Ensemble Docking into Flexible Active Sites. Critical Evaluation of FlexE against JNK-3 and β-Secretase†

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One of the main complicating factors in structure-based drug design is the conformational rearrangement of the receptor upon ligand binding implicating protein flexibility as a crucial component in virtual screening. The FlexE approach allows flexibility through discrete alternative conformations of varying parts of the protein taken from structures having similar backbone traces. Here the performance of FlexE was tested against that of FlexX and FlexX-Pharm, by carrying out virtual screening experiments on two sets of structurally distinct complexes, for the enzymes β-secretase (BACE), and c-jun N-terminal kinase 3 (JNK-3). A large number of incompatible instances occurred between structural elements of the proteins thus loop movements could not be studied in JNK-3 as well as in BACE. The investigation of the side-chain flexibility revealed that at the most FlexE could achieve the enrichment yielded by FlexX in JNK-3 but not in BACE. Although limited side-chain variations (e.g. different protonation states) can be treated by FlexE, docking into protein ensembles remains a practical tool that decreases the average run time for a ligand.

INTRODUCTION

Significant growth in the number of potential therapeutic targets provided by the Human Genome Program has increased the demand for reliable target validation and lead identification. This process can be effectively supported by structure-based drug design tools as key components in the circuitous process of developing new drugs.[1] Objectives of structure-based drug design, especially that of virtual screening, implicate that the accuracy of the predicted geometries and affinities (scores) should be improved. Since protein flexibility is fundamental to understanding the principles of molecular recognition between the ligand and its receptor, it might have a dramatic impact on structure-based virtual screening. Consequently, consideration of receptor flexibility has been identified as a major challenge of structure-based virtual screening.

In most proteins conformational change occurs upon ligand binding, ranging from large-scale domain movements to small side-chain rotations. The following levels of flexibility can be considered during the molecular recognition: (1) large domain motions with extensive changes in backbone and side-chain conformations, (2) loop movements with partial backbone motions and side-chain movements, (3) side-chain conformational changes, and (4) nonsignificant conformational changes.

One way around the problem is to incorporate protein flexibility into the docking program. In contrast to the early docking algorithms which treated both the ligands and the protein as rigid most of the present methods treat the ligands as flexible and still keep the protein structure essentially rigid. Therefore ligands inducing larger changes in the protein conformation do not fit into rigid active sites.[2,3] Since several reports have already demonstrated the impact of protein flexibility in various virtual screening situations,[4−6] a number of efforts have been made to take the protein flexibility into account. In the recent years soft-docking,[7] partial side-chain flexibility,[8,9] continuous side-chain sampling,[10] or rotameric libraries[11] appeared to be the most promising approaches. Although incorporating side-chain flexibility was a straightforward step in all algorithms, current methods should go beyond this point to include backbone rearrangements at least to some extent.

Claussen et al. developed a new approach,[12] which considers multiple side-chain conformations and loop movements with the exception of domain movements. Their main idea behind developing FlexE was to describe the protein structure variations with a set of protein structures that encompassed the flexibility, mutations, or alternative models of the protein. FlexE is based on the united protein description created from the superimposed structures. The various structures are not only considered simultaneously but can also be combined to form a new combined structure. For the combined structure, similar parts of the proteins are merged, whereas dissimilar areas are treated as separate alternatives. FlexE is an extension of FlexX,[13] and concepts used in FlexX (incremental construction algorithm, interaction scheme, scoring function) are also adapted to FlexE. Due to the recombination of protein structures, dependencies between different alternatives in the united protein description might occur. Logical and geometric exclusion can cause dependencies resulting in incompatibility between structure elements of the protein. Valid protein structures are independent sets of alternatives in the incompatibility graph fulfilling certain constraints. During the incremental construction of the ligand optimal independent sets of alternatives with regard to the binding energy are determined for each ligand placement.

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Claussen et al. tested the FlexE using 10 protein structure ensembles containing 105 X-ray structures and one homology model with 60 ligands in total. Significant loop movements (maximal C_α displacement about 1.5 Å) were only investigated in aldose reductase (a loop of 8 residues), carboxypeptidase (a loop of 9 residues), and seryl tRNA synthetase (a loop of 10 residues). In all other proteins loop movements involved less than 5 residues. Three out of the 14 ligands of these proteins (21%) were docked with an rmsd (root-mean-square distance) below 2.0 Å on the first rank. Regarding the top 10 solutions 5 ligands (35%) were docked into the corresponding active site with an rmsd below 2.0 Å. The results of these docking calculations were claimed to be less satisfactory compared to those FlexE runs that involved X-ray structures with inflexible loops. Considering all the target proteins (10) the authors obtained a solution with an rmsd below 2.0 Å in the top 10 solutions for 67% of the test cases. This is comparable with 63% found in the merged ranking list of FlexX but significantly higher than that obtained for proteins with extended loop movements (35%). This discrepancy prompted us to investigate the performance of FlexE on active sites involving more than 5 residues in flexible loop regions.

It has been demonstrated in the original FlexE paper that this new approach successfully represented the protein flexibility (side-chain motions and slight backbone movements), point mutations, and homology models when docking a limited number of ligands. However, FlexE has never been investigated substantially for screening large databases, and its performance has also never been estimated in virtual screening situations. The only reported evaluation was performed by Steffen et al. when screening for inhibitors of cyclin-dependent kinase 2.[14] They first performed a redocking experiment and found that the FlexE/ScreenScore docking scheme could find only about one-third of the examined 8 ligand−protein complexes with a near native binding geometry (rmsd < 2.0 Å) on the first rank. Whereas a solution with an rmsd < 2.0 Å could be found for 75% of the top solutions when using FlexX, the resulting hit lists are subsequently merged. During the subsequent enrichment study 6011 ligands were docked. These authors found that enrichments in different ensembles could not even reach that yielded by FlexX and stated that the enrichment declined sharply with an increasing number of structures in the ensemble.[14]

Here we report a comparative study describing the performance of docking into protein ensembles by FlexE. Since FlexE is incorporated in the FlexX Suite, which is a popular and successful docking algorithm, we aimed to test its applicability in virtual screening situations.[13] We mapped how flexible loops could be involved in an ensemble protein avoiding many incompatible instances occurring between the structural elements of the proteins. Although the application of pharmacophoric constraints is actually not available in FlexE, we also demonstrate the improvements achieved relative to FlexX in our virtual screening studies using FlexX-Pharm.[15]

Our target proteins were c-jun N-terminal kinase 3 (JNK-3) and β-secretase (BACE). JNK-3 undergoes loop rearrangements upon ligand binding. The movements of the glycine rich loop (71−78) and the motions of side chains were first investigated in our virtual screening protocol using FlexE. In practice we found that this loop movement (maximal C_α distance 7.6 Å) could not be handled with FlexE. On the other hand, FlexE with an ensemble involving exclusively those X-ray structures that are free from loop movements provided only a combination of the results given by FlexX. The best enrichment factor given by FlexE (EF=13) was on a par with the enrichment factor given by FlexX (EF=13).

