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The sensitivity of the results of molecular docking to induced fit effects: Application to thrombin, thermolysin and neuraminidase

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Abstract

This paper describes the application of PRO_LEADS to the flexible docking of ligands into crystallographically derived enzyme structures that are assumed to be rigid. PRO_LEADS uses a Tabu search methodology to perform the flexible search and an empirically derived estimate of the binding affinity to drive the docking process. The paper tests the extent to which the assumption of a rigid enzyme compromises the accuracy of the results. All-pairs docking experiments are performed for three enzymes (thrombin, thermolysin and influenza virus neuraminidase) based on six or more ligand-enzyme crystal structures for each enzyme. In 76% of the cases, PRO_LEADS can successfully identify the correct ligand conformation as the lowest energy configuration when the enzyme structure is derived from that ligand's crystal structure, but the methodology only docks 49% of the cases successfully when the ligand is docked against enzyme crystal structures derived from other ligands. Small movements in the enzyme structure lead to an under-prediction in the energy of the correct binding mode by up to 14 kJ/mol and in some cases this under-prediction can lead to the native mode not being recognised as the lowest energy solution. The type of movements responsible for mis-docking are: the movement of sidechains as a result of changes in C^{α} position; the movement of sidechains without changes in C^{α} position; the movement of flexible portions of main chains to facilitate the formation of hydrogen bonds; and the movement of metal atoms bound to the enzyme active site. The work illustrates that the assumption of a rigid active site can lead to errors in identification of the correct binding mode and the assessment of binding affinity, even for enzymes which show relatively small shift in atomic positions from one ligand to the next. A good docking code, such as PRO_LEADS, can usually dock successfully if there is induced fit in relatively rigid enzymes but there remains the need to develop improved strategies for dealing with enzyme flexibility. The work implies that treatments of enzyme flexibility which focus only on sidechain rotations will not deal with the critical shifts responsible for mis-docking of ligands in thrombin, thermolysin and neuraminidase. The paper demonstrates the utility of all pairs docking experiments as a method of assessing the effectiveness of docking methodologies in dealing with enzyme flexibility.

Introduction

Reliable molecular docking and estimation of binding affinities remain an important goal in structure based drug design applications [1–6]. Given a novel inhibitor against an enzyme of known structure, it is important to predict how the ligand is going to bind. Successful prediction allows one to prioritise synthetic resource into producing compounds which are more likely to be useful and biologically active. Docking studies are also important when designing new ligands in order to confirm the binding mode and to form the basis for an energetic assessment of their binding affinity. Docking methodology can be used to screen compound databases [7–9] provided the docking is fast enough and there is a method to provide a reasonably reliable ranking order for the biological activity of the ligands.

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In this paper the focus will be on docking ligands which are flexible but of moderate size, under the assumption that the enzyme/receptor is rigid. The focus on small moderately flexible ligands reflects the desire to apply the method to the design of small organic inhibitors of enzymes. The assumption of a rigid enzyme is an approximation which has not been examined thoroughly in previous docking papers and one of the purposes of this paper is to explore this assumption for enzymes which do not undergo major conformational shifts.

The location of the correct ligand orientation and conformation in a large enzyme active site requires a highly efficient searching procedure. Simulated annealing (SA) [10, 11], evolutionary programming (EP) [12, 13], and genetic algorithms (GA) [14–18] have all been applied to the docking problem together with more specialised sampling techniques [19–23]. In a recent publication [24], we have presented our approach to molecular docking, PRO_LEADS. Four searching algorithms (SA, EP, GA and Tabu Search (TS)) are implemented in PRO_LEADS and it has been found that TS performed at least as well as the other methods, although this conclusion must be tempered by the knowledge that the performance of search algorithms is often highly dependent on the precise implementation. TS [25] has been applied to many problems but to our knowledge this was the first application to the docking problem. The work presented in this paper uses only Tabu search and the approach is briefly described in the Methods section.

The energy function used in PRO_LEADS is based on a simple empirically derived scoring function [26] which should be suitable to estimate the binding affinities of molecules as well as to optimise their binding modes. Its motivation is similar to Böhm's function [27] and Jain's function [28] which have both been used as a basis for docking potentials [9, 22]. The function comprises simple contact terms to estimate lipophilic and metal-ligand binding contributions, a simple explicit form for hydrogen bonds and a term which penalises flexibility. The function was optimised against a large training set of 82 protein-ligand complexes taken from the Protein Data Bank (PDB) [29] for which the binding affinities were known. The function reproduced the binding affinities of the complexes with a cross-validation error of 8.68 kJ/mol and performed well on a separate test set of a further 30 complexes. The function has been amended slightly for the purposes of docking and augmented with suitable penalty terms. Details of the function are given in the following section.

The performance of this function in docking applications has been tested [30] on a database of 50 diverse receptor-ligand complexes for which the binding geometry and the binding affinity of the ligands were known. The ligands ranged in size from 5 to 46 heavy atoms and in flexibility from 0 to 17 rotatable bonds. The lowest energy solution produced by PRO_LEADS was within 1.5 Å heavy atom RMS (Root Mean Square deviation) of the crystallographic solution for 86% of the complexes. After omission of one outlier (biotin in streptavidin – 1STP), the lowest energy produced in each docking run had a standard deviation from the experimental binding affinity of 8.6 kJ/mol and the R^2 value was 0.577. These results indicate that PRO_LEADS provides a good basis for assessing the binding geometry and binding affinity of ligands.

In this work we perform further validation studies with PRO_LEADS. The aim is to explore whether the assumption of a rigid enzyme/receptor structure has severely biased the above conclusions. It is clear that docking methods which employ rigid enzymes will have great difficulty in docking ligands into an enzyme which changes its shape significantly on binding to the ligand. However, even for a relatively rigid enzyme there is still a danger that small changes in its structure will lead one to miss good solutions and fail to rank the binding affinity of different ligands correctly. It is sensitivity to small changes in enzyme structure which is more worrying, since the docking method and associated energy function should be relatively robust to small changes in the binding site. In order to test this, we have studied three enzymes (thrombin, thermolysin and influenza virus neuraminidase) which are normally thought of as being relatively rigid and exhibiting relatively little induced fit with different ligands. The enzymes are all of direct relevance to structure based drug design targets and their associated ligands are small molecules of limited flexibility. The test cases represent exactly the type of application for which we wish to use PRO_LEADS and are therefore a good test for the program. For thrombin, we have chosen 6 thrombin-inhibitor complexes from the PDB for which the binding affinity is known, and docked all the inhibitors into all the enzyme structures (i.e., 36 docking runs in all). This 'all pairs' experiment has also been performed for 9 thermolysin-inhibitor complexes (i.e., 81 docking runs in all) and 6

neuraminidase-inhibitor complexes (i.e., a further 36 docking runs).

The starting point for analysis of the results will focus on the performance of the code on the 'native' docking runs where the ligand is docked against the enzyme structure derived from co-crystallisation against that ligand. The main emphasis will be on the lowest energy solution produced in the native docking run and in particular on its heavy atom RMS to the crystallographic solution, its binding affinity, the energy gap to the next highest scoring conformation and the number of times the correct solution is located in 100 docking attempts. These results will then be compared to those obtained from the 'non-native' docking runs performed in the all pairs experiment to assess and understand any degradation of performance.

