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How to choose relevant multiple receptor conformations for virtual screening: a test case of Cdk2 and normal mode analysis

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Abstract Better treatment of protein flexibility is essential in structure-based drug design projects such as virtual screening and protein-ligand docking. Diversity in ligand-binding mechanisms and receptor conformational changes makes it difficult to treat dynamic features of the receptor during the docking simulation. Thus, the use of pregenerated multiple receptor conformations is applied today in virtual screening studies. However, generation of a small relevant set of receptor conformations remains challenging. To address this problem, we propose a new protocol for the generation of multiple receptor conformations via normal mode analysis and for the selection of several receptor conformations suitable for docking/virtual screening. We validated this protocol on cyclin-dependent kinase 2, which possesses a binding site located at the interface between two subdomains and is known to undergo significant conformational changes in the active site region upon ligand binding. We believe that the suggested rules for the choice of suitable receptor conformations can be applied to other targets when dealing with in silico screening on flexible receptors.

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Conformational changes in protein receptors upon small ligand binding are a very common phenomenon (Cavasotto and Abagyan 2004; Cozzini et al. 2008; Miteva et al. 2010; Teague 2003). Analysis of protein-ligand interactions from a drug discovery perspective over the years has shown that a large number of pharmaceutically relevant targets are flexible (Teague 2003), such as anti-AIDS HIV-1 protease (Hornak and Simmerling 2007), anti-cancer p53 (Bowman et al. 2007a), and anti-viral influenza N1 (Amaro et al. 2007). Hence, even if challenging due to the large number of degrees of freedom that need to be considered (Cavasotto et al. 2005b), addressing protein flexibility is an essential issue in current structure-based drug design and virtual screening endeavors (B-Rao et al. 2009; Cozzini et al. 2008; McInnes 2007; Miteva et al. 2010). Handling protein flexibility would allow structure-based drug design to expand the conformational and chemical space of the hit molecules. Various modeling methods have been developed to incorporate receptor flexibility in molecular docking and virtual screening such as soft docking (Ferrari et al. 2004; Kairys and Gilson 2002), rotamer libraries search (Frimurer et al. 2003), induced fit approximation (Nabuurs et al. 2007; Sherman et al. 2006), Monte Carlo simulations (Cavasotto and Abagyan 2004), molecular dynamics (MD) simulations (Moitessier et al. 2004), or molecular docking on multiple receptor conformations (MRC) using either experimental [multiple crystal (Barril and Morley 2005; Huang and Zou 2007) or NMR ensembles (Bolstad and Anderson 2008)] or computationally generated conformations.

Diversity in ligand-binding mechanisms and receptor conformational changes [from a single side chain to

domain movements (Teague 2003)] makes it difficult to handle dynamic changes in the receptor conformations during the docking simulation, especially when dealing with large chemical libraries. Ligand-binding mechanisms are widely discussed in the literature and nowadays two mechanisms are accepted as most probable (Grant et al. 2010): conformational selection and induced fit. The conformational selection approach is based on the assumption that proteins exist in an equilibrated ensemble of structures, and ligand binding involves an equilibrium shift toward the protein-ligand complex structure (Grant et al. 2010; Ma et al. 2002). The induced fit approximation supposes that the bound-like conformations form after interaction with a ligand due to specific induced structural changes rather than selection from the already existing unbound ensemble. It is still difficult to prove which of them occurs, and it might be possible that both are involved in some cases. The use of predetermined (both experimental or modeled structures) MRC has been accepted today as an attractive practical alternative (Totrov and Abagyan 2008) and is more and more applied in virtual screening experiments (Bowman et al. 2007b; Cheng et al. 2008). In molecular docking/screening studies, the binding energy of the ligand is assessed against all models in a "cross-docking" protocol. A number of investigations employing MRC for binding mode prediction or virtual screening have been reported where the MRC have been generated via MD simulations (Amaro et al. 2008; Cheng et al. 2008; Frembgen-Kesner and Elcock 2006), harmonic dynamics simulations (Tatsumi et al. 2004), or normal mode analysis (NMA)/elastic network analysis (Cavasotto et al. 2005a; Floquet et al. 2006; May and Zacharias 2008; Rueda et al. 2009; Sander et al. 2008; Suhre and Sanejouand 2004).