Table 1. Publicly Available X-ray Structures of c-jun N-Terminal Kinase-3 (JNK-3)

PDB code	resolution (Å)	residues
1JNK	2.3	45−211
		217−373
		379−400
1PMN	2.2	45−211
		217−371
		379−400
1PMQ	2.2	45−212
		217−374
		379−400
1PMU	2.7	45−213
		222−374
		381−400
1PMV	2.5	45−211
		217−374
		379−400

BACE also undergoes rearrangements upon ligand binding. We attempted to investigate first the effect of these movements by FlexE. The effect of various protonation states at catalytic aspartates (Asp-32, Asp-228) of β-secretase has already been studied using FlexX and FlexX-Pharm,[16] and the relevant protonation scheme (Asp-32 protonated and Asp228 ionized) was applied here. Similarly to JNK-3 FlexE has failed to run with ensembles containing loops with a maximal C_α distance of about 7 Å. We show that the results of FlexE (the best EF=22) are far behind those of FlexX (the best EF=43).[16]

FlexE was not able to handle extended loop movements (maximal C_α distance of about 7−7.6 Å) either in BACE or in JNK-3 but proved to be a more practical and faster tool in handling very similar structures simultaneously. Based on our results, the scope of the FlexE approach, its limitations, and possibilities for the future improvements of screening large databases should be discussed.

RESULTS AND DISCUSSION

Analyzing the X-ray Structures. *JNK-3.* Five publicly available X-ray structures were analyzed regarding loop movements and side-chain conformations before virtual screening (Table 1); these comprised 1JNK complexed with 5′-adenylimido-triphosphate,[17] 1PMN with an imidazole-pyrimidine inhibitor, 1PMQ with an imidazole-pyrimidine inhibitor, 1PMU with a phenanthroline inhibitor, and 1PMV with a dihydroanthrapyrazole inhibitor.[18]

The N-terminal domain of JNK-3 (residues 45−149, 379−400) contains mainly β strands, while the C-terminal domain (residues 150−211 and 217−374) is α helical. Between the two domains a deep cleft comprises the ATP-binding site where a glycine rich sequence/loop (71−78) forms a well-defined β-strand-turn-β-strand structure. This loop adopts a significantly different conformation in 1PMU with the maximal C_α displacement of 7.6 Å relative to all of the other structures (Figure 1). In 1PMU the bound inhibitor makes

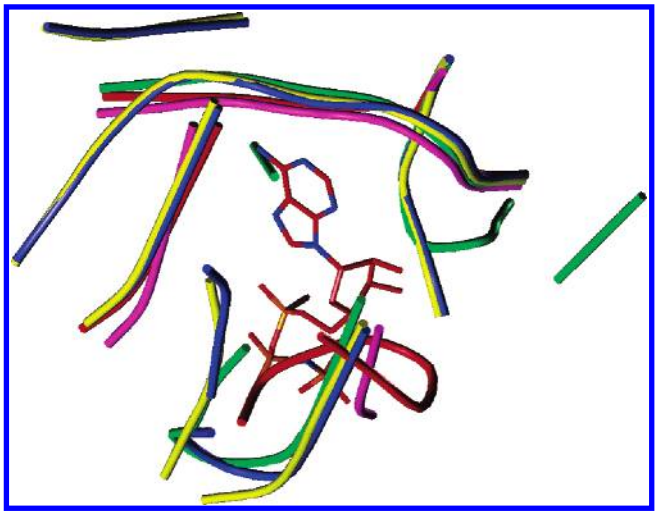


Figure 1. Ribbon representation of the active site of JNK-3. 1JNK, yellow; 1PMN, blue; 1PMQ, green; 1PMU, red; and 1PMV, magenta. 1PMU adopts a different conformation in residues 71−78 (Gly rich loop).

Table 2. Publicly Available X-ray Structures of β-Secretase (BACE)

PDB code	resolution (Å)	residues
1FKN	1.9	1−385
1SGZ	2.0	1−385
1W50	1.75	43−453
1W51	2.55	43−453
1TQF	1.8	43−446
1M4H	2.1	43−385
1XN2	1.9	1−385
1XN3	2.0	1−385
1XS7	2.8	29−385

four H-bonds, one with Met-149, two with Gln-155, and one with Gln-75. This latter interaction is made possible only by a dramatic change in the conformation of the Gly rich loop. 1PMN, 1PMQ, 1PMV, and 1JNK have very similar backbones. The overall root-mean-square distance (rmsd) for all C_α atoms is 0.8 ± 0.2 Å suggesting that these X-ray structures are free from large domain or loop movements.

Side-chain conformations show significant differences among the five X-ray structures. The superposition of the side-chain atoms resulted in an rmsd greater than 2.0 Å for residues 107, 115, 124, 146, 147, 152, and 155 apart from the glycine rich sequence (71−78). The impact of these various side-chain conformations on ligand binding was studied using the FlexE approach.

β-Secretase. To date nine X-ray structures are publicly available in the PDB database. Seven of them are complexed with either a peptidomimetic or small molecule inhibitor. Detailed comparison of the ligand bound and ligand free X-ray structures suggests that BACE can adopt at least two major conformations.[16] Apostructures represent the open form (1SGZ,[19] 1W50[20]), while 1W51,[20] 1FKN,[21] 1M4H,[22] 1TQF,[23] 1XS7,[24] 1XN2,[25] and 1XN3[25] are ligand bound, closed conformations (Table 2).

First we investigated the ligand-bound X-ray structures (Figure 2a and 2b) representing two types; 1FKN, 1M4H, 1XN2, 1XN3, and 1XS7 contain peptidomimetic inhibitors, while 1W51 and 1TQF are crystallized with small molecule inhibitors. The conformational behavior of the so-called FLAP loop (residues 68−74) is similar for both types of

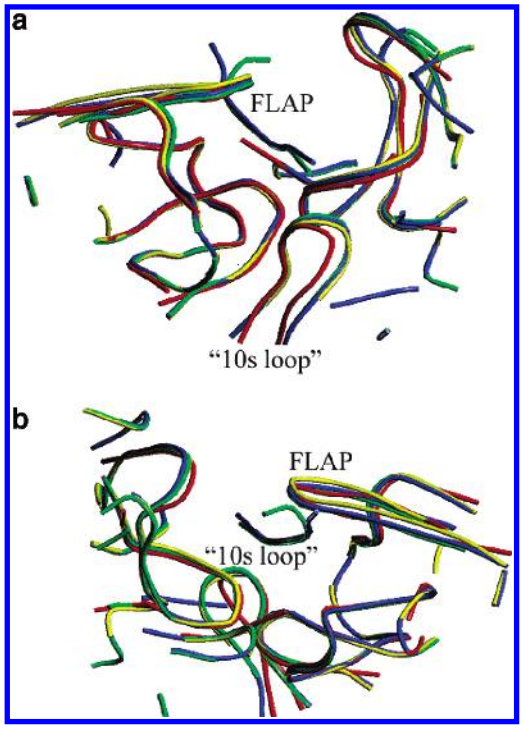


Figure 2. a. Ribbon representation of the active site of BACE. Only 1W51 (colored red) differs in conformation of "10s loop" from the others. b. Ribbon representation of the active site of BACE. Part a is rotated by 180°. Only 1W51 (colored red) differs in conformation of "10s loop" from the others.

complexes. The FLAP region adopts a closed conformation in each of the structures, which is a characteristic feature of the ligand bound structures. In fact 1FKN is very similar to 1M4H (overall rmsd for all atoms is 0.66 Å) and 1XS7 (overall rmsd for all atoms is 0.723 Å). Superposition of these three structures revealed that they have similar backbone traces without any large loop or domain movements; however, these structures demonstrate different side-chain conformations. 1XN2 and 1XN3 were released after the completion of our virtual screening study against β-secretase; therefore, these two structures were only analyzed. Although these latter structures are very similar to each other (overall rmsd for all atoms is 0.548 Å), and to 1FKN (overall rmsd for all atoms is 0.7 Å and 0.823 Å for 1XN2 and 1XN3, respectively), interestingly, chains A and B of 1XN3 adopt an open conformation similar to that of the apo structures 1SGZ and 1W50. The open conformation of chains A and B in 1XN3 was suspected to be a direct consequence of the poor solubility of the inhibitor.[25] Although only chain C is complexed with a ligand, chain D adopts a closed conformation as well.