Materials and methods

The methods used in this paper are very similar to those used in Baxter et al. [30] and will only be described briefly here.

Tabu search

Tabu search maintains only one current solution during the course of a search. At the start of each run, the current solution is initialised by randomising the position, orientation and conformation of the ligand within the bounding box.

In the implementation adopted here, 100 random 'moves' are generated from the current solution and these are scored using the energy function. The lowest energy move is automatically accepted if it has the best energy so far located. Otherwise the moves are judged by their heavy atom RMS to a list of up to 25 previously visited solutions. Moves that are within 0.75 Å RMS of any solution in the list are considered 'tabu' and the lowest energy, non-tabu move is accepted. The accepted move becomes the new current solution and it replaces an existing member of the list of previously visited solutions.

In this work, the Tabu search continues for 4000 iterations so each docking attempt comprises a total of 400 000 energy evaluations. A docking attempt is repeated 100 times in a full docking run on one ligand against one enzyme structure. The full implementation details are given in Baxter et al. [30].

Energy function

The energy function used in this work can be written:

$$\begin{aligned}\Delta G_{\text{binding}} = \Delta G_0 + \\ \Delta G_{\text{hbond}} \Sigma_{iI} g_1(\Delta r) g_2(\Delta\alpha) g_3(\Delta\beta) + \\ \Delta G_{\text{metal}} \Sigma_{aM} f(r_{aM}) + \Delta G_{\text{lipo}} \Sigma_{lL} f(r_{lL}) + \\ \Delta G_{\text{rot}} H_{\text{rot}}. \qquad (1)\end{aligned}$$

There is a constant term; a hydrogen bond term which scores hydrogen bonds between the ligand and receptor taking into account the distances and angles between hydrogen bonding atoms; a lipophilic term which scores lipophilic contact between the ligand and receptor; a metal contact term which scores the contact of ligand atoms with metals in the receptor; and a flexibility penalty which is treated as a constant for all conformations of the same ligand. This empirical energy is augmented with ligand-receptor clash terms, a torsional penalty term for rotatable bonds and a term which punishes ligand atoms which fall outside a user-defined grid. The lipophilic term and clash terms are calculated approximately using a grid method whilst the metal contact and hydrogen bond terms are calculated exactly using a neighbour grid method.

The full form of the empirical energy function used in this work and details on its implementation are given elsewhere [30].

Exclusion of bias from the docking test

The docking experiments described here attempt to simulate the kind of applications which we wish to study with PRO_LEADS. The main difference is that the quality of the results can be judged against experiment. In setting up the docking protocol, it is possible to impose constraints which almost guarantee reproducing the experimental binding mode, but in real applications such constraints may be extremely difficult to define. The purpose of this section is to outline the kinds of constraints which can be imposed in order to improve the quality of docking results and to make clear what we have done to try to eliminate them so as to make the docking experiments as realistic as possible. It should be understood that it is impossible to perform completely objective docking and that some constraints are essential in docking applications.

Definition of the active site. This determines the size of search space considered during the docking run. By

using a small active site, one significantly enhances success rates and reduces the problem of deceptive minima, although some restriction on the size of the active site is unavoidable. In the PRO_LEADS runs described in this paper, the search space is determined by the size of the grid box and relatively large grid boxes are used. The grid boxes are constructed so as to fit *all* the ligands for a particular enzyme with at least about 3 Å to spare on each side of the box. The actual grid boxes used in each test case are given below.

Constraining the translation of the ligand. It is also possible to constrain the movement of the ligand by preventing one of its atoms (or its centre of mass) from moving more than a specified distance from its position in the crystal structure. Clearly if this distance is small then the search space is reduced dramatically and better agreement with experiment will be obtained. This type of constraint is a perfectly valid way of docking molecules especially when one has a good idea of how some portion of the ligand might dock. In contrast to our previous work, no restriction of this type has been used here, although the translation of the ligand is constrained by the definition of the active site.

Fixing degrees of freedom in the ligand. It is known that flexible ligands adopt different torsions when bound to an enzyme or receptor. If any of these torsions are fixed in their receptor bound conformation, this will make it much easier to dock the ligand, both by removing deceptive minima from the potential energy surface and by reducing the dimensionality of the variable space to be searched. It is also possible to introduce bias into the algorithm by only allowing free variables to lie in specified ranges. For example, if torsions are constrained to 60 deg values and the search space contains the bound conformation then this would correspond to a high degree of bias. In the tests presented below, we have tried to include all relevant torsions and all torsion angles are accessible to the search algorithm. Bond lengths, bond angles and torsions associated with non-rotatable bonds are fixed during the docking and this is an approximation. To lessen any bias that might result from using the bound valence geometry of the ligand as the starting point for docking, we minimize the geometry of the ligand in vacuo with the CVFF forcefield [31] prior to docking. PRO_LEADS currently has no way to deal with the flexibility of ring systems except to prepare multiple conformers prior to docking. In some respects, this is an attractive approach since on the fly generation of ring conformations would need a very powerful methodology to ensure that only low energy ring structures were produced.

Initial starting conformation. Before any docking attempt the variables associated with all the degrees of freedom should be randomised as is the case in the current application.

Crystallographic water molecules. Crystal structures for enzyme-ligand complexes usually contain crystallographic water molecules. Some water molecules are known to be conserved across different ligands and in such circumstances, they can be included as part of the receptor structure to be docked against. However, the positions of a large number of crystallographic water molecules vary according to the ligand in the complex. There can be little justification for including these waters in a docking test which is designed to simulate an application for which one does not know the answer. The inclusion of such waters constitutes a severe bias to the test since the waters provide an excluded volume for the docking and significantly reduce the number of deceptive minima. All waters are excluded from the docking tests given in this paper.

Protonation states. In many cases, it is difficult to predict the protonation states of ligand groups and receptor residues in advance. For example, it is not uncommon for aspartates, glutamates and histidines to be protonated in some complexes. Clearly choosing the wrong protonation states will affect the ability of a compound to dock correctly and, so conversely, using the crystallographic coordinates to obtain the correct protonation pattern will bias the docking study to some degree. We have chosen not to address this type of bias and have always been guided by the crystallography papers in choosing protonation states.

Treating the receptor as rigid. The assumption of rigid receptor is an approximation and clearly if the receptor structure changes considerably for different receptor-ligand complexes, the assumption will lead to errors. Simple treatments of receptor flexibility [15, 32] are unlikely to have much impact on such errors. We are more concerned with the effect which relatively small changes in receptor structure could have on docking results. For example, there is a potential for biasing the answers in a docking study when

Table 1. The heavy atom RMS (in Å) for superimpositions of the active sites of the specified enzymes onto the fixed enzyme. For thrombin, the active site was defined as all residues in the 1DWB structure within 3.5 Å of any of the ligands (30 residues with sequence numbers: 57, 60A, 60D, 60F, 94, 97A–99, 102, 174, 189–195, 213–221A and 225–228). For thermolysin, the active site was defined as all residues in the 5TMN structure within 3.5 Å of any of the ligands (24 residues with sequence numbers: 110–116, 129–130, 133, 139, 142–143, 146, 150, 157, 166, 188–189, 192, 202–203, 226, 231). For neuraminidase, the active site was defined as all residues in the 1NSD structure within 4.5 Å of the 1NSD ligand (17 residues with sequence numbers: 115–116, 131, 148–149, 176–177, 220, 222, 225, 244, 274–275, 291, 293, 373, 408)