While considerable progress has been made in ligand docking and screening on MRC, generating a small yet representative set of receptor conformations remains challenging. Some protocols proposing ligand docking on huge numbers of MRC [e.g., inhibitors have been docked on 5,000 structural MD snapshopts of p38 MAP kinase (Frembgen-Kesner and Elcock 2006)] are clearly not applicable to virtual screening experiments. In order to diminish relevantly the number of MRC, several techniques have been utilized such as root mean square deviation (RMSD) clustering on the binding site area of MRC (Cheng et al. 2008) or filtering for conformations with conserved pocket core/geometry (Bolstad and Anderson 2009; Subramanian et al. 2006). For generating MRC by using NMA methods, it has been suggested that only the normal modes impacting the pocket conformation be selected (Cavasotto et al. 2005a). Another possibility for validation/selection of the generated MRC as suitable or not for virtual screening is to analyze known ligand descriptors (Subramanian et al. 2006) and/or the docking reproducibility of known inhibitors (Bisson et al. 2007). In this case, one is supposed to dispose of structural/binding data over at least a small set of compounds in order to calibrate the protocol and select relevant conformations.

The choice of the most appropriate receptor conformations is key for the success of virtual screening experiments. It has been shown that consideration of too many conformations, either experimental or modeled, can lead to reduced performance (Barril and Morley 2005; Polgar and Keseru 2006; Totrov and Abagyan 2008). While docking on MRC may improve the binding mode prediction, too large a number of conformations increases the probability of selecting false positives in virtual screening computations. Along this line, two major issues remain to be addressed properly: first, the quality of the conformational sampling, i.e., the ability of a given process to provide key conformations associated with the binding of true inhibitors; and second, the selection of the most relevant structures among the numerous MRC generated.

To address these key points, we propose an efficient method for a fast generation of multiple receptor conformations via NMA and a new protocol to select several receptor conformations appropriate for virtual screening. Normal modes of vibration are harmonic oscillations characterizing the intrinsic dynamics of the system within an energy minimum (Wilson et al. 1980). Each low-frequency mode describes a collective motion where all particles oscillate with the same characteristic frequency. NMA has been used for more than three decades to study protein flexibility, and a number of studies have found satisfactory agreement between features of the motion predicted by NMA and the observed protein conformational changes (Alexandrov et al. 2005; Kong et al. 2006; Marechal and Perahia 2008; Robert et al. 2004). Despite the harmonic approximation determining NMA, this method can be very powerful not only to characterize large amplitude motions in macromolecules but also to study metastable or intermediate protein structures (Mouawad and Perahia 1996). Recently NMA has been employed in molecular docking studies, where it has been demonstrated that it can be successfully used to improve the accuracy of small ligand docking, taking into consideration the receptor flexibility (Cavasotto et al. 2005a; Floquet et al. 2006; May and Zacharias 2008; Rueda et al. 2009; Sander et al. 2008).

Cdk2 (cyclin-dependent kinase 2) was chosen here as a test protein for the NMA generation of MRC because it is an important anti-cancer target showing significant conformational changes in the active site (ATP-binding pocket). It is worth noting that the ATP-binding site is located at the interface of two subdomains, thus, this protein constitutes a very interesting case for assessing NMA, since this method is known to be appropriate for the

exploration of domain (subdomain) movements. Cdk2 is involved in central cell cycle functions (Morgan 1995) by interacting with cyclins through the S phase and thus participating in the initiation and the progress of the DNA synthesis. Cdk2 has been extensively investigated and a vast number of inhibitors have been discovered (Hardcastle et al. 2004; Sielecki et al. 2000). Further, a number of Cdk2 structures, co-crystallized with different ligands, are available, allowing evaluation of our NMA protocol (Davies et al. 2001; Schulze-Gahmen et al. 1995). All these studies on Cdk2 revealed important conformational changes (Subramanian et al. 2006) due to ligand binding. Recently several ligand docking and virtual screening investigations on Cdk2 have been reported (May and Zacharias 2008; Rueda et al. 2009; Sims et al. 2003; Thomas et al. 2006), allowing further validation of our developed protocol for MRC of Cdk2.