The conformation of the "10s loop" (residues 9−14) is open in 1FKN, 1M4H, 1XN2, 1XS7, and 1SGZ, which all complex peptidomimetic inhibitors (type I complexes). 1TQF and 1W51 contain small molecule inhibitors (type II complexes) having the "10s loop" in closed conformation with the maximal displacement of 6 Å and 5 Å in 1TQF and 1W51, respectively, as compared to 1FKN.

Apo structures (1SGZ and 1W50) are rather similar; the weighted rmsd for all the atoms is 1.48 Å. Regarding the flexible loops, the FLAP region (68−74) has an open conformation in both structures with maximal C_α displacement of around 7 Å compared to ligand-bound structures.

The conformation of the "10s loop" is somewhat different in 1W50; the maximal C_α displacement between the "10s loops" of 1SGZ and 1W50 is around 4.5 Å.

In summary, 1FKN, 1M4H, 1XS7, 1XN2, and 1XN3 (chains C and D) share a common backbone trace with a similar FLAP and the "10s loop" conformation. 1TQF and 1W51 differ from this group of five in the conformation of "10s loop". In 1SGZ the FLAP region adopts an open form and a similar "10s loop" conformation to the group of five structures. 1W50 has a different conformation in the FLAP region and the "10s loop" when compared to the group of five. Analysis of available X-ray structures revealed that the conformation of the FLAP region varies upon ligand binding, while the impact of the "10s loop" movements should be further investigated in subsequent X-ray studies.

Enrichment Studies. The effectiveness of different screening protocols has been investigated in enrichment studies aimed at identifying known actives from screening libraries. Enrichment factors were used to assess the quality of the rankings in each docking run

$$\mathrm{EF}(\%) = \frac{(N_{\mathrm{active}(\%)}/N_{(\%)})}{(N_{\mathrm{active}}/N_{\mathrm{all}})}$$

where EF(%) is given at the % percentage of the ranked database, $N_{\mathrm{active}(\%)}$ is the number of active compounds in a selected subset of the ranked database, $N_{(\%)}$ is the number of compounds in the subset, and N_{active} and N_{all} are the number of active molecules and the number of compounds in the screening database. Enrichment factors were not scaled, i.e., absolute values depend on the ratio of active and inactive molecules and should be compared to the maximum (ideal) achievable EFs. EFs were calculated at 1% of the ranked database as a fixed threshold that enables the comparison of enrichment factors when studying the effectiveness of different screening protocols. EFs at 2%, 5%, and 10% of the ranked database have also been calculated for the best scoring scheme given at 1% of the database. All the enrichment factors are available in the Supporting Information.

Thirty poses were saved from each FlexX, FlexX-Pharm, or FlexE docking run. All poses were scored using five different scoring functions. The best pose for each ligand was selected according to each scoring function separately, to generate a set of poses per scoring function. Each of these sets was then ranked using each of the five scoring functions in turn.

Before virtual screening the molecular weight profiles of the active and inactive compounds were compared. The molecular weight distribution of the JNK-3 inhibitors (mean: 146, SD: 65) is similar in shape to that of the inactive molecules (mean: 335, SD: 95), while the mean value is less for the active than the inactive ones. Smaller compounds generally form fewer interactions and consequently tend to be ranked unfavorably. This might reduce the EFs rather than producing artificial enrichment. The molecular weight distribution of the BACE inhibitors (mean: 570, SD: 111) is similar in shape to that of the inactive molecules (mean: 348, SD: 101). BACE inhibitors are mainly large peptidomimetics or nonpeptidomimetic compounds with a relatively large molecular weight compared to e.g. known drugs. Thus artificial enrichment[26] cannot be ruled out in this case. Nevertheless, the best screening protocols are subsequently evaluated in a comparative study that allowed us to compare the effectiveness of different screening conditions.

The statistical significance of the calculated enrichment factors was investigated taking repeated random samples (12500 times) from the active and inactive test set (preserving the ratio of active and inactive molecules). The results of the analysis are given in the Supporting Information (Table 28).

Virtual Screening for c-jun N-Terminal Kinase-3 Inhibitors. *FlexX*. For 1PMN and 1PMV structures ranking by FlexX and Dock scores yielded the highest enrichment factor (EF=11), for 1JNK the ChemScore beats the others (EF=13), while for 1PMQ Dock, Gold-, and ChemScores (EF=13) ranked the extracted poses the best (Figure 3). The maximal enrichment factor of 21 was achieved by docking into 1PMU and ranking extracted poses by GoldScore or FlexX score. In pose selection PMF and GoldScores outperformed the others for 1PMN and 1PMV. Using 1JNK and 1PMQ structures the PMF score extracted poses the best, while for 1PMU the best enrichment factor was observed when poses were extracted by Dock or GoldScores.

Docking into four (1PMN, 1PMQ, 1PMV, 1JNK) of the five X-ray structures showed very similar performances in virtual screening situations. These structures represent a very similar backbone trace with variable side-chain conformations. This variability in side-chain conformations might be the reason for the slight changes in the EFs and also for the limited overlap of hit lists obtained for the top 1% of the ranked database for the different structures. We assumed that an ensemble involving all the possible side-chain conformations and their combinations can improve the success rate of our virtual screening study because FlexE should find all the actives that the individual FlexX runs identified.

A possible explanation for the higher enrichment obtained in the case of 1PMU might be the similarity of the actives to the cocrystallized ligand of this structure. Low Tanimoto similarity indices (Table 24 in Supporting Information), however, demonstrate that active molecules are dissimilar from each other and from the phenanthroline inhibitor of 1PMU (molecule 20). This particular case can be explained by the unique conformation of the glycine rich loop that facilitates binding due to additional protein interactions (mainly H-bonds) that results in better scores.