Thrombin		Thermolysin		Neuraminidase	
Pcode	RMS to 1DWB (Å)	Pcode	RMS to 5TMN (Å)	Pcode	RMS to 1NSD (Å)
1DWD	0.27	1TMN	0.28	1INF	0.23
1ETS	0.73	2TMN	0.18	1INY	0.58
1ETR	0.76	3TMN	0.29	1IVE	0.47
1ETT	0.90	4TMN	0.20	1NNC	0.43
TMT1	1.00	6TMN	0.11	1NSC	0.11
		4TLN	0.30		
		5TLN	0.28		
		1TLP	0.22		

NAPAP is docked into a thrombin structure derived from the crystallographic structure for the NAPAP-thrombin complex. In a real application, the receptor structure will be derived from a complex with an alternative ligand. It is possible that small adjustments in the receptor conformation could affect the predicted binding affinity and even the predicted binding mode. One of the purposes of this paper is to see if the performance of docking programs is significantly affected by using the 'wrong' receptor structure.

Excluding poor test cases. Even with the best will in the world, it is easy to find excuses to exclude test cases which do not work or to exclude classes of compounds which are 'not appropriate' for the energy function or the docking code. We have not knowingly excluded any ligands from this work, although the choice of the test cases was governed by the need to find enzymes for which a fair number of smallish ligands had been crystallised and for which there was data on binding affinities.

Basis for comparison

When setting up an experiment to test the abilities of a docking code it is important to be clear on how the results are to be judged. Here, PRO_LEADS is used to produce 100 docking solutions for each ligand-receptor complex. The solutions are hierarchically clustered on the basis of heavy atom RMS using a threshold value (1.5 Å) for merging clusters [30]. The clusters are ranked according to the energy of the lowest energy solution in the cluster. The predicted conformation produced by PRO_LEADS is the lowest energy solution of the highest ranking cluster.

The first consideration will be the performance of each ligand against the enzyme from its own crystal structure (i.e., the native docking results). The following criteria will be used to assess the performance of PRO_LEADS in the native docking results.

- Is the predicted conformation within 1.5 Å heavy atom RMS of the crystallographic solution?
- Is there a large energy gap between the predicted conformation and the lowest energy solution from other high ranking clusters?
- How do the predicted binding affinities of the predicted conformations compare with the experimental binding affinities for the ligands? In the case of neuraminidase not all the experimental affinities are available but IC50 information can be used to give a ranking for the ligands.
- How often is the correct conformation located during the 100 docking attempts?
- How do the results compare to those of previous docking studies on the same or similar systems?

For the all pairs docking experiments, we have chosen to present only the energy of the lowest energy solution and its RMS to the crystal structure. This allows us to represent the results from each enzyme in a digestible form. The main focus will be on examining

the degradation in the results and trying to understand why the degradation occurs.

It is our belief that reliance on the heavy atom RMS of the lowest energy solution is the best way to perform an analysis on a large number of docking results because it is objective and allows facile comparison with the work of others. However, there are a number of problems associated with the RMS measure. Firstly, it is poor at distinguishing solutions in which most of ligand is correct but some portion of the ligand is in the wrong orientation, versus poor quality solutions in which most of the molecular recognition is wrong but the ligand is in the right general area. Secondly, small ligands tend to produce lower RMS values – for example, small ligands which bind upside down (and are thus completely wrong) have moderately low RMS's (say 2.5 Å) but it is very difficult to get such low RMS's for large ligands without being 'close' to the right answer. Thirdly, a full heavy atom RMS is inappropriate where some portion of the ligand makes no contact with the enzyme, and in such cases it is advisable to leave out solvent exposed fragments from the RMS calculation. Finally, the heavy atom RMS gives equal weight to the hydrogen bonding and lipophilic portions of the molecule but small discrepancies in the positioning of lipophilic atoms are often acceptable because of the non-specific nature of the molecular recognition. Discrepancies in hydrogen bonding atoms may indicate that the wrong hydrogen bonding pattern has been identified and this could lead to serious errors downstream during the design process.

Test cases

The majority of the enzyme structures used in this work were taken from the prepared database of complexes which was used to construct the empirical scoring function [26]. The protocol used to prepare the complexes has been described before [26]. Briefly, the crystal structures were extracted from the Brookhaven Data Bank [29], hydrogen atoms were added using InsightII [31] and the complex was minimised with Discover [31] using tethers with large force constants to keep heavy atom positions very close to the crystallographically determined positions. In the case of TMT1, the carboxy terminus of the ligand in the PDB file, 1TMT, was removed prior to the minimisation procedure. For each enzyme the different crystal structures were superimposed on each other and information on this procedure is given in Table 1. Waters were removed from the complexes and the ligand was separated from the enzyme. Grid boxes were defined around the ligand positions. Table 2 gives the definition of grid box used for the runs.

Table 2. The box sizes and box positions (in Å) for the thrombin, thermolysin and neuraminidase test cases. The box position is given as the corner of the grid box with the lowest *X*, *Y* and *Z* coordinate and is the frame of reference of 1DWB for thrombin, 5TMN for thermolysin and 1NSD for neuraminidase

		X	*Y*	*Z*
Thrombin	Box Size	16.214	22.969	18.393
Thrombin	Position	23.602	2.984	15.424
Thermolysin	Box Size	19.400	20.200	24.200
Thermolysin	Position	41.062	7.017	−18.316
Neuraminidase	Box Size	18.000	19.400	18.200
Neuraminidase	Position	−12.405	48.603	−18.543

Chemical structures for the thrombin ligands are given in Figure 1 together with the rotatable bonds defined in this study. Figure 2 gives the same information for the thermolysin ligands. Note that some groups in the thermolysin ligands lie completely in solvent and are therefore ignored in the heavy atom RMS calculations, and each atom in these groups has been marked with an asterisk in Figure 2. Also, the 2TMN and 4TMN ligands have had their phosphonamide nitrogens protonated, in line with the original crystallographic papers. Figure 3 gives the chemical structures and rotatable bonds for the neuraminidase ligands. Other aspects of the valence geometry were held rigid during the docking runs, although prior to docking, the valence geometry of the ligands was minimised in vacuo using the CVFF force field [31]. Before every docking run, the rotations about the rotatable bonds and the orientation and position of the ligand in the box were randomised.

