molecules [Jorgensen, 2004; Gohlke and Klebe, 2002]. These energy models will be discussed in Energy Functions (1.4).

Whereas previously, lead compounds were evaluated almost exclusively on binding affinity to

the target protein, recently more emphasis is being placed on identifying hit compounds that satisfy other characteristics besides binding affinity [Bleicher *et al.*, 2003]. It is important to begin to consider other characteristics of the potential drugs earlier in the pre-clinical process, because later it is difficult to make changes affecting characteristics such as solubility without significantly altering the binding affinity of an already highly modified hit compound. As lead compounds are rarely very chemically distinct from the hits from which they were derived, and increasing binding affinity is actually sometimes an easier problem than addressing some of the other characteristics in the “rule of five”, it is reasonable to begin by first trying to optimize hit compounds to satisfy some other criteria and postpone maximizing binding affinity [Proudfoot, 2002].

1.1.2.3 Lead Optimization

In lead optimization the compounds identified by the earlier steps in the process are optimized to drug molecules. Though the objectives of lead optimization overlap heavily with those of the hit-to-lead stage, the compounds at the lead optimization stage are much more plausible as potential drugs. While this can include further increasing binding affinity to the target protein, usually the focus of lead optimization is on other characteristics including selectivity, ease of synthesis, pharmacokinetic properties and intellectual property concerns [Keserű and Makara, 2006]. Computational modeling can not only help identify hit compounds and convert those initial hits into leads, but also help estimate and optimize absorption, distribution, metabolism, elimination, toxicology, sometimes referred to as the ADME characteristics [Kerns and Di, 2008]. Computational models for ADME characteristics usually use regression equations or neural networks to predict these characteristics [Jorgensen, 2004].

Up to one half of all drugs which do not survive clinical trials fail to do so because of lack of efficacy, which is influenced both by binding affinity and the ADME characteristics of the molecule. The number of drugs that fail to make it through clinical trials due to toxicity is similarly high, about 40% [Li, 2001]. Advancing a potential drug to clinical trials represents a very large financial investment, and effective computational screens of lead molecules at this point in the process can reduce the rate of failure in clinical trials, thereby having a very large impact on the final costs of new drugs brought to market.

1.2 Sampling Algorithms

1.2.1 Minimization

Minimization techniques seek to find the lowest energy conformation in a given potential energy well. Generally, they make no attempt to sample outside of that well, and therefore are frequently implemented as a final stage in sampling in order to relieve any unfavorable interactions in proposed structures. There are a large number of different minimization techniques used in molecular mechanics modeling. A thorough review can be found in [Schlick, 2010]. As the basic terms of the general molecular mechanics potential energy function are differentiable, and discounting for the moment the significant effects of solvent, it is possible to solve for the energy gradient, or force on every atom for a given conformation. A few minimizations methods include:

1. Steepest descent, conceptually the simplest minimization algorithm, calculates the gradient of the potential energy at each step of the minimization, and changes the location of each atom by a distance proportional to the magnitude of the gradient at that atom [Levitt and Lifson, 1969; Bixon and Lifson, 1967].
2. Newton methods express the energy gradient as a quadratic function, instead of approximating the gradient as a linear function in a small neighborhood, as in steepest descent. This has been shown to converge to a minimum energy structure more quickly than steepest descent [Ponder and Richards, 1987]. Discrete Newton and Quasi-Newton are variations that use numeric estimation techniques instead of analytically solving for the gradient [Schlick, 2010].
3. Truncated Newton methods find an approximate solution to Newton's equations. The accuracy of the solution is increased as a local minimum is approached, by forcing the residual to approach zero as the series converges [Dembo and Steihaug, 1983].

The majority of molecular mechanics programs use some sort of minimization technique to refine initial structural guesses [Ponder and Richards, 1987; Levitt and Lifson, 1969; Bixon and Lifson, 1967; Zhu *et al.*, 2007b]. In all studies using the Protein Local Optimization Program (PLOP, developed in the Friesner lab and the program used in this thesis) since 2007, a truncated Newton method has been used, as this has been shown to converge more quickly in practice than any of the other methods [Zhu *et al.*, 2007b].

1.2.2 Monte Carlo Sampling

Metropolis Monte Carlo simulation was originally developed in the 1950's to provide rapid sampling of the solution space of many variable problems [Metropolis *et al.*, 1953; Hastings, 1970]. Monte Carlo techniques generate a sequence of states from a distribution by proposing a new state based only on the current state. If the ensemble average is the same as the sequence average, a Monte Carlo Markov chain can be used to estimate ensemble averages, a process known as *ergodicity* [Schlick, 2010]. Another requirement is *detailed balance*: that the probability of transition from a state X_i to a state X_{i+1} is the same as the probability of the reverse transition, i.e. X_{i+1} to X_i . By setting the probability of acceptance to

$$P(x \rightarrow x') = \min \left(1, e^{-\frac{\Delta E}{k_B T}} \right) \quad (1.1)$$

these conditions are met.

In molecular mechanics, Metropolis Monte Carlo provides a very efficient means of sampling conformation space and a simple method of estimating the distribution of states. Modifications on this method include annealing, where the temperature is continuously decreased over the course of the simulation, and umbrella sampling, which attempts to achieve better sampling in cases where a potential energy barrier divides two or more states from each other [Torrie and Valleau, 1977]. While Monte Carlo sampling techniques are very fast to provide new states, the majority of these states reflect higher energy conformations. Since it is of practical biological interest, Monte Carlo minimization has been developed to increase the rate at which minima are sampled [Li and Scheraga, 1987]. This integrates the previously discussed minimization techniques into a Monte Carlo approach, and is the method used in Monte Carlo Minimization in SOM Prediction (chapter 4).

1.2.3 Analytic Loop Closure

Subsequences with regular secondary structures, i.e. α -helices and β -sheets are generally better conserved, and therefore likely to be well covered by simple homology models [Kolodny *et al.*, 2005; Petrey *et al.*, 2003]. The intervening “random coil” or loop regions often play a large role in determining protein specificity for a specific ligand, as in antigen-antibody binding [Bajorath and Sheriff, 1996], small protein toxins to the receptors they target [Wu and Dean, 1996], or transcription

factors to specific DNA sequences [Jones *et al.*, 1999].

Loop closure or prediction is a significant part of homology modeling [Petrey *et al.*, 2003] and building structures consistent with X-ray refraction data. Therefore, in order to accurately predict three dimensional structure through homology models, infer protein binding partners and function, or even build a three dimensional structure consistent with both X-ray data and physical constraints, accurately predicting these loop regions is critical [Fiser *et al.*, 2000].

The loop closure question is, given two fixed endpoints and a flexible loop, or actuator, how does one find a loop conformation, or set of conformations, that connects the two endpoints. Because similar problems are frequently encountered in the field of robotics, a number of loop closure algorithms have been adapted from robotics [Kolodny *et al.*, 2005]. The first of these algorithms is analytical loop closure, where a conformation that satisfies the closure criteria is solved for directly by solving a system of equations. Though this problem can be solved analytically for small loops [Wedemeyer and Scheraga, 1999; Go and Scheraga, 1970; Brucoleri and Karplus, 1985; Palmer and Scheraga, 1991], the difficulty of the problem increases as loop length grows and the number of degrees of freedom of the loop section increases. Additionally, these closure constraints make sampling multiple different conformations more difficult [Cortés and Siméon, 2005], though it is possible to hierarchically solve sub-loops in order to generate conformations for possible complete loop conformations [Wedemeyer and Scheraga, 1999].

1.2.4 Cyclic Coordinate Descent

Another robotics algorithm which has been successfully applied to protein loop closure is Cyclic Coordinate Descent (CCD) [Canutescu and Dunbrack, 2003]. As the length of a flexible loop grows, the number of degrees of freedom increases and the possible solution space grows exponentially. Cyclic coordinate descent seeks to close the loop by adjusting the degrees of freedom, in this case the ϕ and ψ dihedral angles, sequentially and possibly iterating over each degree of freedom multiple times until the loop is closed. This method is able to solve for conformations very quickly, and the probability of failing to find a conformation which successfully joins the two endpoints decreases as the number of degrees of freedom of the system increases.

In cyclic coordinate descent the ϕ and ψ angles of each loop backbone residue are first randomized. Then a loop dihedral is chosen at random, and varied to move the last atom of the loop as near

as possible to its desired position. A new dihedral is chosen and optimized until the loop is closed. While it is possible that this procedure does not converge to a closed state, experiments have shown that this is very unlikely even for extended loops with few degrees of freedom, having less than a 2% failure rate for four residue loops. Solving for the ideal dihedral angle at each step is a simple optimization problem in one dimension, making CCD a very fast algorithm [Wang and Chen, 1991; Canutescu and Dunbrack, 2003]. In experiments CCD produces closed loop candidates in $\sim 1/6$ the time taken by the random tweak method, discussed in the following section.

A variation on cyclic coordinate descent seeks to close the loop by not only requiring atom closure, but by requiring that the entire backbone of the closure residue is superimposed, within some geometric similarity tolerance, between the predicted conformation and the crystal structure. This constraint ensures that the angles and dihedrals of the closure residue are reasonable [Canutescu and Dunbrack, 2003].

1.2.5 Random Tweak

Random tweak, like CCD, is a method of producing and sampling closed loop conformations. It begins in much the same way as CCD, by randomizing ϕ and ψ dihedral angles of the loop backbone. Random tweak seeks to close the loop while retaining dihedral angles as close to the randomized starting structure as possible. By adjusting each dihedral only a small amount at a time and staying in the region where $\sin(\theta) \approx \theta$, it is possible to formulate a set of linear equations to solve for a set of $\Delta\theta_i$, which minimizes the distance between the crystal position of the atom to be closed and the random position. Because the assumption $\sin(\theta) \approx \theta$ only holds for small θ , the maximum change in angle is limited to 10 degrees in the original implementation of the random tweak algorithm. Because almost all structures predicted using the random tweak or cyclic coordinate descent produce closed loops, a much smaller fraction of time is spent sampling loops that do not satisfy the closure criteria, making these algorithms very efficient [Fine *et al.*, 1986; Shenkin *et al.*, 1987].

1.2.6 Rotamer Assembly

Rotamer assembly, or systematic search, shares some similarities with fragment buildup techniques in that it uses a rotamer library to assemble possible loops. This rotamer library contains the

common backbone dihedrals, (ϕ, ψ) pairs, for each amino acid. This method operates by dividing the loop into two pieces, usually in half, and considering all possible half loops that can be built using a rotamer library [Moult and James, 1986]. For each side of the loop a “tree” is considered, in both a physical sense, in that the hemi-loop branches as it grows away from its anchor, and a decision tree sense, in that each residue represents a decision where a single rotamer is selected from the rotamer library. When the hemi-trees for each side of the gap are fully constructed, some closure criteria is applied.

In the case of the original systematic search, geometric agreement is required of the entire mid-residue [Moult and James, 1986], however a more lax criteria is applied in the case of the PLOP where only one atom is required to be approximately superimposed [Jacobson *et al.*, 2004]. By carefully pruning trees during the building process, and biasing the search towards occupied regions of ϕ - ψ space, systematic search can be quite efficient, spending little time sampling implausible regions of conformation space. Additionally, using a multi-peptide rotamer library which captures information about interactions between sequential dihedrals, and building multiple residues at once, this sort of procedure has been used to successfully build loops of twenty or more residues [Zhao *et al.*, 2011].

1.3 Molecular Modeling

Molecular modeling seeks to gain new insights into the real world behavior of molecules by mimicking these molecules using computer simulations. According to the theory of “minimal frustration”, evolution selectively favors proteins sequences which fold to a stable native state, contained within a broad potential energy well, with a minimum number of possible non-native mis-folded conformations [Bryngelson and Wolynes, 1987]. Thus, the prediction of native or native-like conformations focuses on finding those conformations which have a low potential energy. As measuring the true potential energy of a system is very difficult or impossible, computational models seek to reproduce the qualitative behavior of the protein potential energy surface.

Quantum mechanics calculations are often viewed as the gold standard with respect to intramolecular energy calculations. However, despite its accuracy, applying quantum mechanics to large systems such as proteins is currently impossible due to the amount of time necessary to per-

form quantum mechanics calculations on such a large number of atoms, as indicated in figure 1.3. Instead, quantum mechanics calculations have been used to parameterize a majority of the most

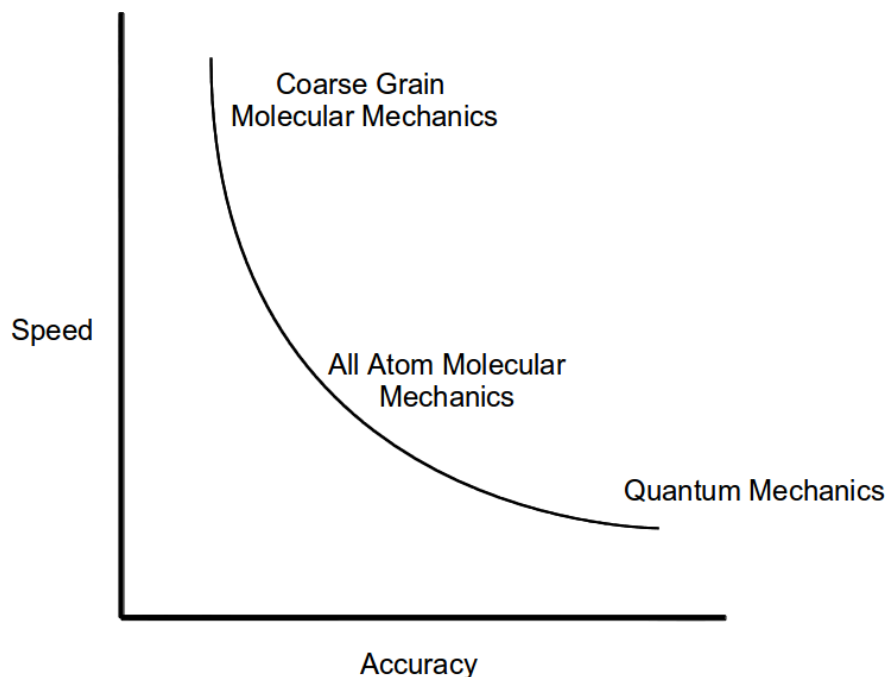


Figure 1.3: To an extent it is always possible to either increase accuracy or decrease running time, i.e. the cost of an experiment. New scientific methods should allow one to increase accuracy while not spending additional time performing computations.

popular molecular mechanics force fields currently in use, including:

1. AMBER [Weiner *et al.*, 1984],
2. OPLS-AA [Kaminski *et al.*, 1994],
3. and CHARMM [MacKerell *et al.*, 2002].

These force fields all include covalent and non-covalent parameters which have been fit to quantum mechanics experiments.

The earliest molecular mechanics force fields were largely coarse grained, modeling groups of atoms as a unit, hydrogens grouped with their bound heavy atom [Jorgensen and Tirado-Rives, 1988], or each residue as a unit [Lee *et al.*, 1999], both to reduce the number of parameters in the model and to increase the speed of computations. Although *ab initio* folding experiments are

theoretically interesting, they are generally not practical because of the difficulty in simulating such a large system for the time-frame necessary to observe behaviors like folding, and also because structural models for many proteins are available either directly as X-ray structures, or indirectly through homology.

Because of the evolutionary cost of misfolding, proteins have been selected to minimize misfolding, making the general shape of the potential energy surface roughly funnel-like, with the native structure at the minimum [Leopold *et al.*, 1992]. Despite this shape, the energy landscape of proteins is a very jagged surface, with a large number of local minima [Tsai *et al.*, 1999]. These shapes, as well as the effect of solvation on smoothing the energy surface are illustrated in figure 1.4.

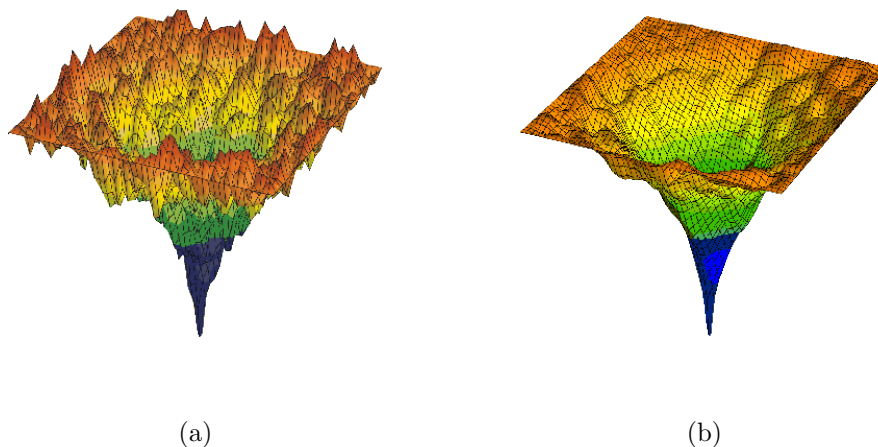


Figure 1.4: Here energy is represented as a function of the two principal components of the protein conformation. In both cases, the approximate funnel shape of the energy surface about the native conformation is very apparent. (a) An energy surface without any solvent effects contains a large number of local minima giving the surface a jagged appearance. (b) A surface including hydration effects appears smooth relative to the dry surface, due to water providing a source of hydrogen bond donors and acceptors such that hydrogen bonds are possible in many side chain conformations. In reality all energy landscapes of larger proteins contain many local minima. Figure from [Chaplin, 2013], used with author's permission.

The potential energy barriers are lowered and smoothed due to the ease with which water molecules can lubricate the movement of the amino acid backbone and side groups by the rapid

formation and exchange of hydrogen bonds

Even the smallest enzyme contains 62 amino acids, and has thousands of degrees of freedom [Chen *et al.*, 1992], and larger enzymes are regularly more than 1000 amino acids in length. The number of degrees of freedom of these systems make any attempt to analytically solve for a global minimum energy conformation impossible, and instead require other methods of generating plausible conformations. In order to compensate for this, a number of different sampling methods have been developed.

1.4 Energy Functions

Some energy models do not seek to accurately rank potential conformations. Fast screening functions instead attempt to quickly differentiate physically impossible conformations from plausible conformations without performing a computationally expensive minimization or energy calculation step. Application of these screening functions has the potential to greatly reduce the number of potential conformations that must be scored using the full detail energy function, greatly decreasing the overall cost of conformation prediction. These screening criteria can be applied either during the sampling procedure, potentially eliminating sampling of a large area of excluded conformation space, or after sampling but before a more expensive energy function is applied to rank conformations. Effective screening criteria have a large impact on the total performance of a structure prediction method.

One of the earliest screening criteria was the hard sphere overlap collision detection [Levinthal, 1966], which continues to be used in many coarse energy models. Other screens include:

1. bounds on bond lengths and angles, as a single bond which deviates significantly from equilibrium can dominate the total energy of a conformation,
2. limitations on ϕ - ψ space occupied by backbone dihedrals corresponding to the Ramachandran plot of the residue,
3. limiting side chain dihedrals to staggered conformations, which correspond to the low energy well of side chain dihedral space [Moult and James, 1986],
4. excluding structures that present excessive solvent accessible surface area and thus high sol-

vation energy, which has a large effect on the conformation of the native state [Chothia and Janin, 1975]

5. limitations on the number of dry cavities, and the number of internal charged residues [Moult and James, 1986]

Application of these and other screens help reduce the amount of time spent sampling implausible parts of conformation space, making sampling methods significantly more efficient. Following application of this sort of screening criteria, a more detailed energy model is used to rank potential conformations.

1.4.1 The General Form of the Energy Model

The form of most molecular mechanics energy potentials is reasonably consistent, following the general form of equation 1.2.

$$E(r^N) = E_{\text{bonds}} + E_{\text{angles}} + E_{\text{dihedrals}} + E_{\text{nonbonded}} \quad (1.2)$$

Bonds and angles are modeled as springs and dihedrals as a Fourier series, as shown in equations below.

$$E_{\text{bonds}} = \sum_{\text{bonds}} K_r (r - r_0)^2 \quad (1.3)$$

$$E_{\text{angles}} = \sum_{\text{angles}} k_\theta (\theta - \theta_0)^2 \quad (1.4)$$

$$E_{\text{dihedrals}} = \sum_{i=1\dots4} \frac{V_i}{2} [1 + \cos(i * (\phi - \phi_0))] \quad (1.5)$$

The non-bonded terms are modeled as a Columbic potential between any point charges and a Lennard-Jones or 6-12 potential between any non-bonded atoms (equation 1.6). These non-bonded atoms are phased in by a “fudge factor” for atoms in a 1-4 configuration.

$$E_{\text{nonbonded}} = \sum_{i>j} f_{ij} \left(\frac{q_i q_j e^2}{r_{ij}} + 4\epsilon_{ij} \left[\left(\frac{\sigma_{ij}}{r_{ij}} \right)^{12} - \left(\frac{\sigma_{ij}}{r_{ij}} \right)^6 \right] \right) \quad (1.6)$$

$$f_{ij} = \begin{cases} 0 & \text{if } i \text{ and } j \text{ are separated by 2 or fewer bonds} \\ 0.5 & \text{if } i \text{ and } j \text{ are separated by 3 bonds} \\ 1.0 & \text{otherwise} \end{cases}$$

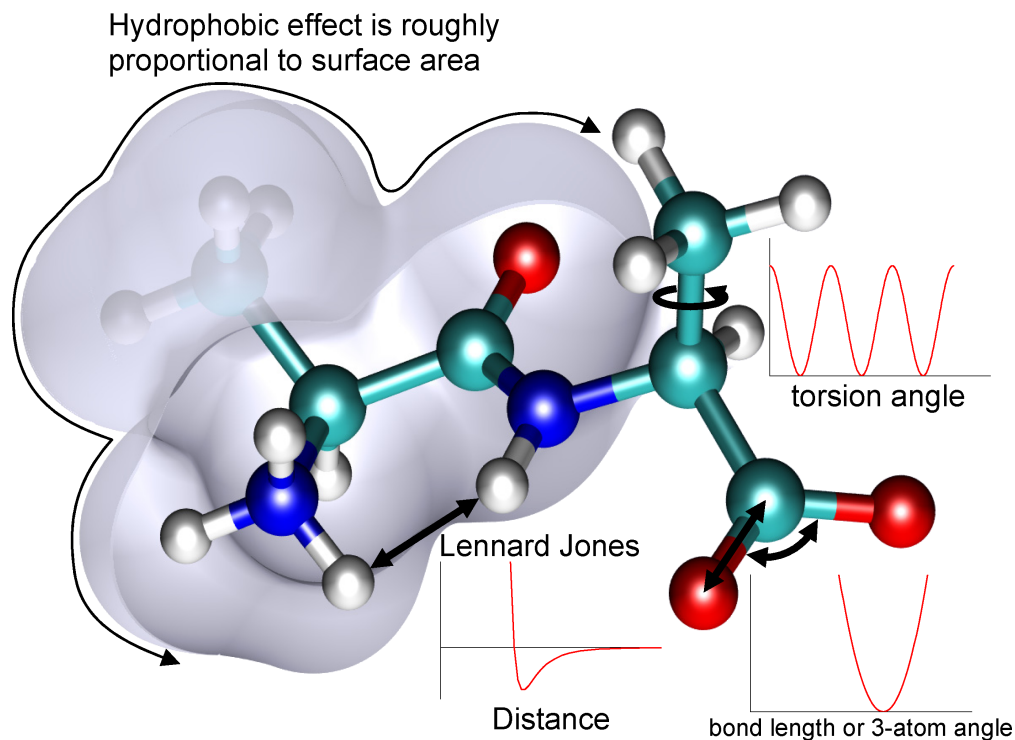


Figure 1.5: A molecule illustrating some of the major terms present in molecular mechanics force fields. Figure adapted from [Boas and Harbury, 2007], and licensed under cc-by-sa 3.0 license.

Where $\sigma_{ij} = \sqrt{\sigma_{ii}\sigma_{jj}}$ and $\epsilon_{ij} = \sqrt{\epsilon_{ii}\epsilon_{jj}}$ [Jorgensen *et al.*, 1996]. The terms of the energy model are illustrated in figure 1.5.

Energy models following this form have a number of desirable characteristics. First, they are reasonably accurate, having been shown to accurately predict a number of different physical phenomena. Second, they are significantly less expensive to compute than quantum mechanical energy formulations. Third, because the terms of the energy model are differentiable, using this form of an energy model is conducive to using one of the minimization techniques discussed in Minimization (1.2.1).

1.4.2 Molecular Surfaces

Central to the discussion of solvent is a discussion of how to formulate the surface of a protein. The most frequently used formulations of surface area include the Van der Waals (VDW) surface,

the solvent accessible surface, and the Connolly surface (also known as the molecular surface).