We started with a structural analysis of cyclin-bound Cdk2 (active Cdk2 form) X-ray structures. The superimposition of nine human holo-forms of Cdk2 bound to various ligands and two apo (nonliganded) forms (the resolution of these X-ray structures is below 2.9 Å) (see Fig. 1) suggests that significant induced fit of the active site occurs upon ligand binding. The two regions involved in conformational changes are the hinge region (GLU81-HIS84) and more importantly the G-loop (ILE10-VAL16), as observed

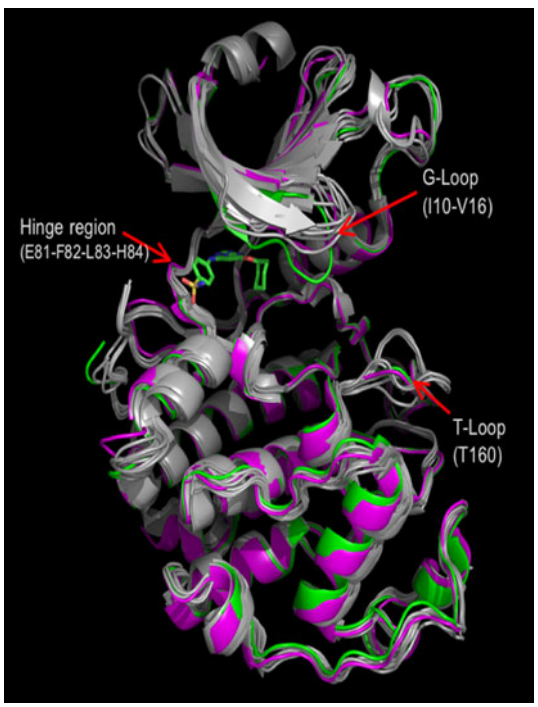

Fig. 1 Superimposition of human Cdk2 X-ray crystal structures: holo: 1fin, 1p5e, 1vyw, 2bkz, 2 bpm, 2i40, 2uzb, 3bht (*in gray*); 2C6T (*green* with the co-crystallized ligand); apo: 2JGZ (*magenta*) and 1w98 (*gray*)

by the differences between the structures 2C6T-holo (green) and 2JGZ-apo (magenta). We have chosen 2JGZ as an initial structure for the NMA because it has an intermediate G-loop conformation across the 11 superimposed active Cdk2 structures and an open pocket conformation.

What is the best manner to use NMA for the generation of MRC for the purpose of docking and virtual screening experiments? Various techniques of NMA computations have been used in molecular docking studies, however in most of them, several limitations can be noticed, e.g., they take into account only Cα backbone atoms (Cavasotto et al. 2005a; May and Zacharias 2008; Sander et al. 2008) or a limited part of the protein receptor around the bound ligand (Rueda et al. 2009).

In these protocols, the fluctuations of all atoms are not represented, which may lead to incorrect simulation of the system flexibility. If the side chain atoms are missing, local changes in the binding site can be affected. Along the same lines, ignoring a large part of the protein might lead to missing global movements that could impact the binding pocket. Thus, we argue that including all atoms in NMA can be critical for a quasi-exhaustive simulation of possible changes that may occur in the binding site. We should emphasize that in our NMA not only were all atoms of Cdk2 involved but also all atoms of cyclin B, the protein activating Cdk2.

The structure of the complex Cdk2-cyclin B considered for the NMA was taken from the PDB structure 2JGZ, an apo-form. We decided to compute the normal modes on an apo-form to simulate a case where no known co-crystallized ligands are available. At the same time the selected apo-form represents an intermediate structure among several holo and apo structures of Cdk2, thus, it is appropriate as a staring point. The hydrogen atoms were first built and the whole complex was energy minimized using the CHARMM program (Brooks et al. 1983) and the force field PARAM22 (MacKerell et al. 1988), using successively Steepest Descent (SD), Conjugate Gradient (CG) and Adopted Basis Newton-Raphson (ABNR) algorithms. To avoid important deviations from the crystal structure, harmonic constraints were applied to all atoms with a force constant that progressively decreased from 250 to 0 kcal mol^{-1} $Å^{-2}$ during the SD minimization. Then the minimization was continued without constraints until an RMS energy gradient of 10^{-5} kcal mol^{-1} $Å^{-2}$ was reached. The normal modes were computed using the DIMB method (Perahia and Mouawad 1995), as implemented in CHARMM. Electrostatic interactions were treated with a 4r-dependent dielectric constant and a short switching function (applied between 6.0 and 8.0 Å) to avoid the shrinkage of the protein. We analyzed the first 25 modes (from 7 to 31) since the lower frequency modes are usually the most responsible for important conformational changes

(Cui et al. 2004; Mouawad and Perahia 1996; Tama et al. 2000).