Results and discussion

Results for native docking

The results for the native docking runs are given in Table 3. The first point is that the lowest energy solution is close to the correct answer for 76% of the native docking runs (see Table 4). This is in line with a previous test on 50 protein-ligand complexes where docking was successful for 86% of the complexes [30]. The main difference with this test was

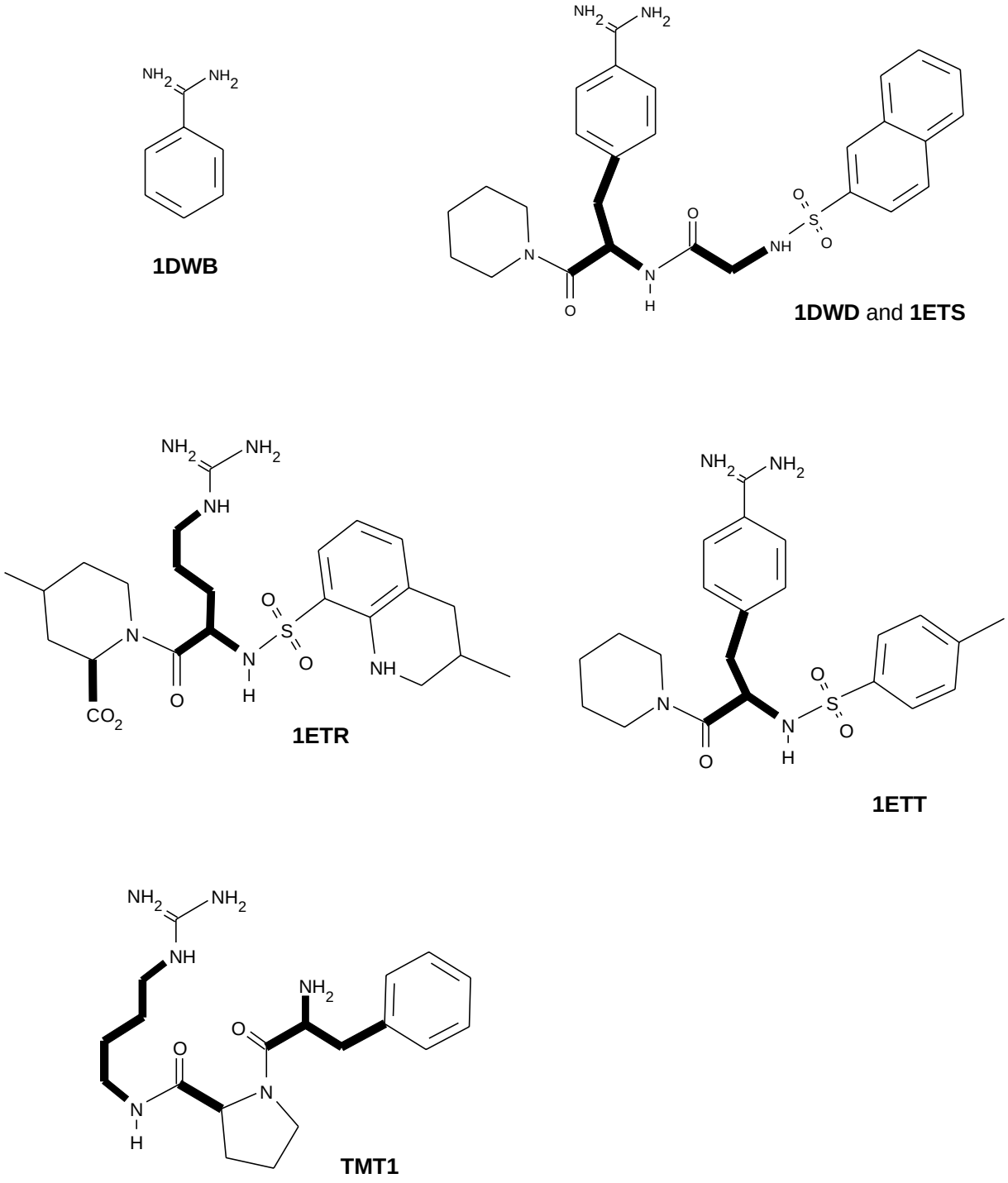

Figure 1. The ligands used in the thrombin docking experiments. The bonds shown in bold are treated as rotatable in the docking runs.

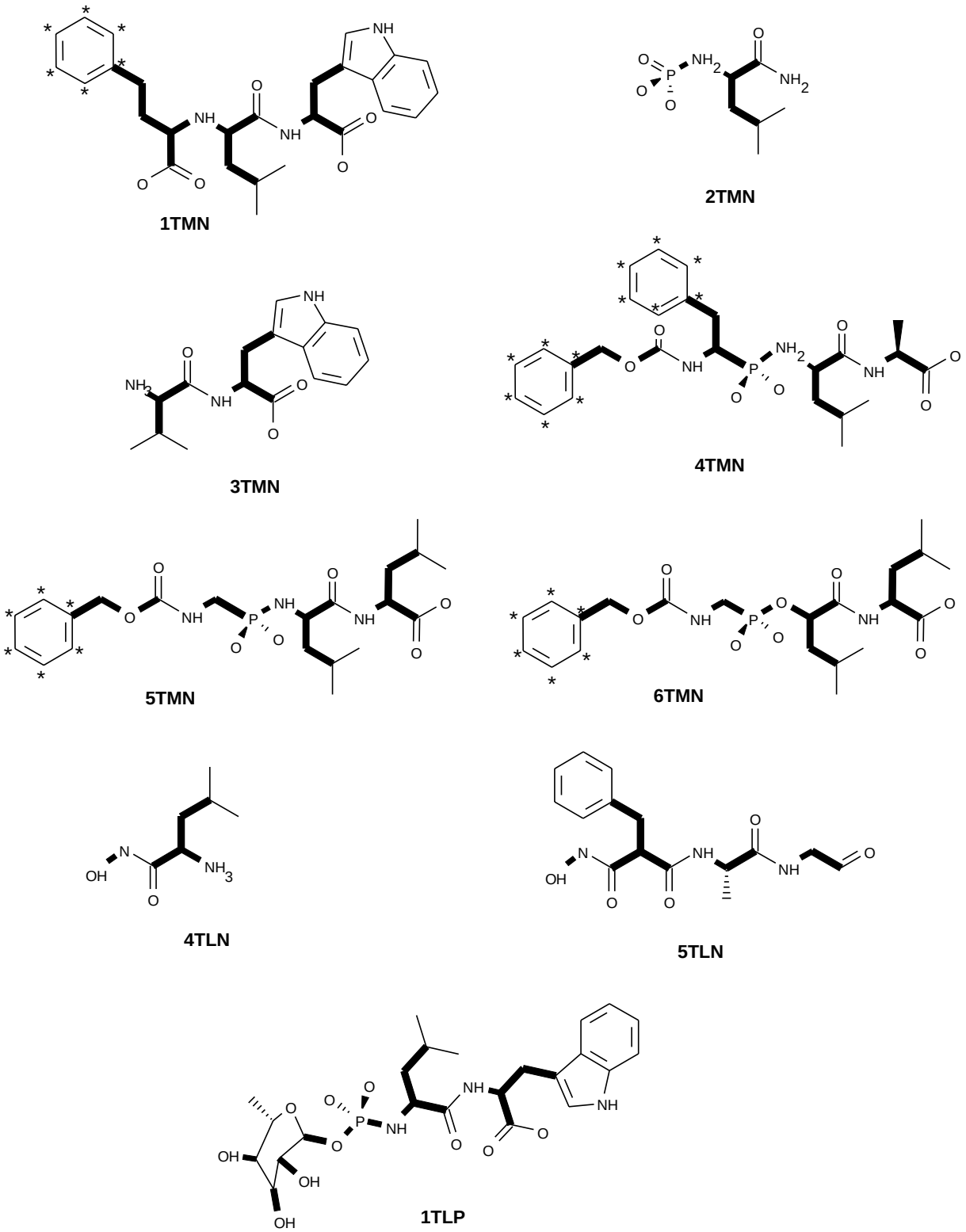

Figure 2. The ligands used in the thermolysin docking experiments. The bonds shown in bold are treated as rotatable. Note that for 5TLN a p-aminonitrobenzene tail has been terminated as an aldehyde since the carbonyl is the last ligand group with coordinates in the PDB structure. Some of the phenyl groups in 1TMN, 4TMN, 5TMN and 6TMN are marked with stars. These phenyl groups make no contact at all with the enzyme and the phenyl conformation has been ignored in most of the heavy atoms RMS calculations.