FlexX-Pharm. Introduction of pharmacophore constraints increased the enrichment factors relative to those given by FlexX (Figure 4). Using 1PMN structure the Gold and FlexX scoring combination gave an enrichment factor of 31, which is almost treble the EF given by FlexX (EF=11). For 1PMQ the maximal enrichment factor of 17 was achieved when poses were selected by PMF and ranked by Dock score. The best two enrichment factors were obtained when compounds were docked into 1PMV or 1PMU structures. In the case of 1PMV, Dock and GoldScores were equally effective in pose selection, while ranking was best performed by only ChemScore (EF=42). For 1PMU the best enrichment factor was observed when poses were selected by Dock, PMF, GoldScore, or FlexX score and were ranked by Chem- or FlexX scores (EF=57). The 1JNK structure has proved to be the poorest target because no enrichment was observed for 21 of the possible 25 scoring combinations. Dock, Gold-,

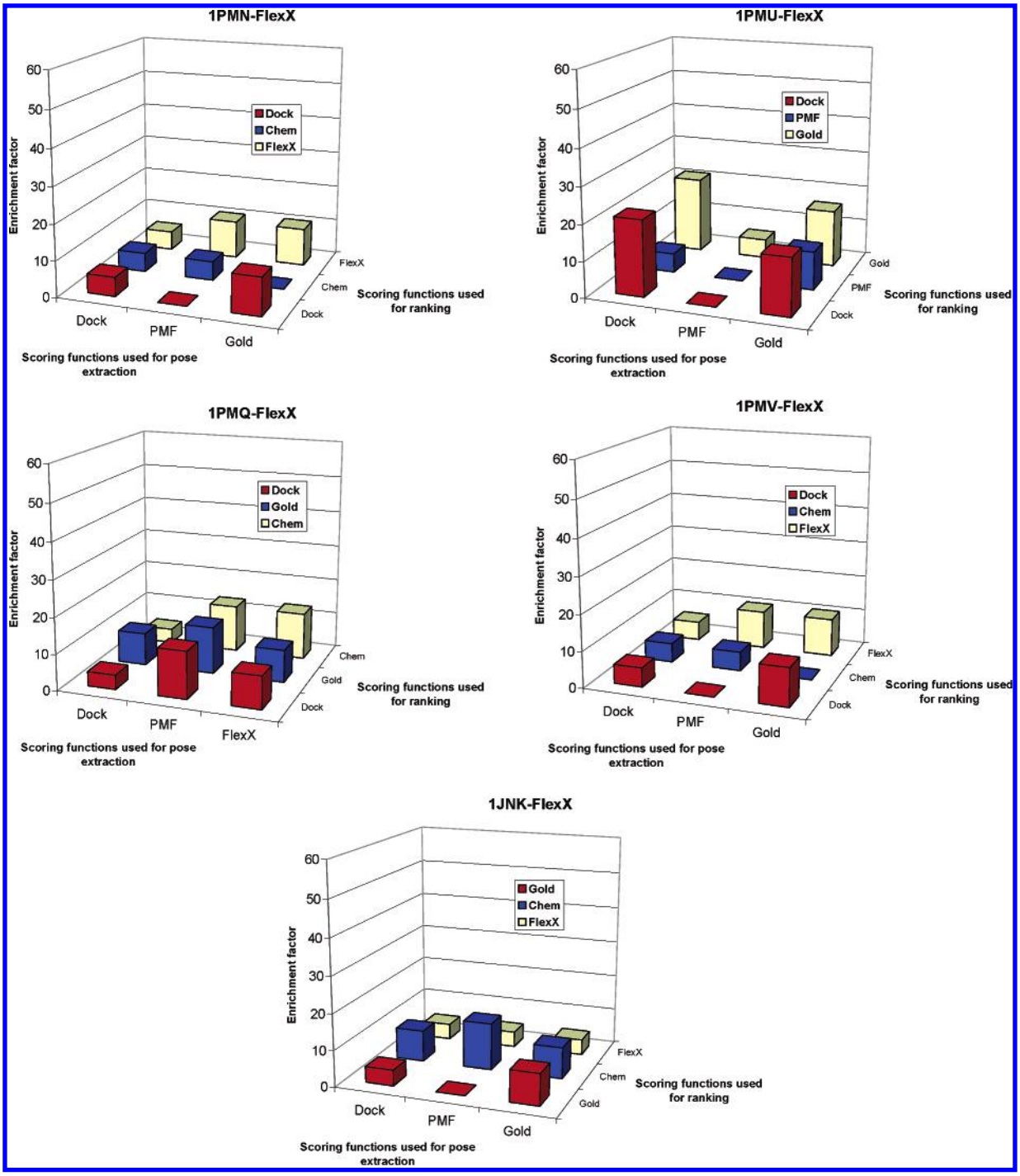


Figure 3. Enrichment factors for JNK-3 as calculated at the top 1% of the ranked database. Docking calculations were performed using FlexX.

Chem-, and FlexX scores selected poses, and only ranking by Dock score resulted in a satisfactory enrichment factor of 16. Introduction of pharmacophore constraints improved the enrichment for each of the five structures because the number of the possible poses was constricted and the inactive drop-out rate was increased.

Enrichment factors for 1PMU outperform the others significantly. In 1PMU the ligand binding is realized mainly through H-bonds, while in the other cases hydrophobic interactions play an important role as well. These latter types of interactions are not so well defined within the active site, and they do not provide such a strict constraint during docking studies. Consequently, this type of interaction is less suitable for defining pharmacophoric constraints and cannot drive docking as accurately as constraints for H-bonds.

FlexE. 1PMN, 1PMQ, 1PMU, 1PMV, and 1JNK X-ray structures were aligned by homology (Figure 1). The alternative loop conformation in 1PMU caused many incompatible instances between structural elements of the protein during the FlexE run, thus docking calculations with an ensemble of the five X-ray structures failed to run. Technically these runs could not be executed. Therefore ensembles of 1PMN, 1PMQ, 1PMV, 1JNK and 1PMN, 1PMQ, and 1PMV were prepared. The saved poses were then scored using the Cscore module of Sybyl 7.0. This