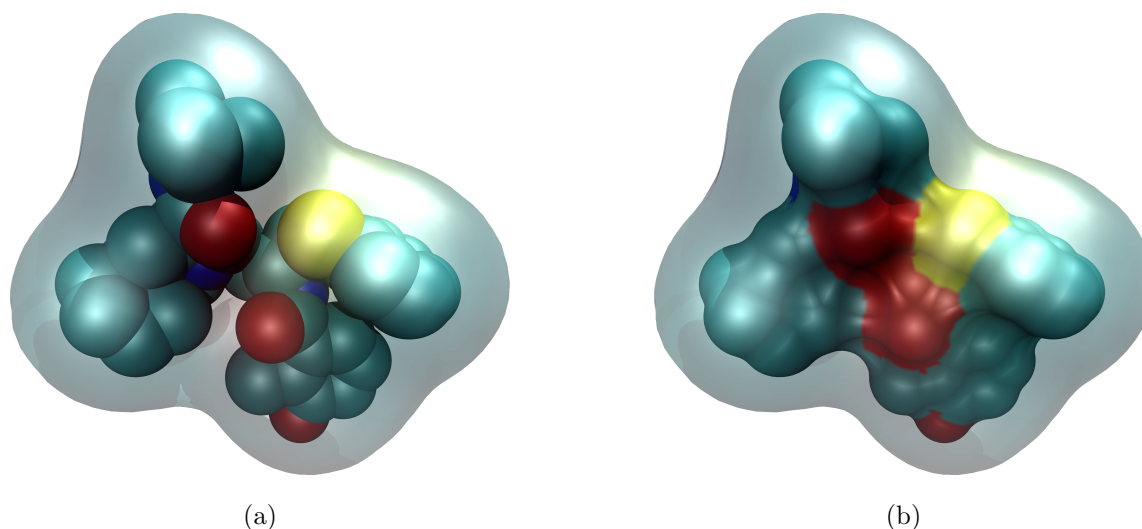


Figure 1.6: (a) The Van der Waals surface of Nelfinavir, defined by the surface of the volume excluded by the VDW radii of the atoms in the structure. (b) The molecular surface, defined as the surface of the volume excluded from a probe of 1.4 angstroms (the radius of a water molecule). Both surfaces are enclosed by an approximate solvent accessible surface, which is defined as the surface traced by rolling a spherical probe over the VDW surface. Figure generated using Nelfinavir structure from PDBid 3EL5 [King *et al.*, 2012], and using VMD and POV-Ray [Humphrey *et al.*, 1996; POV-Ray 3.6, 2004].

1. The Van der Waals (VDW) surface is the surface formed by the VDW radius of each molecule, though the exact radii may vary in different energy functions. Frequently the VDW radii are scaled down in order to reduce the effect of steric clashes and help generate more initial structures [Schulz-Gasch and Stahl, 2003; Halgren *et al.*, 2004]. Clashes which are tolerated using these scaled down radii can later be resolved in minimization. This is illustrated in figure 1.6a.
2. The solvent accessible surface, which is defined as the surface traced by the center of a spherical probe “rolled” over the VDW surface [Richards, 1977]. This idea is very closely related to the idea of the solvent excluded volume, or the shape of the solvent cavity enforced by the VDW surface of the molecule [Richmond, 1984]. An illustration of the solvent accessible surface is shown enclosing both a VDW and molecular surface in 1.6.

3. The molecular surface, or Connolly surface, is composed of the VDW surface in areas where the spherical probe touches the VDW surface, in union with all points on the probe “between” two points on the VDW surface when the probe is contacting multiple atoms [Connolly, 1983] – put another way, the surface of the volume which intersects no possible probe location. This is shown in 1.6b

Frequently, these surfaces are approximated numerically, using the Shrake-Rupley algorithm [Shrake and Rupley, 1973], by considering a spherical mesh about every atom and including only points that satisfy the definition of the surface, or using these points to interpolate a surface.

The effect of surface area on determining protein structure is determined by physical forces. However, the significance of the effect of surface area is well illustrated by the observation that the ratio of total area of a theoretical unfolded, i.e. linearly arranged, protein to its length is almost among proteins, only varying by $\sim 3\%$ between different proteins.

1.4.3 Solvent Models

Beyond covalent and electrostatic terms, solvation effects have the largest contribution to determining protein structure, and the interactions between proteins and small molecules [Chothia and Janin, 1975; Janin *et al.*, 1978]. Therefore, it is critical to accurately model the effect of the solvent on the molecule. While explicitly modeling each water molecule and sampling over possible conformations is the most realistic possible model, doing so requires calculating both a large number of solute-solute interactions as well as sampling extensively different solvent configurations. In this case it is likely that more time will be spent determining the behavior of the solvent than that of the solute. Because of these complexities, even with efficient methods of sampling explicit solvent models, these simulations are too expensive to use on systems the size of proteins [Figueirido *et al.*, 1997; Zhang *et al.*, 2001].

Therefore, there is significant interest in continuum models that accurately describe the mean force of water, without requiring additional sampling or interactions as in explicit models [Zhang *et al.*, 2001; Still *et al.*, 1990; Qiu *et al.*, 1997]. These methods have the potential to be three orders of magnitude, or even more, faster than explicit solvent experiments, and a number of different methods have been shown to accurately describe solvent effects [Zhang *et al.*, 2001].

The total free energy of solvation can be separated into polar and non-polar components, which

correspond to the work done inserting the uncharged solute molecule, or protein, into the solvent and then building the charges to their native values [Roux and Simonson, 1999].

$$E_{solvent} = \Delta W_{non-polar} + \Delta W_{electrostatic} \quad (1.7)$$

According to scaled particle theory the non-polar work done by inserting a sphere into a solvent can be approximated if the radius of the sphere is neither too large nor too small as

$$\Delta W_{np}(s) = \gamma SA(X) \quad (1.8)$$

where γ is the surface tension of the solvent and the surface area corresponds most closely to the Connolly surface.

The electrostatic contribution to the solvent energy is the work necessary to add a charge to a hard sphere atom already in the solvent. The charge density in the solvent can be given by the Poisson-Boltzmann equation

$$\nabla \cdot [\epsilon(r)\nabla\psi(r)] = -4\pi\rho_u(r) \quad (1.9)$$

Though it is possible to solve this at every step of a simulation, it becomes rather expensive, therefore faster approximations are sought [Nicholls and Honig, 1991]. The total work can be approximated by the Born model

$$\Delta W_{electrostatic} = \frac{Q^2}{2R} \left(\frac{1}{\epsilon_v} - 1 \right) \quad (1.10)$$

However, this assumes that the induced charge in the solvent is entirely concentrated on the surface of the ion, which is impossible. Therefore, R , or the Born α radius becomes a fitted parameter, representing the effective radius of a charged sphere in the solvent.

The electrostatic solvation contribution can also be expressed as

$$\Delta W_{electrostatic} = \frac{1}{2} \sum_{i,j} q_i q_j f(x_i, x_j) = \frac{1}{2} \sum_i q_i^2 f(x_i, x_i) + \frac{1}{2} \sum_{i \neq j} q_i q_j f(x_i, x_j) \quad (1.11)$$

where f is a weighting function for the interaction between charges q_i and q_j . Historically there are a variety of methods of approximating this weighting function, however one of the most popular is the generalized Born (GB) [Still *et al.*, 1990]. In the generalized Born approach

$$f(x_i, x_j) = \sqrt{d(x_i, x_j) + R_i R_j e^{\frac{-d(x_i, x_j)^2}{4R_i R_j}}} \quad (1.12)$$

and one of the limiting factors to accuracy becomes obtaining proper estimates of the effective radii, since charges are not uniformly exposed to the solvent [Schaefer and Karplus, 1996].

Another approach is to estimate both the non-polar and electrostatic contributions to solvation as proportional to the surface area, with different proportionality constants for different atoms.

$$\Delta W = \sum_{atoms} \gamma_{atom} SA(atom) \quad (1.13)$$

Although this method is very inexpensive to compute, it can be somewhat difficult to solve for the force on an atom, due to the way the surface changes as atoms move [Roux and Simonson, 1999].

Because in some ways it is simpler to model covalent effects through fitting parameters to quantum data, a large fraction of research on molecular mechanics force fields in the last five years has focused on improving the accuracy and optimizing these solvent contributions. In the next chapter we present an application of a computer graphics algorithm as an optimization of computing the solvation term in a surface area based generalized born solvent model.

Chapter 2

A Cell Based Method for Evaluating Implicit Solvation Effects

2.1 Introduction

Computational protein structure prediction and related areas of research such as target screening and lead optimization continue to be areas of active research in both pure chemistry and pharmaceutical applications [Jorgensen, 2009]. These methods range from identifying leads using chemical similarity metrics, to artificial intelligence methods such as neural networks and support vector machines, to structural based methods [Geppert *et al.*, 2010]. In the recent past, structural methods have contributed to the identification of bioactive drug compounds [Corsino *et al.*, 2009], making computational protein structure prediction highly important to the medical field, and because of its pharmaceutical applications, economically relevant as well.

There are over 81,000 X-ray structures presently in the PDB, more than 8,500 of which have been added in the last 12 months, and the rate at which new structures are determined by X-ray crystallography continues to accelerate (see figure 1.1) [Berman *et al.*, 2007]. The chemical space of small molecules, i.e. potential drug compounds, is essentially unlimited, and at the least too large to effectively screen using conventional experimental methods [Jorgensen, 2009]. Furthermore, computational loop prediction experiments are predicting longer loops, increasingly relying on the output of initial predictions as the input to a later “fixed stage” refinement step that re-predicts some central region of the same loop. This has been shown to increase accuracy [Jacobson *et al.*,

2004] at the cost of an increased number of experiments and corresponding increase in computational cost. Taken together, these factors necessitate the development of more accurate and efficient methods of generating and evaluating protein-protein and protein-small molecule conformations and interactions.

The Protein Local Optimization Program (PLOP), originally developed by Friesner and co-workers, is a popular program used to predict, sample, and evaluate protein conformations [Jacobson *et al.*, 2002a; Jacobson *et al.*, 2002b; Jacobson *et al.*, 2004]. PLOP makes use of the OPLS-AA energy model, an atomic detail force field optimized for organic, including protein, interactions [Jorgensen *et al.*, 1996]. In addition to the terms defined by the OPLS-AA model, it has been shown that solvent effects can make a large contribution to prediction accuracy. The solvent contribution to an energy model can be evaluated either by explicitly modeling and sampling solvent molecules (usually water) or by treating the solvent as a continuous medium, i.e. implicit solvation. PLOP, like many other molecular mechanics programs, makes use of an implicit solvent model. This is largely because explicitly modeling solvent molecules, while possibly very accurate, requires extensive, and therefore very time consuming, sampling of a large number of small molecules [Zhang *et al.*, 2001]. Further, energy errors using explicit solvent models can be due to either unrealistic force field parameters or insufficient sampling of solvent molecule conformations [Zhou, 2003]. Continuum solvation methods, or implicit solvent methods, attempt to address these issues by removing the dependence on sampling of solvent molecules and introducing approximations that reduce the cost of calculating the solvent contribution to energy [Roux and Simonson, 1999].

Among implicit solvent methods, the surface area based generalized Born (S-GB) model is one of the most popular and has been shown to produce results in good agreement with experimental data [Zhang *et al.*, 2001; Gallicchio *et al.*, 2002]. The surface area based generalized Born implicit solvent model provides an approximate solution to the Poisson–Boltzmann equation based on a surface integral [Ghosh *et al.*, 1998]. However, in a naive implementation of this model, the electrostatic contribution is a sum over every charge-charge pair in the system, in this case the protein and its crystal copies.

$$U = \sum_{charges} U_{self}(q_k, r_k) + \sum_{charges, i \neq j} U_{pair}(q_i, q_j, r_i, r_j) \quad (2.1)$$

The electrostatic contribution to solvation free energy can be decomposed into self and pairwise terms. The time complexity of evaluating the pair term is quadratic in the number of charges in the

system [Ghosh *et al.*, 1998]. This means that calculating the electrostatic solvation contribution for a single atom requires a linear search over all other charges in the system, which for large systems becomes the bottleneck of the computation. A common optimization is to assume that the electrostatic contribution to the solvation term for point charges separated by a distance greater than a defined cutoff distance is negligible [Gallicchio and Levy, 2004]. However, making this assumption does not improve the underlying quadratic time complexity of evaluating the solvation term, as it is necessary to compute the inter-charge distance for every charge pair in the system before possibly excluding the interaction. In the implementation of S-GB in the Protein Local Optimization Program, computing the solvation term of a large structure is the rate limiting step of energy calculations, accounting for over 80% of the total time spent computing the energy (see figure 2.2). Therefore, less expensive methods of evaluating the implicit solvent contribution to the energy of a system can allow for increased sampling with the same available resources, thus improving efficiency of computational modeling.

We present an application of a geometric hashing method, grid based spatial indexing, to implicit solvent calculations in PLOP. The hashing method proceeds by dividing space into cubical regions, or cells, and distributing atoms into those cells, while maintaining a list of the contents of each. Retrieval of atoms within a cell can then be performed in constant time, and retrieval of a superset of atoms contained within a region can be performed in time proportional to the number of cells intersecting the region. This efficient geometric lookup allows one to replace a loop over all atoms inside the structure with a loop over only the atoms contained in cells intersecting the sphere with radius corresponding to the distance cutoff of the force in question. While maintaining a list of atoms for each of these grid boxes introduces some overhead when updating atomic coordinates during a simulation, updating atomic coordinates is still a constant time operation. Thus, the benefits outweigh the costs, especially for large systems. As long as the cell size is bounded below, the number of cells necessary to consider when evaluating a fixed distance interaction is constant. Physical limitations provide an upper limit to atom density. Constant time retrieval of the contents of each hash cell, along with upper bounds on the number of atoms per cell and the necessary number of cells to consider for each charge, guarantee constant time lookup of all atoms within a given sphere. This reduces the time complexity of evaluating the electrostatic contribution from $O(n^2)$ to $O(n)$. We show that an implementation of this hashing method can reproduce results obtained

with a non-hash based implementation while providing significant performance improvements.

2.2 Methods

2.2.1 Energy Model

The energy model used in side chain prediction experiments was the optimized variable dielectric model (OVD), sometimes referenced as the variable dielectric surface generalized Born 2.0 model (VSGB2.0). This energy model is based on the OPLS-AA energy model, which in turn gets most of its covalent parameters from the AMBER force field [Jorgensen *et al.*, 1996]. The solvation term used is a surface area based generalized Born formulation, where the internal dielectrics of charged amino acids have been optimized over a set of 2239 single side chain predictions and 100 loop predictions of 11 to 13 residue loops. In addition to the covalent terms from the OPLS-AA model, the current energy model also includes terms to describe $\pi - \pi$ stacking, hydrogen bonding, and a parametrized hydrophobic term for the non-polar free energy of solvation [Li *et al.*, 2011a]. For the electrostatic contribution to solvation free energy, two different molecular surfaces are maintained at different resolutions. A surface mesh is constructed for each atom using the generalized spiral points method [Rakhmanov *et al.*, 1994; Saff and Kuijlaars, 1997; Zhou, 1995]. The number of points for each sphere is 10 for the low resolution surface and 330 for the high resolution surface. An atomic based distance cutoff is used when evaluating the electrostatic contribution to the solvation free energy. Inside a distance of $\sqrt{50}$ angstroms, the high resolution surface is used; between this distance and 20 angstroms, the lower resolution surface is used; and the contribution of atoms outside this distance is assumed to be negligible. The same set of cutoffs is used in both the current implementation in PLOP and the new cell based method described here.

2.2.2 Data Sets

The data set used for energy calculation experiments consisted of large protein structures, containing neither DNA, RNA, or modified residues with molecular masses between 100 and 150 kDa. All samples had resolutions better than 2 angstroms. The data set was filtered at 30% sequence identity, and from this, 20 structures were selected at random, though two were later excluded due

to technical reasons.

The structures used in side chain prediction experiments consisted of high resolution enzyme structures. Structures without an enzyme classification were excluded, as were structures with X-ray resolution less than 1.5 angstroms, or those with modified protein residues. This resolution requirement was imposed to make high resolution side chain prediction comparisons more meaningful. All structures had a molecular mass between 11 kDa and 110 kDa. Structures containing DNA, RNA or modified side chain residues were excluded, as were those that had unreasonable steric clashes either within the canonical structure or with crystal neighbors. Structures containing certain small molecules without energy parameterizations currently defined within PLOP were also excluded.

2.2.3 Structure Preparation

In preparing the crystal structures for energy calculation and side chain prediction experiments, the first step was to add the crystal copies of the protein of interest. PLOP completes this step for all space groups according to the crystal symmetry identified in the PDB file. Before modeling a structure using an all atom force field, it is also necessary to add hydrogens and any missing heavy atoms. When possible, PLOP uses the positions of bonded heavy atoms to build missing atoms into the structure. However, adding hydrogens, especially for titratable residues, is a more complicated problem. To address this, PLOP uses the independent cluster decomposition algorithm (ICDA) to determine the protonation states of any titratable residues, as well as the positions of polar hydrogens [Li *et al.*, 2007]. Generally speaking, this proceeds by dividing titratable and polar residues into independent groups using a distance cutoff, and optimizing each group independently. Structures with unreasonable steric clashes with crystal neighbors were removed from the data set on the basis that such structures are physically unlikely.

2.2.4 Grid-Based Spatial Indexing

Grid-based spatial indexing is a well known algorithm in computer science, especially computer graphics, that allows for efficient lookup based on geometric criteria and also provides fast collision detection [Bentley and Friedman, 1979]. Critical to the present application, it allows constant time retrieval of a superset of atoms guaranteed to contain all atoms within a given Euclidean distance.

In our implementation, the bounding box of the protein and its symmetric copies is subdivided along each of the orthogonal axes to form grid boxes or cells. A simple convention for handling atoms that are positioned along cell boundaries guarantees that each atom is hashed to a unique cell. For a single dimension, d , the cell index, or hash, of a point p is

$$i_d(p) = \text{int} \left(N * \frac{p_d - \text{min}_d(P)}{\text{max}_d(P) - \text{min}_d(P)} \right) \quad (2.2)$$

where $\text{min}_d(P)$ and $\text{max}_d(P)$ are the minimum and maximum coordinates in dimension d over the set of points P , N is the number of cells in dimension d , and p_d is the coordinate of p in d . Following the same procedure in each dimension gives a unique cell location for every atom. In this way, at the beginning of the simulation, each atom is assigned to a specific cell, or grid box. A list of the atoms in each grid box is then maintained over the course of the simulation. When computing the electrostatic contribution to solvent free energy of an atom, a , it is only necessary to loop over the atoms contained in boxes that intersect the sphere corresponding to the distance cutoff around atom a . Beyond that cutoff, charge effects are considered to be negligible [Gallicchio and Levy, 2004]. See figure 2.1 for an illustration of grid based spatial indexing in two dimensions.

In the present implementation, cell size is at first set to 2.745 angstroms, and the number of cells in a given dimension depends on the "length" of the system in that dimension. If the number of cells that this would require is unmanageably large, cells are then grown simultaneously in all dimensions such that the cell size is 1/250th of the longest dimension of the structure.

2.2.5 Experiments

For side chain prediction, the specific side chains used were those which had at least 30% solvent accessible surface area when evaluated in the absence of other chains or crystal neighbors. Glycine and proline residues were also excluded, as they do not have free side chains. Residues missing heavy atoms in the crystal structure were predicted; however, RMSD was not measured for these residues because there is no experimental data. Side chain prediction experiments were performed as described in [Jacobson *et al.*, 2002a].

The experiment in this case consisted of multiple energy calculations, using the modified version of the OPLS-AA force field described in [Li *et al.*, 2011a]. In the control experiments, the same method for evaluating the implicit solvent term was used as in previous works.

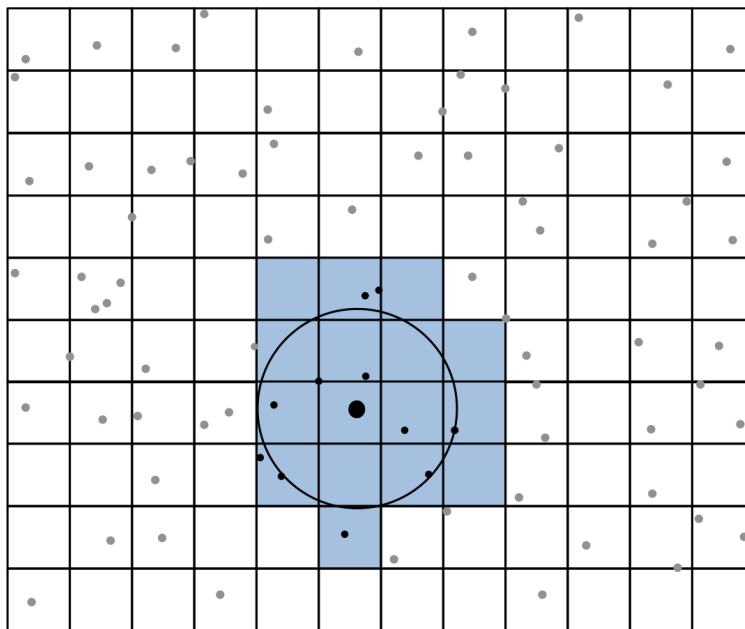


Figure 2.1: This illustrates, in two dimensions, the grid based spatial indexing method. The naive S-GB method would require a distance computation to every other atom in the system. By only considering atoms in cells intersecting the radius of influence, represented here in blue, it is possible to consider far fewer interactions. Although only atoms inside the circle in this illustration contribute to surface charge, it is necessary to compute the distance over all black points. Without using this hashing scheme, it would be necessary to compute the distance to each gray point as well.

2.3 Results

2.3.1 Qualitative Measures of Prediction Quality

For side chain prediction experiments, 85.2% of side chain prediction conformations (9406 of 11030 total) predicted with the new cell based solvation model are within 0.2 angstrom heavy atom RMSD of the prediction using the naive implementation. In other metrics, the quality of prediction is comparable between the two solvent models. Median side chain heavy atom RMSD is 0.567 and 0.558 angstroms for the cell based method and the non-cell based method, respectively. Average RMSD to the crystal structure is similarly close, 1.11 angstroms for both methods, with 79.9% of side chain predictions within 2 angstroms RMSD of the native using the cell based model and

79.4% within two angstroms using the naive approach. Of side chains that are predicted differently by the two implementations there is no correlation between solvation model and prediction quality. The distribution of side chain predictions with respect to RMSD to native is also indistinguishable between the two methods of computing the solvation term.

Data for energy calculations is not presented here because it is identical in every case. This is expected, given that the two models represent two methods of computing the same quantity. Thus, on the whole, prediction accuracy of the hash based model is comparable with the old implementation.

2.3.2 Performance Improvement

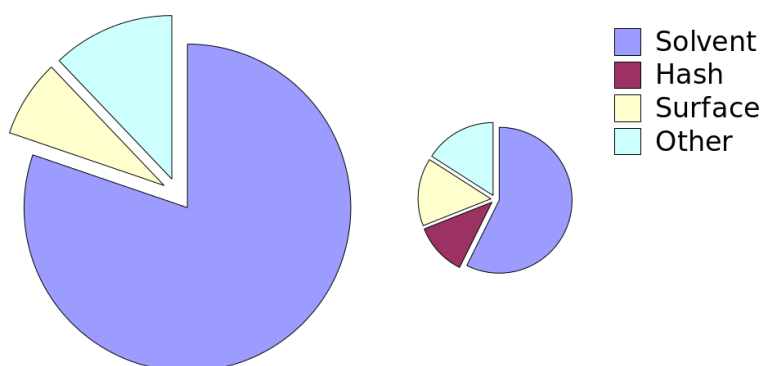


Figure 2.2: Time spent during energy calculations on different parts of the energy model. The left pie represents the non-cell based approach and the right pie the cell based approach. The charts are scaled relative to the total cost of computing the energy. Although some overhead is introduced in maintaining the hash structure, magenta, this significantly reduces the total cost of the solvent term, and as the solvent is such a large contributor to the total, the total cost of computing the energy is also significantly reduced.

The principal goal of the hash based approach is to improve the performance of the implicit solvent models. Thus, the key metric of performance improvement is the speedup over the previous implementation. Energy computations were found to be from 1.6 to 2.5 times as fast, and the trend indicates that even larger improvements would be obtained on calculations on larger system, see figure 2.3. A direct energy calculation experiment, as performed here, represents a “best case” for the expected performance increase of a hash based solvent, as these experiments minimize the fraction of time spent in other types of calculations. Implicit solvent calculations, and energy

calculations in general, compose a smaller fraction of time in simultaneous side chain prediction. Therefore, the observed performance improvement is less than that of energy calculations. The observed performance increase in this sort of experiment is still on the order of 20%. Figure 2.2, illustrates the fraction of time spent during an energy calculation on different terms of the energy model, as well as the improvement, and change in proportions using a grid based model.