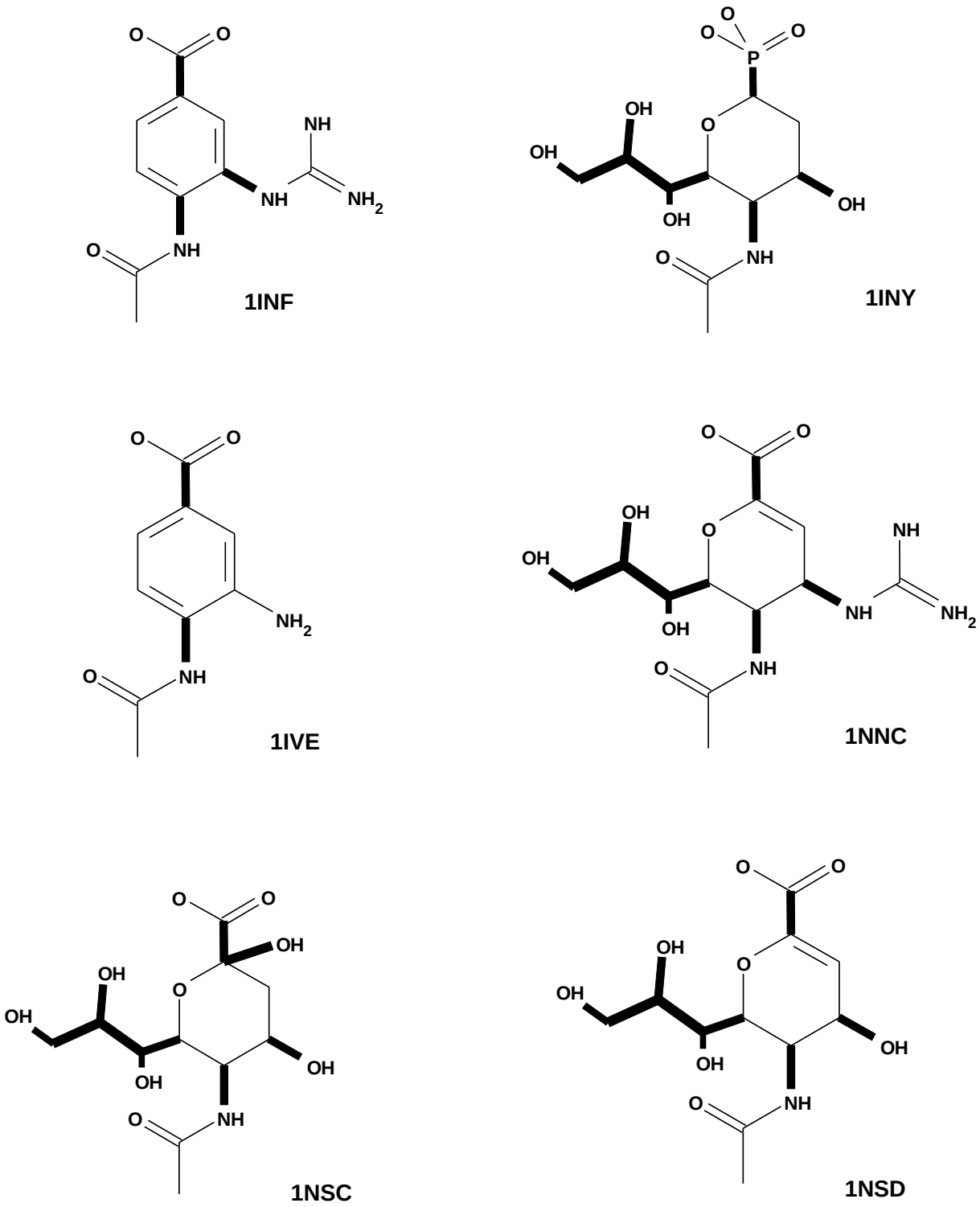

Figure 3. The ligands used in the neuraminidase docking experiments. The bonds shown in bold are treated as rotatable in the docking runs.

that a different protocol was used and in particular, a larger grid box was employed. Ten of the twenty-one complexes in this study were included in the previous test.

In about half the cases, the energy gap between the lowest energy solution and best answer from the second cluster is quite large (>3.0 kJ/mol). Small energy gaps indicate that the docking solution produced is more likely to be in error. For example, native docking is successful for 100% of cases with large energy gaps as compared to 55% of cases with small energy gaps. This trend carries over to the non-native docking: for ligands with large energy gaps in the native docking, 69% of the non-native docking runs are successful compared with only 36% of non-native docking runs for ligands with small energy gaps. It thus appears that the size of the energy gap in a docking run is a good indication of the robustness and reliability of the results.

Figure 4 plots the best energies from the native docking results with experimental binding affinities for the 17 complexes where experimental affinities are available. The correlation is reasonably good as might be expected since only two of the complexes were not included in the training set used to produce the empirical scoring function on which PRO_LEADS docking function is based [26]. For neuraminidase, the experimental binding affinities for four of the ligands could not be obtained although IC50 information can be used to establish an approximate ordering for the potency: 1NSC $<$ 1INY $\approx$ 1IVE $<$ 1INF $\approx$ 1NSD $<$ 1NNC. The potency range spans over 3 orders of magnitude. The scoring function has not done a particularly good job at ranking the potencies and two factors have contributed to this. Firstly the 1NSC ligand (sialic acid) is bound in a boat conformation and PRO_LEADS has no strain energy term to penalize this conformation. Secondly the transition state analogues, 1NNC and 1NSD, are under-predicted owing to a close contact between the Tyr-408 phenolic oxygen and the carbon alpha to the carboxyl group. This contact is probably favourable because of the unusual electrostatic character of this carbon atom but PRO_LEADS treats the contact as a clash and punishes it accordingly.

The number of solutions in the top ranking cluster for each of the docking runs tends to be fairly low, especially for thermolysin. This perhaps indicates that the results would be improved by using a longer or more efficient search protocol. The CPU time for these jobs is considerable and that is the reason why this particular protocol was chosen.

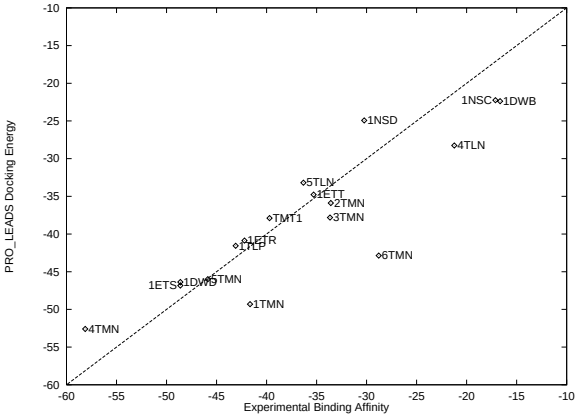

Figure 4. Graph showing the PRO_LEADS docking energy (the lowest energy solution produced in the docking) versus the experimental binding affinity for the 'native' docking runs. The energies are measured in kJ/mol.