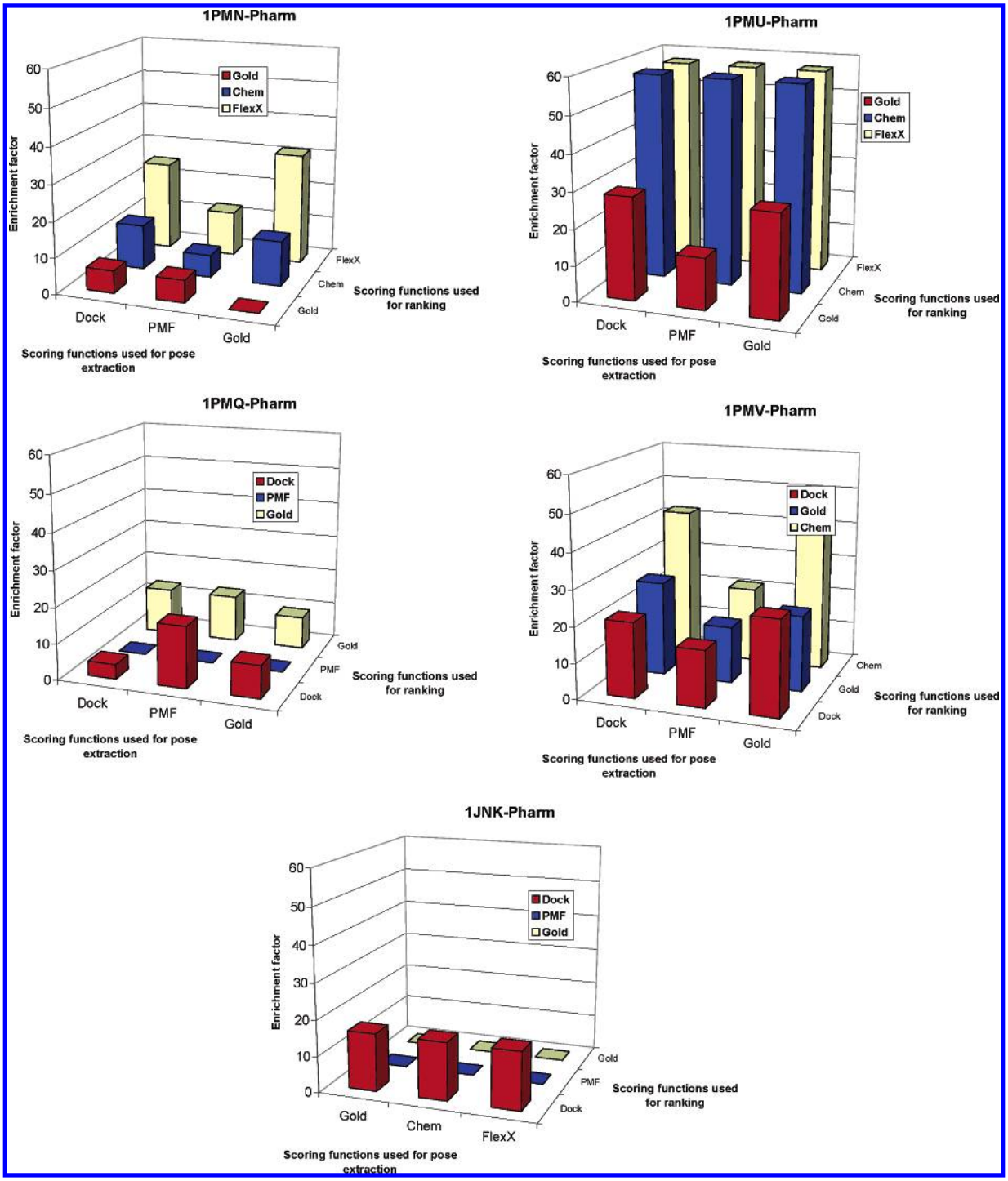


Figure 4. Enrichment factors for JNK-3 as calculated at the top 1% of the ranked database. Docking calculations were performed using FlexX-Pharm.

version of Sybyl did not allow the use of pharmacophoric constraints in combination with FlexE so the impact of the pharmacophore constraints on the FlexE runs could not be investigated. Docking into an ensemble of three X-ray structures (1PMN, 1PMQ, 1PMV) resulted in a maximal enrichment of 4 (Figure 5), which is far below any of the enrichment factors given by FlexX. Thus we made an attempt to improve our enrichment by incorporating a new X-ray structure (1JNK) in the protein ensemble increasing the side-chain variability. In this case the maximal enrichment was achieved when selecting poses by PMF score and ranking them by ChemScore (EF=13).

In our hand, FlexE failed to run on the protein ensemble containing a structure having the alternative loop conformation with a maximal C_α displacement of about 7.6 Å relative to other structures. Therefore our investigation was restricted to side-chain flexibility using three- and four-membered ensembles. The best result of FlexE was the reproduction of the enrichment given by FlexX, and this was limited to the four-membered ensemble. Considering all the possible side-chain combinations from four structures FlexE could not find all the actives identified by FlexX runs performed on the individual structures. Our experience suggests that using protein structure variations in FlexE saves significant com-

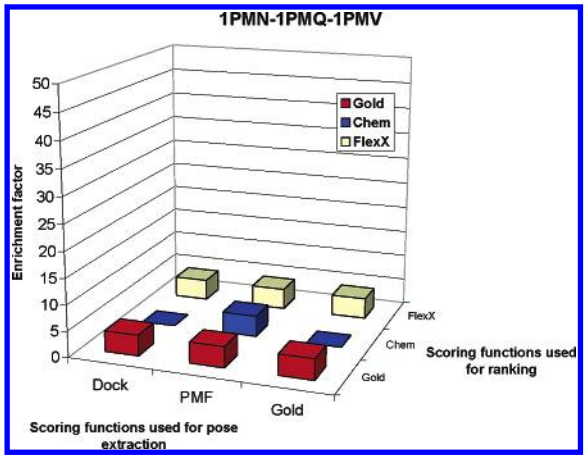


Figure 5. Enrichment factors for JNK-3 as calculated at the top 1% of the ranked database. Docking calculations were performed using FlexE.

Table 3. Average Run Time for One Ligand

PDB code	FlexX-single	FlexX-total	FlexE
1PMN-1PMV-1PMQ	61 s	190 s	82 s
1JNK-1PMN-1PMV-1PMQ	63 s	261 s	120 s
1FKN-1W51-1M4H	52 s	156 s	115s
1FKN-1W51-1M4H-1XS7	49 s	196 s	125 s

putational time (Table 3) but does not necessarily improve the enrichment.

Enrichment factors for the best scoring schemes were calculated at the top 2%, 5%, and 10% of the ranked database (Supporting Information), but the declined enrichment suggests, in this particular case, 1% as the best cutoff for EF calculations.

Virtual Screening for β-Secretase Inhibitors. In our previous investigation we reported a comparative enrichment study using docking algorithms FlexX and FlexX-Pharm on 1FKN and 1SGZ structures.[16] We showed that protonation states of the catalytic residues (Asp-32 and Asp-228) have a major impact on docking results. Asp-32 was found to be preferably protonated, while Asp-228 was ionized so these protonation states were applied here. 1FKN, a ligand bound structure, proved to be a better target compared to 1SGZ (apo structure) for virtual screening. Comparative structural analysis of the available BACE structures showed that the enzyme has flexible chains in the active site (residues ~68−74 and 9−14), thus both the effects of the loop movements and the side-chain variations can be studied by FlexE.

Five X-ray structures, 1FKN, 1SGZ, 1W51, 1XS7, and 1M4H, were selected on the basis of FLAP and "10s loop" conformations for this study. 1XN2 and 1XN3 were omitted because the late release of the X-ray structures that was further supported by the strange loop conformations explained by the suboptimal solubility of the inhibitor crystallized in 1XN2.