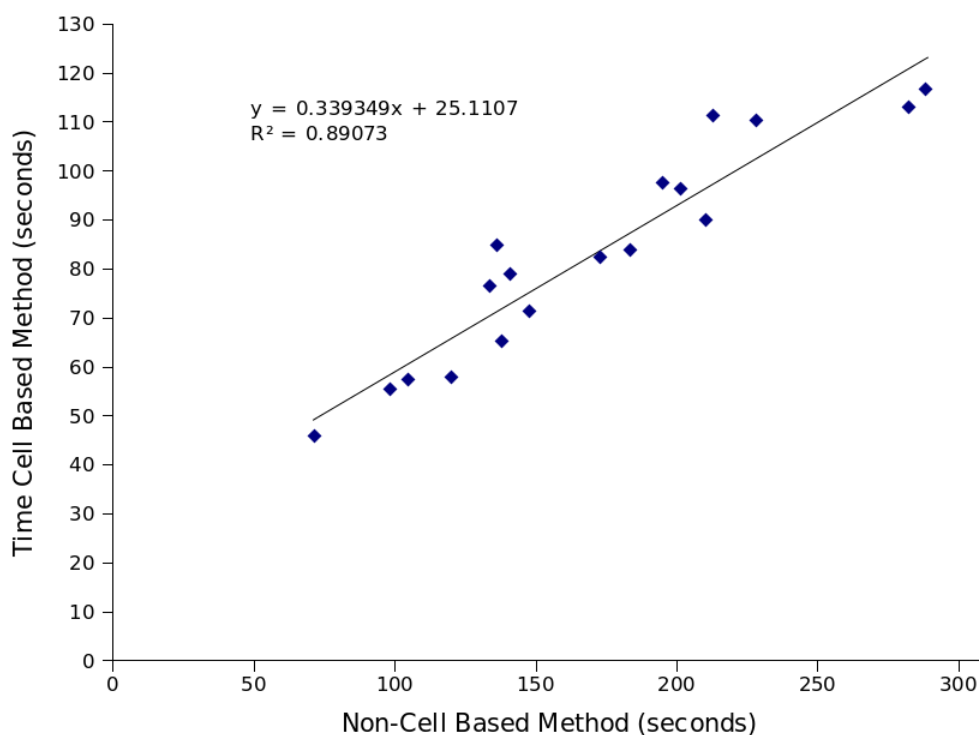


Figure 2.3: The general trend in energy calculation time as a function of system size, each point represents a single system. Energy computations using a grid based method yield approximately a three times performance improvement, slope of 0.339. However, in the case of some very small structures, it is possible that the overhead introduced by maintaining the grid structure outweighs the improvement. Performance for small systems is already very good, and thus the improvement in larger systems is far more valuable than the small penalty paid in small systems.

PDB id	Naive Method	Cell Based Method
1F5Z	201.75	96.37
1H2V	98.31	55.32
1HRD	228.38	110.12
1M1Z	147.83	71.28
1M9X	282.35	113.04
1O60	172.82	82.34
1R0V	210.35	90.0
1XMP	213.25	111.25
2E3Z	141.11	78.79
2H6U	138.25	65.07
2OU1	104.93	57.24
2XI9	71.68	45.73
3AMD	288.58	116.52
3DEL	133.62	76.48
3E1E	183.8	83.8
3FGN	120.38	57.75
3HHP	195.25	97.58
4GVR	136.35	84.78

Table 2.1: The specific experimental times for a series of energy computations presented in Figure 2.3. These examples represent a “best case” scenario, as the majority of time in these experiments is spent computing the solvent contribution, and thus the improvement is more evident.

2.4 Discussion

We have developed an application of a classic computer science grid based hashing algorithm to the implicit solvent model of PLOP. We demonstrate that this application does not affect accuracy of results compared with the previous implicit solvent model implementation in PLOP. Though in a small number of cases, side chain conformations are predicted in widely different conformations by

the hash based method and the old implementation, the two methods are equally likely to predict the more native-like structure, so this variance can be attributed to noise. We have also found in other experiments that the final predicted structure of a minimization is very sensitive to both small changes in pre-minimization coordinates, of a magnitude far less than bond distances, and minimization parameters. It is possible that these effects magnify small differences present early in the experiment, resulting in much larger differences between the final predicted structures. Finally, we present data showing that the reduced computational cost of evaluating the solvent contribution using this hash based approach dramatically reduces the total time spent evaluating the energy model, by a factor of 1.6 to 2.5 on a set of energy calculation experiments.

Note that any such geometric hashing will introduce some overhead for maintaining the data structure. As discussed by Bentley and Friedman, the total storage necessary for the hash structure and the time necessary to sort atoms into cells are both linear in the number of atoms, and placing or updating a single atom in the structure is a constant time operation [Bentley and Friedman, 1979]. The improvement in retrieval using this structure dominates the cost of maintaining the structure, and the difference becomes more pronounced as system size grows. Bentley and Friedman also present a thorough review of the performance characteristics of a number of other geometric hashing techniques, though they compare the algorithms in a data agnostic means. An octree is a similar, though hierarchical, hash structure used in computer graphics for fast location based retrieval. However, because the criteria for “collision” in this case is a fixed distance cutoff, and the data is roughly uniformly distributed, it is efficient to use a fixed cell size [Turk, 1989]. Taking advantage of the characteristics of physical data, in this case atomic coordinates, has some effect on the relative advantages and disadvantages of specific hashing techniques. Specifically, the maximum number of atoms per cell is limited by physical constraints of atomic interactions.

Though the implicit solvent term was initially targeted, because it dominates the time spent in energy calculations, it is possible to apply this method to any pair-pair interaction. However, especially for shorter range interactions it might be beneficial to either maintain a higher resolution hash, implement a hierarchical spatial hash, such as an octree, or maintain an adjacency list. We are also applying this geometric hashing technique to collision detection between simultaneous loop predictions. This will allow efficient screening of neighboring loop prediction candidates, which will be particularly useful in predicting structures with multiple nearby solvent exposed loops, such as

G-protein coupled receptors.

Implicit solvation models offer a very tangible benefit over explicit solvent models, both in performance and experimental complexity. Though explicit solvation is sometimes viewed as a “gold standard”, it has been shown that current implicit solvent models can, at least sometimes, reproduce predictions of explicit solvent models. However, development of implicit solvent models is important because improved performance compared with explicit solvent methods allows modeling of larger systems, longer timescales, and/or improved sampling. The complexity of the experiment is also reduced using implicit solvent models because results are not dependent on sampling of water conformations in addition to protein conformations. However, implicit solvent models can still be very computationally expensive. For instance, in the PLOP implementation of the OPLS-AA energy model with S-GB solvation term, evaluating the solvation term consumes up to 80% of the total time spent in energy calculations, dependent on size of the symmetric system. This is in large part due to the time complexity of evaluating the S-GB solvation term. Thus an algorithm that offers further reduction in experimental cost without a trade off in accuracy represents valuable progress in the development of implicit solvent models.

Because the size of protein systems are limited at some level by physical and biological constraints, the maximum system size expected to be encountered is limited. Therefore, although the new method reduces the time complexity of implicit solvent calculations, the maximum expected speedup is limited to about a factor of three in large systems. The actual speedup depends on both the system size and the amount of time that a given experiment spends evaluating the solvent contribution. The speedup observed in side chain prediction experiments was much less, around 20%, though it is possible that applying a similar method to terms of the gradient during minimization would increase that amount. Nonetheless, even a 20% speedup represents a significant improvement, especially as structure prediction methods continue to depend on parallel prediction and reprediction of the same region as a method of structure refinement [Goldfeld *et al.*, 2013]. Although this algorithm improves on the theoretical time complexity of the S-GB implicit solvent model, parameterizations such as the number and size of cells in the grid structure could have a significant effect on run time. Some effort was made towards choosing reasonable parameters, but they are likely not optimal. Hardware that is optimized for this sort of spatial indexing and collision detection, or proximity detection, exists in modern video cards, and along with general purpose

programming for this sort of hardware, it should be possible to further parallelize computation of implicit solvation effects for even greater performance improvements [Harris, 2008].

Chapter 3

Progress in Computational Mutation Scanning

3.1 Introduction

In order to determine which amino acids in a protein play the largest role in determining binding affinity, it is convenient to compare the binding affinity of the native protein with that of a single residue mutant. Single point mutations with few exceptions, mutations to or from glycine, proline, and depending on the local structure possibly large amino acids, are unlikely to affect the folding of the protein [Illergård *et al.*, 2009; Betts and Russell, 2003]. Alanine is the most frequently occurring amino acid, appearing in both solvent exposed and buried positions [Chothia, 1976; Rose *et al.*, 1985], and is unlikely to disrupt the protein fold in the same way glycine or proline might [Klapper, 1977; Betts and Russell, 2003]. Additionally, because it lacks a charge, it does not interact electrostatically with the ligand. These reasons make it an attractive choice as a “control” amino acid for mutation scanning experiments. Mutation scanning experiments seek to identify the residues that have the largest contributions to binding affinity or “hot spot” residues, by identifying single residue mutants that have a significantly decreased binding affinity when mutated to alanine [Cunningham and Wells, 1989].

While generally viewed as deleterious in biology, there are a few instances in which mutations can be beneficial to an organism. Foremost amongst these is in the immune system, where the immune system maturation response selects antibodies that have a reasonable affinity for an antigen

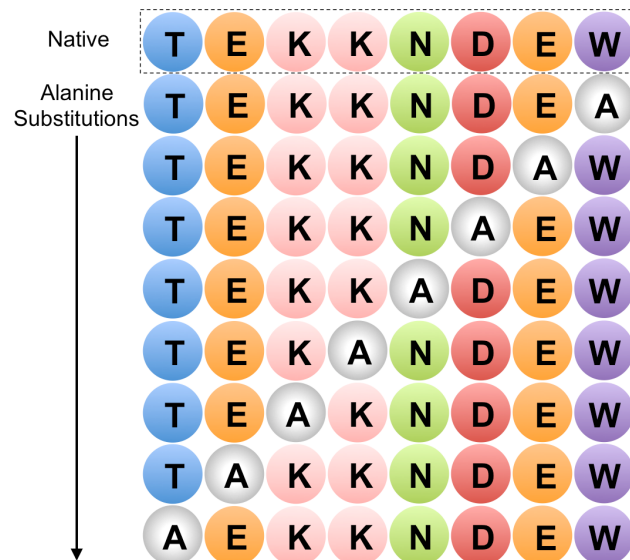


Figure 3.1: The sequences that would be evaluated during an alanine scan for Fc domain of a human IgG for streptococcal protein G. The residues identified here were taken from the AESDB. The native protein is represented in the top row [Sauer-Eriksson *et al.*, 1995; Thorn and Bogan, 2001].

and creates a large number of variants of these antibodies through mutation. The effect of this is that the body produces antibodies with increasing affinity for an antigen some time after the initial exposure [Griffiths *et al.*, 1984]. In vitro affinity maturation attempts to select molecules, frequently antibodies, with high affinity for some target molecule by creating a library of bacteria, that display variants of these antibodies on their cellular surface. This is accomplished by bacteriophage display, which provides a method of pairing the protein represented on a bacteria's surface with the genetic material contained by that bacteria [Smith, 1985]. A bacteria is infected by a library of bacteriophages containing a large number of variants of the antibody of interest. The phage will cause the bacteria to display its specific variant of the antibody on the bacteria surface, allowing sorting of the bacteria according to the affinity of the antigen, through affinity column purification or similar techniques. This step greatly enriches the fraction of antibody variants binding the protein. It is then possible to allow the bacteria to reproduce, sometimes causing more mutations to increase diversity of the antibody library and perform this affinity purification step again. Sequential application of this affinity maturation makes it possible to identify a handful of antibody variants with high affinity from as many as 10^6 different variants [Gram *et al.*, 1992;

Hawkins *et al.*, 1992]. However, the number of possible variants of the complementarity determining region (CDR) of an antibody is many orders of magnitude larger than this.

Computational mutation scanning attempts to replicate the same sort of experiment *in silico*. Making the assumption that the backbone conformation is not altered by mutating a single residue to alanine, computational experiments attempt to identify hot spot residues by measuring the $\Delta\Delta G$ between the bound states of the native and mutated protein. Varying cutoffs for *hot spot* residues are used, usually from 1.0 kcal/mol [Kortemme and Baker, 2002] to 4.0 kcal/mol [Pons *et al.*, 1999]. Mutations at these *hot spot* residues tend to be strongly deleterious leading to above average conservation [Hu *et al.*, 2000; Lichtarge *et al.*, 1996].

In this chapter we present a method of performing mutation scans to arbitrary amino acids and evaluate its performance by predicting $\Delta\Delta G$ binding for a number of single point mutations of hot spot residues to alanine. We show that though the conformations of these side chains are predicted quite accurately, though, no significant correlation of free energy of binding was observed between predicted and experimental values for $\Delta\Delta G$.

3.2 Methods

3.2.1 Entropy-Enthalpy Compensation

Some computational alanine scanning experiments explicitly compute or approximate the entropic contribution to the change in the free energy of binding [Hao *et al.*, 2010; Guerois *et al.*, 2002]. However, other models have achieved good agreement with experimental data while assuming these effects are either accounted for by the correlation between entropy and enthalpy for small changes in protein structure [Sharp, 2001] or to be small relative the entropic changes [Kortemme *et al.*, 2004]. PLOP has not made use of entropic contributions to free energy and has in many cases achieved good agreement with experimental data, so in these experiments it is assumed that contributions due to entropy are small relative entropic contributions.

3.2.2 General Mutation Screening

The generalized mutation screening method implemented in PLOP allows efficient evaluation of a large number of possible mutations to proteins. It accepts as input a set of possible mutations for

each residue, or a set of possible mutations for a set of residues. For instance, tryptophan, tyrosine, and arginine are overrepresented in hot spot residues [Hu *et al.*, 2000], so it may be desirable to consider all mutations in which a set of residues are either left at their native identity or replaced with one of these residues. If desired, the user can also set bounds for the minimum and maximum number of simultaneous mutations allowed. The residues that will be mutated are referred to as *free* residues, as the conformations of the other residues are held fixed throughout the entire process. While the residues are still in their native states, the structure is subjected to some sort of sampling. This is done in order to prevent bias towards predictions that will later be sampled in the same fashion. In the present implementation this consists of predicting the conformation of each free residue and minimizing those residues.

In this side chain sampling, for each free residue, the side chain is initially replaced with a random conformation from a high resolution rotamer library screened for steric clashes with the static part of the protein. The free residues are then examined sequentially, replacing each with the lowest energy conformation present in the rotamer library. This replacement process is continued until the termination condition is met, which is that two or fewer residues are replaced by lower energy conformations during the replacement stage. Five iterations of this procedure, from randomization to a static conformation, are performed and the most frequently selected conformation is chosen for each amino acid [Jacobson *et al.*, 2002b; Jacobson *et al.*, 2002a].

In the mutation stage, each free residue is first updated to its new chemical identity, possibly remaining in the native state, side chains are replaced with the side chain of the desired amino acid, with the corresponding updates to the bond, angle, torsion, and 1-4 interactions. The conformations of free residues are then re-predicted using the same side chain prediction algorithm described for the native conformation.

3.2.3 Alanine Scanning Experiments

Three protein complexes (PDBids: 1FCC, 1BRS, and 1DVF) with both experimental data for binding affinity and crystal structures, were identified using the ASEdb [Thorn and Bogan, 2001; Sauer-Eriksson *et al.*, 1995; Buckle *et al.*, 1994; Braden *et al.*, 1996]. Protonation states and locations of polar hydrogens were assigned for all residues as in [Li *et al.*, 2007]. A crystal context was built for each structure using symmetry data determined by experiment. For each mutation

represented in the alanine scan database, a single residue was mutated to alanine and this side chain prediction was repeated. The resulting structures were examined for side chain conformation agreement with crystal structures and the change in binding free energy to native was recorded.

3.3 Results

A strong correlation between observed and expected $\Delta\Delta G$ was not found for any of the structures nor for the set of mutations taken as a whole. Despite this, single side chain conformations were in very good agreement between predicted side chain locations and crystal structures, suggesting that sufficient sampling was done in the side chain prediction step. Results for mutations in the barstar-barnase complex, first with barnase mutated, then with barstar mutated; antibody anti-antibody Complex; and finally, streptococcal protein G fragment, IgG antibody complex are presented in turn below.

3.3.1 Barstar-Barnase Complex (Barnase Mutated)

The first complex examined is the Barstar-Barnase complex. This complex is frequently used as a case study in protein-protein interaction, as the complex exhibits one of the highest known binding affinities, $K_d = 0.01$ pM [Hartley, 1988; Hartley, 1989; Schreiber and Fersht, 1993]. Experimental affinity data is available from alanine scanning mutations for eight residues in barnase and six residues for barstar. Mutations to each chain were considered independently, and mutations to barstar are covered in the following section. The correlation between predicted and experimental $\Delta\Delta G$ binding was not significant (see figure 3.2, $R^2 = 0.08$).

Table 3.1 shows the computed and experimental $\Delta\Delta G$'s used to generate figure 3.2. Table 3.2 shows the root mean square distance for the side chain conformations predicted during the mutation scan to the crystal structure. All eight side chains are predicted within 1.3 angstroms of the native structure, and the median prediction is 0.359 angstroms, which is a very accurate prediction and sufficiently accurate to reproduce native binding interactions.

Despite the lack of correlation of predicted binding affinities with experimental values, single side chain conformations predicted in the course of the mutation scan were in very good agreement with crystal structures. This indicates that sufficient sampling was done in the side

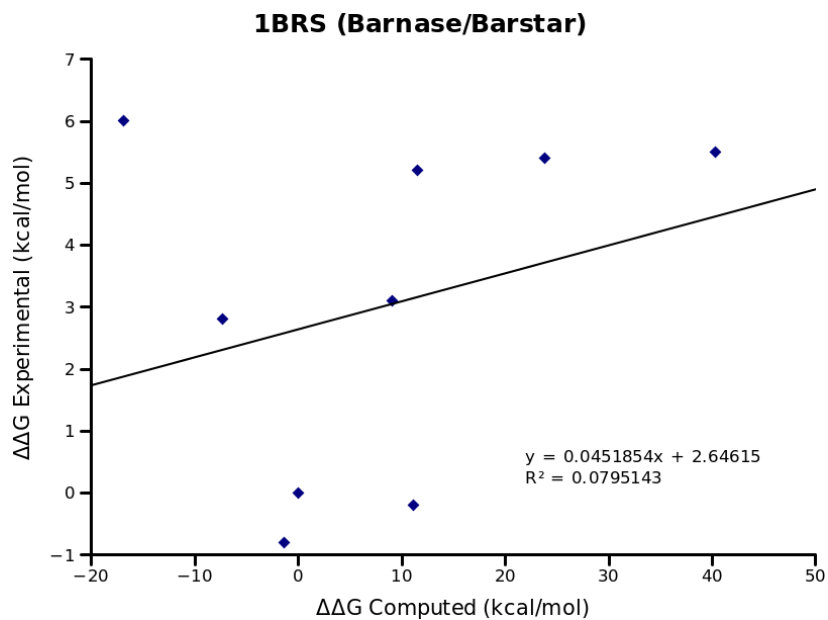


Figure 3.2: Computed versus experimental $\Delta\Delta G$ binding for 8 alanine mutations in the Barstar-Barnase binding pair. Crystal structure used for computations was 1BRS. Specific amino acids mutated were residues 27, 54, 58, 59, 60, 73, 87, and 102, all of chain A. Experimental binding affinity taken from [Thorn and Bogan, 2001].

Residue	$\Delta\Delta G$ calculated	$\Delta\Delta G$ experimental
native	0	0
27	23.82	5.4
54	-1.37	-0.8
58	9.09	3.1
59	11.58	5.2
60	11.15	-0.2
73	-7.28	2.8
87	40.32	5.5
102	-16.83	6

Table 3.1: Calculated and experimental $\Delta\Delta G$ for mutating given residues of barnase (chain A of structure 1BRS) to alanine. Experimental values taken from [Thorn and Bogan, 2001].

Residue	Amino Acid	RMSD
A:27	LYS	1.213
A:54	ASP	0.920
A:58	ASN	0.121
A:59	ARG	0.421
A:60	GLU	0.138
A:73	GLU	0.994
A:87	ARG	0.297
A:102	HIS	0.276

Table 3.2: RMSD of mutated side chains in barnase, in a barnase-barstar complex (chain A of PDBid 1BRS), during the mutation scanning experiments.

chain prediction step of the computation. Figure 3.3 shows the predicted and crystal conformations for Asp 58 of barnase. The conformations are almost identical, differing by only 0.121 angstroms, or less than the resolution of the crystal structure (2.0 angstroms). Figure 3.4 shows a similar comparison for glutamic acid 73, which was one of the least successful predictions in this complex. However, it would still be classified as a successful side chain prediction for many purposes, differing from the crystal structure by 0.993 angstroms.

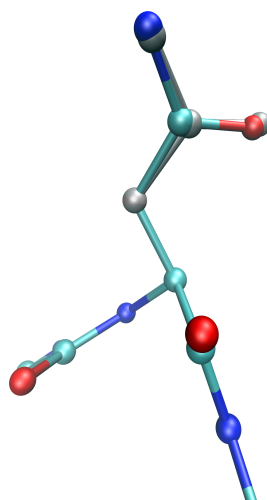


Figure 3.3: Crystal (colored by element) and predicted (gray) side chain conformations for barnase, asparagine 58 of 1BRS. The predicted and crystal conformations are almost identical, differing by only 0.121 angstroms, or less than the resolution of the crystal structure.

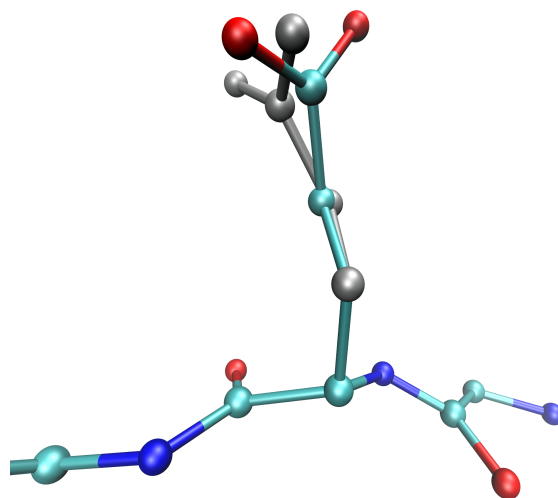


Figure 3.4: Crystal (colored by element) and predicted (gray) side chain conformations of glutamic acid 73 of barnase, chain A of PDBid 1BRS. The two conformations differ by 0.993 angstrom RMSD, which is generally considered a successful side chain prediction.

3.3.2 Barstar-Barnase Complex (Barstar Mutated)

The second example makes use of the same complex, but it is the other partner of the complex, barstar, which is mutated. The results here are similar to the previous case, in which figure 3.5 shows that there is little correlation between computed and experimental $\Delta\Delta G$, $R^2 = 0.21$.

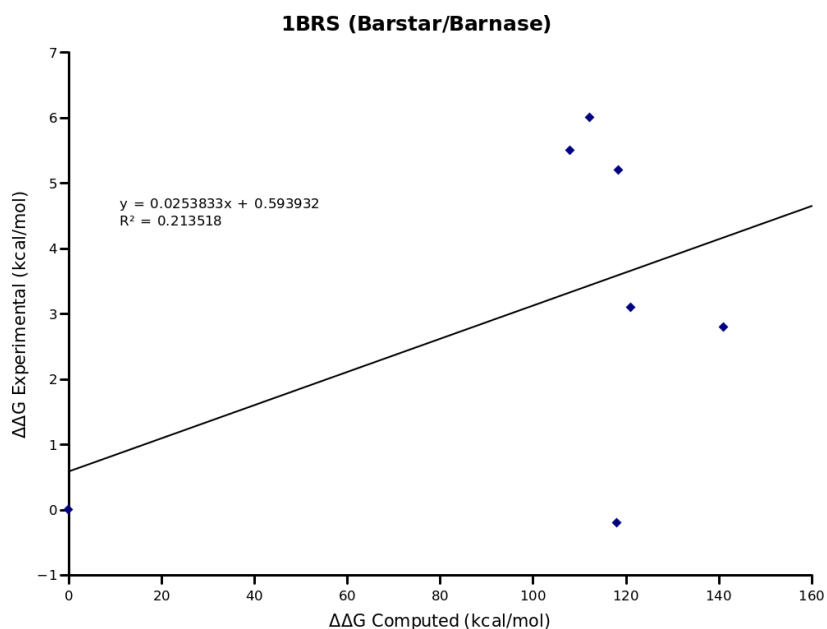


Figure 3.5: Computed versus experimental $\Delta\Delta G$ binding for 6 alanine mutations in the Barstar-Barnase binding pair. Crystal structure used for computations was 1BRS [Buckle *et al.*, 1994]. Specific amino acids mutated were residues 29, 35, 39, 42, 74, and 78, all of chain D. Experimental binding affinity taken from [Thorn and Bogan, 2001].