Comparison with other docking codes

It is not the purpose of this paper to go through each test case in detail and compare the results with other workers. Nevertheless it is important to show that PRO_LEADS is performing well on these test cases in comparison with other workers, because it supports the generality of the conclusions drawn with respect to the non-native docking results. It is our experience that performance of docking codes on a few test cases is a poor guide to their performance on a large set of test cases, so comparison with other workers will only be made where those workers have looked at a considerable number of the test cases studied here (at least four).

The program, GOLD [15, 33], has been used in native docking runs on 1DWD (1.7), 1ETR (4.2), 1TMN (1.7), 2SIM (1.9) and 1IVE (2.2) and the heavy atom RMS's (in Å) to the crystallographic solution for the best answers are given in parentheses. (The test case 2SIM is essentially equivalent to 1NSD since the crystal structures contain the same ligand and enzyme.) PRO_LEADS obtains better RMS's for all but one of the test cases and, more importantly, correctly docks 1ETR yet fails to dock 1NSD. The inferior performance for 1NSD is disappointing but from Table 3 it is noticeable that the second ranking solution is correct and has a very similar energy to the incorrect first ranking solution.

The program, FlexX has been tested on 1DWD (1.0), 1DWC (8.1), 1TMN (0.87) and 4TLN (3.68) in native docking runs [23]. The test case 1DWC is essentially equivalent to 1ETR since the crys-

Table 3. The top 2 ranking clusters obtained in 100 docking attempts for the native docking runs. The best energy, the RMS to the crystal structure of the best energy solution, and number of solutions are given for each cluster. For the thermolysin structures, 1TMN, 4TMN, 5TMN and 6TMN, one or two phenyl groups lie entirely in solvent (see Figure 2) and the RMS's in the table exclude these phenyl groups. For 1TMN, 4TMN, 5TMN and 6TMN, the full heavy atom RMS's for the best energy solution are 0.81, 0.83, 2.62 and 2.52 Å respectively. Also given are the experimental binding affinity and the CPU time in hours taken for each job. The jobs were run on a single processor of the Convex Exemplar which is about 2.4 times slower than a Silicon Graphics R10000 workstation for these applications

PDB code	Binding affinity (kJ/mol)	Rank 1 cluster			Rank 2 cluster			CPU time (h)
		Energy (kJ/mol)	RMS (Å)	Size	Energy (kJ/mol)	RMS (Å)	Size	
1DWB	−16.66	−22.375	0.31	10	−21.810	11.75	77	6
1DWD	−48.62	−46.367	0.71	10	−37.622	2.93	2	16
1ETS	−48.62	−46.830	0.58	17	−38.114	3.81	4	16
1ETR	−42.21	−40.872	0.81	41	−34.381	3.12	3	16
1ETT	−35.30	−34.776	0.48	6	−34.620	3.96	50	13
TMT1	−39.70	−37.893	0.54	15	−34.551	8.65	8	15
1TMN	−41.65	−49.305	0.87	1	−47.946	1.94	2	24
2TMN	−33.58	−35.893	0.68	63	−31.329	2.54	23	15
3TMN	−33.66	−37.817	1.50	71	−34.449	2.74	24	17
4TMN	−58.13	−52.594	0.67	5	−42.927	6.30	28	26
5TMN	−45.87	−45.986	1.22	1	−40.625	5.08	2	24
6TMN	−28.79	−42.856	1.31	2	−38.993	4.89	6	26
4TLN	−21.23	−28.257	2.92	21	−27.160	2.86	58	13
5TLN	−36.32	−33.197	6.98	13	−31.358	2.74	3	18
1TLP	−43.09	−41.563	1.33	1	−39.613	2.07	3	25
1INF	–	−22.308	7.39	40	−21.142	3.82	23	11
1INY	–	−20.496	0.65	29	−19.205	1.35	15	24
1IVE	–	−27.803	1.66	55	−26.151	1.85	6	10
1NNC	–	−29.037	0.44	65	−22.747	2.15	14	24
1NSC	−17.12	−22.247	0.38	6	−21.254	7.24	8	26
1NSD	−30.24	−24.927	5.17	17	−24.800	0.56	15	24

tal structures contain the same ligand and enzyme. PRO_LEADS performs as well or better than FlexX on these complexes and has the distinct advantage of correctly docking 1ETR.

Judson et al. [16] have done a big study of the thermolysin ligands from 1TMN (8.2), 2TMN (3.3), 4TMN (1.4), 5TMN (0.4), 6TMN (1.9), 4TLN (1.6), 5TLN (1.9) and 1TLP (8.8) and in each case the RMS of the *hydrogen bonding atoms* for the lowest energy solution from their favoured protocol is given in parentheses after each test case. The docking runs were all performed using the 5TMN crystal structure and so should be compared with the 5TMN column of Table 6. The RMS of hydrogen bonding atoms is a much less sensitive measure of the accuracy of the ligand conformation than the heavy atom RMS's given in Table 6.

Table 4. The percentage of successful docking runs for the different enzymes. 'Native' means the ligand is docked against its own crystal structure (21 docking runs in all); 'Non-native' means the ligand is docked against an enzyme structure obtained with a different ligand (132 docking runs in all); 'All pairs' means the ligand is docked against all available structures of the appropriate enzyme (153 docking runs in all). A successful docking run is defined as one where the heavy atom RMS of the best scoring ligand conformation is within 1.5 Å heavy atom RMS of the crystallographic solution

	Native	Non-native	All pairs
Thrombin	100%	67%	72%
Thermolysin	78%	47%	51%
Neuraminidase	50%	37%	39%
All enzymes	76%	49%	55%

It is clear that PRO_LEADS is doing a much better job with the thermolysin ligands, docking five out of eight ligands within 1.5 Å RMS compared with only two out of eight from the previous study. Overall it is clear that PRO_LEADS is performing well compared with other docking codes for these test cases.

Results for thrombin non-native docking

The thrombin results are given in Table 5 and summarised in Table 4. It is clear that there is some degradation in performance for thrombin but it is not severe. The best energies are usually produced for the native docking and this reflects the fact that induced fit effects lead to an improvement in affinity. Additionally, any induced fit effects for one ligand are probably going to adversely affect the docking energy of the correct conformation of an alternative ligand, whereas the energies of incorrect conformations are unlikely to increase or decrease in a systematic manner. The practical effect is that the docking of the 1ETT and 1DWB ligands is often unsuccessful against the non-native enzyme structure owing to the low energy of deceptive minima. The difference in energy between the native docking and non-native docking in Table 5 is usually a few kJ/mol, although it can be as much as 10 kJ/mol in extreme cases. We have studied all the docking results in detail and have identified the following changes as being critical in affecting docking performance in the non-native docking studies on thrombin.