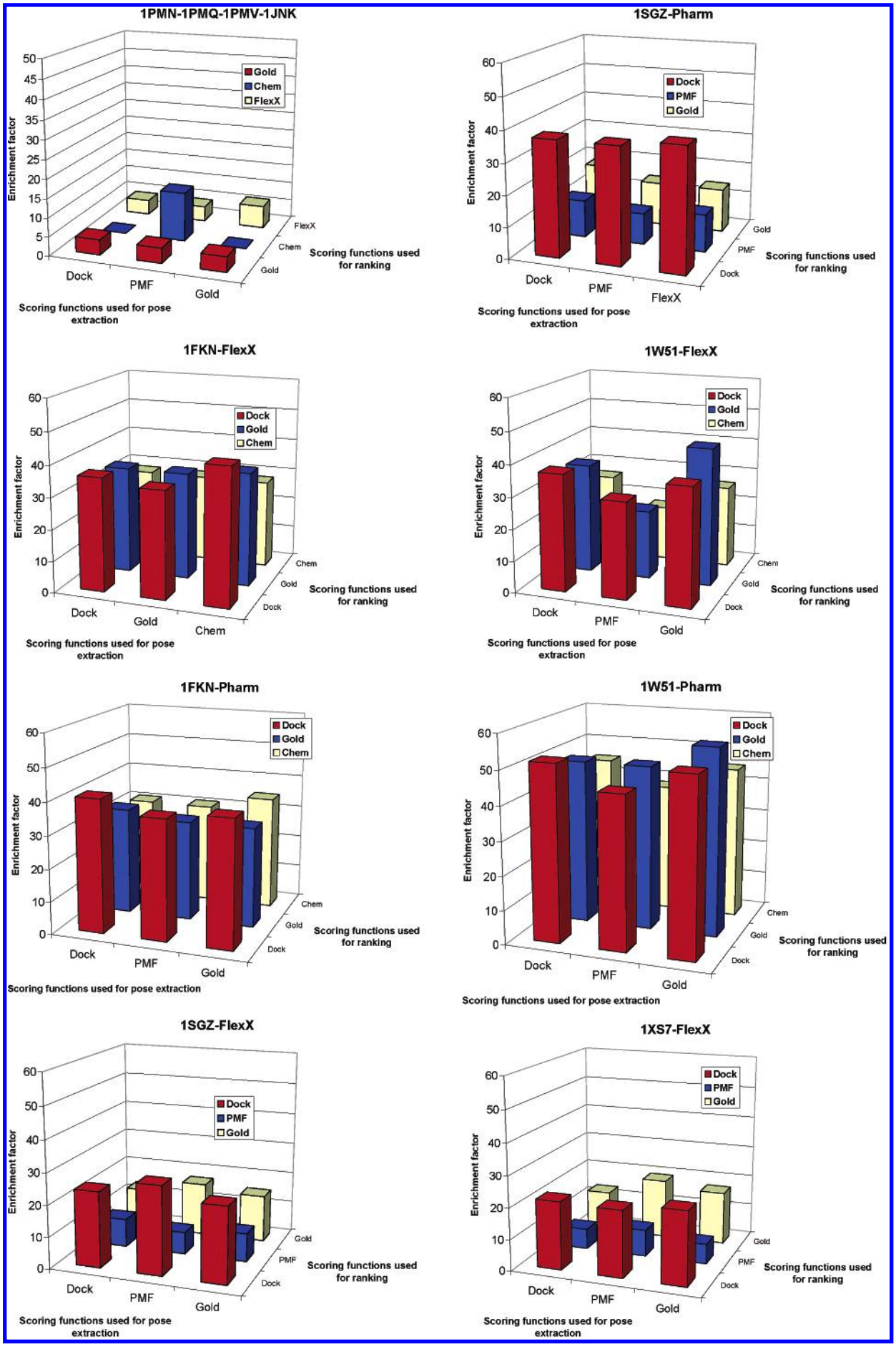
FlexX. Using the apo structure (1SGZ) PMF extracted the poses the best, while Dock score was found to be optimal for ranking yielding an enrichment factor of 28. In the case of 1FKN, the extracted poses by ChemScore were best ranked by Dock score (EF=43) (Figure 6). For 1W51 the best pose extraction and ranking was achieved by the Gold scoring scheme yielding the best EF of 43. When docking into 1XS7 PMF, Gold- and ChemScores extracted poses the best. Dock-, Gold-, and ChemScore ranked the extracted poses resulting an enrichment factor of 23. For 1M4H the PMF/Dock scoring combination gave an EF of 40.

In summary, we obtained reasonable enrichment factors for five protein conformations. The exact placement of the given pose was found to be crucial for ranking. FlexX often finds solutions on the outer surface of the active site and therefore could not always provide sufficiently accurate poses. In fact, these solutions reduced our enrichment. Protonation states of catalytic residues played an important role in positioning and scoring ligands.[16]

FlexX-Pharm. FlexX-Pharm constricted the number of possible poses and increased the inactive drop-out rate. The improvement was significant for 1SGZ but not for the 1FKN structure. Ranking the poses by Dock scores gave the best results for 1FKN (Figure 7). Docking with FlexX-Pharm facilitated the formation of functionally characteristic interactions. Since 1FKN adopts a ligand binding conformation, inhibitors can fit into the preformed active site precisely, and relevant interactions can form readily. In 1SGZ these contacts cannot be formed because its more open active site may preclude the formation of vital interactions. Additionally more false positive molecules that were placed outside the active site of 1FKN were filtered out by FlexX-Pharm. Because of the larger and more open cavity of 1SGZ less false positive compounds were docked on the outer surface of the active site by FlexX, and, consequently, fewer compounds were dropped out by FlexX-Pharm. The impact of pharmacophore constraints was also investigated on docking into 1W51, 1M4H and 1XS7. Using the Gold/Gold scoring combination on 1W51 we observed a significant improvement in enrichment (EF=55). For 1XS7 the Gold/Dock scoring combination yields an EF of 41. Docking into 1M4H the PMF/Dock scoring combination resulted in an improved enrichment (EF=42). Although both FlexX and FlexX-Pharm resulted in reasonable enrichment, a somewhat improved performance of FlexX-Pharm over FlexX could be observed.

FlexE. An ensemble of three (1FKN, 1W51, 1M4H) and four (1FKN, 1W51, 1M4H, 1XS7) X-ray structures was prepared and screened against the screening database. Similarly to JNK-3 the incorporation of the open structure (1SGZ) with an altered loop conformation caused too many incompatible instances, thus these runs could not be executed. Open structures were therefore excluded from further ensembles. The maximal enrichment factor for the ensemble of three structures was achieved with a Gold/Gold pose selecting and ranking combination that resulted in an enrichment factor of 27. Screening with an ensemble of 1FKN, 1W51, 1M4H, and 1XS7 gave the maximal enrichment factor of 22 that was achieved by the PMF/Chem scoring combination. In conclusion, using FlexE the enrichments could not outperform or emulate the individual FlexX runs. Our studies demonstrate that incorporating only side-chain flexibility by FlexE is not sufficient for finding the correct binding mode. Enrichment factors for the best scoring schemes were calculated at the top 2%, 5%, and 10% of the ranked database (Supporting Information). Since the enrichment declined in all cases, we suggest, in this particular case, 1% as the best cutoff for EF calculations.

The effect of loop movements and side-chain flexibility was studied on two target proteins. Claussen et al. studied loop movements on three proteins which is comparable to


1PMN-1PMQ-1PMV-1JNK
Enrichment factor
Gold
Chem
FlexX
Scoring functions used for ranking
Scoring functions used for pose extraction
1SGZ-Pharm
Dock
PMF
Gold
FlexX
1FKN-FlexX
Chem
1W51-FlexX
1FKN-Pharm
1W51-Pharm
1SGZ-FlexX
1XS7-FlexX