Table 3.3 shows the predicted and experimental $\Delta\Delta G$ binding for the barstar-barnase complex, depicted in figure 3.5. Table 3.4 shows the agreement of sampled side chain conformations with the native conformations. Five of six side chains are predicted within 0.4 angstroms of their native conformation, maintaining all native contacts, and supporting the ability of the sampling method to explore sufficiently native-like conformations that the energy model is able to differentiate native-like from non-native like conformations. Figure 3.6 illustrates this in general for the protein-protein interface.

An example of a successful prediction is illustrated in 3.7. The predicted conformation of this aspartic acid is less than 0.1 angstroms RMSD from its crystal conformation.

Residue	$\Delta\Delta G$ calculated	$\Delta\Delta G$ experimental
native	0	0
29	121.07	3.1
35	118.37	5.2
39	118.09	-0.2
42	141.09	2.8
74	107.92	5.5
78	112.14	6

Table 3.3: Calculated and experimental $\Delta\Delta G$ for mutating given residues of barstar (chain D of structure 1BRS) to alanine. Experimental values taken from [Thorn and Bogan, 2001].

Residue	Amino Acid	RMSD
D:29	TYR	0.121
D:35	ASP	0.098
D:39	ASP	0.335
D:42	THR	0.114
D:76	GLU	0.397
D:80	GLU	1.804

Table 3.4: RMSD of mutated side chains in barstar, in a barnase-barstar complex (chain D of PDBid 1BRS), during the mutation scanning experiments.

The prediction that differs by the greatest amount from the crystal structure, Glu 80, is illustrated in 3.8. While this prediction differs from the native by ~ 1.8 angstroms, this is still on the border of what is frequently considered a successful side chain prediction. However, a prediction that differs from the native by this amount makes successfully predicting binding affinity difficult, as the predicted conformation does not accurately recapitulate the contacts formed in the native complex.

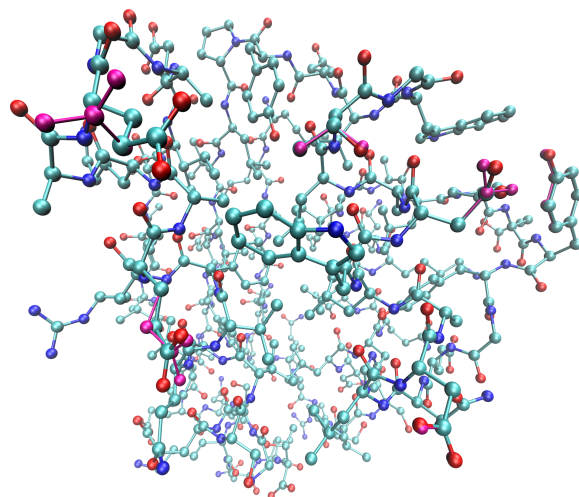


Figure 3.6: Distribution of 6 mutated residues (magenta) on the interface surface of barstar, 1BRS chain D. Five of the six residues are less than 0.4 angstroms RMSD to the crystal structure. The only exception is glutamic acid 80, shown in the upper left of this figure, and also figure 3.8.

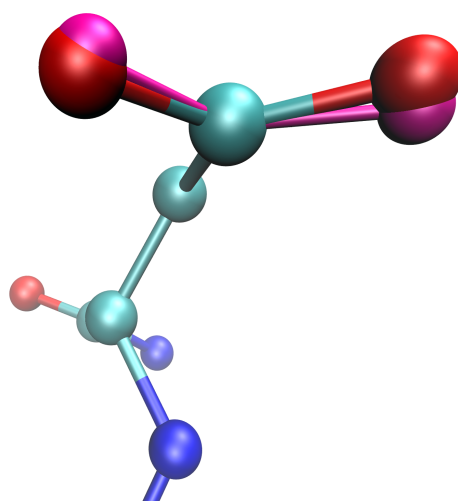


Figure 3.7: Crystal (colored by element) and predicted (magenta) side chain conformations for barstar, chain D of PDBid 1BRS. The distance to the crystal structure is only 0.098 angstroms, or nearly identical.

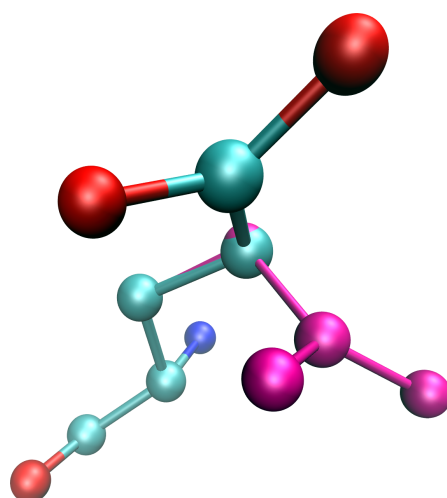


Figure 3.8: Glutamic acid 80 is the only residue on chain D, barstar, of the barnase-barstar complex which was not predicted within 0.4 angstroms of the crystal coordinates during the mutation scanning experiments. The difference between these two conformations is 1.804 angstroms, which while sometimes considered a “successful” prediction, is not sufficiently close to generate the same interactions, making it difficult to accurately predict binding affinities.

3.3.3 Antibody anti-Antibody Complex

The third example is an an antibody, anti-antibody complex. The structure taken from PDBid 1DVF is of anti-hen-egg-white lysozyme antibody complexed with an anti-idiotopic antibody mimicking the hen egg white lysozyme [Braden *et al.*, 1996]. As in the barnase-barstar complex examples, there is no significant correlation between predicted and experimental $\Delta\Delta G$ (figure 3.9). The specific predicted and experimental values of $\Delta\Delta G$ are presented in table 3.3.

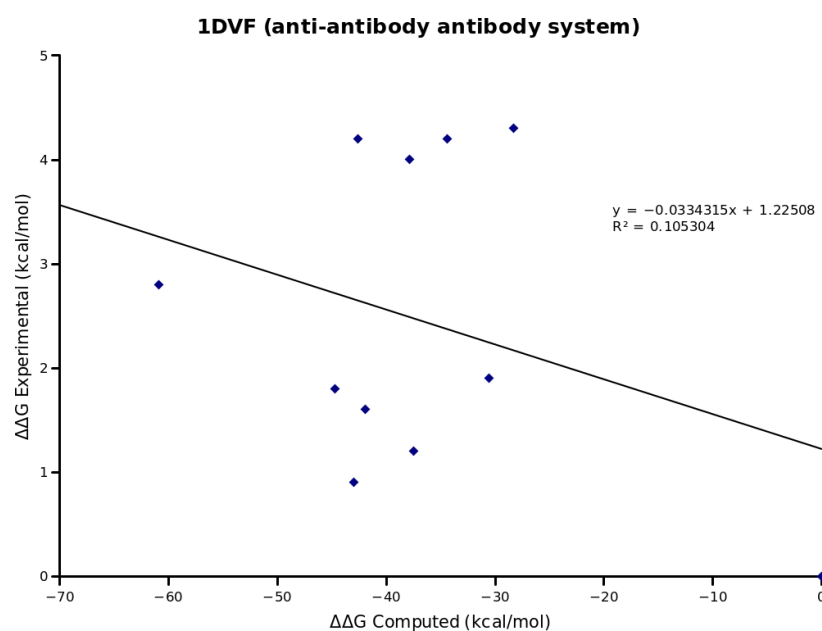


Figure 3.9: Computed versus experimental $\Delta\Delta G$ binding for 10 alanine mutations in the anti-hen-egg-white lysozyme antibody (D1.3) anti-idiotopic antibody (E5.2) complex. Crystal structure used for computations was 1DVF [Braden *et al.*, 1996]. Specific amino acids mutated were residues 30, 32, 52, 54, 56, 58, 98, 99, 100, and 101, all of chain A. Experimental binding affinity taken from [Thorn and Bogan, 2001].

Table 3.6 shows the RMSD of side chains of 1DVF predicted during mutation scanning experiments. The majority of side chains predicted were very close to the native conformation. The most significant exception was aspartic acid 100, shown in figure 3.10.

Two adjacent and successfully predicted side chains are threonine 30 and tyrosine 32, with RMSDs of 0.056 and 0.412 angstroms respectively.

Residue	$\Delta\Delta G$ calculated	$\Delta\Delta G$ experimental
native	0	0
30	-42.93	0.9
32	-44.68	1.8
52	-42.6	4.2
54	-28.29	4.3
56	-37.5	1.2
58	-41.91	1.6
98	-34.38	4.2
99	-30.51	1.9
100	-60.9	2.8
101	-37.84	4

Table 3.5: Calculated and experimental $\Delta\Delta G$ for mutating given residues of anti-idiotopic antibody (chain A of structure 1BRS) to alanine. Experimental values taken from [Thorn and Bogan, 2001].

Residue	Amino Acid	RMSD
B:30	THR	0.056
B:32	TYR	0.412
B:52	TRP	0.391
B:54	ASP	0.442
B:56	ASN	0.238
B:58	ASP	0.159
B:98	GLU	0.182
B:99	ARG	1.137
B:100	ASP	2.577
B:101	TYR	0.340

Table 3.6: RMSD of mutated side chains in 1DVF, anti-hen-egg-white lysozyme antibody (D1.3) complexed with an anti-idiotopic antibody (E5.2), during the mutation scanning experiments.

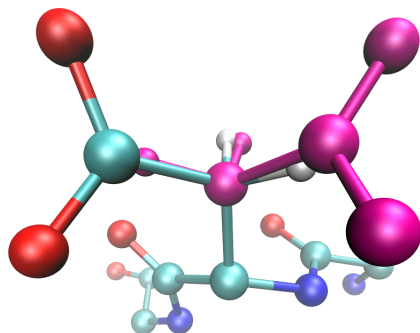


Figure 3.10: An unsuccessful side chain prediction in the antibody antigen complex of PDBid 1DVF. The predicted conformation of this aspartic acid, B:100, differs from the native state by 2.577 angstroms.

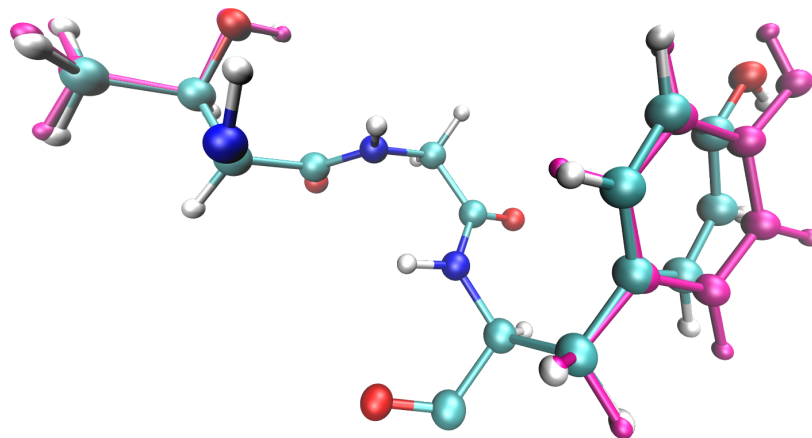


Figure 3.11: Two neighboring successful predictions (magenta) in the same antibody antigen complex. Threonine 30, left, is predicted almost identically to the native structure, at 0.056 angstroms from the crystal coordinates. Tyrosine 32, right, is predicted at 0.412 angstroms RMSD.

3.3.4 Streptococcal Protein G fragment, IgG Antibody Complex

The final complex considered is another antibody antigen complex. PDBid 1FCC depicts a fragment from streptococcal protein G in complex with an IgG antibody. As in the previous cases, there is little agreement between predicted and experimental $\Delta\Delta G$ binding, $R^2 = 0.40$, as shown in figure 3.12.

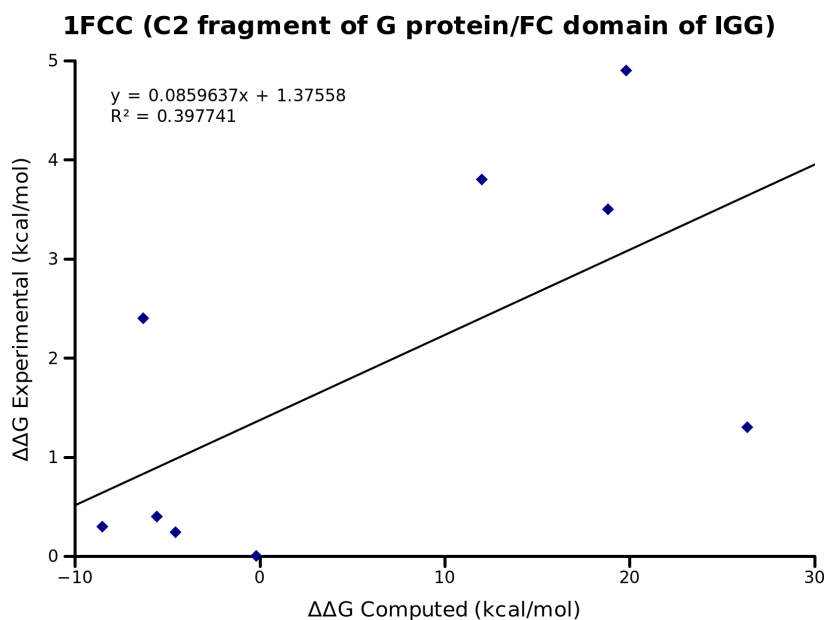


Figure 3.12: Computed versus experimental $\Delta\Delta G$ binding for 8 alanine mutations in the antibody antigen binding pair. Crystal structure used for computations was 1FCC. Specific amino acids mutated were residues 25, 27, 28, 31, 35, 40, 42, and 43, all of chain A. Experimental binding affinity taken from [Thorn and Bogan, 2001].

Table 3.8 shows the RMSD of side chains of 1FCC predicted during mutation scanning experiments. Seven of eight sampled side chains were predicted within 1.3 angstroms of native, and six of eight were within 0.6 angstroms of native. As in the case of 1DVF the incorrect side chain prediction was a carboxylic acid, glutamic acid 42, where the predicted conformation differed from the crystal structure by almost 3 angstroms. The most significant exception was aspartic acid 100, shown in figure 3.10.

This side chain is in close proximity to a number of other hot spot residues on the protein-protein interface and is shown in context in figure 3.13.

Tryptophan 43 is interesting in that despite being critical to protein-protein binding it is largely

Residue	$\Delta\Delta G$ calculated	$\Delta\Delta G$ experimental
native	-0.18	0
25	-4.55	0.24
27	19.8	4.9
28	26.37	1.3
31	18.82	3.5
35	-6.31	2.4
40	-8.51	0.3
42	-5.56	0.4
43	12.0	3.8

Table 3.7: Calculated and experimental $\Delta\Delta G$ for mutating given residues of Fc domain of human IgG (chain A of structure 1FCC) to alanine. Experimental values taken from [Thorn and Bogan, 2001].

Residue	Amino Acid	RMSD
C:25	THR	0.170
C:27	GLU	0.403
C:28	LYS	0.543
C:31	LYS	0.594
C:35	ASN	0.527
C:40	ASP	1.292
C:42	GLU	2.994
C:43	TRP	0.371

Table 3.8: RMSD of mutated side chains in 1FCC, C2 fragment of streptococcal protein G in complex with the Fc domain of human IgG, during the mutation scanning experiments.

buried in a pocket defined by the neighboring protein structure. This interaction is examined in figure 3.17.

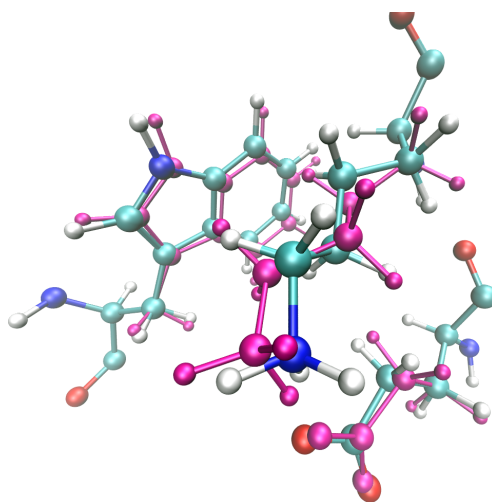


Figure 3.13: Three clustered hot spot residues in another antibody antigen complex, PDBid 1FCC, the C2 fragment of streptococcal protein G in complex with the Fc domain of human IgG. The predicted conformations for glutamic acid 27, lysine 31 and tryptophan 43 are depicted in magenta, with side chain RMSD's of 0.403, 0.594 and 0.371, respectively. These residues are shown in greater detail in other figures, glutamic acid 27 in figure 3.14, lysine 31 in figure 3.15, and tryptophan 43 in figure 3.16.

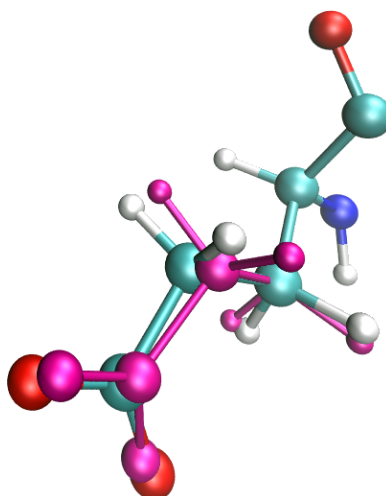


Figure 3.14: Predicted (magenta) and crystal conformations (colored by element) for glutamic acid 27. The side chain RMSD of this prediction is 0.403 angstroms.

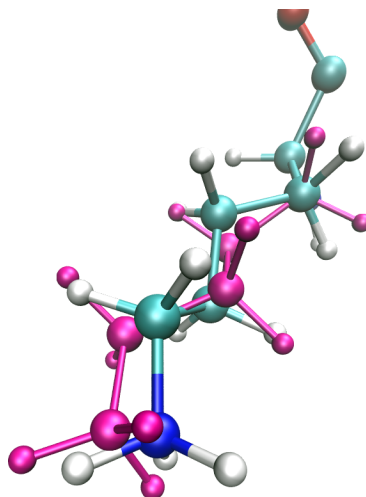


Figure 3.15: The predicted side chain conformation during the course of mutation scanning experiments (magenta) compared to the native conformation (colored by element) for lysine 31. The RMSD of this prediction is 0.594 angstroms.

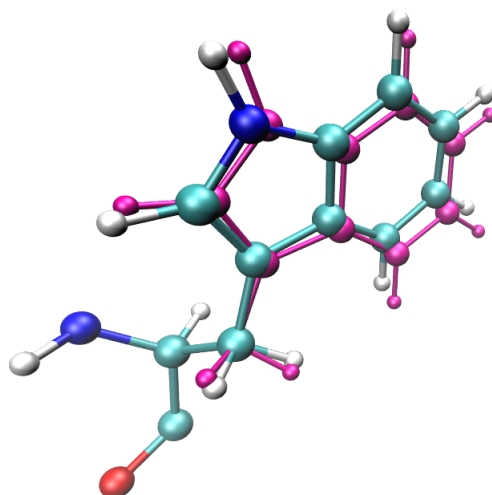


Figure 3.16: Native (colored by element) and predicted (magenta) side chain conformation for tryptophan 43 of 1FCC. The root mean square distance of the predicted conformation to the native is 0.371 angstroms. The effect of the local protein structure on the conformation of this residue is examined in figure 3.17.

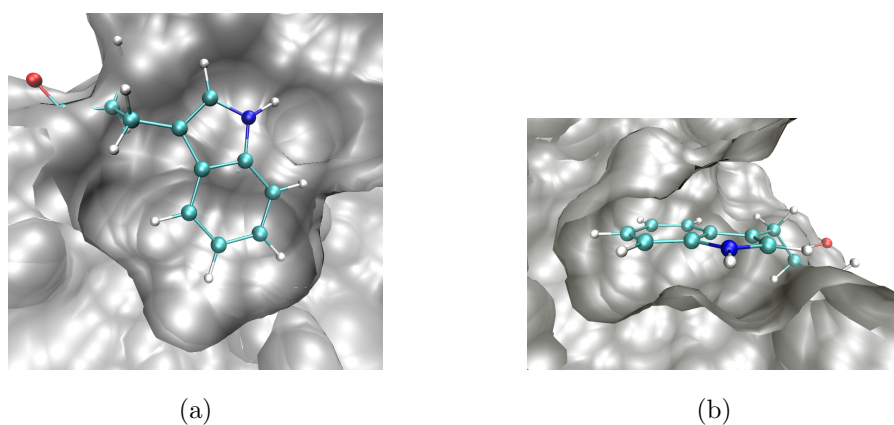


Figure 3.17: The pocket of tryptophan 43 of 1FCC, shown in two orthogonal orientations (a) and (b). Because of conformation of the neighboring protein structure, this residue has very little conformational freedom, and any prediction that successfully locates the side chain in the pocket will be reasonably close to the native state. The conformation predicted in these experiments was very similar, 0.371 angstroms, and is depicted superimposed with the native in figure 3.16.

3.4 Discussion

There are two necessary subproblems in accurately predicting the effect of mutations on local protein structure:

1. prediction of side chain conformations, and
2. accurately describing the energetics of the interactions.

In the work presented here, we have shown that our current methods are able to accurately predict side chain conformations of mutated residues, discussed below in 3.4.1. However, it seems that despite being able to differentiate between native and non-native side chain conformations, we are unable to correlate experimental changes in binding energy with computational predictions. Possible reasons for this discrepancy are addressed in subsection 3.4.2.

3.4.1 Side Chain Prediction Accuracy

Our results indicate that we are able to successfully predict side chain conformations on protein interfaces in the majority of cases examined. Specifically, 29 of 32 side chains predicted over four chains in three independent structures are within 1.5 angstroms of the native conformation, with a significant number of side chains predicted closer to the native crystal conformation than the resolution of the crystal structure. This indicates that both the sampling performed here and the energy model are sufficiently extensive and accurate to reproduce the native conformation, which has been used as a standard metric of success in many previous studies, e.g. loop predictions [Jacobson *et al.*, 2004; Rapp and Friesner, 1999; Zhu *et al.*, 2006; Sellers *et al.*, 2008] and side chain predictions [Jacobson *et al.*, 2002b; Jacobson *et al.*, 2002a; Zhu *et al.*, 2007a]. One of the reasons that RMSD is so popular as a performance metric is the difficulty of obtaining experimental data that can be directly compared to experimental predictions. Binding affinity studies, especially alanine scanning experiments, represent a wealth of data that might be used in training more accurate next generation molecular mechanics energy functions.

3.4.2 Energetic Correlation with Experimental Data

We found that despite predicting side chain conformations approximately correctly, there was generally no correlation between our computed $\Delta\Delta G$ and the experimental $\Delta\Delta G$ from the alanine

scanning database. The relationship between experimental and computationally predicted $\Delta\Delta G$ can be seen in figures 3.2, 3.5, 3.9, and 3.12. It is somewhat surprising that side chain predictions are as accurate as they are without any real correlation in binding affinity. A common assumption is that accuracy of predicted conformations implies accuracy of the energy model. However, this depends on very extensive sampling, such as in full molecular dynamics simulations. It is possible for biased sampling, such as is performed here with the goal of both increasing accuracy and speed of exploring conformation space, to mask shortcomings in an energy model.

Experiments by other groups have demonstrated some success in correlating computational $\Delta\Delta G$ with experimental $\Delta\Delta G$ [Kortemme *et al.*, 2004]. Some of these experiments have made use of energy models that are largely similar to the one implemented in the PLOP program. Despite this, we did not find a significant correlation between experimental data and predictions with respect to $\Delta\Delta G$. It is possible that the interactions at a protein-protein interface are somehow different than intramolecular interactions, which have constituted the majority of the training sets used to develop the PLOP energy model.