In the following, we describe how one can sample simply and rapidly the conformational space of the Cdk2 and model structural changes in the binding site through computation of the normal modes. The atoms of the initial conformation (the minimized X-ray structure of Cdk2, 2JGZmin) were displaced along the first 25 lowest frequency eigenvectors in both directions until reaching a mass-weighted root mean square deviation (MRMSD) of 2Å (or −2Å) with respect to the initial conformation. To distinguish between the two directions of an eigenvector, positive and negative values of MRMSD are used. To obtain energetically relaxed models after this perturbation, short energy minimizations (100 steps of SD with harmonic constraints and followed by CG and ABNR until getting a gradient of 5.10^{-1} kcal mol^{-1} $Å^{-2}$) were performed on the sampled structures. This ultimately yielded 51 structures, 2 per normal mode (in the two directions of an eigenvector) and the initial 1 as well.

From this ensemble we have chosen relevant structures both in terms of conformational diversity and binding site topology. For this purpose, we have chosen some criteria based on the conformation of the binding site to select the usable structures. Indeed, to sample the conformational space of the binding pocket efficiently, we first divided the active site of Cdk2 into three distinct zones or segments shown in red, yellow, and magenta in Fig. 2. Then, we applied rules for the selection of the relevant modes and MRC in terms of conformational changes and properties of binding site as summarized in Fig. 3. To prevent considering unrealistic structures, we discarded all conformations with a displacement of more than 8 Å for at least one atom belonging to any of the three segments with respect to the X-ray minimized structure 2JGZmin (stage II in Fig. 3). In addition, to ensure MRC with sufficient conformational diversity, we selected the conformations that showed distance deviation of at least 1 Å for atom pairs belonging to two distinct segments of each NMA conformation with respect to the 2JGZmin structure. In the case of Cdk2, we ended up with the structures that were displaced along only five modes of interest (modes 8, 9, 10, 12, and 14), satisfying the criteria of stage II.

Stage III (see Fig. 3) involved evaluation of binding site topologies of the retained 10 MRC. This was carried out using the in-house program GP_PASS, based on the program PASS (Brady and Stouten 2000) that performs a pocket detection and evaluates the probes' degrees of burial within the found pocket. GP_PASS score for each binding pocket conformation is obtained by summing up the degrees of burial of each probe in the pocket detected by the program PASS and dividing this sum by the total number of probes within the detected binding site. Thus, by

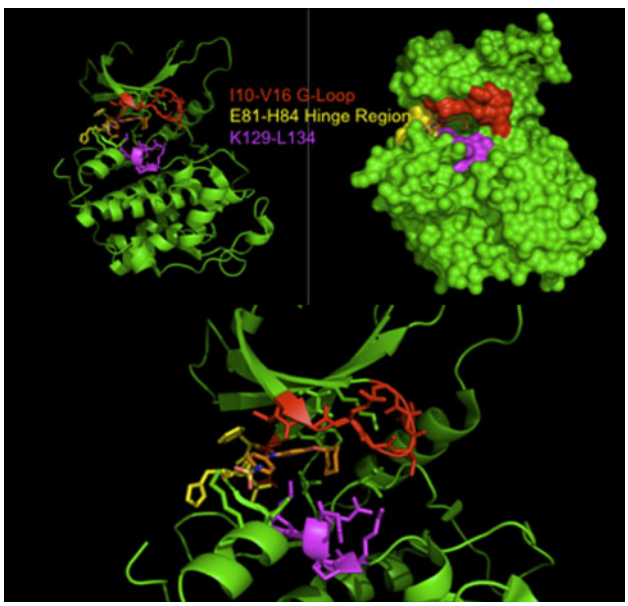

Fig. 2 Binding pocket of Cdk2. The three zones forming the Cdk2 binding pocket are shown in *red*, *yellow*, and *magenta*. The structure of 2C6T and the co-crystallized ligand DT5 are shown in *green* and *orange*, respectively