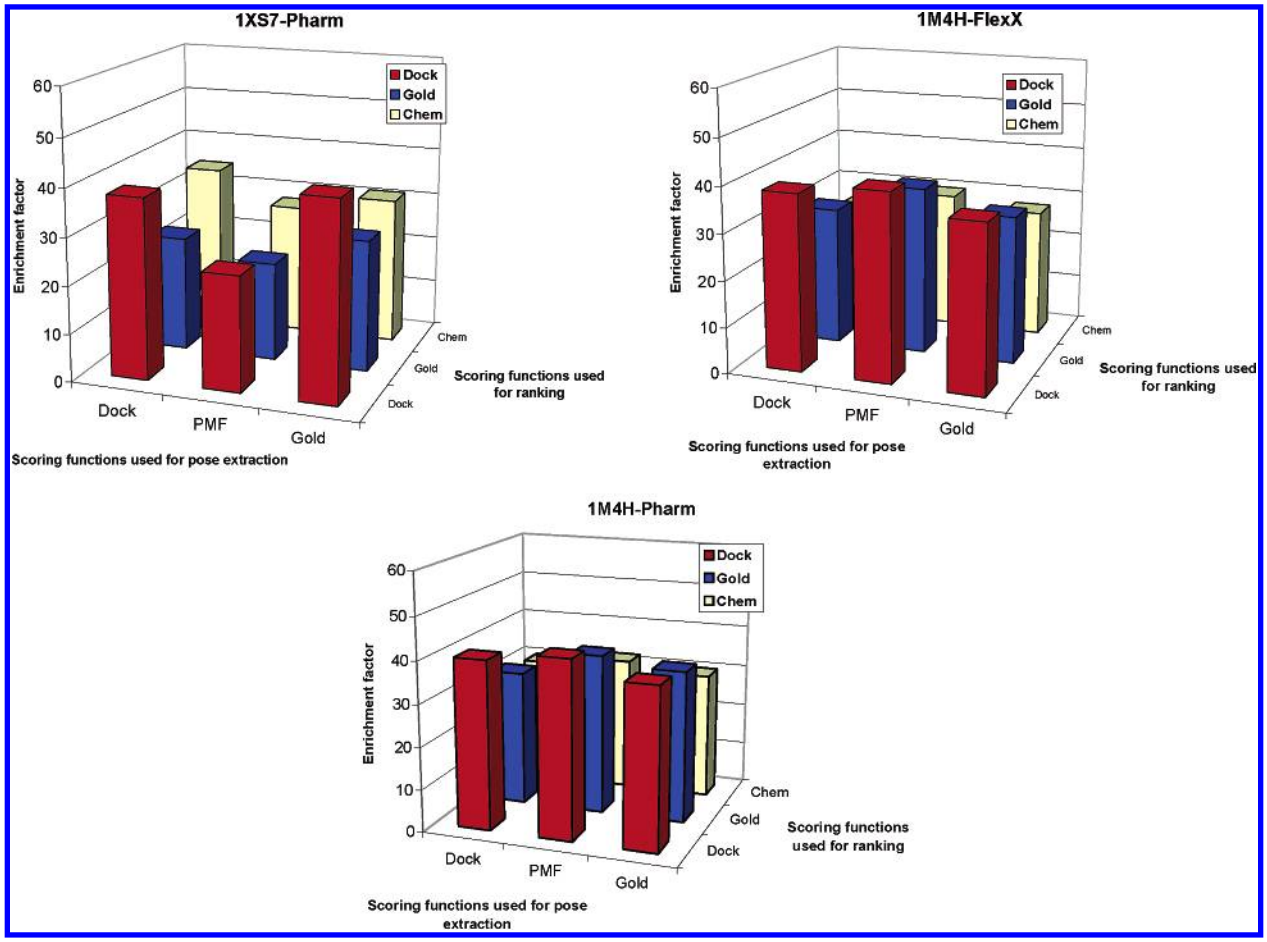


Figure 6. Enrichment factors for 1SGZ, 1FKN, 1W51, 1XS7, and 1M4H as calculated at the top 1% of the ranked database. Docking calculations were performed using FlexX and FlexX-Pharm. Asp32 were protonated and Asp228 were ionized in all runs.

our study with two target proteins. Both of our loop investigations failed because of the vast number of incompatible instances between structural elements of the proteins. The loop movements in Claussen's study represented a maximum C_α distance of around 1.5 Å, whereas loop movements in BACE or JNK-3 reached the maximal C_α distance of around 7−7.6 Å. It is interesting to note, however, that we were able to incorporate 1W51 into the protein ensemble having the FLAP and "10s loop" conformation with a maximal C_α distance of around 2.5 Å. Therefore loop movements up to 2.5 Å $C_\alpha-C_\alpha$ distances can be successfully studied by FlexE that fails for larger $C_\alpha-C_\alpha$ distances (around 7 Å). Investigating different side-chain conformations using the ensemble approach, either resulted in enrichment comparable to or lower than that of FlexX. For these two particular cases FlexE provided poorer results than FlexX, and this is in line with the finding of Steffen et al.[14] These results suggest that incorporating only side-chain flexibility and even slight loop movements cannot make a significant improvement in the success rate of virtual screening.

However, worthy of note that FlexE can be a useful docking tool for investigating structures with limited conformational mobility. One of the potential applications of FlexE is testing the effects of the protonation states considering different protonation schemes in the same structure simultaneously. During docking into BACE the coordinates of the catalytic residues were kept separate with unique charges: Asp32-H and Asp228-, Asp32-H and Asp228-H, Asp32- and Asp228-, Asp32- and Asp228-H. Our studies demonstrated that 100% of the active molecules were docked in a structure containing a protonated Asp-32 and ionized Asp-228. The enrichment factors were comparable to those given by FlexX, when assigning the correct (Asp-32 protonated) protonation state.[16]

In the one hand, the greatest advantage of FlexE was clearly the decreased run times as compared to individual FlexX runs. Table 3 summarizes the run times needed for different screening protocols. CPU times were measured on an Intel Xeon MP CPU 2.50 GHz processor with 3 GB RAM using FlexX, FlexX-Pharm, and FlexE. Comparing accumulated run times of FlexX for all structures in a given ensemble to that of single FlexE runs revealed ensemble docking significantly faster. Two of the four FlexE calculations were rerun saving 60 poses, which is double the quantity of the poses saved during all of our studies. The enrichment factors calculated at 1% of the ranked database and the average run times are collected in Tables 25−27 (Supporting Information). The results further support our findings that FlexE still provides similar or lower enrichment even with doubled sampling space.

In our previous publication[29] a virtual screening study on glycogen synthase kinase 3β (GSK-3β) has been described using both the FlexX and FlexE docking algorithms. With this case only side-chain conformational changes were investigated by FlexE. The enrichment factors calculated at 1% of the ranked database are collected in Table 27 (Supporting Information). Structural analysis and the devel-