Additionally, some hot spot residues are tightly constrained in conformation by neighboring protein structure, making prediction more similar to placing a jigsaw piece than searching for a low energy conformation among many possibilities. This sort of situation was especially prevalent in tyrosine 43 of 1FCC. In this case there were only two conformations from the side chain rotamer library that were not eliminated in the initial screening process. The conformation of tyrosine 43 in the protein context is shown in 3.17.

Finally, because the rotamer library used here biases sampling towards experimentally observed side chain conformations, it is possible that the energy model is capable of correctly ranking conformations present in the library by energy, but it performs less well for areas of conformational space outside the rotamer library. In order to test this hypothesis it would be necessary to use a non-rotamer based approach in a similar set of experiments. Conveniently, the side chain sampling method used in the minimization Monte Carlo experiments described in chapter 4 can be used to perform such an experiment.

3.4.3 Future Directions

Current data indicates that the sampling method introduced here is sufficiently exhaustive to identify native like conformations. Thus, it would be logical for future work to focus initially on exploring and improving the energy model. As mentioned above, one possible means of doing this would be to implement an energy model used in similar experiments, such as CHARMM, used by the Baker lab in their hot spot identification experiments, in which they were able to successfully predict hot-spot residues, and those that did not contribute significantly to the binding affinity [Kortemme *et al.*, 2004; Lazaridis and Karplus, 1999]. This would allow for testing of the sampling method independently of the energy model used, to support the success of or reveal potential shortcomings in the sampling method.

Testing the performance of the energy model on protein-protein surfaces and classifying the errors would be a necessary step in improving the correlation between predicted and experimental $\Delta\Delta G$. It would also be very enlightening to be able to compare the performance of the PLOP sampling methods using a known energy model implemented in a different molecular mechanics toolkit.

The sampling introduced in these experiments is very modular in nature. Because of this, it would be possible to modify the sampling procedure used in the code, or even to specify the sampling method in the input file. This flexibility will hopefully allow testing of many different sampling methods in order to find a method that provides a good balance of time and coverage of conformational space.

Because the method implemented here is capable of not only alanine scanning, but also generalized mutation scanning, it is possible to computationally screen interactions between a large number of variants of similar proteins. If the correlation between predicted and experimental binding affinities can be further improved, there would be a number of extremely valuable applications for such a method. First, it would be possible to screen variants of an antibody in order to help accelerate affinity maturation. Second, it might also be possible to test the efficacy of a number of drugs targeting highly variable proteins, such as HIV protease [Watkins *et al.*, 2003], by screening the drug against a number of possible mutations.

Chapter 4

Application of Monte Carlo Minimization Sampling to Prediction of P450 Sites of Metabolism

*Parts of this chapter are adapted from “IDSite: An Accurate Approach to Predict P450-Mediated Drug Metabolism” [Li, J.; Schneebeli, S.; **Bylund, J.**; Farid, R.; Friesner, R.; 2011]. My contribution to the paper was principally in the minimization Monte Carlo method described and the small molecule library used for the ligands.*

4.1 Introduction

The most common method of drug clearance among currently prescribed drugs is metabolism, which is the primary method of clearance for approximately 75% of the top 200 most commonly prescribed drugs in the United States [Williams *et al.*, 2004]. Cytochrome p450 is critical to drug metabolism, being active in approximately 75% of drugs cleared in this method [Guengerich, 2007]. Of the human isoforms of P450, Cytochrome P450 2D6 (CYP2D6) is frequently involved metabolism of xenobiotics [Williams *et al.*, 2004]. There are high resolution crystal structures available for CYP2D6 [Rowland *et al.*, 2006], it was used as a test case for this study. As covered in 1.1.2.3, accurately predicting absorption, distribution, metabolism, and excretion (ADME) characteristics of drug compounds can be a critical factor in determining drug efficacy, performance in clinical

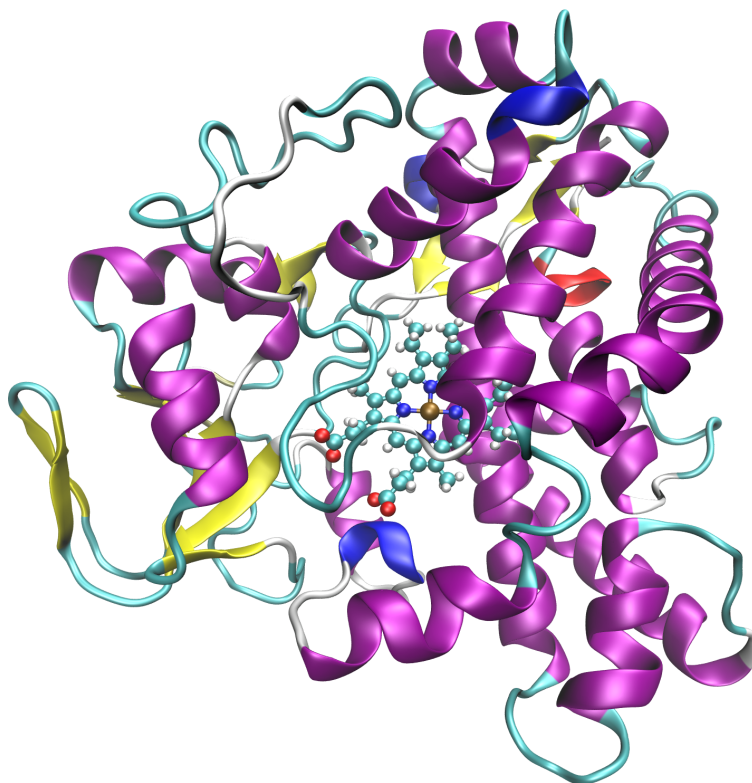
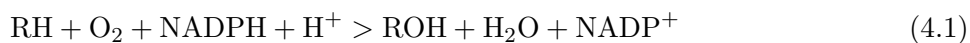


Figure 4.1: The structure of cytochrome P450, taken from PDBid 1JFB, shown in cartoon representation. The bonded heme group, shown as ball and stick model, is visible in the center. The brown iron atom is chelated by four deep blue nitrogen atoms.

development stages, and the overall costs of bringing new drugs to market. Because of the ubiquity of P450 in metabolic reactions of drugs, there is no other single enzyme family as significant to determining ADME as P450. The general form of the reaction most frequently catalyzed by P450 is



The specific locations of sites of metabolism (SOM) on small molecules can have a profound effect on the ADME characteristics of a small molecule. Some cancer drugs, such as epipodophylotoxins, ifosphamide, tamoxifen, taxol and vinca alkaloids, are converted into their active states by oxygenation at specific locations by P450 [Kivisto *et al.*, 1995]. P450 is the body's primary defense against toxicity, usually catalyzing the conversion of toxic compounds into harmless products [Gonzalez, 2005; Guengerich, 2001]. However, in certain cases, such as acetaminophen, it is possible

for P450 to convert a harmless reactant into a toxic product [Chen *et al.*, 1998], although usually these compounds would be eliminated during the clinical trial stages. Additionally, the different metabolites of a compound may be differentially cleared by the body, having significant effects on bioavailability. Because of the costs associated with testing ADME parameters in live organisms, accurate computational predictions can significantly decrease both costs and times associated with drug development.

Because of its central role in drug metabolism, P450 has already been a subject of a number of studies attempting to predict sites of metabolism and chemical metabolites [Afzelius *et al.*, 2007]. A number of different classes of methods for predicting sites of metabolism by P450 have been developed. Broadly speaking, these can be classified into: quantitative structure-activity relationship (QSAR) based, pharmacophore-based, structure-based (docking), reactivity-based, and rule-based methods [Cruciani *et al.*, 2005]. Rule based and pharmacophore based methods make predictions based on a subset of the drug structure, and it is possible for elements of the drug far from a possible site of metabolism to either prevent or promote metabolism at that location. QSAR based approaches work best when the set of reactions being catalyzed are very similar. However, P450 catalyzes a very broad range of reactions, so these approaches are likewise somewhat limited in the case of P450. Reactivity based methods are both very expensive to compute, being unsuited for screening a large database, and do not take into account the structure of the P450 isoform [Singh *et al.*, 2003; Chen *et al.*, 1997; de Visser *et al.*, 2002]. MetaSite, an approach which makes use of structural information of both the ligand and the P450 isoform process has achieved an 84.3% prediction accuracy (296 of 351 total sites of metabolism correctly predicted), and the primary site of metabolism is identified in the top 3 ranked sites in over 90% of cases [Cruciani *et al.*, 2005]. However, the sampling of P450 conformations done by MetaSite is quite limited, because it pre-computes a number of low energy conformations and then docks the substrate into each of those.

We have developed a similar approach which provides significantly more thorough sampling of the P450 substrate complex. The new method, IDSite, makes use of the structures of both the P450 and the substrate, as well as evaluating the intrinsic reactivity of the possible site of metabolism.

4.2 Methods

Prediction of sites of metabolism is a three stage procedure:

1. Initially a number of different ligand conformations are generated, and these are docked into a rigid protein, with soft VDW terms using Glide [Halgren *et al.*, 2004; Friesner *et al.*, 2004].
2. The docked conformations are refined using a Monte Carlo Minimization (MMC) approach which samples degrees of freedom in both the ligand and protein.
3. Refined conformations are classified into reactive site or non-reactive site on the basis of the energy of the refined conformations and the intrinsic reactivity of the site. [Li *et al.*, 2011b]

4.2.1 Docking

In the initial docking stage of the IDSite protocol, Glide is used to generate a number of proposed docked conformations for each ligand. Glide (standard precision) is used to generate a number of different ligand conformations by sampling conformations of freely rotatable bonds and rings. A bounding box, which will be used for a grid search, is defined, centered at the centroid of the ligand with an edge length of 10 angstroms. Because the crystal structure used for CYP2D6 (PDBID: 2F9Q) does not have a ligand, the centroid of residues Glu216, Asp301, Thr309, and Phe483 was used instead in this case. Because the steric clashes present in many proposed docked conformations can be relieved using a simple minimization procedure, reduced Van der Waals radii are used in the docking stage for non-polar atoms. The VDW radii used for P450 are scaled by a factor of 0.4, and the scaling for the ligand starts at 0.8. If an insufficient number of poses, in this case fewer than four, are found using these scaling factors for the radii, the scaling of the ligand is stepped down until at least four poses are found. Additional filtering of possible high energy conformations was also skipped in order to ensure the greatest diversity of docked poses reached the refinement stage. The collection of docked poses are then clustered according to the RMSD of the ligand, and each pose is minimized. The top sixty ranked poses according to the Glide SP metric are then passed on to the next screening stage.

The conformations generated from the Glide docking procedure are then screened using a number of different criteria. A hard sphere overlap screen is used to remove poses with obvious steric

clashes that were not removed during the minimization procedure. A conserved feature of CYP2D6 ligand complexes is a salt bridge with Glu216 or Asp301. In order to reduce sampling cost, IDSite only considers structures with at least one hydrogen-bond donor within 4 angstroms of the centroid of these two residues and Ser304. The sphere defined by these residues is illustrated along with the bounding box used for sampling in Figure 4.3. A number of other rule based geometric screens are used to remove structures that are unlikely to react. Structures meeting any of the following criteria are removed:

1. The distance of the basic nitrogen to the ferryl oxygen is less than 5.0 angstroms;
2. The distance of the basic nitrogen to the negative charged oxygen (in Glu216 or Asp301) is greater than 5.5 angstroms;
3. More than 2 heavy atoms from the ligands are farther than 16.0 angstroms away from the heme iron;
4. More than 1 heavy atom from the ligand are closer than 1.0 angstroms to the receptor;
5. More than 6 heavy atoms from the ligand are closer than 1.8 angstroms to the receptor;
6. No heavy atom in the ligand is within 5.0 angstroms to the heme iron;

If the number of structures at this point is too low, the VDW scaling factors of the non-polar atoms of the ligand are stepped down, and the process is repeated. If four or more poses are found at this point, these poses are passed onto the next stage of the IDSite procedure, the Monte Carlo Minimization refinement stage.

4.2.2 Monte Carlo Minimization Refinement

Since the emphasis in IDSite sampling is efficient sampling of low energy conformations, as only the lowest energy conformations are passed on to the next stage of prediction, Monte Carlo Minimization was used instead of a more traditional Monte Carlo simulation because it provides more efficient sampling of low energy conformations (see 1.2.2). The Monte Carlo Minimization sampling used by IDSite for refinement incorporates three different types of steps: side chain motions, rigid body transformations, and hybrid Monte Carlo simulations. For each Monte Carlo step, one of three

types of motions is selected according to the weighted probabilities, which are different for the two different PLOP sampling stages (see table 4.1). Using the chosen method, a new conformation is

	PLOP Sampling Stage	
	First	Second
Number of Residues Sampled	12	40
Number of Structures Advanced to Next Stage	$\max(n*8,24)$	$\max(n*20,60)$
P(side chain step)	0.5	0.7
P(rigid body step)	0.1	0.2
P(HMC)	0.4	0.2

Table 4.1: The number of residues sampled as well as the number of structures advanced to the next stage from each of the sampling stages. Also, the relative probabilities of selecting each of the different sampling steps during a Monte Carlo minimization sampling stage.

proposed and minimized before the Metropolis acceptance criteria (equation 1.1) is applied to the proposed state, using a temperature of 300 K. All atoms of all residues with any atom within 5 angstroms of the ligand in the starting crystal structure were allowed to move during Monte Carlo moves, including the ligand itself.

During the minimization Monte Carlo sampling stages of the IDSite procedure, artificial constraints are used to guide the sampling towards a transition state like conformation. These constraints create artificial bond or angle potentials, which affect the minimization but are not used in the Monte Carlo acceptance test. For each of the minimization Monte Carlo sampling stages of the IDSite procedure, two different sets of constraints are applied depending on the hybridization of the carbon atom at the possible site of metabolism, for a total of four possible different sets of constraints. In the first minimization Monte Carlo stage, two constraints are applied, shown in figures 4.4b and 4.4a:

1. The sulfur-iron-carbon angle is constrained to 145 degrees, with 20 degrees of “slack”, or a flat bottom to the potential well (denoted as 145 ± 20 degrees). The spring constant of this constraint is about 25 kcal/mol/degree², or ~40% the strength of a carbon-carbon-carbon angle.

2. A “dummy” oxygen atom is placed above the plane of the heme group, in the same position that it would occupy if an oxygen molecule was bound to the heme. This dummy atom has no interactions with other atoms, but is used as the anchor of a distance constraint for the carbon at the site of metabolism. The carbon-dummy oxygen distance is constrained to 2.5 ± 0.5 angstroms. The spring constant of this constraint is $100 \text{ kcal/mol/angstrom}^2$, approximately 1/3rd the strength of a carbon-carbon bond.

In the second minimization Monte Carlo sampling stage, the constraints are different for sp^2 and sp^3 carbons. These constraints are illustrated in figures 4.5b and 4.5a. For sp^3 sites:

1. the hydrogen bound to the carbon at the possible site of metabolism is constrained to a distance of 1.25 ± 0.1 angstroms and a spring constant of $20 \text{ kcal/mol/angstrom}^2$,
2. the carbon in question is constrained to 2.2 ± 0.8 angstroms and a spring constant of $10 \text{ kcal/mol/angstrom}^2$,
3. the heme iron-hydrogen-carbon angle is constrained to 138 ± 5 degrees and a spring constant of $20 \text{ kcal/mol/degree}^2$.

For sp^2 sites:

1. the carbon at the possible site of metabolism is constrained to 1.8 ± 0.1 angstroms and a spring constant of $20 \text{ kcal/mol/angstrom}^2$,
2. both adjacent carbons are also constrained to the dummy oxygen atom, at a distance of 2.5 ± 0.1 angstroms and a spring constant of $20 \text{ kcal/mol/angstrom}^2$, and
3. finally, the hydrogen bonded to the carbon at the possible site of metabolism is constrained to the oxygen atom at a distance of 2.0 ± 0.1 angstroms and a $20 \text{ kcal/mol/angstrom}^2$ spring constant.

As CYP2D6 forms a conserved salt bridge with the substrate with either glutamate 216 or aspartate 301 [Paine *et al.*, 2003], this was also incorporated as a constraint during the sampling stages. In the first sampling stage, this salt bridge is enforced by introducing a harmonic constraint of 3.0 ± 0.3 angstroms between the basic nitrogen of the substrate and each of the side chain oxygen atoms in GLU216, ASP301, and SER304 (see figure 4.6). The spring constants of these constraints

are 15.0, 8.0, and 4.0 kcal/mol/angstrom² for GLU216, ASP301, and SER304, respectively. Additionally, an angle constraint of 150.0 ± 30.0 degrees with spring constant of 5.0 kcal/mol/degree² is applied to each of the N-H-O angles. In the second sampling stage, four separate trajectories are calculated for each of the four carboxalate oxygens of GLU216, ASP301 (shown in figure 4.7). In each trajectory a constraint of 1.9 ± 0.1 angstroms is applied between the hydrogen attached to the basic substrate nitrogen and one of the four carboxalate oxygens. Additionally, the angle of the hydrogen bond is constrained to 168 ± 12 degrees, with a spring constant of 5.0 kcal/mol/degree².

Three different methods of sampling, or moves, were implemented in order to sample different protein-ligand conformations. A flow chart presenting the minimization Monte Carlo procedure is presented in figure 4.8.

1. Side chain motions seek to sample different conformations of the neighboring side chains and flexible moieties attached to a static constrained central core of the ligand. Because side chain conformations are locally highly correlated, neighboring side chains are sampled simultaneously. First, a number of side chains to be sampled is selected. For this study, the possible cluster sizes were from one to three residues, all with equal probability. Once a cluster size is selected, a single side chain is selected at random. Until the desired cluster size is reached, additional residues are selected, and rejected if the β carbon is greater than 6 angstroms from any of the β carbons of the residues already included in the cluster.

Once the side chains have been selected, three different moves are possible for each side chain:

- (a) rotamer library based moves,
- (b) random perturbations of a single dihedral, and
- (c) random perturbation of all dihedrals.

For 75% of side chains, the rotamers are replaced with rotamers drawn at random from a high resolution rotamer library. The rotamer states for protein residues is the same library used in other places in PLOP, constructed from a set high resolution protein X-ray crystal structures [Xiang and Honig, 2001]. The rotamers for the ligand are constructed by sampling rotatable bonds at 10 degree resolution and screening this set for steric clashes. In addition to the rotamer selected, a small amount of “noise”, which is random, and less than the resolution of the side chain library, is added to each rotamer.

For the remaining 25% of side chains, moves are distributed equally between perturbations of a single dihedral and perturbations of all dihedrals. The perturbation for each dihedral is constructed of both a small and a large perturbation. The small perturbation is chosen at random in the range $[-d,d]$, where d is the resolution of the side chain library, in this case 10 degrees. This term corresponds to the “noise” term in the case of rotamer moves. The large perturbation is a multiple of 60 degrees, which corresponds to the minima of both sp^2 and sp^3 carbons, though the null large perturbation is weighted more heavily. The effect of the union of these two terms is that dihedrals that are expected to yield higher acceptance probabilities are selected more frequently, though with the large perturbations this is balanced with large changes in conformation.

After a set of dihedral angles has been proposed for all residues in the cluster the atomic locations are updated. Finally, before testing for acceptance of the new coordinates a screen is applied. A reduced radius, 0.6x scaling factor, hard sphere overlap screening criteria is applied for all possible side chain moves before a possibly costly minimization step. If the proposed structure does not pass the screening stage another side chain step is performed to obtain a new proposed conformation.

2. Rigid body translation and rotation were also implemented for non-covalently linked moieties, such as ligands. Rigid body steps are implemented as a multiple time scale Monte Carlo simulation. In inner steps a more lenient screening criteria is applied, and only short range interactions are updated. An inner step consists of a translation and a rotation. For translations the ligand is translated a random distance between 0 and 0.5 angstroms in a direction is selected at random. Additionally, the ligand is allowed to rotate, this is accomplished by choosing another random vector through the center of mass, and rotating by an angle distributed at random between -60 and 60 degrees. Coupling rotations and translations allows sampling of concerted movement of the ligand. During inner steps the atomic radii are scaled by 0.7 when testing for atomic collisions. 1000 attempted inner steps are performed for each outer step. In the outer Monte Carlo step the full energy is calculated and the unscaled Lennard-Jones radii are used.

This multi-scale sampling method increases the ability to escape from tight spacial bottle-

necks. This increases the conformational freedom and therefore sampling of the inner steps, at a cost of decreasing the acceptance probability in the outer loop. However, because a minimization was performed before testing for acceptance the acceptance criteria for rigid body steps was still 0.1 to 0.4 depending on the size of the ligand. Each rigid MC step consisted of 1000 inner steps, and only one outer step, meaning that only one minimization occurred each time rigid body Monte Carlo was selected as the move step. The rigid body move usually takes 20 to 40 seconds per move.

3. The Hybrid Monte Carlo (HMC) [Duane *et al.*, 1987] step is a velocity verlet molecular dynamics simulation. This simulation allows all atoms in both the ligand and residues containing atoms within 5 angstroms of the ligand to move. Initial velocities are taken from a Maxwell-Boltzmann distribution at 900 K. Bonded and short range interactions are evaluated every 1 nanosecond inner time step, and long range potentials are assumed to be fixed over inner steps. Five inner steps compose each outer HMC step. In the outer step, the molecular surface, long range interactions, and Born alphas are updated before computing the energy and applying the Metropolis acceptance criteria at a temperature of 900 K after each molecular dynamics run. Taking up to 15 minutes per move, the HMC is the most expensive among all three types of moves in PLOP.

4.2.3 Evaluation

Both a parameterized and an unparameterized model were used to classify potential sites of metabolism. IDSite makes the assumptions that all intermediates before the rate determining step are at equilibrium [Wang *et al.*, 2007], that hydrogen abstraction is the rate limiting step for hydroxylation of aliphatic carbons, and electrophilic attack is the rate limiting step for hydroxylation of aromatic rings [Guengerich, 2001; Shaik *et al.*, 2005]. With these assumptions, the rate of metabolism at each possible site of reaction is affected by the free energy of binding in order to put that site in the site of reaction, as well as the free energy barrier of rate determining step, or

$$\Delta G_{\text{total}} = \Delta G_{\text{binding}} + \Delta G_{\text{barrier}} \quad (4.2)$$

The $\Delta G_{\text{binding}}$ above is calculated using a PLOP evaluation of the refined pose. The intrinsic reactivity for the system is computed from density functional theory calculations on a simplified system,

replacing the heme with a methoxy radical, and using a linear relationship between $IR(\text{heme})$ and $IR(\text{methoxy radical})$ to estimate the true reactivity for the heme system. This linear relationship is illustrated in 4.9.

Model compound	Site of Metabolism	Heme model (kcal/mol)	Methoxy model (kcal/mol)
Benzene		20.51	8.66
Anisole	Ortho-	16.29	5.31
	Meta-	18.76	8.55
	Para-	16.01	4.71
	Beta-	16.18	6.63
Dimethylether		15.03	3.9
Dimethylanisole	Meta-	16.54	6.07
	Para-	17.51	7.35
Ethane		21.58	10.37
Ethanol	1	12.73	2.36
	2	17.35	6.9
Propane		18.31	7.8
Toluene	Ortho-	17.15	6.22
	Meta-	18.86	8.27
	Para-	18	7.58
	Alpha-	15.72	5.16
t-Butylbenzene	Beta-	20.82	9.33

Table 4.2: Quantum mechanics (density functional theory) calculated values for internal reactivity of various compounds with either methoxy radical (compound I) or heme system. Correlation between these values is illustrated in Figure 4.9.

$$IR(\text{heme}) = 1.117 * IR(\text{methoxy radical}) + C \quad (4.3)$$

Since this constant C is identical for each state, it has no effect on the relative differences in ΔG_{site} or the relative rate of metabolism at possible sites.

$$E = \langle 1.117 * IR(\text{methoxyradical}) + C + E_{\text{TS}} \rangle - kT \ln(N_H) \quad (4.4)$$

Since the ligand is forced to assume a different conformation in order to react, the energy of this transition state conformation, E_{TS} , is also computed using PLOP. As the relative abundance of different metabolites is determined by differences in ΔG per site rather than absolute reactivities, the constant in equation 4.4 does not affect which metabolites are produced. A site of possible metabolism is classified as positive if it is observed in greater than 0.1% yield, which corresponds to a $\Delta\Delta G$ of ~ 4.75 kcal/mol between the most favored state and the cutoff for negative predictions.

The second classifier is similar however:

1. a different constant is used to estimate $IR(\text{heme})$ from $IR(\text{methoxy radical})$, namely 1.071,
2. if the binding energy of the transition state complex of a pose is within 5.26 kcal/mol of the lowest pose, it is set to the binding energy of the lowest pose. Otherwise the difference is scaled by 0.58,
3. and the cutoff for an active prediction is changed from 4.75 kcal/mol to 1.46 kcal/mol.