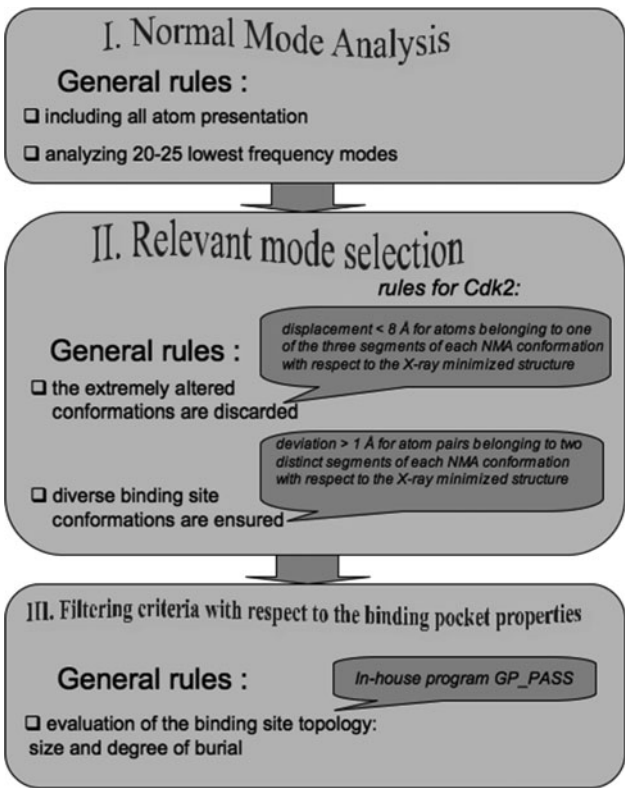

Fig. 3 Protocol for selection of relevant MRC via NMA. Several general rules are shown and particular ones for the Cdk2 active site as well

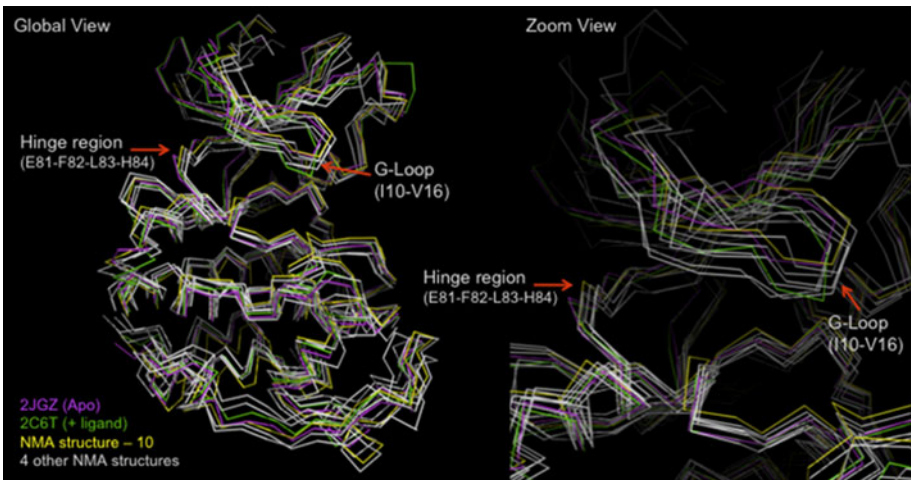

Fig. 4 Superimposition of the five best NMA conformations (four in *gray*), including the NMA structure "10_open" in *yellow*, showing a good exploration of the conformational changes of binding site compared to the experimental structures 2JGZ (apo in *magenta*) and 2C6T (holo in *green*)

the number of probes we evaluate the binding pocket size and by the GP_PASS score its deepness, factors that are known to be important for ligand binding (Brady and Stouten 2000; Kuntz et al. 1982). From the population of 10 structures generated along the five modes cited above, the GP_PASS score allowed us to choose five structures; these were also visually inspected (Fig. 4). The five selected structures correspond to an open conformation that provides larger space within the binding core of the protein for potential ligands.