- the movement of sidechains to make the size of an enzyme pocket slightly smaller. For example, the thrombin P pocket is slightly smaller for some ligands through the movement of the Trp-60D sidechain. The indole ring moves by over 1.5 Å and the movement is derived from a shift in the C^{α} position for this residue. This movement means that it is not possible to position the other ligands properly in the thrombin P pocket without incurring a clash penalty.
- The movement of sidechains to obscure entry into pockets. For example, the Glu-192 sidechain is highly mobile in thrombin (and other trypsin-like serine proteases) and in some of the thrombin structures it blocks some angles of entry for the ligand into the S1 pocket. The movement is mainly in the sidechain itself rather than being caused by shifts in the C^{α} position. The result is that some ligands incur a clash penalty if they are to enter the S1 pocket correctly.
- The movement of flexible portions of main chains to facilitate the formation of hydrogen bonds to the protein backbone. For example, the presentation of the backbone carbonyl of Gly-216 changes significantly in some structures so that hydrogen bonds can be formed.
- The trivial movement of a hydroxyl group in a sidechain to form hydrogen bonds with appropriate inhibitors. For example, the Ser-195 hydroxyl group can rotate to form hydrogen bonds with the 1ETR ligand.

Results for thermolysin non-native docking

The thermolysin results are given in Table 6 and summarised in Table 4. The results for 4TLN and 5TLN ligands are best ignored since it is clear that PRO_LEADS has some difficulty in positioning the hydroxamic acid moiety correctly and thus fails to dock these ligands against any of the enzyme structures. PRO_LEADS also fails to dock 3TMN and 1TLP in all cases except the native docking. The 3TMN ligand is the only ligand which does not bind to the metal atom and proves a difficult test case for PRO_LEADS with only a small separation between the correct answer and competing minima. However 3TMN ligand does dock moderately well against the 1TMN and 5TLN receptors only differing from the crystallographic conformation in the orientation of the indole ring which is partially in solvent. The 1TLP ligand is an extremely challenging test case since the sugar moiety forms few direct contacts with the enzyme and is locked in position via an internal hydrogen bond (which would be treated as a clash by PRO_LEADS). Nevertheless PRO_LEADS still gets reasonably close in a number of the non-native docking tests.

The remaining ligands (1TMN, 2TMN, 4TMN, 5TMN, 6TMN) are usually docked correctly. Such behaviour would be expected since the heavy atom RMS of the active site of all the enzyme structures (see Table 2) is very low. Even so, there is still some variation particularly with the energies which often vary in the non-native docking by over 10 kJ/mol. The main reason for the variation is that the zinc atom in the enzymes moves a significant amount. In 2TMN, 4TMN, 5TMN, 6TMN and 5TMN the metal is in virtually the same position and this arrangement allows carboxylate groups to form a dual contact with the metal. In 4TLN, 3TMN, 1TLP and 1TMN, the metal is displaced from this position by 0.67, 0.68, 0.69

Table 5. Results from the all-pairs docking experiments on thrombin. For each docking run, the energy in kJ/mol of the lowest energy solution is given. Also given in parentheses is the heavy atom RMS (in Å) of the lowest energy solution compared to the crystal structure

Ligand PDB code	Receptor PDB code					
	1DWB	1DWD	1ETS	1ETR	1ETT	TMT1
1DWB	**−22.4**	−22.0	−22.7	−22.1	−23.7	−23.0
	(0.31)	(0.43)	(11.3)	(0.39)	(10.9)	(0.55)
1DWD	−40.4	**−46.4**	−46.9	−46.7	−44.1	−45.5
	(0.71)	**(0.71)**	(0.55)	(0.86)	(0.67)	(0.74)
1ETS	−40.3	−45.5	**−46.8**	−46.1	−44.0	−44.9
	(0.84)	(0.81)	**(0.58)**	(0.85)	(0.79)	(0.89)
1ETR	−36.2	−35.3	−31.7	**−40.9**	−33.9	−30.5
	(3.32)	(3.36)	(0.89)	**(0.81)**	(3.42)	(9.29)
1ETT	−34.9	−35.3	−34.8	−33.9	**−34.8**	−29.1
	(4.02)	(4.02)	(1.42)	(1.28)	**(0.48)**	(6.96)
TMT1	−34.2	−34.8	−34.3	−33.9	−34.9	**−37.9**
	(1.10)	(0.56)	(0.85)	(1.48)	(7.89)	**(0.54)**

Table 6. Results from the all-pairs docking experiments on thermolysin. For each docking run, the energy in kJ/mol of the lowest energy solution is given. Also given in parentheses is the heavy atom RMS (in Å) of the lowest energy solution compared to the crystal structure. For 1TMN, 4TMN, 5TMN and 6TMN, the RMS excludes atoms which lie entirely in solvent (see Figure 2)

Ligand PDB code	Receptor PDB code								
	1TMN	2TMN	3TMN	4TMN	5TMN	6TMN	4TLN	5TLN	1TLP
1TMN	**−49.3**	−52.9	−46.2	−54.7	−55.2	−55.0	−41.5	−47.6	−50.0
	(0.81)	(0.71)	(0.98)	(0.59)	(0.72)	(0.76)	(0.64)	(0.69)	(0.79)
2TMN	−29.9	**−35.9**	−31.9	−36.7	−38.0	−38.4	−26.5	−30.8	−32.6
	(0.89)	**(0.68)**	(1.03)	(0.88)	(0.90)	(0.90)	(0.80)	(0.72)	(0.74)
3TMN	−36.3	−38.5	**−37.8**	−38.3	−38.2	−38.3	−34.1	−36.9	−35.2
	(1.57)	(7.46)	**(1.50)**	(7.47)	(6.58)	(8.77)	(8.97)	(1.61)	(6.53)
4TMN	−44.0	−48.2	−42.1	**−52.6**	−52.1	−52.0	−38.1	−39.7	−42.2
	(0.59)	(0.62)	(0.91)	**(0.67)**	(0.50)	(0.87)	(0.91)	(0.43)	(0.48)
5TMN	−35.3	−44.2	−39.1	−46.6	**−46.0**	−47.2	−36.4	−44.0	−44.1
	(5.62)	(0.89)	(1.74)	(1.83)	**(1.22)**	(0.96)	(1.45)	(0.92)	(1.29)
6TMN	−36.5	−37.9	−31.5	−42.9	−41.6	**−42.9**	−30.6	−36.5	−39.0
	(0.83)	(1.32)	(1.75)	(1.51)	(1.41)	**(1.31)**	(4.80)	(0.93)	(0.89)
4TLN	−31.4	−31.5	−28.4	−31.7	−31.1	−30.4	**−28.3**	−28.1	−30.4
	(2.70)	(2.92)	(2.80)	(3.08)	(3.00)	(2.95)	**(2.92)**	(3.08)	(2.95)
5TLN	−33.6	−35.7	−29.8	−36.2	−36.5	−36.5	−30.4	**−33.2**	−36.5
	(6.95)	(6.18)	(2.04)	(6.36)	(6.37)	(6.26)	(7.01)	**(6.98)**	(6.26)
1TLP	−33.7	−44.1	−35.8	−44.3	−41.8	−45.2	−32.0	−38.1	**−41.6**
	(6.06)	(1.57)	(1.94)	(1.95)	(2.39)	(2.32)	(6.14)	(1.75)	**(1.33)**

Table 7. Results from the all-pairs docking experiments on neuraminidase. For each docking run, the energy in kJ/mol of the lowest energy solution is given. Also given in parentheses is the heavy atom RMS (in Å) of the lowest energy solution compared to the crystal structure