These parameters were decided upon by maximizing the positive predictive value of the model, defined as $\frac{\text{true positives}}{(\text{false positives} + \text{true positives})}$, on a training set of 36 compounds [Altman and Bland, 1994].

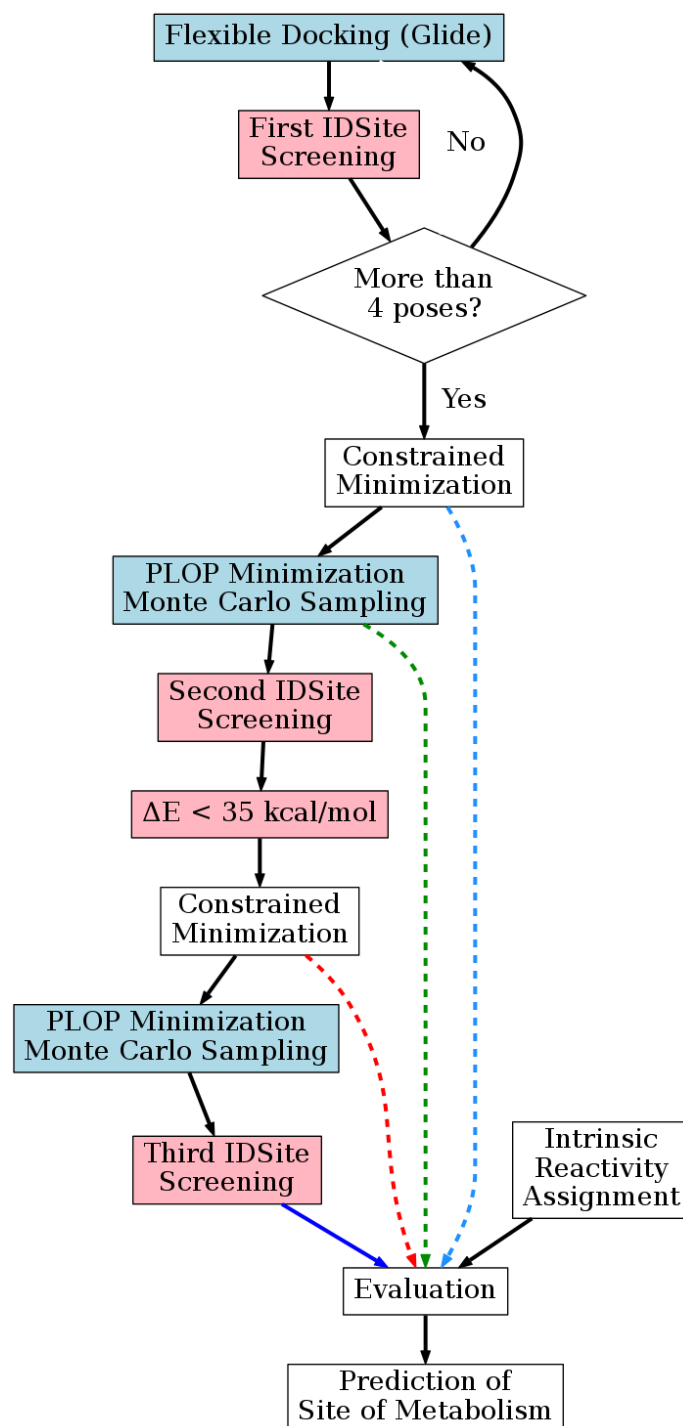


Figure 4.2: An overview of the entire IDSite procedure. The dotted lines represent abbreviated versions of the full procedure. Receiver operating characteristic graphs for the full version, and these abbreviated versions, are presented in 4.13. Series colors on ROC graphs correspond to arrow colors here.

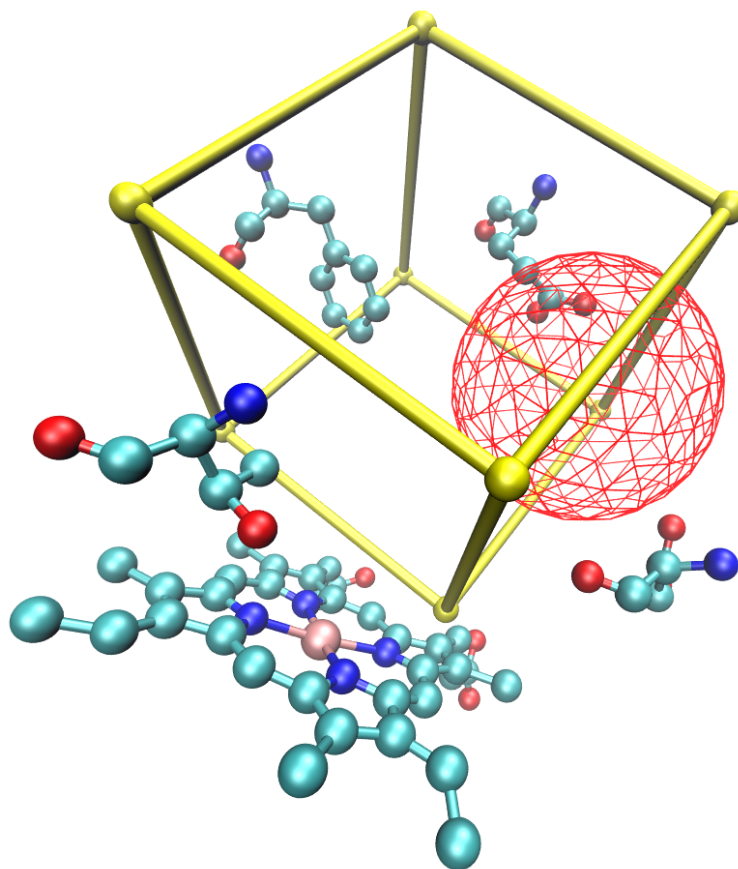


Figure 4.3: The bounding box used by Glide in order to generate the initial set of docked poses. The docking procedure also requires at least one hydrogen bond donor be found within 4 angstroms of the centroid of Glu216, Asp301, and Ser304 is also shown. The sphere representing this constraint is also shown.

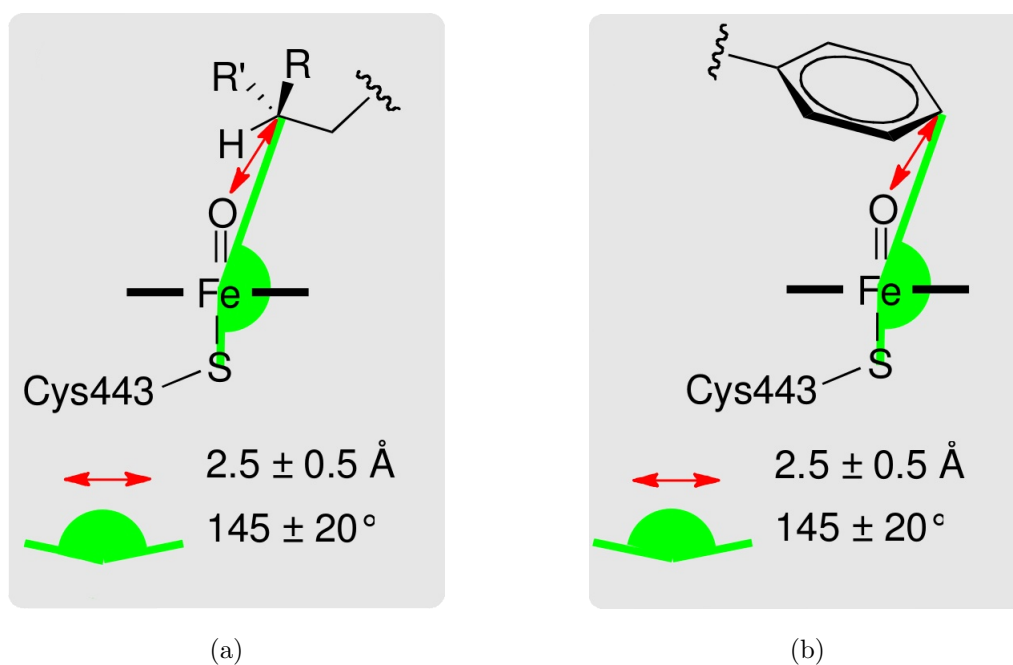


Figure 4.4: The constraints applied to (a) sp^3 and (b) sp^2 atoms during the constrained minimization and first minimization Monte Carlo sampling stage. The spring constant of the bond constraint (red arrow) is $100 \text{ kcal/mol/angstrom}^2$, and that of the angle constraint is $25 \text{ kcal/mol/degree}^2$. The oxygen atom depicted in this figure is a “dummy” atom and does not interact with any other atoms in the structure except through the constraint.

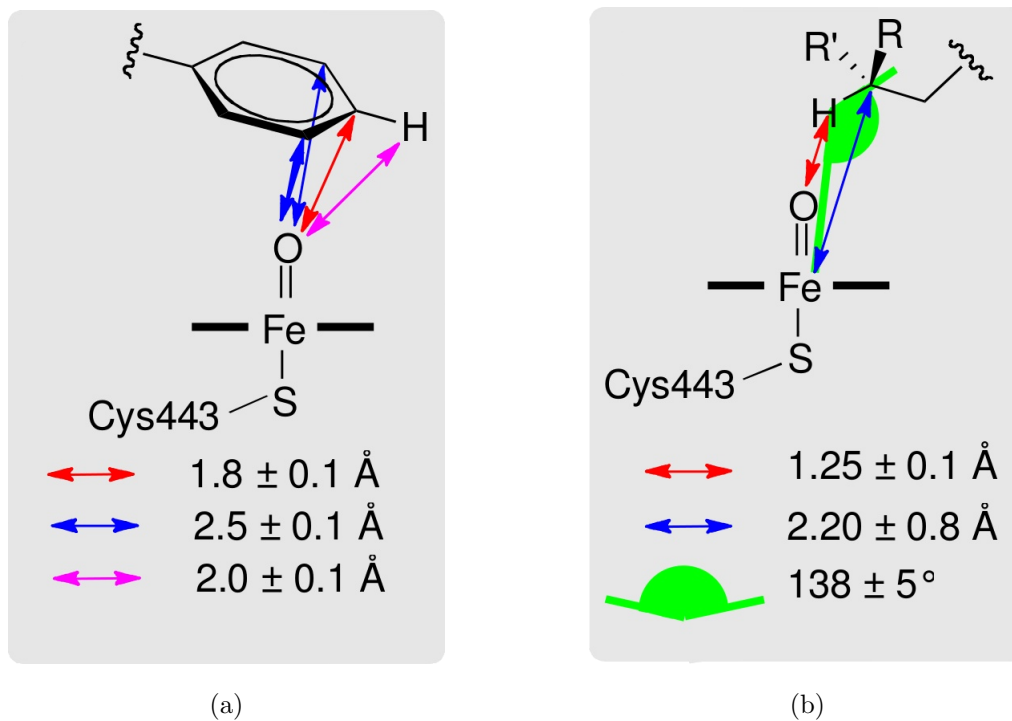


Figure 4.5: The constraints applied to (a) sp^2 and (b) sp^3 atoms during the constrained minimization and second minimization Monte Carlo sampling stage.

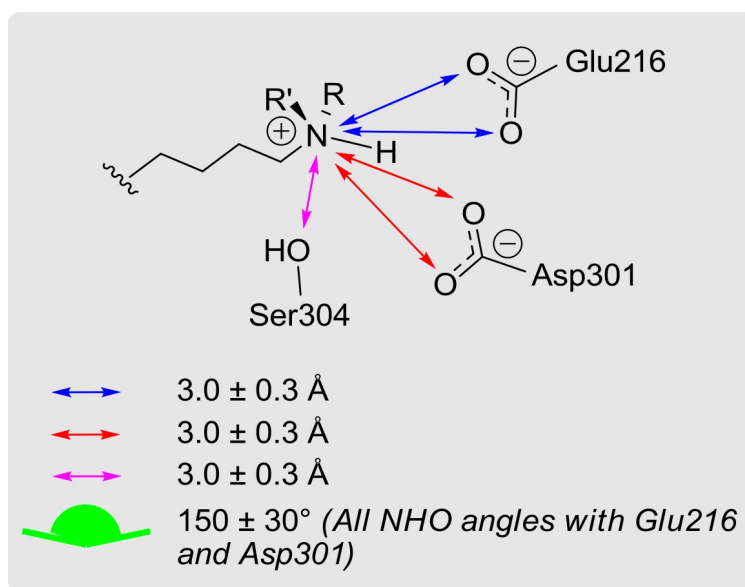


Figure 4.6: The constraints applied to the salt bridge region of CYP2D6 during the *first* minimization Monte Carlo sampling stage.

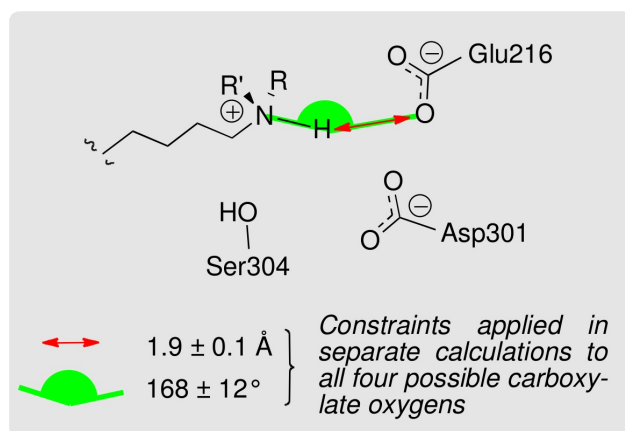


Figure 4.7: The constraints applied to the salt bridge region of CYP2D6 during the *second* minimization Monte Carlo sampling stage.

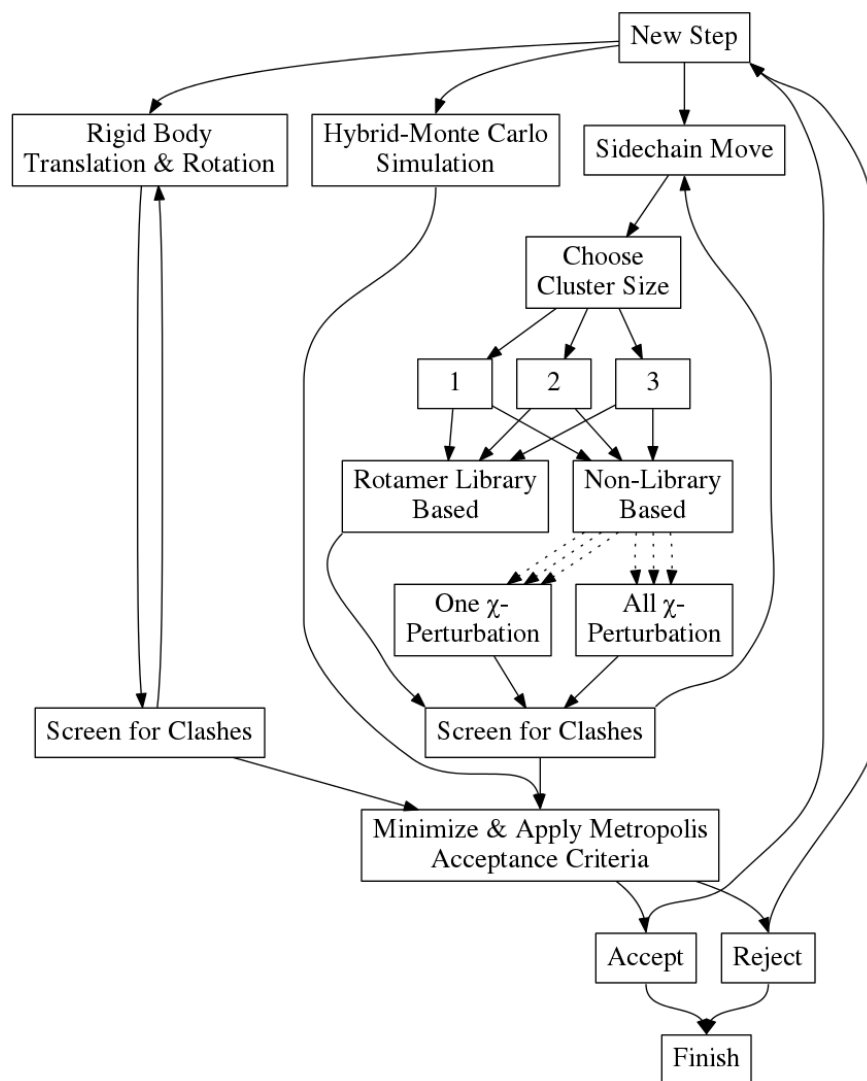


Figure 4.8: An outline of the Monte Carlo minimization refinement stages in PLOP.

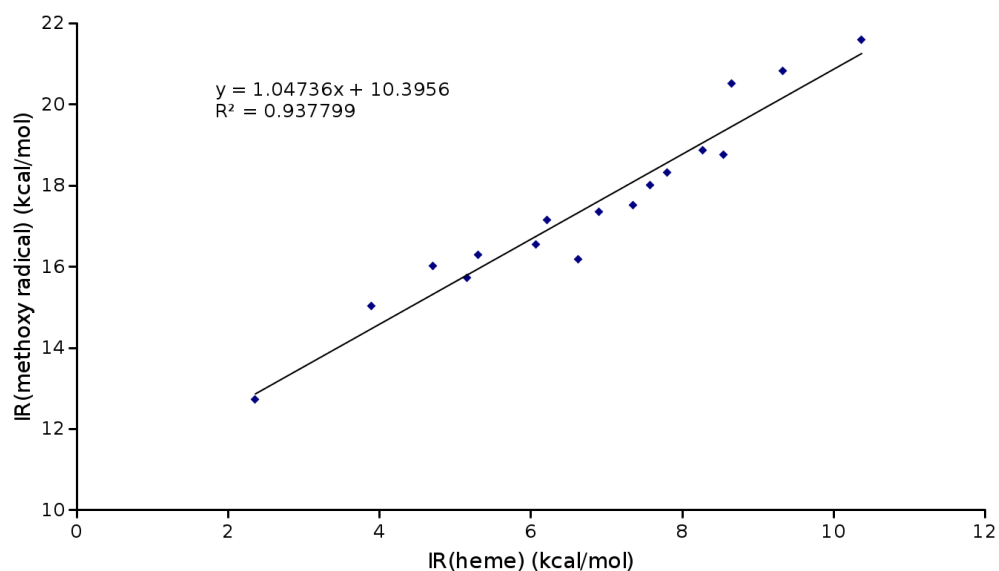


Figure 4.9: The linear relationship between the calculated intrinsic reactivity of the methoxy radical complex and that of the heme complex. Adapted from [Li *et al.*, 2011b] with minor correction. In the original manuscript, the slope of the regression was reported as 1.117 and that number was used throughout. This difference should not significantly affect the physical IDSite classifier results, and does not affect the results of the fit model. In the rest of this text, the value from the original publication of 1.117 will be used. The values used in this plot are presented in table 4.2.

4.3 Results

Both physical and fitted IDSite were able to achieve promising results predicting CYP2D6 sites of metabolism. The physical model correctly identified 68 of 82 active sites of metabolism for a sensitivity of 0.829. For inactive sites this model correctly identified 1054 of 1075 inactive sites with a specificity of 0.980. The fit model performed similarly, and even slightly better, identifying 52 of 57 sites of metabolism (sensitivity of 0.912) in the training set and 25 of 25 in the test set (sensitivity of 1.0).

$$\text{specificity} = \frac{TN}{TN + FP} = \frac{\# \text{ of true sites of non-metabolism identified}}{\# \text{ experimental sites of non-metabolism}} \quad (4.5)$$

$$\text{sensitivity} = \frac{TP}{TP + FN} = \frac{\# \text{ of true sites of metabolism identified}}{\# \text{ experimental sites of metabolism}} \quad (4.6)$$

The fit model also correctly identified 709 of 717 inactive sites in the training set (specificity of 0.989) and 352 of 358 inactive sites in the test set (specificity of 0.983). As the performance of the fit model is similar to that of the physical model, it does not appear that the fit model is over-parametrized to the training set. Specific results for both models are presented in Tables 4.3 and 4.4, and the specific sites identified by both models as well as experiments are illustrated in Figures 4.11 and 4.12.

We believe that the parameters help account for some degree of noise in the molecular mechanics calculations. The scaling of the binding energy difference, either to zero inside a window about the minimum energy pose, or by a factor of 0.58 decreases the relative weight of the molecular mechanics contribution relative the quantum contribution to the classifier. This might imply that some sites are not being classified as active because they are not in the lowest energy conformation around the docked pose, suggesting that additional molecular mechanics sampling might further improve results. However, as will be discussed later, the molecular mechanics stage already dominates the total time necessary for an IDSite prediction, and the current molecular mechanics procedure takes about 450 hours.

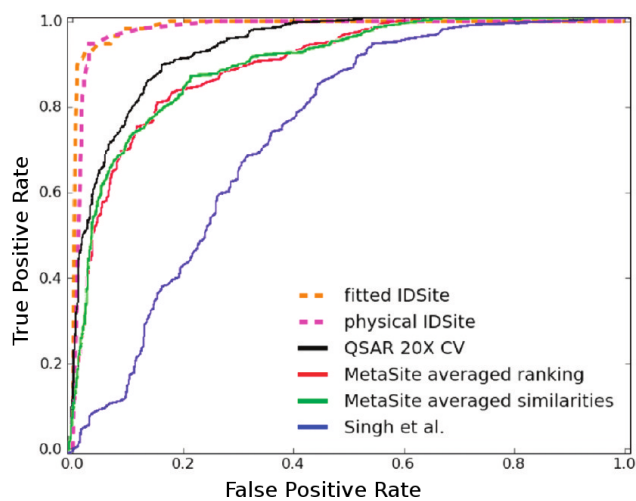


Figure 4.10: A comparison of the performance of IDSite with a variety of other methods of predicting P450 sites of metabolism. IDSite obtains the best performance, followed by a quantitative structure-activity relationship based method [Sheridan *et al.*, 2007]. Adapted from [Sheridan *et al.*, 2007].

Compound #	Compound	Physical			Fitted		
		TP	FP	FN	TP	FP	FN
1	4-methoxyamphetamine	1	0	0	1	0	0
2	Amitriptyline	2	2	0	2	0	0
3	Aprindine	4	0	1	5	0	0
4	Brofaromine	1	0	0	1	0	0
5	Bufuralol	0	1	1	1	0	0
6	Carvedilol	1	0	2	2	0	1
7	Cinnarizine	0	2	1	0	2	1
8	Clomipramine	1	0	1	1	0	1
9	Codeine	1	0	0	1	0	0
10	Desipramine	2	0	0	2	0	0
11	Dextromethorphan	1	0	0	1	0	0
12	Dihydrocodeine	1	1	0	1	0	0
13	Ethylmorphine	1	0	0	1	0	0
14	Flunarizine	1	0	0	1	0	0
15	Fluperlapine	1	0	0	1	0	0
16	Hydrocodone	1	0	0	1	0	0
17	Imipramine	2	0	0	2	0	0
18	Indoramine	1	0	0	1	0	0
19	MDMA	1	0	0	1	0	0
20	Methamphetamine	1	0	0	1	2	0
21	Methoxyphenamine	2	0	0	2	0	0
22	Metoprolol	1	0	1	2	0	0
23	Mexiletine	2	0	1	2	0	1
24	Mianserin	1	0	0	1	0	0
25	Mirtazapine	0	1	1	1	1	0
26	Nortriptyline	1	1	0	1	0	0
27	Ondansetron	2	0	0	1	0	1
28	Paroxetine	1	0	0	1	0	0
29	Perhexiline	2	0	0	2	0	0
30	Propafenone	1	1	0	1	1	0
31	Propranolol	2	2	0	2	1	0
32	Tamoxifen	1	0	0	1	0	0
33	Terfenadine	3	0	0	3	0	0
34	Tiracizine	1	2	0	1	1	0
35	Tropisetron	2	0	1	3	0	0
36	Venlafaxine	1	0	0	1	0	0
	Total	47	13	10	52	8	5

Table 4.3: Results of physical and fitted IDSite on training set of 36 compounds.