Finally, in order to validate the protocol presented above (Fig. 3), we evaluated the quality of the selected MRC of Cdk2 by assessing their capacity: first, to correctly dock cognate inhibitors, and second, to distinguish between active and inactive compounds during virtual screening experiments using the docking program Surflex (Jain 2007). For this purpose, we constructed a set of 52 Cdk2 inhibitors taken from the DUD data set (Huang et al. 2006) and 35,804 drug-like compounds (decoys) taken from the diversity set of the ChemBridge database (http://chembridge.com/chembridge) both filtered for ADME/tox properties using FAF-Drugs2 (Lagorce et al. 2008) and generated in 3D using DG-AMMOS (Lagorce et al. 2009). The 52 inhibitors were docked into the five NMA conformations, the X-ray structures 2C6T (holo) and 2JGZ (apo), and the minimized X-ray structure, 2JGZmin.

The calculated RMSD between the docked ligands and the co-crystallized reference ligand for the three best NMA structures are given in Table 1 (10_open, 9_open, and 14_open showed the best RMSD among the five selected NMA structures; "open" refers to the displacement along the mode in the direction that opens the cavity). These three NMA structures correspond to three different relative subdomain motions that modify the overall structure of the protein and slightly change the conformation of the binding pocket (see the GP_PASS scores in Table 2). It can be seen that the cognate inhibitors can be better docked within an NMA conformation, namely 10_open structure (70.4% of the docked poses have an RMSD < 2 Å with respect to the crystal structure), than in both the holo and apo X-ray structures. We should note that we achieved RMSD < 1 Å for 30.1% and RMSD < 1 Å for 24.2% of the 52 docked inhibitors when docking on the holo X-ray structure. One can expect better docking performance on the holo X-ray structure. However, as seen in Fig. 1, the Cdk2 holo X-ray structures strongly depend on the bound ligands, e.g., strong induced fits are observed. Thus, for 45.7% of the tested 52 inhibitors, the binding site of the 2C6T holo structure is not suitable to dock them properly, possibly because the structure seems strongly influenced by its bound ligand. Similar phenomena have been observed for other protein targets with extreme induced fit presenting strong memory of their cognate ligands (Rueda et al. 2009). In such cases it might be possible that a modeled or an experimental "intermediate" apo receptor conformation represents a better conformation for cross docking, as opposed to a highly specific holo X-ray structure. In the same context, various docking accuracies are reported in

Table 1 Rate of successful docking (in %) on different receptor X-ray and NMA conformations based on RMSD between the docked ligands and the experimental co-crystallized ligand structures

Receptor conformation	X-ray holo (2C6T)	X-ray apo (2JGZ)	X-ray apo minimized (2JGZmin)	NMA best no. 1 (10_open)	NMA best no. 2 (9_open)	NMA best no. 3 (14_open)
RMSD $<$ 1Å	30.1%	25.0%	8.3%	27.9%	25.1%	21.1%
1Å $<$ RMSD $<$ 2Å	24.2%	33.1%	34.5%	42.5%	30.6%	34.1%

Table 2 GP_PASS scores vs virtual screening enrichments

NMA and X-ray receptor structures	Pocket conformation	GP_PASS score	Enrichment (% actives at 5% of the ranked dataset)	Enrichment (% actives at 10% of the ranked dataset)
8	Open	21	13.5	19.2
9	Open	37	17.3	28.8
10	Open	34	15.4	25.0
12	Open	22	3.8	15.4
14	Open	30	11.5	25.0
2C6T (holo)	Intermediate	44	26.9	46.2
2JGZ (apo)	Open	39	25.0	46.2
2JGZmin (apo)	Open	35	13.5	19.2
Correlation GP_PASS vs enrichment: R^2			0.84	0.82