Ligand	Receptor PDB code					
PDB code	1INF	1INY	1IVE	1NNC	1NSC	1NSD
1INF	**−22.3**	−24.2	−25.6	−24.9	−29.4	−29.8
	(7.39)	(6.13)	(0.73)	(3.13)	(0.69)	(0.65)
1INY	−18.9	**−20.5**	−18.1	−21.7	−21.2	−22.2
	(6.72)	**(0.65)**	(0.84)	(3.82)	(1.55)	(4.85)
1IVE	−24.8	−27.8	**−27.8**	−28.7	−29.7	−29.3
	(2.55)	(1.79)	**(1.66)**	(2.27)	(1.90)	(1.90)
1NNC	−20.0	−22.2	−21.6	**−29.0**	−24.0	−23.5
	(6.69)	(3.35)	(1.35)	**(0.44)**	(0.57)	(0.40)
1NSC	−15.9	−14.5	−15.9	−19.0	**−22.2**	−19.9
	(8.01)	(2.01)	(7.15)	(5.72)	**(0.38)**	(0.49)
1NSD	−19.5	−21.2	−21.4	−25.5	−25.6	**−24.9**
	(6.01)	(1.56)	(1.12)	(0.45)	(0.60)	**(5.17)**

and 0.97 Å, respectively, and allows only one contact to form and restricts access to the metal in general. In some of the non-native dockings this movement is enough to prevent correct docking of the ligand.

No other shifts in enzyme structure could be categorically assigned as affecting docking performance. The thermolysin results are somewhat 'noisy' and it is clear from Table 3 that the number of correct solutions located is low even for favourable cases. The results would probably be improved by longer protocols. We also believe that the results would be improved by a better treatment of metal-ligand interactions and this remains a focus of our current work.

Results for neuraminidase non-native docking

The results for the native docking on neuraminidase generally are not as good as for the other two enzymes (see Table 4). The degradation in performance in going from native to non-native docking is similar to that observed with thrombin and thermolysin. The interpretation of the results is hampered by the small energy gaps between the correct conformation and false minima. This means that very small changes in enzyme structure can change the ranking of correct and incorrect ligand conformations. Nevertheless it is possible to identify some differences in the enzyme structures which limit the success of the non-native docking runs. The following features are found to affect the success of the results:

- The movement of sidechains to accommodate larger ligands. For example in 1INY the guanidine on Arg-373 moves back nearly an Ångstrom to accommodate the large phosphate group. The movement is mainly derived from a shift in the C^{α} atom of the residue.
- The movement of sidechains to form hydrogen bonds with one ligand but clashing with another ligand. For example in 1NNC, the movement of Asp-189 facilitates the formation of hydrogen bonds with guanidine on the ligand, but other enzyme structures have a different positioning for this residue which clashes with the correct ligand conformation for 1NNC. The movement is over an Ångstrom and is mainly derived from a shift in the Cα position for the residue.

Implications for docking

Most docking experiments take an enzyme-ligand crystallographic complex, remove the ligand and water molecules from the complex, and then attempt to dock the ligand flexibly back into the enzyme structure. Most docking experiments either do not treat the enzyme as flexible or treat very few degrees of freedom on the enzyme flexibly. This kind of 'native'

docking experiment introduces a bias into the docking experiment. Table 4 gives an indication how this bias improves the quality of the results for enzymes which are normally thought of as comparatively rigid. The results are not especially bad but it is clear that there is a significant bias. It would be extremely interesting to see how other docking methods perform on this kind of experiment, especially methods which use a partially flexible receptor. Unless all relevant degrees of freedom in the receptor are free in a docking test then methods which use a semi-flexible receptor will also be liable to significant bias in native docking experiments. In this study we have identified that the following movements in the enzyme structure can lead to misdocking using PRO_LEADS.

1. Movement of sidechains as a result of movement of the C^{α} atom either to adapt the size of an enzyme pocket or to form additional hydrogen bonds.
2. Movement of sidechains without movement of corresponding main chain atoms to obscure entry into pockets or to form hydrogen bonds.
3. Movement of metal atoms chelated in the active site.
4. Movement of flexible portions of main chains to facilitate the formation of hydrogen bonds to the protein backbone.

The importance of each of these terms is highly case dependent but in the three cases studied here, the more important structural changes would be quite difficult to incorporate in a simplified treatment of receptor flexibility. Only type-2 movement could be addressed by a simplified treatment of sidechain mobility and in our examples only the type-2 movement of Glu-192 in thrombin seriously affects the docking results. In the vast majority of trypsin-like serine proteases residue 192 is highly mobile but rarely forms hydrogen bonds with ligands and we routinely change the orientation of this residue in docking studies so that it cannot interfere with ligand binding in the S1 pocket. In our view this is a more reliable and practical way of dealing with this particular residue than a complex algorithm for incorporating receptor flexibility. The results in this paper would tend to suggest that for a flexible receptor docking method to be successful, it must try to take into account all the degrees of freedom listed above. It is a challenging theoretical problem to take into account so many potential degrees of freedom without proliferating the number of competing minima and decreasing their energy. It will also be difficult to restrict the enzyme movement to native-like conformations and to balance the penalisation of unfavoured but accessible receptor conformations. It is interesting to consider other strategies for minimizing the impact of receptor flexibility in docking studies.

One strategy, where multiple complexes are available, is to dock the ligand against all the enzyme structures or to use some kind of average structure for the docking studies [34]. Another useful approach would be to subject some of the docked solutions to minimisation and/or low temperature molecular dynamics using a molecular mechanics energy function. This would allow the complexes to relax and they could then be re-scored with the empirical scoring function. This procedure would work better if the relaxation of the enzyme structure could be integrated into the identification of the binding mode. A final strategy might be to change the scoring function so that it was more robust to small changes in the enzyme structure. Currently, a soft clash term is employed and making it softer would probably not help the identification of native-like binding modes. The lipophilic term is long-range and therefore already insensitive to small changes in enzyme structure. The hydrogen bond term is short range and is highly sensitive to the precise geometry of interaction. Our experience in adapting the hydrogen bond term and making it more tolerant of alternative geometries suggests that this leads to a large number of competing minima and has a detrimental effect on the RMS of the predicted solution relative to the crystallographically determined geometry. Perhaps the most promising changes to the scoring function would centre on trying to increase the energy gap between the correct solution and competing minima because this energy gap is correlated with robust docking performance.

Conclusions

This paper has applied a flexible docking program, PRO_LEADS, to thrombin, thermolysin and neuraminidase. In the native docking runs, PRO_LEADS successfully docks 76% of the complexes whereas only 49% of the docking runs are successful in the non-native docking runs. The degradation in performance can be ascribed to the movement of sidechains; the movement of flexible portions of main chains to facilitate the formation of hydrogen bonds and the movement of metal atoms bound to the enzyme active site. It is found that the sidechain movements responsible for mis-docking in these examples are usually

associated with the movement of the main chain C^{α} atoms. The results have implications on the type of strategies that might be adopted to take into account enzyme flexibility in docking studies.

The all-pairs docking experiments have shown themselves a useful tool in assessing the likely errors that can be expected from using a rigid enzyme structure and such experiments should prove a good tool in future for assessing the effectiveness of strategies for incorporating enzyme flexibility into docking methods. The conclusion is that PRO_LEADS can usually dock successfully if there is induced fit in the relatively rigid enzymes studied here but there remains the need to develop improved strategies for dealing with enzyme flexibility.

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