Compound #	Compound	Physical			Fitted		
		TP	FP	FN	TP	FP	FN
37	Atomoxetine	0	1	1	1	2	0
38	Bicifadine	1	2	0	1	0	0
39	Bupranolol	1	0	0	1	0	0
40	Carteolol	1	1	0	1	0	0
41	Chlorpromazine	1	0	0	1	0	0
42	EMAMC	1	0	0	1	0	0
43	Encainide	1	1	0	1	1	0
44	Harmaline	1	0	0	1	0	0
45	Harmine	1	1	0	1	1	0
46	Ibogaine	1	0	0	1	0	0
47	MAMC	1	0	0	1	0	0
48	MMAMC	1	0	0	1	0	0
49	MOPPP	1	0	0	1	0	0
50	Oxycodone	1	0	0	1	0	0
51	Spirosulfonamide	2	0	0	2	0	0
52	Timolol	2	0	2	4	0	0
53	Tolterodine	0	1	1	1	1	0
54	Tramadol	1	1	0	1	1	0
55	Tyramine	2	0	0	2	0	0
56	Zotepine	1	0	0	1	0	0
	Total	21	8	4	25	6	0

Table 4.4: Results of physical and fitted IDSite on a test set of 20 compounds. Note that for the physical model there is no training performed, so results in the text are presented in a unified fashion for the training and test set.

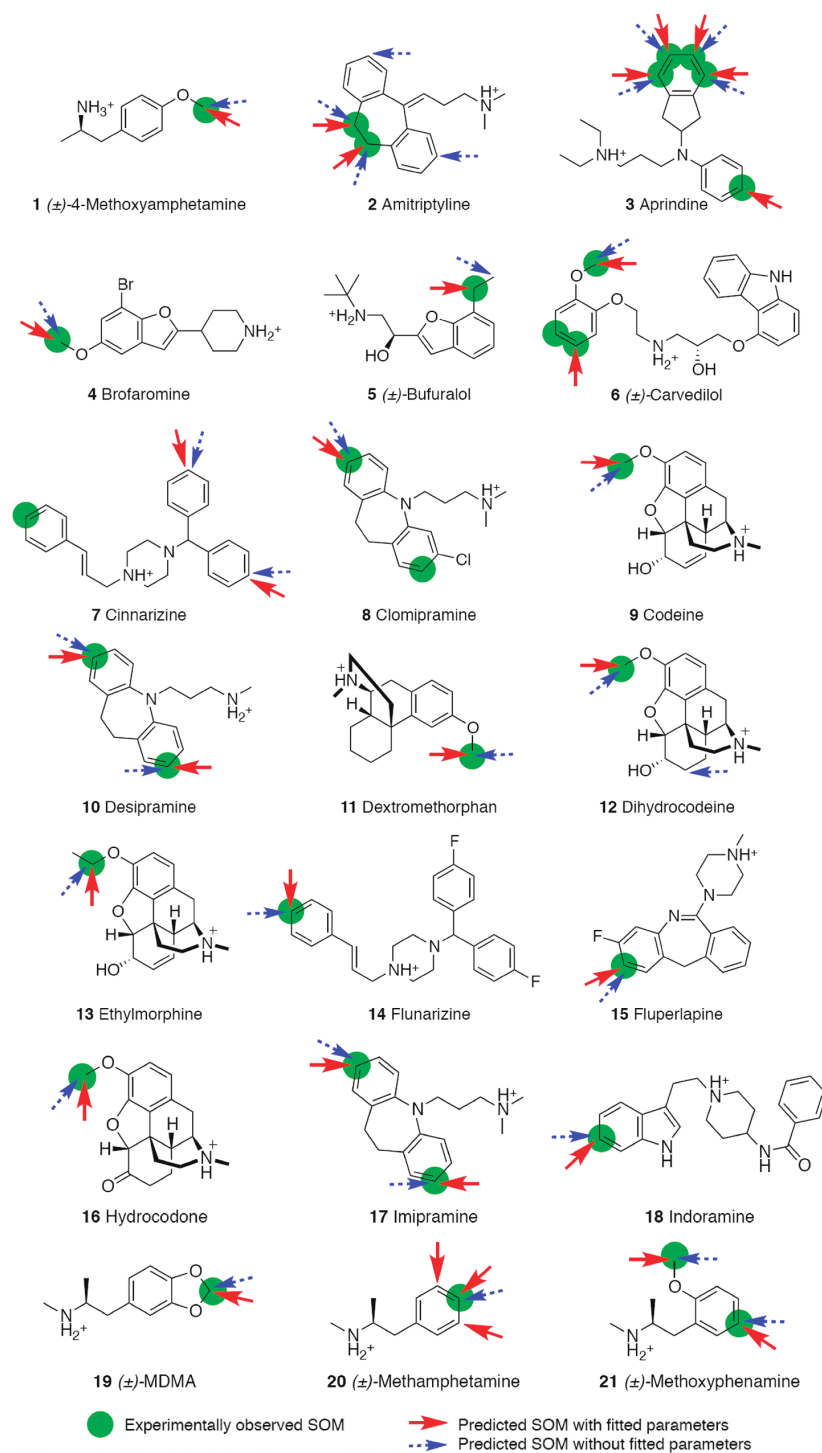


Figure 4.11: Physical and fitted IDSite predictions of sites of metabolism on the training set.

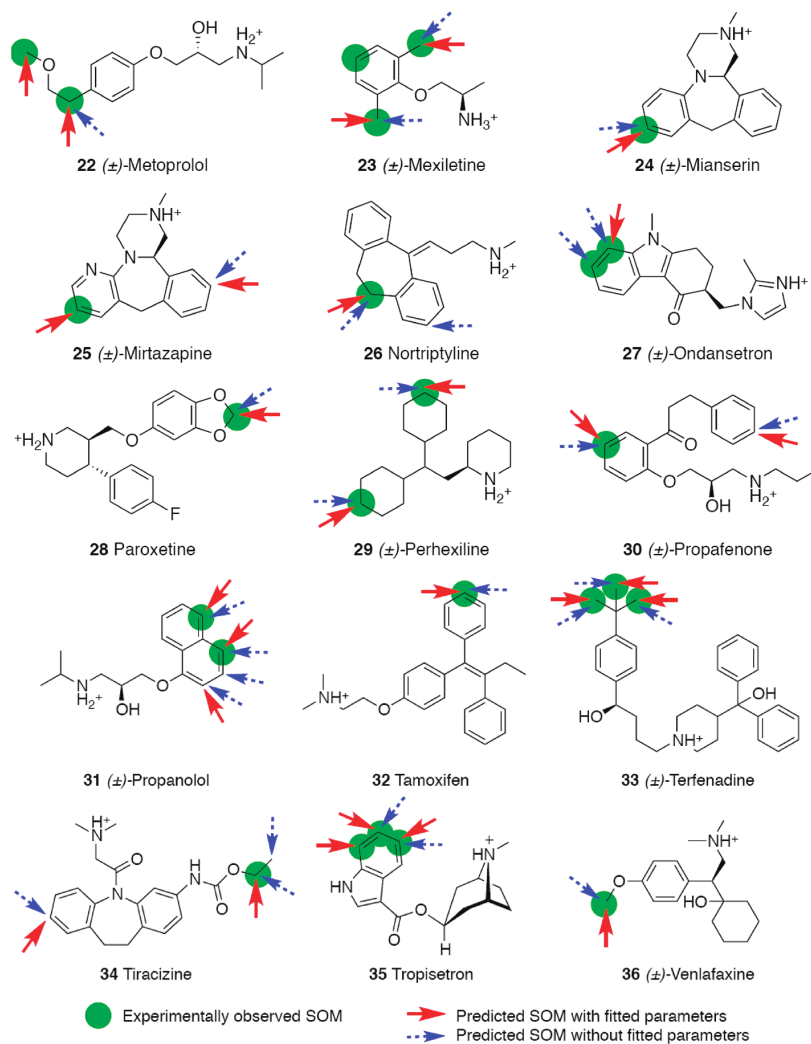


Figure 4.11: (continued)

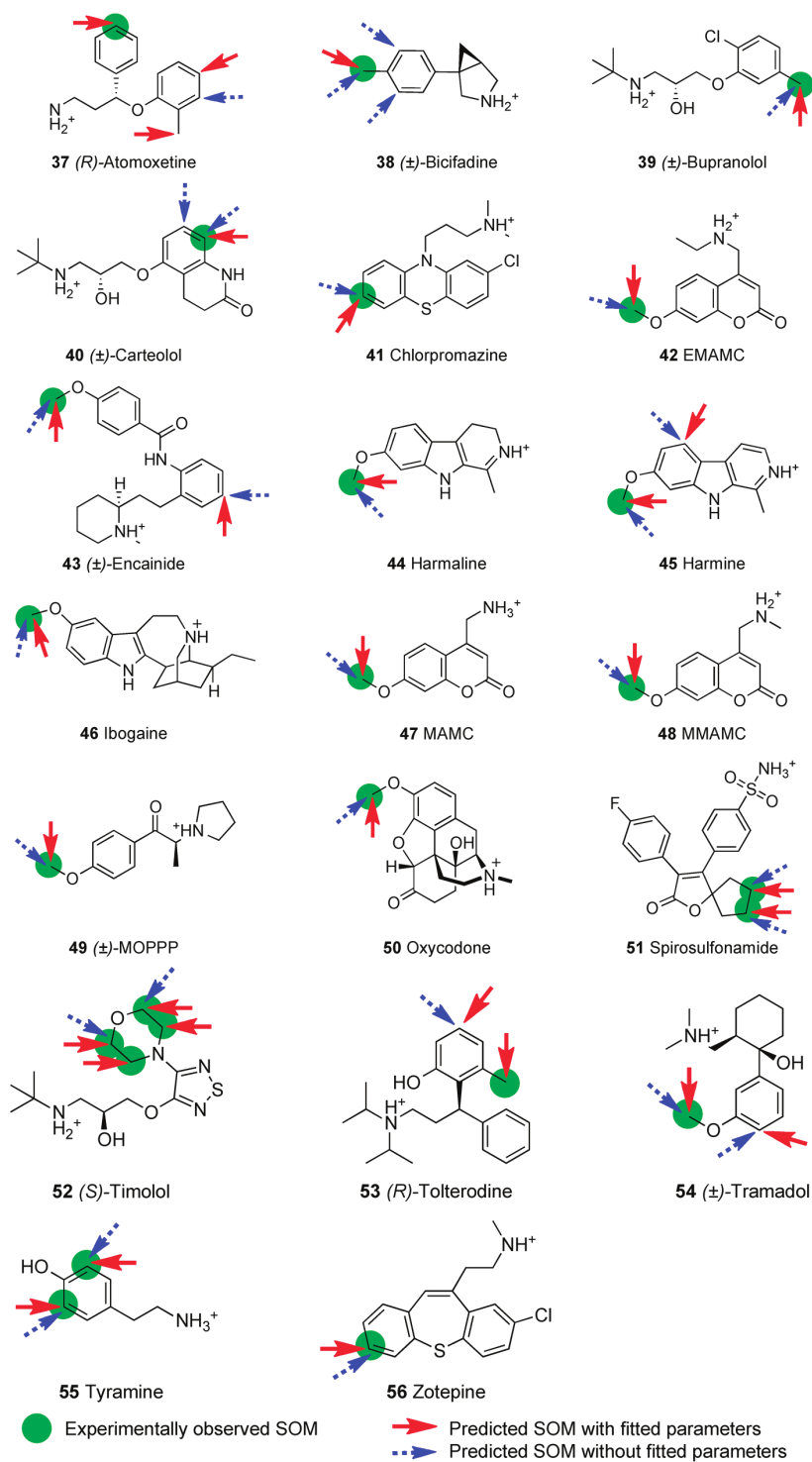


Figure 4.12: Physical and fitted IDSite predictions of sites of metabolism on the test set.

4.4 Discussion

The physical score performs nearly as well as the fitted score, indicating that the model is not over-fit to the training set. Figure 4.10 presents a comparison of both IDSite models (physical and fit) to a number of other methods for prediction of P450 sites of metabolism. Though the data sets used are not identical, they are similar, and overlapping for some compounds. At all sensitivities, IDSite is clearly the best performing protocol, with the physical and fit model performing very nearly identically.

At 90% sensitivity, the QSAR method included roughly 20% false positives, and MetaSite included 40% false positives. At the same sensitivity, IDSite has a ~1% false positives rate. This represents a significant improvement in prediction accuracy that has the potential to have a large impact on drug development.

4.4.1 Significance of Sampling Stages

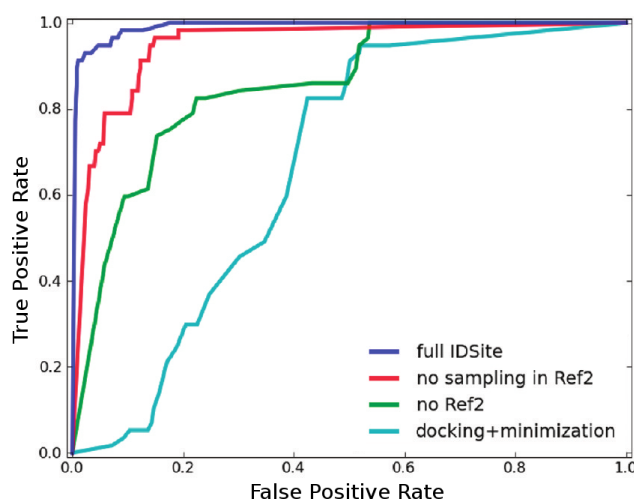


Figure 4.13: The effect of additional sampling on prediction of site of metabolism by P450. The light blue series describes only performing the initial Glide docking stage followed by minimization. The green series is obtained by using the set of structures obtained in the first minimization Monte Carlo sampling stage. The red series is obtained by screening the structures obtained in the first sampling stage, and minimizing these structures using the constraints specified in Figures 4.5a and 4.5b. The blue series makes use of the entire IDSite procedure. The color scheme of these series corresponds to the colors of edges in Figure 4.2.

As shown in Figure 4.13, at a given specificity, sensitivity increases with additional sampling. For some compounds, either no conformations predicted via the docking procedure have the site of metabolism is sufficiently close to the heme oxygen to react or the Glide scoring metric prefers conformations that are far from the reactive site over more reactive conformations. In these cases the sampling stages and constraints are useful in guiding initial docked poses into more active conformations.

The trajectory followed during the minimization Monte Carlo sampling stages differs appreciably for small and large ligands. For smaller ligands, in many cases the poses after the first constrained minimization were among the lowest energy poses explored. For about 40% of these small ligands (24/56) the same predictions were obtained after the first minimization Monte Carlo sampling stage as after the full IDSite procedure. However, for large ligands, the constraints and sampling continued to decrease the energy over the course of the sampling stages, indicating that especially for large compounds the second refinement stage is critical to obtaining accurate predictions.

4.4.2 Induced Fit Effects

The catalytic site of cytochrome P450 is flexible, changing conformation to accommodate a large variety of different substrates [Li *et al.*, 2004; Scott *et al.*, 2004]. The Monte Carlo sampling stages reflect this flexibility, allowing the substrate to settle into the active site, guided by harmonic constraints, and allows the side chains to alter their conformations to better accommodate and interact with the ligand. Side chain dihedrals were found to change less for smaller ligands, illustrating that less rearrangement of the active site was necessary in order to bind the substrate in an active conformation. In larger ligands, some side chain dihedrals changed by larger amounts, especially for bulkier amino acids such as phenylalanine, with Phe120 and Phe483 changing by up to 40 and 60 degrees respectively for some ligands.

4.4.3 Balancing Structural Contribution and Reactivity

As reflected in equation 4.2, the binding energy and intrinsic reactivity are the determining factors in predicting sites of metabolism. The IDSite procedure evaluates and uses both of these quantities in classifying possible sites of metabolism. The combination of structural information and information about the intrinsic reactivity of a site allows IDSite to correctly predict cases which would not be

possible using either of these methods alone.

One case of such a prediction is nortriptyline (ligand 26), since the two sites on the seven-membered aliphatic ring are difficult to distinguish only with their intrinsic reactivity, as they are almost equally reactive. However, experiments show that only the (E)-10 site of nortriptyline is metabolized [Linnet and Olesen, 1997]. IDSite ranks the poses with the (Z)-10 site close to the ferryl oxygen at least 10 kcal/mol higher in energy than those with the (E)-10 atom close to the ferryl oxygen. This is a sufficiently large energy gap for IDSite to correctly predict the (E)-isomer as the only metabolite. While structural effects are clearly very important to determine nortriptylene's site of metabolism, the intrinsic reactivities also play a key role. A purely structure based method would have predicted an aromatic hydroxylation due to the favorable energy of the corresponding poses. It is only through the combination of information about binding energy and intrinsic reactivity that IDSite is able to correctly predict these sites of metabolism.

Another case illustrating the importance of balancing reactivity and structural information is brofaromine (ligand 4). Experiments show that the major site of metabolism by CYP2D6 is O-demethylation [Feifel *et al.*, 1993]. The intrinsic reactivity of the site of metabolism (4.7 kcal/mol) is very close to those of sites on the aromatic rings (non-sites of metabolism, 3.3 to 4.9 kcal/mol). Due to the receptor geometry, it is impossible for the atoms on the furan ring to get close to the ferryl oxygen while still attaining the salt bridge with either Glu216 or Asp301. Therefore, no poses that would have predicted reaction on the furan ring satisfied the geometric constraints, and those sites were correctly predicted as sites of non-metabolism. Although poses that satisfied the geometric constraints and placed the benzene ring in the metabolic site were generated, these poses are strongly disfavored energetically by more than 20 kcal/mol. This indicates that taking the interactions between the ligand and the receptor into account, IDSite is able to make the prediction of the site of metabolism for brofaromine in good agreement with the experimental observation.

Chapter 5

Other Improvements in the Protein Local Optimization Program

5.1 Regression Testing

A number of common problems inherent in large programming projects are identifying bugs or regressions. A common means of minimizing the cost of maintaining a project is regression testing. Regression testing identifies a number of test cases that ideally provide good coverage of the code base and are able to identify changes that may have adversely affected established features of the program [Wong *et al.*, 1997]. When testing is performed continuously, the time spent testing can account for as much as half of all development time [Leung and White, 1989]. However, in cases where this testing is neglected, large software projects can easily reach the point where it is no longer feasible to repair or reverse-engineer [Weide *et al.*, 1995].

We have implemented a regression testing framework for the Protein Local Optimization Program with the goal of making the program more maintainable. Using this tool, after each modification, a number of tests are performed and the results are compared with results from a known reference. All of this is performed in parallel, and without the users' interaction. This allows good code coverage without requiring an unwieldy amount of time. The regression testing framework implemented here is outlined in figure 5.1.

Tests can be submitted as new features are developed, hopefully maintaining good code coverage. Each test file contains three pieces of information:

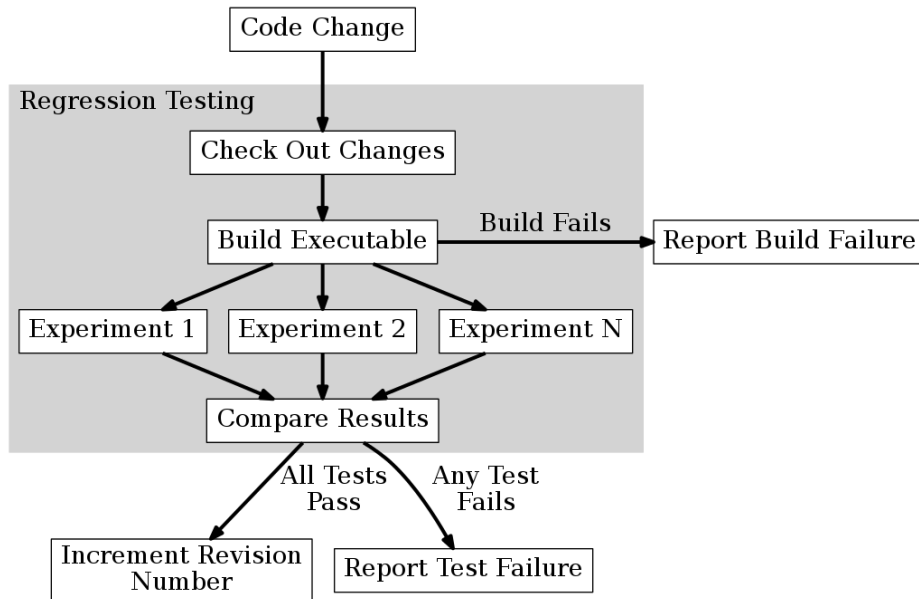


Figure 5.1: An overview of the regression testing procedure.

1. an experiment to be run,
2. a set of regular expressions matching the values to be compared between code changes,
3. for numeric comparisons, a threshold, or tolerance for change in a test before it is classified as a failure.

These test files are submitted to the version control system (e.g. CVS, subversion, Mercurial, git...). The regression tester checks out a copy of these tests along with the code necessary to build an executable. The executable is then built, logging any warnings or errors, and assuming the build is successful, the experiments described in the test files are run, saving all output files in an archive. The results for each test, identified using a set of patterns in the test file, are then compared to the last “passing” set of results in the archive. If there is no old copy of a result, as in the case of testing a new feature, that test is assumed to pass. PLOP uses the major.minor.revision numbering scheme, and after a set of tests runs to completion and successfully reproduces the previous results, within the threshold, the revision number is changed. If a qualitative change in behavior is desired, i.e. larger than the threshold defined by the test files, incrementing the version number will allow the test to pass. This guarantees that results between two different revisions differ by no more than the threshold for each of the tests specified, which theoretically allows one

to reproduce results simply by using the same major and minor version.

At the present, functionality tested includes: loop predictions, side chain prediction, truncated Newton minimization, a number of different energy functions, protonation state prediction, the minimization Monte Carlo sampling procedure described in 4.2.2, loading from sequence, retrieval of structures from the PDB, the knowledge based dihedral potential described in 5.4, and every space group present in the PDB as of 2012. An approximate performance based comparison is also included, which ensures that the time required for each test increases by no more than 10% between successive revisions.

Presently 159 tests are performed, which takes about three hours. However, because the tests can be performed in parallel with a sufficient number of CPUs available, the time is limited by the execution of the longest experiment. Using an eight core machine, the total execution time for these tests is about 20 minutes.

Although in a perfect world all changes would be tested thoroughly enough such that they have no adverse affects on disparate parts of the program, this is not possible due to practical considerations. This allows developers to spend their time working on relevant and related parts of code, and leave tedious and slow, but important, testing to an automated process.

This was originally implemented because fragmentation had led to an inability to reproduce results between different versions of the program. By performing a scan over time the specific changes that caused the difference between two versions were able to be quickly identified and addressed. Performing the same task by hand might have required reviewing all the code changes over a three year period, an unreasonably time-consuming project.

5.2 Perturbed Minimization

The specific minimization method used in PLOP is a modified version of the TNPACK, truncated Newton minimization package [Schlick and Fogelson, 1992a; Schlick and Fogelson, 1992b]. This has been shown to have very good performance on high dimensional minimization problems. However, as the energy surface of large structures is very rough, having a large number of local minima, simple minimization methods tend to be sensitive to initial conformations, and occasionally small changes in starting conformations, or internal parameters can have a large effect on the final minimized en-

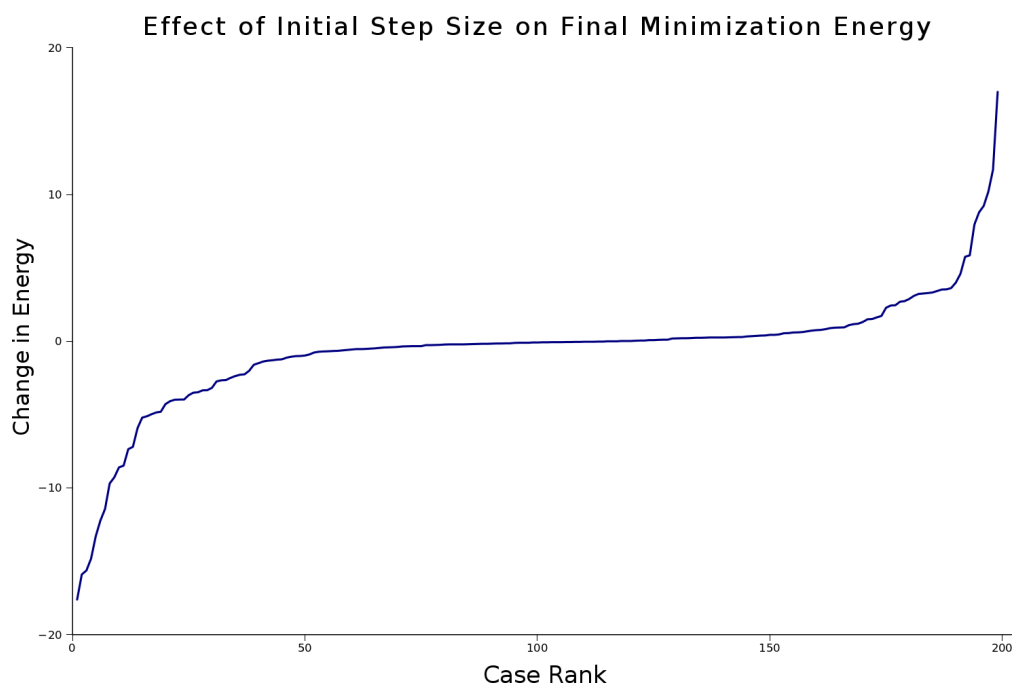


Figure 5.2: The effect of varying the initial step size in the line search step of the minimization. Theoretically this should have little to no effect on the result of the minimization, as the ideal step size would be constant. It was found that that was not the case, though the effect of the initial guess in the line search seems random. The cases which were effected to the greatest and least degree showed significant differences in final minimized energy.

ergy, 5.2. One such approach to many minima problems has been simulated annealing [Kirkpatrick *et al.*, 1983; Černý, 1985]. However, this performs significantly more sampling, and this requires appreciably more time than a simple minimization method. Therefore, it is desirable to find a minimization method, which is both somewhat tolerant to local minima and insensitive to initial conformations and quickly arrives upon a solution. We have performed some initial experiments with such a method.