the literature when cross docking is performed on diverse Cdk2 inhibitors within different X-ray Cdk2 structures. For instance, it has been demonstrated that docking of 34 ligands on 49 X-ray Cdk2 structures (Barril and Morley 2005) yielded on average 33% ligands docked correctly within RMSD of 2 Å. It has been also reported that a success rate of 84% has been achieved for correct ligand pose reproduction when 340 ligands were docked on 20 X-ray Cdk2 structures (Thomas et al. 2006). In a different manner, it has recently been shown (May and Zacharias 2008) that docking on Cdk2 MRC generated by elastic network normal modes and rotamer side chain search significantly improved the docking accuracy, yet only six Ckd2 inhibitors have been tested on the modeled MRC issued from the corresponding six X-ray Cdk2 structures, suggesting that the robustness of the method used has to be assessed further. Our NMA procedure resulted in several "good" Cdk2 conformations, one of which (10_open) docked 70.4% of the 52 Cdk2 inhibitors within a RMSD of 2 Å. This can be considered an excellent result keeping in mind that MRC conformations were generated from NMA of an apo protein structure. In most cases, when working on a new target without known actives, the only available structure is in an apo state, therefore this procedure may be successfully applied for predicting ligand-binding modes. The two other best NMA conformations show similar RMSD results compared to both the holo and the apo X-ray structures. The worst docking accuracy results were obtained for the minimized X-ray structure 2JGZmin, similarly to that described by Barril and Morley (2005) who reported that geometry optimization/energy minimization can alter the binding site and cause a wrong positioning of some ligands.

Docking methodology is widely employed for virtual screening with the goal of identifying new hit molecules. In this context, a docking/virtual screening protocol should be able to retrieve the true actives in a short list of potential binders suggested for experimental assays. Regarding this issue, the percentages of the retrieved actives at the top 5 and 10% of the ranked chemical compounds' dataset when screened on the best NMA and X-ray Cdk2 structures with Surflex are given in Table 2. One can observe that the best performance is achieved when screening the holo or the apo X-ray structures, with 26.9 and 25% retrieved actives, respectively, at 5% of the ranked dataset. Among the individual NMA conformations, 9_open and 10_open provided the best enrichment results with retrieved 17.3 and 15.4%, which can be considered as an encouraging result with respect to the X-ray structures. Regarding the so-called global enrichment (in this study combining the virtual screening results of the five selected NMA conformations in a single enrichment by including the best score of each ligand for all MRC), the results were degraded in comparison with the best individual NMA conformations. This is in agreement with an often observed drawback in treating receptor flexibility, as for example reported by Barril and Morley (2005) and Rueda et al. (2009). There, it was found that the enrichment can sometimes be degraded when combining a large set of MRC due to the increasing number of false positives, possibly highlighting the need to develop specific scoring approaches when using MRC.

It is worth addressing another issue on analyzing the MRC binding pockets. In a recent analysis of prediction of inhibitors binding on several Cdk2 X-ray structures, Barril and Morley (2005) suggested applying a compensatory term to the pose scores depending on the binding pocket properties by evaluation of the pocket internal energy. We propose a more simple method for pocket assessment. In Table 2, we report the GP_PASS scores for the binding site of each Cdk2 conformation along with the levels of enrichment (% of retrieved actives at the corresponding percentage of the ranked chemical compounds' dataset). It is important to note that one can observe a significant tendency of the enrichment at both 5 and 10% to correlate ($r^2_{5\%} = 0.84$; $r^2_{10\%} = 0.82$) with the GP_PASS score. Further, the two X-ray structures have the best level of

enrichment and also the best GP_PASS score. At this stage, additional computations should be performed on a larger set of conformations to establish a rigorous correlation of the GP_PASS score and the docking/enrichment success of MRC, yet we suggest that such analysis is promising and relatively straightforward to implement.

In conclusion, here we have elaborated an efficient protocol to choose relevant structures appropriate for subsequent ligand docking and virtual screening based on NMA. Successful docking experiments that reproduce correctly binding modes on the best selected MRC are shown. Although further improvement of this protocol might be required for virtual screening studies, the enrichment results presented here are encouraging. Cdk2 is chosen as a test protein receptor because its active site is located at the interface of two subdomains, thus, it is a very interesting structure to test NMA methods that were suggested to be appropriate for exploration of domain (subdomain) movements. It should be noted that NMA might not be appropriate to explore all kinds of binding site flexibilities, for instance in cases where one only expects side-chain conformational changes or when extreme induced fit of a cognate ligand is present [as was recently observed (Rueda et al. 2009) for 3 of 28 tested proteins]. Although validated only on Cdk2, we believe that the suggested protocol in this study can serve when dealing with docking on flexible receptors showing relatively large movements where the NMA has proved to be a very efficient method. In addition, the selection rules might be worth applying when MD is utilized for similar MRC generation.

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