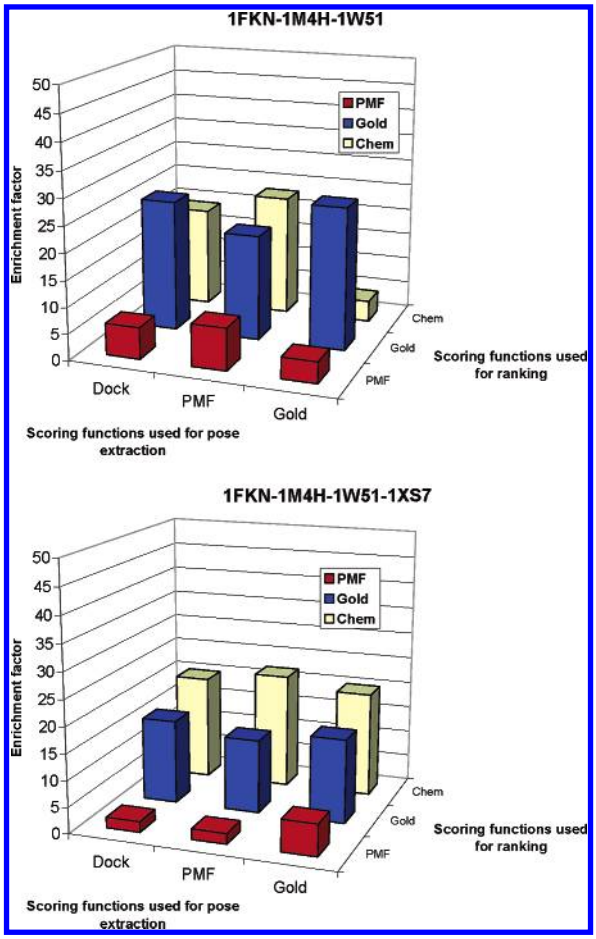


Figure 7. Enrichment factors for BACE as calculated at the top 1% of the ranked database. Docking calculations were performed using FlexE.

opment of the virtual screening protocol are fully detailed elsewhere.[29] Since here we recall this study as a further example supporting our findings, only the results of the enrichment studies are discussed. Virtual screens were performed for 1UV5, 1Q3D, and 1Q4L X-ray structures using FlexX and FlexE. The best enrichment factor (18) was achieved using FlexX for 1Q4L, when poses were extracted with PMF, Dock, or FlexX score and ranked by GoldScore. Docking into an ensemble comprising 1UV5, 1Q3D, and 1Q4L X-ray structures the Dock/GoldScore combination provided the best enrichment factor (14). Again, FlexE could not outperform the performance of FlexX. The average run time of FlexE is about a factor of 2 lower than the accumulated time needed by FlexX for placing one ligand into the active site.

CONCLUSIONS

In this study we investigated the performance of FlexE in virtual screening against protein active sites with moderate to large loop movements and side-chain movements. Consideration of loops with large maximal C_α distance (around 7 Å) failed because of the vast number of incompatible instances between structural elements of the proteins. Moderate loop movements represented by the maximum C_α distance of around 2.5 Å could be considered by FlexE; however, enrichments were lower than that obtained by individual FlexX runs.

FlexE could be used to investigate side-chain variations when docking to protein ensembles. Since conformations of side chains were extracted from X-ray structures, this solution could not be considered as a realistic treatment of side-chain flexibility, where all the possible conformations can be formed; only a group of the possible conformations was tested. Backbone movements were also not considered here. We found that FlexE cannot provide a good approximation for the problem of protein flexibility. Nonetheless FlexE was successful at treating limited side-chain variations and therefore docking into protein ensembles with a decreased average run time for each ligand, although in none of the cases (for JNK-3, BACE, and GSK-3β) did FlexE outperform FlexX.

METHODS

Preparing Protein Structures. *β-Secretase*. 1FKN (OM99-2, 1.9 Å), 1M4H (OM00-3, 2.1 Å), 1W51 (L01, 2.55 Å), 1XS7 (cycloamide-urethane inhibitor, 2.8 Å), and 1SGZ (apo form, 2.0 Å) structures of β-secretase were evaluated and selected for virtual screening studies.

The protonation state of catalytic aspartates was treated as follows: Asp-32 was protonated, while Asp-228 was ionized. The active sites of 1FKN, 1SGZ, 1M4H, 1XS7, and 1W51 were defined as the collection of residues within 6.5 Å of the bound inhibitor. The active site of the ensemble was defined by the union of all ligands of the ensemble. All atoms located less than 6.5 Å from any ligand atom were considered in ensembles. The bound inhibitor was not included in the docking runs. 1FKN was used as a reference structure for the united protein preparation.

JNK-3. Enrichment studies were performed on 1PMN (cyclopropyl-{4-[5-(3,4-dichlorophenyl)-2-[(1-methyl)piperidin]-4-yl-3-propyl-3H-imidazol-4-yl]-pyrimidin-2-yl}amine, 2.2 Å), 1PMU (9-(4-hydroxyphenyl)-2,7-phenanthroline, 2.7 Å), 1PMV (2,6-dihydroanthra[1,9-cd]pyrazol-6-one, 2.2 Å), 1JNK (5′-adenylimido-triphosphate, 2.3 Å), and 1PMQ (phosphoaminophosphonic acid-adenylate ester cyclohexyl-{4-[5-(3,4-dichlorophenyl)-2-piperidin-4-yl-3-propyl-3h-imidazol-4-yl]-pyrimidin-2-yl}amine, 2.2 Å) X-ray structures of JNK-3. The active site of the individual structures was defined as the collection of residues within 6.5 Å of the bound inhibitor. The active site of the ensemble was defined by the union of all ligands of the ensemble. All atoms located less than 6.5 Å from any ligand atom were considered in ensembles. The bound inhibitor was not included in the docking run. 1JNK was used as a reference structure for the united protein preparation.

Preparing the Screening Database. Our screening library includes a subset of World Drug Index (WDI) as inactive molecules that were specifically designated to reduce artificial enrichment.[26] WDI was first filtered in order to eliminate compounds having molecular weight lower than 200 and greater than 800, logP larger than 7, and rotatable bonds more than 15. The remaining 37 843 WDI compounds were subjected to diverse selection based on 2D UNITY fingerprints. Dissimilarity selection performed by the Selector module of Sybyl resulted in 5300 compounds with a maximum Tanimoto index of 0.65 and 9950 compounds with a maximal Tanimoto index of 0.69 that were defined as inactive sets.

Our active set was complied by the diverse selection of 25 JNK-3 and 50 β-secretase inhibitors from the total of 138 and 218 inhibitors, respectively, which are available in Prous Integrity Drugs&Biologics database.[27]

The final screening library was comprised of both active and inactive sets, which were stored as a Sybyl SLN list and converted to Sybyl mol2 format by means of Concord. This library of 5325/10 000 compounds has an active content of ~0.5% that mimics real-life screening situations.

Docking Protocol. Virtual screening experiments were performed using FlexX 1.13.2, FlexX-Pharm, and FlexE. Standard parameters were used as implemented in the SYBYL 7.0 package.[28] Thirty docking solutions (poses) for each docked molecule were scored and saved for further analysis. In two further cases, 60 docking solutions for each docked molecule were scored and saved for further analysis. In all cases the "place particles" option and formal charges were used. All stored poses were rescored using the CScore module of SYBYL 7.0 comprising five different scoring functions including Dock, Chem, FlexX, PMF, and Gold.

Pharmacophore constraints applied in FlexX-Pharm involved optional interaction constraints for residues Glu-147, Met-149, Asn-152, and Ser-193 in JNK-3 as derived from the analysis of X-ray structures, for Asp-32, Asp-228, Gly-34, and Gly-230 in BACE as derived from the pharmacophore published by Vertex.[16] Accepted poses fulfilled at least two of these constraints simultaneously in both of the cases (JNK-3, BACE).

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Supporting Information Available: EFs calculated at 1, 2, 5, and 10% of the ranked database, average run times and EFs when saving 60 poses, minimal and maximal Tanimoto similarity indices calculated between the JNK-3 active molecules, and descriptive statistics of the $EFs_{1\%}$. This material is available free of charge via the Internet at http://pubs.acs.org.

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