Our method, perturbed minimization, combines iterative minimization from slightly varied starting conformations with an adaptive stopping rule in order to minimize time spent sampling after finding a reasonably minimum. The stopping rule, inspired by solutions to the classic game theory secretary problem balances continuing to sample while new lower energy conformations are likely to be found, with efficient stopping after sampling ceases to be productive [Freeman, 1983; Chow *et al.*, 1964].

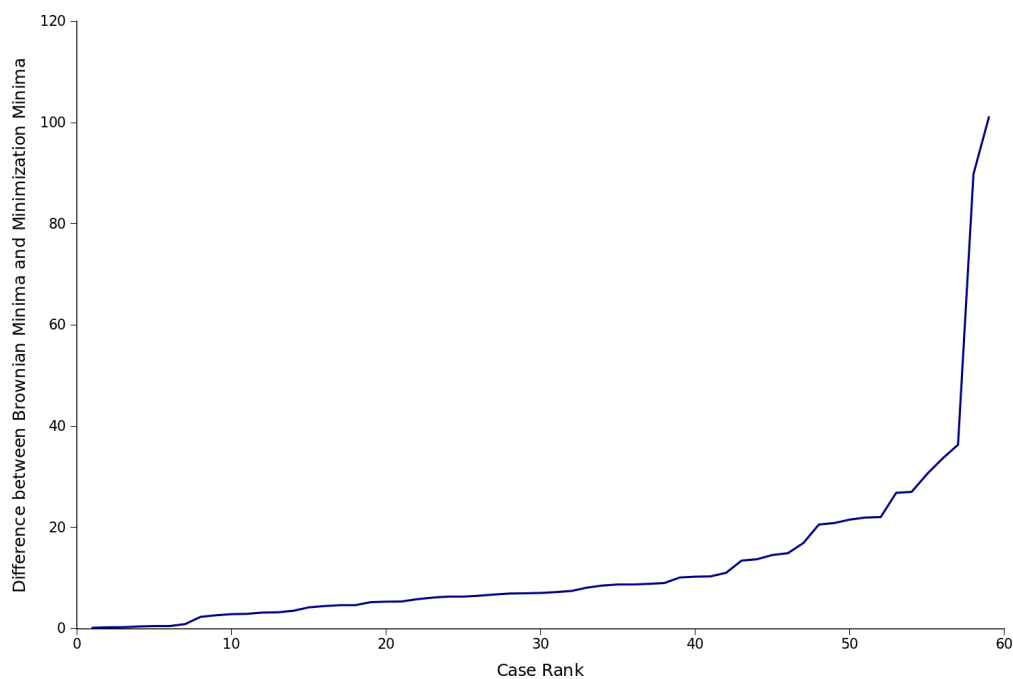


Figure 5.3: The difference in minimized energy using the TNPACK truncated newton minimizer and the perturbed sampling routine on a set of 60 small, less than 100 residue, peptides, ordered by rank. Though for a majority of cases the difference in final energy between a single minimization and multiple perturbed minimization is less than 10 kcal/mol, for approximately 10% of cases the energy is much higher, up to 60 kcal/mol, far more than the claimed resolution of the current energy model implemented in PLOP.

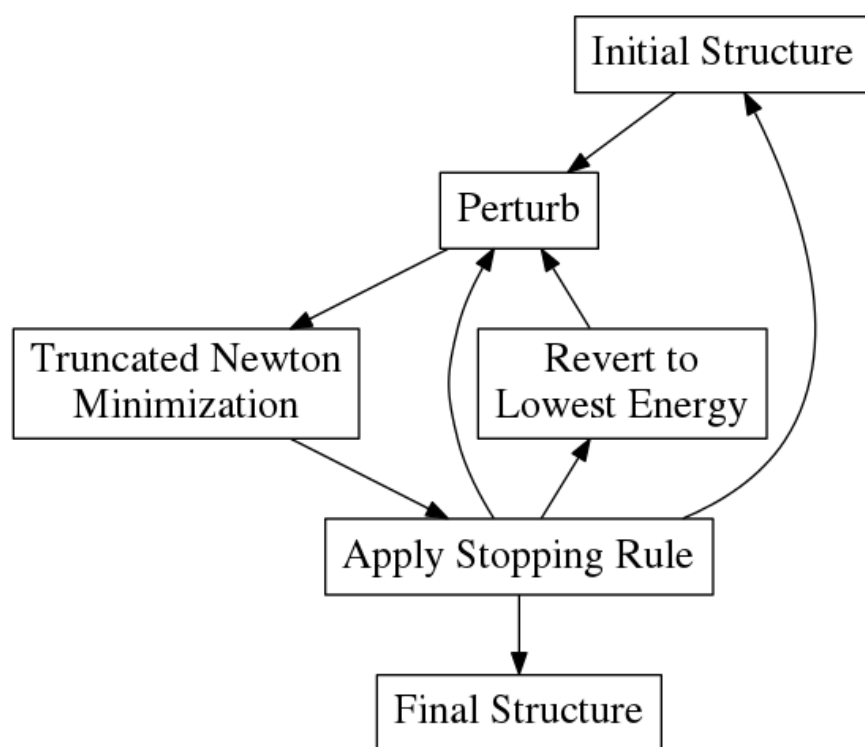


Figure 5.4

Specifically, the initial structure is perturbed before applying any minimization. The new perturbed structure is minimized, and if the energy is lower than the current best energy the new low energy conformation along with the energy are saved. The structure is then reverted to its initial conformation and the procedure is repeated until more than a specified number of steps are performed without finding a new lowest energy structure. This is effectively the successive non-candidate heuristic solution to the secretary problem applied in reverse (as in this case it is possible to “call back” a candidate). This procedure is described graphically in 5.4 This method has been applied to a number of test sets with promising initial results, though it is not clear at the moment in which circumstances this sort of sampling may be necessary.

5.3 Small Molecule Library

PLOP was originally developed to predict protein side chain and loop conformations. Many of the studies performed using this program have explicitly avoided cases in which proteins were near metal ions or ligands. Generally, cases in which a charged ligand is closer than 6.5 angstroms, or a neutral ligand is closer than 4 angstroms is rejected [Goldfeld *et al.*, 2011; Miller *et al.*, 2013]. This is partially because energy models have been trained using proteins, but also because for many ligands parameters for the various terms of the energy function are not available. At present, the PDB contains 15,612 small molecule ligands, and previous experiments have used a limited library of 4,321 small molecules. Additional ligands have either been

1. removed from structures, under the assumption that they are sufficiently far from the region of interest so as to not affect the prediction (because cases with nearby ligands are filtered in an earlier stage), or
2. had parameters assigned through a process requiring much intervention on a per case basis.

In order to improve upon this situation we have created a library of parameters for 11,837 small molecule ligands (for the remainder parameters could not be assigned either due to OPLS-AA lacking parameters or technical reasons). Protonation states and tautomers for each ligand were identified using Schrodinger’s epik. Parameters for bonded and non-bonded terms were assigned using the OPLS 2005 force field using Macromodel. A static ligand “core” is identified and groups

with rotatable bonds attached to this core are identified. For these flexible groups, a rotamer library is constructed at a uniform resolution of 10 degrees to allow sampling of flexible ligands. Using these rotamer libraries, it is possible to sample conformations of flexible ligands by treating flexible groups in a fashion similar protein side chains. However, due to lack of experimental conformations, these rotamer libraries are not knowledge based as in the case of protein side chains.

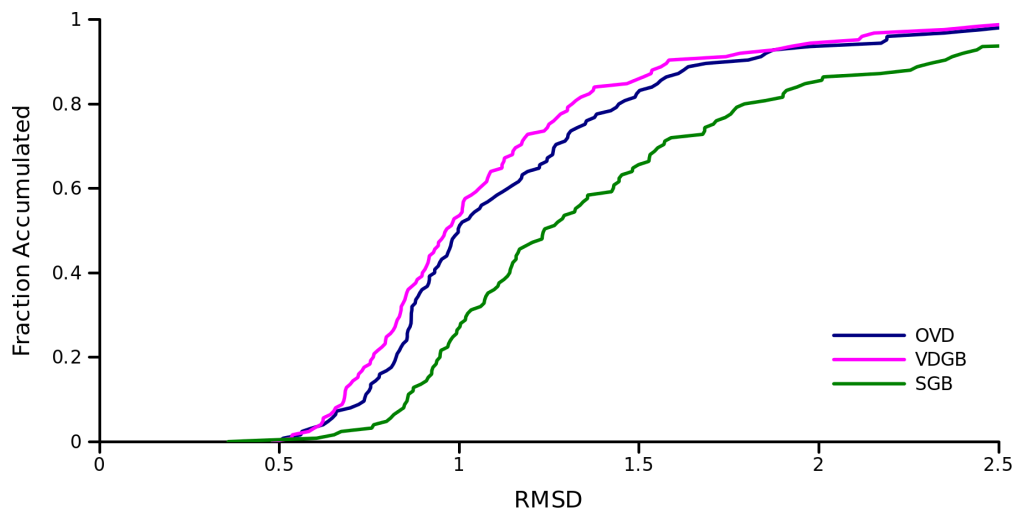


Figure 5.5: The fraction of minimized structures found within a given RMSD to native. The newer energy models, optimized variable dielectric (OVD or VSGB2.0) and variable dielectric surface generalized Born (VSGB) perform better than the original surface generalized Born model, however there is not significant differentiation between the two.

The performance of a selection of the energy models implemented in PLOP was compared using a subset of these ligands found in CDK2 crystal structures. Minimized crystal structures starting from the crystal coordinates were consistently closer to native conformations using the variable dielectric generalized Born solvation model than using a constant internal dielectric. However, there was no differentiation found between the newer and significantly more complex energy model, variously named optimized variable dielectric (OVD) and variable dielectric surface generalized Born 2.0 (VSGB2.0) [Li *et al.*, 2011a], which implements a number of terms to explicitly describe hydrogen bonding, $\pi - \pi$ interactions, and a number of other organic interactions.

In addition to adding support for a large number of small molecule ligands, a number of problems that plagued PDB loading have been corrected. These included lacking support for a number of crystal symmetry groups, and erroneously loading modified amino acids as non-covalently bonded

zwitterions. This latter problem frequently led to steric clashes, as the free amino acid would clash with the free ends of the protein chain to which it should have been covalently bonded. The energy involved in this sort of interaction is so severe that it would “drown out” small changes elsewhere in the system, though occasionally this could be worked around by freezing the coordinates of the problematic part of the protein structure.

5.4 Knowledge Based Backbone Dihedral Penalty

Though the systematic search method of sampling closed loops uses a rotamer of (ϕ, ψ) backbone dihedral pairs, it is still possible for amino acids to fall outside of the permitted dihedral space. These disfavored dihedrals can be introduced at least two different ways:

1. If the loop closure criteria is approximate superposition of a single atom, any dihedrals containing this atom can fall outside the allowed area, and the angle centered at the atom can also be very high energy.
2. If the initial loop conformation, including the predicted side chains, is strained, it is possible for any loop dihedrals to be pushed out of the permitted space.

Because some loop closure techniques, such as random tweak, and the systematic search implemented in PLOP, possibly introduce disfavored dihedrals, many loop sampling techniques apply filtering criteria to remove these dihedrals [Fine *et al.*, 1986; Shenkin *et al.*, 1987].

It has also been observed that sequential (ϕ, ψ) pairs are correlated in protein structure. This correlation has been used in PLOP to create a dipeptide library that is smaller than the product of the sequential peptide libraries. This has the effect of accelerating sampling by reducing sampling in areas of the $(\phi_1, \psi_1, \phi_2, \psi_2)$ space, which are unlikely to be occupied. Using this information, the predictive ability of PLOP was extended from thirteen to seventeen residue loops [Zhao *et al.*, 2011].

Using a similar, idea we sought to filter proposed loop conformations that contained unfavorable $(\phi_1, \psi_1, \omega_1, \phi_2, \psi_2)$ combinations. The dipeptide rotamer frequency-based scoring term employed a dipeptide rotamer library constructed from ~ 7500 high-quality PDB structures. Frequently occurring rotamers, corresponding to very populated regions in the higher dimensional equivalent to the Ramachandran plot, are weighted according to their frequency in this subset of the PDB.

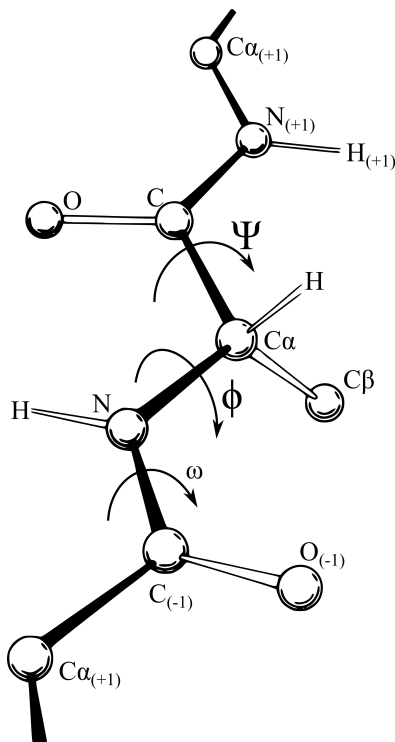


Figure 5.6: Labels assigned to protein backbone dihedrals. The dihedrals used in the knowledge based penalty term are $(\phi_1, \psi_1, \omega_1, \phi_2, \psi_2)$. Image licensed under cc-by-sa license by Jane Richardson.

Two criteria determine whether a penalty will be applied to the dipeptide:

1. If the Euclidean distance between the loop dipeptide and the nearest rotamer in the library is greater than a certain, empirically determined cutoff, or
2. If the total population of rotamers within a set radius of the loop dipeptide is below a certain threshold. The form of this penalty term, its implementation, and its successes in improving loop prediction in crystal structure and homology model environments will be discussed in detail in an upcoming publication.

An illustration of these criteria is presented in figure 5.7.

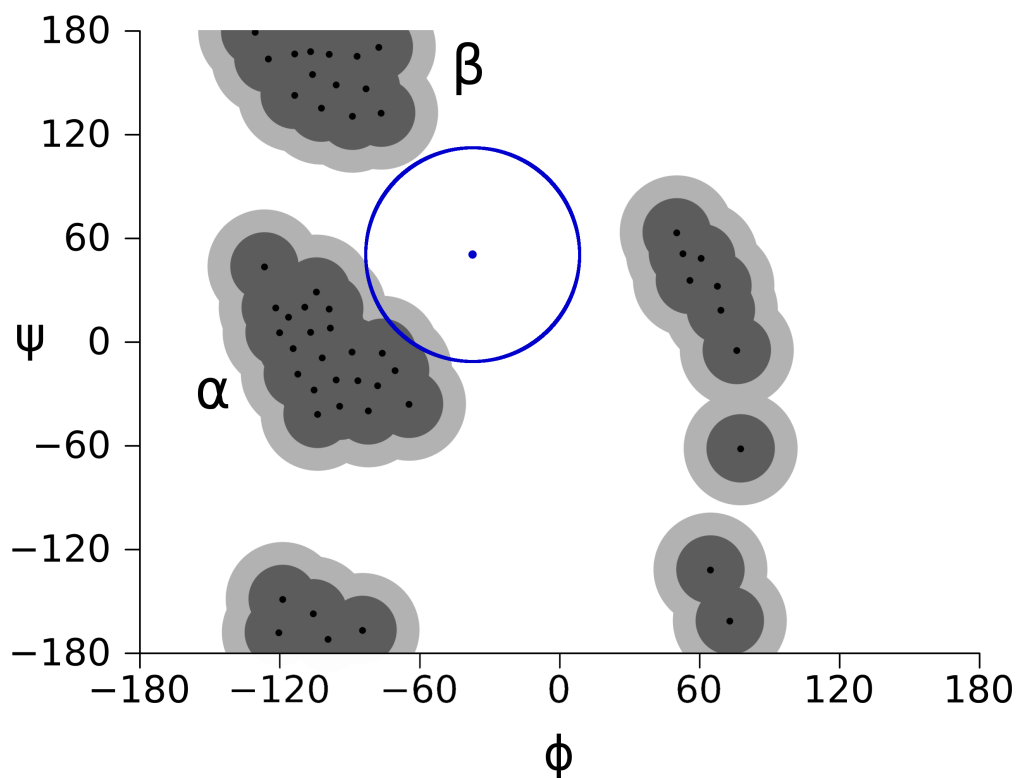


Figure 5.7: A simulated Ramachandran plot illustrating a similar scoring surface in two dimensions. Dark gray areas, or those very near a library rotamer, are considered native like and are never penalized. Light gray areas are pseudo-native like and are penalized at a linear rate if fewer than 30 library rotamers are found within a Euclidean distance D of the conformation. Rotamers in the white area are penalized according to how many library rotamers are contained within the distance D of the rotamer, illustrated by a blue circle in the figure. If 30 or more library rotamers are found within the blue circle, the conformation is not penalized at all. If the blue circle contains between 5 and 30 library rotamers, the conformation is assigned a penalty proportional to the distance from the nearest rotamer. If the blue circle contains fewer than 5 library rotamers, the penalty is proportional to the square of the distance to the nearest rotamer.

5.5 Call Graphs

With a project the size and complexity of PLOP, it becomes difficult for developers to understand the flow of the program without the assistance of external tools. At the moment there are over thirteen thousand different subroutines in more than 750 different source files. Because of this it is very difficult for new students to start contributing to development. Combined with the turnover intrinsic to an academic lab, students are almost constantly working on learning how existing code works, leading to little new development. Addressing this issue alone would greatly contribute to increasing lab productivity.

One popular tool to help in the analysis of a large program such as this is a call graph [Graham *et al.*, 1982]. In order to facilitate analysis of PLOP, we have implemented a tool to automatically generate call graphs making use of the DOT graph language [Koutsofios *et al.*, 1991]. It accepts as arguments the source files to be processed and outputs a directed call graph for the subroutines contained in or directly connected to subroutines contained in the enumerated source files. Figure 5.8 is a directed graph representing the call stack for each subroutine in a few source files related to mutation scanning in PLOP. This tool is available for all source files within PLOP, to assist developers, whether they be novices or veterans, in efficiently working on the project.

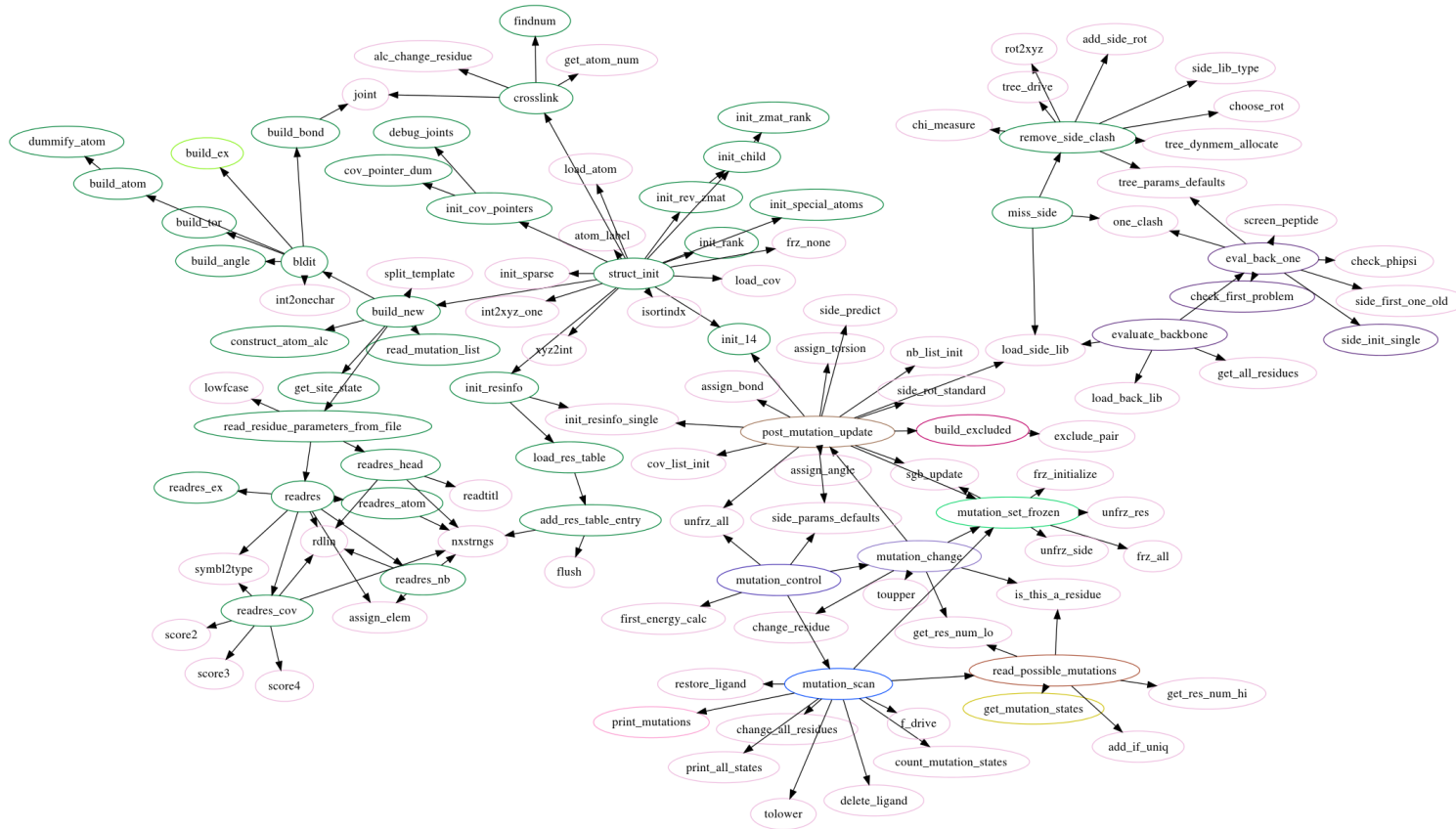


Figure 5.8: An example call graph generated for the code implementing mutation scanning and certain files having to do with building a structure in memory. The call graph of the entire program is both too large to display, and too complicated to be useful in development.

5.6 Conclusion

Maintaining the current rate of progress in drug development is a challenging problem. This is despite advancements in the rate at which new protein structures are being determined. Perhaps more important, however, is improving the efficiency of discovering successful new drugs, as failures along the drug discovery pathway represent serious economic and health-related costs both to industry and patients. Computer assisted drug development has the potential not only to maintain, but to improve this progress by helping focus research on more likely drug molecules and allowing efficient screening of large libraries of small molecules.

The work presented here has focused on development in the Protein Local Optimization Program (PLOP), which has in the past been used as a tool to predict side chain and loop conformations. We have focused on adding additional support for small molecules and mutation scanning within PLOP and demonstrate a significant algorithmic improvement in computation of implicit solvent models. The latter improvement greatly enhances performance of PLOP on large systems, allowing more detailed sampling to be performed more efficiently. We have also demonstrated successful side chain conformation predictions in protein-protein interfaces in our alanine scanning experiments, although predicted binding affinities were not well correlated with experimental data, an area for possible further investigation. Monte Carlo minimization was successfully implemented and applied to refine docked structures to transition state like complexes, which allowed for successful prediction of sites of metabolism. This, especially, represents a utilization of PLOP that is highly relevant to the medical community. Finally, we have made additional progress on a variety of supporting tools that have greatly helped development on the PLOP project in a number of areas. Taken together, this work has moved the PLOP project forward a great deal, making it more efficient, accurate, and reliable.

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